DESIGN, SYNTHESIS AND DEVELOPMENT OF PDE5 INHIBITORS

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Design, Synthesis and Development of PDE5 Inhibitors

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Summary

This research project is concerned with the design, synthesis and development of new phosphodiesterase 5 (PDE5) inhibitors with improved selectivities and lower toxicities.

Two series of a 5 member and a 6 member ring fused heterocyclic compounds were designed, and synthesized. By alteration of starting materials and fragments, two virtual libraries, each is consisted of close to hundred compounds, were obtained successfully. The screening of sexual stimulation activity with rabbits demonstrated both groups of compounds were able to stimulate rabbit penile erection significantly.

The following toxicitiy studies revealed 2-(substituted–sulfonylphenyl)-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one group possessed an unacceptable toxicity with oral LD_{50} about 200mg/kg; while 2-(substituted–sulfonylphenyl)-pyrrolo[2,3-d]pyrimidin -4-one group showed an acceptable toxicity with oral LD_{50} over 2000mg/kg.

The continued bioactivity studies showed yonkenafil, the representative of 2-(substituted–sulfonylphenyl)-pyrrolo[2,3-d]pyrimidin-4-one group, has a better selectivity towards PDE5 and PDE6 than sildenafil and a better overall profile of sexual stimulation on animals than sildenafil.

Chronic toxicitiy studies of yonkenafil further confirmed yonkenafil did not cause any serious side effect and damage on animal models and most actions were explainable.

Based on evidences of the above studies, yonkenafil were recommended to enter clinical trials by the regulation authority of China, SFDA. Currently yonkenafil has been through the Phase I clinical trials and ready to progress into Phase II. Hopefully, yonkenafil will provide an alternative to the ED patients in the future.

Keywords: PDE5, PDE5 inhibitors, erectile dysfunction, 5 and 6-fused heterocyclic, yonkenafil, bioactivity, toxicity.

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Abbreviations

PDE/PDEs	phosphodiesterase/ phosphodiesterases
SFDA	State Food and Drug Administration of P. R. China
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
ATP	adenosine triphosphate
GTP	guanosine triphosphate
NO	nitric oxide
PKA	protein kinase A
PKG	protein kinase G
AC	adenylyl cyclase
AKAPs	A kinase anchor proteins
CREB	cAMP Response Element Binding Protein
GC	guanylyl cyclase
COPD	chronic obstructive pulmonary disease
PEST	Parameter Estimation by Sequential Testing
SMC/SMCs	smooth muscle cell/smooth muscle cells
KATP	ATP sensitive potassium
PGI2	prostacyclin
NOD	non-obese diabetic
RA	rheumatoid arthritis
CIA	collagenII-induced arthritis
SCW	streptococcal cell wall
IC_{50}	50% inhibition concentration
ED	erectile dysfunction
FSD	female sexual dysfunction
GMP	guanosine monophosphate
CGRP	calcitonin gene-related peptide
VIP	vasoactive intestinal polypeptide
MUSE	medicated urethral system for erection
FDA	Food and Drug Administration
IIEF	International Index of Erectile Function
HRMS	high resolution mass spectra
TEA	triethylamine
THF	tetrahydrofuran
HMDS	hexamethyldisilane
DCM	dichloromethane
MS	Mass spectra

NMR	Nuclear Magnetic Resonance
AMMS	academy of military medical
NICPBP	national institute for the control of pharmaceutical and biological products
I.p.	intraperitoneal
T	testosterone
RBC	red blood cell count
LD_{50}	median lethal dose
HGB	hemoglobin
HCT	hematocrit
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin
MCHT	mean corpuscular hemoglobin concentration
WBC	white blood cell
LYM	lymphocyte
MO	monocyte series
GRAN	granulocyte count
PLT	platelet count
GPT	glutamic pyruvic transaminase
GOT	glutamic oxaloacetic transaminase
ALP	alkaline phosphatase
TBIL/BIL	total bilirubin/ bilirubin
TG	triglyceride
CHO	cholesterol
TP	total protein
ALB	albumin
GLO	globulin
GLU	glucose
CK	creatine kinase
LDH	lactate dehydrogenase
UA	uric acid
BUN	blood urea nitrogen
CR	creatinine
TT	thrombin time
PT	prothrombin time
APTT	activated partial thromboplastin time
FIB	fibrinogen
KET	ketone
BLO	occult blood
PRO	protein

URO	urobilinogen
NIT	nitrite
LEU	leukocyte
ESI	electrospray ionization
LLOQ	lower limit of quantification
QC	quality control
EMS	Enhanced Ms Scan
SIM	Selected Ion Monitoring
NL	Neutral Loss Experiment
PIS	Precursor Ion Scanning
MRM	Multiple Reaction Monitoring

Chapter 1

Introduction

1.1 Phosphodiesterases (PDEs) and PDEs inhibitors

1.1.1 PDEs

Phosphodiesterases (PDEs) are a superfamily of enzymes that degrade cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) *(Beavo,1995)*.There are now 11 known PDE members and further more subtypes are being identified, many of which exist as splice variants *(Beavo et al.,1994)*. The cAMP-specific enzymes include PDE4, 7 and 8. The cGMP-specific PDEs are PDE5, 6 and 9, whereas PDE1, 2, 3, 10 and 11 use both cyclic nucleotides *(Mehats et al., 2002)*. PDEs influence a vast array of pharmacological processes, including proinflammatory mediator production and action, ion channel function, muscle contraction, learning, differentiation, apoptosis, lipogenesis, glycogenolysis and gluconeogenesis. As essential regulators of cyclic nucleotide signalling with diverse physiological functions, PDEs have become recognized as important drug targets for the treatment of various diseases, such as heart failure, depression, asthma, inflammation and erectile dysfunction *(Mehats et al., 2002; Torphy, 1998)*.

Both cAMP and cGMP are ubiquitous second messengers responsible for transductions of various extracellular signals, including hormones, light and neurotransmitters. These cyclic nucleotides are formed from ATP and GTP by the catalytic reactions of adenylyl cyclase and guanylyl cyclase, respectively. Adenylyl cyclase can be activated by forskolin and guanylyl cyclase by nitric oxide (NO). Through cell-surface receptors such as β-adrenoreceptor and prostaglandin E2, these

enzymes can also be activated indirectly *(Torphy, 1998)*.

As the intracellular concentrations of the cyclic nucleotides rise, they bind to and activate their target enzymes, protein kinase A (PKA) and protein kinase G (PKG). These protein kinases phosphorylate substrates such as ion channels, contractile proteins and transcription factors, which regulate key cellular functions. Phosphorylation alters the activity of these substrates and thus changes cellular activity. Obviously, altering the rate of cyclic nucleotide formation or degradation will change the activation state of these pathways *(Krebs & Beavo, 1979)*.

1.1.1.1 Classification and structural biology

Table 1.1 shows the current classification scheme for cyclic nucleotide PDEs. Eleven distinct members are differentiated functionally on the basis of substrate specificity and sensitivity to endogenous/exogenous regulators and genetically on the basis of sequence homology (three additional members have been reported, but the data available at this time are insufficient for their inclusion in Table 1.1) *(Soderling et al., 1998)*. Most of PDEs include more than 1 gene product (subtype, designated with a letter following the member number—eg. PDE4D). Multiple splice variants (isoforms, designated with a number of following the subtype letter—eg. PDE4D1), each with different functional and regulatory characteristics, may exist for any given gene product. The number of specific human PDEs is thus well over 50.

Table 1.1 Human cyclic nucleotide phosphodiesterase isozymes

Types (no. of genes)	Characteristics	Tissue distribution
PDE1 (3)	Ca^{2+}/calmodulin-stimulated	brain, heart, skeletal muscles, liver, vascular muscles, visceral muscles
PDE2 (1)	cGMP-stimulated	adrenal cortex, corpus cavernosum, heart, visceral muscles, brain, skeletal muscles
PDE3 (2)	cGMP-inhibited, cAMP-selective	corpus cavernosum, heart, vascular and visceral muscles, blood platelets, liver, adipose tissue, kidney
PDE4 (4)	cAMP-specific, cGMP-insensitive	brain, testes, thyroid gland, kidney, lung, mast cells, skeletal muscles, vascular and visceral muscles
PDE5 (1)	cGMP-specific	corpus cavernosum, vascular and visceral muscles, blood platelets
PDE6 (3)	cGMP-specific	retina (cones, rods)
PDE7 (2)	cAMP-specific, high-affinity	skeletal muscles, heart, lymphocytes
PDE8 (2)	cAMP-selective, IBMX insensitive	widespread; e.g. testes, ovaries, bowel
PDE9 (1)	cGMP-specific, IBMX insensitive	widespread; most strongly expressed in the spleen, small intestine and brain
PDE10 (1)	cGMP-sensitive, cAMP-selective	Brain (putamen and caudal nerve), testes, thyroid gland
PDE11 (1)	cGMP-sensitive, dual specificity	Skeletal muscles, heart, vascular muscles and visceral muscles (corpus cavernosum, prostate), pituitary gland, testes, liver and kidneys

1.1.1.2 Pharmacology

The general scheme for the formation and degradation of cyclic nucleotides is depicted in Figure 1.1. Cyclic AMP and guanosine 3', 5' -cyclic guanosine monophosphate (cGMP) are formed from their respective triphosphates by the

catalytic activity of adenylyl cyclase (AC) or guanylyl cyclase (GC), respectively. These cyclases exist in multiple isoforms (9 for AC; 11 for GC). Each isoform has a specific tissue and cellular distribution, and each couples specifically to discrete sets of receptors for signal transduction. Some isoforms are regulated by transactivators such as calmodulin, whereas others are sensitive to phosphorylation. Thus, adenylyl and guanylyl cyclase are able to integrate a vast array of direct and cross talk signals and to do so differently in different cell types.

Cyclic nucleotides are degraded by PDE-catalyzed hydrolytic cleavage of the 3'-phosphodiester bond, resulting in formation of the corresponding inactive 5'-monophosphate. PDE inhibitors block this hydrolytic activity, causing accumulation of cyclic nucleotides corresponding to the type-specificity of the inhibitor used (eg, accumulation of cAMP when inhibiting a cAMP specific PDE such as PDE4). Interestingly, activation of AC or GC fails to induce more than transient alterations in cyclic nucleotides because of compensatory increases in PDE activity; however, concomitant cyclase activation and PDE inhibition produce synergistic effects on cyclic nucleotide steady-state levels *(Torphy et al., 1995)*.

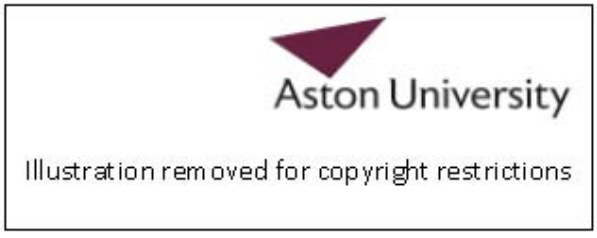


Figure 1.1 Cyclic nucleotide signaling and homeostasis *(David & Essayan, 2001)*

Specific PDE isoforms display remarkably selective cellular and subcellular localizations *(Jin et al., 1998)*. Thus, compartmentalization of PDE activities possessing discrete pharmacokinetic and regulatory characteristics likely plays an important role in the maintenance of cellular homeostasis. This type of compartmentalization may also contribute to rapid and reversible cross talk between PDE families and isoforms; a local shift in cyclic nucleotide content within one of these subcellular pools through the activity of one PDE might affect allosteric regulation or active site competition on another PDE *(Whalin et al,. 1991)*. Clearly, pharmacologic regulation of PDEs must account for a complex set of variables.

Cyclic nucleotides transduce signals through direct protein binding. The best characterized of these protein targets are the cyclic nucleotide–dependent protein kinases, cAK and cGK. Each is composed of 2 major subtypes (cAK I and cAK II; cGK I and cGK II); each subtype is composed of several isoforms *(Scott et al., 2000)*. Cyclic AK and cGK are serine-threonine kinases, each a tetramer of 2 regulatory subunits coupled to 2 catalytic subunits. When activated through cooperative binding of 4 cyclic nucleotide molecules, the 2 regulatory domains disengage from the 2 catalytic domains. These catalytic domains are then free to translocate within the cell and phosphorylate specific targets including transcription regulators, ion channels, and signaling proteins *(Coghlan et al., 1995)*. This translocation is not a random process. In the case of cAK, subcellular targeting is enhanced by the family of A kinase anchor proteins (AKAPs, at least 20 isoforms, each differentially regulated and expressed in specific tissues). AKAPs' hydrophobic docking sites bind the catalytic subunits of cAK, and their coiled coil regions interact with various structural or functional proteins to complete the targeting process *(Glantz et al., 1993)*. Interestingly, targets for regulation by cAK and cGK include AC and GC, several AKAPs, and several PDEs. Thus, multiple levels of degeneracy, cross talk, and feedback create a cyclic nucleotide–mediated signaling network of extreme complexity.

Two final points concerning cyclic nucleotide signaling should be underscored. First, the cyclic nucleotide signaling cascade engages in cross talk with several other critical signaling pathways. For example, cAK targets Raf-1 in the RAS/mitogen-activated protein kinase pathway, whereas Erk2 kinase targets several isoforms of PDE4.21 Moreover, several pathways may independently regulate cyclic nucleotide downstream effectors (a process called *convergence*). For example, both cAK and the immunoglobulin Fc signaling cascades target the transcription factor CREB at different sites on the molecule and exert different effects *(MacKenzie & Houslay, 2000)*. The outcome of cross talk or convergence may be either inhibition or enhancement of signaling. Second, the cyclic nucleotide pathway undergoes autoregulation. For example, transcriptions of several PDE genes are under the control of cyclic nucleotide regulatory elements, and several PDE proteins have cAK and/or cGK phosphorylation sites *(Alvarez et al., 1995)*. The outcome of autoregulation may be either positive or negative feedback.

1.1.2 PDEs inhibitors

By the late 1970s and early 1980s it became clear that kinetically distinct PDEs could indeed be inhibited selectively by a variety of small organic molecules *(Wells et al., 1981)*, in addition to well-known nonselective inhibitors include caffeine, theophylline, pentoxifylline, and 3-isobutyl-1-methylxanthine. Table 1.2 lists some of commonly used selective inhibitors for each of the PDEs. Although selective inhibitors offer the potential for specificity of pharmacologic effect, the structural similarities and apparent functional redundancy within subtypes and isoforms have renewed concerns for the identification and utility of more highly selective Agents *(Baillie et al., 2000)*.

Table 1.2 Selective PDEs inhibitors

PDE isoenzyme	Possible functional significance of PDE	Examples of inhibitors
PDE 1	Vascular muscular weakness, taste, olfaction	KS-505a Vinpocetine
PDE 2	Olfaction, adrenocorticosteroid production	EHNA (MEP-1)
PDE 3	Myocardial contractility; insulin secretion; lipolysis, glucoseproduction, platelet aggregation	Cilostamide Enoxamone Milrinone Siguazodan
PDE 4	Inflammation; vascular and visceral muscle tone; depression, thyroid gland secretion, reproduction	CDP840 Rolipram SB 207499 Tibenelast
PDE 5	Erection; smooth muscle tone platelet aggregation	Dipyridamole MY-5445 Sildenafil Zaprinast
PDE 6	Signal transduction in vision	Dipyridamole Zaprinast
PDE 7	T-cell activation; skeletal muscles; metabolism	Several in development
PDE 8	T-cell activation	None selective
PDE 9	?	None selective
PDE 10	Dopamine signal transmission	None selective
PDE 11	?	None selective

PDE inhibitors reduce the hydrolysis of cAMP/cGMP, and hence elevate the intracellular level of cAMP/cGMP. Thus, PDE inhibitors will change the activation state of cyclic nucleotide signaling pathways, resulting in the regulation of various physiological functions. An important issue in the development of an inhibitor for single PDE is specificity for the rest. The molecular diversity of the PDE inhibitors and structure-based design of PDE inhibitors may provide opportunities for development of more selective inhibitors. This section will update the recent progress of the development of PDE inhibitors.

1.1.2.1 PDEs inhibitors effect in human inflammatory/accessory cells

Inflammatory cells are exquisitely sensitive to alterations in cyclic nucleotide levels; interestingly, modulation of immune cell function by selective PDE inhibitors is limited to specific PDE types, probably as a result of compartmentalization of cyclic nucleotide and PDE pools. In immune cells, elevation of intracellular cAMP, mediated predominantly through inhibition of PDE4, results in a wide range of anti-inflammatory effects.

- **Basophils and mast cells**

Human basophils contain primarily PDE3, PDE4, and PDE5. Inhibition of PDE4 has been shown to downregulate anti-IgE–induced histamine release and leukotriene C4 (LTC4) generation, platelet-activating factor–induced histamine release, and dust mite antigen–induced IL-4 and IL-13 generation from human basophils. Although PDE3 and PDE5 inhibitors show little independent efficacy in regulating these functions, PDE3 inhibitors may potentiate the activity of PDE4 inhibitors on several of these activities. Finally, functional immunomodulation is closely correlated with intracellular cAMP levels; synergy is observed with PDE4 inhibitors and forskolin. To date, there are no studies probing the potential utility of inhibitors targeting PDE7-PDE11 *(Weston et al., 1997)*.

Human mast cells contain both PDE3 and PDE4. Interestingly, although some investigators have reported that PDE4 inhibitors downregulate IgE-mediated calcium fluxes, histamine release, and cytokine generation, several groups have reported resistance of mast cell mediator release to PDE4 inhibition and have correlated this resistance with a lack of PDE4-mediated increases in intracellular cAMP *(Shichijo et al., 1999)*. Differences in cellular substrate for these studies (skin and lung fragments vs. purified mast cells vs cultured mast cells) may account for some of these discrepancies. This remains an area of unresolved debate.

- **Eosinophils**

Human eosinophils contain both PDE3 and PDE4. PDE4 inhibitors block a variety of eosinophil functions, including superoxide generation, LTC4 production, CD11b/CD18 upregulation and L-selectin shedding, chemotaxis, and survival; inhibitors of PDE3 show no independent efficacy in downregulating eosinophil functions *(Hatzelmann & Schudt, 2001)*. Several recent reports suggest both synergy between AC activators and PDE4 inhibitors in downregulating various eosinophil functions and reversal of the effects of PDE4 inhibitors on freshly isolated human eosinophils by cAK antagonists. The effects of PDE4 inhibitors on eosinophil-derived cytokine generation and the potential roles of PDE7-PDE11 have not been reported.

- **Lymphocytes**

B lymphocytes contain PDE3, PDE4, and PDE7. Several authors have described complex relationships between intracellular cAMP, IL-4, and IgE synthesis. Unfortunately, these studies have been performed in mixed cell populations in which the concomitant effects of T cells and accessory cells are not adequately controlled *(Coqueret et al,. 1997)*. However, even studies with purified B cells have yielded seemingly contradictory results. Whereas proliferation of polyclonally stimulated, purified B lymphocytes is enhanced by PDE4 inhibitors, cAK activation results in inhibition of B-cell receptor–mediated proliferation and enhanced apoptosis that is subject to rescue by IL-4. Finally, although PDE4 inhibitors have been shown to reduce spontaneous IgE release from mononuclear cells isolated from allergic subjects, these effects have not been observed with purified B cells *(Chan et al,. 1993)*. The effects of PDE inhibition on synthesis of other immunoglobulin classes, B-cell differentiation, and cytokine generation have not been reported. This is an area with great research potential.

In addition to PDE3, PDE4, and PDE7, T lymphocytes express PDE1, PDE2, PDE5, and PDE8. The distribution of PDE types is nearly identical in CD4+ and CD8+ lymphocytes, but subtype and isoform expression may vary among T-cell phenotypes and in disease states *(Giembycz et al., 1996)*. In general, PDE4 inhibitors downregulate mitogen-, antigen- , and allogeneic HLA class II–induced blastogenesis and T-cell proliferation. Although PDE3 inhibitors show little or no independent efficacy in these models, they do synergize with the PDE4 inhibitors. PDE5 inhibitors are ineffective *(Giembycz et al., 1996)*. PDE4 inhibitors also downregulate mitogen- or antigen-induced production of many TH1- and TH2-derived proinflammatory cytokines, including IL-2, IL-4, IL-5, IL-13, IFN-γ, TNF, and GM-CSF; interestingly, upregulation of IL-10 has also been reported *(Giembycz et al., 1996)*. Once again, although PDE3 inhibitors show no independent efficacy in these models, they do synergize with PDE4 inhibitors. More recently, PDE4 inhibitors were shown to downregulate adhesion molecule expression on T cells and promote neoantigen tolerance in murine T cells *(Kasyapa et al., 1999)*. Finally, inhibition of PDE7 through use of antisense technology has demonstrated efficacy in downregulating anti-CD3-anti-CD-28–mediated proliferation and IL-2 production. The functional role of PDE8-PDE11 in T lymphocytes has not been pursued.

- **Monocytes/macrophages/dendritic cells**

Human monocytes contain PDE3 and PDE4; macrophages retain PDE4 and upregulate PDE3 while gaining PDE1 and PDE5; dendritic cells downregulate PDE4, upregulate PDE3, and gain only PDE5. These data underscore the importance of PDEs in the differentiation and maintenance of immunologic phenotypes. Inhibition of PDE4 markedly downregulates TNF-α production and arachidonic acid release/LT generation from monocytes *(Hichami et al., 1996)*. Interestingly, PDE4 inhibitors do not modulate superoxide, nitric oxide, IL-6, or IL-8 production from human monocytes. Although the data concerning PDE4 inhibitor effects on IL-1β production from monocytes are conflicting, PDE4 inhibitors consistently upregulate IL-10

production from these cells. Inhibition of PDE4 in macrophages is associated with weak downregulation of TNF-α and IL-1β production and arachidonic acid release *(Verghese et al., 1995)*. Interestingly, whereas PDE3 inhibitors display only minimal independent efficacy and variable synergy with PDE4 inhibitors in monocytes, inhibition of PDE3 in macrophages is associated with significant independent suppression of TNF-α production, nitric oxide generation, and IL-10 secretion *(Verghese et al., 1995)*. These differences between monocytes and macrophages may be attributable, in part, to maturation-induced changes in PDE profile. PDE4 inhibitors also downregulate T-cell help and cytokine generation from dendritic cells; although PDE3 inhibitors show marginal independent efficacy in dendritic cells, they show marked synergy with PDE4 inhibitors. The effects of PDE inhibitors on adhesion molecule expression and antigen presentation have not been reported; the potential roles of PDE1, PDE5, and PDE7 through PDE11 have not been explored.

- **Neutrophils**

Neutrophils contain predominantly PDE4. Inhibitors of PDE4 enhance intracellular calcium resequestration and decrease degranulation, superoxide and LT production, elastase and IL-8 secretion, and CD11b surface expression *(Anderson et al., 1998)*. Interestingly, PDE4 inhibitors also antagonize proapoptotic signals in neutrophils. Whereas PDE3 and PDE5 inhibitors show little or no efficacy in modulating these cellular functions, AC inducers act in an additive or synergistic fashion with PDE4 inhibitors in downregulating most neutrophil cellular functions *(Anderson et al., 1998)*. The potential roles of PDEs other than PDE3, PDE4, and PDE5 have not been explored.

- **Endothelial cells**

Endothelial cells contain predominantly PDE2, PDE3, and PDE4, with lesser contribution of PDE1, PDE5, and PDE7 *(Rousseau et al., 1994)*. Whereas PDE4

inhibitors decrease calcium fluxes and growth factor secretion, PDE3 inhibitors decrease monocyte chemoattractant protein 1, and the combination of PDE3 and PDE4 inhibitors downregulates hyperpermeability, VCAM-1 expression, and E-selectin expression (but not ICAM-1 expression) *(Blease et al., 1994)*.

1.1.2.2 PDEs as therapeutic targets

Given the multitude of cellular responses that cAMP and cGMP can elicit, it is clear that to achieve specificity of signal transduction, cells must be able to tightly regulate the magnitude and duration of cAMP/cGMP elevation, and also in specific cellular locations. Mammalian cells have evolved a complex and highly conserved complement of enzymes in order to generate, recognize and inactivate cyclic nucleotides. Inactivation of cAMP/cGMP is achieved by hydrolysis of the 3'-ester bond catalyzed by the PDEs, of which more than 50 have been identified. If cells did not possess PDEs, intracellular cAMP levels should rapidly become uniform. These enzymes therefore provide a key ability for the cell to generate nonuniform intracellular distribution of cAMP/cGMP, and hence differentially activate distinct compartmentalized protein kinase species.

Recent advances in the molecular pharmacology of PDE isoenzymes support new applications of PDE inhibitors for various diseases. Genomics and proteomics research may provide new rationales or possibilities for PDEs to be exploited for new drug applications. Many new pathways are being elucidated that involve specific PDE isoenzymes. Validation studies of PDEs as drug targets that use model animals and specific inhibitors are also in progress. The potential adverse effects of PDE inhibitors must be considered.

- **Pulmonary diseases (asthma, COPD, pulmonary hypertension)**

Several reports suggest that PDEs play important roles in the development and maintenance of pulmonary hypertension. PDE activity is increased in pulmonary arteries of rats with chronic hypoxia-induced pulmonary hypertension, and this is correlated with a decrease in intracellular cAMP and cGMP levels *(MacLean et al., 1996)*. The majority of PDE4 inhibitor patent claims address use of compounds as treatment for inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). Pretreatment with PDE4 inhibitors reduces antigen-induced bronchoconstriction in guinea pigs *(Howell et al., 1993)*, rabbits and cynomolgus monkeys *(Turner et al., 1994)*, primarily due to inhibition of mast cell degranulation. PDE4 inhibitors also abolish antigen-driven eosinophil infiltration in models of pulmonary inflammation, including guinea pig *(Howell et al., 1993)*, rat, rabbit and monkey *(Turner et al., 1994)*. Additional beneficial effects of PDE4 inhibitors in vivo include their ability to reduce airway hyperreactivity *(Howell et al., 1993; Turner et al., 1994)* and pulmonary microvascular leakage, induced by a number of challenges and in a number of species. These studies indicate that PDE4 inhibitors are active in a wide spectrum of pulmonary inflammation models. Recently, combined inhibition of PDE3 and 4 was found to be effective in pulmonary hypertension. It was shown that PDE3 and 4 inhibitors promote acute pulmonary vasodilation in experimental models of pulmonary hypertension *(Schermuly et al., 2000)*.

- **Neurodegenerative diseases**

PDE1A2 is predominantly expressed in brain, and its inhibition by deprenyl (selegeline hydrochloride) and amantadine can lead to enhanced intracellular levels of cAMP. It has been reported that in patients having Parkinson's disease with dementia, there is a significant decrease in cAMP. It was demonstrated that PDE1A2 is inhibited by antiparkinsonian agents, suggesting a potential role of PDE1 in Parkinson's disease *(Kakkar et al., 1996)*. On the other hand, isoenzyme PDE1A2 has a PEST motif and acts as a substrate for m-calpain. In brain, calpains are implicated in synaptic

modification, neurite pruning, receptor characteristics, neurofilament turnover and neural differentiation *(Sorimachi et al., 1997)*. Several reports indicate that calpains are involved in axonal neurofilament degradation, motorneuronal degradation, neuronal ischemia and other neurodegenerative diseases, including Alzheimer's and epilepsy *(Sorimachi et al., 1997)*. The proteolysis of PDE1A2 by m-calpain results in a CaM-independent form which in turn could decrease the intracellular levels of cAMP. These studies suggest that PDE1 isoenzymes may be useful targets for therapeutic intervention with respect to disorders of the central nervous system.

Alterations of PDE7 and 8 isoenzyme messenger RNA (mRNA) expression in Alzheimer's disease brains indicate that the expression of specific cAMP PDE isoforms may be selectively regulated in Alzheimer's disease and associated with different stages of the disease. Their differential regulation in AD brains suggests that the isoenzymes of these two families could be implicated in neurodegenerative and inflammatory diseases *(Pérez-Torres et al., 2003)*.

- **Vascular disease**

Atherosclerotic lesions occur in the context of endothelial cell dysfunction and involve activation, migration and proliferation of smooth muscle cells (SMCs). Endothelial derived relaxing factors, such as NO or prostacyclin (PGI2), relax blood vessels and inhibit the proliferation and migration of SMCs by increasing synthesis of the cyclic nucleotides cAMP or cGMP. In fact, cAMP and cGMP inhibit the proliferation of arterial SMCs, and elevation of cyclic nucleotides reduces neointimal formation after angioplasty in animal models.

Oral administration for 3–21 days of milrinone (0.3–3.0 mg/kg), a bipyridine derivative that specifically inhibits PDE3, suppressed intimal thickening by up to 56% in a dose- and time-dependent manner in a mouse model of photochemically induced vascular injury. In this model, oral administration of milrinone decreased the number

of activated SMC and consequently suppressed intimal thickening by preventing SMC proliferation within the media (*Kondo et al., 1999).*

- **Diabetes**

Type 2 diabetes mellitus is characterized by impaired insulin secretion and peripheral insensitivity to the hormone. Treatment of type 2 diabetes is currently unsatisfactory, and new agents are needed. One approach is to develop non-sulfonylurea drugs that will augment insulin secretion through mechanisms other than blocking KATP channels. Agents increasing islet beta-cell camp have potential as therapeutic agents, and GLP-1 and its derivatives have been shown to normalize insulin responses to glucose and nearly normalize overnight and daytime glucose concentrations. However, GLP-1 has the disadvantages associated with peptides, namely rapid degradation and inactivity by the oral route. Selective inhibition of PDE3 in the islet beta cell might augment meal-related insulin secretion, due to amplification of the effect of incretin factors, particularly GLP-1. Thus, PDE3 offers a potential target for developing drugs for the treatment of type 2 diabetes mellitus. Development of PDE3 inhibitors for this purpose will require their selectivity for islet beta-cell PDE3, as PDE3 also seems to be an important isoenzyme in the liver and adipose tissue, where its activation mediates some of the effects of insulin *(Reinhardt et al., 1995).*

Another intriguing possibility lies in the potential of PDE inhibitors to prevent beta-cell loss in both type 1 and type 2 diabetes. The non-selective PDE inhibitor pentoxifylline and the PDE4-selective agent rolipram were shown to reduce insulitis and prevent diabetes in non-obese diabetic (NOD) mice. These results suggest the importance of PDEs as therapeutic targets for treatment of diabetes.

- **Cancer**

The possible utility of PDE inhibitors as anti-cancer drugs has been proposed. Potent

inhibitors of PDE could elevate intracellular levels of cAMP. There are some indications that high intracellular levels of cAMP could arrest growth, induce apoptosis and attenuate cancer cell migration *(Santibanez et al., 2003)*. It has been known that elevation of cAMP levels functions as another stimulus that can induce growth arrest or cell death (or both) in many cultured lymphoid cells, including resting B cells, germinal centre B cells, T lymphocytes and thymocytes *(Kizaki et al., 1990)*. cAMP also induces cell death in cells derived from lymphoid malignancies, including murine lymphoma cell line S49.1, B-CLL cells and multiple myeloma cells *(Krett et al., 1997)*,. These results suggest that screening of PDE inhibitors could open a possibility to improved chemotherapeutic cancer treatments with reduced undesired side effects.

- **Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a highly inflammatory joint disorder that affects 1–2% of the U.S. population. In vitro and in vivo evidence suggesting that PDE4 inhibitors would be expected to be beneficial in the treatment of RA has recently been summarized. PDE4 inhibitors have been found to be efficacious in several animal models of arthritis. Rolipram has been shown to exhibit anti-inflammatory effects in carrageenan-induced paw edema and the adjuvant arthritis model. It has also been shown to ameliorate collagen II-induced arthritis (CIA) in mice. Recent studies in the adjuvant arthritis model demonstrated that rolipram abrogated edema formation and significantly inhibited hyperalgesia. Inhibition of cellular influx and inhibition of bone and cartilage destruction were also achieved. The efficacy of rolipram in the streptococcal cell wall (SCW)-arthritis model also has been documented *(Francischi et al., 2000)*. Several PDE4 inhibitors have also been evaluated in animal models of arthritis, particularly in models which involve LPS-induced TNF release *(He et al., 1998)*.

- **Osteoporosis**

Osteoporosis is a disease characterized by an imbalance between bone re-sorption and formation. The elevation of cAMP in osteoporosis has been shown to enhance bone formation, and therefore suggests that agents which elevate cAMP levels could have the potential to increase bone mass. Since PDE4 inhibitors are able to inhibit the production of TNF-α and are also able to elevate cAMP, atherapeutic effect in osteoporosis would be predicted *(Waki et al., 1999)*. Several recent studies provide evidence to support this hypothesis. Recently the effects of rolipram and pentoxifylline in normal mice were investigated. Both compounds were able to increase significantly both cortical and cancellous bone mass, predominantly by the acceleration of bone formation *(Waki et al. ,1999)*. It has also been suggested that the disease-modifying effects of PDE4 inhibitors in animal models of rheumatoid arthritis are related to their ability to suppress osteoporosis *(Souness & Foster., 1998)*.

- **Depression**

Impairment of signal transduction that regulates neuroplasticity and cell survival is thought to be an important mechanism contributing to major depressive disorders. In particular, cAMP-mediated signaling appears to have a key role in the pathophysiology and pharmacotherapy of depression. Elevating intracellular cAMP, either via inhibition of PDE4, which specifically catalyzes the hydrolysis of cAMP, or stimulation of adrenergic receptors, produces antidepressant-like effects in animal models *(O'Donnell &. Frith, 1999)*. PDE4 is particularly important for controlling intracellular cAMP concentrations and is considered to be a prime target for therapeutic intervention in a range of disorders such as depression and impaired cognition *(Zhang & O'Donnell, 2000)*. Notably, PDE4 is the predominant mediator of hydrolysis of cAMP formed by stimulation of β-adrenergic receptors, which are involved in the mediation of the effects of antidepressant drugs. Consistent with this, Inhibition of PDE4 by rolipram produces antidepressant-like and memory enhancing effects in animals *(Zhang & O'Donnell, 2000)*.

Among all the PDEs inhibitors, PDE5 is a cGMP-binding cGMP-specific PDE *(Corbin & Francis, 1999)*. PDE5 inhibitors are structurally similar to cGMP and compete with cGMP at the catalytic site of PDE5. Many PDE5 inhibitors were applied in clinical therapy such as treatment of erectile dysfunction by sildenafil. In next section, we reviewed the PDE5 inhibitor in its distribution and biological functions.

1.1.3 PDE5 inhibitors

PDE5 is abundant in the corpus cavernosum as the predominant PDE in this tissue. Although other PDEs are present in the corpus cavernosum*(Ballard et al.,1998)*, they do not appear to significantly modulate changes in cGMP levels associated with the ability to achieve penile erection. Immunohistochemical studies have demonstrated the presence of PDE5 in vascular and bronchial smooth muscle and in platelets, whereas negligible amounts of PDE5 have been detected in myocardial contractile cells and cardiac conducting tissue, although this remains under some debate *(Ballard et al., 1998)*.

PDE5 catalyzes the hydrolysis of cGMP with absolute specificity. The enzyme is active as a homodimer, which has a molecular mass of approximately 200 kDa. Either PKA or PKG can phosphorylate PDE5, and this has resulted in a significant increase in PDE5 activity. PDE5 is the primary cGMP-hydrolyzing activity in human corpus cavernosum tissue. Erection is largely a hemodynamic event which is regulated by vascular tone and blood-flow balance in the penis. Because cGMP levels modulate vascular tone, PDE5 is an obvious target for therapeutic intervention in the process. Oral PDE5 inhibitors can increase the cGMP, smooth muscle relaxation in the penis and, thus, penis election. Similar mechanisms appear to be involved in genical vasodilatation in the human female *(Rosen & Mckenna, 2002)*. This, coupled with its specificity for cGMP, has identified PDE5 as a target of considerable interest for the

pharmaceutical industry.

PDE5 inhibitors are structurally similar to cGMP and compete with cGMP at the catalytic site of PDE5 *(Corbin & Francis, 1999)*. Elevation of cGMP by a PDE5 inhibitor occurs secondary to reduced cGMP degradation by PDE5 when cGMP synthesis is concomitantly increased. This is the rationale for pharmacologic inhibition of PDE5 as a therapeutic approach for inducing penile erection in men with ED (erectile dysfunction).

Sildenafil is a highly selective and competitive inhibitor of PDE5, showing a higher degree of selectivity for PDE5 than for other PDEs, as evidenced by the much lower concentration of sildenafil needed to inhibit 50% (IC_{50}) of the activity of PDE5 compared with the IC_{50} concentrations against other PDEs (Table 1.3). Published IC_{50} values for sildenafil inhibition of PDE5 vary considerably, primarily because of the use of different tissue sources of PDE enzymes (eg, bovine vs human), variable purity of PDE5 preparations and buffer ingredients, and different assay conditions (eg, different substrate concentrations) *(Corbin & Francis,1999;Kim et al.,2001)*.

Table 1.3 Inhibition of phosphodiesterases (PDEs) by sildenafil

PDEs	PDE Tissue Source	Geometric Mean IC_{50} (μmol/L) (95% Confidence Intervals)
1	Human cardiac ventricle	0.28 (0.22–0.36)
2	Human corpus cavernosum	>30
3	Human corpus cavernosum	16.2 (9.50–27.8)
4	Human skeletal muscle	7.68 (5.51–10.7)
5	Human corpus cavernosum	0.0035 (0.0025–0.0048)
6	Bovine retina rods	0.033 (0.022–0.048)
7	Recombinant human enzyme expressed in *Sf9* cells	21.3 (16.5–27.4)
8	Recombinant human enzyme expressed in *Sf9* cells	29.8 (17.0–52.5)
9	Recombinant human enzyme expressed in *Sf9* cells	2.61 (1.39–4.91)
10	Recombinant human enzyme expressed in *Sf9* cells	9.80 (6.30–15.3)
11	Recombinant human enzyme expressed in *Sf9* cells	2.73 (2.46–3.04)

The usage of PDE 5 inhibitors in the treatment of ED is very successful. In addition, these drugs also exert positive effects on other pathological applications. For example, the impact of sildenafil and vardenafil on the memory performance in object recognition tasks was analysed by Prickaerts and co-workers *(Prickaerts et al.,2002)*. They orally administered both compounds to rats and observed immediately improved object discrimination performance. Compared with sildenafil, vardenafil appeared to be even more potent because it produced a high discrimination performance at a lower dose. Taken together, these results suggest that the inhibition of PDE 5 improves object recognition memory. Sarfati et al. indicated a functional role for sildenafil and vardenafil in apoptosis in chronic lymphocyctic leukaemia cells. By testing these drugs *in vitro*, they found that vardenafil was up to 30 times more potent in inducing apoptosis than sildenafil. Interestingly, normal B cells isolated from control donors were totally resistant to phosphodiesterase-induced apoptosis. Vardenafil and sildenafil might thus be considered in the treatment of chronic lymphocyctic leukaemia patients. In two animal models (rats and mice) it was shown that the co-administration of sildenafil significantly enhanced the antinociceptive effect of morphine *(Jain et al.,2003)*. Furthermore, sildenafil produced antinociception per se and increased the response of morphine, probably through the inhibition of cGMP degradation.

There are other PDE5 inhibitors in earlier stages of clinical development, and, on the basis of an evaluation of patent publications, it seems that several companies have preclinical discovery programs. Pfizer has reported that a 'second-generation' PDE5 inhibitor, UK357903, is now in phase II trials for ED. Tanabe is investigating avanafil in phase II trials for ED and FSD. Dong-A Pharmaceutical entered DA-8159 into phase II clinical trials for ED. DA-8159 is a pyrazolopyrimidinone that has shown erectogenic activity after oral administration of 0.3-1.0 mg kg^{-1} to rats. In anesthetized dogs, intravenous administration of 1–300 μg kg^{-1} resulted in an increase in intracavernosal pressure in a dose-related manner. Eisai Pharmaceutical entered E-8010 into phase I clinical trials for ED.

1.2 Erectile dysfunction (ED) and PDE5 inhibitors

1.2.1 Physiology of penile erection

Penile erection is a neurovascular event modulated by psychological factors and hormonal status. On sexual stimulation, nerve impulses cause the release of neurotransmitters from the cavernous nerve terminals and of relaxing factors from the endothelial cells in the penis, resulting in the relaxation of smooth muscle in the arteries and arterioles supplying the erectile tissue and a several fold increase in penile blood flow. At the same time, relaxation of the trabecular smooth muscle increases the compliance of the sinusoids, facilitating rapid filling and expansion of the sinusoidal system (Fig. 1.2). The subtunical venular plexuses are thus compressed between the trabeculae and the tunica albuginea, resulting in almost total occlusion of venous outflow. These events trap the blood within the corpora cavernosa and raise the penis from a dependent position to an erect position, with an intracavernous pressure of approximately 100mm Hg (the phase of full erection).

Figure 1.2 Anatomy and mechanism of penile erection *(Tom et al., 2000)*

During masturbation or sexual intercourse, both of which trigger the bulbocavernous reflex, the ischiocavernous muscles forcefully compress the base of the blood-filled corpora cavernosa and the penis becomes even harder, with an intracavernous pressure reaching several hundred millimeters of mercury (the phase of rigid erection). During this phase, the inflow and outflow of blood temporarily cease *(Lue et al., 1988)*. Detumescence can be the result of a cessation of neurotransmitter release, the breakdown of second messengers by PDEs, or sympathetic discharge during ejaculation. Contraction of the trabecular smooth muscle reopens the venous channels, the trapped blood is expelled, and flaccidity returns.

The cavernous nerves (autonomic), which travel posterolaterally to the prostate, enter the corpora cavernosa and corpus spongiosum to regulate penile blood flow during erection and detumescence. The dorsal nerves (somatic), which are branches of the pudendal nerves, are primarily responsible for penile sensation. The mechanisms of erection and flaccidity are shown in the upper and lower inserts, respectively. During erection, relaxation of the trabecular smooth muscle and vasodilatation of the arterioles results in a severalfold increase in blood flow, which expands the sinusoidal spaces to lengthen and enlarge the penis. The expansion of the sinusoids compresses the subtunical venular plexus against the tunica albuginea. In addition, stretching of the tunica compresses the emissary veins, thus reducing the outflow of blood to a minimum. In the flaccid state, inflow through the constricted and tortuous helicine arteries is minimal, and there is free outflow via the subtunical venular plexus.

1.2.1.1 Neurophysiology of penile erection

The penis is innervated by autonomic and somatic nerves. In the pelvis, the sympathetic and parasympathetic nerves merge to form the cavernous nerves, which enter the corpora cavernosa, corpus spongiosum, and glans penis to regulate blood

flow during erection and detumescence. The somatic component, the pudendal nerve, is responsible for penile sensation and the contraction and relaxation of the extracorporeal striated muscles (bulbocavernous and ischiocavernous).

1.2.1.2 Penile flaccidity

The maintenance of the intracorporeal smooth muscle in a semicontracted state results from three factors: intrinsic myogenic activity, adrenergic neurotransmission, and endothelium-derived contracting factors such as prostaglandin and endothelins.

1.2.1.3 Penile erection

Nitric oxide released during nonadrenergic, noncholinergic neurotransmission and from the endothelium is probably the principal neurotransmitter mediating penile erection. Within the muscle, nitric oxide activates a soluble guanylyl cyclase, which raises the intracellular concentration of cyclic guanosine monophosphate (GMP). Cyclic GMP in turn activates a specific protein kinase, which phosphorylates certain proteins and ion channels, resulting in the opening of potassium channels and hyperpolarization of the muscle-cell membrane, sequestration of intracellular calcium by the endoplasmic reticulum, and blocking of calcium influx by the inhibition of calcium channels. The consequence is a drop in cytosolic calcium concentrations and relaxation of the smooth muscle. During the return to the flaccid state, cyclic GMP is hydrolyzed to GMP by PDE5. Other PDEs are also found in the corpus cavernosum, but they do not appear to have an important role in erection. Communication among smooth-muscle cells takes place through gap junctions in the membranes of adjacent cells, which allow the passage of ions and second messengers to synchronize muscle activity *(Christ et al., 1993)*.

After released from nerve endings and vascular endothelial cells, NO diffuses to

neighboring vascular and trabecular smooth muscle cells and binds to guanylate cyclase. This induces a conformational change in the enzyme and subsequent catalytic production of 3',5'-cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate. The intracellular cGMP and cAMP levels are finely tuned by PDEs *(Beavo et al., 1994)*.

Table 1.4 Classification and common causes of erectile dysfunction.

Category of erectile dysfunction	Common disorders	Pathophysiology
Psychogenic	Performance anxiety, Relationship problems, Psychological stress, Depression	Loss of libido, over inhibition, or impaired nitric oxide release
Neurogenic	Stroke or Alzheimer's disease, Spinal cord injury, Radical pelvic surgery, Diabetic neuropathy, Pelvic injury	Failure to initiate nerve impulse or interrupted neural transmission
Hormonal	Hypogonadism, Hyperprolactinemia	Loss of libido and inadequate nitric oxide release
Vasculogenic (arterial or cavernosal)	Atherosclerosis, Hypertension, Diabetes mellitus, Trauma, Peyronie's disease	Inadequate arterial flow or impaired venoocclusion
Drug-induced	Antihypertensive and antidepressant drugs, Antiandrogens, Alcohol abuse, Cigarette smoking	Central suppression, Decreased libido, Alcoholic neuropathy, Vascular insufficiency
Caused by other systemic diseases and aging	Old age, Diabetes mellitus, Chronic renal failure, Coronary heart disease	Usually multifactorial, resulting in neural and vascular dysfunction

1.2.2 Pathophysiology of erectile dysfunction

Erectile dysfunction can be classified as psychogenic, organic (neurogenic, hormonal, arterial, cavernosal, or drug-induced), or mixed psychogenic and organic (Table 1.4). The last form is the most common.

1.2.2.1 Psychogenic erectile dysfunction

Common causes of psychogenic erectile dysfunction include performance anxiety, a strained relationship, lack of sexual arousability, and overt psychiatric disorders such as depression and schizophrenia. The strong association between depression and erectile dysfunction has been confirmed in two recent studies. In men with schizophrenia, decreased libido is the main problem reported; neuroleptic drugs improve libido but lead to difficulties with erection, orgasm, and sexual satisfaction *(Aizenberg et al., 1995)*.

1.2.2.2 Neurogenic erectile dysfunction

Neurologic disorders such as Parkinson's disease, Alzheimer's disease, stroke, and cerebral trauma often cause erectile dysfunction by decreasing libido or preventing the initiation of an erection. In men with spinal cord injuries, the degree of erectile function depends largely on the nature, location, and extent of the lesion. Sensory involvement of the genitalia is essential to achieve and maintain reflexogenic erection, and this becomes more important as the effect of psychological stimuli abates with age.

1.2.2.3 Hormonal causes of erectile dysfunction

Androgen deficiency decreases nocturnal erections and libido. However, erection in response to visual sexual stimulation is preserved in men with hypogonadism, demonstrating that androgen is not essential for erection. Hyperprolactinemia from any cause results in both reproductive and sexual dysfunction because prolactin inhibits central dopaminergic activity and therefore the secretion of gonadotropin-releasing hormone, resulting in hypogona- dotropic hypogonadism.

1.2.2.4 Vascular causes of erectile dysfunction

Common risk factors associated with generalized penile arterial insufficiency include hypertension, hyperlipidemia, cigarette smoking, diabetes mellitus, and pelvic irradiation. Focal stenosis of the common penile artery most often occurs in men who have sustained blunt pelvic or perineal trauma (e.g., from bicycling accidents). In men with hypertension, erectile function is impaired not by the increased blood pressure itself but by the associated arterial stenotic lesions *(Hsieh et al., 1989)*.

Failure of the veins to close during an erection (veno-occlusive dysfunction) can cause erectile dysfunction. Veno-occlusive dysfunction may be caused by the formation of large venous channels draining the corpora cavernosa, degenerative changes to the tunica albuginea (due to Peyronie's disease, old age, or diabetes mellitus) or traumatic injury (penile fracture), structural alterations of the cavernous smooth muscle and endothelium, poor relaxation of trabecular smooth muscle (in anxious men with excessive adrenergic tone) *(Christ et al., 1990)*, and shunts acquired as a result of operative correction of priapism.

1.2.2.5 Drug-induced erectile dysfunction

Many drugs have been reported to cause erectile dysfunction. Central neurotransmitter pathways, including serotonergic, noradrenergic, and dopaminergic pathways involved in sexual function, may be disturbed by antipsychotic, antidepressant, and centrally acting antihypertensive drugs.

Cigarette smoking may induce vasoconstriction and penile venous leakage because of its contractile effect on the cavernous smooth muscle. Alcohol in small amounts improves erection and increases libido because of its vasodilatory effect and the suppression of anxiety; however, large amounts can cause central sedation, decreased libido, and transient erectile dysfunction. Chronic alcoholism may cause hypogonadism and polyneuropathy, which may affect penile nerve function.

Cimetidine, a histamine H_2-receptor antagonist, has been reported to decrease libido and cause erectile failure; it acts as an antiandrogen and can cause hyperprolactinemia *(Wolfe, 1979)*. Other drugs known to cause erectile dysfunction are estrogens and drugs with antiandrogenic action, such as ketoconazole and cyproterone acetate.

1.2.2.6 Erectile dysfunction due to other systemic diseases and aging

Sexual function progressively declines in healthy aging men. For example, the latent period between sexual stimulation and erection increases, erections are less turgid, ejaculation is less forceful, the ejaculatory volume decreases, and the refractory period between erections lengthens. There is also a decrease in penile sensitivity to tactile stimulation, a decrease in the serum testosterone concentrations *(Kaiser et al., 1988)*, and an increase in cavernous muscle tone.

About 50 percent of men with chronic diabetes mellitus have erectile dysfunction. In addition to affecting small vessels, diabetes may affect the cavernous nerve terminals and endothelial cells, resulting in a deficiency of neurotransmitters. Chronic renal failure has frequently been associated with diminished erectile function, impaired libido, and infertility. The mechanism is probably multi-factorial, involving low serum testosterone concentrations, vascular insufficiency, use of multiple medications, autonomic and somatic neuropathy, and psychological stress. Men with angina, myocardial infarction, or heart failure may have erectile dysfunction due to anxiety, depression, or concomitant penile arterial insufficiency.

1.2.3 The practical therapy of ED before PDE5 inhibitors

In the 1960s and early 1970s it was widely assumed that almost all sexual disorders, including erectile dysfunction (ED), had a psychogenic basis. Therefore sex or behaviour therapies were the only possible therapeutic strategies for ED *(Masters &*

Johnson, 1976). In conclusion, in the early 1970s, there was no drug therapy besides psychogenic and machine aided therapy. However, it is now generally believed that the majority of patients with ED have an underlying organic disorder that contributes, at least in part, to the erectile problem. In other words, this disease originates in complicated reasons, including both organic function and psychogenic factors.

In the 1980s, intracavernosal injections of vasoactive substances and assistant therapy of the vacuum constriction device were progressively introduced. The latter is a safe and effective form of therapy. It essentially consists of a plastic cylinder connected to a vacuum pump that allows a negative pressure to induce erection by increasing corporal blood flow. Erections are thereafter prolonged by a constricting ring applied to the base of the penis. Although vacuum constriction devices might improve erection adequately for sexual intercourse in the majority of cases, they do not meet the expectations of the patient or his partner and therefore the long-term satisfaction rate is not high. Problems with vacuum devices include pain from the constriction ring, lack of spontaneity, decrease in the quality of orgasm and ejaculatory discomfort.

Vasoactive substances act on the tuned pathway of cAMP and cGMP to increase their cellular level. Among agents that increase cGMP levels, NO donors represent the most physiological candidate for the ideal drug. However, despite their effects in vitro, contrasting results have been obtained in vivo by directly injecting NO donors into the corpora cavernosa *(Truss et al., 1994)*; therefore, NO donors are not widely used in the therapy of ED. Similarly inconsistent results were obtained with the intracavernosal administration of neurotransmitters that mediate their effect through an increase in the cAMP-mediated pathway, such as CGRP, VIP and adenosine *(Kilic et al., 1994)*. Conversely, interesting results were obtained by blocking cAMP breakdown using a nonspecific inhibitor of PDE, such as papaverine. Since the first report by Virag in 1982, papaverine have been used extensively because of effectiveness (success rate 55%) and low cost. The usual dosage is 20-80mg. However, important local side effects such as penile fibrosis (6%) and prolonged erection (5%)

limited its popularity.

Up to now PGE1 (alprostadil) has given the best results as a monodose pharmacotherapy for ED. In a 6-month study of intracavernosal self-injection with alprostadil in 683 men, the study participants and their partners reported a satisfactory sexual activity for almost 90% of the injections, with relatively few side effects. The most common was a burning sensation during injection and painful erection, occurring in 15% of patients. In contrast to papaverine, alprostadil injections carry a small risk of prolonged erection and fibrosis at the injection site (1-2%). The general dosage is 5-40μg. The initial testing dose is usually 10mg, although in patients with neurological disease it should be lowered to 5μg. Although the average success rate of alprostadil injection can be as high as 73%, this type of treatment is only rarely chosen as initial therapy, and up to 50% of men eventually discontinue treatment for reasons relating to pain, lack of confidence in self-administration, loss of effectiveness or loss of spontaneity of love-making. Hence the initial enthusiasm for intracavernosal alprostadil was in fact dampened by the number of patients that refused or discontinued therapy. This is the rationale for alternative, local, routes of administration of alprostadil. Although topical gel containing PDE1 is been proposed, its effectiveness needs to be further validated. An interesting alternative route introduced recently was MUSE (medicated urethral system for erection). It is based on the ability of alprostadil to diffuse from the corpus spongiosum of the urethra to the corpora cavernosa via vascular interconnections. Alprostadil is deposited in the urethra through a syringe-like device containing a pellet of the vasoactive substance. In placebo controlled studies, nearly 60% of the patient achieved efficient erections*(Padma-Nathan et al.,1997)*. Another study reported a lower success rate. The reported risk of fibrosis and priapism is very low; however, some patients are reluctant to accept this treatment because of burning sensation at the site of applicator insertion. Among the oral agents currently available, sildenafil represents the cornerstone for ED therapy.

1.2.4 Treatment of erectile dysfunction with sildenafil

Sildenafil is a selective inhibitor of PDE5, which inactivates cyclic GMP. In human corpus cavernosum the presence of several members of PDE family have been demonstrated, including the cAMP/cGMP non-selective PDE2, the cAMP-selective PDE2 and PDE4 and cGMP-selective PDE5. Sildenafil analogues are selective inhibitors of PDE5. They induced penile erection by inhibition of hydrolysis of cGMP, which tunes NO-cGMP signal transduction (see Figure 1.3) *(Zhang et al.,2004)*.

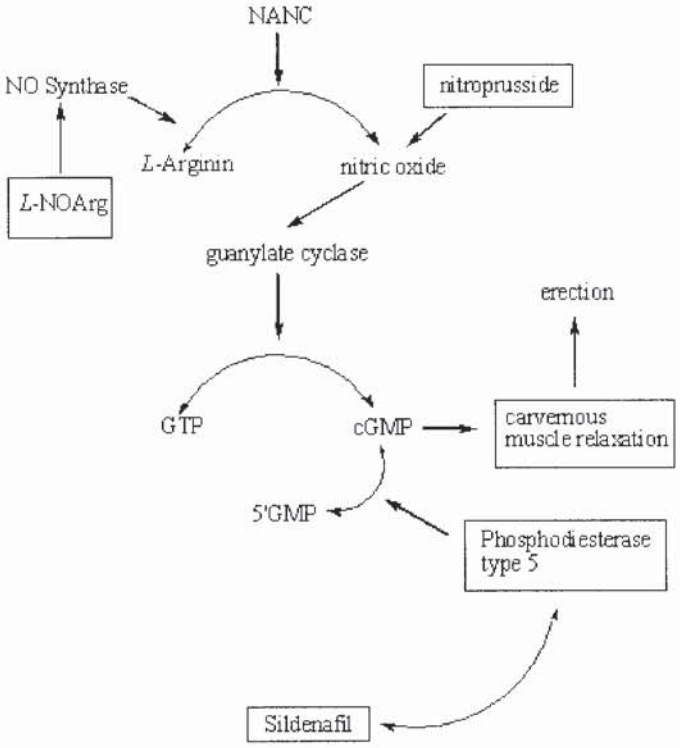


Figure 1.3 Activation mechanism of sildenafil in treating erectile dysfunction

Since its release in March 1998, sildenafil has become the drug of choice for most men with erectile dysfunction. When sexual stimulation releases nitric oxide into the penile smooth muscle, inhibition of PDE5 by sildenafil causes a marked elevation of cyclic GMP concentrations in the glans & penis, corpus cavernosum, and corpus spongiosum, resulting in increased smooth-muscle relaxation and better erection. Sildenafil has no effect on the penis in the absence of sexual stimulation, when the concentrations of nitric oxide and cyclic GMP are low.

Sildenafil has been evaluated in 21 clinical trials of up to six months' duration in over 3000 men and in 10 open-label extension studies. In most of the men in these studies, erectile dysfunction was caused by organic or combined organic and psychogenic factors. The results were based mainly on the reports of the men (and sometimes the partners) and scores on the International Index of Erectile Function. This index is a validated questionnaire with five domains: erectile function (six questions), orgasmic function (two questions), sexual desire (two questions), satisfaction with intercourse (three questions), and overall sexual satisfaction (two questions). In these studies, the number of erections and the rates of penile rigidity, orgasmic function, and overall sexual satisfaction were significantly higher with sildenafil than with placebo *(Goldstein et al., 1998)*. However, sildenafil had little effect on libido.

Among more than 3700 men with 1631 patient years of exposure to sildenafil, most adverse events were mild to moderate and self-limited in duration. Among men taking 25 to 100 mg of sildenafil, 16 percent reported headache, 10 percent flushing, 7 percent dyspepsia, 4 percent nasal congestion, and 3 percent abnormal vision (described as a mild and transient colour tinge or increased sensitivity to light). Among men taking 100 mg of sildenafil, these side-effects occurred as high as two-fold comparing with men who were taking lower doses. The visual effect is probably related to inhibition of PDE6 in the retina. No chronic visual impairment has been reported, and the incidence of visual side effects was similar in diabetic and nondiabetic men. Nevertheless, because of the short duration of the clinical trials and the difficulty in detecting subtle retinal changes, the long-term safety of sildenafil treatment is still unknown. In men with retinal diseases, an ophthalmologic consultation may be warranted before sildenafil treatment is initiated.

Adverse cardiovascular events (nasal congestion, headache, and flushing) were mild and transient in the majority of men. The rate of serious cardiovascular events (angina and coronary-artery disorder) was 4.1 per 100 man-years of treatment among those

taking sildenafil and 5.7 per 100 man-years for those taking placebo. The rates of myocardial infarction were 1.7 and 1.4 per 100 man-years for the sildenafil and placebo groups, respectively. However, because most of the studies excluded men taking nitrates and those with concomitant medical conditions, the incidence of serious cardiovascular events could be expected to be higher in the general population. From late March to mid-November 1998, more than 6 million outpatient prescriptions of sildenafil were dispensed (about 50 million tablets) to more than 3 million men. During the same period, 130 deaths associated with sildenafil therapy were reported to the Food and Drug Administration (FDA).

Sexual activity was thought to be a likely contributor to myocardial infarction in only 0.9 percent of 858 men in one study. Thus, the absolute increase in risk caused by sexual activity is low (one chance in a million for a healthy man). According to data from the National Centre for Health Statistics and the Framingham Heart Study, the rate of death from myocardial infarction or stroke for men in the age range in which erectile dysfunction is common is approximately 170 per million men per week. Therefore, it appears that sildenafil therapy is safe for most men. Nevertheless, given that most of the men who died had underlying cardiovascular syndrome; cardiovascular status should be carefully assessed before treatment. The combination of nitrates and sildenafil has resulted in severe hypotension and 16 deaths in the United States. Therefore, nitrate therapy is an absolute contraindication to sildenafil therapy. In response to the concern of physicians, the American Heart Association has published a guideline for sildenafil therapy *(Cheitlin et al., 1999)*.

Sildenafil is absorbed well during fasting, and the plasma concentrations are maximal within 30 to 120 minutes (mean, 60). It is eliminated predominantly by hepatic metabolism, and the terminal half-life is about four hours. The recommended starting dose is 50 mg taken one hour before sexual activity. The maximal recommended frequency is once per day. On the basis of effectiveness and side effects, the dose may be increased to 100 mg or decreased to 25 mg.

1.3 Discovery and development of sildenafil type of PDE5 Inhibitors

1.3.1 The origin of sildenafil

The design of sildenafil is based on the research of zaprinast (Fig. 1.4, I-1). Originally, it was designed to selectively inhibit PDE in order to treat hypertension and angina. During the clinical trial of this case, it was not expectably notable in therapy result. However, the effect of penile erection was significant. Therefore, people devoted their attention to its clinical function of therapy ED. At first, people were not confident of its clinical application. After a period, it was found many men attended clinical trial couldn't be living normal without sildenafil. Even after the clinical experiment, Pfizer had to supply sildenafil to them. Till that time, the confidence of sildenafil had been increasing. Based on the drug design and lead compound modification and evolution, the design and development of sildenafil is worth being studied.

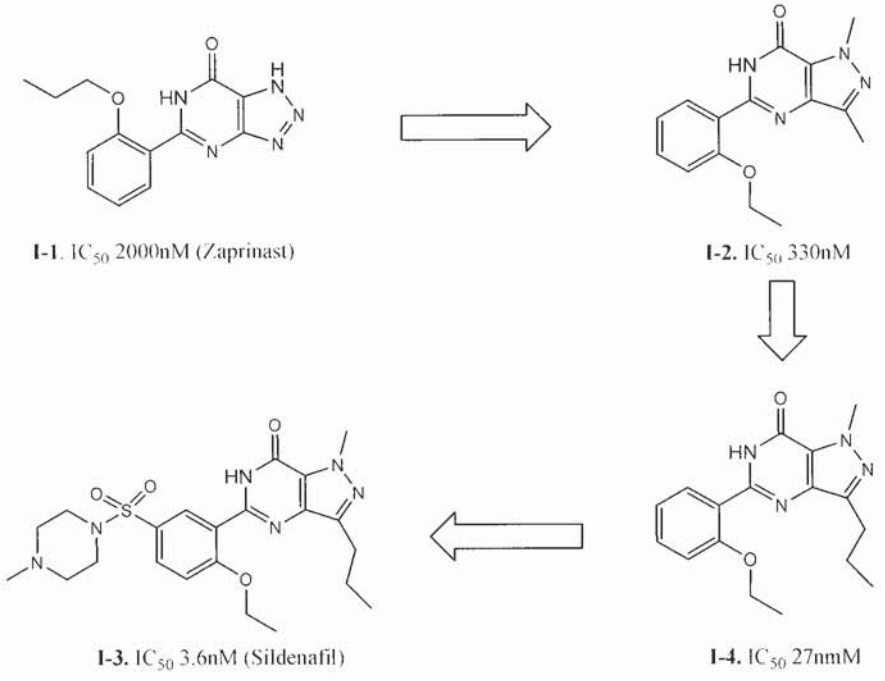


Figure 1.4 The research and development process of sildenafil

Zaprinast (**I-1**; IC_{50}=2200nM), a kind of antianaphylaxis substance, has weak activity to PDE5, moderate affinity to cGMP hydrolase and no specificity to PDE1. Pfizer scientists found pyrazolo[4,3-d]pyramidine-7-one (**I-2**; IC_{50}=330nM) analogues show potent inhibition activity to PDE5, after screening a series of five fused six-membered heterocyclic derivatives.

Computer analysis has shown that pyrazolo[4,3-d]pyramidine-7-one (**I-2**) were very similar with guanosine cGMP in size and dipole-dipole distance. Based on this, Pfizer scientists considered that the substituent of 3'-position could act as ribose part when it binds to enzyme. At the same time, the substituent of 5'-position could act as phosphate part when it binds to enzyme. It proved that when 3-methyl was substituted by propyl, the activity to PDE5 was dramatic increased (**I-3**; IC_{50}=27nM). As to 5'-substituent, Pfizer scientists believed a polar group should be introduced. On one hand the structure is more similar to phosphate, on the other hand the water solubility could be increased. Therefore, sulfonylamino was chosen. Later experiment result proves the completely accurate of this design. The obtained compound, sildenafil (Fig. 1.4 **I-4**; IC_{50}=3.6nM), showed an increased affinity to enzyme as well as a greatly improved solubility.

Afterwards, the substituents on sildenafil were modified and screened. Although the activities and specificities have some improvements, all the new derivatives were no much better than sildenafil in aggregative index number.

1.3.2 The other PDE5 inhibitors of sildenafil analogues

Following the discovery of sildenafil, many pharmaceutical companies focused on nucleotide derivatives with five- and six-member fused heterocyclic compounds in order to develop more advanced sexual dysfunction therapy drugs. Bayer developed and marketed another PDE5 inhibitor vardenafil. The structure of vardenafil is very

similar to sildenafil, except the position of N atoms on the nucleus (Fig. 1.5 **I-5**). The activation mechanisms of them are same.

Other pharmaceutical companies also joined this competition. Taking advantage of the defect of the patent of sildenafil, DONG Pharmaceutical Company of Korea developed monosulfonyl substituents of sildenafil (Fig. 1.5 **I-6**).

SK Chemical Company (Korea) also developed a series of amido substituted derivatives of sildenafil (Fig. 1.5 **I-7**). However, this series of compounds had little specific selectivity to PDE5 and PDE6.

GlaxoSmithKline designed and synthesized pyrazolo[3,4-d]pyramid analogues (Fig. 1.5 **I-8**) and investigated their inhibition activities to PDE5 *(Dumaitre &. Dodic, 1996).*

Starting from the structure of xanthine, Novartis designed and synthesized a series of compounds *(Ruth et al., 2002)*. The activity to PDE5 of most of Navartis compounds was increased, but the selectivity of PDE5 to PDE6 is decreased. The compound with the highest selectivity is **I-9** in Fig. 1.5, the inhibition to PDE5 is IC_{50}=16nM, and its selectivity of PDE5/PDE6 is 72.

Schering-Plough also designed and synthesized a series of xanthine compounds (Fig. 1.5 **I-10**). The effect is similar to sildenafil and the selectivity of PDE5/PDE6 was increased. This kind of compound can be rapidly absorbed and cleared *(Wang et al., 2002).*

Frenchmen synthesized a series of pyrazolo[3,4-b]pyrazine analogues (Fig. 1.5 **I-11**). The activity of these analogues was 10 times lower than sildenafil *(Marina et al.,2001).*

Tanabe Seiyaku of Japan obtained a series of PDE5 inhibitors (Fig. 1.5 **I-12**) based on the modification of PDE4 inhibitor compounds with 1-phenyl substitute naphthalene centre *(Ukita et al., 1999)*.

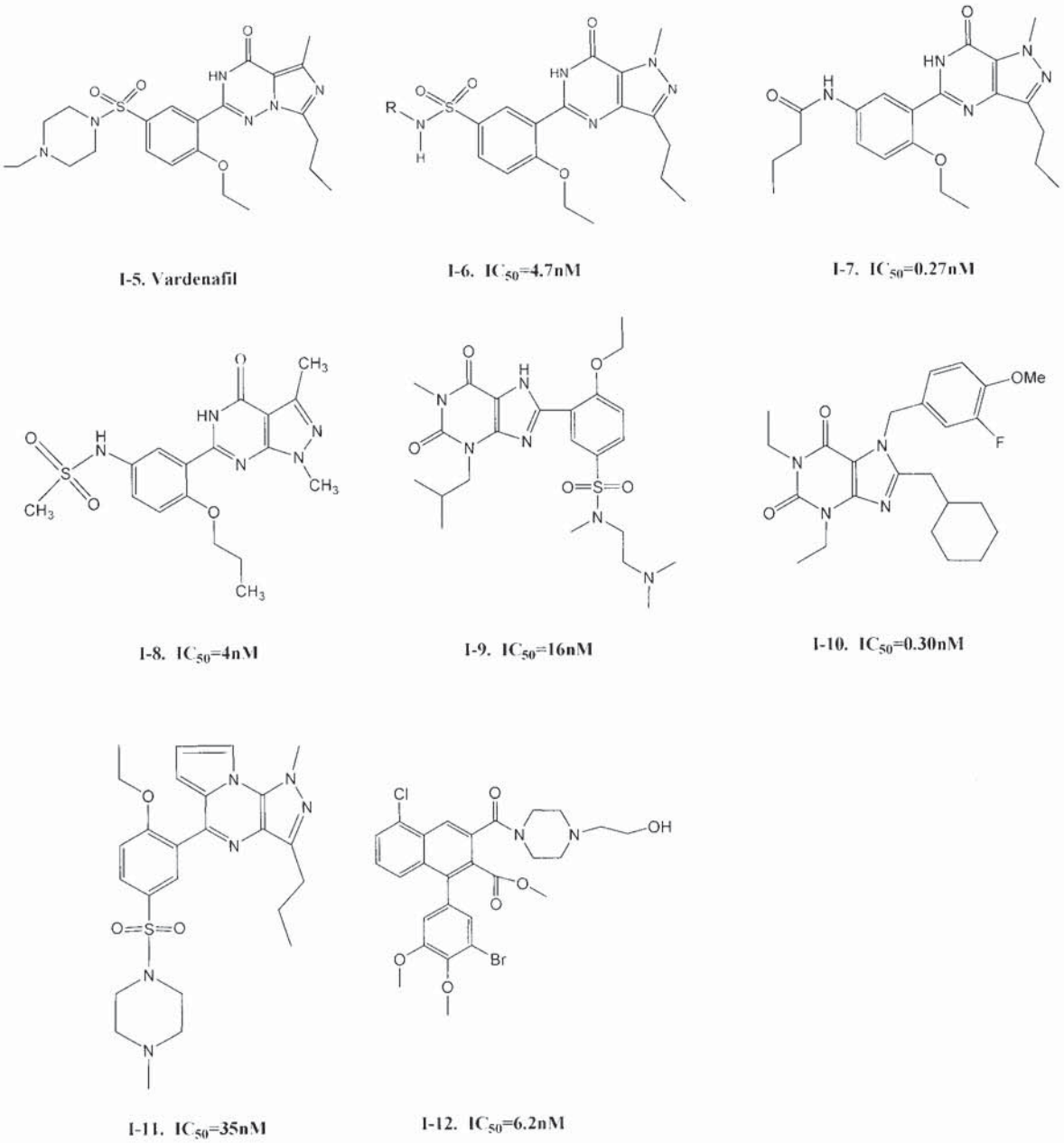


Figure 1.5 PDE5 inhibitors of sildenafil analogues

Based on the analysis of cGMP conformation, they designed and synthesized some new derivatives (Fig. 1.6 **I-16**) which had similar structure with compound **I-13** *(Ukita et al., 2001)*. PDE5/PDE6 activity ratio of **I-16** (T-1032) is 28. T-1032 has been

selected to do more physiological and pharmacokinetic research.

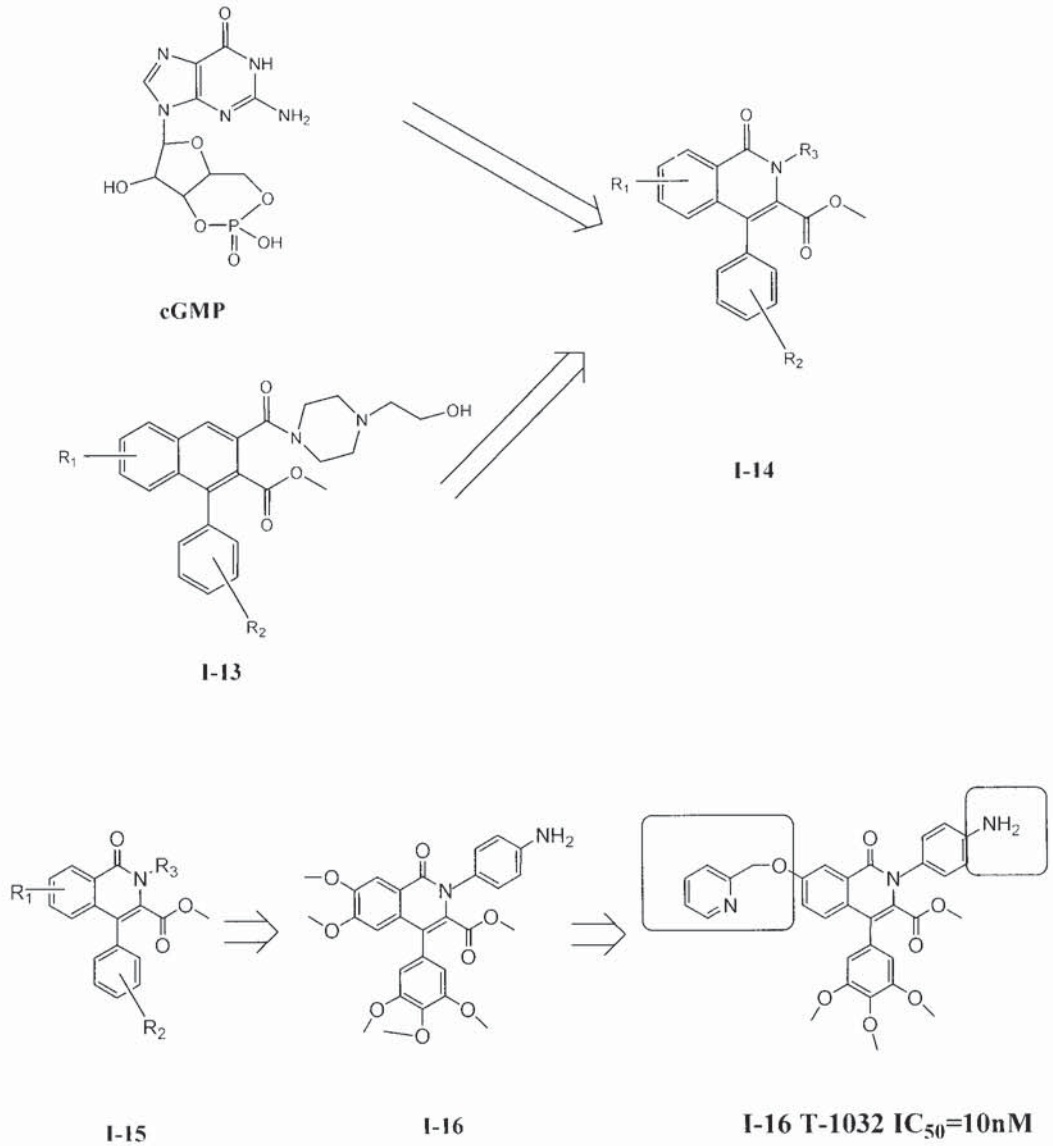
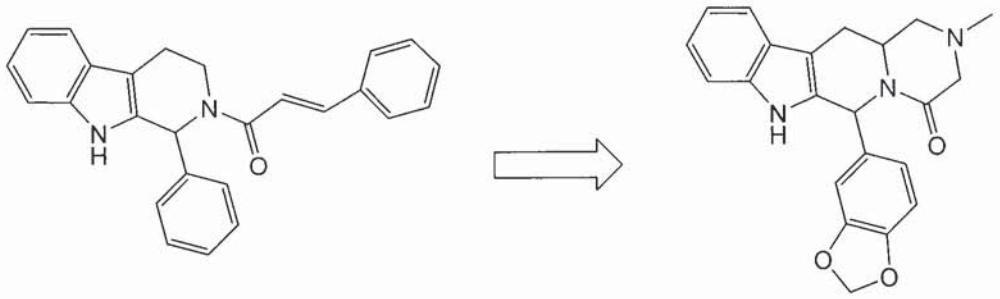


Figure 1.6 Evolvement process of I-16 (T-1032) from I-13

1.3.3 Carboline derivatives and their tricyclic derivatives---tadalafil

The development of tadalafil (Fig. 1.7 **I-18**) also experienced a hard process. At first, it was designed for hypertension and angina therapy. In 1997, GlaxoSmithKline bought its development right and began to investigate the therapeutic effectiveness of treatment to sexual dysfunction. Later, GlaxoSmithKline gave up its right in 1998. Eli

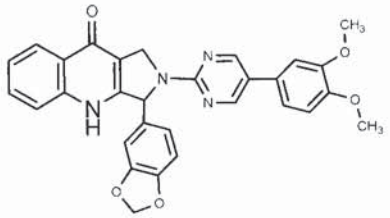
Lilly bought in the right in the following year to continue research and produced today's tadalafil. Tadalafil have good selectivity and rapid effect. However, specialists remind that because of the different structure of sildenafil and vardenafil, their pharmacokinetic activity and safety are demanded long-term observation. In addition, tadalafil also act on PDE11, which is distributing in many important organs such as heart, brain, spermary and pituitary. Therefore, the possible clinical side effects are required to be watched. The effect time of tadalafil is 30-120 min, but the activation can be durative examined till 24 hours. In old men groups, the drug can be examined even after 6 days. The long actuation duration and slow excretion may bring unpredictable effect to the body. So it needs long-term examination for getting the final conclusion. In the clinical experiment, the drug withdrawal of tadalafil is a very long duration. It is 7% in 10mg dosage's experiment, 10% in 25mg, 19% in 50mg and 29% in 100mg.



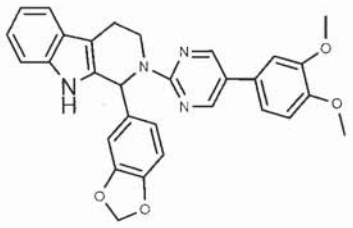
I-17 β-carboline **I-18 Tadalafil**

Figure 1.7 Structure evolution of PDE5 inhibitor from carboline to tadalafil

Johnson & Johnson Co. Ltd. worked hard on developing sexual dysfunction therapy drugs based on β-carboline as a lead compound (Fig. 1.8). At first, they want to keep the 6-5-6 tricycle structure of β-carboline. However, the rearrangement side product with 6-6-5 tricycle structure was discovered by accident during synthesis of β-carboline derivatives. Structural analysis show 6-6-5 tricycle structure has potential activity. Therefore, they synthesized two serious of compounds *(Sui et al,.2003)*. Unfortunately, although these compounds have similar enzyme inhibition activity in *vitro*, but less in *vivo* activity, none of them can be selected to further research.



I-19



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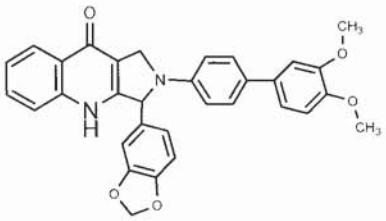

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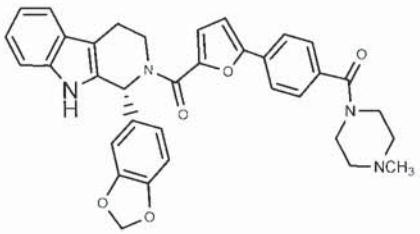


I-22

6-6-5 tricycle structure

6-5-6 tricycle structure

Figure 1.8 PDE5 inhibitors of carboline derivatives and their tricyclic derivatives

Bristol-Myers Squibb developed another tricycle structural compound (Fig. 1.9 **I-23**). In vivo and in vitro experiments of this compound showed a similar activity to sildenafil. Bristol-Myers Squibb had progressed this compound into clinical trial. In clinical trial, the healthy people can bear 50mg dosage.

The development of this kind of compound experiences such a complex process, which we can learn from it. The synthesis of piperidine derivatives was based on the leading compound M-5445 (**I-25**), which is PDE active compound. It was found that benzyl could be substituted by penta-heterocycle, as a result, 5-6 fused heterocycle was obtained. From the structural analysis (**I-27**), it can be clearly seen that the hydrogen bond formed between H atom of imido group and O atom of carbonyl group increased the selectivity to PDE5. Based on this, compound **I-23** was designed and synthesized *(Yu et al., 2003)*.

Bristol-Myers Squibb screened a new tricycle system. This tricycle system can be seen as a phenyl was inserted between 5 and 6 cycles of sildenafil (Fig. 1.9 **I-29&I-30**). This kind of analogues has a higher inhibition activity to PDE5 than sildenafil and same selectivity *(David et al., 2000)*. No more reports have been seen.

Bristol-Myers Squibb also presented third tricycle system (Fig. 1.9 **I-24**) and showed their inhibition activity to PDE5 is better than sildenafil, but the selectivity to PDE5 was a little improved *(Bi et al., 2001)*.

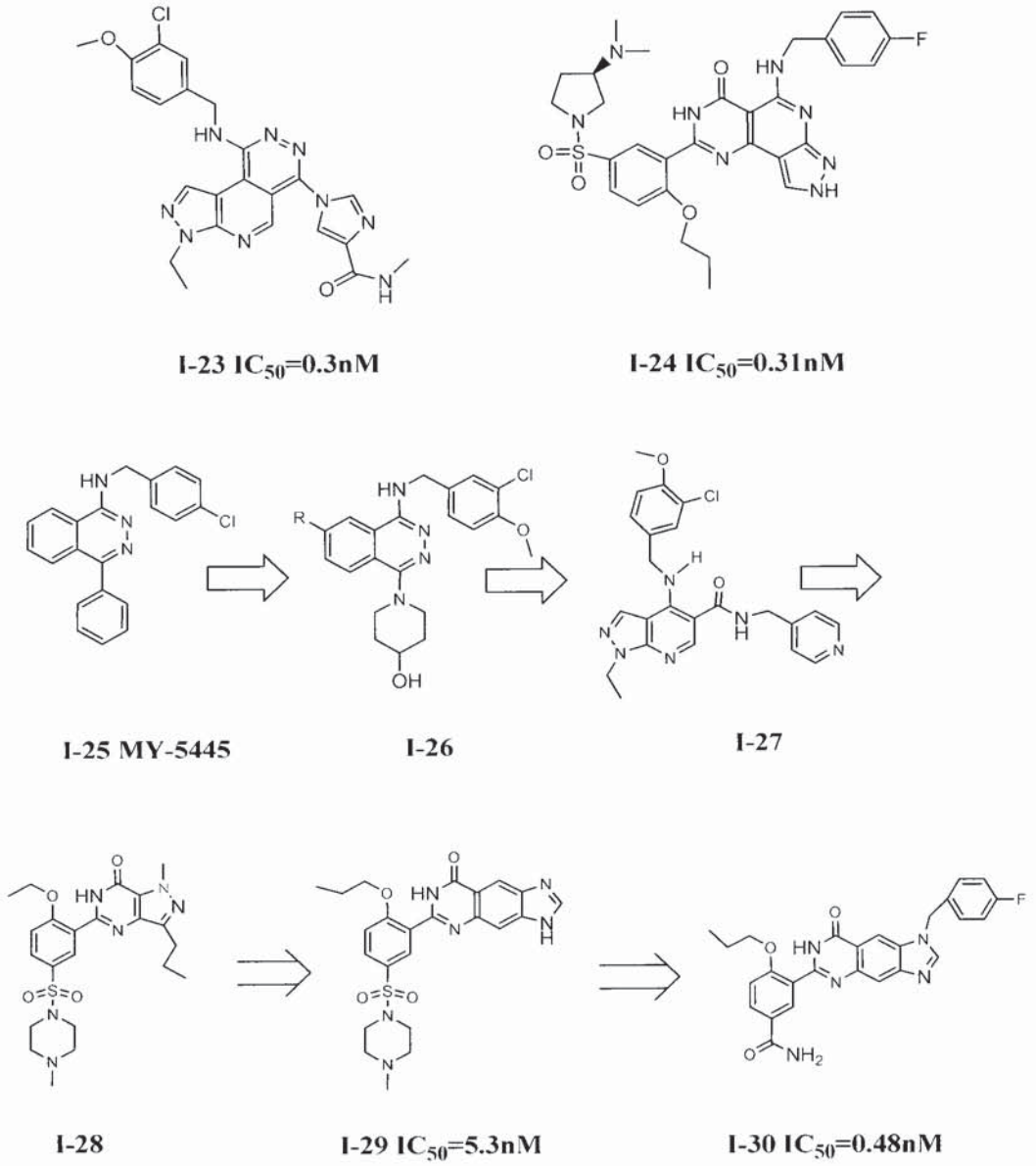


Figure 1.9 PDE5 inhibitors with tricycle centre developed by American B-M Squibb

1.4 Comparison of three primary PDE5 inhibitors: sildenafil, vardenafil and tadalafil

1.4.1 Onset of effect and duration of effect

In humans, the mean onset of effect of sildenafil takes place approximately 27 minutes after ingestion; in the rabbit vardenafil acts approximately 20 minutes afterwards and the maximum vardenafil effect takes place after 45 to 90 minutes. An initial onset of action 16 minutes after ingestion has been described for tadalafil in a highly pre-selected population; for sildenafil, efficacy has been demonstrated only 12 minutes after ingestion. Peak plasma tadalafil levels are reached after two hours on average. In one study of tadalafil (25 mg) in 61 men, adequate efficacy was found in the majority of patients after 30 to 120 min. However, 24 h after ingestion of tadalafil (10 and 20 mg respectively) some effect was still apparent *(Padma-Nathan et al., 2001)*.

1.4.2 Efficacy

According to the study results available to date, all three PDE-5 inhibitors are effective. Owing to the lack of direct comparative studies, any comparison of the clinical effect of the substances relies on the comparison of different, not directly comparable studies, which is difficult because the target criteria and patient selection criteria are not uniform. In some cases, sildenafil non-responders were excluded from studies with vardenafil *(Porst et al., 2001)* and tadalafil, which makes it practically impossible to compare response rates. For this reason, a sensible comparison criterion would appear to be a change in erectile function versus the baseline value in comparison with placebo, which was recorded uniformly in all of the studies using the

IIEF (International Index of Erectile Function) questionnaire. Treatment with vardenafil in a dose of 20 mg produced an improvement in the ability to achieve an erection in 80 % of ED patients *(Porst et al., 2001)*. In a comparable study of sildenafil (100 mg dose), 84 % of ED patients were successfully treated. Treatment with tadalafil 25 mg produced an improvement in the ability to achieve an erection in 81 % of ED patients. Therefore, sildenafil, at 84 %, is slightly more effective than vardenafil at 80 % and tadalafil at 81 %. If efficacy is compared versus placebo, the differences between sildenafil and the two other substances are even clearer. In comparison with placebo, treatment with 100 mg sildenafil leads to a 20-fold improvement in IIEF question 3 (when you attempted sexual intercourse, how often were you able to penetrate your partner?), treatment with 20 mg vardenafil to a 7.5-fold improvement, and treatment with 25 mg tadalafil to a 1.4-fold improvement.

1.4.3 Tolerance

Typical side effects of PDE-5 inhibitors are headache, facial flushing, nasal congestion, and dyspepsia. According to the current publication situation, desired and undesired effects can be assumed to be similarly frequent, similarly severe and similarly dose-dependent for all three PDE-5 inhibitors. As yet, the published data on the two more recent substances remain insufficient to reach a conclusive assessment of the adverse effects. In particular, there is a lack of large, double-blind randomised comparative studies. It would be particularly interesting to establish whether the relatively long elimination half-life of tadalafil or the low bioavailability of vardenafil is associated with a greater number of adverse effects. Both sildenafil and vardenafil weakly inhibit PDE-6. Changes in vision have been rarely described for both substances in relatively high dosages. Experience with sildenafil has shown that only a few patients discontinue treatment for this reason. Long-term studies with sildenafil have produced no evidence of more extensive or permanent disturbances of the visual system as a result of occasional PDE-6 inhibition. Similar data is not yet available for

vardenafil. In the case of sildenafil, patients with retinitis pigmentosa, a rare hereditary disease, are excluded from treatment for drug safety reasons. Tadalafil is a potent PDE-11 inhibitor and PDE11 are found, amongst other places, in the smooth muscles of the internal organs, cardiac and skeletal muscles, pituitary gland, Leydig's cells and germ cells in the testes. The physiological significance of the enzyme and the possible consequences of its short-term or medium-term inhibition have not yet been established. The back and muscle pain reported relatively frequently with tadalafil may be associated with this. Tolerability of tadalafil is problematic: with daily ingestion, 7 % of patients in the 10 mg group discontinued treatment owing to side effects, 10 % at 25 mg, 19 % at 50 mg and 29 % at 100 mg. With ingestion on demand, tadalafil caused muscle and back pain in over 10 % of those treated and dyspepsia and headache in over 25 %.

1.4.4 Interactions

All PDE-5 inhibitors act in a similar way via the NO/cGMP mechanism described at the beginning. For this reason, tadalafil and vardenafil can be assumed to potentiate the hypotensive and anticoagulant effect of nitrates and NO donors, as is already known to occur with sildenafil. Vardenafil and tadalafil potentiate the vasodilatory effect of NO donors. In patients who are being treated with them, none of the three PDE-5 inhibitors is advisable. For sildenafil detailed information is published in the Standard Product Characteristics (EU-SPC Viagra). Since all 3 substances are broken down mainly via cytochrome P450 CYP3A4, a dose adjustment should be considered when given in combination with CYP3A4 inhibitors (e.g. HIV protease inhibitors, erythromycin, ketoconazole).

1.4.5 High-risk groups

Like sildenafil, vardenafil has a slightly hypotensive effect, maximal 5-10 mmHg

average. An increase in the heart rate has been described at a vardenafil dose of 40 mg. Since peak vardenafil levels are 30 % higher in elderly patients and the half-life is 25% longer, low dosages should first be used in this patient group. At the high dose of 20 mg vardenafil, adverse effects were reported twice as frequently in elderly patients with 63%. Tadalafil, too, exhibits a 28% longer half-life, i.e. 22 hours, in elderly patients. In elderly men, the substance was still detectable 6 days after ingestion. Impaired hepatic and renal function also produced an additional lengthening of the half-life of the substance. Furthermore, smoking and the body mass index had a weak effect on the pharmacokinetics of tadalafil whereas food intake had no effect. Both diabetes mellitus and sexual intercourse are associated with an increased risk to get a cardiovascular event. For vardenafil serious adverse events have been reported in diabetes patients (placebo: 1%, 10 mg: 2%, 20 mg: 3%). The availability of the new PDE5 inhibitors has led to an intensive discussion of the differences in efficacy and the side effect rate in comparison to sildenafil, particularly with respect to the frequency of any cardiovascular events. In the first phase of the Prescription Event Monitoring study, it became clear that myocardial infarctions and deaths as a result of coronary heart disease were not observed more frequently in sildenafil users than in the general population, even in widespread use - when prescribed by doctors in general practice without any formal inclusion and exclusion criteria. Age standardised mortality and morbidity rates produce no evidence of an increased risk of myocardial infarction or deaths as a result of coronary heart disease in sildenafil patients. In a FDA publication, a conclusive evaluation of spontaneous reports of deaths associated with sildenafil was undertaken. The authority came to the conclusion that no evidence of an increased mortality rate among sildenafil users compared to the general population could be deduced from the spontaneous reports. In fact, fewer deaths associated in time with the ingestion of sildenafil were reported than might have been expected purely statistically on the basis of the normal mortality rate for men in this age group.

1.4.6 Contraindications

For all three substances contraindications are similar. Patients whose sexual activity is not advisable on medical reasons (e.g. patients with severe cardiovascular disease) should not be given ED treatment. In some circumstances, abstaining from sexual activity may save the lives of these patients - even though they have to forego some of the pleasure of sexual activity. For the same reason, patients who have recently experienced a heart attack or stroke should be excluded from ED treatment. In patients receiving nitrate or NO-donors treatment with PDE-5 inhibitors is contraindicated. Since both sildenafil and vardenafil have moderate vasodilatory and hypotensive effects, they should not be given in the presence of marked arterial or orthostatic hypotension, and should only be administered with caution in aortic stenosis or hypertrophic obstructive cardiomyopathy. Further studies are needed to clarify whether tadalafil affects the circulatory system less owing to its slower pharmacokinetics. Patients with retinitis pigmentosa should not be treated with a PDE-5 inhibitor. Patients receiving nitrate or NO-donor treatment, and those who have experienced significant cardiac events in the previous six months, suffer from proliferative retinopathy or retinitis pigmentosa have been excluded from studies with vardenafil, something which is scientifically logical and ethically correct. Even more caution has been exercised in studies with tadalafil - all patients with signs of clinically relevant liver, kidney or coronary artery disease, cardiovascular disease or CNS disturbances in the last six months have been excluded. This is to be acknowledged as morally and ethically positive, but it does make it more difficult to compare the new substances with sildenafil, which has been studied in considerably wider-ranging patient populations. There are sufficient data on sildenafil to confirm that it does not lead to an increase in the mortality rate compared with the general population. It is not possible to comment on vardenafil and tadalafil in this regard, owing to the lack of study data.

The all comparisons of these three compounds are listed in Table 1.5.

Table 1.5 Comparison of phosphodiesterase 5 (PDE5) inhibitors

	Viagra (sildenafil)	Cialis (tadalafil)	Nuviva (vardenafil)
Manufacturer	Pfizer	Lilly ICOS	Bayer/GlaxoSmithKline
Status	Marketed (1998)	Marketed (2005)	Marketed (2007)
Duration	4 hours	36 hours	4 hours
Onset	30–60 minutes	16 minutes	40 minutes
Food interaction	Less efficacious after consumption of certain meals	None	No data reported
Side effects	Headache, indigestion, blue-tinged vision and facial flushing	Headache and indigestion	Headache, facial flushing and indigestion
Selectivity for PDE5 compared with:			
PDE1	>80	>10,000	>200
PDE2	>1,000	>10,000	>14,000
PDE3	4,000	>10,000	>3,000
PDE4	>1,000	>10,000	>5,000
PDE6	9	780	>200
PDE7,8,9,10	No data reported	>10,000	No data reported

1.5 Design PDE5 inhibitors with 3H-imidazo[1,5-a][1,3,5] triazin-4-one and 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one nuclei

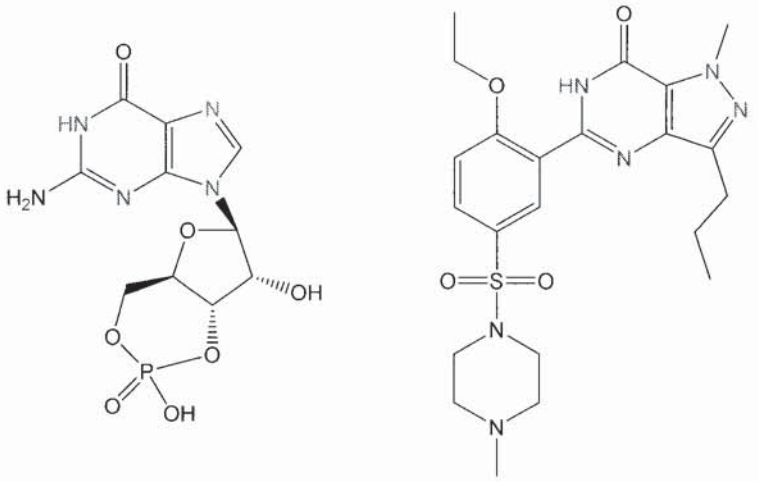
As described above, each of three currently available drugs for ED has variable side effects, the present project aims to design, synthesize and develop a group of new compounds with better selectivities among PDEs to overcome the side effects, particularly vision side effect of sildenafil.

1.5.1 Importance of the fused six-member with five-member heterocyclic ring nucleus in PDE5 inhibitors

PDE5 is a cGMP-binding cGMP-specific PDE. Interaction of cGMP or a PDE5 inhibitor at the catalytic site of PDE5 stimulates cGMP binding at its allosteric sites, which leads to phosphorylation of PDE5 regulatory domain by PKG. Phosphorylation of PDE5 increases the affinity of the catalytic site and the allosteric binding sites for cGMP. These changes result in negative feedback regulation of cGMP. These effects also imply that by elevating cGMP, PDE5 inhibitor increases its own binding affinity, thus providing positive feedback for effectiveness of the drug.

PDE5 inhibitors are structurally similar to cGMP and compete with cGMP at the catalytic site of PDE5 (Fig. 1.10). Elevation of cGMP by PDE5 inhibitors occurs secondary to reduced cGMP degradation by PDE5 when cGMP synthesis is concomitantly increased. This is the rationale for pharmacologic inhibition of PDE5 as a therapeutic approach for inducing penile erection in men with ED.

We know the chemical structure of sildenafil was evolved from cGMP, so the centres of these two compounds are a five- and a six-membered ring fused heterocyclics, in another word guanine and its derivatives (Fig. 1.10, drawn with red line). Sildenafil and cGMP are also similar in molecular size and dipole-dipole distance, so they have homothetic binding activities and both stimulate the phosphorylation of PDE5.



cGMP **Sildenafil**

Figure 1.10 The structures of sildenafil and cyclic guanosine monophosphate (cGMP)

To compare the reported PDE5 inhibitors, we found that most of them have a five- and a six-membered ring fused heterocycle guanine nucleus (Fig. 1.11). The differences, for most of them, lie in numbers of nitrogen (N) atoms and positions of N atoms in rings. The heterocyclic nuclei of these compounds are very similar to cGMP molecule. This genuine structural comparability suggests that a five- and a six-membered ring fused heterocyclic centre is one of the essential chemical fragments for a PDE5 inhibitor. In some of new developed PDE inhibitors, this chemical fragment can still be found though they have become a three even a four fused heterocyclic central structure.

Therefore, we decided to start with a five- and a six-membered fused guanine derivative as our PDE inhibitor heterocyclic centre to look for higher activity and selectivity.

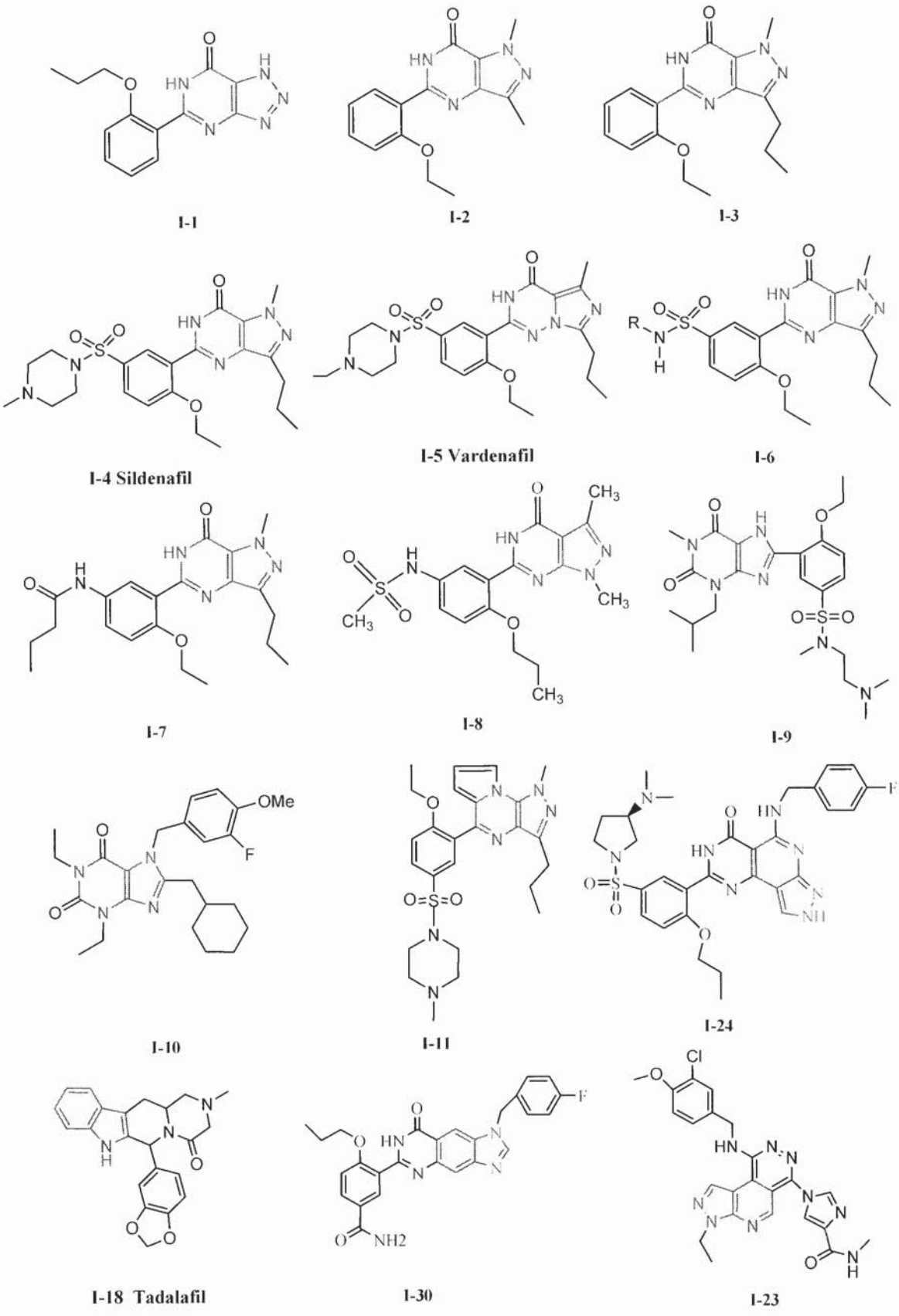
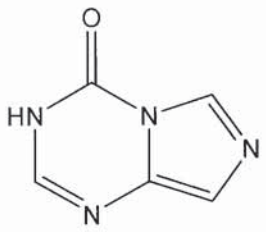


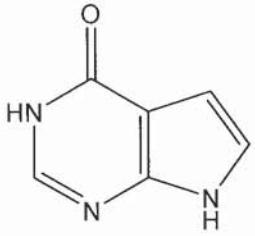
Figure 1.11 The PDE5 inhibitors with 5- and 6-member fused heterocycle (red lines)

1.5.2 Design PDE5 inhibitors with 3H-imidazo[1,5-a][1,3,5] triazin-4-one and 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one nucleus

In the process of literature searching and structural comparison, we found two guanine derivatives had potentials for the development as new PDE5 inhibitors. They are imidazo[1,5*a*]-[1,3,5]-triazine-4(3*H*)-one (Fig. 1.12 **I-31**), and 3H-pyrrolo[2,3-d]-pyrimidin-4(7H)-one (Fig. 1.12 **I-32**). In the six-membered rings of these derivatives, the guanine features are retained and in the five-membered rings of them, well located functional atoms allow a minimum structure modification to generate groups of structurally diversified derivatives for biological evaluations.



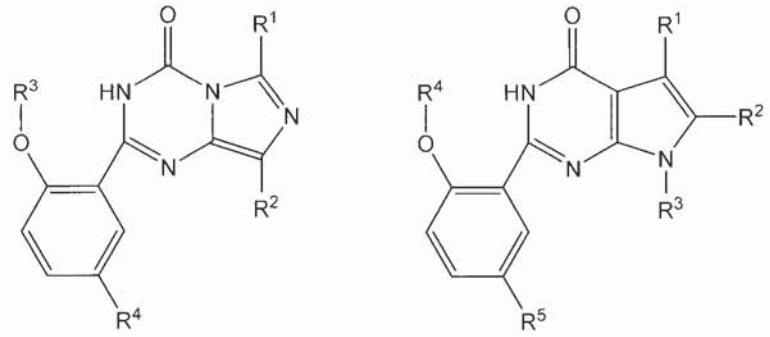
imidazo[1,5*a*]-[1,3,5]-triazine-4(3*H*)-one(I-31)



3*H*-pyrrolo[2,3-*d*]-pyrimidin-4(7*H*)-one(I-32)

Figure 1.12 The selected active nuclei with 5- and 6-member fused heterocycles.

Taken successful stories of sildenafil and vardenafil into account, we felt better to start with 2-substituted phenyl-6,8-dialkyl-3*H*-imidazo[1,5-a]-[1,3,5]-triazine-4-one (Fig. 1.13 **I-33**), which has a centre of imidazo[1,5*a*]-[1,3,5]-triazine-4(3*H*)-one, and 2-substisuted phenyl-5,7-dialkyl-3,7-2*H*-pyrrolo[2,3-d]pyrimidine-4-one (Fig. 1.13 **I-34**), which has a centre of 3H-pyrrolo[2,3-d]-pyrimidin-4(7H)-one.



I-33 **I-34**

Figure 1.13 The molecular structures of two series of new PDE5 inhibitors with two new active heterocyclic centres.

Chapter 2

Synthesis of PDE5 Inhibitors of 2-(substituted-sulfonylphenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-ones

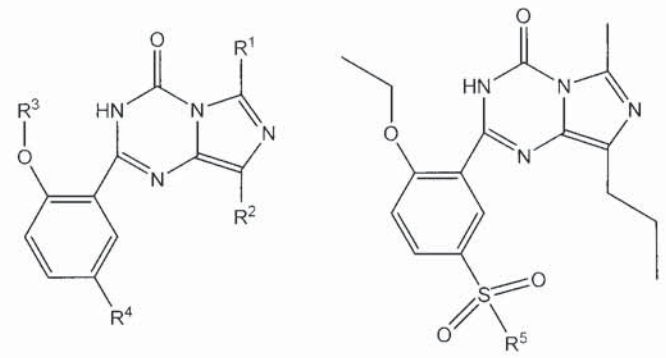
2.1 Introduction

The relaxation of smooth muscle is caused by cGMP, consequently resulting in the penile erectile. Phosphodiesterase 5 could deactivate cGMP by degradation the cyclic nucleotide and result in erectile dysfunction. Therefore, a synthesis PDE5 inhibitor could become a drug for treatment of ED. In this project, we focused on development of new compounds as PDE5 inhibitors to overcome erectile dysfunction. As described in the previous chapter we had designed two groups of compounds as our targeted candidates to explore. In this chapter, The synthetic routes for 2-(substituted-sulfonyl-phenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-ones will be grouped and described in details.

2.2 Design of synthetic routes for 3H-imidazo[1,5-a][1,3,5] triazin-4-one

As described in the previous chapter, we have chosen 2-substituted-phenyl-6,8-dialkyl-3*H*-imidazo[1,5-a]-[1,3,5]-triazine-4-one (Fig. 1.13 **I-33**) as a start compound to develop new PDE5 inhibitors, in which R^1, R^2, R^3 and R^4 represent the different substituents. In comparison with the successful drugs sildenafil, vardenafil and tadalafil we felt that I-33 5-sulfonyl derivative, 2-(substituted-sulfonylphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one, was a

good start point to explore, wherein the substituent R^4 is a sulfonyl group $-SO_2R^5$ (Fig. 2.1, **II-1**).



I-33 **II-1**

Figure 2.1 Molecular structures of I-33 and II-1

2.2.1 A general synthetic route for imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (I-33)

Imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (I-33) represents a series of possible PDE inhibitors resulting from introducing different R^1 R^2, R^3, and R^4 groups and alternating their combinations.

According to the literature methods (*English et al., 1946*), we foresaw a feasible synthetic route for imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (I-33) derivatives. Firstly, a condensation reaction between cyano and ester groups in compound II-2 with benzamidine II-3 to give pyrimidine-4-one II-4, followed by a rearrangement of pyrimidine-4-one II-4 to imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (I-33).

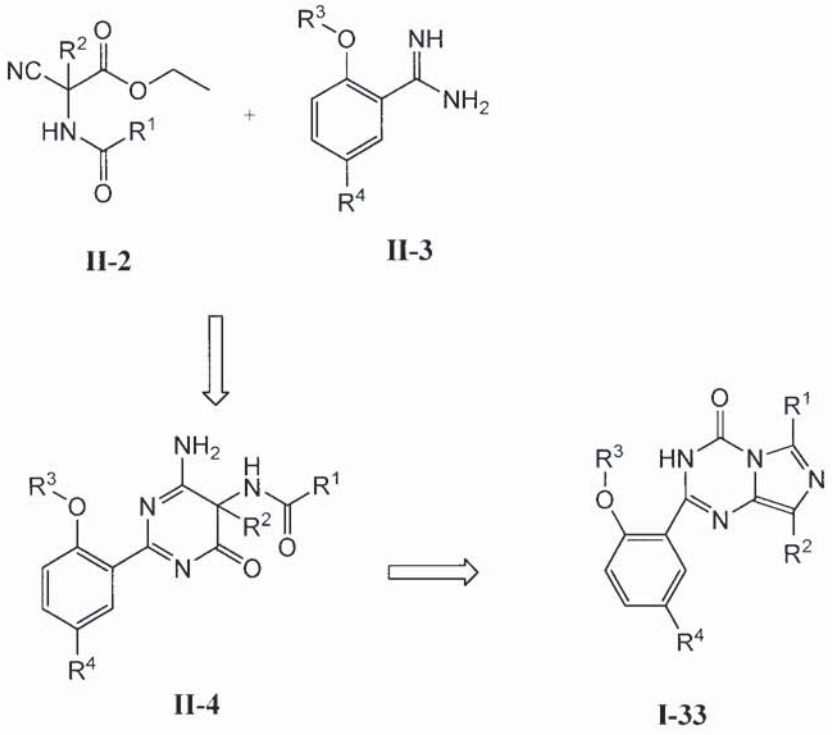


Figure 2.2 Synthetic route to I-33

2.2.2 A general synthetic route to 2-(substituted-sulfonylphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-1)

Ethyl 2-cyano-2-(hydroxyimino)acetate (II-6) was synthesized from ethyl 2-cyanoacetate (II-5) with sodium nitrite under an acidic condition, followed by Zn reduction, acylation and alkylation to give the key precursor ethyl 2-acetamido-2-cyanopentanoate (II-8). 5-Acetamido-6-amino-4,5-dihydro-2-(2-ethoxyphenyl)-5-propylpyrimidine-4-one (II-10) was obtained from the condensation of ethyl 2-acetamido-2-cyanopentanoate (II-8) and 2-ethoxybenzamidine (II-9), as shown in Fig. 2.3. Conversion of II-10 into II-11 was initialized by an attack of the amino of 2-ethoxybenzamidine (II-9) on the carbonyl carbon of the acylamido group of ethyl 2-acetamido-2-cyanopentanoate (II-8) with the capturing of the oxide by trimethyl-chlorosilane followed by a rearrangement (Fig. 2.4). Finally, sulfonation of

II-11 with chlorosulfonic acid and followed by a reaction with an amine compound gave the final product II-1 (Fig. 2.3).

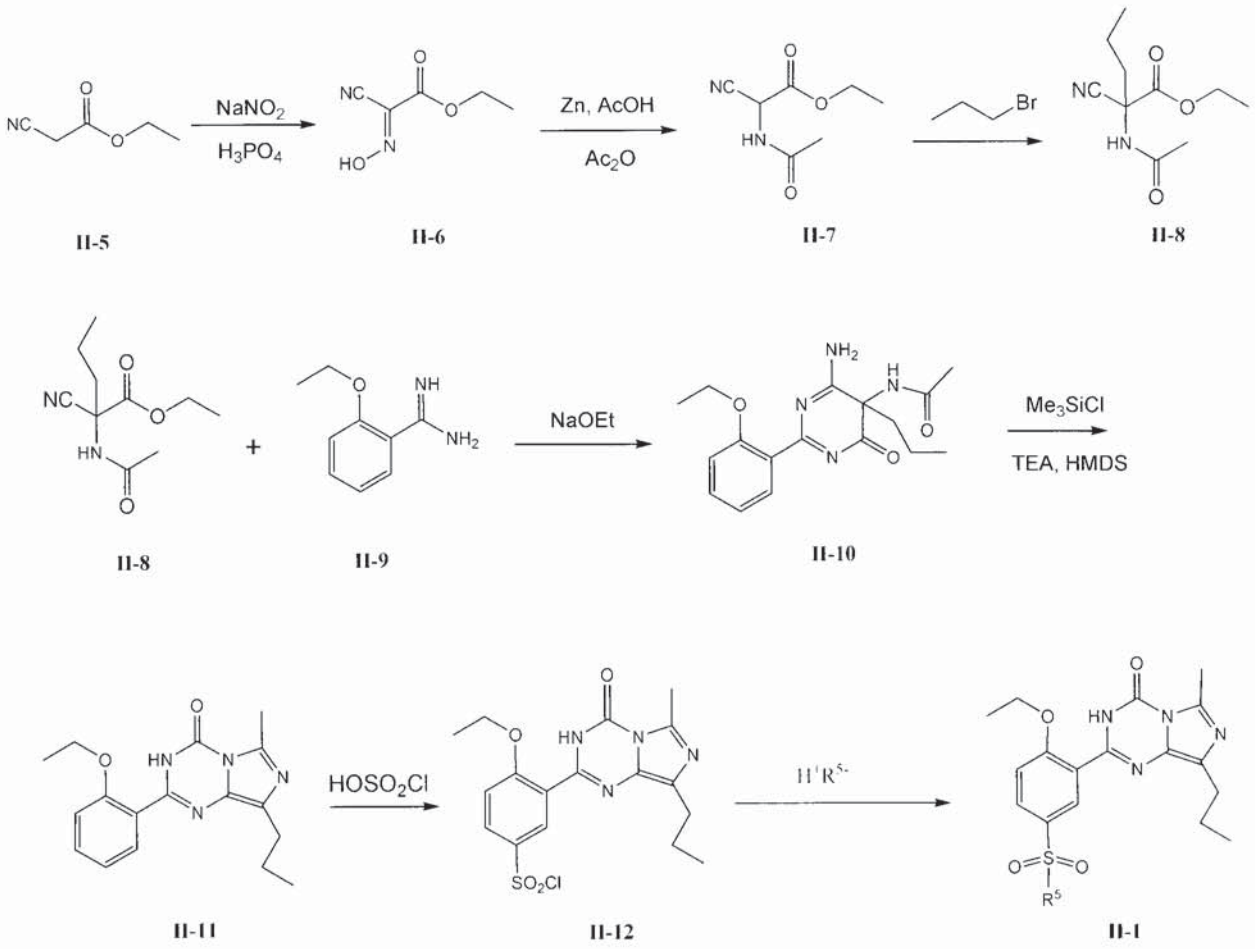


Figure 2.3 The general synthetic route of 2-[2-ethoxy-5-(substituted-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-1) compounds.

List of compounds in this reaction:

II-5: ethyl 2-cyanoacetate;

II-6: ethyl 2-cyano-2-(hydroxyimino)acetate;

II-7: ethyl 2-acetamido-2-cyanoacetate;

II-8: ethyl 2-acetamido-2-cyanopentanoate;

II-9: 2-ethoxybenzamidine;

II-10: 5-acetamido-6-amino-4,5-dihydro-2-(2-ethoxyphenyl)-5-propylpyrimidine-4-one;

II-11: 2-(2-ethoxyphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one;

II-12: 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride;

Table 2.1 Parameters of compounds in synthetic process of II-1.

Compounds	Molecular weight	Molecular formula	Element components
II-5	113.12	$C_5H_7NO_2$	C=53.09%; H=6.24%; N=12.38%; O=28.29%
II-6	142.12	$C_5H_6N_2O_3$	C=42.26%; H=4.26%; N=19.71%; O=33.77%
II-7	170.17	$C_7H_{10}N_2O_3$	C=49.41%; H=5.92%; N=16.46%; O=28.21%
II-8	212.25	$C_{10}H_{16}N_2O_3$	C=56.59%; H=7.60%; N=13.20%; O=22.61%
II-9	164.21	$C_9H_{12}N_2O$	C=65.83%; H=7.37%; N=17.06%; O=9.74%
II-10	330.39	$C_{17}H_{22}N_4O_3$	C=61.80%; H=6.71%; N=16.96%; O=14.53%
II-11	312.37	$C_{17}H_{20}N_4O_2$	C=65.37%; H=6.45%; N=17.94%; O=10.24%
II-12	410.88	$C_{17}H_{19}ClN_4O_4S$	C=49.70%; H=4.66%; Cl=8.63%; N=13.64%; O=15.58%; S=7.80%

Conversion of II-10 into II-11 was the crucial step in this synthetic sequence. The reaction was initiated with a condensation of amino on benzamidine (II-9) with the cyano and the carbonyl carbon of ethyl 2-acetamido-2-cyanopentanoate (II-8), with the resulting oxide was captured by trimethyl-chlorosilane, to form the imdazopyrimidine precursor ($[M_3]^*$). With extra energy supplied by heating-up, the ketene ($[M_4]^*$) was formed through electrons rearrangement within pyrimidine ring, followed by acylation on more energetic favourable nitrogen of the imidazol ring and elimination of the siloxane to give the key imidazo[1,5-a]-1,3,5-triazine precursor (II-11) (Fig. 2.4).

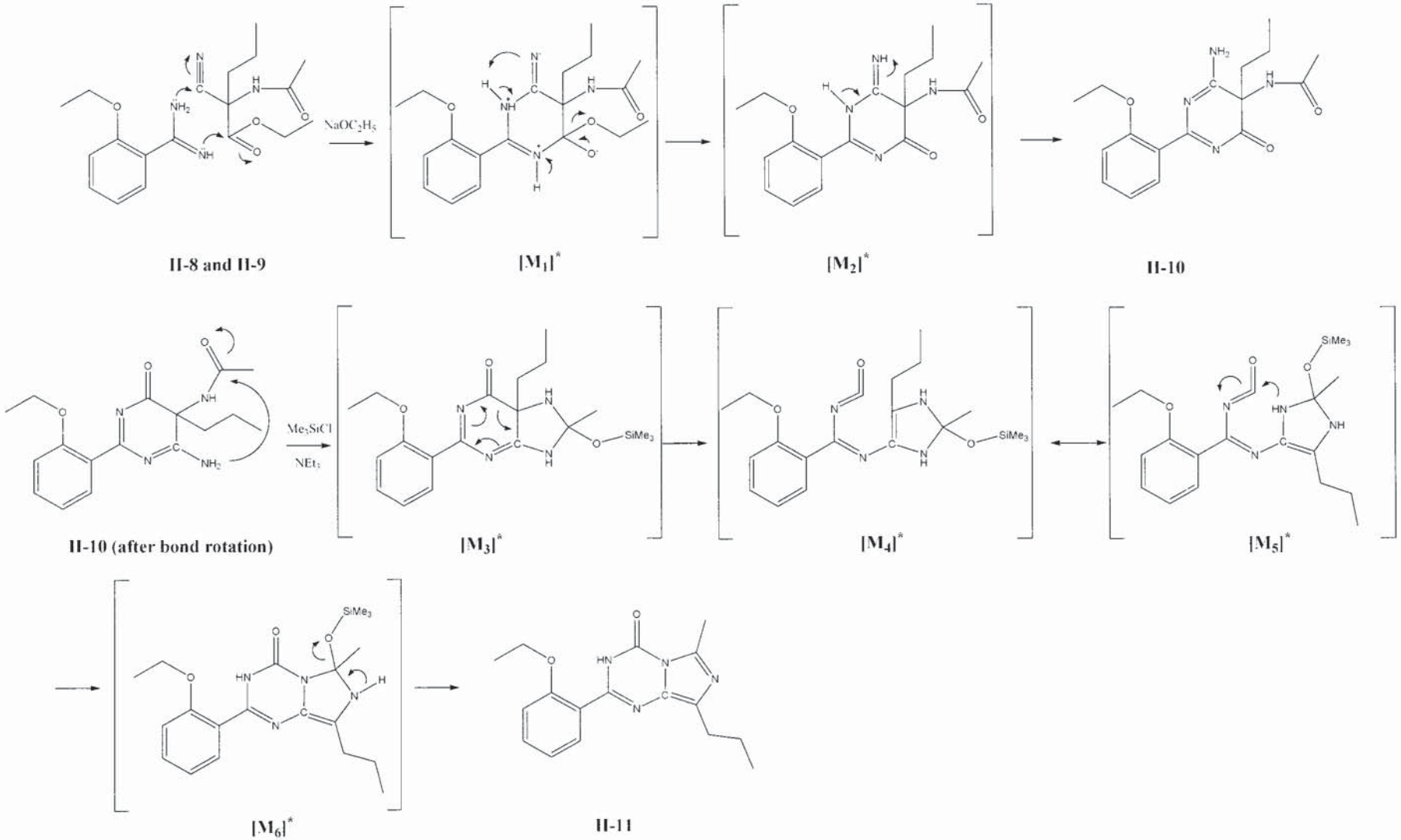


Figure 2.4 The mechanism of cyclization and rearrangement reaction from compound II-8 to compound II-11

Sulfonation of 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-12) with an amine compound gave a designed candidate compound for screening PDE5 inhibition activity. It is clear there are a variety of amine compounds available for this purpose, therefore we selected a group of repetitive amine compounds for the sulfonation to produce a series of 2-(substituted-sulfonylphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one derivatives. At the same time, we have altered R^1, R^2 and R^3 groups on imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (I-33). By doing so, combinations of variable amine compounds and R^1, R^2 and R^3 groups resulted in range structural diversities among the imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one derivatives produced in this approach.

2.3 Experiments section

2.3.1 Equipments, materials and general methods

Melting points (M.p.) were determined on a Reichert-Jung Microthermal apparatus which was uncorrected. NMR spectra were recorded on a Bruker AC250 Spectrometer at ^1H (250.1 MHz) and ^{13}C (62.9 MHz). Chemical shifts are downfield of tetramethylsilane. Mass spectroscopic analysis was carried out on a Hewlett Packard 5989B MS engine with an HP 5998A API Electrospray LC/MS interface; the LC was taken on Agilent HPLC meter with HP1100 system and autosampler. High resolution mass spectra (HRMS) were measured on a Finnigan MAT 900 XLT high resolution double focusing mass spectrometer using an electrospray method. Infrared spectra were recorded on a Mattson 3000 FTIR Spectrometer; solid samples were prepared as KBr discs and liquids as thin films between sodium chloride plates. Flash column chromatography was performed using Sorbsil C60 silica gel. TLC was carried out using aluminum backed Merck Silica Gel 60 F254 plates and visualized under UV (254 nm). Potassium permanganate was used where appropriate to develop TLC

plates. Elemental analyses (C, H, N) were performed on a Leeman 440 analyzer. Dry THF and dry ether were prepared by refluxing with sodium metal chips and distillation. Magnesium powder was activated using 3%HCl, washed with distilled water then acetone and dried in vacuum. The reagents used as mobile phase in chromatographic measurement were HPLC grade. Other reagents used in organic synthesis without especially mentioned were all commercial analysis grade.

2.3.2 Example of synthesis of 2-(substituted-sulfonylphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-1) derivatives

Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13)

***Ethyl 2-cyano-2-(hydroxyimino)acetate (II-6)*:** Ethyl 2-cyanoacetate (II-5, 33.9g, 300mmol) was suspended in 240ml aqueous solution of sodium nitrite（19.5g, 283mmol）. The mixture was stirred at 35-40°C for 30 min. Phosphoric acid (85% aq) was added dropwise to adjust the mixture pH to 4.5. The reaction mixture was stirred continuously for one more hour at 30°C. Hydrochloric acid (conc., 25ml) was added and the temperature raised to about 50°C. The resulting mixture is cooled to –10°C. The precipitates was collected by filtration and dried in vacuum. The filtrate was extracted with 50ml ether to reclaim the remaining product. 35g of the combined product was obtained, yield 82%. M.p.: 129-131°C (Lit.: 129-131°C) *(Parker, 1962)*

***Ethyl 2-acetamido-2-cyanoacetate (II-7)*:** Ethyl 2-cyano-2-(hydroxyimino)acetate (14.2g, 100mmol) was dissolved in a mixture of acetic acid (100ml) and acetic anhydride (28.4ml, 300mmol), 4.5g zinc powder was added. The reaction mixture was kept stirring at room temperature for 20 hours. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was recrystallized in

ethyl acetate to give 8.0g white crystals, yield 47%. M.p.: 123-124°C (Lit.: 129°C) *(Wilson, 1948)*

***Ethyl 2-acetamido-2-cyanopentanoate (II-8)*:** Ethyl 2-acetamido-2-cyanoacetate (17.0g, 100mmol) and 1-bromopropane (10.0ml, 110mmol) was added to a preparation of sodium (2.3g, 100mmol) in 50ml absolute ethanol. The mixture is stirred and heated at reflux for 3hr. After cooling down to ambient temperature, water (150ml) was added to the resulting solution. The solids were collected by filtration, after drying in air 13.6g product was afforded, yield 64%. M.p.: 118-119°C (Lit.: 118°C) (*English et al., 1946)*

***2-Ethoxybenzamidine (II-9)*:** Ammonium chloride (145mg, 2.72mmol) was dissolved in cold toluene. A solution of trimethyl aluminum in hexane (2M, 1.36 ml, 2.72mmol) was added slowly to the mixture. The mixture is stirred at room temperature for 2 hours. After heated to 80°C, 2-ethoxybenzonitrile (200mg, 136mmol) was added, and the reaction mixture was kept at 80°C for 2 days. The resulting mixture was slowly poured into a suspension of silica gel (5g) in chloroform (100ml). The resulting mixture was filtered and washed by a mixture of methanol and water (1:1). The filtrate was evaporated and the residue was dissolved in 30ml DCM. This solution was extracted by 5% sodium hydroxide. After dried with anhydrous sodium sulfate, the organic phase was evaporated to dryness to give the product as white solids, which was used in the step reaction without further purification *(Garigipati, 1990)*.

***5-Acetamido-6-amino-4,5-dihydro-2-(2-ethoxyphenyl)-5-propylpyrimidine-4-one (II-10)*:** 2-Ethoxybenzamidine (0.83g, 5.00mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-acetamido-2-cyanopentanoate (1.06g, 5.00mmol) had been added, the solution was heated at reflux with stirring for 20 minutes. The system pH value is adjusted to 5.5 using acetic acid and the mixture was evaporated to dryness. The residue was extracted with DCM and filtered. The crude

product of II-10 was afforded by evaporating of the filtrate, which was used directly to next step.

2-(2-Ethoxyphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-11): 5-Acetamido-6-amino-4,5-dihydro-2-(2-ethoxyphenyl)-5-propylpyrimidine-4-one was mixed with triethylamine (20ml) and trimethylchlorosilane (1.26ml, 10mmol), the mixture was stirred at room temperature for 30 minutes. Hexamethyldisilane (2.09ml, 10mmol) was added and the mixture was heated at reflux for 10 minutes. The solvent was evaporated under reduced pressure. Absolute methanol (50ml) was added to the residue, and the mixture was stirred for 45 minutes. The white product 1.25g was gained by evaporating the methanol solution under reduced pressure, yield is 80%. M.p.: 141-142°C. IR (cm^{-1}): 3311, 2981, 2931, 2865, 1738, 1604, 1585, 1510, 1473, 1452, 1352, ,1297, 1238, 1164, 1125, 1111, 1032, 752, 729, 677; ^{1}H NMR ($CDCl_3$) δ: 0.98(t, 3H), 1.57(t, 3H), 1.68-1.83(m, 2H), 2.76(t, 2H), 2.84(s, 3H), 4.25(q, 2H), 6.98 (d, 1H), 7.12(t, 1H), 7.44(m, 1H), 8.35(dd, 1H), 10.30(brs, 1H); ^{13}C NMR ($CDCl_3$) δ (CH_3): 13.9, 14.5, 16.1; (CH_2): 22.4, 28.2, 65.1; (CH): 112.6, 121.6, 130.3, 132.4; (C): 118.4, 132.0, 132.4, 138.6, 143.5, 144.9, 146.2, 156.5; Elemental analysis calculated(%) for $C_{17}H_{20}N_4O_2$: C 65.37, H 6.45, N 17.94; found(%): C 65.39, H 6.44, N 17.92.

4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-12): 2-(2-ethoxyphenyl)-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (1.25g, 3.83mmol) was added to a solution of chlorosulfonic acid (4ml) in ethyl acetate (20ml) by portions with stirring at 0°C. The mixture was kept stirred at room temperature for 4 hours. After it had been stirred at 0°C for another 30 minutes, the resulting solution was poured into a 0.1L mixture of ice water and DCM (1:1). The organic phase was washed by 5ml ice water and dried over anhydrous sodium sulfate. It was evaporated to give 1.2g yellow solids, yield 73%. This product is pure enough to be used in the next step.

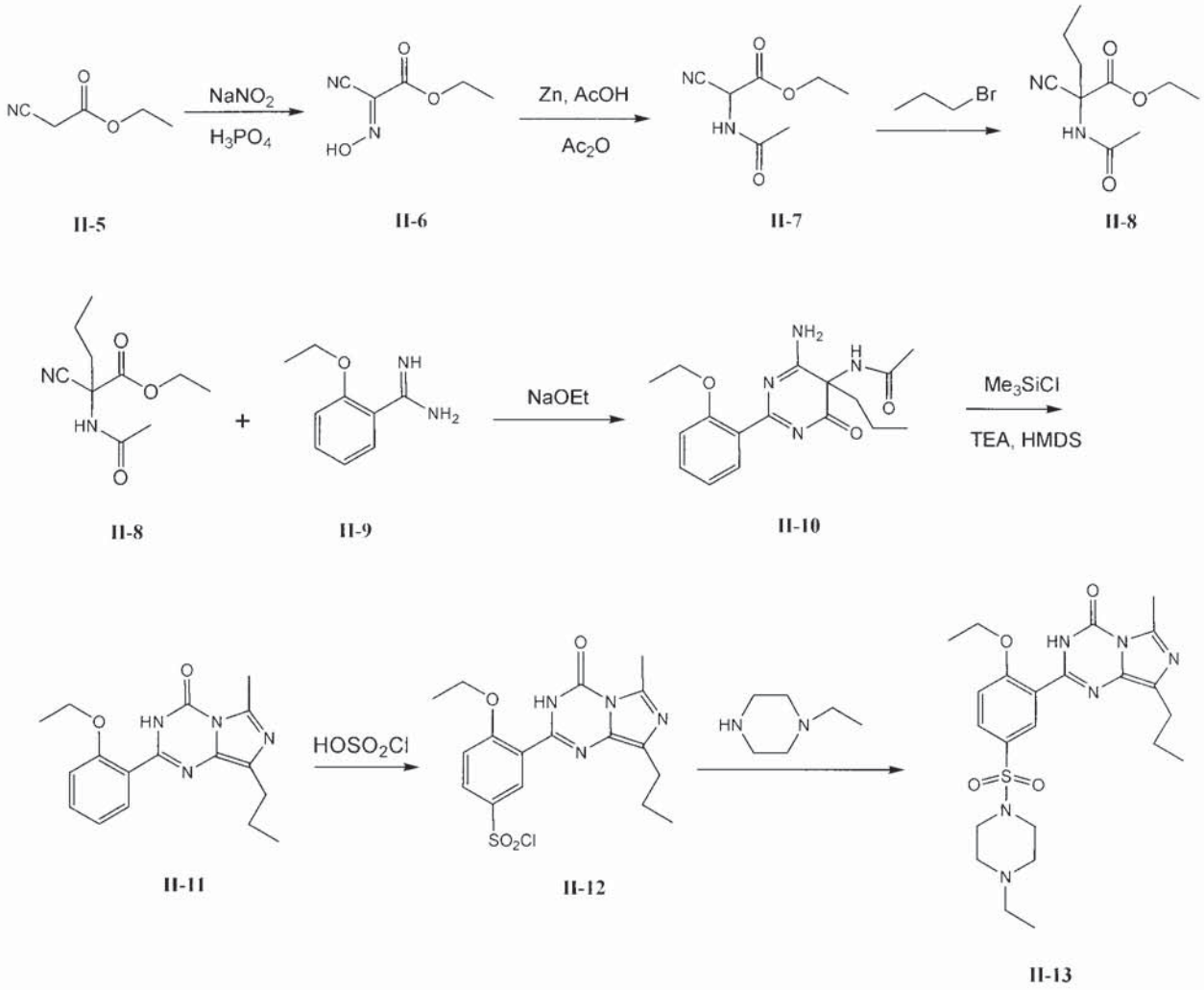


Figure 2.5 Synthetic route of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13).

2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-13): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (1.00g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 1-Ethylpiperazine (0.78ml, 6.10mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then reacted at room temperature for two hours. The resulting solution was washed by water and dried by anhydrous sodium sulfate, then evaporated to get 1.2g yellow solids. The final pure yellow product (II-13, 0.73g) was gained by chromatography (ethyl acetate: methanol=20:1-10:1 as eluents) and recrystallization from ethyl acetate (5ml), yield is 59%. M.p.: 177-179°C; IR (cm^{-1}): 2964, 2935, 2869, 2811, 1741, 1726,

1672, 1604, 1463, 1356, 1265, 1172, 1147, 950, 740, 617; ^{1}H NMR ($CDCl_3$) δ: 0.94-1.04 (m, 6H), 1.61(t, 3H), 1.71-1.80(m, 2H), 2.37(q, 2H), 2.52(m, 4H), 2.75(dd, 2H), 2.83(s, 3H), 3.07(brs, 4H), 4.33(q, 2H), 7.10 (d, 1H), 7.79(dd, 1H), 8.68(d, 1H), 9.96(brs, 1H); ^{13}C NMR ($CDCl_3$) δ (CH_3): 11.8, 13.8, 14.3, 16.1; (CH_2): 22.2, 28.2, 45.9, 51.6, 51.7, 66.0; (CH): 113.1, 130.3, 131.7; (C): 119.6, 128.3, 132.0, 133.1, 139.2, 141.8, 144.7, 159.4; MS (ES^+): m/z 506 ($M+NH_4$); Elemental analysis calculated(%) for $C_{23}H_{32}N_6O_4S$: C 56.54, H 6.60, N 17.20; found(%): C 56.51, H 6.59, N 17.21.

2.3.3 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with a different R^5 group

***2-[2-Ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-14)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8- propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86 mmol), was dissolved in 45ml DCM with stirring at 0°C. 1-Methylpiperazine dihydrochloride (2.1g, 12mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for two hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get yellow solids. The final pure yellow product (II-14, 1.85g) was gained by chromatography (ethyl acetate: methanol=18:1 as eluent) and recrystallization from ethyl acetate. ^{1}H NMR (D_2O) δ: 0.96 (t, 3H), 1.62 (t, 3H), 1.75 (m, 2H), 2.31(s, 3H), 2.53(m, 4H), 2.75(t, 2H), 2.84 (s, 3H), 3.07 (m, 4H), 4.34 (q, 2H), 7.12 (d, 1H), 7.78(dd, 1H), 8.66 (d, 1H); MS(ES^+) m/z 492 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{31}N_6O_4S$ $[M+H]^+$ 475.21275, found 475.21268.

2-[2-Ethoxy-5-(4-ethoxycarbonylpiperazine-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-15): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86 mmol), was dissolved in 45ml DCM with stirring at 0°C. Ethyl piperazine-1-carboxylate (1.8g, 11.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for four hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get yellow solids. This crude product was recrystallized by ethyl acetate and methanol (1:1) to get the pure product. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.27 (t, 3H), 1.63 (t, 3H), 1.80 (m, 2H), 2.77(t, 2H), 2.86(s, 3H), 3.05 (m, 4H), 3.51 (m, 4H), 4.03 (q, 2H), 4.41 (q, 2H), 7.12 (d, 1H), 7.81 (d, 1H), 8.67 (s, 1H); MS(ES^+) m/z 550 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{33}N_6O_6S$ $[M+H]^+$ 533.21823, found 533.21829.

2-[2-Ethoxy-5-(4-hydroxyethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-16): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43 mmol), was dissolved in 20ml DCM with stirring at 0°C. N-hydroxyethylpiperazine (0.72g, 5.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with saturated brines and dried over anhydrous sodium sulfate, then evaporated to get yellow solids. This crude product was recrystallized from ethyl acetate and methanol (1:1) to get the pure product II-16. ^{1}H NMR ($CDCl_3$) δ: 0.96 (t, 3H), 1.61(t, 3H), 1.72(m, 2H), 2.52-2.56 (m, 6H), 2.75(t, 2H), 2.82(s, 3H), 3.07(brs, 4H), 3.67(m, 2H), 4.33(q, 2H), 7.11 (d, 1H), 7.79(dd, 1H), 8.68(d, 1H); MS(ES^+) m/z 522 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{33}N_6O_5S$ $[M+H]^+$ 505.22331, found 505.22326.

2-[2-Ethoxy-5-(N-pyrrolidinyl-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-17): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12,

1.0g ,2.43 mmol), was dissolved in 20ml DCM with stirring at 0°C. The Lewis base reagent pyrrolidine (0.43g, 6mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then reacted at room temperature for 3.5 hours. The resulting solution was washed by water and dried over anhydrous sodium sulfate, then evaporated to get yellow solids. This crude product was recrystallized by acetone to get the pure product II-17. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.62 (t, 3H), 1.79 (m, 6H), 2.80 (t, 2H), 2.92 (s, 3H), 3.27 (m, 4H), 4.35 (q, 2H), 7.13 (d, 1H), 7.90 (dd, 1H), 8.72 (d, 1H); MS(ES^+): m/z 463 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{28}N_5O_4S$ $[M+H]^+$ 446.18620, found 446.18625.

2-{2-Ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl]aminosulfonyl}-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-18): 4-Ethoxy-3-(6-methyl -4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12,1.0g , 2.43mmol), was dissolved in 20ml DCM with stirring at 0°C. 3-(2-oxo-1-pyrrolidinyl)propylamine (0.87g ,6.1mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethyl acetate and methanol (1:1) to get the pure product II-18. ^{1}H NMR ($CDCl_3$) δ: 0.95 (t, 3H), 1.56 (t, 3H), 1.72 (m, 4H), 2.00 (m, 2H), 2.31 (t, 2H), 2.74 (t, 2H), 2.83 (s, 3H), 2.86 (m, 2H), 3.09 (m, 2H), 3.32 (m, 2H), 4.30 (q, 2H), 7.08 (d, 1H), 7.92 (dd, 1H), 8.67 (d, 1H); MS(ES^+): m/z 534 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{33}N_6O_5S$ $[M+H]^+$ 517.22331, found 517.22338.

2-{2-Ethoxy-5-[N-(2-pyrrolidinylethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-19): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol), was dissolved in 45ml DCM with stirring at 0°C. 2-Pyrrolidinylethylamine (1.42g, 12.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The

resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone and methanol (1:1) to get the pure product II-19. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.57 (t, 3H), 1.78 (m, 4H), 1.8-2.2 (m, 6H), 2.78 (t, 2H), 2.90 (s, 3H), 3.39 (m, 2H), 3.89 (m, 2H), 4.31 (q, 2H), 7.15 (d, 1H), 7.51 (br, 1H), 8.02 (d, 1H), 8.67 (s, 1H); MS(ES^+): m/z 506 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{33}N_6O_4S$ $[M+H]^+$ 489.22840, found 489.22828.

2-[2-Ethoxy-5-(morpholinosulfonyl)phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-20): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g ,4.86mmol), was dissolved in 45ml DCM with stirring at 0°C.Morpholine (1.03g,11.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 4 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-20. ^{1}H NMR ($CDCl_3$) δ: 0.98 (t, 3H), 1.64 (t, 3H), 1.78 (m, 2H), 2.78 (t, 2H), 2.92 (s, 3H), 3.03 (m, 4H), 3.75 (m, 4H), 4.37 (q, 2H), 7.18 (d, 1H), 8.20 (d, 1H), 8.68 (s, 1H), 10.12 (br, 1H); MS(ES^+) m/z 479 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{28}N_5O_5S$ $[M+H]^+$ 462.18111, found 462.18122.

2-{2-Ethoxy-5-[N-(3-morpholinopropyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-21): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol), was dissolved in 20ml DCM with stirring at 0°C. 3-Morpholinopropan-1-amine (0.84g, 5.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-21. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.61 (t, 3H),

1.74 (m, 4H), 2.54 (m, 6H), 2.75 (t, 2H), 2.78 (s, 3H), 3.11 (t, 2H), 3.75 (m, 4H), 4.34 (q, 2H), 7.13 (d, 1H), 7.92 (d, 1H), 8.76 (s, 1H); MS(ES^+): m/z 536 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_6O_5S$ $[M+H]^+$ 519.23896, found 519.23885.

***2-{2-Ethoxy-5-[N-(2-morpholinoethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-22)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8 -propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol), was dissolved in 40ml DCM with stirring at 0°C. 2-Morpholinoethanamine (1.53g, 11.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-22. ^1H NMR ($CDCl_3$) δ: 0.98 (t, 3H), 1.61 (t, 3H), 1.74 (m, 2H), 2.32 (m, 4H), 2.44 (t, 2H), 2.76 (t, 2H), 2.84 (s, 3H), 3.03 (t, 2H), 3.61 (m, 4H), 4.34 (q, 2H), 7.12 (d, 1H), 7.92 (d, 1H), 8.79 (s, 1H); MS(ES^+) m/z: 522 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{33}N_6O_5S$ $[M+H]^+$ 505.22331, found 505.22337.

***2-[2-Ethoxy-5-(2,6-dimethylmorpholinosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-23)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl -3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol), was dissolved in 40ml DCM with stirring at 0°C. 2,6-Dimethyl-morpholine (1.33g ,11.6mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethanol to get the pure product II-23. ^1H NMR ($CDCl_3$) δ: 0.98 (t, 3H), 1.18 (s, 3H), 2.21 (s, 3H), 1.63 (t, 3H), 1.73 (m, 2H), 2.77 (t, 2H), 2.87 (s, 3H), 3.04 (dd, 1H), 3.25 (m, 1H), 3.71 (m, 4H), 4.35 (q, 2H), 7.16 (d, 1H), 7.80 (dd, 1H), 8.66 (d, 1H), 9.98 (br, 1H); MS(ES^+): m/z 507 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{32}N_5O_5S$ $[M+H]^+$ 490.21241, found 490.21246.

***2-[2-Ethoxy-5-(1-benzylpiperidine-4-aminosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-24)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 1-Benzylpiperidin-4-amine (1.1g, 5.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-24. ^{1}H NMR ($CDCl_3$) δ: 0.95 (t, 3H), 1.51-2.04 (m, 13H), 2.73 (t, 2H), 2.80-2.96 (m, 4H), 3.46 (s, 2H), 4.31 (q, 2H), 7.07 (d, 1H), 7.21-7.27 (m, 5H), 7.92 (dd, 1H), 8.78 (d, 1H); MS(ES^+): m/z 582 ($M+NH_4$); HRMS (CI) calcd for $C_{29}H_{37}N_6O_4S$ $[M+H]^+$ 565.25970, found 565.25982.

***2-[2-Ethoxy-5-(2-piperidinylethylamino-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-25)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (1.0g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 2-Piperidinylethylamine (0.74g, 5.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from methanol to get the pure product II-25. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.35 (m, 2H), 1.56-1.78 (m, 9H), 2.59 (m, 4H), 2.71-2.76 (m, 4H), 2.82 (s, 3H), 3.15 (t, 2H), 4.32 (q, 2H), 7.13 (d, 1H), 7.95 (d, 1H), 8.74 (s, 1H); MS(ES^+): m/z 520 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_6O_4S$ $[M+H]^+$ 503.24405, found 503.24418.

***2-[2-Ethoxy-5-(4-benzylpiperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-26)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imi-dazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound

II-12,1.0g,2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 4-Benzylpiperazine (0.99g, 5.6mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then reacted at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-26. ^{1}H NMR ($CDCl_3$) δ: 0.94 (t, 3H), 1.62 (t, 3H), 1.75 (m, 2H), 2.52 (m, 4H), 2.70 (t, 2H), 2.84 (s, 3H), 3.09 (m, 4H), 3.47 (s, 2H), 4.34 (q, 2H), 7.11 (d, 1H), 7.21-7.29 (m, 5H), 7.78 (dd, 1H), 8.65 (d, 1H); MS(ES^+): m/z 568 ($M+NH_4$); HRMS (CI) calcd for $C_{28}H_{35}N_6O_4S$ $[M+H]^+$ 551.24405, found 551.24391.

***2-[2-Ethoxy-5-(4-phenylpiperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-27)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 1-Phenylpiperazine (1g, 6.2mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 4 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone to get the pure product II-27. ^{1}H NMR ($CDCl_3$) δ: 0.99 (t, 3H), 1.63 (t, 3H), 1.77 (m, 2H), 2.78 (t, 2H), 2.86 (s, 3H), 3.22 (m, 8H), 4.36 (q, 2H), 6.84-6.91 (m, 3H), 7.13-7.21 (m, 3H), 7.84 (dd, 1H), 8.74 (d, 1H), 10.00 (br, 1H); MS(ES^+): m/z 554 ($M+NH_4$); HRMS (CI) calcd for $C_{27}H_{33}N_6O_4S$ $[M+H]^+$ 537.22840, found 537.22848.

***2-[2-Ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-tria-zine-4-(3H)-one (II-28)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g ,2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. Piperazine (0.52g, 6.0mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 4 hours. The resulting solution was washed with water

and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from methanol to get the pure product II-28. ^{1}H NMR ($CDCl_3$) δ: 0.94 (t, 3H), 1.61(t, 3H), 1.75 (m, 2H), 2.65(m, 4H), 2.75(dd, 2H), 2.83(s, 3H), 3.10(brs, 4H), 4.33(q, 2H), 7.11 (d, 1H), 7.76(dd, 1H), 8.67(d, 1H), 9.90(brs, 1H); MS(ES^+): m/z 478 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{29}N_6O_4S$ $[M+H]^+$ 461.19710, found 461.19717.

2-[2-Ethoxy-5-(4-benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-29): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g , 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 1-(Benzo[d][1,3] dioxol-2-ylmethyl)piperazine (1.32g, 6.0mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 4 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethanol to get the pure product II-29. ^{1}H NMR ($CDCl_3$) δ: 0.95 (t, 3H), 1.62 (t, 3H), 1.73 (m, 2H), 2.49 (m, 4H), 2.71 (t, 2H), 2.84 (s, 3H), 3.11 (m, 4H), 3.43 (s, 2H), 4.35 (q, 2H), 5.92 (s, 2H), 6.65-6.80 (m, 3H), 7.11 (d, 1H), 7.78 (dd, 1H), 8.6 (d, 1H); MS(ES^+): m/z 612 ($M+NH_4$); HRMS (CI) calcd for $C_{29}H_{35}N_6O_6S$ $[M+H]^+$ 595.23388, found 595.23403.

2-{2-Ethoxy-5-[4-(3-phenylpropane-1-yl)piperidine-1-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-30): 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzene-sulfonyl chloride (compound II-12, 2.0g , 4.86mmol) was dissolved in 45ml DCM with stirring at 0°C. 4-(3-Phenylpropyl)piperidine (2.4g, 11.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 4 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethanol to get the pure product II-30. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.29-1.31 (m,

6H), 1.58-1.80 (m, 8H), 2.50-2.67 (m, 6H), 2.76 (t, 2H), 2.85 (s, 3H), 4.33 (q, 2H), 7.08-7.29 (m, 6H), 7.80 (dd, 1H), 8.66 (d, 1H); MS(ES^+): m/z 595 ($M+NH_4$); HRMS (CI) calcd for $C_{31}H_{40}N_5O_4S$ $[M+H]^+$ 578.28010, found 578.28015.

***2-[2-Ethoxy-5-(N-propylaminosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-31)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 40ml DCM with stirring at 0°C. Propylamine (0.68g, 11.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethanol to get the pure product II-31. ^1H NMR ($CDCl_3$) δ: 0.94-0.99 (m, 6H), 1.51 (q, 2H), 1.61(t, 3H), 1.75 (m, 2H), 2.75 (t, 2H), 2.85 (s, 3H), 2.96 (q, 2H) 4.34 (q, 2H), 7.11 (d, 1H), 7.92 (dd, 1H), 8.78 (d, 1H); MS(ES^+): m/z 451 ($M+NH_4$); HRMS (CI) calcd for $C_{20}H_{28}N_5O_4S$ $[M+H]^+$ 434.18620, found 434.18611.

***2-{2-Ethoxy-5-[N,N-bis(2-hydroxyethyl)aminosulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-32)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 40ml DCM with stirring at 0°C. N,N-bis(2-hydroxyethyl)amine (1.27g, 12.1mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethyl acetate to get the pure product II-32. ^1H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.62 (t, 3H), 1.79 (m, 2H), 2.78 (t, 2H), 2.85 (s, 3H), 3.33 (t, 4H) 3.87 (t, 4H), 4.35 (q, 2H), 7.12 (d, 1H), 7.89 (dd, 1H), 8.71 (d, 1H); MS(ES^+): m/z 497 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{30}N_5O_6S$ $[M+H]^+$ 480.19168, found 480.19172.

2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-methyl]aminosulfonyl-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-33): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. The Lewis base reagent 2-(methylamino) ethanol (0.45g, 6.0mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethyl acetate to get the pure product II-33. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.62 (t, 3H), 1.76 (m, 2H), 2.77 (t, 2H), 2.86 (s, 6H), 3.21 (t, 2H) 3.78 (t, 2H), 4.35 (q, 2H), 7.15 (d, 1H), 7.86 (dd, 1H), 8.71 (d, 1H); MS(ES^+): m/z 467 ($M+NH_4$); HRMS (CI) calcd for $C_{20}H_{28}N_5O_5S$ $[M+H]^+$ 450.18111, found 450.18106.

2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-ethyl]aminosulfonyl-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-34): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol) was dissolved in 20ml DCM with stirring at 0°C. 2-(Ethylamino)ethanol (0.53g, 6.0mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethyl acetate to get the pure product II-34. ^{1}H NMR ($CDCl_3$) δ: 0.98 (t, 3H), 1.18 (t, 3H), 1.62 (t, 3H), 1.77 (m, 2H), 2.78 (t, 2H), 2.86 (s, 3H), 3.31 (m, 4H) 3.79 (t, 2H), 4.35 (q, 2H), 7.12 (d, 1H), 7.91 (dd, 1H), 8.76 (d, 1H); MS(ES^+): m/z 481 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{30}N_5O_5S$ $[M+H]^+$ 464.19676, found 464.19671.

2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-n-butyl]aminosulfonyl-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-35): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 45ml DCM with stirring at 0°C.

2-(Butylamino)ethanol (1.38g, 11.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed by water and dried by anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from ethyl acetate to get the pure product II-35. ^{1}H NMR ($CDCl_3$) δ: 0.92-1.00 (m, 6H), 1.29-1.41 (t, 4H), 1.64 (t, 3H), 1.75 (m, 2H), 2.76 (t, 2H), 2.84 (s, 3H), 3.11 (m, 2H) 3.26 (m, 2H), 3.77 (t, 2H), 4.34 (q, 2H), 7.11 (d, 1H), 7.89 (dd, 1H), 8.74 (d, 1H); MS(ES^+): m/z 509 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{34}N_5O_5S$ $[M+H]^+$ 492.22806, found 492.22818.

***2-[2-Ethoxy-5-(4-ethoxycarbonylaniline-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imiazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-36)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 45ml DCM with stirring at 0°C. 4-Ethoxycarbonylaniline (1.85g, 11.2mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from methanol and acetone (1:1) to get the pure product II-36. ^{1}H NMR ($CDCl_3$) δ: 0.96 (t, 3H), 1.30 (t, 3H), 1.54 (t, 3H), 1.72 (m, 2H), 2.72 (t, 2H), 2.85 (s, 3H), 4.29 (m, 4H), 6.62 (d, 2H), 7.09 (d, 1H), 7.82 (d, 2H), 7.95 (dd, 1H), 8.76 (d, 1H); MS(ES^+): m/z 557 ($M+NH_4$); HRMS (CI) calcd for $C_{26}H_{30}N_5O_6S$ $[M+H]^+$ 540.19168, found 540.19161.

***2-[2-Ethoxy-5-(o-benzoylaniline-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-37)*:** 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 1.0g, 2.43mmol), was dissolved in 20ml DCM with stirring at 0°C. *o*-Benzoylaniline (1.14g, 5.8mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3.5 hours. The resulting solution was washed with water

and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from methanol to get the pure product II-37. ^{1}H NMR ($CDCl_3$) δ: 0.96 (t, 3H), 1.54 (t, 3H), 1.72 (m, 2H), 2.72 (t, 2H), 2.85 (s, 3H), 4.29 (m, 2H), 6.60-6.72 (m, 2H), 7.11 (d, 1H), 7.25-7.45 (m, 5H), 7.79 (d, 2H), 8.05 (dd, 1H), 8.80 (d, 1H); MS(ES^+): m/z 589 ($M+NH_4$); HRMS (CI) calcd for $C_{30}H_{30}N_5O_5S$ $[M+H]^+$ 572.19676, found 572.19668.

2-{2-Ethoxy-5-[N2-acetohydrazide-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-38): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl -3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 40ml DCM with stirring at 0°C. Acetohydrazide (0.78g, 10.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed by water and dried by anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from methanol to get the pure product II-38. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.61(t, 3H), 1.76 (m, 2H), 2.02 (s, 3H), 2.76 (t, 2H), 2.87 (s, 3H), 4.34 (q, 2H), 7.11 (d, 1H), 7.92 (dd, 1H), 8.78 (d, 1H); MS(ES^+): m/z 466 ($M+NH_4$); HRMS (CI) calcd for $C_{19}H_{25}N_6O_5S$ $[M+H]^+$ 449.16071, found 449.16078.

2-[2-Ethoxy-5-(2-dimethylaminoethylamine-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-39): 4-Ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (compound II-12, 2.0g, 4.86mmol) was dissolved in 40ml DCM with stirring at 0°C. 2-Dimethylamino-ethylamine (1.0g, 11.5mmol) was added slowly. The mixture was firstly stirred at 0°C for 5 minutes and then at room temperature for 3 hours. The resulting solution was washed with water and dried over anhydrous sodium sulfate, then evaporated to get slight yellow solids. This crude product was recrystallized from acetone and methanol (1:1) to get the pure product II-39. ^{1}H NMR ($CDCl_3$) δ: 0.97 (t, 3H), 1.61(t, 3H), 1.75 (m, 2H), 2.11 (s, 3H), 2.25 (s, 3H), 2.54 (m, 2H), 2.79 (t, 2H),

2.93 (s, 3H), 3.02 (m, 2H), 4.34 (q, 2H), 7.11 (d, 1H), 7.94 (dd, 1H), 8.79 (d, 1H); MS(ES^+): m/z 480 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{31}N_6O_4S$ $[M+H]^+$ 463.21275, found 463.21277.

2.3.4 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with a different R^3 group

It has been foreseen that alternation of R^3 group may affect affinity and binding force or docking between the molecule I-33 and PDE5. Therefore, a selective group of alkyl fragments was chosen as alternatives of R^3 group to produce a further group derivatives of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13). In the above general synthetic route (Fig. 2.5), ethyl R^3 group was introduced into the target compound with 2-ethoxybenzamidine (II-9). Replacement of ethyl group with its alkyl analogues in 2-ethoxybenzamidine (II-9) followed by condensation with ethyl 2-acetamido-2-cyanopentanoate (II-8) in the same way should give a group of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with a different R^3 group.

2.3.4.1 Synthesis of 2-[2-methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-40)

2-Methoxybenzamidine (II-41): Ammonium chloride (145mg, 2.72mmol) was dissolved in cold toluene (30ml). A solution of trimethyl aluminum in hexane (2M,

1.36 ml, 2.72mmol) was added slowly to the mixture. The mixture is stirred at room temperature for 2 hours. After heated to 80°C, 180mg 2-methoxybenzonitrile (1.36mmol) was added, and then the reaction mixture was kept at 80°C for 2 days. The resulting mixture was slowly poured into a suspension of silica gel (5g) in chloroform (100ml). The resulting mixture was filtered and washed with methanol and water (1:1). The filtrate was evaporated and the residue was dissolved in 30ml DCM. This solution was extracted by 5% sodium hydroxide. After dried over anhydrous sodium sulfate, the organic phase was evaporated to dryness to give the white product 2-methoxybenzamidine (171mg, yield 84%).

***5-Acetamido-6-amino-4,5-dihydro-2-(2-methoxyphenyl)-5-propylpyrimidine-4-one (II-42)*:** 2-Methoxybenzamidine (0.9g, 5.00mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol. The mixture was stirred at room temperature for 10 minutes. After ethyl 2-acetamido-2-cyanopentanoate (1.06g, 5.00mmol) was added, the solution was heated at reflux with stirring for 0.5 hour. The system pH value is adjusted to 5.5 by acetic acid and the mixture was evaporated to dryness. The residue was extracted with 15ml DCM and filtered. The crude product was afforded by evaporating of the filtrate, which was used directly to next step.

The above crude product was directly introduced into the following arrangement and sulfonylation reactions under the same conditions as described in preparation of 2-(2-ethoxyphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-11) and preparation of 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-12). The corresponding intermediate 4-methoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-43) was afforded, which reacted with 1-ethylpiperazine to gain the compound II-40. ^{1}H NMR ($CDCl_3$) δ: 0.96-1.04 (m, 6H), 1.76(m, 2H), 2.38(q, 2H), 2.52(m, 4H), 2.74(t, 2H), 2.84(s, 3H), 3.06(br, 4H), 4.04(s, 3H), 7.11(d, 1H), 7.78(dd, 1H), 8.67(d, 1H); MS(ES^+): m/z 492 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{31}N_6O_4S$ $[M+H]^+$ 475.21275, found 475.21278.

2.3.4.2 Synthesis of 2-[2-propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-44):

***2-propoxybenzamidine (II-45)*:** Ammonium chloride (214mg, 4mmol) was dissolved in 50ml cold toluene. A solution of trimethyl aluminum in hexane (2M, 2ml, 4mmol) was added slowly to the mixture. The mixture is stirred at room temperature for 2 hours. After heated to 80°C, 322mg 2-propoxybenzonitrile (2mmol) was added, and the reaction mixture was kept at 80°C for 2 days. The resulting mixture was slowly poured into a suspension of silica gel (5g) in chloroform (100ml). The resulting mixture was filtered and washed with methanol and water (1:1). The filtrate was evaporated and the residue was dissolved in 30ml DCM. This solution was extracted by 5% sodium hydroxide. After dried over anhydrous sodium sulfate, the organic phase was evaporated to dryness to give the white product 2-propoxylbenzamidine (0.3g, yield 84%).

***5-acetamido-6-amino-4,5-dihydro-2-(2-propoxyphenyl)-5-propylpyrimidine-4-one (II-46)*:** 2-Propoxybenzamidine (1.25g, 5.00mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-acetamido-2-cyanopentanoate (1.06g, 5.00mmol) was added, the solution was heated at reflux with stirring for 0.5 hour. The system pH value is adjusted to 5.5 with acetic acid and the mixture was evaporated to dryness. The residue was extracted with 15ml DCM and filtered. The crude product was afforded by evaporating of the filtrate, which was used directly to next step.

The above crude product was directly introduced into the following arrangement and sulfonylation reactions under the same conditions as described in preparation of 2-(2-ethoxyphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-11)

and preparation of 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-12). The corresponding intermediate 4-propoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-47) was afforded, which then reacted with 1-ethylpiperazine to give the compound II-44. ^{1}H NMR ($CDCl_3$) δ: 0.95-1.03 (m, 6H), 1.19 (t, 3H), 1.73 (m, 2H), 2.01 (m, 2H), 2.38(q, 2H), 2.51 (m, 4H), 2.73 (dd, 2H), 2.82(s, 3H), 3.08 (br, 4H), 4.21 (t, 2H), 7.10 (d, 1H), 7.80(dd, 1H), 8.68 (d, 1H); MS(ES^+): m/z 520 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_6O_4S$ $[M+H]^+$ 503.24405, found 503.24412.

2.3.4.3 Synthesis of 2-[2-allyloxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-48):

***2-Allyloxybenzamidine (II-49)*:** Ammonium chloride (214mg, 4mmol) was dissolved in 50ml cold toluene. A solution of trimethyl aluminum in hexane (2M, 2ml, 4mmol) was added slowly to the mixture. The mixture is stirred at room temperature for 2 hours. After heated to 80°C, 318mg 2-allyloxybenzonitrile (2mmol) was added, and the reaction mixture was kept at 80°C for 2 days. The resulting mixture was slowly poured into a suspension of silica gel (5g) in chloroform (100ml). The resulting mixture was filtered and washed with methanol and water (1:1). The filtrate was evaporated and the residue was dissolved in 30ml DCM. This solution was extracted by 5% sodium hydroxide. After dried over anhydrous sodium sulfate, the organic phase was evaporated to dryness to give the white product 2-allyloxylbenzamidine (275mg, yield 78%).

***5-Acetamido-6-amino-4,5-dihydro-2-(2-allyloxyphenyl)-5-propylpyrimidine-4-one (II-50)*:** 2-Allyloxybenzamidine (1.30g , 5.00mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-acetamido-2-cyanopentanoate (1.06g,

5.00mmol) was added, the solution was heated at reflux with stirring for 0.5 hour. The system pH value is adjusted to 5.5 with acetic acid and the mixture was evaporated to dryness. The residue was extracted with 15ml DCM and filtered. The crude product was afforded by evaporating of the filtrate, which was used directly to next step.

The above crude product was directly introduced into the following arrangement and sulfonylation reactions under the same conditions as described in preparation of 2-(2-ethoxyphenyl)-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-11) and preparation of 4-ethoxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-12). The corresponding intermediate 4-allyloxy-3-(6-methyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-51) was afforded, which then reacted with 1-ethylpiperazine to give the compound II-48. ^{1}H NMR ($CDCl_3$) δ: 0.96-1.04 (m, 6H), 1.74 (m, 2H), 2.37 (q, 2H), 2.52 (m, 4H), 2.75 (t, 2H), 2.83 (s, 3H), 3.07 (br, 4H), 4.65 (m, 2H), 5.25 (m, 2H), 5.90 (m, 1H), 7.12 (d, 1H), 7.79 (dd, 1H), 8.69 (d, 1H), 9.97 (br, 1H); MS(ES^+): m/z 518 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{33}N_6O_4S$ $[M+H]^+$ 501.22840, found 501.22844.

2.3.5 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4- (3H)-one (II-13) with a different R^1 group

R^1 group of the compound I-33 was also considered as a biological important. The length of alkyl R^1 chain could affect the molecule binding with and docking the active pocket of the enzyme. In the general synthetic process described in Fig. 2.3, it is clear that the R^1 group was introduced with ethyl 2-acetamido-2-cyanoacetate (II-7), wherein 2-acetamido fragment was resulted from acetic anhydride acylation of ethyl 2-cyanoglycine, the reduction product of ethyl 2-cyano-2-(hydroxyimino)acetate (compound II-6) Therefore by application of a selected series of anhydrides into the

reaction to replace acetic anhydride, a group of 2-alkylamido homologous of ethyl 2-acetamido-2-cyanoacetate (II-7) would be obtained, followed by the established synthetic sequence in Fig 2.3, the group of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with a different R^1 group was produced.

2.3.5.1 Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-52)

***Ethyl 2-propionamido-2-cyanopentanoate (II-53)*:** Ethyl 2-cyano-2-(hydroxyl-imino)acetate (14.2g , 100mmol) was dissolved in acetic acid (100ml) and 4.5g zinc powder was added. With stirring, 39.4ml propionic anhydride (300mmol) was slowly added into reaction system. The reaction mixture was kept stirring at room temperature for 20 hours. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was recrystallized in ethyl acetate to give 9.8g white product ethyl 2-cyano-2-propion-amidoacetate, yield 53%, which reacted with 1-brompropane to get the main intermediate ethyl 2-propionamido-2-cyanopentanoate.

2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-52): 2-Ethoxybenzamidine (1.00g, 5.0mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-propionamido-2-cyanopentanoate (1.13g, 5.0mmol) had been added, the solution was heated at reflux with stirring for 20 minutes. The system pH value is adjusted to 5.5 by acetic acid and the mixture was evaporated to dryness. The residue was extracted with DCM and filtered. The crude product was used directly to following steps under same conditions as described above. The corresponding compound

4-ethoxyl-3-(6-ethyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenes ulfonyl chloride (II-54) was afforded. It reacted with 1-ethylpiperazine to gain compound II-52. ^{1}H NMR ($CDCl_3$) δ: 0.94-1.04 (m, 6H), 1.56-1.60(m, 6H), 1.75 (m, 2H), 2.37(q, 2H), 2.53(m, 4H), 2.75-2.88(m, 4H), 3.06 (m, 4H), 4.34(q, 2H), 7.11 (d, 1H), 7.78(dd, 1H), 8.69(d, 1H), 9.95 (br, 1H); MS(ES^+): m/z 520 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_6O_4S$ $[M+H]^+$ 503.24405, found 503.24417.

2.3.5.2 Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-morpholino-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-55)

***Ethyl 2-cyano-2-(2-morpholinoacetamido)pentanoate (II-56)*:** Ethyl 2-cyano-2-(hydroxyimino)acetate (14.2g ,100mmol) was dissolved in acetic acid (100ml) and 4.5g zinc powder was added. With stirring, 2-morpholinoacetic and acetic mix-anhydride (56.1g, 300mmol) was slowly added into reaction system. The reaction mixture was kept stirring at room temperature for 20 hours. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was recrystallized in ethyl acetate to give 9.12g white product ethyl 2-cyano-2-(2-morpholinoacetamido)acetate, yield 38%. This product was reacted with 1-brompropane to get the main intermediate ethyl 2-cyano-2-(2-morpholinoacetamido)pentanoate.

***2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-morpholino-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-55)*:** 2-Ethoxybenzamidine (1.00g, 5.0mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-cyano-2-(2-morpholinoacetamido)pentanoate (1.42g, 5.0mmol) had been added, the solution was heated at reflux with stirring for 20 minutes. The system pH value is adjusted to 5.5 by acetic acid and the mixture was evaporated to dryness. The residue was extracted with DCM and filtered. The crude product was used

directly to following steps under same conditions as described above. The corresponding compound 4-ethoxyl-3-(6-morpholinomethyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-57) was afforded. It reacted with 1-ethylpiperazine to gain compound II-55. ^{1}H NMR ($CDCl_3$) δ: 0.94-1.04 (m, 6H), 1.61(t, 3H), 1.74 (m, 2H), 2.34-2.55 (m, 10H), 2. 75 (t, 2H), 3.07 (m, 4H), 3.68 (m, 4H), 3.94 (s, 2H), 4.34(q, 2H), 7.12 (d, 1H), 7.78(dd, 1H), 8.70(d, 1H); MS(ES^+): m/z 587 ($M+NH_4$); HRMS (CI) calcd for $C_{27}H_{40}N_7O_5S$ $[M+H]^+$ 574.28116, found 574.28121.

2.3.5.3 Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-(2-pyrimi-dinyl-methyl)-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4- (3H)-one (II-58)

***Ethyl 2-cyano-2-[2-(pyrimidin-2-yl)acetamido]pentanoate (II-59)*:** Ethyl 2-cyano -2-(hydroxyimino)acetate (14.2g, 100mmol) was dissolved in acetic acid (100ml) and 4.5g zinc powder was added. With stirring, 2-(pyrimidin-2-yl)acetic and acetic mix-anhydride (54g, 300mmol) was slowly added into reaction system. The reaction mixture was kept stirring in room temperature for 20 hours. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was recrystallized in acetone to give 9.8g white product ethyl 2-cyano-2-[2-(pyrimidin-2-yl)acetamido]acetate, yield 42%. This product was reacted with 1-brompropane to get the main intermediate ethyl 2-cyano-2-[2-(pyrimidin-2-yl) acetamido]pentanoate.

***2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-(2-pyrimidinyl-methyl)-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-58)*:** 2-Ethoxybenzamidine (1.00g, 5.0mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-cyano-2-[2-(pyrimidin- 2-yl)acetamido]pentanoate (1.38g, 5.0mmol) had been

added, the solution was heated at reflux with stirring for 20 minutes. The system pH value is adjusted to 5.5 by acetic acid and the mixture was evaporated to dryness. The residue was extracted with DCM and filtered. The crude product was used directly to following steps under same conditions as described above. The corresponding compound 4-ethoxyl-3-[6-(2-pyrimidinyl-methyl)-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl]-benzenesulfonyl chloride (II-60) was afforded. It reacted with 1-ethylpiperazine to gain compound II-58. ^{1}H NMR ($CDCl_3$) δ: 0.94-1.04 (m, 6H), 1.60 (t, 6H), 1.75 (m, 2H), 2.37(q, 2H), 2.53(m, 4H), 2.75 (t, 2H), 3.06 (m, 4H), 4.01 (s, 2H), 4.35(q, 2H), 7.11-7.15 (m, 2H), 7.77 (dd, 1H), 8.68-8.71(m, 3H); MS(ES^+): m/z 570 ($M+NH_4$); HRMS (CI) calcd for $C_{27}H_{35}N_8O_4S$ $[M+H]^+$ 567.25020, found 567.25011.

2.3.6 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5- triazine-4-(3H)-one (II-13) with a different R^2 group

Referenced to the synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13), we made ethyl 2-acetamido-2-cyanoacetate (II-7) reacted with different halohydrocarbons instead of 1-bromopropane used in synthetic process of ethyl 2-acetamido-2-cyanopentanoate (II-8) to get the corresponding alkylated compound. Followed by the established synthetic sequence in Fig 2.3, the group of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with a different R^2 group was produced.

2.3.6.1 Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-yl-sulfonyl)-phenyl]-6-methyl-8-allyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-61)

***Ethyl 2-acetamido-2-cyanopent-4-enoate (II-62)*:** Ethyl 2-acetamido-2-cyanoacetate (17.0g, 100mmol) and 3-bromoprop-1-ene (13.5g, 110mmol) was added to a preparation of sodium (2.3g, 100mmol) in 50ml absolute ethanol. The mixture is stirred and heated at reflux for 3hr. After cooling down to ambient temperature, water (150ml) was added to the resulting solution. The solids are collected by filtration. After drying in vacuum, 12.3g product is afforded, yield 58%.

***2-[2-ethoxy-5-(4-ethyl-piperazine-1-yl-sulfonyl)-phenyl]-6-methyl-8-allyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (II-61)*:** Ethyl 2-acetamido-2-cyanopent-4-enoate (II-62) reacted with 2-ethoxybenzamidine (II-9) under the same conditions as synthesis of II-10, II-11 and II-12 to get the relative sulfonated compound. This compound reacted with 1-ethylpiperazine to afford compound II-61. ^{1}H NMR ($CDCl_3$) δ: 1.04 (t, 3H), 1.59 (t, 3H), 2.37(q, 2H), 2.52(m, 4H), 2.81(s, 3H), 3.09 (br, 4H), 3.18 (m, 2H), 4.31 (q, 2H), 5.68 (m, 1H), 5.01 (dd, 1H), 4.96 (dd, 1H), 7.10 (d, 1H), 7.80 (dd, 1H), 8.70 (d, 1H), 9.86(br 1H); MS(ES^+): m/z 504 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{31}N_6O_4S$ $[M+H]^+$ 487.21275, found 487.21262.

2.3.7 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5- triazine-4-(3H)-one (II-13) with altering two or more of R^1, R^2 or R^3 groups at the same time

In previous sections, by replacements of R^1, R^2, R^3 or R^5 group of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with analogue alkyl groups, produced three series derivatives have been prepared. In order to explore effects of R^1, R^2 or R^3 group on biological activities further, in this section, a few examples of 2-[2-ethoxy-5-(4-ethyl-piperazine-

1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) with altering two or more of R^1, R^2 or R^3 groups at once were presented.

2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1, 5-a]- 1,3,5-triazine-4-(3H)-one (II-63) in which R^1 and R^3 groups are different from those in 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]- 1,3,5-triazine-4-(3H)-one (II-13)

In the synthesis of II-63, relative to synthesis of II-13, we change the reaction compound ethyl 2-acetamido-2-cyanopentanoate (II-8, fig. 2.3) to ethyl 2-propionamido-2-cyanopentanoate (II-64, fig. 2.6) and change the reaction compound 2-ethoxy-benzamidine to 2-propoxybenzamidine (II-65, fig. 2.6). A new potential PDE5 inhibitor will be synthesized. The synthetic details were illustrated below.

2-Propoxybenzamidine (0.9g, 5.00mmol) was added into a 5ml solution of sodium metal (0.23g, 10mmol) in absolute ethanol, the mixture was stirred at room temperature for 10 minutes. After ethyl 2-propionamido-2-cyanopentanoate (1.13g, 5.00mmol) had been added, the solution was heated at reflux with stirring for 20 minutes. The system pH value is adjusted to 5.5 using acetic acid and the mixture was evaporated to dryness. The residue was extracted with DCM and filtered. The crude product of 5-propionamino-6-amino-4,5-dihydro-2-(2-propoxyphenyl)-5-propyl-pyrimidine-4-one (II-66, Fig. 2.6) was afforded by evaporating of the filtrate, which was used directly to next step. Under the same conditions as synthesis of II-11 and II-12, 4-propoxyl-3-(6-ethyl-4-oxo-8-propyl-3H-imidazo[1,5-a]-1,3,5-triazine-2-yl)-benzenesulfonyl chloride (II-67) was afforded. Followed by the reaction with 1-ethylpiperazine, the target compound II-63 was given. ^{1}H NMR ($CDCl_3$) δ: 0.94-1.05 (m, 9H), 1.54 (t, 3H), 1.76-1.82 (m, 4H), 2.40 (q, 2H), 2.52 (m, 4H), 2.75-2.86 (m, 4H), 3.07 (br, 4H), 4.28 (q, 2H), 7.11 (d, 1H), 7.78 (dd, 1H), 8.67 (d, 1H); MS(ES^+): m/z 534 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{37}N_6O_4S$ $[M+H]^+$

517.25970, found 517.25977.

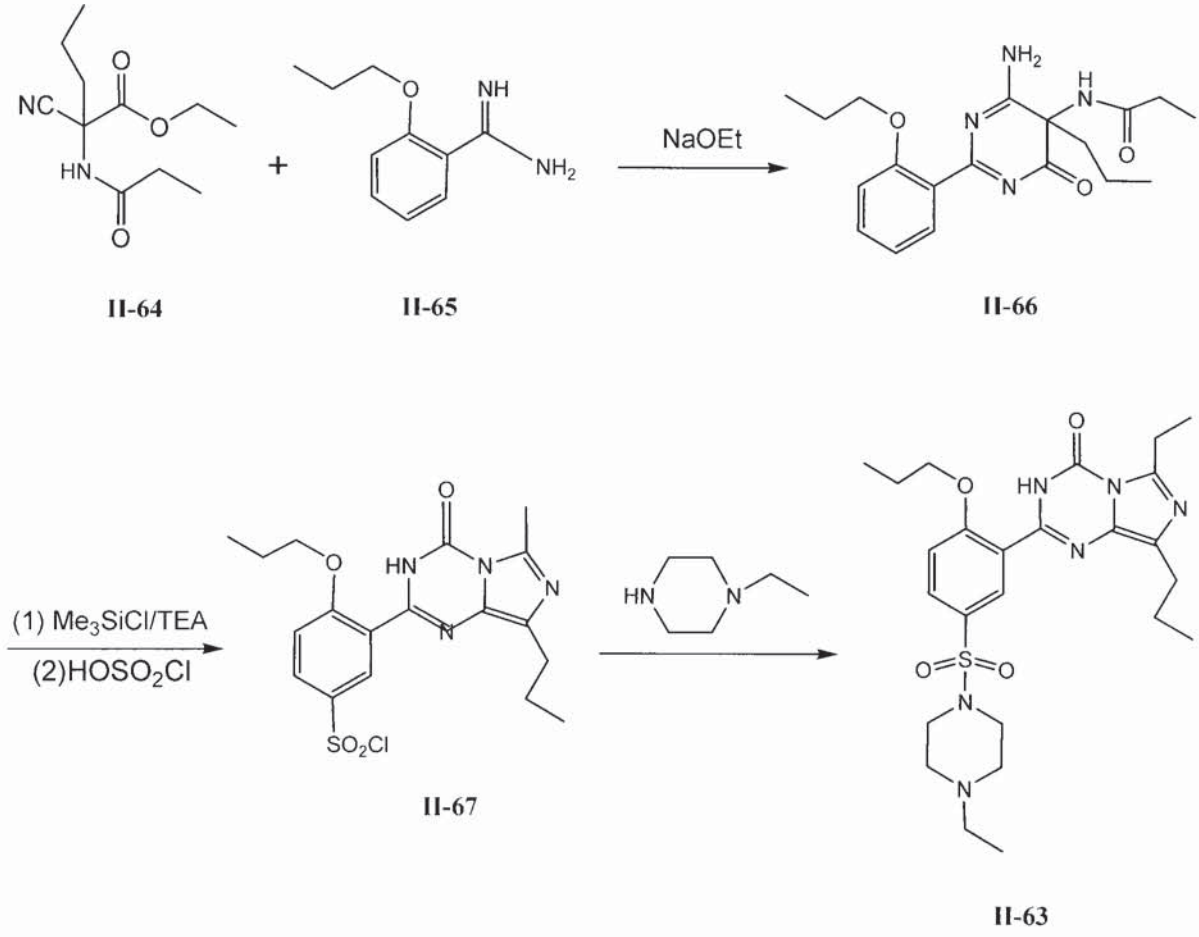


Figure 2.6 Synthetic route of II-63

***Synthesis of different R^1, R^3 and R^5 substituted II-13 analogues of 2-[2-propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-68)*:** The reaction of ethyl 2-propionamido-2-cyanopentanoate (II-64) with 2-propoxyl- benzamidine (II-65) under the same conditions as synthesis of II-63 (fig. 2.6) gave the corresponding compound II-67. This compound reacted with 1-methylpiperazine to afford compound II-68. ^{1}H NMR ($CDCl_3$) δ: 0.96 (t, 6H), 1.56-1.60(m, 3H), 1.75 (m, 4H), 2.28 (s, 3H), 2.53(m, 4H), 2.75-2.88(m, 4H), 3.06 (m, 4H), 4.34(q, 2H), 7.11 (d, 1H), 7.78(dd, 1H), 8.69(d, 1H); MS(ES^+): m/z 506 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_6O_4S$ $[M+H]^+$ 503.24405, found 503.24416.

2.3.8 Preparation of water-soluble salts of 3H-imidazo[1,5-a][1,3,5]triazin-4-one analogues

Syntheses of the group of 2-(substituted-sulfonylphenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-ones derivatives for developments of new PDE5 inhibitors were described in the previous section. In order to facilitate their oral administration, it would be better to turn them into their corresponding water-soluble salts with an organic or inorganic acid. Here we demonstrated their conversions from the basic forms into corresponding mono- or bi-acid salts.

Synthesis of compound II-13•HCl salt 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate: 2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) (1.00g, 2.05mmol) was dissolved in ether (10ml) and DCM (10ml). 4M solution of hydrochloric acid-dioxane (0.51ml, 2.04mmol) was diluted by 10ml ether and added dropwish into the stirred solution. After stirring at room temperature for 20 minutes, the solid product was filtered and dried to give 1.02g hydrochlorate, yield 95%. M.p.: 219-221°C; IR (cm^{-1}): 2954, 2937, 2867, 2433, 1745, 1604, 1462, 1357, 1284, 1245, 1166, 1153, 731, 611; ^1H NMR (D_2O) δ: 0.85 (t, J=7.1 Hz, 3H), 1.29 (t, J=7.0Hz, 3H), 1.47-1.58 (m, 5H), 2.49 (m, 2H), 2.58 (s, 3H), 3.23 (q, 2H), 3.42 (brs, 8H), 4.22 (m, 2H), 7.20 (d, 1H), 7.79(d, 1H), 8.05(s, 1H); ^1H NMR ($CDCl_3$) δ: 0.98 (t, 3H), 1.44 (t, 3H), 1.65 (t, 3H), 1.79 (q, 2H), 2.76 (t, 2H), 2.86 (s, 3H), 3.06 (q, 2H), 3.18 (b, 2H), 3.46 (b, 2H), 3.86 (b, 4H), 4.35(dd, 2H), 7.14 (d, 1H), 7.75 (d, 1H), 8.65 (s, 1H), 10.01 (b, 1H); HRMS (CI) calcd for $C_{23}H_{34}ClN_6O_4S$ $[M+H]^+$ 525.20508, found 525.20511.

Synthesis of compound II-13•2HCl salt 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one bi-hydrochlorate:2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-

propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) (2.00g, 4.10mmol) was dissolved in ether (20ml) and DCM (20ml). 4M solution of hydrochloric acid-dioxane (2.05ml, 8.20mmol) was diluted by 20ml ether and added dropwish into the stirred solution. After stirring at room temperature for 20 minutes, the solid product was filtered and dried to give 2.08g bi-hydrochlorate, yield 95%. M.p.: 213-215°C; IR (cm^{-1}): 2960, 2931, 2871, 2723, 2684, 2607, 1766, 1614, 1488, 1458, 1274, 1159, 1036, 951, 725, 582; ^1H NMR (D_2O) δ: 0.93 (t, J=7.1 Hz, 3H), 1.29 (t, J=7.0Hz, 3H), 1.46 (t, J=6.4Hz, 3H), 1.69-1.77 (m, 2H), 2.84 (m, 4H), 3.03 (s, 3H), 3.20 (m, 4H), 3.64-3.69 (d, 2H), 3.90-3.95 (d, 2H), 4.31 (m, 2H), 7.38 (d, 1H), 7.98(d, 1H), 8.09(s, 1H); HRMS (CI) calcd for $C_{23}H_{35}Cl_2N_6O_4S$ $[M+H]^+$ 561.18175, found 561.18179.

Synthesis of Compound II-14•2HCl salt 2-[2-ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one bi-hydrochlorate: 2-{2-Ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl]amino-sulfonyl}-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-14) (1.03g, 2.0mmol) was dissolved in ether (10ml) and DCM (10ml). 4M solution of hydrochloric acid in dioxane (1.0ml, 4.0mmol) was diluted by 20ml ether and added dropwish into the stirred solution. After stirring at room temperature for 20 minutes, the solid product was filtered and dried to give 1.02g bi-hydrochlorate, yield 93%. ^1H NMR (D_2O) δ: 0.85 (t, 3H), 1.38 (t, 3H), 1.63 (dd, 2H), 2.78 (m, 4H), 2.83 (s, 3H), 2.96 (s, 3H), 3.19 (t, 2H), 3.52 (d, 2H), 3.84 (d, 2H), 4.22 (m, 2H), 7.34 (d, 1H), 7.92(d, 1H), 8.02 (s, 1H); HRMS (CI) calcd for $C_{22}H_{33}Cl_2N_6O_4S$ $[M+H]^+$ 547.16610, found 547.16625.

Synthesis of compound II-15•2HCl salt 2-[2-Ethoxy-5-(4-ethoxycarbonylpiperazine -N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one bi-hydrochlorate: 2-[2-Ethoxy-5-(4-ethoxycarbonylpiperazine-N-sulfonyl)-phenyl] -6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one(II-15) (1.06g, 2.0mmol) was dissolved in ether (10ml) and DCM (10ml). 4M solution of hydrochloric acid in dioxane (1.0ml, 4.0mmol) was diluted by 20ml ether and added dropwish into the

stirred solution. After stirring at room temperature for 30 minutes, the solid product was filtered and dried to give 1.04g bi-hydrochlorate, yield 94%.^{1}H NMR ($CDCl_3$) δ: 1.00 (t, 3H), 1.18 (t, 3H), 1.65 (t, 3H), 1.91 (m, 2H), 2.99 (m, 5H), 3.20 (s, 4H), 3.56 (m, 4H), 4.05 (dd, 2H), 4.16 (m, 2H), 7.19 (d, 1H), 7.86 (d, 1H), 8.55 (s, 1H), 10.94 (b, 1H); HRMS (CI) calcd for $C_{24}H_{35}Cl_2N_6O_6S$ $[M+H]^+$ 605.17158, found 605.17152.

2.4 Conclusions

A series of 2-(substituted-sulfonylphenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one analogues was prepared, they are structurally similar to cGMP and sildenafil. Compound II-13 was synthesized successfully in a seven-step method. By employing different starting materials, different R^1, R^2, R^3 and R^4 substituted II-13 analogues which will be used in anti ED study were synthesized. The water soluble salt forms of these compounds were also synthesized.

Chapter 3

Synthesis of PDE5 Inhibitors of 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one

3.1 Introduction

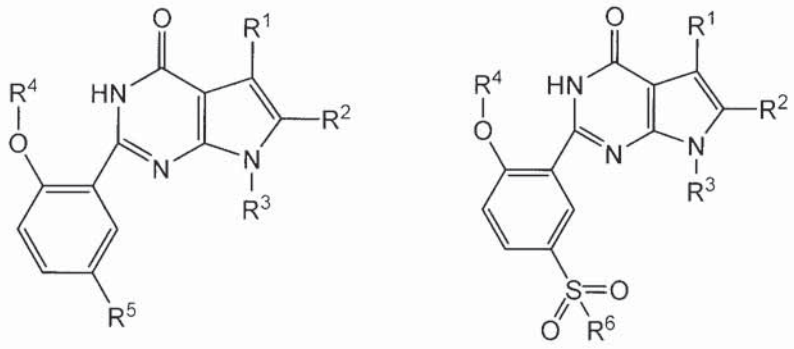
As described in the chapter 1, in comparison with sildenafil and vardenafil, 3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (I-34) was considered as another desired six fused five-membered ring to explore for new potential PDE5 inhibitors. In this chapter, we discussed selection of synthetic strategies, synthetic routes, characterizations and preparation details for 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one derivatives.

3.2 Design of synthetic routes to 2-(substituted-sulfonylphenyl)-3,7-dihydro–pyrrolo[2,3-d]pyrimidin-4-ones

3.2.1 The route to 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (I-34)

The selection of 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (I-34) as the nucleus structure of new PDE5 inhibitors to explore because it possesses similar number positions to allow substituents to be introduced onto the rings to build up a overall scaffolding same to sildenafil and vardenafil (Fig. 3.1). This will reduce a great deal of dilemma in selections of R^1, R^2, R^3, $R^4$and R^5 groups from a pool of substitutes,

because we could follow the successful tracks of sildenafil and vardenafil to introduce dedicated number of substituents on purpose, particularly the R^5 was sulfonyl group $-SO_2R^6$ (III-1, Fig. 3.1) definitely.



I-34 III-1

Figure 3.1 The molecular structures of I-34 and III-1

3.2.2 A general synthetic route for 3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (I-34)

Retro-synthesis analysis indicated that the pyrimidine ring in I-34 could be constructed from condensation of the carbamoyl group and the amido group in III-5, in which the carbamoyl group was resulted from the reduction of the cyano group in III-4. Acylation between 2,5-substituted benzoyl chloride (III-2) and the 2-amino-3-cyano-1,4,5-sustituted pyrrole (III-3) was one preferred choice when R^3 is not H, because providing less opportunities for acylation on the pyrrole ring (Fig. 3.2). However, by controlling the reaction conditions carefully, acylation of 1-H 2-amino-3-cyano-4,5-substituted pyrrole (III-6) with 2,5-substituted benzoyl chloride (III-2) was performed well, and it provided an alternative strategy to synthesize 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (I-34) (Fig. 3.3).

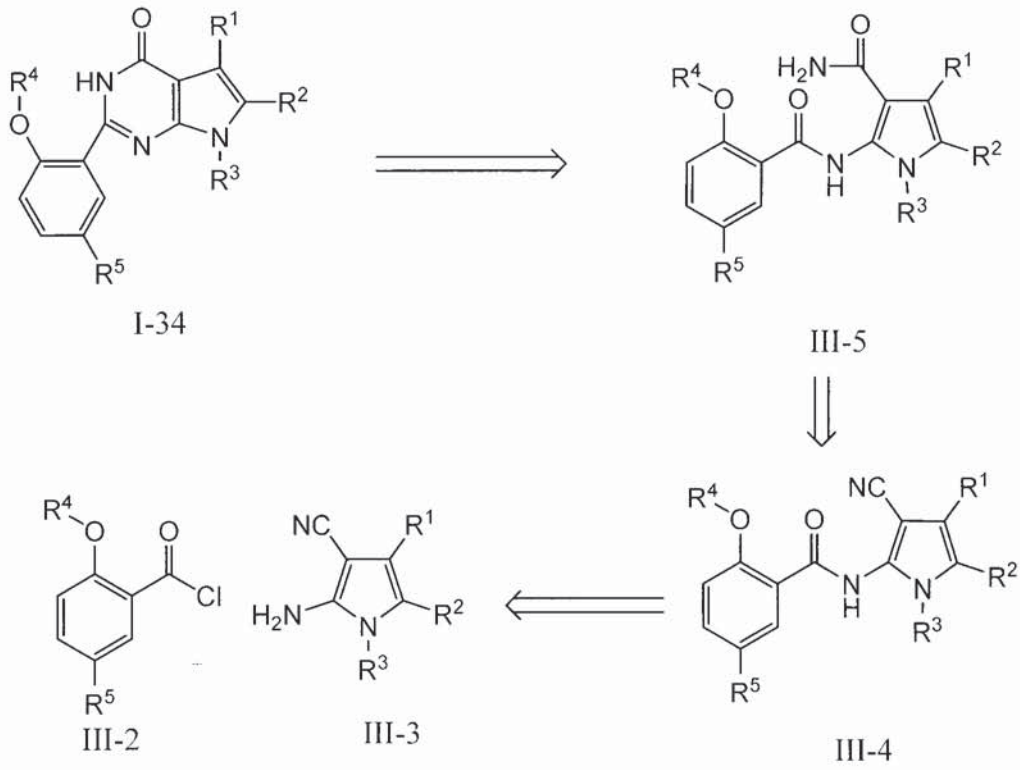


Figure 3.2 The general synthetic route of I-34

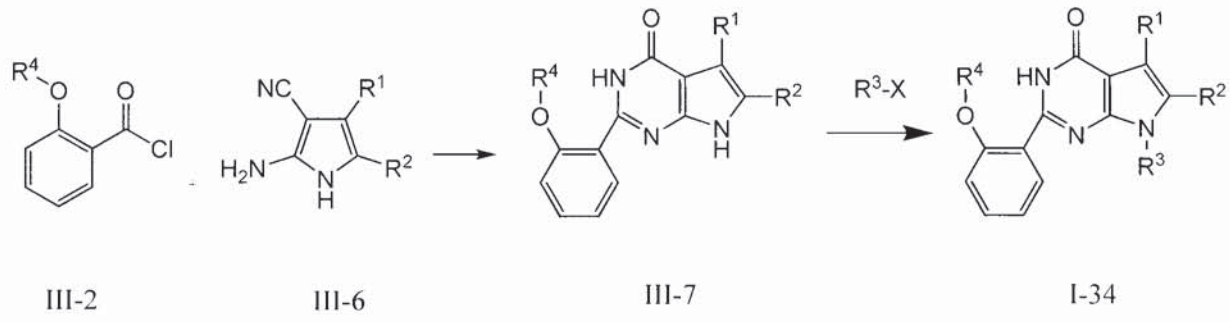


Figure 3.3 Specific synthetic route which could directly react from III-7 to I-34

3,7-Dihydro-pyrrolo[2,3-d] pyrimidin-4-one (I-34) reacts with chlorosulfonic acid to give the sulfonic acid derivative (III-1) (Fig. 3-4), which is the key precursor for making 2-[2-alkoxy-5-substitutedsulphonyl]phenyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-ones through sulfonation with corresponding amine derivatives as described in Chapter 2.

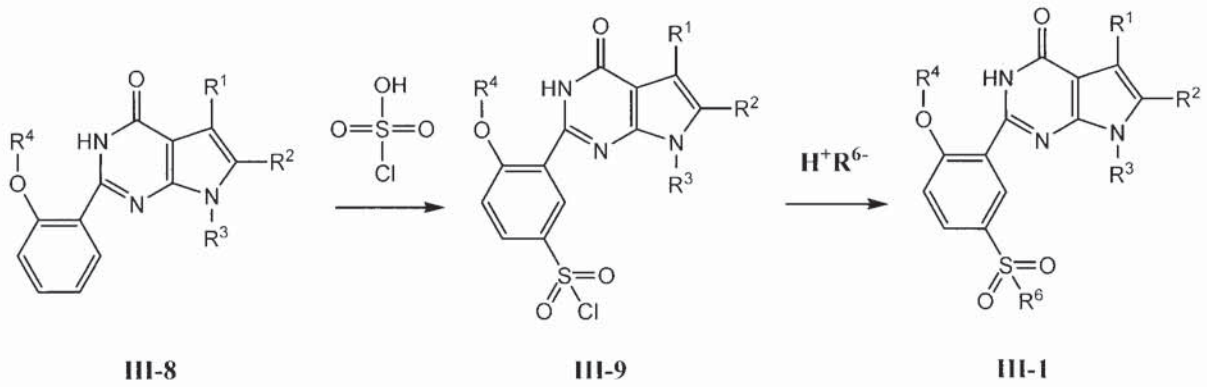


Figure 3.4 Specific synthetic route which could give specific structure of III-1

The predicted total synthetic route for 2-[2-alkoxy-5-substitutedsulphonyl] phenyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-ones was showed in Fig. 3.5.

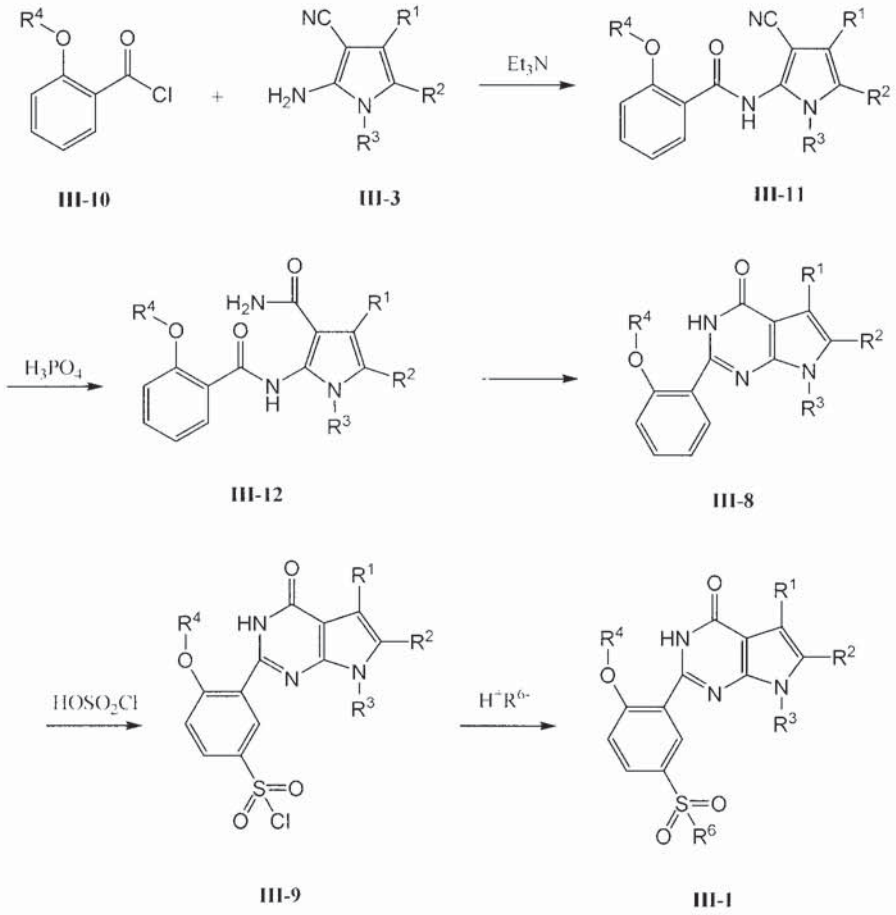


Figure 3.5 The general synthetic route of III-1 compounds

3.3 Experiments section

3.3.1 Equipment, materials and General Methods

Melting points were determined on a Reichert-Jung Microthermal apparatus which was uncorrected. NMR spectra were recorded on a Bruker AC250 Spectrometer at ^{1}H (250.1 MHz) and ^{13}C (62.9 MHz). Chemical shifts are downfield of tetramethylsilane. Mass spectroscopic analysis was carried out on a Hewlett Packard 5989B MS engine with an HP 5998A API Electrospray LC/MS interface; the LC was taken on Agilent HPLC meter with HP1100 system and autosampler. High resolution mass spectra (HRMS) were measured on a Finnigan MAT 900 XLT high resolution double focusing mass spectrometer using an electrospray method. Infrared spectra were recorded on a Mattson 3000 FTIR Spectrometer; solid samples were prepared as KBr discs and liquids as thin films between sodium chloride plates. Flash column chromatography was performed using Sorbsil C60 silica gel. TLC was carried out using aluminium backed Merck Silica Gel 60 F254 plates and visualised under UV (254 nm). Potassium permanganate was used where appropriate to develop TLC plates. Elemental analyses (C, H, N) were performed on a Leeman 440 analyzer. Dry THF and dry ether were prepared by refluxing with sodium metal chips and distillation. The reagents used as mobile phase in chromatographic measurement were HPLC degree. Other reagents used in organic synthesis without especially mentioned were all commercial analysis degree.

3.3.2 An example of synthesis of 2-(substituted-sulfonylphenyl) -5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-1) derivatives

Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20)

The synthetic details and conditions of III-1 series compounds will be exhaustively illustrated in the following example of synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20), as shown below in Fig. 3.6.

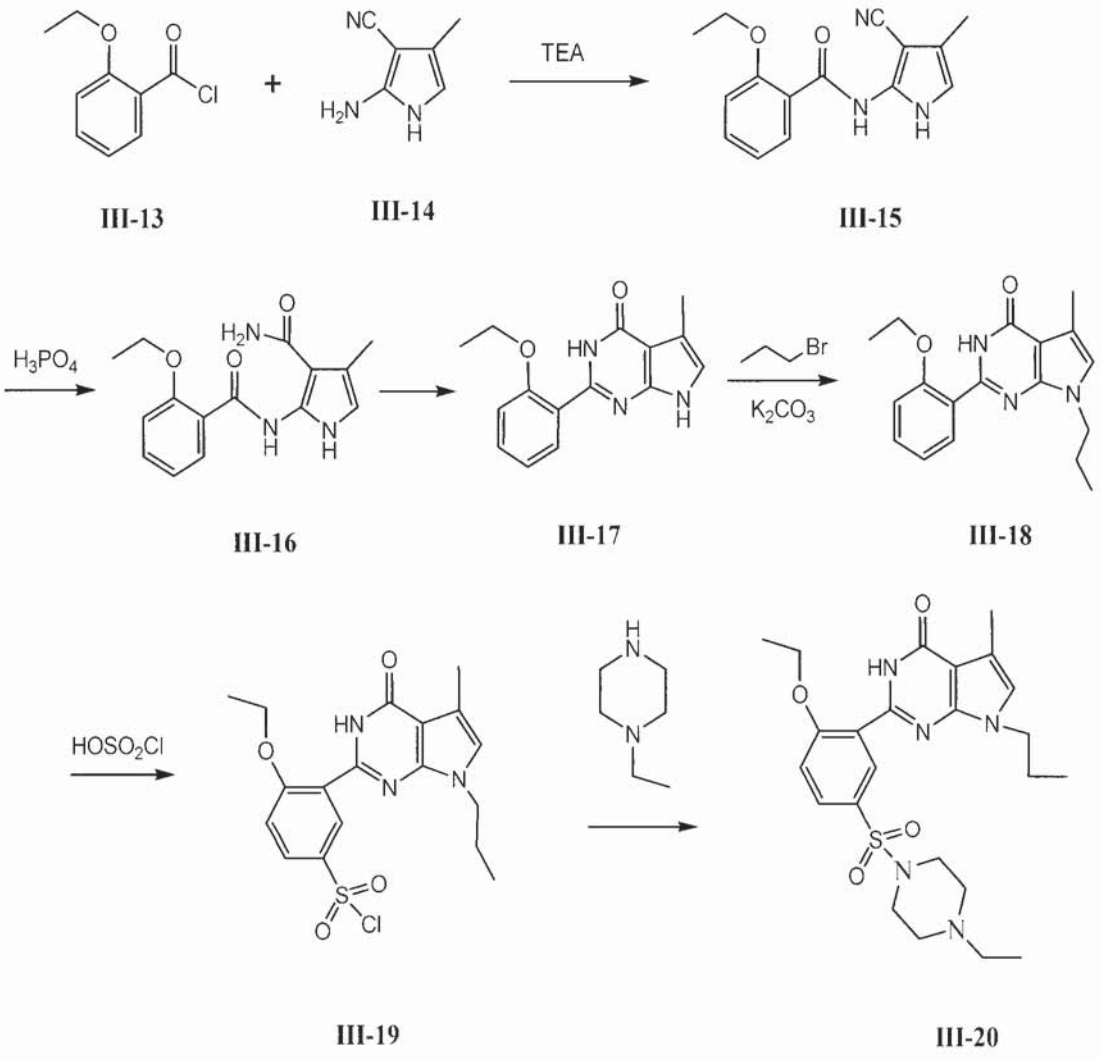


Figure 3.6 Synthetic route of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20)

List of compounds in this reaction:

III-13: 2-ethoxybenzoic chloride;

III-14: 2-amino-3-cyano-4-methylpyrrole;

III-15: N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-ethoxy-benzamide;

III-16: 2-(2-ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide;

III-17: 2-(2-ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one;

III-18: 2-(2-ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one;

III-19: 4-ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d] pyrimidin-2-yl)-benzenesulfonyl chloride;

III-20: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one.

Table 3.1 Parameters of compounds in synthesis process of III-20

Compounds	Molecular weight	Molecular structure	Element components
III-13	184.03	$C_9H_9ClO_2$	C=58.55%; H=4.91%; Cl=19.20%; O=17.33%
III-14	121.06	$C_6H_7N_3$	C=59.49%; H=5.82%; N=34.69%
III-15	269.12	$C_{15}H_{15}N_3O_2$	C=66.90%; H=5.61%; N=15.60%; O=11.88%
III-16	287.13	$C_{15}H_{17}N_3O_3$	C=62.71%; H=5.96%; N=14.63%; O=16.71%
III-17	269.12	$C_{15}H_{15}N_3O_2$	C=66.90%; H=5.61%; N=15.60%; O=11.88%
III-18	311.16	$C_{18}H_{21}N_3O_2$	C=69.43%; H=6.80%; N=13.49%; O=10.28%
III-19	409.09	$C_{18}H_{21}ClN_3O_4S$	C=52.74%; H=4.92%; Cl=8.65%; N=10.25%; O=15.61%; S=7.82%
III-20	487.22	$C_{24}H_{33}N_5O_4S$	C=59.12%; H=6.82%; N=14.36%; O=13.12%; S=6.58%

N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-ethoxy-benzamide (III-15): 2-ethoxybenzoic acid (III-13 10.0g, 60.2mmol) was heated at reflux with thionyl chloride (20ml) for 40min. The excess thionyl chloride was evaporated and the residue was dissolved in DCM (150ml). The 2-amino-3-cyano-4-methylpyrrole (III-14 7.0g, 56.8mmol) was dissolved in THF (80ml), Et_3N (8.5ml, 61.0mmol) was added. The mixture was stirred

in an ice-water bath. The above 2-ethoxybenzoic chloride in DCM solution was added dropwise in 30min, the reaction was carried out at 0°C for another 1hr. The resulting mixture was washed with water, filtered and the filtrate was evaporated at 40°C with 20g silica gel. The product was washed down from silica gel (80g) using DCM to give 7.5g solids, yield 48%. Analytical sample was prepared by chromatography (DCM: Hexane=1:2 as eluent) and recrystallisation from DCM /Hexane=1/5. M.p.: 183-184°C (sublime 162°C); IR (cm^{-1}): 3326, 3309, 2981, 2938, 2915, 2854, 2208, 1647, 1593, 1471, 1309, 1302, 1232, 1039, 923, 727, 655, 648; ^1H NMR ($CDCl_3$): δ 1.70 (t, J=7.0Hz, 3H), 2.15 (s, 3H), 4.32 (q, J=7.0Hz, 2H), 6.24 (s, 1H), 7.04(d, 1H), 7.10 (m, 1H), 7.51(dd, 1H), 8.20(dd, J=7.9 and 1.8 Hz, 1H), 10.69 (brs, 1H), 10.80 (s, 1H); ^{13}C NMR ($CDCl_3$): δ (CH_3) 10.6, 15.0; (CH_2) 65.7; (CH) 110.3, 112.3, 121.4, 132.1, 134.2 ; (C) 78.7, 115.6, 119.2, 119.4, 136.7, 157.0, 163.2; MS (ES^+): m/z 287 ($M+NH_4$).

***2-(2-ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide (III-16)*:** N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-ethoxy-benzamide (2.00g, 7.44mmol) was mixed with 85% phosphorus acid (14.8ml). The mixture was stirred and heated to 130°C for 20min. The resulting mixture was cooled and poured into crushed ice (80g). The precipitate was filtered and dried to give 1.7g dark red powders, yield 80%. This product is pure enough to be used in next step.

***2-(2-ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-17)*:** 2-(2-Ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide (III-16) (7.0g, 25.5mmol) was heated at reflux with sodium hydroxide (16g, 400mmol) in ethanol (100ml) for 14hr. Ethanol was evaporated under reduced pressure. The residue was extracted with DCM, the DCM layer was washed by water, and dried over sodium sulfate. The solution was evaporated and worked up by hexane to give 6.0g product, yield 91%. M.p.: 219-221°C; IR (cm^{-1}): 3187, 3114, 3062, 2978, 2923, 1658, 1587, 1460, 1321, 1292, 1250, 1044, 771, 763; ^1H NMR (DMSO-d_6): δ 1.35 (t, J=6.9Hz, 3H), 2.29 (s, 3H), 4.13 (q, J=7.0Hz, 2H), 6.79 (s, 1H), 7.05 (t, 1H), 7.14(d,

1H), 7.45(dd, 1H), 7.76 (dd, 1H), 11.35 (brs, 1H), 11.54 (brs, 1H); ^{13}C NMR (DMSO-d_6): δ (CH_3) 11.2, 14.5; (CH_2) 64.2; (CH) 113.0, 118.0, 120.6, 130.1, 131.9; (C) 105.0, 113.6, 121.9, 148.5, 149.8, 156.5, 159.2; MS (ES^+): m/z 287 ($M+NH_4$).

2-(2-ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-18): 2-(2-Ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (1.5g, 5.57 mmol), 1-bromopropane (2.0g, 16.3mmol) and potassium carbonate (5g, 36.2mmol) were mixed in acetone (15ml). This mixture was heated at reflux for 15hr. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was submitted to chromatography (DCM as eluent) to give 0.6g final product, yield 35%. M.p. 124-127°C; IR (cm^{-1}): 3234, 3184, 3141, 3103, 3056, 2956, 2943, 2869, 1654, 1595, 1567, 1468, 1311, 1267, 1243, 1191, 1118, 1047, 758; ^1H NMR ($CDCl_3$): δ 0.88 (t, J=7.5Hz, 3H), 1.23 (t, 3H), 1.80 (q, 2H), 2.42 (s, 3H), 4.08 (t, J=7.2Hz, 2H), 4.22 (q, 2H), 6.60 (s, 1H), 7.01(d, J=8.3Hz, 1H), 7.08 (t, 1H), 7.40 (m, 1H), 8.35 (dd, J=8.0 and 1.9Hz, 1H), 11.02 (brs, 1H).

4-ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19): 2-(2-Ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (1.25g, 4.01mmol) was added in two portions to chlorosulfonic acid (4ml) at 0°C with stirring. The mixture was stirred at 0°C for 30min and room temperature for 3 hr. The resulting solution was poured into a mixture of ethyl acetate (50ml) and ice-water (50ml). The organic layer was separated and washed with cold water (5ml). Evaporation under reduced pressure give the product as 1.33g yellow foam, yield 81%. This product was used directly to next step.

2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20): 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (1.00g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-Ethylpiperazine (0.78ml, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 5min,

then at room temperature for 5hr. The resulting solution was washed with water, dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow foam which was submitted to chromatography (EtOAc/MeOH 20/1 as eluent) to give 0.89g light yellow solids, yield 75%. M.p. 174-176°C (EtOAc); IR (cm^{-1}): 3324, 2960, 2923, 2869, 2862, 2767, 1682, 1560, 1458, 1355, 1282, 1247, 1172, 1149, 739, 615, 588, 555; ^1H NMR ($CDCl_3$): δ 0.89 (t, J=7.4Hz, 3H), 0.99 (t, J=7.2Hz, 3H), 1.61 (t, J=7.0Hz, 3H), 1.77-1.86 (m, 2H), 2.35 (m, 2H), 2.41 (s, 3H), 2.50 (brs, 4H), 3.05 (brs, 4H), 4.08 (t, J=7.0Hz, 2H), 4.29-4.37 (q, 2H), 6.61 (s, 1H), 7.11(d, J=8.8Hz, 1H), 7.77 (dd, J=8.7 and 2.2 Hz, 1H), 8.74(d, J=2.2, 1H), 10.63 (brs, 1H); ^{13}C NMR ($CDCl_3$): δ (CH_3) 11.0, 11.3, 11.8, 14.3; (CH_2) 23.8, 45.9, 46.1, 51.6, 51.7, 65.8; (CH) 112.9, 121.1, 130.6, 131.3; (C) 105.7, 114.6, 121.4, 127.8, 146.8, 147.3, 159.3, 159.6; MS (ES^+): m/z 505 ($M+NH_4$); FAB-MS m/z: 537.2289 (M+H); Elemental analysis calculated(%) for $C_{24}H_{33}N_5O_4S$: C 59.12, H 6.82, N 14.36; found(%): C 59.16, H 6.79, N 14.38. HRMS (CI) calcd for $C_{24}H_{34}N_5O_4S$ $[M+H]^+$ 488.23315, found 488.23321.

3.3.3 Synthesis of the analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) with a different R^6 group

***2-[2-Ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-21)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.00g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-Methylpiperazine (0.61g, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water, dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow foam which was submitted to chromatography (EtOAc/MeOH 20/1 as eluent)

to give 0.91g light yellow solids of III-21, yield 75%. ^{1}H NMR (D_2O) δ: 0.90 (t, 3H), 1.62 (s, 3H), 1.78 (m, 2H), 2.15(s, 3H), 2.41(s, 3H), 2.48(brs, 4H), 3.04(brs, 4H), 4.08(t, 2H), 4.34(q, 2H), 6.61(s, 1H), 7.09(d, 1H), 7.74(dd, 1H), 8.76(d, 1H); MS (ES^+): m/z 491 ($M+NH_4$); HRMS (CI) calcd for $C_{23}H_{32}N_5O_4S$ $[M+H]^+$ 474.21750, found 474.21757.

***2-[2-Ethoxy-5-(4-ethoxycarbonyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-22)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g , 2.44mmol) was dissolved in DCM (20ml) at 0°C. Ethyl piperazine-1-carboxylate (0.96g, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water, dried over sodium sulfate. Evaporation of the solvent gave 1.3g crude product. This crude product was recrystallized with ethanol to give 0.96g light yellow solids of III-22, yield 79%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.25 (t, 3H), 1.62 (t, 3H), 1.83 (m, 2H), 2.41 (s, 3H), 2.48 (brs, 4H), 3.07 (brs, 4H), 4.05 (q, 2H), 4.32 (q,2H), 4.41 (q, 2H), 6.62(s, 1H), 7.12 (d, 1H), 7.81 (d, 1H), 8.67 (s, 1H); MS (ES^+): m/z 549 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{34}N_5O_6S$ $[M+H]^+$ 532.22298, found 532.22310.

***2-[2-Ethoxy-5-[4-(2-hydroxy-ethyl)-piperazine-1-sulfonyl]-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-23)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 2-(Piperazin-1-yl) ethanol (0.79g, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water, dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow crude product. This crude product was recrystallized with ethanol to give 0.89g light yellow solids of III-23, yield 74%. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, 3H), 1.61(t, 3H), 1.72(m, 2H), 2.41(s, 3H), 2.49-2.58 (m, 6H), 3.07(brs, 4H), 3.67(m, 2H),

4.08(t, 2H), 4.33(q, 2H), 6.61(s, 1H), 7.10 (d, 1H), 7.76(dd, 1H), 8.69(d, 1H); MS (ES^+): m/z 521 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{34}N_5O_5S$ $[M+H]^+$ 504.22806, found 504.22803.

***2-[2-Ethoxy-5-(pyrrolidine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-24)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. Pyrrolidine (0.43g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water, dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow crude product. This crude product was recrystallized with ethanol to give 0.89g light yellow solids of III-24, yield 76%.^{1}H NMR($CDCl_3$) δ: 0.89 (t, 3H), 1.65 (t, 3H), 1.81 (m, 6H), 2.41(s, 3H), 3.25 (m, 4H), 4.05(t, 2H), 4.37 (q, 2H), 6.62(s, 1H), 7.10 (d, 1H), 7.80 (dd, 1H), 8.77 (d, 1H). MS (ES^+): m/z 462 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{29}N_4O_4S$ $[M+H]^+$ 445.19095, found 445.19104.

***2-{2-Ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl]aminosulfonyl}-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-25)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-(3-Amino-propyl)-pyrrolidin- 2-one (0.87g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.4g light yellow crude product. This crude product was recrystallized with methanol to give 0.92g light yellow solids of III-25, yield 79%. ^{1}H NMR($CDCl_3$) δ: 0.90 (t, 3H), 1.65 (t, 3H), 1.73 (m, 4H), 2.02 (m, 2H), 2.32 (t, 2H), 2.40(s, 3H), 2.75 (t, 2H), 2.86 (m, 2H), 3.09 (m, 2H), 4.04(t, 2H), 4.30 (q, 2H), 6.60(s, 1H), 7.08 (d, 1H), 7.85 (dd, 1H), 8.71 (d, 1H); MS (ES^+): m/z 533 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{34}N_5O_5S$ $[M+H]^+$ 516.22806, found 516.22804.

***2-{2-Ethoxy-5-{N-[2-(2-oxy-1-pyrrolidinyl)ethyl]aminosulfonyl}-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-26)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19. 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-(2-Aminoethyl)pyrrolidin-2-one (0.78g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light yellow crude product. This crude product was recrystallized with acetone to give 1.01g light yellow solids of III-26, yield 82%. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, 3H), 1.62 (t, 3H), 1.79 (m, 4H), 1.83-2.20 (m, 6H), 2.41 (s, 3H), 3.40 (m, 2H), 4.05 (t, 2H), 4.37 (q, 2H), 6.60(s, 1H), 7.12 (d, 1H), 7.60 (dd, 1H), 8.75 (s, 1H); MS (ES^+): m/z 505 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{32}N_5O_5S$ $[M+H]^+$ 502.21241, found 502.21244.

***2-[2-Ethoxy-5-(morpholine-4-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-27)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19. 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. Morpholine (0.53g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.92g light yellow solids of III-27, yield 73%.^{1}H NMR ($CDCl_3$) δ: 0.91 (t, 3H), 1.64 (t, 3H), 1.77 (m, 2H), 2.42(s, 3H), 3.06 (m, 4H), 3.74 (m, 4H), 4.05(t, 2H), 4.37 (q, 2H), 6.60(s, 1H), 7.12 (d, 1H), 8.00 (d, 1H), 8.77 (s, 1H), 10.33 (br, 1H); MS (ES^+): m/z 478 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{29}N_4O_5S$ $[M+H]^+$ 461.18587, found 461.18582.

***2-[2-Ethoxy-5-(3-(morpholin-4-yl)-propylamine-N-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-28)*:** 4-Ethoxy-3-(5-methyl-4-

oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 3-(Morpholin-4-yl)-propylamine (0.88g , 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light yellow crude product. This crude product was recrystallized with acetone to give 0.96g light yellow solids of III-28, yield 75%. ^{1}H NMR($CDCl_3$) δ: 0.90 (t, 3H), 1.61 (t, 3H), 1.74 (m, 4H), 2.41(s, 3H), 2.55 (m, 6H), 3.11 (t, 2H), 3.75 (m, 4H), 4.07(t, 2H), 4.34 (q, 2H), 6.60(s, 1H), 7.13 (d, 1H), 7.88 (d, 1H), 8.79 (s, 1H); MS (ES^+): m/z 535 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{36}N_5O_5S$ $[M+H]^+$ 518.24371, found 518.24374.

2-[2-Ethoxy-5-(2-(morpholin-4-yl)-ethylamine-N-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-29): 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 2-(Morpholin-4-yl)-ethylamine (0.79g , 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow crude product. This crude product was recrystallized with methanol:DCM=1:1 to give 0.96g light yellow solids of III-29, yield 73%. ^{1}H NMR($CDCl_3$) δ: 0.90 (t, 3H), 1.62 (t, 3H), 1.74 (m, 2H), 2.32 (m, 4H), 2.41 (s, 3H), 2.74 (t, 2H), 3.03 (t, 2H), 3.61 (m, 4H), 4.06(t, 2H), 4.36 (q, 2H), 6.62(s, 1H), 7.12 (d, 1H), 7.87 (d, 1H), 8.79 (s, 1H); MS (ES^+): m/z 521 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{34}N_5O_5S$ $[M+H]^+$ 504.22806, found 504.22813.

2-[2-Ethoxy-5-(2,6-dimethylmorpholine-4-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-30): 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C.

2,6-Dimethylmorpholine (0.70g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with methanol:DCM=1:1 to give 0.91g light yellow solids of III-30, yield 71%. ^{1}H NMR($CDCl_3$) δ: 0.89 (t, 3H), 1.19 (s, 3H), 2.21 (s, 3H), 1.61 (t, 3H), 1.73 (m, 2H), 2.41(s, 3H), 3.01 (dd, 1H), 3.26 (m, 1H), 3.74 (m, 4H), 4.05(t, 2H), 4.35 (q, 2H), 6.60(s, 1H), 7.10 (d, 1H), 7.80 (dd, 1H), 8.57 (d, 1H), 10.10 (br, 1H); MS (ES^+): m/z 506 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{33}N_4O_5S$ $[M+H]^+$ 489.21717, found 489.21722.

***2-[2-Ethoxy-5-(1-benzylpiperidine-4-aminosulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-31)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-Benzylpiperidin-4-amine (1.16g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.5g light yellow crude product. This crude product was recrystallized with methanol:DCM=1:1 to give 1.2g light yellow solids of III-31, yield 80%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.51-2.04 (m, 8H), 2.43(s, 3H), 2.85-2.97 (m, 4H), 3.47 (m, 3H), 4.05(t, 2H), 4.31 (q, 2H), 6.61(s, 1H), 7.08 (d, 1H), 7.21-7.27 (m, 5H), 7.92 (dd, 1H), 8.79 (d, 1H); MS (ES^+): m/z 581 ($M+NH_4$); HRMS (CI) calcd for $C_{30}H_{38}N_5O_4S$ $[M+H]^+$ 564.26445, found 564.26451.

***2-{2-Ethoxy-5-[2-(piperidine-1-yl)-ethylaminosulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-32)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 2-(Piperidin-1-yl) ethanamine (0.78g, 6.10 mmol) was added slowly to this solution. The mixture was

stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light yellow crude product. This crude product was recrystallized with ethanol to give 1.0g light yellow solids of III-32, yield 79%. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, 3H), 1.35 (m, 2H), 1.55-1.80 (m, 9H), 2.43(s, 3H), 2.59 (m, 4H), 2.74 (t, 2H), 3.15 (t, 2H), 4.06(t, 2H), 4.32 (q, 2H), 6.61(s, 1H), 7.14 (d, 1H), 7.90 (d, 1H), 8.80 (s, 1H); MS (ES^+): m/z 519 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{36}N_5O_4S$ $[M+H]^+$ 502.24880, found 502.24886.

***2-[2-Ethoxy-5-(4-benzylpiperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-33)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1.07g 1-Benzylpiperazine (6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.5g light yellow crude product. This crude product was recrystallized with ethanol to give 1.3g light yellow solids of III-33, yield 82%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.66 (t, 3H), 1.75 (m, 2H), 2.41(s, 3H), 2.52 (m, 4H), 3.09 (m, 4H), 3.47 (s, 2H), 4.05(t, 2H), 4.35 (q, 2H), 6.61(s, 1H), 7.10 (d, 1H), 7.21-7.29 (m, 5H), 7.78 (dd, 1H), 8.78 (d, 1H); MS (ES^+): m/z 567 ($M+NH_4$); HRMS (CI) calcd for $C_{29}H_{36}N_5O_4S$ $[M+H]^+$ 550.24880, found 550.24886.

***2-[2-Ethoxy-5-(4-phenylpiperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-34)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-Phenylpiperazine (1.0g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light

yellow crude product. This crude product was recrystallized with ethanol to give 1.12g light yellow solids of III-34, yield 78%. ^{1}H NMR ($CDCl_3$) δ: 0.94 (t, 3H), 1.64 (t, 3H), 1.78 (m, 2H), 2.41 (s, 3H), 3.16-3.24 (m, 8H), 4.05(t, 2H), 4.36 (q, 2H), 6.61(s, 1H), 6.84-6.91 (m, 3H), 7.13-7.27 (m, 3H), 7.84 (dd, 1H), 8.74 (d, 1H), 10.20 (br, 1H); MS (ES^+): m/z 553 ($M+NH_4$); HRMS (CI) calcd for $C_{28}H_{34}N_5O_4S$ $[M+H]^+$ 536.23315, found 536.23321.

***2-[2-Ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-35)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g ,2.44mmol) was dissolved in DCM (20ml) at 0°C. Piperazine (0.52g , 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.86g light yellow solids of III-35, yield 77%.^{1}H NMR ($CDCl_3$) δ: 0.91 (t, 3H), 1.66 (t, 3H), 1.77-1.86 (m, 2H), 2.41 (s, 3H), 2.65 (brs, 4H), 3.10 (brs, 4H), 4.08 (t, 2H), 4.29-4.35 (q, 2H), 6.61 (s, 1H), 7.14(d, 1H), 7.80 (dd, 1H), 8.66(d, 1H), 10.44 (s, 1H); MS (ES^+): m/z 477 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{30}N_5O_4S$ $[M+H]^+$ 460.20185, found 460.20178.

***2-[5-(4-Benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-2-ethoxy-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-36)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzene-sulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 1-(Benzo[d][1,3]dioxol- 2-ylmethyl)piperazine (1.33g ,6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.4g light yellow crude product. This crude product was recrystallized with ethanol to give 1.1g light yellow solids of III-36, yield 75%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.65 (t, 3H), 1.73 (m, 2H), 2.41(s, 3H), 2.48 (m,

4H), 3.08 (m, 4H), 3.43 (s, 2H), 4.08(t, 2H), 4.34(q, 2H), 5.92 (s, 2H), 6.61 (s, 1H), 6.60-6.80 (m, 3H), 7.10 (d, 1H), 7.75 (dd, 1H), 8.76 (d, 1H); MS (ES^+): m/z 611 ($M+NH_4$); HRMS (CI) calcd for $C_{30}H_{36}N_5O_6S$ $[M+H]^+$ 594.23863, found 594.23866.

***2-[2-Ethoxy-5-[4-(3-phenyl-propyl)-piperidine-1-sulfonyl]-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-37)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 4-(3-Phenylpropyl)piperidine (1.24g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light yellow crude product. This crude product was recrystallized with ethanol to give 1.05g light yellow solids of III-37, yield 74%. ^1H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.23-1.31 (m, 5H), 1.58-1.85 (m, 8H), 2.41(s, 3H), 3.10-3.21 (m, 4H), 4.05(t, 2H), 4.34 (q, 2H), 6.61 (s, 1H), 7.08-7.29 (m, 6H), 7.80 (dd, 1H), 8.66 (d, 1H); MS (ES^+): m/z 594 ($M+NH_4$); HRMS (CI) calcd for $C_{32}H_{41}N_4O_4S$ $[M+H]^+$ 577.28485, found 577.28484.

***2-(2-Ethoxy-5-propylaminosulfonyl-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-38)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. Propylamine (0.36g ,6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.92g light yellow solids of III-38, yield 86%. ^1H NMR ($CDCl_3$) δ: 0.94-0.99 (m, 6H), 1.52 (q, 2H), 1.65(t, 3H), 1.73 (m, 2H), 2.40 (s, 3H), 2.92 (q, 2H) 4.05 (t, 2H), 4.34 (q, 2H), 6.61 (s, 1H), 7.10 (d, 1H), 7.89 (dd, 1H), 8.79 (d, 1H); MS (ES^+): m/z 450 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{29}N_4O_4S$ $[M+H]^+$ 433.19095, found 433.19083.

2-{2-Ethoxy-5-[N,N-bis(2-hydroxyethyl)aminosulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-39): 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44 mmol) was dissolved in DCM (20ml) at 0°C. N,N-bis(2-hydroxyethyl)amine (0.63g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow crude product. This crude product was recrystallized with ethanol to give 0.94g light yellow solids of III-39, yield 79%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H),1.65 (t, 3H), 1.74 (m, 2H), 2.41 (s, 3H), 3.32 (t, 4H) 3.85 (t, 4H), 4.05 (t, 2H), 4.35 (q, 2H), 6.60 (s, 1H), 7.14 (d, 1H), 7.80 (dd, 1H), 8.75 (d, 1H); MS (ES^+): m/z 496 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{31}N_4O_6S$ $[M+H]^+$ 479.19643, found 479.19635.

2-{2-ethoxy-5-[N-(2-hydroxyethyl)-N-methyl]aminosulfonyl-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-40): 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. 2-(Methylamino)ethanol (0.46g, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.89g light yellow solids of III-40, yield 78%. ^{1}H NMR ($CDCl_3$) δ: 0.90(t, 3H),1.64 (t, 3H), 1.78 (m, 2H), 2.41 (s, 3H), 2.70(s, 3H), 3.24 (t, 2H) 3.77 (t, 2H), 4.05 (t, 2H), 4.35 (q, 2H), 6.61 (s, 1H), 7.16 (d, 1H), 7.87 (dd, 1H), 8.73 (d, 1H); MS (ES^+): m/z 466 ($M+NH_4$); HRMS (CI) calcd for $C_{21}H_{29}N_4O_5S$ $[M+H]^+$ 449.18587, found 449.18596.

2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-ethyl]aminosulfonyl-phenyl}-5-methyl-7-

***propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-41)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44 mmol) was dissolved in DCM (20ml) at 0°C.2-(Ethylamino)ethanol (0.54g ,6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.92g light yellow solids of III-41, yield 79%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.18 (t, 3H), 1.65 (t, 3H), 1.76 (m, 2H), 2.41 (s, 3H), 3.30 (m, 4H) 3.75 (t, 2H), 4.08 (t, 2H), 4.35 (q, 2H), 6.61 (s, 1H), 7.10 (d, 1H), 7.86 (dd, 1H), 8.66 (d, 1H); MS (ES^+): m/z 480 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{31}N_4O_5S$ $[M+H]^+$ 463.20152, found 63.20155.

***2-{2-Ethoxy-5-[N-butyl-N-(2-hydroxyethyl)]aminosulfonyl-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-42)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44 mmol) was dissolved in DCM (20ml) at 0°C. 2-(Butylamino)ethanol (0.71g, 6.10mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.96g light yellow solids of III-42, yield 79%. ^{1}H NMR ($CDCl_3$) δ: 0.92-0.99 (m, 6H), 1.29-1.40 (t, 4H), 1.64 (t, 3H), 1.75 (m, 2H), 2.40 (s, 3H), 3.12 (m, 2H) 3.29 (m, 2H), 3.78 (t, 2H), 4.07 (t, 2H), 4.34 (q, 2H), 6.61 (s, 1H), 7.12 (d, 1H), 7.78 (dd, 1H), 8.76 (d, 1H); MS (ES^+): m/z 508 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{35}N_4O_5S$ $[M+H]^+$ 491.23282, found 491.23286.

***2-[2-Ethoxy-5-(4-ethoxycarbonylphenylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-43)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl

chloride (III-19, 1.0g , 2.44 mmol) was dissolved in DCM (20ml) at 0°C. Ethyl 4-aminobenzoate (1.01g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.3g light yellow crude product. This crude product was recrystallized with ethanol to give 1.08g light yellow solids of III-43, yield 80%.^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 3H), 1.29 (t, 3H), 1.60(t, 3H), 1.72 (m, 2H), 2.42 (s, 3H), 4.05 (t, 2H), 4.25-4.37 (m, 4H), 6.62 (m, 3H), 7.08 (d, 1H), 7.78 (d, 2H), 7.97 (dd, 1H), 8.79(d, 1H); MS (ES^+): m/z 556 ($M+NH_4$); HRMS (CI) calcd for $C_{27}H_{31}N_4O_6S$ $[M+H]^+$ 539.19643, found 539.19649.

***2-[2-Ethoxy-5-(2-benzoylphenylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-44)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. *o*-Benzoylaniline (1.20g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.5g light yellow crude product. This crude product was recrystallized with ethanol to give 1.26g light yellow solids of III-44, yield 78%. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, 3H), 1.54 (t, 3H), 1.72 (m, 2H), 2.43 (s, 3H), 4.05 (t, 2H), 4.29 (m, 2H), 6.60-6.72 (m, 3H), 7.11 (d, 1H), 7.25-7.45 (m, 5H), 7.80 (d, 2H), 8.07 (dd, 1H), 8.77 (d, 1H); MS (ES^+): m/z 588 ($M+NH_4$); HRMS (CI) calcd for $C_{31}H_{31}N_4O_5S$ $[M+H]^+$ 571.20152, found 571.20155.

***2-{2-Ethoxy-5-[N2-acetohydrazide-sulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-45)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44mmol) was dissolved in DCM (20ml) at 0°C. Acetohydrazide (0.45g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C

for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.1g light yellow crude product. This crude product was recrystallized with ethanol to give 0.92g light yellow solids of III-45, yield 80%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.62(t, 3H), 1.76 (m, 2H), 2.02 (s, 3H), 2.41 (s, 3H), 4.05 (t, 2H), 4.34 (q, 2H), 6.61(s, 1H), 7.10 (d, 1H), 7.90 (dd, 1H), 8.74 (d, 1H); MS (ES^+): m/z 465 ($M+NH_4$); HRMS (CI) calcd for $C_{20}H_{26}N_5O_5S$ $[M+H]^+$ 448.16546, found 448.16549.

***2-[2-Ethoxy-5-(2-dimethylaminoethylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-46)*:** 4-Ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-19, 1.0g, 2.44 mmol) was dissolved in DCM (20ml) at 0°C. 2-Dimethylamino-ethylamine (0.54g, 6.10 mmol) was added slowly to this solution. The mixture was stirred at 0°C for 10 min, then at room temperature for 5 hrs. The resulting solution was washed with water and dried over sodium sulfate. Evaporation of the solvent gave 1.2g light yellow crude product. This crude product was recrystallized with ethanol to give 0.89g light yellow solids of III-46, yield 76%. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H), 1.61 (t, 3H), 1.75 (m, 2H), 2.10 (s, 3H), 2.24 (s, 3H), 2.44 (s, 3H), 2.54 (m, 2H), 3.02 (m, 2H), 4.05 (t, 2H), 4.34 (q, 2H), 6.61(s, 1H), 7.11 (d, 1H), 7.87 (dd, 1H), 8.77 (d, 1H); MS (ES^+): m/z 479 ($M+NH_4$); HRMS (CI) calcd for $C_{22}H_{32}N_5O_4S$ $[M+H]^+$ 462.21750, found 462.21758.

3.3.4 Synthesis of the analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) with a different R^4 substituent

It has been foreseen that alternation of R^4 group may affect affinity and binding force

or docking between the molecule I-34 and PDE5. Therefore, a selective group of alkyl fragments was chosen as alternatives of R^4 group to produce a further group derivatives of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one (III-20). In the above general synthetic route (Fig. 3.5), ethyl R^4 group was introduced into the target compound with 2-ethoxybenzoic chloride (III-13). Replacement of ethyl group with its alkyl analogues in 2-ethoxybenzoic chloride (III-13) followed by condensation with 2-amino-3-cyano- 4-methylpyrrole (III-14) in the same way gave a group of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5- triazine-4-(3H)-one (III-20) with a different R^4 group.

3.3.4.1 Synthesis of 2-[2-methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimi-din-4-one(III-47)

N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-methoxy-benzamide (III-48): 2-Methoxy -benzoic acid (9.12g, 60mmol) was heated at reflux with thionyl chloride (20ml) for 40min. The excess thionyl chloride was evaporated and the residue was dissolved in DCM (150ml). 2-Amino-3-cyano-4-methylpyrrole (III-14) (7.0g, 56.8mmol) was dissolved in THF (80ml), and Et_3N (8.5ml, 61.0mmol) was added. The mixture was stirred in an ice-water bath. The above 2-methoxybenzoic chloride in DCM solution was added in this reaction system dropwise in 30min, the reaction was carried out at 0°C for another 1hr. The resulting mixture was washed with water, filtered and the filtrate was evaporated at 40°C with 20g silica gel. The product was washed down from silica gel (80g) using DCM to give 8.8g solids, yield 58%.

The above crude product was directly introduced into the following reduction, cyclization and sulfonylation reactions under the same conditions as described in

preparation of 2-(2-ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide (III-16), 2-(2-ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-17) and 2-(2-ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-18). The corresponding intermediate 2-[2-methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-49) was afforded, which reacted with 1-ethylpiperazine to gain the compound III-50. Mp. 180-182°C; ^{1}H NMR($CDCl_3$) δ: 0.86(t, 3H), 0.99(t, 3H), 1.73(m, 2H), 2.32(q, 2H), 2.40(s, 3H), 2.48(brs, 4H), 3.04(brs, 4H), 4.08(t, 2H), 4.14(s, 3H), 6.60(s, 1H), 7.10(d, 1H), 7.74(dd, 1H), 8.77(d, 1H). MS (ES^+): m/z 491 ($M+NH_4$); FAB-MS m/z: 474.2178 (M+H); HRMS (CI) calcd for $C_{23}H_{32}N_5O_4S$ $[M+H]^+$ 474.21750, found 474.21753.

3.3.4.2 Synthesis of 2-[2-propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-50)

N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-propoxy-benzamide (III-51): 2-Propoxy -benzoic acid (10.8g, 60mmol) was heated at reflux with thionyl chloride (20ml) for 40min. The excess thionyl chloride was evaporated and the residue was dissolved in DCM (150ml). 2-Amino-3-cyano-4-methylpyrrole (III-14) (7.0g, 56.8mmol) was dissolved in THF (80ml), and Et_3N (8.5ml, 61.0mmol) was added. The mixture was stirred in an ice-water bath. The above 2-propoxybenzoic chloride in DCM solution was added in this reaction system dropwise in 30min, the reaction was carried out at 0°C for another 1hr. The resulting mixture was washed with water, filtered and the filtrate was evaporated at 40°C with 20g silica gel. The product was washed down from silica gel (80g) using DCM to give 7.8g solids, yield 48%.

The above crude product was directly introduced into the following reduction, cyclization and sulfonylation reactions under the same conditions as described in

preparation of 2-(2-ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide (III-16), 2-(2-ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-17) and 2-(2-ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-18). The corresponding intermediate 2-[2-propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-52) was afforded, which reacted with 1-ethylpiperazine to gain the compound III-50. M.P. 168-170°C; ^{1}H NMR($CDCl_3$) δ: 0.87 (t, 3H), 1.00(t, 3H), 1.16 (t, 3H), 1.70-1.79 (m, 4H), 2.36(q, 2H), 2.42(s, 3H), 2.50(brs, 4H), 3.07(brs, 4H), 4.08(t, 2H), 4.34(t, 2H), 6.60(s, 1H), 7.09 (d, 1H), 7.81(dd, 1H), 8.71 (d, 1H). MS (ES^+): m/z 519 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{36}N_5O_4S$ $[M+H]^+$ 502.24880, found 502.24871.

3.3.4.3 Synthesis of 2-[2-allyloxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-53)

***N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-allyoxy-benzamide (III-54)*:** 2-Allyl-oxybenzoic acid (10.68g, 60mmol) was heated at reflux with thionyl chloride (20ml). After one hour, the excess thionyl chloride was evaporated and the residue was dissolved in DCM (150ml). Compound III-14 (7.0g, 56.8mmol) was dissolved in THF (80ml), and Et_3N (8.5ml, 61.0mmol) was added. The mixture was stirred in an ice-water bath. The above 2-allyloxybenzoic chloride in DCM solution was added in this reaction system dropwise in 30min, the reaction was carried out at 0°C for another 1hr. The resulting mixture was washed with water, filtered and the filtrate was evaporated at 40°C with 20g silica gel. The product was washed down from silica gel (80g) using DCM to give 8.0g solids, yield 50%.

The above crude product was directly introduced into the following reduction, cyclization and sulfonylation reactions under the same conditions as described in

preparation of 2-(2-ethoxy-benzoylamino)-4-methyl-1H-pyrrole-3-carboxylic acid amide (III-16), 2-(2-ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-17) and 2-(2-ethoxy-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-18). The corresponding intermediate 2-[2-allyoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-55) was afforded, which reacted with 1-ethylpiperazine to gain the compound III-53. Mp162-165°C; ^{1}H NMR($CDCl_3$) δ: 0.83 (t, 3H), 0.97 (t, 3H), 1.82 (m, 2H), 2.37(q, 2H), 2.41(s, 3H), 2.52(brs, 4H), 3.07(brs, 4H), 4.07(t, 2H), 4.62 (m, 2H), 5.24 (m, 2H), 5.83 (m, 1H), 6.62(s, 1H), 7.12 (d, 1H), 7.79(dd, 1H), 8.69(d, 1H), 9.97 (br, 1H). MS (ES^+): m/z 517 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{34}N_5O_4S$ $[M+H]^+$ 500.23315, found 500.23323.

3.3.5 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-20) with a different R^1 substituent

R^1 on the compound III-1 was considered to another biological important group. The difference in the chain length and possible introducing a function group could affect bioactivities and enzyme binding specificities. In fig. 3.6, the particular synthetic process of III-1 had been described through a specific compound of III-20. In the synthetic process of N-(3-cyano-4-methyl-1H-pyrrol-2-yl)-2-ethoxy-benzamide (compound III-15), the compound 2-amino-3-cyano-4-methylpyrrole III-14 was reacted with compound III-13. If we selected a series of analogues of III-14 with different R^1 substituents to apply into the reaction, we will gain a series of homologous compound of III-15. Followed by established synthetic methods, the new group of compounds with a different R^1 substituent have been produced. Some of them were listed below and their synthetic details were described.

***2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-56)*:** The title compound was prepared from 2-amino-3-cyano-4-ethylpyrrole (III-57) and 2-ethoxy-benzoic chloride. With the same condition of III-20 synthetic process, through the relative intermediate, 4-ethoxy-3-(5-ethyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-58) was gained. It was treated with 1-ethylpiperazine to afford the final product III-56. ^{1}H NMR ($CDCl_3$) δ: 0.90-.095 (m, 6H), 1.56-1.60(m, 6H), 1.75 (m, 2H), 2.37 (q, 2H)2.49 (brs, 4H), 2.53(q, 2H), 3.06 (m, 4H), 4.05(q, 2H), 4.34(q, 2H), 6.61(s, 1H), 7.10 (d, 1H), 7.75(dd, 1H), 8.79(d, 1H), 9.90 (br, 1H); MS (ES^+): m/z 519 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{36}N_5O_4S$ $[M+H]^+$ 502.24880, found 502.24893.

***2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-(pyrimidin-2-ylmethyl)-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-59)*:** The title compound was prepared from 2-amino-4-(pyrimidin-2-ylmethyl)-2H-pyrrole-3-carbonitrile (III-60) and 2-ethoxybenzoic chloride. With the same condition of III-20 synthetic process, through the relative intermediate, 4-ethoxy-3-[5-(pyrimidin-2-ylmethyl)-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl]-benzenesulfonyl chloride (III-61) was gained. It was treated with 1-ethylpiperazine to afford the final product III-59. ^{1}H NMR ($CDCl_3$) δ: 0.90-1.00 (m, 6H), 1.60 (m, 6H), 1.75 (m, 2H), 2.37(q, 2H), 2.53(m, 4H), 3.06 (m, 4H), 4.01 (s, 2H), 4.07 (t, 2H), 4.35(q, 2H), 6.69(s, 1H), 7.11-7.15 (m, 2H), 7.77 (dd, 1H), 8.68-8.71(m, 3H); MS (ES^+): m/z 569 ($M+NH_4$); HRMS (CI) calcd for $C_{28}H_{36}N_7O_4S$ $[M+H]^+$ 566.25495, found 566.25503.

***2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-(morpholin-4-ylmethyl)-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-62)*:** The title compound was prepared from 2-amino-4-(morpholin-4-ylmethyl)-2H-pyrrole-3-carbonitrile (III-63) and 2-ethoxybenzoic chloride. With the same condition of III-20 synthetic process,

through the relative intermediate,4-ethoxy-3-[5-(morpholin-4-ylmethyl)-4-oxo-7-propyl-4,7-dihydro-3*H*-pyrrolo[2,3-d]pyrimidin-2-yl]-benzenesulfonyl chloride (III-64) was gained. It was treated with 1-ethylpiperazine to afford the final product III-62. ^{1}H NMR ($CDCl_3$) δ: 0.90-1.00 (m, 6H), 1.61(t, 3H), 1.74 (m, 2H), 2.32-2.56 (m, 10H), 3.06 (m, 4H), 3.67 (m, 4H), 3.94 (s, 2H), 4.07 (t, 2H), 4.34(q, 2H), 6.55(s, 1H), 7.15 (d, 1H), 7.78(dd, 1H), 8.75(d, 1H); MS (ES^+): m/z 576 ($M+NH_4$); HRMS (CI) calcd for $C_{28}H_{41}N_6O_5S$ $[M+H]^+$ 573.28591, found 573.28585.

3.3.6 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-20) with a different R^3 substituent

Synthesis of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-allyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-65)

***2-(2-Ethoxy-phenyl)-5-methyl-7-allyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-66)*:** 2-(2-Ethoxy-phenyl)-5-methyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-18, 1.5g, 5.57 mmol), 3-bromoprop-1-ene (2.0g, 16.3mmol) and potassium carbonate (5g, 36.2mmol) were mixed in acetone (15ml). This mixture was heated at reflux for 15hr. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure. The residue was submitted to chromatography (DCM as eluent) to give 0.8g final product, yield 48%.

Through the relative intermediate, 4-ethoxy-3-(5-methyl-4-oxo-7-allyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-67) was gained. It was treated with 1-ethylpiperazine to afford the final product III-65. ^{1}H NMR ($CDCl_3$) δ: 1.00 (t, 3H), 1.65 (t, 3H), 2.37(q, 2H), 2.41(s, 3H), 2.50(m, 4H), 3.09 (br, 4H), 4.31

(q, 2H), 4.95 (m, 2H), 4.96 (dd, 1H), 5.01 (dd, 1H), 5.68 (m, 1H), 6.61(s, 1H), 7.11 (d, 1H), 7.82(dd, 1H), 8.71 (d, 1H), 9.77(br 1H); MS (ES^+): m/z 503 ($M+NH_4$); HRMS (CI) calcd for $C_{24}H_{32}N_5O_4S$ $[M+H]^+$ 486.21750, found 486.21755.

3.3.7 Synthesis of analogues of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) with altering two or more of R^1, R^4 or R^6 groups at the same time

In previous sections, by replacements of R^1, R^3, R^4 or R^6 group of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) with analogues alkyl groups, produced three series derivatives have been prepared. In order to explore effects of R^1, R^4 or R^6 group on biological activities further, in this section, a few examples of 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) with altering two or more of R^1, R^4 or R^6 groups at the same time were presented.

2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one (III-68) in which R^1 and R^4 groups are different from those in 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro- pyrrolo[2,3-d]pyrimidin-4-one (III-20)

In the synthesis of III-68, relative to synthesis of III-20, we change the reaction compound 2-ethoxybenzoic chloride (III-13, fig. 3.6) to 2-propoxy-benzoic chloride (III-69, fig. 3.7) and change the reaction compound 2-amino-3-cyano-4-methylpyrrole to 2-amino-3- cyano-4-ethylpyrrole (III-70, fig. 3.7). A new potential PDE5 inhibitor will be synthesized. The synthetic details were illuminated below. 10.8g

2-propoxybenzoic acid (60.0 mmol) was heated at reflux with thionyl chloride (20ml) for 40min. The excess thionyl chloride was evaporated and the residue was dissolved in DCM (150ml). The 2-amino-3-cyano-4- ethylpyrrole (8.1g, 60mmol) was dissolved in THF (80ml), Et_3N (8.5ml, 61.0mmol) was added. The mixture was stirred in an ice-water bath. The above 2-ethoxybenzoic chloride in DCM solution was added dropwise in 30min, the reaction was carried out at 0°C for another 1hr. The resulting mixture was washed with water, filtered and the filtrate was evaporated at 40°C with 20g silica gel. The crude product of N-(3-cyano-4-ethyl-1H-pyrrol-2-yl)-2-propoxy-benzamide (III-71) was washed down from silica gel (80g) using DCM to give 8.2g solids, yield 51%. Analytical sample was prepared by chromatography (DCM:Hexane 1:1 as eluent) and recrystallisation from DCM /Hexane=1/2. Under the same conditions as synthesis of III-16, III-17 and III-18, 4-ethoxy-3-(5-methyl-4-oxo-7-propyl-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)-benzenesulfonyl chloride (III-72) was afforded. Followed by the reaction with 1-ethylpiperazine, the target compound III-68 was given. ^{1}H NMR($CDCl_3$) δ: 0.88 (t, 3H), 0.99(t, 3H), 1.04(t, 3H), 1.52 (t, 3H), 1.74-1.84 (m, 4H), 2.35(q, 2H), 2.46(brs, 4H), 2.68(q, 2H), 3.05(brs, 4H), 4.09(t, 2H), 4.36(t, 2H), 6.61(s, 1H), 7.09 (d, 1H), 7.81(dd, 1H), 8.71 (d, 1H); MS (ES^+): m/z 533 ($M+NH_4$); HRMS (CI) calcd for $C_{26}H_{38}N_5O_4S$ $[M+H]^+$ 516.26445, found 516.26454.

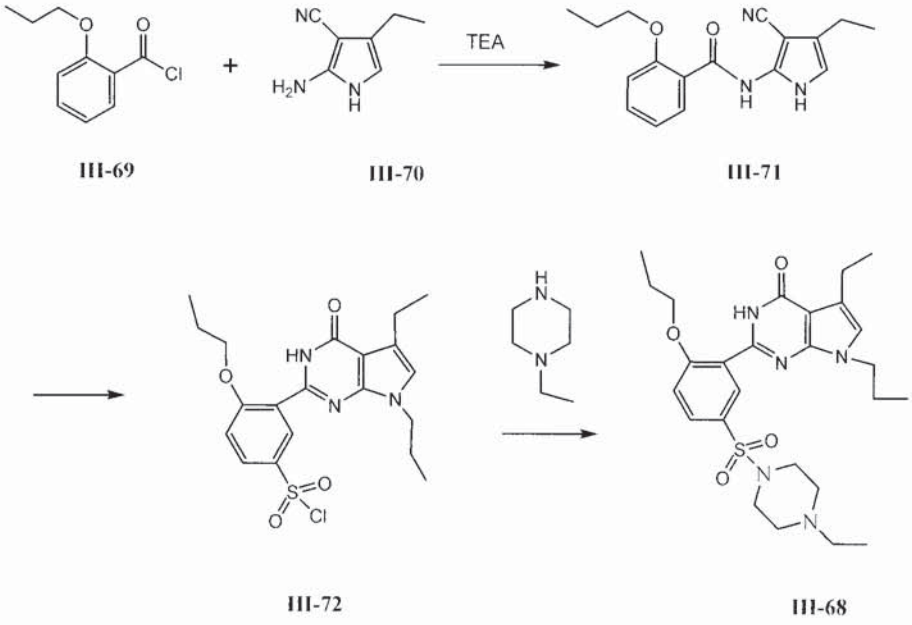


Figure 3.7 Synthetic route of III-68.

2-[2-Propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-73) in which R^1 R^4 and R^6 groups are different from those in 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro -pyrrolo[2,3-d]pyrimidin-4-one (III-20)

The reaction of 2-propoxybenzoic chloride (III-69) with 2-amino-3-cyano-4-ethylpyrrole (III-70) under the same conditions as synthesis of III-71 (fig. 3.7) gave the corresponding compound III-72. This compound reacted with 1-methylpiperazine to afford compound III-73. ^{1}H NMR ($CDCl_3$) δ: 0.92 (t, 3H), 1.54-1.64(m, 6H), 1.75 (m, 4H), 2.14(s, 3H), 2.53(m, 6H), 3.04 (brs, 4H), 4.09(t, 2H), 4.37(q, 2H), 6.62(s, 1H), 7.11(d, 1H), 7.80(dd, 1H), 8.76(d, 1H); MS (ES^+): m/z 505 ($M+NH_4$); HRMS (CI) calcd for $C_{25}H_{36}N_5O_4S$ $[M+H]^+$ 502.24880, found 502.24886.

3.3.8 Preparation of water-soluble salts of 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-ones

Syntheses of the group of 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-ones derivatives for developments of new PDE5 inhibitors were described in the previous section. In order to facilitate their oral administration, it would be better to turn them into their corresponding water-soluble salts with an organic or inorganic acid. Here we demonstrated their conversions from the bases into corresponding mono- or bi-acid salts.

***Synthesis of compound III-20·HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one mono-hydrochloride*:** The free base afforded in preparation of III-20 (1.00g, 2.05mmol) was dissolved in a mixture of ether (10ml) and DCM (10ml). 4M solution of hydrochloric acid-dioxane (0.51ml, 2.04mmol) was diluted by 10ml ether and added dropwise into stirred solution. After stirring at room temperature for 20 minutes, the solid product

was filtered and dried to give 1.01g hydrochloride, yield 94%. M.p. 147-150°C; IR (cm^{-1}): 2964, 2931, 2675, 2599, 2462, 1668, 1574, 1456, 1348, 1167, 933, 588; ^1H NMR (D_2O) δ: 0.72 (m, 3H), 1.24 (t, J=7.3Hz, 3H), 1.45 (m, 3H), 1.59 (m, 2H), 2.04 (s, 3H), 2.77-3.81 (all brs, 8H), 3.20 (q, 2H), 3.75 (m, 2H), 4.20 (m, 2H), 6.62 (m, 1H), 7.17(m, 1H), 7.73 (m, 1H), 8.22(s, 1H); HRMS (CI) calcd for $C_{24}H_{35}ClN_5O_4S$ $[M+H]^+$ 524.20983, found 524.20991.

Synthesis of Compound III-20·2HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one bi-hydro-chloride: The free base afforded in preparation of III-20 (1.00g, 2.05mmol) was dissolved in a mixture of ether (10ml) and DCM (10ml). 4M solution of hydrochloric acid-dioxane (1.02ml, 4.10mmol) was diluted by 20ml ether and added dropwise into stirred solution. After stirring at room temperature for 20 minutes, the solid product was filtered and dried to give 1.12g hydrochloride, yield 95%. M.p.: 177-180°C; IR (cm^{-1}): 2962, 2929, 2677, 2597, 2456, 1652, 1569, 1458, 1357, 1276, 1162, 1093, 1027, 939, 731, 582; ^1H NMR (D_2O) δ: 0.64 (t, J=7.4Hz, 3H), 1.23 (t, J=7.3Hz, 3H), 1.40 (t, J=6.9Hz, 3H), 1.51 (m, 2H), 1.98 (s, 3H), 2.74 (m, 2H), 3.12 (m, 2H), 3.19 (t, 2H), 3.56 (m, 2H), 3.65 (t, 2H), 3.78 (d, 2H), 4.12 (q, 2H), 6.43 (s, 1H), 7.10(d, J=9.1Hz, 1H), 7.68 (dd, J= 8.8 and 2.3Hz, 1H), 8.16(d, J=2.3Hz, 1H); HRMS (CI) calcd for $C_{24}H_{36}Cl_2N_5O_4S$ $[M+H]^+$ 560.18651, found 560.18656.

3.4 Conclusions

A series of 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one compounds was designed. They are structurally similar to cGMP and sildenafil. Compound III-20 was synthesized successfully in a six-step method. By employing different starting material, different R^1, R^2, R^3, R^4 and R^6 substituted III-20 analogues which will be used in anti ED study were synthesized. The water soluble salt forms of these compounds were also synthesized.

Chapter 4

Preliminary Bioactivity and Toxicity Studies

4.1 Introduction

In the chapters 2 and 3, I have presented syntheses of two series of compounds containing 3H-imidazo[1,5-a][1,3,5]triazine-4-one and 3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-one nuclei as candidates for development of PDE5 inhibitors. Though in the literature, there are a number of assays for evaluations of PDE5 inhibition activities, mainly either enzymatic assay or animal assay. It is clear that enzymatic assay is robotic for screening a large number of compounds and convenient for a general bioassay laboratory. However commercial PDEs were difficult to obtain and are expensive. It is a sensitive issue to perform an animal assay because of ethnical concerns, animal rights, and cost for animals affair. However, we found the reported conscious-rabbit model *(Bischoff & Schneider, 2001)* was a convenient and straight forward assay for screening a medium size library of compounds.The experiment is easy to perform and does not damage animals, as well as the activity is easy to measure. Therefore, the reported rabbit model was adopted in this project as a screening method to evaluate PDE5 inhibitions. Based on the screening results, promising candidates were chosen to further bioactivity studies using mice models. In line with bioactivity studies, acute toxicity studies of the compounds were carried out to guide the selecting process for PDE5 inhibitor candidates.

4.2 Screening penile erection activity with the conscious-rabbit model

4.2.1 Materials and methods

***Animals*:** Three adult male rabbits with an average weight of 2.0 ±2.5 kg were used for each compound. The rabbits were housed individually in cages, food and drink were available freely for 2 h a day, and the animals were kept in the animal facility for at least 1 week after arrival before being tested. Room lights were on from 8 am to 6 pm, and room temperature was 22 - 24°C.

The compounds and their dosage: The tested compounds were dissolved in saline and were administered intravenously 3mg/kg doses into the ear vein of conscious rabbits. The compounds tested in this experiment were listed below.

II-13•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-13•2HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one bi-hydrochlorate;
II-14•HCl: 2-[2-ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-15•HCl: 2-[2-ethoxy-5-(4-ethoxycarbonylpiperazine-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-18•HCl: 2-{2-ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl]aminosulfonyl}-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-19•HCl: 2-{2-ethoxy-5-[N-(2-pyrrolidinylethyl)amino-sulfonyl]-phenyl}-6-methyl-8- propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-20•HCl: 2-[2-ethoxy-5-(morpholinosulfonyl)phenyl]-6-methyl-8-propyl-imidazo [1,5-a]- 1,3,5-triazine-4-(3H)-one monohydrochlorate;
II-22•HCl: 2-{2-ethoxy-5-[N-(2-morpholinoethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl- imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-24•HCl: 2-[2-ethoxy-5-(1-benzylpiperidine-4-aminosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-28•HCl: 2-[2-ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-29•HCl: 2-[2-ethoxy-5-(4-benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-30•HCl: 2-{2-ethoxy-5-[4-(3-phenylpropane-1-yl)piperidine-1-sulfonyl]-phenyl}-6-methyl-8- propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-32•HCl: 2-{2-ethoxy-5-[N,N-bis(2-hydroxyethyl)aminosulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-35•HCl: 2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-n-butyl]aminosulfonyl-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-40•HCl: 2-[2-methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-44•HCl: 2-[2-propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl- imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-52•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-61•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-yl-sulfonyl)-phenyl]-6-methyl-8-allyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-63•HCl: 2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5- a]-1,3,5-triazine-4-(3H)-one monohydrochlorate;

II-68•HCl: 2-[2-propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate.

III-20•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-20•2HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one bi-hydrochlorate;

III-21•HCl: 2-[2-ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-22•HCl: 2-[2-ethoxy-5-(4-ethoxycarbonyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-25•HCl: 2-{2-ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl]aminosulfonyl}-phenyl}-5- methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-26•HCl: 2-{2-ethoxy-5-{N-[2-(2-oxy-1-pyrrolidinyl)ethyl]aminosulfonyl}-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-30•HCl: 2-[2-Ethoxy-5-(2,6-dimethylmorpholine-4-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-33•HCl: 2-[2-ethoxy-5-(1-benzylpiperidine-4-aminosulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-35•HCl: 2-[2-ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-36•HCl: 2-[5-(4-benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-2-ethoxy-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-38•HCl: 2-(2-ethoxy-5-propylaminosulfonyl-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-41•HCl: 2-{2-ethoxy-5-[N-(2-hydroxyethyl)-N-ethyl]aminosulfonyl-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-43•HCl: 2-[2-ethoxy-5-(4-ethoxycarbonylphenylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-46•HCl: 2-[2-Ethoxy-5-(2-dimethylaminoethylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-47•HCl: 2-[2-methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;

III-53•HCl: 2-[2-allyloxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;
III-56•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;
III-65•HCl: 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-allyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate;
III-68•HCl: 2-[2-propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro- pyrrolo[2,3-d] pyrimidin-4-one monohydrochlorate;
III-73•HCl: 2-[2-propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate.

***Method*:** The compounds were given into the rabbit ear vein, followed by a small volume of saline. The time was recorded and the animals were gently removed from the cage and the length of any uncovered penile mucosa was measured using a calliper at the defined time intervals. Between measurements, the animals were returned to their cages and were given access to water and food. Each animal was used multiple times for different compounds with adequate washout periods between the experiments. The minimal time between each experiment for each animal was 10 days. Each compound was repeated three times on three different animals.

***Activity measurements*:** Penile erection was evaluated by measuring the length to the nearest millimetre of the uncovered penile mucosa with a sliding calliper at 0, 30, 60, 90, and 120 minutes after administration of the test compounds. The controls were performed with the corresponding solvent. Physiological parameters of rabbits, such as heart rate and blood pressure, were not measured during the experiments.

4.2.2 Results

Table 4.1 Effect of penile erectile of rabbits administrated 3H-imidazo[1,5-a][1,3,5]triazine-4-one compounds

Compounds	Penile length of rabbits at the time intervals after the injection					
	Before administration	0 (min)	30 (min)	60 (min)	90 (min)	120 (min)
II-13•HCl	0	6.3	10	6.7	2.3	0.0
II-13•2HCl	0	2.3	2.3	3.0	2.0	2.0
II-14•HCl	0	6.0	2.7	1.3	0.3	0.0
II-15•HCl	0	1.3	3.0	2.0	1.3	0.0
II-18•HCl	0	1.8	2.6	1.0	0.6	0.0
II-19•HCl	0	2.0	1.2	1.0	1.0	0.0
II-20•HCl	0	3.2	2.2	1.0	1.0	0.0
II-22•HCl	0	1.2	1.9	1.0	0.7	0.0
II-24•HCl	0	2.3	2.3	1.5	1.0	0.0
II-28•HCl	0	4.3	4.5	2.6	2.0	0.0
II-29•HCl	0	1.4	2.4	1.7	0.9	0.0
II-30•HCl	0	1.9	2.5	2.0	1.2	0.0
II-32•HCl	0	0.8	2.2	0.9	0.9	0.0
II-35•HCl	0	2.5	2.0	1.3	0.9	0.0
II-40•HCl	0	1.6	2.8	2.4	1.2	0.0
II-44•HCl	0	3.1	2.1	1.4	1.0	0.0
II-52•HCl	0	2.8	2.8	1.8	0.9	0.0
II-61•HCl	0	1.2	1.2	0.8	0.0	0.0
II-63•HCl	0	1.4	2.6	1.6	0.7	0.0
II-68•HCl	0	2.5	3.7	1.1	0.6	0.0

Table 4.2 Effect of penile erectile of animal administrated 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one compounds

Compounds	Penile length of rabbits the time intervals after the injection					
	Before administration	0 (min)	30 (min)	60 (min)	90 (min)	120 (min)
III-20•HCl	0	8.3	5.7	5.3	2.0	1.3
III-20•2HCl	0	1.3	3.0	2.0	1.0	1.0
III-21•HCl	0	0.0	5.3	2.7	1.7	0.0
III-22•HCl	0	2.3	1.7	1.3	0.7	0.0
III-25•HCl	0	2.1	2.8	1.2	1.2	0.0
III-26•HCl	0	1.8	2.6	1.4	0.6	0.0
III-30•HCl	0	3.0	2.0	1.2	1.2	0.0
III-33•HCl	0	1.7	4.0	4.3	4.0	0.0
III-35•HCl	0	2.8	1.6	1.0	1.0	0.0
III-36•HCl	0	3.5	2.1	1.2	0.8	0.0
III-38•HCl	0	4.5	3.7	1.5	1.5	0.0
III-41•HCl	0	1.2	2.3	1.6	1.0	0.0
III-43•HCl	0	1.3	3.1	2.0	1.1	0.0
III-46•HCl	0	3.8	3.2	2.1	0.8	0.0
III-47•HCl	0	2.0	1.3	1.3	0.5	0.0
III-53•HCl	0	2.5	3.0	1.8	1.0	0.0
III-56•HCl	0	3.2	1.5	0.8	0.8	0.0
III-65•HCl	0	2.4	2.8	1.6	0.9	0.0
III-68•HCl	0	1.2	2.1	2.1	0.9	0.0
III-73•HCl	0	1.7	2.2	1.5	0.6	0.0

Erection was assessed by measuring the length of uncovered penile mucosa before and after the intravenous administration of the compounds using the literature method *(Bischoff & Schneider, 2001)*.The results of the effect of 3H-imidazo[1,5-a][1,3,5]

triazine-4-one and 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one series compounds to rabbit penile were shown in Table 4.1 and Table 4.2. Table 4.1 showed the penile length variation of the rabbits which were administrated hydrochlorides of 3H-imidazo[1,5-a][1,3,5]triazine-4-one series compounds. The penile length was measured at 0, 30, 60, 90 and 120 minutes after administration. Table 4.2 showed the penile length variation of the rabbits which were administrated hydrochlorides of 3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one series compounds.

Intravenous administration of saline vehicle to the animals did not cause any penile erections over the experiment period of 3-h. However, intravenous administrations of tested compounds showed increases in the length of penile mucosa exposed, respectively. Overall, the profiles of penile erection activities between two groups of compounds seem to be no significant differences; however a big difference was observed among the compounds in the each group. In the group compounds of 3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one, 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate (III-20•HCl) and 2-[2-ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate (III-21•HCl) showed most active in stimulating rabbit penile erection. While in group of 3H-imidazo[1,5-a][1,3,5]triazine-4-one compounds, 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate (II-13•HCl) and 2-[2-ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate (II-14•HCl) demonstrated the best results. In comparison of the substituents on these four compounds, it was clearly showed that they barred a great deal of similarity. Therefore, it could be concluded the combinations of substituents R^1, R^2, R^3 and R^5 on these four compounds are probably among the best choices for the compounds of 3H-imidazo[1,5-a][1,3,5]triazine-4-one and 3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one groups for stimulating rabbit penile erections.

4.2.3 Conclusions

Both series of the designed compounds demonstrated penile erection activity on conscious rabbit models, especially compounds 2-[2-ethoxy-5-(4-ethyl-piperazine -1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate (II-13•HCl) and 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl) -phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one monohydrochlorate (III-20•HCl).

4.3 Inhibitory activity of II-13•HCl and III-20•HCl against PDE 1-6

4.3.1 Objective

To investigate selective inhibitions of II-13•HCl and III-20•HCl against phosphodiesterase 1-6 in order to obtain information for possible toxicity and potential therapeutical index of the compounds.

4.3.2 Materials

Phosphodiesterase PDE 1-4 and 6 were commercial products from Amersham Biosciences; Recombinant phosphodiesterase PDE 5 of Bovine was from Calbiochem. Compound II-13•HCl and III-20•HCl were light yellow powder, easily soluble in water and methanol, but slightly soluble in ethanol or acetonitrile.

4.3.3 Methods – enzyme assays

146000 phosphodiesterase PDE1

Source:	Bovine heart
Substrate:	1.01 μM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 10U / ml Calmodulin
Quantitation method:	Quantitation of [^{3}H] Adenosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

148000 phosphodiesterase PDE2

Source:	Human platelets
Substrate:	25.1 μM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$
Quantitation method:	Quantitation of [^{3}H] Adenosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

152000 phosphodiesterase PDE3

Source:	Human platelets
Substrate:	1.01 μM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$
Quantitation method:	Quantitation of [^{3}H] Adenosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

154000 phosphodiesterase PDE4

Source:	Human U 937 cells
Substrate:	1.01 µM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$
Quantitation method:	Quantitation of [^{3}H] Adenosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

156000 phosphodiesterase PDE5

Source:	Human platelets
Substrate:	1.01 µM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$
Quantitation method:	Quantitation of [^{3}H] Guanosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

156100 phosphodiesterase PDE6

Source:	Bovine retinal rod outer segments
Substrate:	100 µM ([^{3}H] cAMP + cAMP)
Vehicle	99% H_2O
Pre-incubation time / temp:	15 minutes at 25°C
Incubation time / temp:	20 minutes at 25°C
Incubation buffer:	50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$
Quantitation method:	Quantitation of [^{3}H] Guanosine
Significance Criteria:	≧ 50% of max stimulation or inhibition

4.3.4 Results

The inhibitory activity of II-13•HCl and III-20•HCl against PDE 1-6 was determined by MDS Pharm Services – Taiwan Ltd. Pharmacology Laboratories.

To ensure the reliability and repeatability of the experiments, the reported literature methods were employed and the literature results were used as reference standard to analyze the experiments. The used reference enzymes were shown in Table 4.3. Biochemical assays of the results were expressed as % inhibition of the activity of PDE and the half maximal inhibitory concentration IC_{50}.

Table 4.3 Reference compounds for PDEs inhibition

CAL =	PDE	Reference enzyme	MIC				
			IC_{50} (μM)	Ki	nH	Patch	IC_{50} (μM)
146000	PDE1	IBMX	3.8			136834	6.57
148000	PDE2	EHNA	1.8			136835	1.79
152000	PDE3	Cilostamide	0.05			136836	0.0839
154000	PDE4	Ro 20-1724	1.1			136837	1.14
		Ro 20-1724	1.1			137190	1.13
156000	PDE5	Zaprinast	1.1			136838	1.25
		Zaprinast	1.1			137467	0.758
156100	PDE6	Zaprinast	0.22			136839	0.246
		Zaprinast	0.22			137192	0.382

Table 4.4 showed the reported PDE 1-6 inhibition chart from MDS Pharm Services for II-13•HCl and III-20•HCl (10μM each). Comparison of PDE 1-6 inhibitory activity of III-20•HCl and II-13•HCl with established PDE 5 inhibitors was summarized in Table 4.5*(Gresser & Gleiter,2002;Ballard et al.,1998;Hyounmie et al.,2002)*.

Table 4.4 Percents(%)inhibition of II-13•HCl and III-20•HCl against PDE1-6

	COMPOUND CODE	PT NUMBER	BATCH	SPP.	n=	CONC.	†% INHIBITION %	IC$_{50}$	K_I	n_H	R
	146000 Phosphodiesterase PDE1										
	WZA-001.HCl*	1058905	136834 bov	2		10 µM	36				
	WZA-002.HCl**	1058906	136834 bov	2		10 µM	38				
	148000 Phosphodiesterase PDE2										
	WZA-001.HCl	1058905	136835 hum	2		10 µM	5				
	WZA-002.HCl	1058906	136835 hum	2		10 µM	15				
	152000 Phosphodiesterase PDE3										
	WZA-001.HCl	1058905	136836 hum	2		10 µM	27				
	WZA-002.HCl	1058906	137465 hum	2		10 µM	34				
	154000 Phosphodiesterase PDE4										
♦	WZA-001.HCl	1058905	136837 hum	2		10 µM	55				
♦			137190 hum	2		100 µM	83	12 µM			
♦				2		30 µM	66				
				2		10 µM	46				
				2		3 µM	27				
♦	WZA-002.HCl	1058906	136837 hum	2		10 µM	52				
♦			137190 hum	2		100 µM	83	13.6 µM			
♦				2		30 µM	65				
				2		10 µM	47				
				2		3 µM	19				
	156000 Phosphodiesterase PDE5										
♦	WZA-001.HCl	1058905	136838 hum	2		10 µM	106				
♦			137467 hum	2		0.1 µM	82	0.014 µM			
♦				2		0.03 µM	75				
				2		10 nM	45				
				2		3 nM	3				
♦	WZA-002.HCl	1058906	136838 hum	2		10 µM	103				
♦			137467 hum	2		0.1 µM	93	8.59 nM			
♦				2		0.03 µM	80				
♦				2		10 nM	53				
				2		3 nM	26				
	156100 Phosphodiesterase PDE6										
♦	WZA-001.HCl	1058905	136839 bov	2		10 µM	104				
♦			137192 bov	2		3 µM	94	0.266 µM			
♦				2		1 µM	80				
♦				2		0.3 µM	52				
				2		0.1 µM	27				
♦	WZA-002.HCl	1058906	136839 bov	2		10 µM	102				
♦			137192 bov	2		1 µM	95	0.125 µM			
♦				2		0.3 µM	71				
				2		0.1 µM	43				
				2		0.03 µM	19				

* WZA-001•HCl is the compound III-20•HCl.

**WZA-002•HCl is the compound II-13•HCl.

♦ Denotes item meeting criteria for significance.

†Results with ≥50% stimulation or inhibition are highlighted. (Negative values correspond to stimulation of binding or enzyme activity)

R=Additional Comments; bov=bovine; hum=human.

Table 4.5 IC_{50} of II-13•HCl and III-20•HCl against PDE1-6

PDE types	IC_{50} (nM)				
	III-20•HCl	**II-13•HCl**	**vardenafil**	**sildenafil**	**udenafil**
PDE1	>10000	>10000	70	281	870
PDE2	>10000	>10000	6200	>30000	101,000
PDE3	>10000	>10000	>1000	16200	52,000
PDE4	>10000	>10000	6100	7680	No reference
PDE5	2.01-14	8.59	0.14	3.5-8.5	5
PDE6	266	126	3.5	37	53

4.3.5 Conclusions

This assay showed PDE5 is the most selective target for II-13•HCl and III-20•HCl and the activity of two compounds towards to PDE5 is almost equal, though which is 100 times lower than vardenafil, which is almost equal to udenafil and sildenafil. Same to the established PDE5 inhibitors, PDE6 is the second most selective target for II-13•HCl and III-20•HCl. It was believe the activity of sildenafil toward to the PDE6 is the reason for sight side effect of sildenafil. Therefore the selectivity or the ratio of the inhibition between PDE5 and PDE6 became another ruler for judge whether or not a PDE5 inhibitor could become a drug. The selectivity of sildenafil towards PDE5 and PDE6 is about 10 times, while III-20•HCl is 16 and II-13•HCl 15, both higher than sildenafil.

4.4 Preliminary toxicity

4.4.1 Introduction

In the preliminary bioactivity experiments, the compounds II-13•HCl and III-20•HCl showed best activity in stimulating the penile erectile on conscious rabbit models;

therefore they were selected to do further drug suitability studies. First is to examine their safety, by evaluating acute toxicity and mortality of mice by oral administration and i.p. administration.

4.4.2 Oral administration Acute Toxicity

4.4.2.1 Materials and Method

Drugs: In the following experiments, we gave a codename of wanzaonafil to compound II-13•HCl and a codename of yonkenafil to compound III-20•HCl. These two compounds were slightly yellow crystal powders, and were soluble in water. The drugs were dissolving in distilled water to the required concentration before use.

Animals: Kunming mice, weighing 19~23g (half female and half male), were provided by Laboratory Animal Centre, Peking University Health Science Centre (Certification No. SCXK-(Jing) 2002-001). Mice were housed in the constant temperature (21-23°C) and constant humidity (45-65%) room. They were kept under a reversed 12-h light/dark cycle. Every 5 same sexual mice were fed in one cage. They were had the granule feed and free access to water.

Methods: 60 male and 60 female Kunming mice were randomly assigned to 12 groups (10 mice per group). Six groups were oral administrated different concentrations of yonkenafil and the rest were oral administrated wanzaonafil. After fasting for 12 hr (free access to water), the mice were orally administered 0.2ml/10g yonkenafil or wanzaonafil by gavage. Observation was continuously for 2 weeks and the mice's behaviour was recorded, including activity, body weight, food consumption, faces and death. After 14 days, all surviving animals were subjected to a gross necropsy.

4.4.2.2 Results

A few of mice died in the first two days. Necropsy performed on the mice showed that no significant changes in their viscus (heart, live, spleen, lung and kidney). A few of animals in the first 2 days manifested activity reduction, impotence, rough hair, food consumption decreasing and body weight gained slowly. Remained mice were survival for 2-14 days. No significant behaviour changes were found in the surviving mice. Their behaviour, hair, food consumption, faces and body weight become normal. Calculate LD_{50} and 95% confidence level by modified Korbor method. The results were shown in Table 4.6 and Table 4.7.

Table 4.6 Results of acute toxicity of mice per oral administration of yonkenafil

Dose	Animals	Observe days (day) / Death (number.)					Mortality	LD_{50} (mg/kg) 95%
(mg/kg)	(number)	1d	2d	3d	7d	14d	(%)	Confidence level
3926	10	9	9	9	9	9	90	
2804	10	6	6	6	6	6	60	
2003	10	5	5	5	5	5	50	2000
1431	10	3	3	3	3	3	30	(1610-2483)
1022	10	1	1	1	1	1	10	
730	10	1	1	1	1	1	10	

Table 4.7 Results of acute toxicity of mice per oral administration of wanzaonafil

Dose	Animals	Observe days (day) / Death (number.)					Mortality	LD_{50} (mg/kg) 95%
(mg/kg)	(number)	1d	2d	3d	7d	14d	(%)	Confidence level
393	10	9	9	9	9	9	90	
280	10	6	6	6	6	6	60	
200	10	5	5	5	5	5	50	200
143	10	3	3	3	3	3	30	(161-248)
102	10	1	1	1	1	1	10	
73	10	1	1	1	1	1	10	

4.4.2.3 Conclusions

The LD_{50} value of mice oral administration of yonkenafil was 2000 mg/kg and 95% confidence level was 1610-2486 mg/kg. The LD_{50} value of mice oral administration of wanzaonafil was 200 mg/kg and 95% confidence level was 161-248 mg/kg. Yonkenafil had a low acute toxicity than wanzaonafil. The LD_{50} value of yonkenafil was ten times higher than that of wanzaonafil. This result showed that yonkenafil possesses a better opportunity to become a new anti-ED drug, while wanzaonafil is too toxic to deserve a continuous study.

4.4.3 Acute toxicity of intraperitoneal (I.p.) injection administration of yonkenafil and wanzaonafil

4.4.3.1 Materials and methods

***Drugs*:** Yonkenafil and wanzaonafil were slightly yellow crystal powders, and were soluble in water. The drugs were dissolving in distilled water to the required concentration before use.

***Animals*:** Kunming mice, weight 19~23g (half female and half male), were provided by Laboratory Animal Centre, Peking University Health Science Centre (Certification No. SCXK-(Jing) 2002-001). Mice were housed in the constant temperature (21-23°C) and constant humidity (45-65%) room. They were kept under a reversed 12-h light/dark cycle. Every 5 same sexual mice were fed in one cage. They were had the granule feed and free access to water.

***Methods*:** 60 male and 60 female Kunming mice were randomly assigned to 12 groups (10mice/group). Six groups were intraperitoneal administrated different

concentrations of yonkenafil and the rest groups were administrated wanzaonafil. After fasting for 12 hr (free access to water), the mice were i.p. injection administered 0.2 ml/10g yonkenafil or wanzaonafil. Observation was continuously for 2 weeks and the mice's behaviour was recorded, including activity, body weight, food consumption, faces and death. After 14 days, all surviving animals were subjected to a gross necropsy.

4.4.3.2 Results

Death of mice were happened in most of injection groups in the first 2 days after administration. Necropsy performed on the mice showed no significant changes in their viscus (heart, live, spleen, lung and kidney). No lethality was observed at the dosage of 400mg/kg in yonkenafil groups. Some of animals in the first 2 days manifested activity reduction, rough hair and food consumption decreasing. Remained mice were survival for 2-14 days. No significant behaviour changes were found in the surviving mice. Their behaviour, hair, food consumption, faces and body weight become normal. Calculate LD_{50} and 95% confidence level was calculated by modified Korbor method. The results were shown in Table 4.8 and Table 4.9.

Table 4.8 Results of acute toxicity of mice i.p. administration of yonkenafil

Dosage	Animals	Observe days (day) / Death (number.)					Mortality	LD_{50} (mg/kg)
(mg/kg)	(No.)	1d	2d	3d	7d	14d	(%)	95% Confidence level
995	10	9	9	9	9	9	90	
829	10	7	8	8	8	8	80	
691	10	6	6	6	6	6	60	634
576	10	4	5	5	5	5	50	(566~710)
480	10	2	2	2	2	2	20	
400	10	0	0	0	0	0	0	

Table 4.9 Results of acute toxicity of mice i.p. administration of wanzaonafil

Dosage	Animals	Observe days (day) / Death (number.)					Mortality	LD_{50} (mg/kg)
(mg/kg)	(No.)	1d	2d	3d	7d	14d	(%)	95% Confidence level
75	10	9	9	9	9	9	90	
62	10	7	8	8	8	8	80	
52	10	5	6	6	6	6	60	48
43	10	4	4	5	5	5	50	(42~53)
36	10	1	2	2	2	2	20	
30	10	0	0	0	0	0	0	

4.4.3.3 Conclusions

The LD_{50} value of mice single i.p. administration of yonkenafil was 634 mg/kg and 95% confidence level was 566-710 mg/kg. The LD_{50} value of mice single i.p. administration of wanzaonafil was 48 mg/kg and 95% confidence level was 42-53 mg/kg. The results were same to the above oral administration; therefore yonkenafil was selected to do further research while wanzaonafil was abandoned at this stage.

4.5 Evaluation of sexual stimulation with mice model

4.5.1 Introduction

Based on above activities of penile erections of two groups of the compounds and acute toxicities of the representatives of two series, Yonkenafil and wanzaonafil, we concluded that the group of the compounds with 2-substituted-phenyl-3,7 -dihydro-pyrrolo[2,3-d]pyrimidin-4-one feature probably have potential to become new PDE5 inhibitors, while the group compounds with 2-substitutedphenyl -imidazo[1,5-a]-1,3,5-triazine-4-(3H)-ones feature could not be drugs because their therapeutic window is too narrow. Therefore, we selected a few samples of 2-substituted-phenyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one group to conduct

further evaluation of their bioactivities using mice model.

4.5.2 Materials and methods

***Drugs and reagents*:** Yonkenafil and its 2-substituted-phenyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one derivatives are slightly yellow powder. Positive control drug sildenafil(raw material) is a white powder. Estradiol benzoate, standard product, a white powder, was purchased from National Institute for the Control of Pharmaceutical and Biological Products, China.

***Animals*:** Male and female sexual matured Kunming mice weighing 30-34g were provided by Laboratory Animal Centre, Academy of Military Medical Sciences (Certification No. SCXK-(Martial) 2002-001). The ratio of male and female mice is 1 to 3. Female mice were treated with subcutaneous injection of estradiol benzoate solution (20μg/mouse). Test was begun after 48 hours of injection.

4.5.3 Experiments

The male and female mice treated by estrogen are placed in the same cage for 2 days and the male mice obtain sexual experience. Then taken out the female mice from cage and let the male mice rest for 5 days. Test groups were orally administered with 24mg/kg yonkenafil, III-21•HCl and III-26•HCl respectively. The positive control group is administered 24mg/kg of sildenafil and the control group is administered with the equal volume of physiological saline (0.1ml/10g). After 50min, the male mice were put into the observation containers (diameter: 24cm; height: 24cm) for 5min, and then two female mice treated with estrogen were introduced into the containers. Use Panasonic WVCP410/G monitor to observe the behaviour of male mice for 20 min without disturbance. Bestriding latent time, bestriding times, mating latent time and mating times were recorded.

4.5.4 Results

As shown in Table 4.10, in comparison with the physiological saline group, sexual function indexes of the animals were significantly increased after orally administered sildenafil, for example the bestriding and the mating latent period was markedly shortened and the mating times were markedly increased. The results of orally administered yonkenafil were similar to that of the sildenafil group, however yonkenafil showed some advantages such as the mating times were markedly increased ($P<0.05$). Sexual function indexes of the mice had no obvious change after orally administered III-21•HCl as compared with that of the physiological saline group. III-26•HCl could markedly shorten the mating latent period of the mice, but had little effect on other indexes.

Table 4.10 Effect of test samples on male mice sexual function indexes (mean±SD)

Groups	Animal number	Bestriding latent period (min)	Bestriding times	Mating latent period (min)	Mating times
Physiological saline	17	8.9±3.69	7.1±3.62	16.2±4.02	3.4±4.64
Yonkenafil	9	2.6±1.13**	7.6±2.70	7.1±5.62**	13.1±8.77$^{**\#}$
III-21•HCl	9	6.1±5.42	8.7±3.87	10.7±6.10	7.6±7.33
III-26•HCl	8	6.4±3.07	8.0±3.93	8.8±5.85*	9.0±8.50
Sildenafil	19	5.0±4.74*	12.7±7.18	8.2±4.02**	8.2±5.25*

Compared with physiological saline group: * $P<0.05$, ** $P<0.01$;
compared with sildenafil group: $^{\#}$ $P<0.05$

4.5.5 Conclusions

Yonkenafil can markedly increase mice sexual function and overall sexual profile is better than that of sildenafil; III-21•2HCl has no obvious effects on mice sexual function indexes; III-26•2HCl can markedly shorten mating latent period of the mice.

4.6 Comparison of inhibition activity of yonkenafil and sildenafil against PDE5

4.6.1 Materials and methods

Phosphodiesterase-[^{3}H]cGMP-SPA Enzyme Assay (Amersham Biosciences), cGMP specific phosphodiesterase 5 (Bovine, Recombinant, Calbiochem) were commercial products. Yonkenafil is yellow light powder, easily soluble to water and methanol, but slightly soluble to alcohol or acetonitrile. It was diluted to the needed concentration with 2% DMSO before use. Positive control drug sildenafil was a commercial product of Pfizer. It was dissolved with 2% DMSO before use. TopCount events-per-unit-time meter used in this experiment was produced by Packard BioSciece Company.

SPA (Scintillation Proximity Assay) was used to carry out this study. Its rudiment is that the PDE5 can make the cGMP remove its annuli to become GMP, and the GMP can combine to the SPA-YS superbead. Because the cGMP has been marked with ^{3}H(^{3}H-cGMP), and ^{3}H can retain in the GMP(^{3}H-GMP), it will photogenesis when the GMP combine to the SPA-YS superbead (the wavelength between 350-500 nm). The luminous intensity is correlated to the contents that the ^{3}H-GMP combined to the SPA-YS superbead. Then we can use TopCount events-per-unit-time meter to detect the luminous intensity and analyze the contents of 3H-GMP in the solution. According to the results, we can calculate the activity of PDE5, and then to analyze the effects of inhibitor to the activity of PDE5.

The determination of enzyme activity refer to the description of phosphodies-terase-[^{3}H]cGMP-SPA enzyme assay (Table 4.11) . The reaction system includes: 50mM Tris-Hcl (PH7.5), 8.3mM $MgCl_2$, 1.7mM EGTA, the recombination of PDE5 Unit; inhibitors (different concentration, 10μl), the control tubes with the same

volume of solvent, each of them is to be parallel tube. After the application of sample, took them to preheat in 30°C for 10 minutes and added up 10μl ^{3}H-cGMP(1mCi/ml, 10-30Ci/mmol, 37MBq/ml, diluted to 200times). Then made it begin to react under 30°C and the total volume of 100μl. After reacted 30 min, 50μl SPA superbead (contain superbead 18mg/ml and 18mM ZnSO4) was added to terminate the reaction. Finally, it was transferred to the prepared 96-well plates and stabilized 30 min. Then the TopCount events-per-unit-time meter was used to count when the superbeads have been settled.

Table 4.11 Assay protocol conditions were used

	Control	Sample	Blank
10x assay buffer	10μl	10μl	10μl
PDE enzyme	10μl	10μl	-
Water	60μl	60μl	70μl
Inhibitor sample	-	10μl	-
Inhibitor diluent	10μl	-	10μl
[^{3}H]cAMP or cGMP	10μl	10μl	10μl
Incubate for a defined time at 30°C			
SPA beads	50μl	50μl	50μl
Mix well and allow to stand at room temperature for 20 minutes			
Count tube/trays/plates in a scintillation counter			

4.6.2 Results

The concentration of the yonkenafil and sildenafil in this trial are 10^{-10}-10^{-4} mol/L. The results of the inhibition ratio detected were recorded in Table 4.12.

From to Table 4.12, it can be seen that both yonkenafil and sildenafil showed strong inhibition effects on the PDE5. When the concentration reaches to 10^{-6}mol/L, yonkenafil can inhibit the activity of PDE5 in the solution completely, while the

sildenafil can not inhibit completely With the statistical analysis, the needed concentration to inhibit the half of activity of the PDE5 (IC_{50}) for yonkenafil is 2.01nM, while the sildenafil is 4.46nM, which is within the range of reported results 3.5-8.5nM. It is obviously that the IC_{50} of yonkenafil is much lower than that of sildenafil. So it indicated that the inhibitive activity of yonkenafil against PDE5 is stronger than sildenafil.

Table 4.12 The inhibition of yonkenafil and sildenafil with different conc. to PDE5

Drug Conc. (mol/L)	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Inhibition of Yonkenafil (%)	0.00	14.44	66.87	95.53	100	100	100
Inhibition of Sildenafil (%)	0.00	19.44	70.37	79.61	96.52	100	100

4.6.3 Conclusions

The results of this assay demonstrated that yonkenafil and sildenafil have strong inhibition effects against PDE5, moreover the effect of yonkenafil seemed to be stronger than the sildenafil (Table 4.12) and yonkenafil showed some advanced aspects; therefore, it was worth to conduct further investigation to see if yonkenafil is druggable.

Chapter 5

Studies of the Pharmacodynamics and General Pharmacology of Yonkenafil

5.1 Introduction

In this project, we designed and synthesized two series of new compounds as PDE5 inhibitors for treatments of ED. The preliminary studies of bioactivities and toxicities of the compounds had been presented in the preceding chapters. The results demonstrated that 2-[2- ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (Fig. 5.1, yonkenafil) possesses the highest activity and lowest toxicity among those compounds; therefore it was selected to be a candidate drug for preclinical studies. In addition to chemistry, pharmaceutical studies, pharmacodynamic study is the key element in the preclinical studies. In this chapter, the studies of pharmacodynamics of yonkenafil in rats and rabbits and its general pharmacology in mice and dogs were presented.

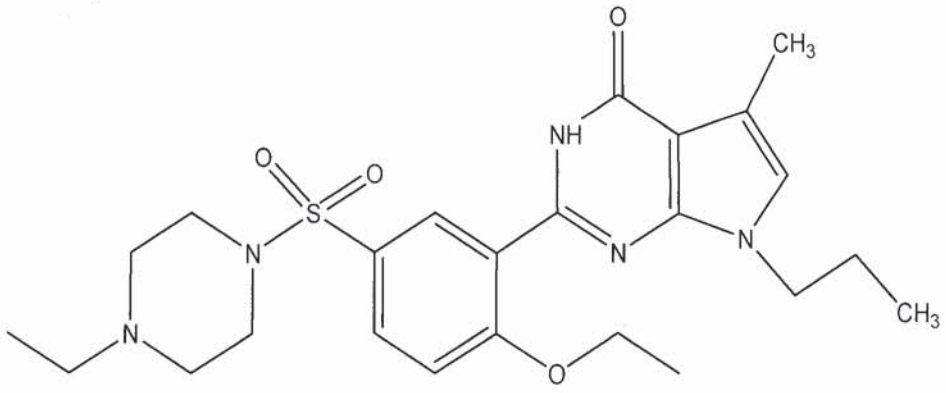


Figure 5.1 Structure of yonkenafil

5.2 Effects of yonkenafil on male mice sexual function after a single administration

5.2.1 Purpose

In the previous chapter, the activities of yonkenafil on stimulating animal sexual function and against PDEs were described. In order to confirm the inhibitive effects of yonkenafil on PDE5, the ex vivo experiments with mice were designed in comparison with sildenafil, the positive control agent.

5.2.2 Reagents and animals

Yonkenafil is yellow light powder, diluted to the needed concentration with distilled water before use; sildenafil, white powder, was purchased from Pfizer Inc; estradiol benzoate, white powder, was bought from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), China; progesterone injection (10mg/ml) was purchased from Xianju Pharmaceutical Co., Ltd. Zhejiang China. Animals: Kunming mice (weight: 30-34g; Certificate NO. SCXK-2002-001) were purchased from The Academy of Military Medical Sciences (AMMS); the ratio of male and female mice is 1 to 3.

5.2.3 Methods and Principle

The female mice were given a subcutaneous injection of estradiol benzoate oil solution 48h before they were used in tests. The mice were housed separately in the course of experiments.

In order to make the male mice obtain the sex experience, they were housed with the

female mice for two days, and then the female mice were taken out from the cages to make the male mice have rest.

At first, yonkenafil or sildenafil at the dose of 24mg/kg was given orally to the mice, respectively, and the same volume of sodium chloride (0.1ml/10g) as the indicated drugs to the blank control animals. Fifty minutes after administration, male mice were put into a container for 5min to adapt. Then put two female mice administered estradiol benzoate oil solution to the container. A monitor (Panasonic, WVCP410/G) was used to observe and record the male mice sexual behaviour. The animals' incubation period of straddle, the frequency of straddle, the incubation period of copulation and the frequency of copulation were recorded in 20 min.

5.2.4 Results of yonkenafil on male mice sexual function

The sexual function parameters of male mice were shown in Table 5.1. According to the Table 5.1, it is clear that the sexual functional parameters in the positive control group were strengthened obviously after administered sildenafil 50min. The incubation period of straddle and the incubation period of copulation were decreased, and the frequencies of copulation were increased. The animals could accomplish the copulation under the impulse, so the frequency of straddle had no obvious changes compared with the control group. These findings implicated that yonkenafil bore similarity in potency with that of the sildenafil; however overall profile of sexual functions afforded by yonkenafil were obviously stronger than the positive agent, for example, the frequency of copulation of the mice in the yonkenafil group was increased significantly than that in the positive control group ($P<0.05$).

The results demonstrated that yonkenafil can strengthen the male mice sexual function obviously, and it is much more effective than the sildenafil at the same dose.

Table 5.1 The sexual function parameters of male mice after administered yonkenafil, sildenafil and blank control (X±SD).

Groups	Amounts of animals	Incubation period of straddle（min）	Frequency of Straddle	Incubation period of copulation (min)	Frequency Of copulation
Sodium Chloride	17	8.9±3.69	7.1±3.62	16.2±4.02	3.4±4.64
Yonkenafil	9	2.6±1.13**	7.6±2.70	7.1±5.62**	13.1±8.77**$^{\#}$
Sildenafil	19	5.0±4.74*	12.7±7.18	8.2±4.02**	8.2±5.25*

To compare with the blank control group: * P<0.05, **P<0.01,
To compare with the positive control group: $^{\#}$P<0.05.

5.3 Effects of yonkenafil on male rats sexual function after a single administration

5.3.1 Purpose

In the previous chapter, the effects of yonkenafil on stimulating animal sexual function and against PDEs were described. In order to confirm the inhibitive activities of yonkenafil to PDE5, the ex vivo experiments with rats were designed in comparison with sildenafil, the positive control agent.

5.3.2 Materials and methods

Yonkenafil, yellow light powder, was diluted to the needed concentration with distilled water before use; sildenafil was purchased from Pfizer Inc; estradiol benzoate, white powder, was bought from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), China; progesterone injection (10mg/ml) was purchased from Xianju Pharmaceutical Co., Ltd. Zhejiang China. Animals: Wistar rats

(male: 250-280g; female: 220-260g; Certificate NO. SCXK-2003-0001), purchased from Experimental Animal Centre at the Basic Medical of Jilin University.

5.3.3 Experiments

At first, the ovaries of 50 healthy female rats weighing 220-260g were removed after etherization by surgical operation. Then they were successively given an i.m. injection of penicillin G(2U/kg) once in 3 days. Three weeks after the operation, they were used in experiments. 48 hours before the experiments, they were given an subcutaneous injection of Estradiol Benzoate (20μg/per one), and progesterone(500μg/per one) was injected respectively 4 hours before the experiment.

Then, 50 healthy male rats were divided into 5 groups randomly, blank control group, positive control group(sildenafil 10mg/kg), test group I (yonkenafil 7.5mg/kg), test group II (yonkenafil 15mg/kg) and test group III (yonkenafil 30mg/kg). Each group of animals were administered by oral administration. One hour after administration, the male rats were separated alone. 5 Minutes later, a female rat was put into the cage and the following data were recorded in 20 minutes: □ The time that the male rat first time to capture the female rat (the incubation period of capture); □The frequency that the male rat to capture the female rat (the frequency of capture).

Date analysis: The experiment results were expressed by 'X±SD' and analyzed by interclass T-test.

5.3.4 Results and discussions

According the Table 5.2, it has shown that the yonkenafil shorten incubation period of capture of the male rats and increased the frequency of capture. In comparison with the blank control or sildenafil groups, 15mg/kg yonkenafil group took significant

advantage in stimulating rats sexual function.

Table 5.2 Effects of yonkenafil on the rats' copulation function (X±SD)

Group	Dosage (mg/kg)	Animals	Incubation period Of capture (sec)	Frequency of capture
Control Group	-	10	95.5±42.4	13.6±7.0
Sildenafil	10	10	25.9±17.1***	22.8±8.3*
	30	10	59.1±32.1*	24.1±13.6*
Yonkenafil	15	10	19.4±11.6***	45.8±14.6***
	7.5	10	24.7±14.7***	21.6±9.6*

To compare with the control group: * $P < 0.05$,** $P < 0.01$,***$P < 0.001$.

5.4 Effects of yonkenafil in treatments of rat erectile dysfunction induced by paroxetine

5.4.1 Purpose

In the previous chapter, the activities of yonkenafil to stimulate animal sexual function and against PDEs were described. In order to confirm the inhibitive effects of yonkenafil to PDE5, the ex vivo experiments with erectile dysfunction rats induced by paroxetine were designed in comparison with sildenafil, the positive control group

5.4.2 Materials and methods

Yonkenafil, yellow light powder, was diluted to the needed concentration with distilled water before use; sildenafil, purchased from Pfizer Inc; paroxetine: purchased from Huahai Pharmaceutical Co., Ltd. Zhejiang China. Animals: Wistar rats (male:

250-280g; female: 220-260g; Certificate NO. SCXK-2003-0001), purchased from Experimental Animal Centre at the Basic Medical of Jilin University. Instrument: YSD-4 Polygraph (Anhui China).

5.4.3 Experiments

Sixty healthy male rats were divided into 6 groups. One of these group is normal control group with an intragastric administration of distilled water and the other 5 were groups with a tail intravenous injection of paroxetine(10mg/kg) followed by intragastric administration of yonkenafil(used the dosages were listed in the Table 5.2). One hour after administration of yonkenafil, the polygraph's stimulating electrode was used to stimulate the penis of the trial rats. The stimulation parameters were set as 'successive A, the frequency is 64 Hz, the wave width is 2ms and the voltage is 10V". The period from the initiation of stimulation to the penile erection (the incubation period of penile erection) was recorded and analyzed.

5.4.4 Results and discussions

Paroxetine is a selective 5-HT reuptake inhibitor. One of its side effects is that it can cause erectile dysfunction. In this study, first trial rats were injected with paroxetine to create ED model, and then were administrated yonkenafil or sildenafil. The treated animals were observed carefully to see their reaction to the drugs and then data were collected for assessments of the therapeutic effect against ED. The experiment results were shown in Table 5.3.

According to the Table 5.3, it can be seen that in the three groups treated with yonkenafil, including high-dose group, middle-dose group and low-dose group, the incubation period of erectile dysfunction were shorten obviously in comparison with the untreated group. In comparing with sildenafil-treated rats, it was shorten the

incubation period of erectile dysfunction of the rats administrated by yonkenafil at the high or middle dose, even the improvement of the yonkenafil low dose group was assessments equal to that of sildenafil group.

Table 5.3 Effects of yonkenafil on the rats' erectile dysfunction induced by paroxetine

Group	Dosage (mg/kg)	Animals	Incubation of penile erection (seconds)
Normal control group	--	10	9.2±3. 22
Paroxetine	10	10	37.6±16. 52
Sildenafil + Paroxetine	10+10	10	20.7±5. 16
	30+10	10	12.4±4. 95
Yonkenafil+Paroxetine	15+10	10	12.8±6. 86
	7.5+10	10	21.4±8. 82

To compare with the Paroxetine group: $P < 0.05$, $P < 0.01$, $P < 0.001$.

5.5 Effects of yonkenafil on male rabbit sexual function after a single administration

5.5.1 Purpose

In the previous chapter, the activities of yonkenafil on stimulating animal sexual function and against PDEs were described. In order to confirm the inhibitive effects of yonkenafil to PDE5, the ex vivo experiments of a single administration of yonkenafil into rabbits were designed in comparison with sildenafil, the positive control group.

5.5.2 Materials and methods

Yonkenafil, yellow light powder, was diluted to the needed concentration with distilled water before use; sildenafil , purchased from Pfizer Inc. Animals: flap-eared

rabbits (a combination of male and female, 3-3.5kg), were purchased from Experimental Animal Centre at PLA 208 Hospital, Certificate No. is SCXK-2002-005.

5.5.3 Experiments

Before the experiments, 60 experimental rabbits (male: female=1:1) were acclimated for one week. Then the male rabbits were divided into 5 groups, blank control group (oral administered by the same volume of distilled water), positive control group (oral administered by sildenafil (6mg/kg)), yonkenafil low-dose group (4mg/kg), yonkenafil middle-dose group (8mg/kg) and yonkenafil high-dose group (16mg/kg). After administered sildenafil or yonkenafil one hour, put a male rabbit and a female one into a cage together to observe and record the incubation period of capture (the time that the male first time to capture the female) and the frequency of capture in 20 minutes.

5.5.4 Results and discussions

Table 5.4 Effects of yonkenafil on the rabbits' capture capability (X±SD)

Group	Dosage (mg/kg)	Animals	Incubation period of capture (minute)	Frequency of capture
Blank Control Group	-	8	2.75±2.38	9.5±4.8
Sildenafil	6	8	0.75±1.75	16.4±7.3*
	16	8	0.25±0.46**	22.1±13.8*
Yonkenafil	8	8	0.62±1.06*	19.8±9.2**
	4	8	0.88±1.46	14.8±9.9

To compare with the blank control group : *P < 0. 05 , **P < 0. 01, ***P < 0. 001.

In the Table 5.4, it can be found that the yonkenafil can shorten the male rat incubation period of capture and increase the frequency of capture significantly in

comparison with the blank control group. The sexual stimulation effects of yonkenafil were increased in line with the increase of its dosage. In this experiment, the sexual stimulation effects of sildenafil group were similar to the low dosage group of yonkenafil, reflecting promising of development of yonkenafil into a drug for treatments of ED.

5.6 Effects of yonkenafil on the level of rabbit sexual hormone

5.6.1 Purpose

In the previous chapter, the activities of yonkenafil to stimulate animal sexual function and against PDEs were described. In order to confirm the inhibitive effects of yonkenafil to PDE5, the ex vivo experiments for measurements of level of rabbit sexual hormone after administration of yonkenafil were designed in comparison with sildenafil, the positive control group

5.6.2 Materials and methods

Yonkenafil, yellow light powder, was diluted to the needed concentration with distilled water before use; sildenafil was purchased from Pfizer Inc. Radio-immunity Kit (E2, T) was purchased from Furui Biotechnology Company. Animals: flap-eared rabbit (male, 3-3.5kg) was purchased from Experimental Animal Centre at PLA 208 Hospital, Certificate No. is SCXK-2002-005.

5.6.3 Experiments

Before the experiment, 60 experimental rabbits (male: female=1:1) were acclimated

for one week. Then the male rabbits were divided into 5 groups randomly, blank control group, positive control group, yonkenafil low-dose group, yonkenafil middle-dose group and yonkenafil high-dose group. The rabbits were first blood sampled from auricular vein for 2ml per rabbit. The next day, experimental rabbits were administrated dosage as following: the positive control group with sildenafil (6mg/kg), yonkenafil low-dose group (yonkenafil 4mg/kg), yonkenafil middle-dose group (yonkenafil 8mg/kg), yonkenafil high-dose group (yonkenafil 16mg/kg), and the blank control group with distilled water (the same volume as other groups). One hour after administration, the bloods were sampled from the rabbit aural-vain, and then the testosterone (T) and estrogen (E2) in the blood were monitored and determined by radio-immunity method. The result expressed by the percentage that the level of rabbit sexual hormone change (change%=(the level of pre-administration subtract the level of post-administration)×100/the level of pre-administration).

5.6.4 Results and discussion

Table 5.5 Effects of yonkenafil on the rabbits' hormone (X±SD)

Group	Dosage (mg/kg)	Animals	Change of testosterone (T%)	Change of estrogen (E2%)
Blank Control Group	-	6	23.23±83.26	3.45±42.69
Sildenafil	6	6	51.09±131.11	-0.38±17.59
	16	6	9.17±82.53	-7.19±17.48
Yonkenafil	8	6	28.93±1.32	0.25±25.13
	4	6	96.3±109.17	2.74±27.7

From the Table 5.5, it can be seen that in comparison with the blank control group, the animal testosterone and estrogen levels of both sildenafil and yonkenafil groups have no significant change. The levels of estrogen in yonkenafil groups seemed to show a descended tendency with increasing of dosages of the drug; however, there was no statistically significant.

5.7 Studies on the general pharmacology of yonkenafil

5.7.1 Purpose

The general pharmacology mainly refers to safety pharmacology. It mainly studies on the potential drug adverse effects on physiological function beyond the effective therapy dosage. The results of pharmacology will provide a reference dosage and method to toxicity studies.

5.7.2 Materials and methods

Yonkenafil, yellow light powder, was diluted to the needed concentration with normal saline before use. Heparin sodium parenteral solution was purchased from Biochemistry Pharmaceutical Factory of Tianjin. Pentobarbital was purchased from Sinopharm Chemical Reagent Co., Ltd. NaCl parenteral solution was a product of Shandong Hualu Drug Manufactory Co., Ltd. Kunming sexual maturity mice (amount: 40, weight: 18-22g) were provided by Experimental Animal Centre at the Medical Department of Peking University Certificate No. SCXK-(jing)2002-001. It is a combination of female and male, and the ratio of them is 1 to 1. Beagle dogs, weight 7~10 kg, the number of 20, is provided by Experimental Animal Centre of Beijing Tianshoushan, Certificate NO. SCXK-(jing)2003-001.

MP150 model of polygraph was made by BIOPAC Company, USA. DA100C model of biological amplifier and TSD160A respiration energy transducer were used as respiration recording equipments in this experiment. DA100C model of biological amplifier and TSD104A blood pressure transducer were used as blood pressure recording apparatus in this experiment. ECG100C model electrocardio magnifying apparatus were used as electrocardio recording equipment. XZ–4 mice spontaneous movement recorder, product of Institute of Materia Medica, Chinese Academy of

Medical Sciences.

5.7.3 Experiments

5.7.3.1 Dosage group and administration methods

Before the experiment, we refer to the pharmacodynamics of rabbit by yonkenafil treated (set up different dosage: 16 mg/kg, 8 mg/kg and 4mg/kg). The dogs were divided into three groups, 90 mg/kg, 45 mg/kg and 22.5mg/kg. The dosage of correspond mice spontaneous movement experiment were 432mg/kg, 216mg/kg and 108mg/kg.

5.7.3.2 The determination of mice spontaneous movement

40 Kunming mice, half female and half male were used and were randomly divided into four groups, they were yonkenafil high-dose group (432mg/kg), middle-dose group (216mg/kg), and low-dose group (108mg/kg) and solvent control group. Each group was consisted of ten mice. According to above-mentioned dosage, the drug was orally given and the solvent control group was given saline. The spontaneous movement frequency of mice was recorded for 5min prior to drug administration. After drug administration, mice spontaneous movement was recorded at 30min, 60min, 90min, 120min and 180min. At the same time, the behaviour disorder of mice will be recorded.

5.7.3.3 The effects of yonkenafil on the heart rate, electrocardio and respiration of anaesthesia dog

20 Beagle dogs were divided into 3 dosage groups of yonkenafil 90mg/kg, 45mg/kg,

22.5mg/kg and a solvent control groups. Each group was consisted 5 dogs. According to above-mentioned dosages 90mg/kg, 45mg/kg and 22.5mg/kg, yonkenafil was administrated by esophago-cannula and the solvent control group was given isovolumic saline.

According the dog weight, sodium pentobarbital (30mg/kg) was intravenous injected to anaesthetize the dogs. Then operations were carried out on dog neck to full expose trachea and left common carotid artery, followed by tracheal intubation and to link aspiration energy transducer. After that, arterial cannula (before the activity will prefill 0.1% solution heparin in arterial cannula) was adapted and then pressure transducer was connected. After that, II-lead electrocardiogram electrode, respiration energy transducer and pressure transducer were linked to polygraph then to prepare for treatments with drugs. After post-operation 30min, blood pressure (contractive pressure, diastolic pressure and mean blood pressure), electrocardiogram, heart rate, respiratory frequency and extent will be recorded. Then yonkenafil was administrated. At the point of 15min, 30 min, 45 min, 60min, 90 min, 120 min, 150 min and 180 min after drug treated, the above-mentioned index were recorded again (polygraph collect the breathing signal as flow ml/s, computer peak and minimum value respiratory wave as respiration extent, unit also is ml/s).

5.7.3.4 Treatment of data

The experiment results were expressed by 'X±SD'. The spontaneous movement experiment of mice was analyzed by T-test to compare drug treated group with control group. Anaesthetic dog experiment was analyzed by interclass T-test at different time point.

5.7.4 Results and discussions

5.7.4.1 Results of mice spontaneous movement experiment

The effect of yonkenafil on spontaneous movements of mice was shown in Table 5.6. From the Table 5.6, it can be seen that the spontaneous movement frequency of mice in control group was decreased with the lapse of time. It was due to mice gradually adaptation to the internal environment of recorder, so exploratory activity reduced. This phenomenon was considered as normal to animals. But in contrast with the control group, the groups of animals administrated yonkenafil behaved so differently and showed some kind of dose related. After 30min of administration of yonkenafil, the frequency of mice locomotor activities in 432mg/kg dosage group was reduced from 240 to 83 per min. The mice locomotor activities were obviously inhibited and the inhibition was significant difference from the control group. After this period, the mice autonomic activities increased gradually to the experiment ended (after treated 3hours) the mice autonomic activities were recovered to the level of the control group. In 216mg/kg dosage group, at the first 30min, the behaviours of mice autonomic activities were similar to the control group. After that the mice autonomic activities were increased, at 1 hour point the difference between the control group and the treated group reaching 70, and this trend was kept strengthening by the 2 hour point, the difference was widened to 2 times. This tendency was maintained to the end of experiment.

The results of 108mg/kg dosage group showed some similarity to that of the 216mg/kg dosage group. The excitement of the animals started after administration of yonkenafil and was maintained to the end of the experiment; however there a degree difference of excitement between the groups. The excitement of the higher dose group was kept growing the end of the experiment, while the low dose group, the excitement was kept at the same level through the first two hours and by the end it came down to the control group level almost. These results clearly demonstrated the mice spontaneous movements was dose related to the yonkenafil. In addition to above

observation, in the group of 432mg/kg yonkenafil dosage, we have observed mice ptosis, activity inhibiting, but no mice gait apraxia and dyscoria, and no salivation. Muscle trembling was manifested also. The above observations showed that yonkenafil had some periaqueductal gray stimulation to central nervous system. But in high dosage group, the inhibitive effect was appeared obviously. This is owing to the augmentation of medicine stimulation.

Table 5.6 Effects of yonkenafil on spontaneous movements in mice

(Times in 5min, X±SD, n=10)

Group	Dosage (mg/kg)	Before treated	After treated				
			30	60	90	120	180
Control	--	249±35	203±58	176±69	152±78	160±89	132±68
Yonkenafil	430	240±47	83±83**	108±94	115±104	112±111	133±122
	216	251±51	213±94	243±98	278±90**	313±111**	331±132**
	108	251±40	211±77	223±69	213±53	215±72	166±96

vs control group, * P<0.05, ** P<0.01

5.7.4.2 Results of heart rate, electrocardio and respiration of anesthesia dog

The pharmacodynamics behaviours on blood pressure of anesthesia dog administrated yonkenafil were shown in Table 5.7 to 5.9. The Table 5.7 to 5.9 showed blood pressure of the anesthetize dog was slightly increased during this experiment in comparison with the control group. This may be caused by anesthesia. The change of contractive pressure in the treatment group was shown in Table 5.7. In the 90mg/kg dosage group, the contractive pressure was declined immediately after administration of yonkenafil and the drop was extended to the greatest 20mmHg at 15 minutes. The low level of the pressure was kept through the period of three hours to the end of experiment. Over this period at the points of 45, 90, 120, 150, 180min, there was

significant difference of the contractive pressures between the control group and the treatment group. In contrast, there were no changes of the contractive pressure of dogs in the 45mg/kg dosage and 22.5mg/kg dosage was observed in the period of three hours experiments. In these two groups, contractive pressure took on rising to similar with control group.

Table 5.8 showed the results of diastolic blood pressure change caused by yonkenafil. The effects of yonkenafil on the dog diastolic blood pressure were similar to that of the contractive pressure. The decline of the diastolic blood pressure of dogs in 90mg/kg dosage group was rapidly after administration of the drug, taking about 15 min to get the lowest level. The diastolic blood pressures were kept at the same low level through three hours experiment and no recovering trend was observed. In comparison with the control group significant difference was recorded at any measuring point. The profiles of the diastolic blood pressure of 45mg/kg and 22.5mg/kg dosage groups were similar to that of the control group, showing a slight rising tendency at the end of experiments. But in the 45mg/kg dosage group, before administration of the drug, the foundation level had significant difference from control group (no statistical significance), so the result of diastolic blood pressure is significant difference with control group after 45min of administration of the drug. Mean blood pressure as the whole reflect between contractive pressure and diastolic blood pressure had the similar results.

Table 5.7 Effects of yonkenafil on contractive pressure in anesthetized beagles (mmHg, X±SD, n=5)

Group	Dosage (mg/kg)	Before treated	After treated							
			15	30	45	60	90	120	150	180
Contral	--	148±14	150±16	153±17	152±17	151±17	151±15	153±16	156±19	161±21
Yonkenafil	90	146±21	127±20	126±21	126±18*	130±20	126±13*	126±12*	124±9*	124±8**
	45	145±16	148±16	150±19	151±23	152±24	151±20	153±19	150±15	157±22
	22.5	151±13	149±17	148±20	147±18	148±20	154±23	153±21	153±18	155±27

vs control group, * $P<0.05$, ** $P<0.01$

Table 5.8 Effects of yonkenafil on diastolic pressure in anesthetized dogs

(mmHg, X±SD, n=5)

Group	dosage (mg/kg)	Before treated	After treated							
			15	30	45	60	90	120	150	180
Contral	--	109±18	110±13	112±10	113±11	111±13	113±11	113±13	116±12	117±14
Yonkenafil	90	101±13	89±13*	86±13**	85±12**	86±13*	82±12**	80±11**	78±5**	77±8**
	45	92±9	98±6	98±10	97±9*	98±12	98±11	98±10	100±13	101±13
	22.5	95±16	95±17	95±19	95±17	95±18	101±22	101±21	100±18	102±23

vs control group, * P<0.05, ** P<0.01

The effect of yonkenafil to animals on heart rate was recorded in Table 5.10. According to Table 5.10, it can be seen that the highest, 90mg/kg, dose group the heart rate of the animals were reduced significantly after administration of the drug. The reducing trend was kept through three hours and at the end of the experiment the trend seemed to keep going down and no recovery sign. For the groups of 45mg/kg and 22.5mg/kg, no significant difference was observed in comparison with the control group.

According to Table 5.11, it can be seen, before given the drug , there is significant individual difference on dog breathing frequency (it is relevant to individual and also to narcotization). From Table 5.12, there is some fluctuate of breathing when given the medicine to anesthetized dog and existed no significant difference between medication administration team and blank control group.

Table 5.9 Effects of yonkenafil on mean blood pressure in anesthetized dogs (mmHg, X±SD, n=5)

Group	Dosage (mg/kg)	Before treated	After treated							
			15	30	45	60	90	120	150	180
Contral	--	122±16	124±12	125±12	126±12	125±13	125±12	126±13	129±14	131±16
	90	116±16	102±15*	100±15*	98±14*	101±15*	96±12**	95±11**	93±6**	93±6**
Yonkenafil	45	110±9	115±9	116±13	115±13	116±16	115±13	116±12	117±13	120±6
	22.5	113±14	113±16	112±19	112±17	113±18	118±22	118±21	117±17	120±24

vs control group, * P<0.05, ** P<0.01

Table 5.10 Effects of yonkenafil on heart rate in anesthetized dogs (times/min, X±SD, n=5)

Group	Dosage (mg/kg)	Before treated	After treated							
			15	30	45	60	90	120	150	180
Contral	--	162±21	166±24	166±27	156±24	160±29	154±29	153±29	158±41	152±42
	90	162±19	141±25	138±24	140±20	144±19	137±13	131±10	125±17	122±26
Yonkenafil	45	165±39	160±35	158±43	155±42	158±48	163±58	164±59	161±56	162±57
	22.5	156±25	154±24	152±25	149±25	148±26	151±34	149±35	146±34	150±43

Table 5.11 Effects of yonkenafil on frequency of respiration in anesthetized dogs(time/min，X±SD, n=5)

Group	Dosage (mg/kg)	Untreated group	treated group							
			15	30	45	60	90	120	150	180
Contral	--	20±10	20±7	22±9	18±4	19±5	19±6	18±7	22±16	15±9
	90	21±17	23±17	22±15	28±15	30±15	23±13	23±8	17±3	15±5
Yonkenafil	45	20±3	24±15	25±19	24±21	26±20	25±14	36±29	31±18	27±18
	22.5	15±7	16±8	16±8	15±7	15±7	17±9	14±7	15±8	15±8

Table 5.12 Effects of yonkenafil on the extent of respiration in anesthetized dogs (ml/s, X±SD, n=5)

Group	Dosage (mg/kg)	Untreated group	treated group							
			15	30	45	60	90	120	150	180
Contral	--	2879±849	3418±1739	3115±1659	2825±1091	3289±1314	2783±991	2820±530	3143±1086	2893±912
	90	3567±1562	2270±1034	2597±1653	2975±1605	3779±2016	3330±1536	2995±1465	2917±2161	3191±2935
Yonkenafil	45	2539±1110	2110±881	2619±1175	2746±1465	3097±2380	2706±1432	3040±2132	2431±877	3188±1518
	22.5	2152±906	2134±712	2097±864	2298±1340	2337±1487	2448±1314	2532±1221	2431±1519	2711±1937

5.8 Conclusion

5.8.1 Pharmacodynamics experiment

Yonkenafil could obviously strengthen the male mice sexual function, and it is much more effective than the sildenafil. Yonkenafil also showed significant advantages in stimulating rat sexual functions. For the rats with erectile dysfunction (caused by injection of paroxetine), yonkenafil could shorten the incubation period of penile erection. Yonkenafil could shorten the male rabbits incubation period of capture and increase the frequency of capture significantly without changing their sexual hormone level.

5.8.2 Pharmacology experiment

In neural pharmacology, 216mg/kg yonkenafil dosage group showed some exciting effect to central nervous system of testing mice. In anesthesia dog experiment, the blood pressure of dogs in 90mg/kg dosage group of yonkenafil was decreased obviously. It appeared a little toxicity in the high dosage of yonkenafil.

Chapter 6

Toxicity Studies of Yonkenafil

6.1 Introduction

In the process of new drug research and development, preclinical safety evaluation plays an important role. The purpose of preclinical safety evaluation is to visualize the toxic activities on the experimental animals after administrated a test compound. The new drug safety evaluations include acute toxicity test (one-dosage administration toxicity), long term toxicity test (repeated administration toxicity), fertility and general reproductive toxicity tests and genetic toxicity test. The tests could evaluate the new drug safety from different aspects. In this chapter, we will describe the toxicity tests of the potential PDE5 inhibitor drug yonkenafil.

6.2 Study on the acute toxicity of yonkenafil

6.2.1 Introduction

Acute toxicity test (also called single dose toxicity test) is to study adverse reactions of test animals administrated a single density dose of a test substance. It is the early state of safety evaluation. The test results will guide the long term toxicity studies in dosage.

6.2.2 Study on the single oral administration acute toxicity of yonkenafil to beagle dogs

6.2.2.1 Purpose

To observe the acute toxicity reactions and numbers of death of beagle dogs after a single oral administration of yonkenafil, and to gain the approximate lethal dose or the dosage range of yonkenafil to beagle dogs. The results will serve as key information for design of the administration dosage of long-term toxicity tests.

6.2.2.2 Reagents and animals

Reagents: Yonkenafil is yellow light powder. It was diluted to the needed concentration with distilled water before using. **Animals:** Beagle dogs (7-9kg, 6-12 months, male, certificate no. SCXK (Jing) 2002-0005, provided by Beijing Keyu Animal Cultivation Centre). The animals were housed in a constant temperature (21-23°C), constant humidity and reversed 12h light/dark cycle room.

6.2.2.3 Experiments and methods

Dosage and Grouping: According to approximate lethal dose method, the preparation was given by gavage with solution and per os with capsule individually. For gavage, the administration volume was 20ml • kg^{-1}, and the dose of each animal was shown in Table 6.1 and Table 6.2 .

Dosing Procedure: The doses for all animals were calculated based on the body weight.

Clinical Observations: All study animals were observed for toxic indexes (times of onset recovery, time of death, agonal vs. non-agonal signs) at 30 minutes after administration, then be observed 1 hour every 4 hours for a total of 14 days. When an animal died during the study, the time of death was estimated as closely as possible and recorded, and the animal was weighed and dissected as soon as possible.

Body Weights: Body weights were recorded prior to dose administration and on the day of D8, D14 for the calculation of body weight changes.

Necropsy: After 14 days, Animals were subjected to a full gross necropsy, which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents. The following organs will be examined from all animals at necropsy: liver, kidney, spleen, heart, and lung. The abnormal organs would be taken for further histopathology examine.

6.2.2.4 Results and discussions

The dosage of oral gavage administration and the related toxicity behaviours of beagle dogs were shown in Table 6.1.

Table 6.1 Toxicological reactions of beagle dogs given yonkenafil by oral gavage

Animal number	Weight (kg)	Dosage ($mg \cdot kg^{-1}$)	Observed results
2	8.3	30	no abnormal seen
3	9.0	152	vomit about 10min later
4	9.4	341	vomit about 10min later, again at nearly 1h, saliva, recovery about 2h later
5	8.4	1726	vomit about 5min later, again at nearly 30min
6	9.7	2586	vomit about 5min later, gait apraxia , discontinuous vomit for nearly 2h, saliva 2h later, recovery about 6h later

For oral gavage administration, the dogs vomited at about 10mins after given yonkenafil at the dose of 152 mg/kg. It seemed dogs had a dose-related emesis reaction to the drug. The higher dosage, the earlier to vomit. And the vomit was mainly yonkenafil solution. The animal given yonkenafil at the dose of 341 mg/kg,

726 mg/kg, 2586 mg/kg also salivate. All dogs recovered 6h later and no adverse reaction was observed in followed days. The results were shown in Table 6.2.

Table 6.2 Observation results of beagle dogs after oral gavage administration of yonkenafil

Animal number	Day (8)		Day (14)		Day (15)
	Weight (kg)	Result	Weight (kg)	Result	Gross anatomy
2	9.4	normal	9.8	normal	no abnormality seen in viscera
3	10.2	normal	10.2	normal	no abnormality seen in viscera
4	9.6	normal	10.4	normal	no abnormality seen in viscera
5	8.9	normal	9.2	normal	no abnormality seen in viscera
6	10.4	normal	10.6	normal	no abnormality seen in viscera

In this study, the dogs vomited at about 10mins when oral gavage dosage of yonkenafil given was 152mg/kg. When the dosage was increased the vomiting frequency was increased. No other toxicology reaction was seen except emesis. As the solution of yonkenafil had some special smell, so we decided to per os capsule of yonkenafil to dogs in order to make sure whether it was the special smell of solution or yonkenafil itself that make the dog vomit, the results were shown in Table 6.3 and Table 6.4.

Table 6.3 showed the intraday reaction after administration yonkenafil per os capsule. No adverse reaction was seen when dog given yonkenafil at the dose of 81 $mg \cdot kg^{-1}$. Emesia, male genital erection, saliva dribbling were observed at the dosage of 152 $mg \cdot kg^{-1}$, 227$mg \cdot kg^{-1}$, 767$mg \cdot kg^{-1}$ and 1150$mg \cdot kg^{-1}$. Extremities shiver, acratia also occurred with the dog given yonkenafil at 1150$mg \cdot kg^{-1}$, but no adverse response were seen with the dog given yonkenafil at 511 $mg \cdot kg^{-1}$. All dogs recovered about 8h later and no adverse reaction was observed in followed days.

Table 6.3 Toxicological reaction of Beagle dogs administrated yonkenafil by per os capsule

Animal number	Weight (kg)	Dosage ($mg \cdot kg^{-1}$)	Result
5	9.1	81	No abnormal seen
1	8.6	152	Light vomit about 20min after dosing, genital organ erection
3	10.0	227	Light vomit about 50min after dosing, saliva dribbling about 3h, recovered about 6.5h
4	10.7	511	No abnormal seen
2	10.5	767	Just Vomit about 35min after dosing
6	10.6	1150	Vomit about 25min after dosing, extremities shudder , acratia about 5h later ,recovered about 8h later。

Table 6.4 Reaction of Beagle dogs after per os capsule administration of yonkenafil

Animal number	Day(8)		Day(14)		Day(15)
	Weight (kg)	Result	Weight (kg)	Result	Gross anatomy
1	9.2	normal	9.3	normal	No abnormality seen in viscera
2	11.3	normal	10.8	normal	No abnormality seen in viscera
3	11.0	normal	10.8	normal	No abnormality seen in viscera
4	11.4	normal	11.3	normal	No abnormality seen in viscera
5	9.9	normal	9.9	normal	No abnormality seen in viscera
6	11.3	normal	11.2	normal	No abnormality seen in viscera

Comparing between the two oral administration methods, it was found that the time to vomit of per os capsule was a little longer than oral gavage. Dribbling can be seen in both ways.

In conclusion, under the current experiment conditions, the approximate lethal dose of yonkenafil to beagle dogs given by oral was ≥2586 $mg \cdot kg^{-1}$. The mainly toxic reaction was emesis, saliva and extremities shiver.

6.2.3 Study on the single oral administration acute toxicity of yonkenafil to mice

6.2.3.1 Purpose

To observe the acute toxicity reactions and the median lethal dose (LD_{50}) value in mice that after a single peroral-administration of the yonkenafil.

6.2.3.2 Reagents and animals

Test article: Yonkenafil is yellow light powder. Diluted it to the needed concentration with distilled water before use it. **Animals:** Kunming mouse (19-23g, male: female=1:1, certificate no. SCXK (Jing) 2002-0001, provided by the experimental animal department of Beijing university). The animals were housed in a constant temperature (21-23°C), constant humidity and reversed 12h light/dark cycle room. And 5 homogeneity mice were kept in the same cage and had free access to food (whole nutrition drops animal feeds) and water.

6.2.3.3 Experiments and methods

60 prepared mice were divided into 6 groups, A, B, C, D, E and F。 Then they were kept to fast diet, but offer water 12 hours before the experiment. The animals were gave a single intragastric-administration (0.2ml/10g) with yonkenafil at dosage of 3926mg/kg for group A, 2804mg/kg B, 2003mg/kg C, 1431mg/kg D, 1022mg/kgE

and 730mg/kg F respectively, and then kept observing for 2 weeks. The dosage interval is 1.4:1. The behaviour, activity, weight, appetite, dejecta and the number of deaths of experimental animals were recorded. At last, they were put to death and to autopsy by naked eyes.

6.2.3.4 Results and discussions

Under the given dosage of yonkenafil, there were mice death in every group during the first day of the experiments; the death mice can be observed by naked eyes no abnormality seen in the major organs, including heart, liver, spleen, lung and kidney. After administration the drug, the animals in each group showed some abnormalities, such as the activity of mice was depressed movement, fur rough, ingest appetite decreased, weight grow up slow. Using the reformed Karber's method, we can calculate the median lethal dose (LD_{50}) value, when yonkenafil was given by single time to mice through oral administration and its 95% confidence limit and the results were listed in the Table 6.5 .

Table 6.5 Mice acute toxicity by oral administration of yonkenafil

Group dosage (mg/kg)	**Animals (numbers)**	**After treated time (day) / death of animals (numbers)**					**Death date (%)**	**LD_{50} (mg/kg) 95% confidence limit**
		1d	**2d**	**3d**	**7d**	**14d**		
3926	10	9	9	9	9	9	90	
2804	10	6	6	6	6	6	60	
2003	10	5	5	5	5	5	50	2000
1431	10	3	3	3	3	3	30	(1610-2483)
1022	10	1	1	1	1	1	10	
730	10	1	1	1	1	1	10	

From the results, we can conclusion that the median lethal dose (LD_{50}) value is 2000mg/kg, when yonkenafil was given by once to mice by oral administration. The 95% confidence limit value is 1610-2483mg/kg.

6.2.4 Study on single intraperitoneal injection administration acute toxicity of yonkenafil to mice

6.2.4.1 Purpose

To observe the acute toxicity reaction and the median lethal dose (LD_{50}) value in mouse that after a single intraperitoneal injection administration of the yonkenafil.

6.2.4.2 Reagents and animals

Reagents: Yonkenafil, yellow light powder, is easily dissolved in water and methanol, slightly dissolved in ethanol and acetonitrile. It was diluted to the needed concentration with distilled water before use. **Animals:** Kunming mouse (19-23g, male: female=1:1, certificate no. SCXK (Jing) 2002-0001, provided by the experimental animal department of Beijing University). The animals were housed in a constant temperature (21-23°C), constant humidity (45-65%) and reversed 12h light/dark cycle room. And 5 homogeneity mice were kept in the same cage and had free access to food (whole nutrition drops animal feeds) and water.

6.2.4.3 Experiment and method

At first, 60 prepared mice were divided into 6 groups, which were A, B, C, D, E and F. All of them were administered with yonkenafil and the dosage is 995mg/kg, 829mg/kg, 691mg/kg, 576mg/kg, 480mg/kg and 400mg/kg respectively. The dosage interval is 1.2:1. Then gave them a single intragastric-administration (0.2ml/10g) and to observe 2 weeks successively. The behaviour, activity, weight, appetite, dejecta and the number of deaths of the experimental animals were recorded. At last, they were put to death and to autopsy by naked eyes.

6.2.4.4 Results and discussions

Above-mentioned the dosage groups of yonkenafil 995mg/kg, 829mg/kg, 691mg/kg, 576mg/kg and 480mg/kg, during two days experiments, there were mice death in every group. The death mice can be observed by naked eyes. No abnormality was found in the major organs, including heart, liver, spleen, lung and kidney. There is no death mouse in the group of yonkenafil dosage 400mg/kg. After administration, abnormalities were observed in each group, such as the activity of mice was depressed movement, fur rough, ingest appetite decreased, weight grow up slow. Use the reformed Karber's method to calculate the median lethal dose (LD_{50}) value, when yonkenafil was given by a single dose to mice through intraperitoneal injection administration and its 95% confidence (Table 6.6).

Table 6.6 Mice acute toxicity by a single dose intraperitoneal injection of yonkenafil

Group dosage (mg/kg)	**Animals (numbers)**	**After treated time (day) / death of animals (numbers)**					**Death date (%)**	**LD_{50} (mg/kg) 95% confidence limit**
		1d	**2d**	**3d**	**7d**	**14d**		
995	10	9	9	9	9	9	90	
829	10	7	8	8	8	8	80	
691	10	6	6	6	6	6	60	634
576	10	4	5	5	5	5	50	(566-710)
480	10	2	2	2	2	2	20	
400	10	0	0	0	0	0	0	

From these results, we concluded that the median lethal dose (LD_{50}) value is 634mg/kg, when yonkenafil was given by once to mice through intraperitoneal injection. The 95% confidence limit value is from 566 to 710mg/kg.

6.3 Study on the long-term toxicities of yonkenafil

6.3.1 Introduction

Long-term toxicity study (repeated dosing toxicity) is the core of drug non-clinical safety evaluation. The purpose of long-term toxicity is to predict potential side-effect to human body and to reduce the drug risk on clinical volunteers. It is an important process from drug pre-clinical research to clinical research. The long-term toxicity of yonkenafil on rats was studied in this experiment.

6.3.2 Long-term toxicity studies on rats after repeated dosing yonkenafil

6.3.2.1 Purpose

To observe the long-term toxicity reactions and the toxicity degrees of SD rats after repeated administration of yonkenafil for 90 days, and to establish the relationship of toxicity dosage and pharmacodynamics, as well as to find the target organ of yonkenafil and reversibility of its damage, provide the information to design a clinical administration safe dosage and to monitor clinical toxicity side-effects.

6.3.2.2 Reagents and animals

Reagents: Yonkenafil is yellow light powder. It was diluted to the needed concentration with distilled water before use. Pentobarbital sodium (bio-degree) was used as anesthetic; sodium citrate (bio-degree) was used as anticoagulant. **Animals:** 120 SD rats (160-180g), male: female=1:1, certificate no. SCXK (Jing) 2002-0003, were provided by Beijing Weili tonghua experimental animal company. The animals

were housed in a constant temperature (23.1±1.2°C), constant humidity and reversed 12h light/dark cycle room. And 5 homogeneity rats were kept in the same cage and had free access to food (whole nutrition drops animal feeds) and water.

6.3.2.3 Experiments and methods

Dosage and Grouping: According to approximate lethal dose in the acute toxicity tests of mice (2000mg·kg^{-1}) and the rat pharmacodynamics dosage (12mg·kg^{-1}), we divided the experimental rats into three administration groups which were low- med- and high-dosage groups and one blank groups with half male and female. These three dosing group were respectively administrated yonkenafil for 20mg·kg^{-1}, 60mg·kg^{-1} and 180mg·kg^{-1}

Dosing Procedure: The animals were dosed yonkenafil by gavage. In every week, a one-day break was taken after continuously administrated yonkenafil for six days. The administration was continued for 90 days, the dosing volume was 1.0ml/100g.

Clinical Observations: 24 hours after the last time administration, ten female and ten male rats were picked up from each group. The haematology index was measured first with sampling from tail vein. After anaesthetized with intraperitoneal injection pentobarbital sodium (45mg·kg^{-1}), blood sample was collected from the abdominal aorta. One part of blood sample was anticoagulated by sodium citrate, and the coagulation index was mensurated after plasma separation. The odd part of blood sample was separated serum, and then the biochemistry index was determined. At last, the animals were anatomized to collect related organs. The pathological examination was processed then. For a note, the animals were forbidden to take food 16 hours before blood sampling.

Following observation: After 90 days' toxicity examination, the residual animals (5

female and 5 male rats in each group) were continuously observed for 30 days and then were sacrificed. The animals were blood sampling and anatomized with same method as general toxicity observations. It was help us to research the recovery condition after toxicity reaction and the possible undiscovered toxicity.

6.3.2.4 Results and discussions

Directly observing beside the cage: No abnormality, including in behavioural activity, appearance and signs, colour and shape of dejecta, was found in both the administration and the control groups of animals. The weight of administration group animals were no changes against the control group, the feed wastage was also no changes.

Haematology indices: The change of haematology indices were shown in Table 6.7. Contrasting with the control group, the haematology indices of high-dosage group animals had a little change in comparison with the control group. The red blood cell count (RBC), hemoglobin content (Hb) and hematocrit were decreased with the control group ($p \leq 0.01$, $p \leq 0.05$, $p \leq 0.05$), and the changes of RBC and Hb were relational with the dose of yonkenafil. Other haematology indices in administration group were no difference from the control group. After 30days recovery, the indexes were resumed to the normal level.

Serum biochemical indices: The serum biochemical indices results were listed in Table 6.8 and 6.9. Table 6.8 was the data of male rats and Table 6.9 was of female. For male rats, after 90 days of administration, of the drug, the serum glucose parameters for $60mg \cdot kg^{-1}$ and $180mg \cdot kg^{-1}$ groups were a little higher than that of the control group and were resumed to normal level after recovery days. For female rats, after administration 90 days, for $180mg \cdot kg^{-1}$ group the serum total cholesterol concentration was a little higher than that of the control group and were resumed to normal level after recovery days.

Blood electrolyte indices: Table 6.10 gave the result of blood electrolyte of female rats. The concentration of Na^+ and Cl^- in the groups 20mg•kg^{-1} and 60mg•kg^{-1} were a little higher than that of the control group. After 30 days' recovery, they all resumed to normal value. Table 6.11 showed the result of blood electrolyte of male rats. The concentration of Cl^- in the group 20mg•kg^{-1} and the concentration of K^+ in the group 20mg•kg^{-1} and 60mg•kg^{-1} were a little higher than that of the control group. After 30 days' recovery, they all resumed to normal values.

Table 6.7 The haematology index changes of SD rats after administrated yonkenafil for 90 days and recovered for 30 days ($\bar{x} \pm s$)

Period	Index	Group			
		Control group	20 mg•kg^{-1} group	60mg•kg^{-1} group	180mg•kg^{-1} group
After administration (n=10△)	RBC(10^{12}/L)	8.4±0.6	7.8±0.6	7.7±0.4	7.2±0.6**
	HGB(g/L)	166.5±10.1	157.8±12.2	155.5±8.5	147.5±11.0*
	HCT(%)	41.6±2.2	39.0±3.6	39.1±1.6	36.8±3.7*
	MCV(fL)	49.8±1.8	49.7±1.2	50.8±1.4	51.1±2.4
	MCH(pg)	19.9±0.7	20.2±0.5	20.2±1.0	20.6±1.1
	MCHC(g/L)	400.2±11.5	405.1±12.3	398.0±17.4	402.8±23.0
	WBC(10^9/L)	12.6±2.5	13.6±3.6	13.0±3.0	14.3±3.8
	LYM(%)	52.2±4.5	51.0±6.3	55.0±3.8	52.0±4.9
	MO(%)	13.9±2.6	13.6±2.9	13.9±1.7	13.3±3.0
	GRAN(%)	34.0±4.1	35.4±4.1	31.2±3.4	34.7±4.0
	PLT(10^9/L)	409.3±58.2	399.6±59.6	363.6±35.8	363.5±41.4
After recovery (n=5)	RBC(10^{12}/L)	8.2±1.4	8.3±0.5	6.8±4.6	7.5±1.5
	HGB(g/L)	159.0±26.3	163.6±10.6	135.2±32.2	148.6±27.9
	HCT(%)	40.1±6.3	42.3±2.4	34.3±8.2	37.2±7.6
	MCV(fL)	49.3±1.7	50.8±0.7	50.2±1.1	49.7±1.6
	MCH(pg)	19.5±0.7	19.7±0.4	19.8±0.6	19.9±0.7
	MCHC(g/L)	396.2±9.0	386.6±10.3	394.4±18.6	400.6±14.8
	WBC(10^9/L)	8.8±1.7	10.3±1.5	8.4±2.3	9.0±1.6
	LYM(%)	51.4±3.0	57.7±7.3	52.5±2.2	50.1±5.7
	MO(%)	10.6±1.9	9.0±2.2	11.0±1.8	11.3±2.2
	GRAN(%)	37.9±2.4	33.3±5.1	36.5±2.0	38.6±4.4
	PLT(10^9/L)	368.0±68.0	360.2±72.2	307.8±126.5	302.2±65.1

Note: *p≤0.05; ** p≤0.01; △ the rats number in 180mg•kg^{-1} group was 8 after administration

Table 6.8 Serum biochemical indices of female rats after administration ($\bar{x} \pm s$)

Period	Index	Group			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10△)	GPT(U/L)	41.4±2.4	40.2±12.2	34.5±3.5***	48.5±13.9
	GOT(U/L)	143.6±10.6	121.5±38.7	122.0±22.9	136.0±77.9
	ALP(U/L)	38.8±10.7	36.2±9.7	35.0±8.4	39.4±4.3
	TBIL(μmol/L)	10.6±0.7	9.9±1.1	9.8±1.2	9.3±0.4***
	TG(mmol/L)	0.43±0.17	0.30±0.11	0.28±0.064	0.24±0.084
	CHO(mmol/L)	1.6±0.2	1.7±0.5	2.0±0.3	2.1±0.4*
	TP(g/L)	68.6±2.8	66.7±8.5	72.2±2.6	70.7±3.3
	ALB(g/L)	38.9±1.5	36.3±4.9	39.3±1.6	37.9±1.8
	GLO(g/L)	29.7±1.5	30.4±4.3	32.8±1.6**	32.9±2.2*
	A/G	1.3±0.05	1.2±0.1	1.2±0.1	1.2±0.1
	GLU(mmol/L)	5.9±0.7	5.9±1.0	6.0±0.4	6.4±1.4
	CK(U/L)	757.4±161.4	644.6±382.5	627.3±258.9	524.3±162.9
	LDH(U/L)	969.4±100.1	775.7±365.4	790.6±221.8	597.3±77.8***
	UA(μmol/L)	48.6±10.8	42.8±13.3	39.7±10.7	48.3±22.0
	BUN(mmol/L)	6.9±0.6	5.8±1.2	6.9±1.0	7.1±0.5
	Cr(μmol/L)	64.9±5.7	58.2±10.6	65.1±6.0	65.6±7.9
After recovery (n=5)	GPT(U/L)	56.0±27.7	46.4±26.7	47.2±9.9	37.8±7.9
	GOT(U/L)	208.6±52.3	163.0±60.9	152.2±14.6	100.2±13.0*
	ALP(U/L)	22.2±3.0	26.0±12.4	22.2±7.9	18.8±4.5
	TBIL(μmol/L)	6.8±1.4	6.1±0.5	6.9±0.9	7.0±1.1
	TG(mmol/L)	0.48±0.18	0.56±0.17	0.33±0.074	0.50±0.080
	CHO(mmol/L)	1.9±0.1	2.1±0.4	2.1±0.2	2.4±0.4
	TP(g/L)	66.2±4.3	68.8±4.2	67.3±2.8	68.2±2.1
	ALB(g/L)	35.5±1.3	37.3±1.8	35.5±0.7	35.9±0.8
	GLO(g/L)	30.7±3.1	31.5±0.5	31.8±3.1	32.3±1.4
	A/G	1.2±0.1	1.2±0.1	1.1±0.1	1.1±0.04
	GLU(mmol/L)	6.1±1.3	6.0±0.8	7.3±0.7	7.5±0.8
	CK(U/L)	1216.4±297.6	762.0±165.5	854.8±359.1	344.6±113.0**
	LDH(U/L)	1199.4±137.8	880.8±130.4*	869.8±169.8	369.4±135.8***
	UA(μmol/L)	39.2±7.0	33.4±6.6	42.2±19.7	44.2±7.0
	BUN(mmol/L)	6.0±0.7	6.6±0.5	6.2±0.2	5.5±0.7
	Cr(μmol/L)	61.3±3.7	59.8±2.0	59.2±4.6	58.2±3.2

Note: *p≤0.05, ** p≤0.01, *** p≤0.001, △ the rats number in 180mg•kg^{-1} group was 8 after administration

Table 6.9 Serum biochemical indices of male rats after administration ($\bar{x} \pm s$)

Period	Index	Group			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10^)	GPT(U/L)	52.2±7.5	47.2±4.9	55.7±16.7	38.9±5.0**
	GOT(U/L)	175.4±16.6	136.7±36.0	147.4±27.2	92.6±16.3***
	ALP(U/L)	66.9±7.2	83.4±27.1	80.2±11.1	72.6±10.8
	TBIL(μmol/L)	12.8±1.4	12.3±0.7	12.8±1.1	12.5±1.0
	TG(mmol/L)	0.34±0.15	0.29±0.11	0.20±0.068	0.17±0.090*
	CHO(mmol/L)	1.3±0.2	1.5±0.2	1.2±0.3	1.2±0.2
	TP(g/L)	64.2±3.3	64.3±2.1	64.4±4.1	63.7±1.9
	ALB(g/L)	35.7±1.2	35.0±1.5	35.7±1.5	35.4±0.9
	GLO(g/L)	28.5±2.3	29.3±0.8	28.7±2.7	28.2±1.6
	A/G	1.3±0.1	1.2±0.05	1.2±0.1	1.2±0.1
	GLU(mmol/L)	5.2±0.4	5.8±0.8	6.8±0.6***	6.7±0.7***
	CK(U/L)	993.2±212.5	677.7±380.6	608.5±165.6**	329.8±149.0***
	LDH(U/L)	1203.8±178.6	875.4±467.0	856.6±197.0**	375.8±254.0***
	UA(μmol/L)	32.9±7.8	38.2±12.8	34.6±13.5	40.3±5.6
	BUN(mmol/L)	5.9±0.8	6.2±0.6	6.4±0.7	6.4±0.8
	Cr(μmol/L)	55.6±5.7	52.5±4.3	53.6±4.2	51.8±3.6
After recovery (n=5)	GPT(U/L)	50.4±6.4	51.3±10.1	96.4±93.4	47.0±6.6
	GOT(U/L)	190.0±46.2	175.0±29.5	199.6±76.3	95.8±4.5*
	ALP(U/L)	47.0±6.2	52.5±6.7	40.2±3.3	45.2±7.7
	TBIL(μmol/L)	6.6±0.5	6.1±0.7	6.1±0.7	6.2±0.7
	TG(mmol/L)	0.66±0.24	0.42±0.20	0.56±0.23	0.38±0.20
	CHO(mmol/L)	1.9±0.2	1.8±0.2	1.8±0.4	2.0±0.4
	TP(g/L)	62.4±2.4	63.5±1.8	65.5±2.7	61.2±3.1
	ALB(g/L)	33.3±1.2	33.7±0.5	34.4±0.9	33.0±0.6
	GLO(g/L)	29.1±1.3	29.8±1.3	31.1±1.9	28.2±2.6
	A/G	1.1±0.02	1.1±0.03	1.1±0.04	1.2±0.1
	GLU(mmol/L)	5.9±0.9	6.2±1.0	7.6±0.5	7.6±0.7
	CK(U/L)	1476.2±497.7	1338.5±411.9	854.6±342.6	367.2±82.2*
	LDH(U/L)	1380.8±447.3	1232.3±353.4	887.8±358.3	267.0±112.1*
	UA(μmol/L)	34.8±11.2	27.5±16.6	37.4±13.4	29.0±20.3
	BUN(mmol/L)	5.0±0.7	6.3±1.0*	5.4±0.5	5.7±0.6
	Cr(μmol/L)	56.9±3.9	55.9±2.9	56.9±2.7	60.1±4.8

Note: *p≤0.05, ** p≤0.01, *** p≤0.001, △the rats number in 20mg•kg^{-1} group was 9 after administration and 4 after recovery.

Coagulation indices: There were no differences of coagulation indices of male rats between the administration groups and the control group. The differences of coagulation indices of female rats were shown in Table 6.12. TT of 60mg•kg^{-1} group and FIB of 180mg•kg^{-1} group were increased than the control group. After 30 days' recovery, these indices had resumed to the same level as the control group.

Urinalysis: The male results of urinalysis were shown in Table 6.13, in which showed that urine protein value of 60mg•kg^{-1} group was increased than the control group. From Table 6.14, the results of female rats urinalysis, we compared data and found that urine protein values of all three dosage groups were higher than that of the control group, urine erythrocyte of 20mg•kg^{-1} and 180mg•kg^{-1} groups and urine protein of 60mg•kg^{-1} group were also higher than that of the control group and were recovered after 30days.

Table 6.10 Blood electrolyte indices of female rats after administration ($\bar{x} \pm s$)

Period	Index	Group			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10△)	Na(mmol/L)	138.4±1.4	140.0±0.8*	140.6±1.4*	140.0±1.5
	K(mmol/L)	4.0±0.2	4.2±0.2	3.9±0.2	4.1±0.2
	Cl(mmol/L)	103.5±1.4	107.0±0.9***	105.8±1.5*	105.8±2.0
After recovery (n=5)	Na(mmol/L)	134.8±1.6	137.0±1.6	137.0±1.9	136.0±1.2
	K(mmol/L)	4.1±0.1	4.2±0.2	4.1±0.4	4.0±0.2
	Cl(mmol/L)	97.6±1.1	98.6±0.5	100.8±3.2	100.8±1.3*

Note: *p≤0.05, *** p≤0.001, △the rats no. in 180mg•kg^{-1} group was 8 after administration

Table 6.11 Blood electrolyte indices of male rats after administration ($\bar{x} \pm s$)

Period	Index	Group			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10$^{\triangle}$)	Na(mmol/L)	141.0±1.9	142.9±2.4	141.9±2.1	141.0±1.9
	K(mmol/L)	4.7±0.2	4.3±0.2*	4.3±0.2*	4.4±0.3
	Cl(mmol/L)	106.4±1.6	108.4±2.1*	107.6±1.6	107.9±1.6
After recovery (n=5)	Na(mmol/L)	138.2±1.1	137.8±0.5	140.4±0.5*	138.8±0.8
	K(mmol/L)	4.8±0.3	5.1±0.2	4.7±0.2	4.5±0.2
	Cl(mmol/L)	100.6±1.9	101.0±0.8	103.4±1.1	102.8±1.3

Note: *p≤0.05, ** p≤0.01, *** p≤0.001, △the rats number in 20mg•kg^{-1} group was 9 after administration and 4 after recovery.

Table 6.12 Coagulation indices of female rats after administration($\bar{x} \pm s$)

Period	Index	Groups			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10$^{\triangle}$)	TT(s)	40.0±3.4	41.8±2.5	48.0±3.0***	43.4±7.2
	PT(s)	18.5±0.8	18.2±1.0	18.6±0.9	18.5±0.5
	APTT(s)	17.4±2.9	16.9±3.1	18.1±1.3	17.3±2.5
	FIB(mg/dl)	190.3±17.2	209.0±20.2	209.2±11.4	209.3±6.6*
After recovery (n=5)	TT(s)	40.1±4.1	39.8±1.1	43.9±4.6	41.5±1.9
	PT(s)	17.0±1.1	15.8±1.3	15.4±1.5	15.4±2.0
	APTT(s)	16.8±1.5	17.8±1.3	17.7±0.7	17.5±0.9
	FIB(mg/dl)	192.3±12.3	176.0±13.5	200.6±15.7	197.3±21.4

Note: *p≤0.05, *** p≤0.001; △the rats no. in 180mg•kg^{-1} group was 8 after administration

Table 6.13 Urinalysis indices of male rats after administration ($\bar{x} \pm s$)

Period	Index	Groups			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10$^{\triangle}$)	GLU	10(-)	9(-)	10(-)	10(-)
	BIL	9(-); 1(+)	8(-); 1(+)	9(-) ; 1(+)	10(-)
	KET	5(-); 2(+); 3(++)	5(+); 4(++)	3(-); 3(+); 4(++)	2(-); 5(+); 3(++)
	BLO	1(-); 7(+); 1(++); 1(++++)	1(-); 4(+); 2(+++); 2(++++)	4(+); 2(++); 2(+++); 2(++++)	6(+); 2(++); 1(+++); 1(++++)
	PH	1(6); 4(6.5); 3(7); 1(7.5); 1(8)	1(6.5); 6(7); 2(7.5)	2(6); 4(6.5); 4(7)	1(5.5); 3(6); 3(6.5); 2(7); 1(8)
	PRO	6(-); 1(+/-); 3(+)	2(-); 4(+/-); 3(+)	1(-); 1(+/-) 7(+); 1(++)*	3(-); 1(+/-) 5(+); 1(++)
	URO	10(-)	9(-)	10(-)	10(-)
	NIT	10(-)	9(-)	10(-)	10(-)
	LEU	4(-); 5(+); 1(++)	1(-); 7(+); 1(++)	2(-); 8(+)	3(-); 7(+)
After recovery (n=5)	GLU	5(-)	4(-)	5(-)	5(-)
	BIL	5(-)	4(-)	5(-)	5(-)
	KET	2(-); 2(+);1(++)	2(-); 2(+)	3(-); 2(+)	5(-)
	BLO	2(+); 1(++) 2(++++)	3(+); 1(++)	4(+); 1(+++)	3(++); 2(+++)
	PH	1(6.5); 3(7.5); 1(7)	2(6.5); 2(7)	1(7); 1(7.5) 2(8); 1(8.5)	2(6.5); 3(7)
	PRO	2(-); 1(+/-); 1(+);1(++)	2(-); 1(+); 1(++)	4(-); 1(+/-)	5(-)
	URO	5(-)	4(-)	5(-)	5(-)
	NIT	5(-)	4(-)	5(-)	5(-)
	LEU	2(-); 3(+)	3(-); 1(+)	4(-); 1(+)	3(-); 2(+)

Table 6.14 Urinalysis indices of female rats after administration ($\bar{x} \pm s$)

Period	Index	Groups			
		Control group	20 mg•kg^{-1}	60mg•kg^{-1}	180mg•kg^{-1}
After administration (n=10$^{\triangle}$)	GLU	10(-)	10(-)	10(-)	8(-)
	BIL	10(-)	10(-)	9(-) ; 1(+)	8(-)
	KET	10(-)	8(-); 2(+)	8(-) ; 2(+)	6(-); 2(+)
	BLO	5(-); 4(+); 1(++) ;	5(+); 1(++); 2(+++); 2(++++)**	3(-); 4(+); 1(+++); 2(++++)	3(+); 2(+++): 3(++++)**
	PH	2(6.5); 4(7); 3(7.5); 1(8)	1(6.5); 3(7); 4(7.5); 2(8)	1(5.5);1(6); 1(6.5) 3(7); 2(7.5); 2(8)	4(6.5); 2(7) 2(7.5)
	PRO	9(-); 1(+/-)	4(-); 3(+/-); 3(+)*	4(+/-); 5(+); 1(++)***	4(-); 3(+); 1(+++)*
	URO	10(-)	10(-)	10(-)	8(-)
	NIT	10(-)	10(-)	10(-)	8(-)
	LEU	9(-); 1(+)	6(-); 4(+)	3(-); 4(+); 3(++)**	6(-); 1(+); 1(++++)
After recovery (n=5)	GLU	5(-)	5(-)	5(-)	5(-)
	BIL	5(-)	5(-)	5(-)	5(-)
	KET	5(-)	5(-)	5(-)	5(-)
	BLO	2(-); 2(+); 1(++)	2(-); 2(+); 1(+++)	3(+); 1(+++); 1(++++)	1(++); 2(+++) 2(++++)*
	PH	1(5); 1(5.5) 2(7); 1(7.5)	2(6.5); 1(7) 2(7.5)	1(6.5); 2(7) 2(7.5)	2(7) 3(7.5)
	PRO	4(-); 1(+/-)	4(-); 1(+)	5(-)	5(-)
	URO	5(-)	5(-)	5(-)	5(-)
	NIT	5(-)	5(-)	5(-)	5(-)
	LEU	5(-)	5(-)	5(-)	5(-)

Note: *p≤0.05, ** p≤0.01, *** p≤0.001.

△ the rats number in 20mg•kg^{-1} group was 9 after administration and 4 after recovery.

6.4 Conclusions

In acute toxicity and long term toxicity studies, yonkenafil embodied out the low to none toxicity. This showed that it may be a safety drug to treat ED. The safety evaluation of yonkenafil was verified.

Chapter 7

Conclusions

7.1 Design and synthesis of new PDE5 inhibitors

In this project, I firstly analyzed and compared the structures of some marketed PDE5 inhibitors, such as sildenafil, tadalafil and vardenafil, and some literature compounds with the structure of cGMP. According to their similarity on structures, we designed two series of new compounds as potential PDE5 inhibitors. One is the series of 2-(substituted-sulfonylphenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one compounds and another is series of 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-ones compounds. As a targeted compound of 2-(substituted-sulfonylphenyl)-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one analogues, 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one (II-13) was synthesized successfully in seven-steps method. By employing different starting material and different amine compounds, a group of II-13 analogues with different R^1, R^2, R^3 and R^4 substituents were successfully synthesized. For the other series of 2-(substituted-sulfonylphenyl)-3,7-dihydro-pyrrolo[2,3-d] pyrimidin-4-ones compounds, 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl] -5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one (III-20) was chosen as a target. It was synthesized successfully in six-steps. By employing different starting materials and different amine compounds, III-20 analogues with different R^1, R^2, R^3, R^4 and R^6 substituents were successfully synthesized. These two new series of compounds were structurally different from the existing PDE5 inhibitors. To make these compounds water soluble for bioactivity tests, hydrochloridesalt form of some of these compounds were prepared.

7.2 Preliminary bioactivity and toxicity screening for the new compounds

In penile erection activity assay, both series of the compounds demonstrated erection activity on conscious rabbit models, especially compounds 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one monohydrochlorate (II-13•HCl, wanzaonafil) and 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimi-din-4-one monohydrochlorate (III-20•HCl, yonkenafil). In PDE1-6 selectivity experiment, the results showed that PDE5 is the most selective target for wanzaonafil and yonkenafil and the activity of these two compounds towards to PDE5 is almost equal to udenafil and sildenafil, but about 100 times lower than vardenafil. Same to the established PDE5 inhibitors, PDE6 was the second most selective target for wanzaonafil and yonkenafil. It was believed that the activity of sildenafil toward to the PDE6 is the reason for its sight side effect. Therefore the selectivity or the ratio of the inhibition between PDE5 and PDE6 became another ruler for judging whether or not a PDE5 inhibitor could become a drug. The difference of the activity of sildenafil towards PDE5 and PDE6 is about 10 times, while yonkenafil is 16 and wanzaonafil 15, much bigger than that of sildenafil.

Preliminary toxicity experiment showed the LD_{50} value of mice oral administration of yonkenafil was 2000 mg/kg and 95% confidence level was 1610-2486 mg/kg. The LD_{50} value of mice oral administration of wanzaonafil was 200 mg/kg and 95% confidence level was 161-248 mg/kg. The LD_{50} value of mice single i.p. administration of yonkenafil was 634 mg/kg and 95% confidence level was 566-710 mg/kg, while the LD_{50} value of mice single i.p. administration of wanzaonafil was 48 mg/kg and 95% confidence level was 42-53 mg/kg. The results were same to the above oral administration. Therefore yonkenafil was selected to do further research while wanzaonafil was abandoned at this stage.

Yonkenafil can significantly increase mice sexual function and overall sexual profile is better than that of sildenafil.

7.3 Pharmacodynamics research on yonkenafil

Yonkenafil can strengthen the male mice sexual function significantly, and it is more effective than the sildenafil. Yonkenafil also showed significant advantages in stimulating rat sexual functions. For the rats with erectile dysfunction (caused by injection of paroxetine), yonkenafil can shorten the incubation period of penile erection. Yonkenafil can shorten the male rabbits incubation period of capture and increase the frequency of capture significantly without changing their sexual hormone level. These results showed that yonkenafil was effective for treatment of ED.

7.4 Toxicity research on yonkenafil

In acute toxicity studies, the results showed that, the median lethal dose (LD_{50}) value is 2000mg/kg，when yonkenafil was given by a single dose to mice orally; the median lethal dose (LD_{50}) value is 634mg/kg，when yonkenafil was given to mice through a single intraperitoneal injection. The LD_{50} value of yonkenafil was in a safety level to be a drug. In chronic toxicity studies, we investigated haematology indices, serum biochemical indices, blood electrolyte, coagulation indices and urinalysis of rats in a period for 90 days continuously administrated yonkenafil. The results showed that yonkenafil embodied out the low to none toxicity. Therefore yonkenafil may be a safety drug for treatment of ED.

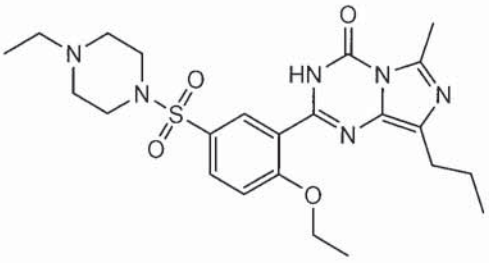
7.5 Conclusions of my research work

In my research, I designed and synthesized two series of new compounds as potential

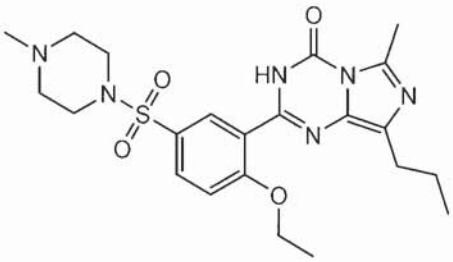
PDE5 inhibitors.These compounds was structurally similar to cGMP and sildenafil. Furthermore, we investigated their bioactivities and toxicities. A typical compound, yonkenafil, which exhibited lower toxicity and higher bioactivity was chosen to do further research. Its pharmacodynamics, pharmacology, toxicity were studied.The results showed that yonkenafil could be a new safe and effective drug for treatment of PDE5 related syndromes.

Appendix I

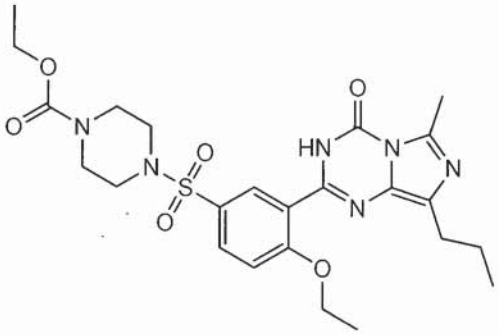
Structures of synthesized potential PDE5 inhibitors



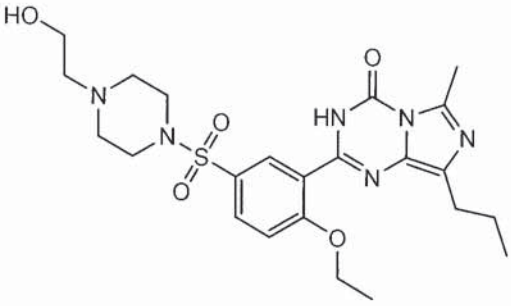
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-13)**



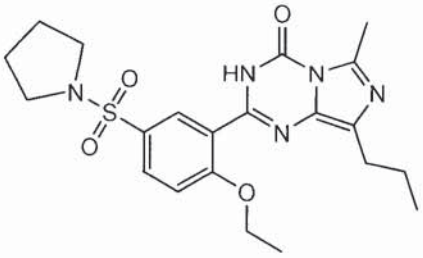
2-[2-Ethoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-14)**



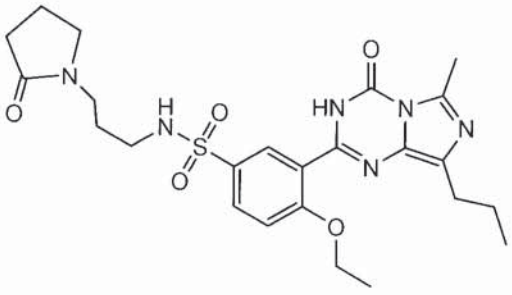
2-[2-Ethoxy-5-(4-ethoxycarbonylpiperazine-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-15)**



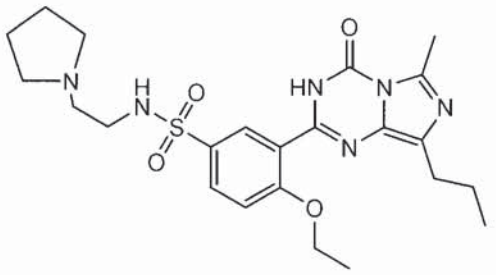
2-[2-Ethoxy-5-(4-hydroxyethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-16)**



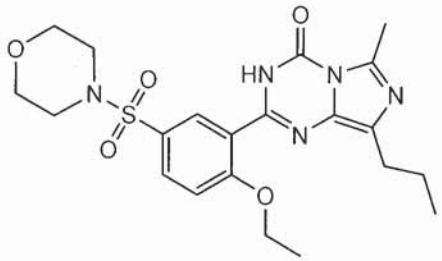
2-[2-Ethoxy-5-(N-pyrrolidinyl-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-17)**



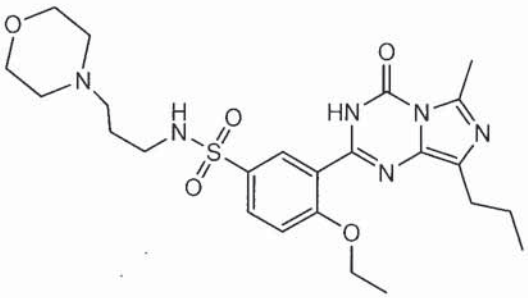
2-{2-Ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl] aminosulfonyl}-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-18)**



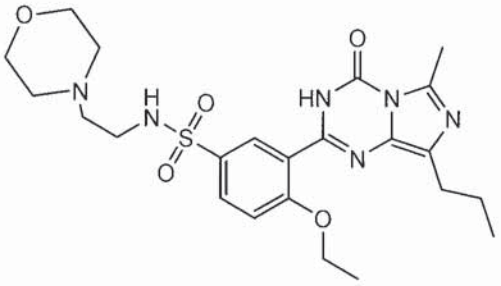
2-{2-Ethoxy-5-[N-(2-pyrrolidinylethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl- imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-19)**



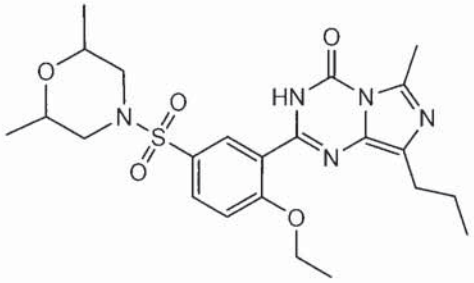
2-[2-Ethoxy-5-(morpholinosulfonyl) phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-20)**



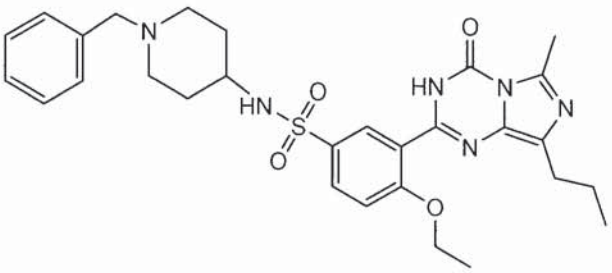
2-{2-Ethoxy-5-[N-(3-morpholinopropyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl- imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-21)**



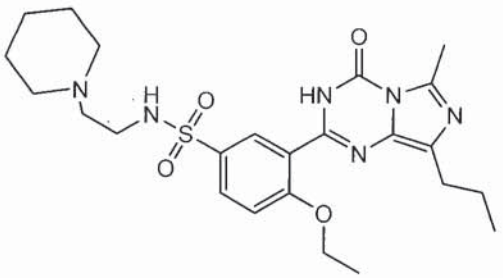
2-{2-Ethoxy-5-[N-(2-morpholinoethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl- imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-22)**



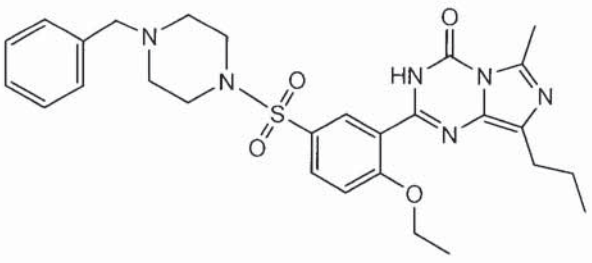
2-[2-Ethoxy-5-(2,6-dimethylmorpholinosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-23)**



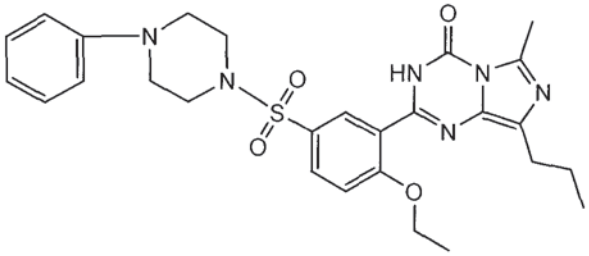
2-[2-Ethoxy-5-(1-benzylpiperidine-4-amino-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-24)**



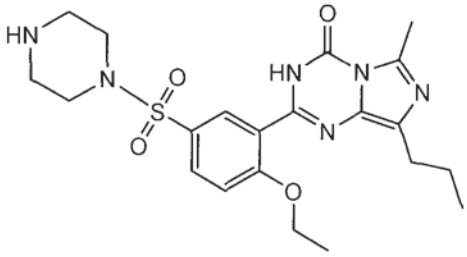
2-[2-Ethoxy-5-(2-piperidinylethylamino-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-25)**



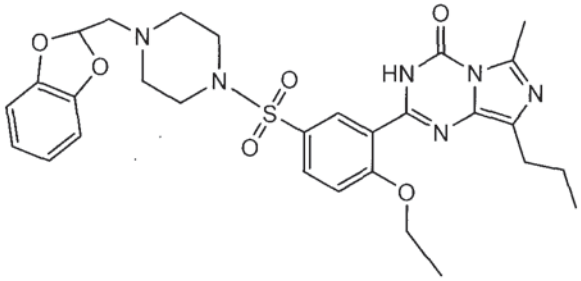
2-[2-Ethoxy-5-(4-benzylpiperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-26)**



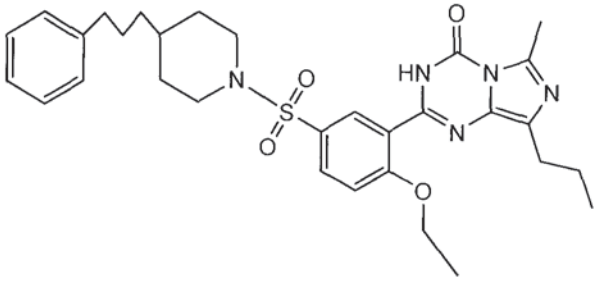
2-[2-Ethoxy-5-(4-phenylpiperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-27)**



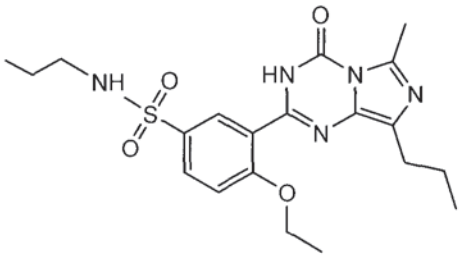
2-[2-Ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-28)**



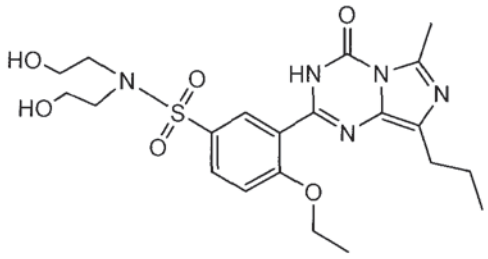
2-[2-Ethoxy-5-(4-benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-phenyl]-6- methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-29)**



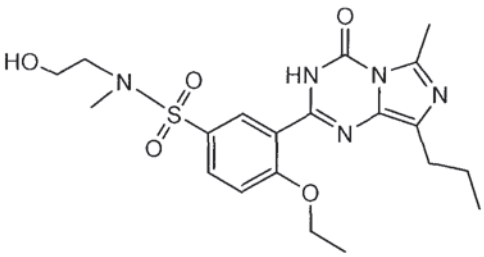
2-{2-Ethoxy-5-[4-(3-phenylpropane-1-yl)piperidine-1-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-30)**



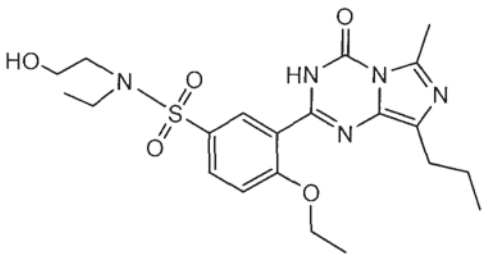
2-[2-Ethoxy-5-(N-propylaminosulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-31)**



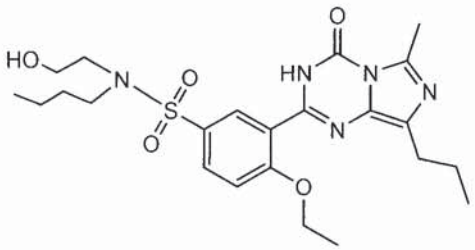
2-{2-Ethoxy-5-[N,N-bis(2-hydroxyethyl)amino-sulfonyl]-phenyl}-6-methyl-8-propyl- imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-32)**



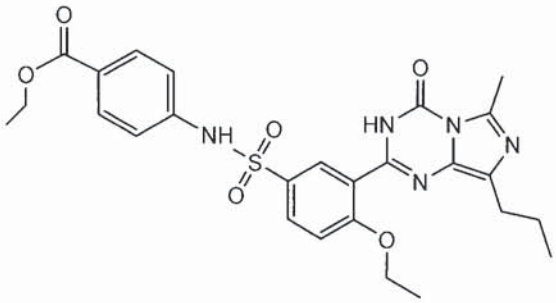
2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-methyl] aminosulfonyl-phenyl}-6-methyl-8- propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-33)**



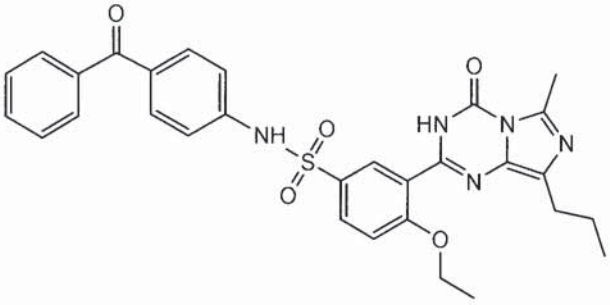
2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-ethyl] aminosulfonyl-phenyl}-6-methyl-8- propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-34)**



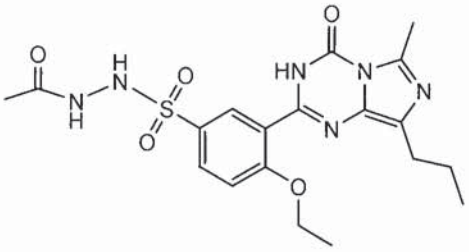
2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-n-butyl] aminosulfonyl-phenyl}-6-methyl-8- propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-35)**



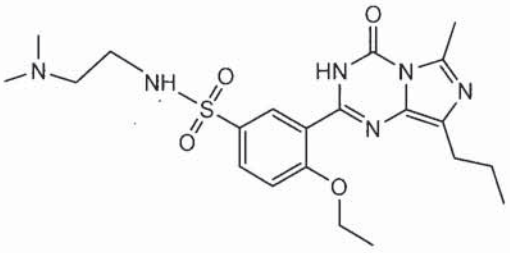
2-[2-Ethoxy-5-(4-ethoxycarbonylaniline-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imiazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-36)**



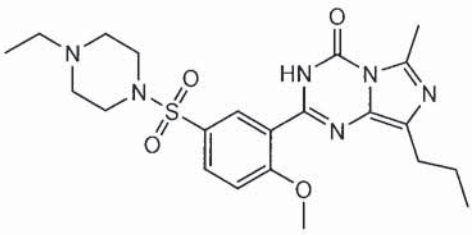
2-[2-Ethoxy-5-(o-benzoylaniline-N-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-37)**



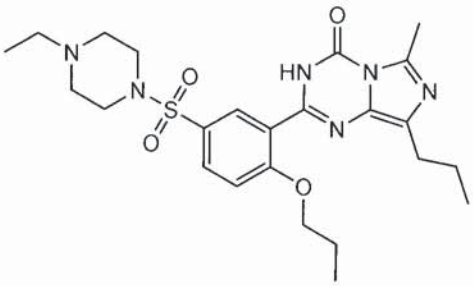
2-{2-Ethoxy-5-[N2-acetohydrazide-sulfonyl]-phenyl}-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-38)**



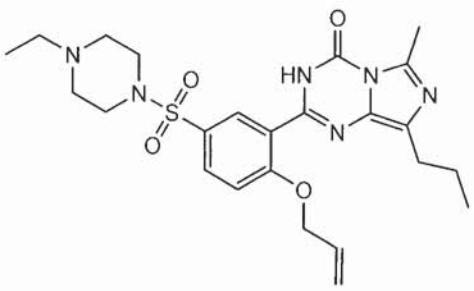
2-[2-Ethoxy-5-(2-dimethylaminoethylamine-N-sulfonyl)-phenyl]-6-methyl-8-propyl- imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-39)**



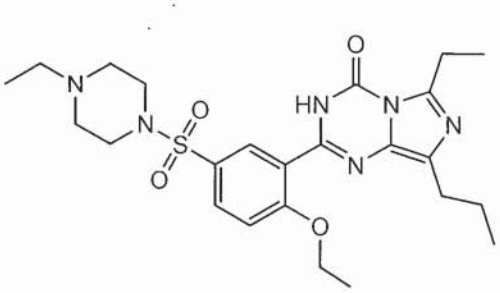
2-[2-Methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-40)**



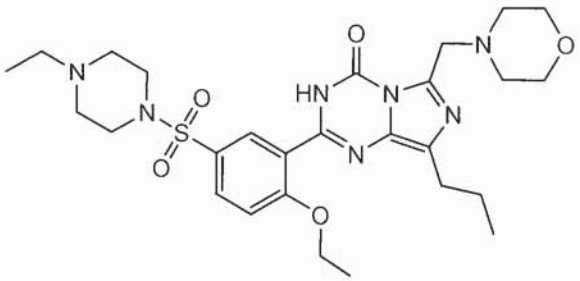
2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-44)**



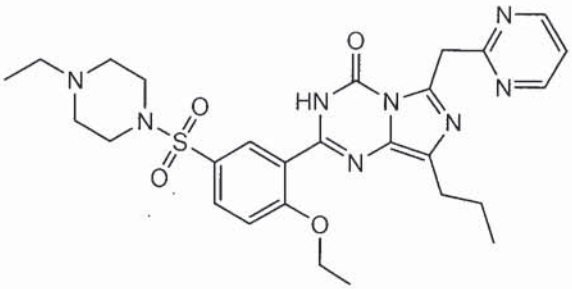
2-[2-Allyloxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-propyl-imidazo [1,5-a]-1,3,5-triazine-4-(3H)-one **(II-48)**



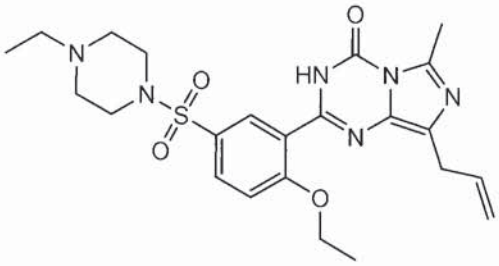
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-52)**



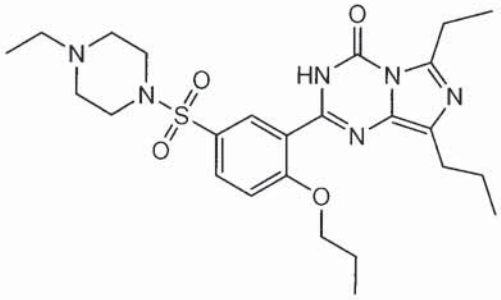
2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-morpholino-methyl-8-propyl-imidazo [1,5-a]-1,3,5- triazine-4-(3H)-one **(II-55)**



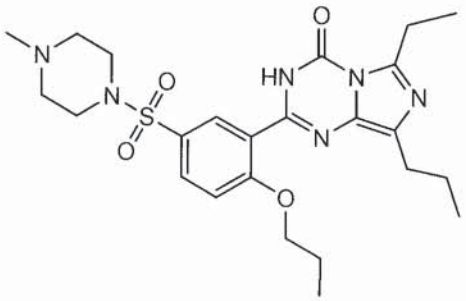
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-(2-pyrimidinyl-methyl)-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4- (3H)-one **(II-58)**



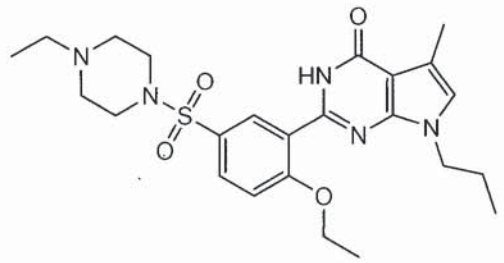
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-methyl-8-allyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-61)**



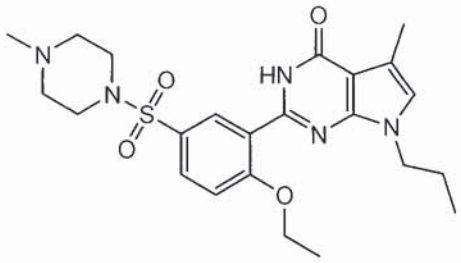
2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-63)**



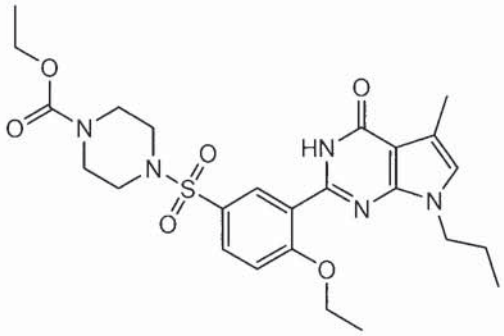
2-[2-Propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-6-ethyl-8-propyl-imidazo[1,5-a]-1,3,5-triazine-4-(3H)-one **(II-68)**



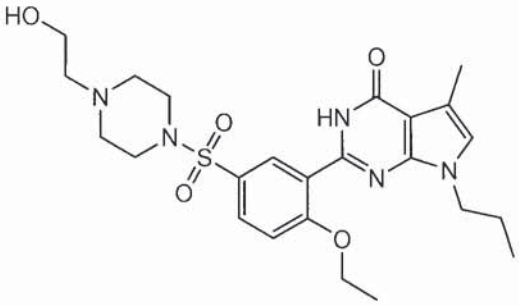
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-20)**



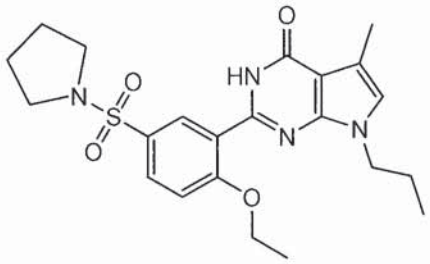
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-21)**



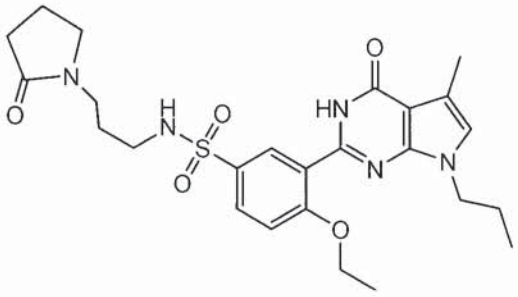
2-[2-Ethoxy-5-(4-ethoxycarbonyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-22)**



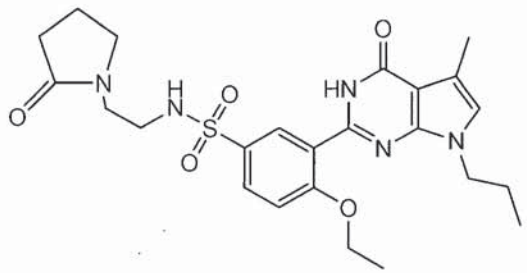
2-[2-Ethoxy-5-[4-(2-hydroxy-ethyl)-piperazine-1-sulfonyl]-phenyl]-5-methyl-7-pro- pyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-23)**



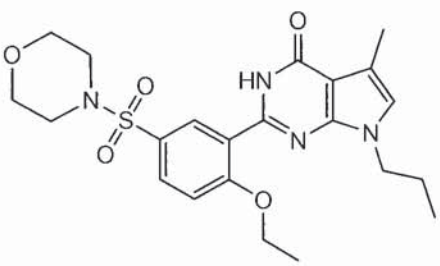
2-[2-Ethoxy-5-(pyrrolidine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro- pyrrolo [2,3-d]pyrimidin-4-one **(III-24)**



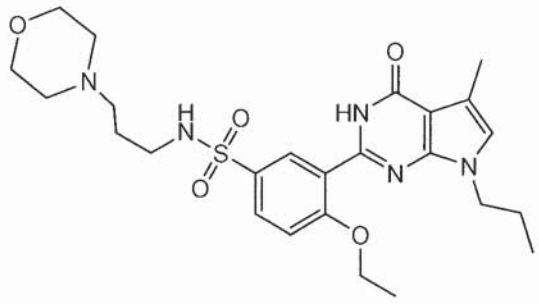
2-{2-Ethoxy-5-{N-[3-(2-oxy-1-pyrrolidinyl)propyl] aminosulfonyl}-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-25)**



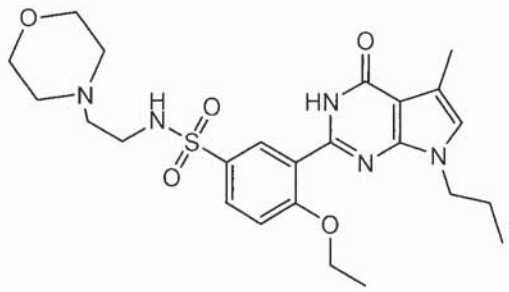
2-{2-Ethoxy-5-{N-[2-(2-oxy-1-pyrrolidinyl)ethyl] aminosulfonyl}-phenyl}-5-methyl- 7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-26)**



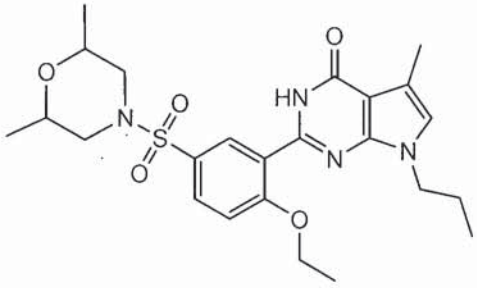
2-[2-Ethoxy-5-(morpholine-4-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-27)**



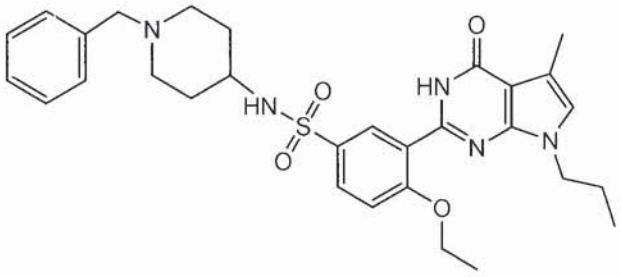
2-[2-Ethoxy-5-(3-(morpholin-4-yl)-propylamine-N-sulfonyl)-phenyl]-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-28)**



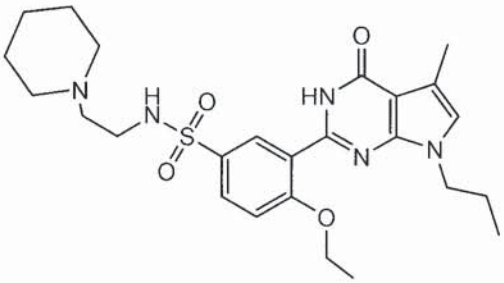
2-[2-Ethoxy-5-(2-(morpholin-4-yl)-ethylamine-N-sulfonyl)-phenyl]-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-29)**



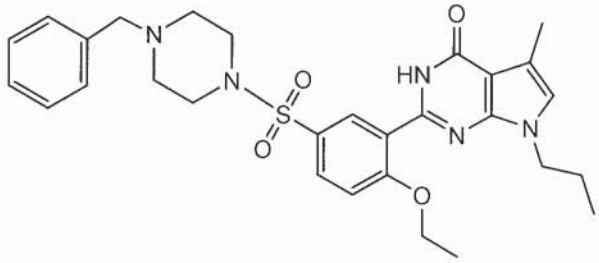
2-[2-Ethoxy-5-(2,6-dimethylmorpholine-4-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-30)**



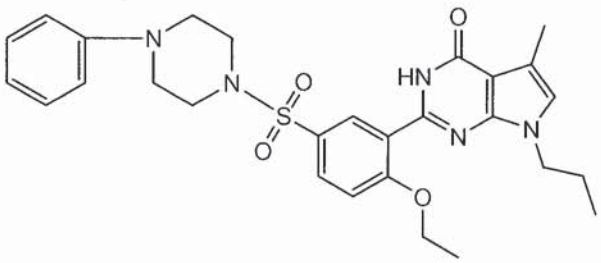
2-[2-Ethoxy-5-(1-benzylpiperidine-4-amino-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-31)**



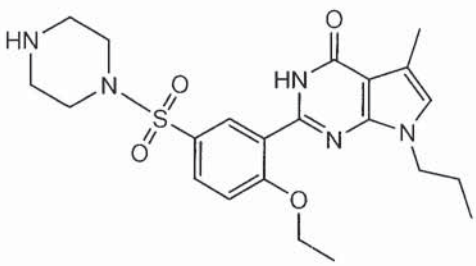
2-{2-Ethoxy-5-[2-(piperidine-1-yl)-ethylamino-sulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-32)**



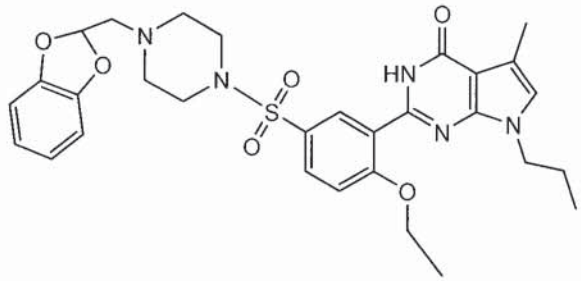
2-[2-Ethoxy-5-(4-benzylpiperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-33)**



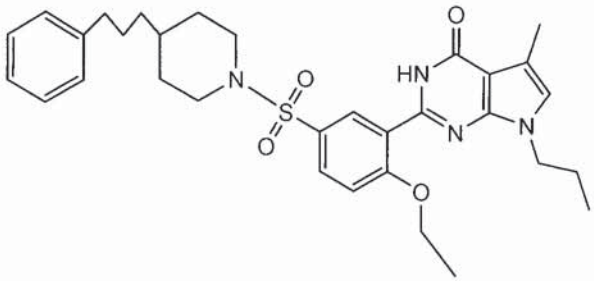
2-[2-Ethoxy-5-(4-phenylpiperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7- dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-34)**



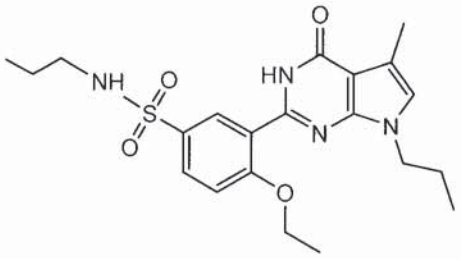
2-[2-Ethoxy-5-(piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-35)**



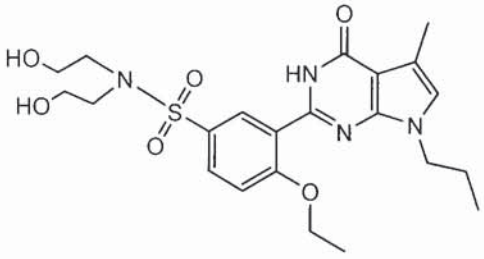
2-[5-(4-Benzo[1,3]dioxolanylmethyl-piperazine-1-sulfonyl)-2-ethoxy-phenyl]-5- methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-36)**



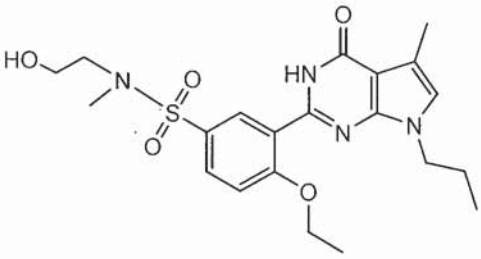
2-[2-Ethoxy-5-[4-(3-phenyl-propyl)-piperidine-1-sulfonyl]-phenyl]-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-37)**



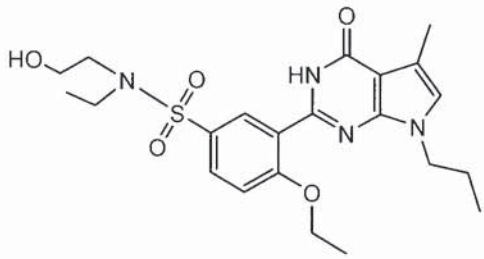
2-(2-Ethoxy-5-propylaminosulfonyl-phenyl)-5-methyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-38)**



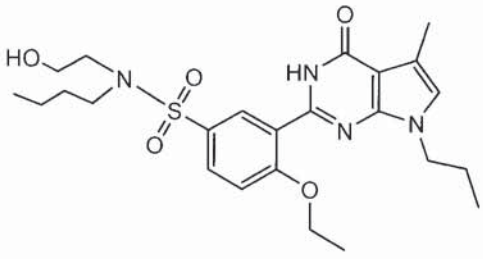
2-{2-Ethoxy-5-[N,N-bis(2-hydroxyethyl)amino-sulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-39)**



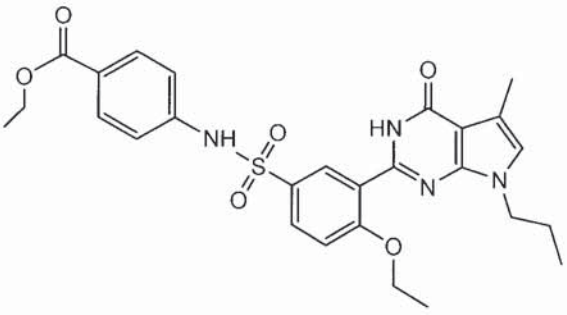
2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-methyl] aminosulfonyl-phenyl}-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-40)**



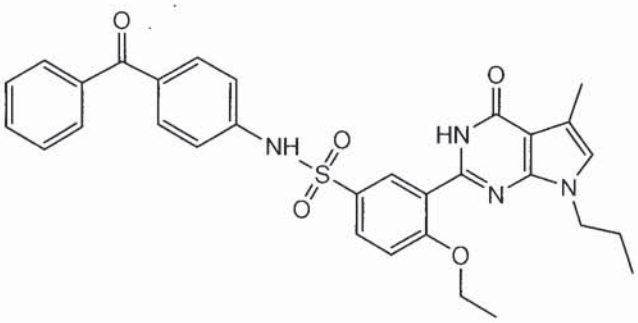
2-{2-Ethoxy-5-[N-(2-hydroxyethyl)-N-ethyl] aminosulfonyl-phenyl}-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-41)**



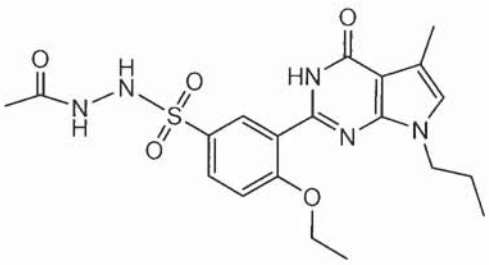
2-{2-Ethoxy-5-[N-butyl-N-(2-hydroxyethyl)] aminosulfonyl-phenyl}-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-42)**



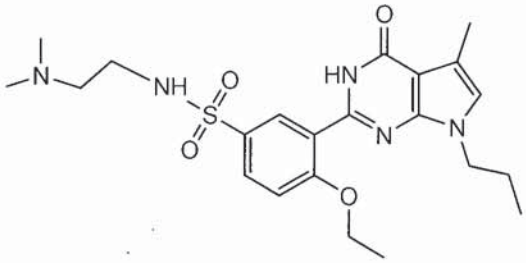
2-[2-Ethoxy-5-(4-ethoxycarbonylphenylamine)-N-sulfonyl-phenyl]-5-methyl-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-43)**



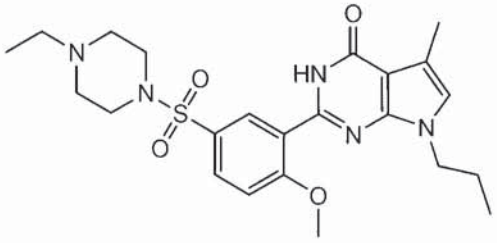
2-[2-Ethoxy-5-(2-benzoylphenylamine)-N-sulfonyl-phenyl]-5-methyl-7-propyl-3,7- dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-44)**



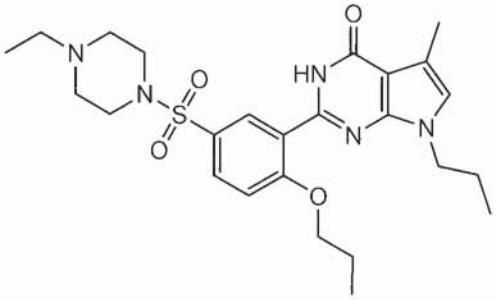
2-{2-Ethoxy-5-[N2-acetohydrazide-sulfonyl]-phenyl}-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-45)**



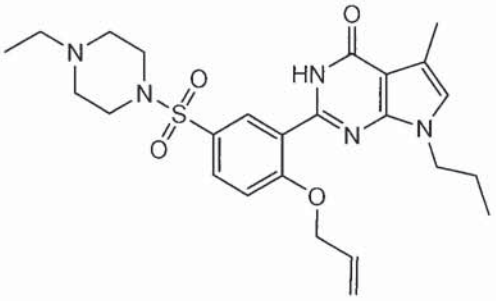
2-[2-Ethoxy-5-(2-dimethylaminoethylamine-N-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-46)**



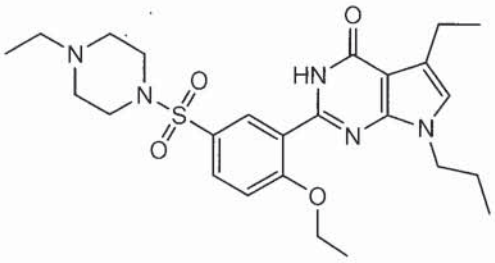
2-[2-Methoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-47)**



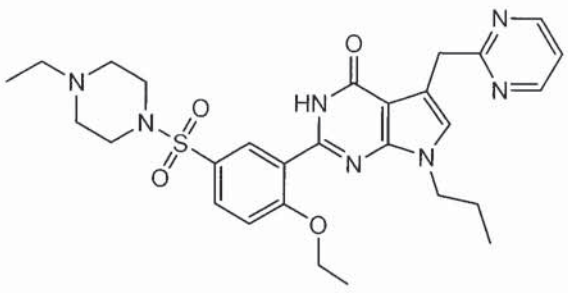
2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-50)**



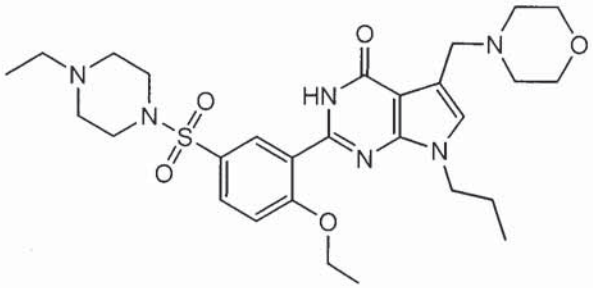
2-[2-Allyloxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-53)**



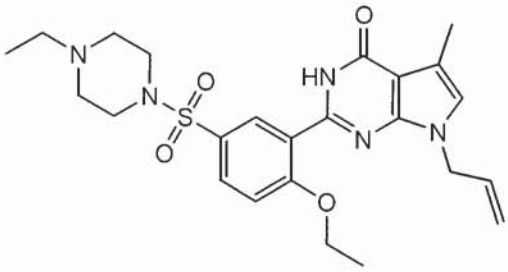
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-56)**



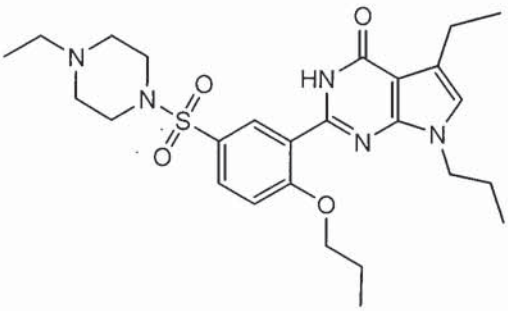
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-(pyrimidin-2-ylmethyl)-7- propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-59)**



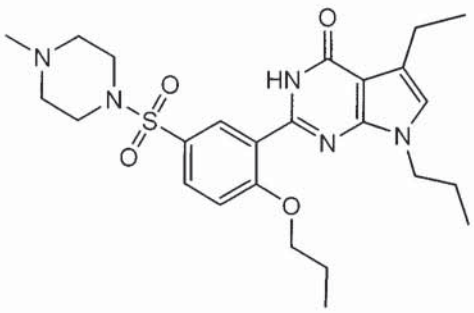
2-[2-Ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-(morpholin-4-ylmethyl)-7-propyl-3,7-dihydro-pyrrolo[2,3-d]pyrimidin-4-one **(III-62)**



2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-allyl-3,7-dihydro-pyrrolo [2,3-d]pyrimidin-4-one **(III-65)**



2-[2-Propoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-di- hydro-pyrrolo [2,3-d] pyrimidin-4-one **(III-68)**



2-[2-Propoxy-5-(4-methyl-piperazine-1-sulfonyl)-phenyl]-5-ethyl-7-propyl-3,7-dihy-dro-pyrrolo [2,3-d]pyrimidin-4-one **(III-73)**

Appendix II

Pharmacokinetics Studies of Yonkenafil

1 Introduction

Yonkenafil, a sildenafil analogue, is a novel inhibitor of the human cGMP-specific phosphodiesterase type 5. It is a new drug to treat with erectile dysfunction (ED). The high validity and safety of yonkenafil have been examined in *vitro* and in *vivo* experiments. As required for a new drug application *(Guide for chemicals preclinical pharmacokinetics study, 2002)*, its non-clinical pharmacokinetics should be investigated. So the absorption, distribution, metabolism and excretion studies of yonkenafil were performed in rats and dogs *(Shah et al.,1992;Xu et al.,2002)*. In this chapter, we described the pharmacokinetics process of yonkenafil in *vivo*.

2 Materials and animals

Materials: Testing sample of yonkenafil was synthesized in our lab. Diazepam (Batch No.: 1225-9601, purity: 99%), as internal standard, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Methanol and acetonitrile was HPLC grade as eluent, while ethylether and dichloromethane were analytical grade. They were purchased from Concord Technology Co., Ltd (Tianjin, P.R. China). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals were analytical grade and purchased from Fuchen Chemicals and Reagents Company (Tianjin, P.R. China) and Beijing Chemical Reagent Company (Beijing, P.R. China). Bio-materials used in the experiments were listed as followings. Filter bag: EQ-D005, perimeter 50mm, antilinear 10000 units, was purchased from Boke Chemical Industry

Co., Ltd (Shenyang, P.R. China). Dialysate was 0.1mol·l^{-1} phosphate buffered solution containing 0.034 mol·l^{-1} sodium chloride (PH 7.0). Blank plasma was freshly collected from Wistar rats.

Animals: Wistar rats (half male and half female) weighting 250.0±10.0g were obtained from Gaoxin Medical Animal Research Centre (Changchun, P.R. China). Beagle dogs (half male and half female) weighting 10.0±1.0kg were obtained from Kexin Experimental Animals Ltd. (Fuoshan, P.R. China). Qualified permit numbers of rats and dogs were SCXK (Ji) 2003-004 and SCXK (Yue) 2003-0006, respectively.

3 Experiments and methods

3.1 Preparation of solutions

The reserving solution (100μg/ml) of yonkenafil was prepared by weighting 5 mg of yonkenafil into 50ml volumetric flask and filling the flask to the volume scale with methanol: water (50:50, v/v). A series of yonkenafil standard solutions were prepared by dilutions of aliquots of the reserving solution with water. The reserving solution (100 μg/ml) of diazepam was prepared by weighting 5 mg of diazepam into 50ml volumetric flask and filling the flask to the volume scale with methanol: water (50:50, v/v). The internal standard solutions containing diazepam at different concentrations were prepared by dilutions of aliquots of the reserving solution with water. The solutions were stored at 4°C. The i.v. injection solutions of yonkenafil were prepared by dissolving and diluting yonkenafil with physiologic saline to the concentrations needed. The oral solutions of yonkenafil were prepared by dissolving and diluting yonkenafil with water to the concentrations needed.

3.2 Administration and sample collection

3.2.1 Administration and sample collection in the absorption of yonkenafil

I.v. administration to rats: Six Wistar rats (three males and three females) weighting 250.0 ± 10.0g used in the experiment were allowed free access to drinking water and eating food. Single, 16.0mg · kg^{-1} i.v. doses of yonkenafil were administered via vena caudalis to the rats (administration volume was 2ml · kg^{-1}). Blood samples (0.25ml) were collected from the venous plexus behind the eyeball into heparinized tubes prior to dosage and at the following times after dosing: 0.08, 0.17, 0.33, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0 and 12.0 h. After centrifugation, plasma samples were stored in polypropylene tubes at -20°C.

Intragastric administration to rats: Eighteen Wistar rats weighting 250.0 ± 10.0g were randomly divided into three groups (three males and three females in each group). The rats were starved overnight until completion of drug administration but retained free access to drinking water at all times. 8.0, 16.0 and 32.0mg · kg^{-1} dosages of yonkenafil were intragastric administered to the three groups of rats, respectively (administration volume was 8ml · kg^{-1}). Blood samples (0.25ml) were collected from the venous plexus behind the eyeball into heparinized tubes prior to dosing and at the following times after dosing: 0.03, 0.10, 0.17, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0 and 12.0 h. After centrifugation, plasma samples were stored in polypropylene tubes at -20°C.

I.v. administration to dogs: Six beagle dogs (three males and three females) weighting 10.0 ± 1.0kg used in the experiment were starved overnight until completion of drug administration but retained free access to drinking water. Single, 6.0 mg · kg^{-1} i.v. dose of yonkenafil was administered via a front leg vein to the dogs (administration volume was 0.2ml · kg^{-1}). Blood samples (1.0ml) were collected from another front leg vein into heparinized tubes prior to dosing and at the following times after dosing: 0.08, 0.17, 0.33, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0 and 12.0 h. After centrifugation, plasma

samples were stored in polypropylene tubes at -20°C.

Oral administration to dogs: Eighteen beagle dogs weighting 10.0±1.0kg were randomly divided into three groups (three males and three females in each group). The dogs were starved overnight until completion of drug administration but retained free access to drinking water at all times. 3.0, 6.0 and 12.0mg·kg^{-1} dosages of yonkenafil were oral administrated to the three groups of dogs, respectively (administration volume was 8ml·kg^{-1}). Blood samples (1.0ml) were collected from a front leg vein into heparinized tubes prior to dosing and at the following times after dosing: 0.08, 0.17, 0.33, 0.50, 1.0, 2.0, 4.0, 6.0, 8.0 and 12.0 h. After centrifugation, plasma samples were stored in polypropylene tubes at -20°C.

3.2.2 Administration and sample collection in the disposition of yonkenafil

Thirty-two Wistar rats were randomly divided into four groups (half males and half females per group). The rats were starved for 12h before intragastric administration at 16.0mg·kg^{-1} (administration volume was 8ml·kg^{-1}). Four groups of rats were sacrificed, respectively, at 5 min, 20 min, 1h and 6h post-dose. Consequently, brain, heart, lungs, liver, spleen, kidneys, stomach, fat, testes, ovaries, bladder, pancreas, uterus, small intestine, stomach contents and wash, intestinal contents and wash, skeletal muscle and abdominal wall smooth muscle were collected. After centrifugation, plasma samples were stored in polypropylene tubes at -20°C. Tissues were processed excised, rinsed with physiological saline, blotted dry, weighed and placed on ice until transferred to a freezer. Finally, they were stored at -20°C.

3.2.3 Administration and sample collection in the metabolism of yonkenafil

Four Wistar rats were starved for 12 h before the bile intubation experiment but retained free access to drinking water at all times. The rats were allowed free access to eating food 2 h after intragastric administration (32 $mg \cdot kg^{-1}$). Bile sample in 0-12 h post-dose was collected and stored at -20°C till analysis.

3.2.4 Administration and sample collection in the excretion of yonkenafil

Excretion of urine and feces: Eight Wistar rats (half males and half females) were brood in metabolic cages and were starved for 12 h but retained free access to drinking water before intragastric administration at 16.0$mg \cdot kg^{-1}$ (administration volume was 8 $ml \cdot kg^{-1}$). Blank urine and feces were collected at the same time. The rats were allowed to eat food 2 h after administration. The urine and feces were collected at 0-4, 4-8, 8-12, 12-24, 24-36, 36-48, 48-60, 60-72, 72-84, 84-96, 96-108 and 108-120 h post-dose. The feces samples at 96-108 and 108-120 h post-dose were put together. The volumes of urine and weights of feces after dry were recorded. Samples were stored at -20°C till analysis.

Excretion into bile: Eight Wistar rats (half males and half females) were starved for 12 h before the bile intubation experiment but retained free access to drinking water at all times. The rats were allowed free access to eating food 2 h after intragastric administration (16$mg \cdot kg^{-1}$). Bile before administration and 0-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-24 and 24-36 h post-dose was collected and stored at -20°C till analysis. The volumes of bile were also recorded.

3.3 Sample measurement and instrument analysis

3.3.1 Measurement of plasma sample in the absorption experiment

Plasma samples of rats and dogs were determined by an LC-MS/MS method. The LC-MS/MS system consisted of an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) binary pump and an autosampler connected to Zorbax extend C18 column (5 μm, 150 mm×4.6 mm I.D from Agilent Technologies) and an Applied Biosystems Sciex Q-trapTM mass spectrometer (Concord, Ontario, Canada) using electrospray ionization (ESI) and Applied Biosystems Analyst version 1.3.2 software. The mobile phase is consisted of methanol: 10mM ammonium acetate (85:15, v/v) delivered at 1.0 ml/min (approximately 1:1 split). Column temperature was 30°C, and 20 ml of prepared samples were injected into the LC-MS/MS system. The Applied Biosystems Sciex Q-trapTM mass spectrometer (Applied Biosystems Sciex, Ontario, and Canada) with an electrospray ionization (ESI) source was used for mass analysis and detection. ESI was performed in the positive ion mode with nitrogen as the nebulizer, heater and curtain gas. Optimized parameters were as follows: nebulizer, heater and curtain gas (N_2) 55, 40 and 15 psi, respectively; source temperature 450°C; Ionspray voltage 1500 V. Declustering Potential and Collision Induced Dissociation voltage were, respectively, 85 V and 39 V for yonkenafil and 85 V and 44 V for diazepam. The detector was operated at unite resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of yonkenafil at m/z 488.1→ m/z 310.1 and diazepam at m/z 285.2 → m/z 193.2.

The plasma samples were frozen stored and thawed at ambient temperature before measurements. An aliquot of plasma (100μl) was placed in a 10 ml glass tube followed by 100μl internal standard solution (250ng·ml^{-1}), 100μl methanol: water (50:50, v/v) and 50μl 1M NaOH. The mixture was extracted with 3ml ether-dichloromethane (3:2, v/v) by vortex-mixing for 30 s and shaking for 10 min (240 times·min-1). After centrifugation at 3500r/min for 5 min, the organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream

of nitrogen. The residue was reconstituted in 200μl mobile phase and 20μl injected into the LC-MS/MS system.

3.3.2 Measurement of tissue sample in the disposition experiment

The tissue samples were determined by LC-MS/MS method too. The chromatography conditions and mass spectrometry conditions were same to the conditions in plasma sample measurement.

Tissue samples were vortex-mixed before preparation. An aliquot of tissue samples (100μl) was placed in a 10ml glass tube followed by 100μl internal standard solution (250ng·ml^{-1}), 100μl methanol: water (50:50, v/v) and 50 ml 1M NaOH. The mixture was extracted with 3 ml ether-dichloromethane (3:2, v/v) by vortex-mixing for 1min and shaking for 10min (240 times·min^{-1}). After centrifugation at 3500r/min for 5min, the organic phase was transferred to another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200ml mobile phase and 20ml injected into the LC-MS/MS system.

3.3.3 Measurement of bile sample in the metabolism experiment

The metabolites in bile of rats were qualified by LC/MS method. The mobile phase is consisted of methanol: 10mM ammonium acetate (65:35, v/v) delivered at 1.0 ml/min (approximately 1:1 split). Column temperature was 30°C, and 20 ml of prepared samples were injected into the LC-MS/MS system. For MS conditions, optimized parameters were as follows: nebulizer, heater and curtain gas (nitrogen) 55, 40 and 15 psi, respectively; source temperature 450°C; Ionspray voltage 1500 V; Declustering Potential 85 V. The detector was operated at medium resolution in the positive ion mode. Scanner modes include: Enhanced MS Scan (EMS), Selected Ion Monitoring (SIM), Neutral Loss Experiment (NL), Precursor Ion Scanning (Prec), Multiple

Reaction Monitoring (MRM).

An aliquot of bile (500ml) was placed in a 10 ml glass tube followed by 100μl 1M NaOH. The mixture was extracted with 4ml ether-dichloromethane (3:2, v/v) by vortex- mixing for 30 s and shaking for 10 min (240 times·min^{-1}). After centrifugation at 3500r/min for 5min, the organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200ml mobile phase and 20ml injected into the LC-MS/MS system.

3.3.4 Measurement of samples in the excretion experiment

The samples were determined by LC-MS/MS method too. The chromatography conditions and mass spectrometry conditions were same to the conditions in plasma sample measurement.

Frozen urine or bile samples were thawed at ambient temperature and vortex-mixed. An aliquot of samples (100ml) was placed in a 10ml glass tube followed by 100ml I.S. solution (250ng·ml^{-1}), 100ml methanol-water (50:50, v/v), and 50ml 1M NaOH. The mixture was extracted with 3ml ether-dichloromethane (3:2, v/v) by vortex-mixing for 30s and shaking for 10 min (240 times·min^{-1}). After centrifugation at 3500g for 5 min, the organic phase was transferred to another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200ml mobile phase and 20ml injected into the LC-MS/MS system. A certain amount of feces (≤0.5g) were collected and grinded. Six times (V/W) of methanol-water (50:50, v/v) solution were added to the feces samples. After homogenate, ultrasound and centrifugation for 10 min, 100μl of supernatant was transferred to a 10ml glass tube followed by 100μl I.S. solution (250ng·ml^{-1}), 100μl methanol-water (50:50, v/v) and 50μl 1M NaOH. The mixture was extracted with 3ml ether-dichloromethane (3:2, v/v) by vortex-mixing for 30s and shaking for 10 min (240 times·min^{-1}). After centrifugation

at 3500rpm for 5 min, the organic phase was transferred to another tube and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 200μl mobile phase and 20μl injected into the LC-MS/MS system.

4 Results and discussions

4.1 Absorption experiment

4.1.1 Plasma concentrations and pharmacokinetic parameters in rats

I.v. Administration: The plasma concentrations and pharmacokinetic parameters of yonkenafil in rats after i.v. administration (16.0 $mg \cdot kg^{-1}$) are shown in Table 1. The mean plasma concentration-time profile is shown in Fig. 1.

Table 1 Pharmacokinetic parameters of yonkenafil in rats after a single, i.v. administration (16.0 $mg \cdot kg^{-1}$)

Animals	Gender	Pharmacokinetic parameters						
		$t_{1/2}$ (h)	k_e (h^{-1}*100)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	MRT (h)	CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	m	3.20	21.7	8263	8839	3.19	30.2	8.35
2	m	1.75	39.6	16796	16928	2.28	15.8	2.39
3	m	2.29	30.2	4017	4072	2.18	65.5	13.0
4	f	1.32	52.6	10674	10678	0.89	25.0	2.85
5	f	1.17	59.4	21939	21954	1.38	12.1	1.23
6	f	2.74	25.3	5667	5780	1.93	46.1	11.0
Mean		2.08	38.1	11226	11375	1.98	32.5	6.47
S.D.		0.81	15.2	6896	6846	0.79	20.2	4.98

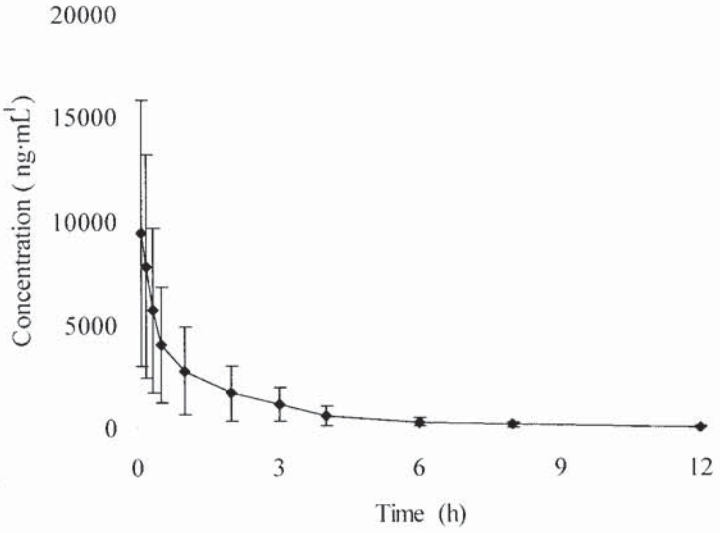


Figure 1 Mean plasma concentration-time profile of yonkenafil in rats after a single, i.v. administration (16.0 mg · kg^{-1})

Pharmacokinetic parameters of yonkenafil in rats after i.v. administration (16.0 mg · kg^{-1}) were: $t_{1/2}$ 2.08 ± 0.81 h; AUC_{0-t} 11226 ± 6896 ng · h · ml^{-1}; $AUC_{0-\infty}$ 11375 ± 6846 ng · h · ml^{-1}; CL 32.5 ± 20.2 ml·min^{-1}·kg^{-1}; V_d 6.47 ± 4.98 L · kg^{-1}. The results processed by Topfit 2.0 showed that the dynamics process of yonkenafil in rats after i.v. administration fitted the two-compartment model.

Intragastric administration: The pharmacokinetic parameters of yonkenafil are shown in Table 2, Table 3 and Table 4. The mean plasma concentration-time profiles are shown in Fig. 2, Fig. 3 and Fig. 4. The plasma concentrations of yonkenafil at three concentrations were processed by Topfit 2.0, respectively. The dynamics process of yonkenafil in rats after intragastric administration fitted the single-compartment model.

The absolute bioavailability of yonkenafil in rats calculated by the AUC_{0-t} ratio between intragastric administration (16.0 mg · kg^{-1}) and i.v. administration (16.0 mg · kg^{-1}) was 29.5%.

Table 2 Pharmacokinetic parameters of yonkenafil in rats after a single intragastric administration (8.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} ($ng \cdot ml^{-1}$)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	*MRT* (h)	*CL* ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	M	3.99	20.4	1.00	572	2281	2474	4.06	53.9	15.8
2	M	2.88	24.1	0.50	328	952	991	2.48	135	33.5
3	M	1.83	37.8	0.50	548	1591	1621	3.11	82.3	13.1
4	F	1.71	40.6	0.50	823	2472	2495	2.91	53.4	7.89
5	F	1.22	57.0	0.50	390	862	863	1.90	154	16.2
6	F	1.62	42.9	0.50	438	948	951	1.88	140	19.6
Mean		2.21	37.1	0.58	517	1518	1566	2.72	103	17.7
S.D.		1.03	13.3	0.20	176	717	761	0.83	45.4	8.68

Table 3 Pharmacokinetic parameters of yonkenafil in rats after a single intragastric administration (16.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} ($ng \cdot ml^{-1}$)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	*MRT* (h)	*CL* ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	M	1.52	45.5	0.25	2925	4897	4909	1.60	54.3	7.16
2	M	2.68	25.9	1.00	792	4086	4288	3.67	62.2	14.4
3	M	1.99	34.7	0.50	1445	4476	4555	2.78	58.5	10.1
4	F	2.44	28.4	0.17	767	1882	1948	2.92	137	28.9
5	F	1.91	36.3	0.50	1980	3017	3045	1.85	87.6	14.5
6	F	2.72	30.6	0.50	730	1484	1499	1.81	178	34.9
Mean		2.21	33.6	0.49	1440	3307	3374	2.44	96.3	18.3
S.D.		0.48	7.01	0.29	880	1410	1431	0.81	50.5	11.0

Table 4 Pharmacokinetic parameters of yonkenafil in rats after a single intragastric administration (32.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} ($ng \cdot ml^{-1}$)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	MRT (h)	CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	M	1.70	40.7	0.50	4760	18559	18744	3.14	28.5	4.20
2	M	2.56	27.1	0.25	3435	8570	8801	2.62	60.6	13.4
3	M	1.80	38.5	0.50	2260	5016	5053	1.86	106	16.5
4	F	2.36	29.3	0.50	830	1582	1607	1.72	332	67.8
5	F	2.27	30.5	0.25	960	2005	2053	2.78	260	51.1
6	F	2.67	26.0	0.50	2005	3392	3449	1.67	155	35.7
Mean		2.23	32.0	0.42	2375	6521	6618	2.30	157	31.5
S.D.		0.40	6.12	0.13	1507	6418	6485	0.63	118	24.6

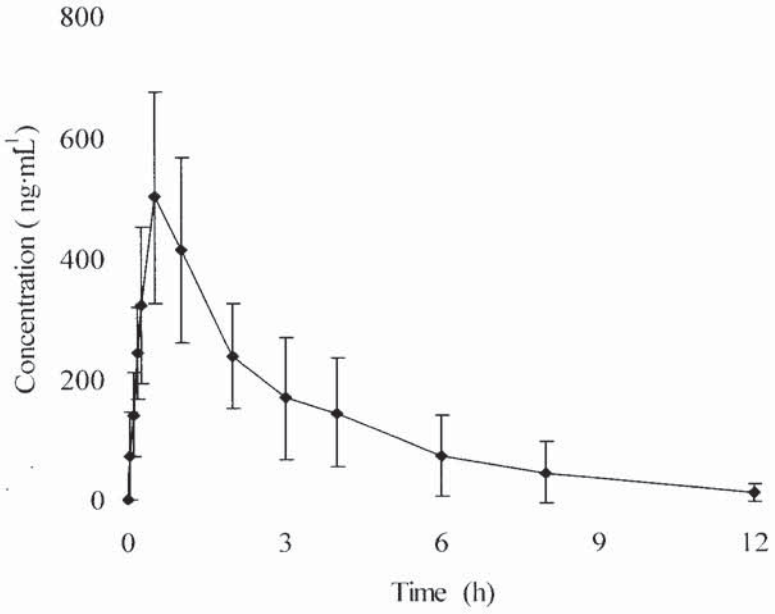


Figure 2 Mean plasma concentration-time profile of yonkenafil in rats after a single intragastric administration (8.0 mg·kg^{-1})

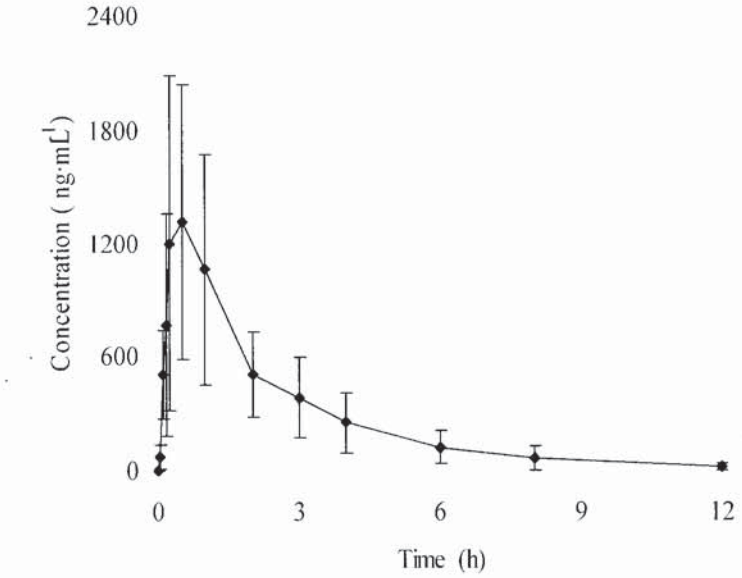


Figure 3 Mean plasma concentration-time profile of yonkenafil in rats after a single intragastric administration (16.0 mg · kg^{-1})

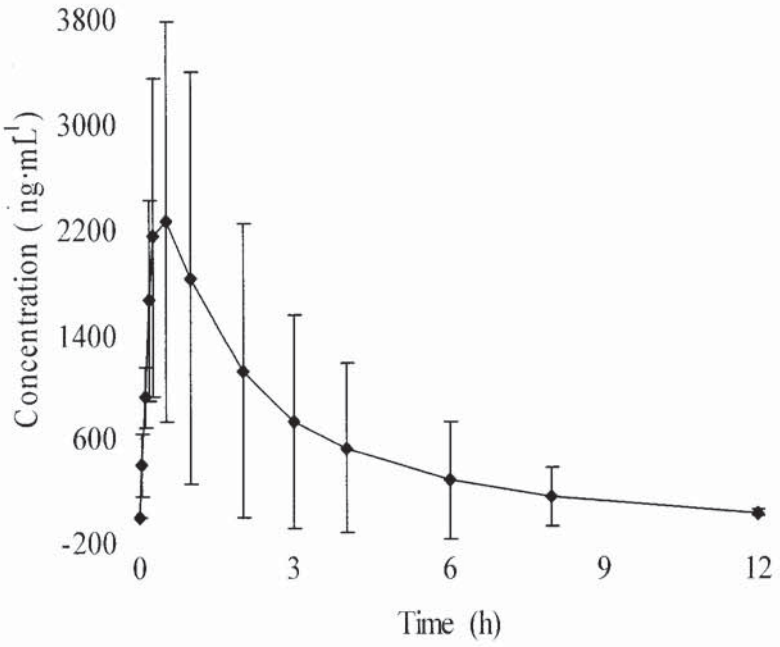


Figure 4 Mean plasma concentration-time profile of yonkenafil in rats after a single intragastric administration (32.0 mg · kg^{-1})

4.1.2 Plasma concentrations and pharmacokinetic parameters in dogs

I.v. Administration: The pharmacokinetic parameters of yonkenafil in dogs after i.v. administration (6.0 mg · kg^{-1}) are shown in Table 5, respectively. The mean plasma concentration-time profile is shown in Fig. 5. Pharmacokinetic parameters of yonkenafil in dogs after i.v. administration (6.0 mg · kg^{-1}) were: $t_{1/2}$ 2.46±0.26 h; AUC_{0-t} 3852±1985ng · h · ml^{-1}; $AUC_{0-\infty}$ 4128±2140 ng · h · ml^{-1}; CL 27.4±8.99ml · min^{-1} · kg^{-1}; V_d 3.56±0.95 L · kg^{-1}. The results processed by Topfit 2.0 showed that the dynamics process of yonkenafil in dogs after i.v. administration fitted the two-compartment model.

Table 5 Pharmacokinetic parameters of yonkenafil in dogs after a single i.v. administration (6.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters						
		$t_{1/2}$ (h)	k_e (h^{-1}*100)	AUC_{0-t} (ng·h·ml^{-1})	$AUC_{0-\infty}$ (ng·h·ml^{-1})	MRT (h)	CL (ml·min^{-1}·kg^{-1})	V_d (L·kg^{-1})
1	m	2.48	27.9	2683	2944	2.21	34.0	7.30
2	m	2.33	29.8	2345	2581	1.65	38.2	7.70
3	m	2.02	34.3	3539	3798	1.65	26.3	4.60
4	f	2.49	27.9	3303	3582	1.87	27.9	6.01
5	f	2.70	25.7	3452	3462	1.89	25.8	6.02
6	f	2.71	25.5	7790	8401	2.9	11.9	2.80
Mean		2.46	28.5	3852	4128	2.03	27.4	5.74
S.D.		0.26	3.25	1985	2140	0.47	8.99	1.81

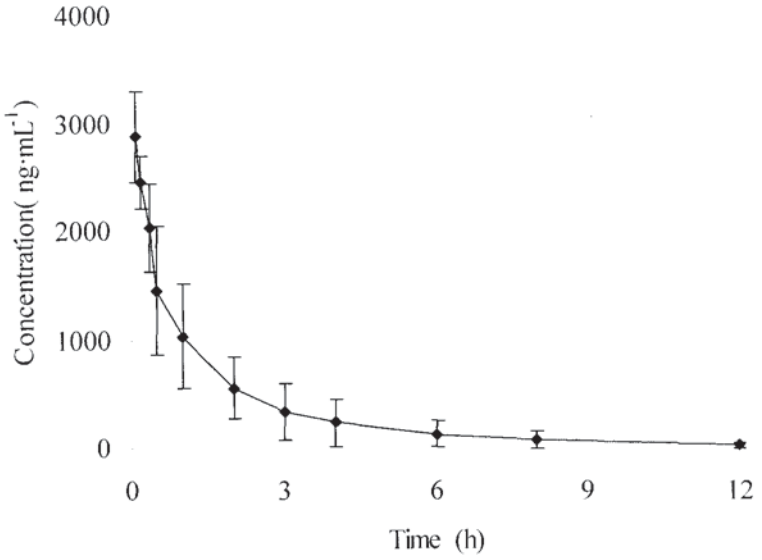


Figure 5 Mean plasma concentration-time profile of yonkenafil in dogs after a single i.v. administration (6.0 mg · kg^{-1})

Oral administration: The pharmacokinetic parameters are shown in Table 6, Table 7 and Table 8. The mean plasma concentration-time profiles are shown in Fig. 6, Fig. 7 and Fig. 8. Pharmacokinetic parameters of yonkenafil in dogs after oral administration (3.0, 6.0 and 12.0 mg · kg^{-1}) were, respectively: C_{max} 167±32.0, 274±107 and 1000±104ng · ml^{-1}; T_{max} 0.83±0.26, 0.92±0.58 and 1.00±0.00h; $t_{1/2}$ 2.53±0.45, 2.87±0.16 and 2.57±0.03h; AUC_{0-t} 576±181, 913±213 and 3659±544ng · h · ml^{-1}; $AUC_{0-\infty}$ 605±196, 961±226 and 3956±377ng · h · ml^{-1}. The plasma concentrations of yonkenafil at three concentrations were processed by Topfit 2.0, respectively. The dynamics process of yonkenafil in dogs after oral administration fitted the single-compartment model.

The absolute bioavailability of yonkenafil in dogs calculated by the AUC_{0-t} ratio between oral administration (6.0 mg · kg^{-1}) and i.v. administration (6.0 mg · kg^{-1}) was 23.7 %.

Table 6 Pharmacokinetic parameters of yonkenafil in dogs after a single oral administration (3.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} ($ng \cdot ml^{-1}$)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	MRT (h)	CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	M	2.13	32.6	1.00	174	569	610	3.02	85.0	15.1
2	M	2.37	29.3	0.50	112	309	318	2.81	157	32.3
3	M	1.96	35.3	0.50	203	480	485	2.23	103	17.5
4	F	2.78	24.9	1.00	185	712	751	3.38	66.6	16.1
5	F	3.16	22.0	1.00	144	552	591	3.59	84.6	23.1
6	F	2.78	25.0	1.00	184	829	876	3.59	57.1	13.7
Mean		2.53	28.2	0.83	167	575	605	3.10	92.0	19.6
S.D.		0.45	5.10	0.26	33.0	181	196	0.53	35.5	7.00

Table 7 Pharmacokinetic parameters of yonkenafil in dogs after a single oral administration (6.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} ($ng \cdot ml^{-1}$)	AUC_{0-t} ($ng \cdot h \cdot ml^{-1}$)	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	MRT (h)	CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	V_d ($L \cdot kg^{-1}$)
1	M	2.97	23.3	0.50	318	1114	1190	3.34	84.0	21.6
2	M	2.93	23.7	0.50	444	1175	1230	2.58	81.3	20.6
3	M	2.57	26.9	2.00	182	786	819	3.24	122	27.2
4	F	2.88	24.1	0.50	217	864	912	3.43	110	27.3
5	F	2.82	24.6	1.00	321	938	982	2.90	102	24.8
6	F	3.02	23.0	1.00	161	599	632	3.19	158	41.3
Mean		2.87	24.3	0.92	274	913	961	3.11	110	27.1
S.D.		0.16	1.41	0.58	107	213	226	0.32	28.3	7.48

Table 8 Pharmacokinetic parameters of yonkenafil in dogs after a single oral administration (12.0 mg · kg^{-1})

Animals	Gender	Pharmacokinetic parameters								
		$t_{1/2}$ (h)	k_e (h^{-1} *100)	t_{max} (h)	C_{max} (ng·ml^{-1})	AUC_{0-t} (ng·h·ml^{-1})	$AUC_{0-\infty}$ (ng·h·ml^{-1})	MRT (h)	CL (ml·min^{-1}·kg^{-1})	V_d (L·kg^{-1})
1	M	2.6	26.7	1.00	881	4154	4356	3.66	45.9	10.3
2	M	2.57	26.9	1.00	1070	3076	3606	2.79	55.5	12.4
3	M	2.54	27.3	1.00	1050	3746	3906	3.65	51.2	11.2
4	F	2.96	23.4	1.00	624	1967	2076	3.32	96.3	24.7
5	F	2.71	25.5	0.50	1050	3007	3139	3.27	63.7	15.0
6	F	2.57	27	0.50	818	2274	2354	2.89	84.9	18.9
Mean		2.66	26.1	0.83	916	3037	3240	3.26	66.3	15.4
S.D.		0.16	1.48	0.26	176	834	891	0.37	20.1	5.51

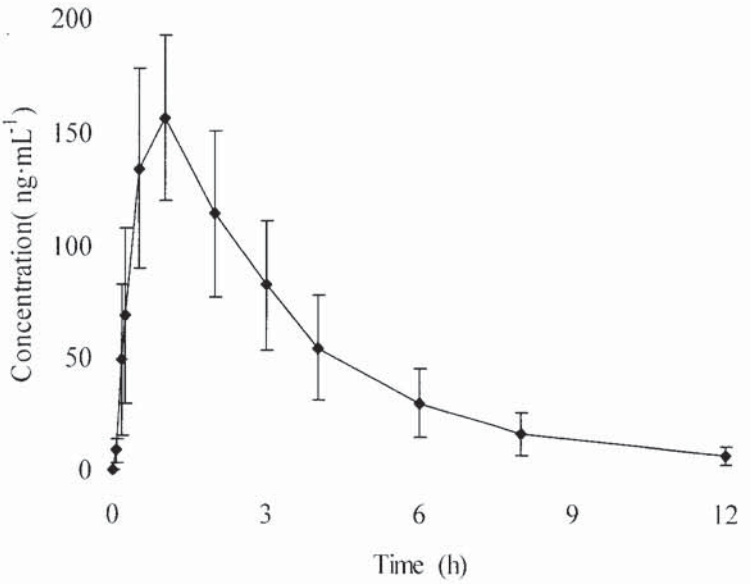


Figure 6 Mean plasma concentration-time profile of yonkenafil in dogs after a single oral administration (3.0 mg · kg^{-1})

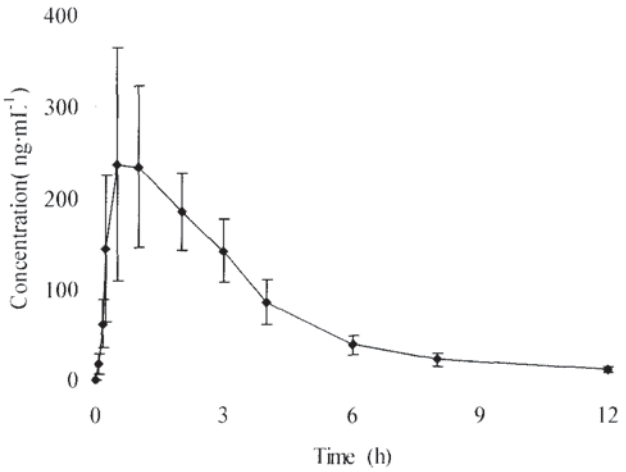


Figure 7 Mean plasma concentration-time profile of yonkenafil in dogs after a single oral administration (6.0 mg · kg^{-1})

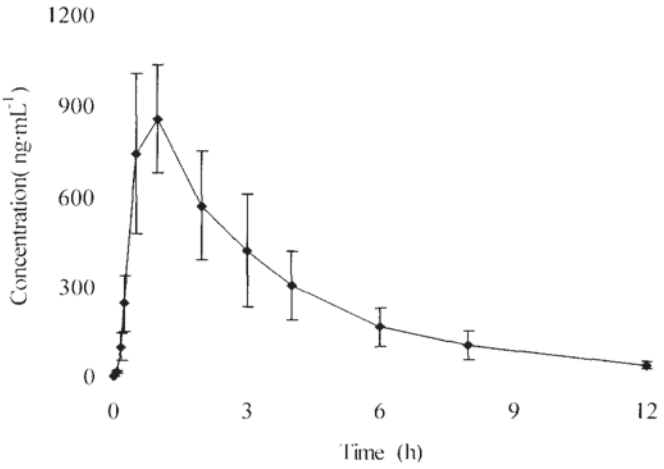


Figure 8 Mean plasma concentration-time profile of yonkenafil in dogs after a single oral administration (12.0 mg · kg^{-1})

4.2 Distribution experiment

The concentrations of yonkenafil in different tissues were measured in MS/LC method. The tissue disposition bars of yonkenafil at different time points after administration were shown in Fig. 9. From Fig. 9, we can find the main distribution

organ of yonkenafil. Yonkenafil distributed fast after intragastric administration. T_{max} was between 20 min and 1h. The concentrations of yonkenafil at 6 h post-dose in all tissues, except intestinal contents, were smaller than 10% of C_{max}, which illustrated that yonkenafil was not cumulated in tissues. The concentrations of yonkenafil in intestinal contents, stomach contents and wash, intestine, stomach, lungs and liver were relative high at 1 h post-dose.

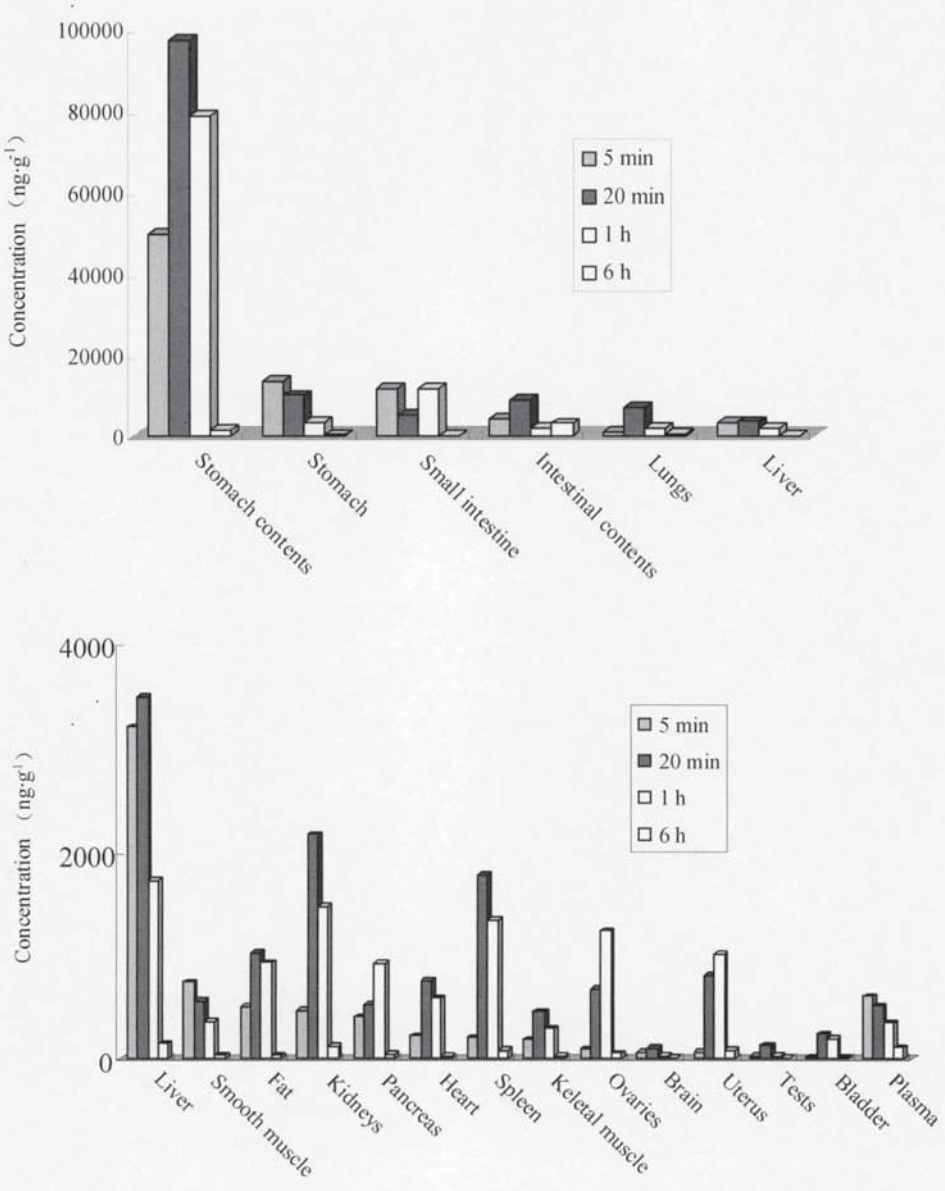


Figure 9 Tissue disposition bars of yonkenafil at 5 min, 20 min, 1 h and 6 h after intragastric administration (16 $mg \cdot kg^{-1}$)

4.3 Metabolism experiment

4.3.1 Metabolism way analysis

Rat bile samples before and after administration (0-12 h) of the drug were collected and detected by LC/MS method. The comparison between EMS chromatograms of rat bile samples before and after administration showed that there was a series of new relative ions except the product ion *m/z* 488.1 in the bile samples after administration. According to the structure of yonkenafil, split rules and the metabolic pathway of sildenafil (structural analogue of yonkenafil) *(Walker et al., 1999)*, the metabolites and metabolic pathway of yonkenafil were supposed (Fig. 10). Then the metabolites were identified and quantified thought EMS, SIM and EPI *et al* scanner modes.

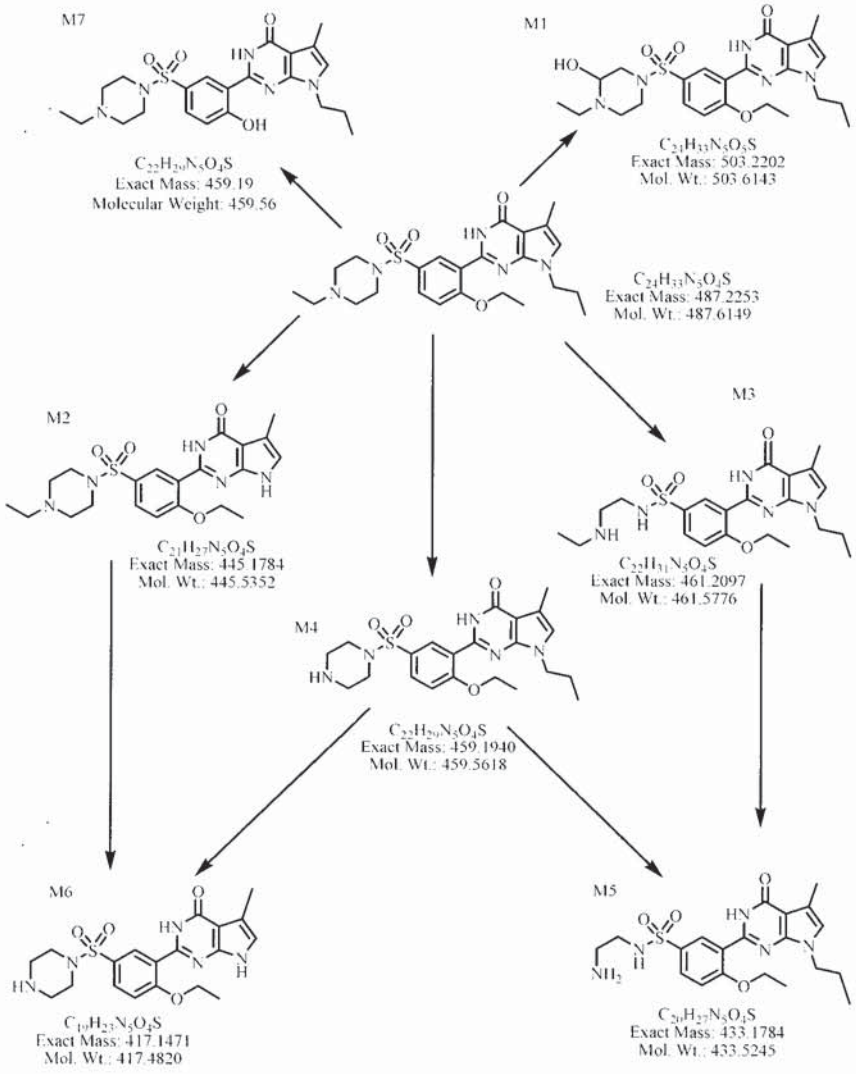
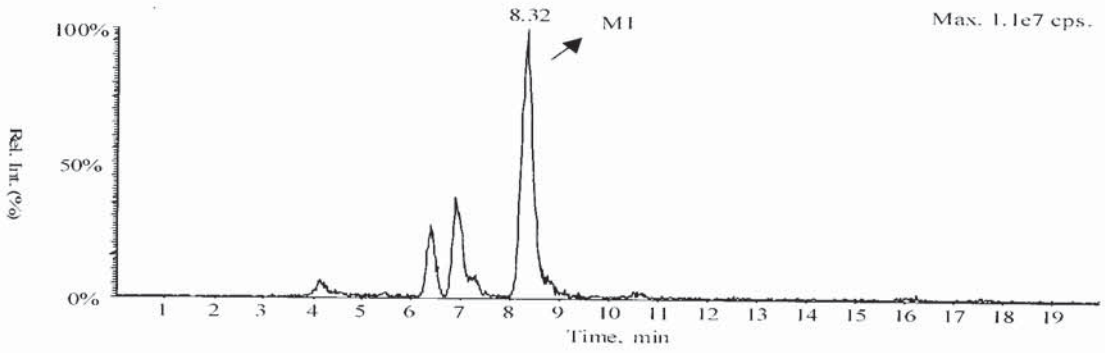


Figure 10 Metabolites and metabolic pathway of yonkenafil in rats

The metabolites of yonkenafil were mainly excreted through bile and feces. Three of six metabolites proposed were identified. M3 was the metabolite of N-deethylation yonkenafil (N in piperazine loop, ethyl is in piperazine loop); M4 was the metabolite of N-deethylation yonkenafil (N in piperazine loop, ethyl is out of piperazine loop); M5 was the metabolite of M4 deethylation (ethyl is in piperazine loop). There was no metabolite found in urine after administration.

4.3.2 The metabolites analysis

All metabolites except M6 shown in Fig. 11 were isolated from the bile samples post-dose. According to the SIM chromatograms of metabolites standard, the chromatographic and spectrometric characters of M3, M4 and M5 in bile samples were accord with the relative synthetic standard. Thus, M3 was the metabolite of N-deethylation yonkenafil (N in piperazine loop, ethyl is in piperazine loop); M4 was the metabolite of N-deethylation yonkenafil (N in piperazine loop, ethyl is out of piperazine loop); M5 was the metabolite of M4 deethylation (ethyl is in piperazine loop). The other metabolites proposed in the assay were not identified because of the absence of reference synthetic standards. There was no metabolite found in urine after administration through SIM scanner mode.



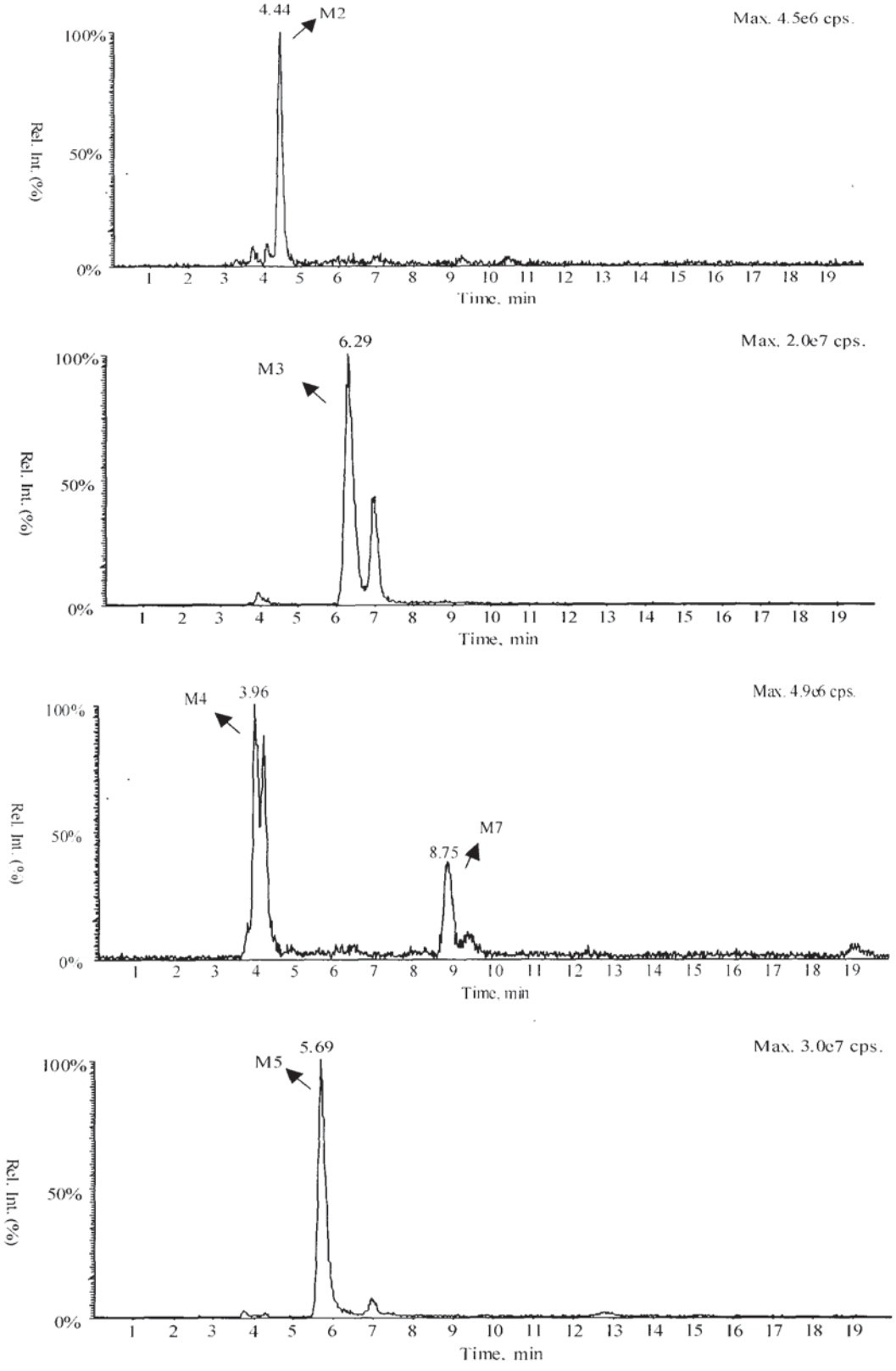


Figure 11 SIM chromatograms of yonkenafil and metabolites

4.4 Excretion experiment

4.4.1 Excretion of yonkenafil in urine and feces in rats

The excretion rate of yonkenafil in urine and feces were shown in Table 9 and Table 10. The relative profiles were in Fig. 12 and Fig. 13. The excretion studies in rats showed that 2.18 ± 1.34% of an intragastric dose of yonkenafil (16.0mg·kg^{-1}) was excreted into urine and feces over the 0-120 h collection period. And 0.05 ± 0.03% of the dose was excreted into urine after 120 h collection period, while 2.13 ± 1.31% of the dose was excreted into feces.

Table 9 Excretion rate of yonkenafil into urine after intragastric administration (16.0 mg·kg^{-1})

Time (h)	△t (h)	Amount of excretion (ng)	Meso-time (h)	Excretion rate (ng/h)
0		0.000		
4	4	1430	2	357
8	4	1722	6	431
12	4	1845	10	461
24	12	1918	18	160
36	12	1943	30	162
48	12	1959	42	163
60	12	1970	54	164
72	12	1978	66	165
84	12	1986	78	166
96	12	1992	90	166
108	12	1998	102	166
120	12	2001	114	167

Table 10 Excretion rate of yonkenafil into feces after intragastric administration (16.0 mg · kg^{-1})

Time (h)	△t (h)	Amount of excretion (ng)	Meso-time (h)	Excretion rate (ng/h)
0		0.0000		
4	4	270.16	2	67.54
8	4	2428.4	6	607.1
12	4	27391	10	6848
24	12	58243	18	4854
36	12	84319	30	7027
48	12	84751	42	7063
60	12	84796	54	7066
72	12	84881	66	7073
84	12	84973	78	7081
96	12	85055	90	7088
120	24	85364	108	3557

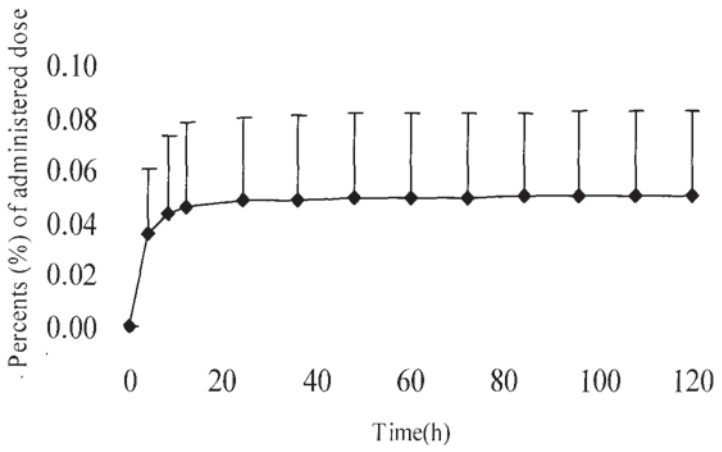


Figure 12 Percents (%) of administered dose profile of excreted into urine in rats after intragastric administration (16.0 mg · kg^{-1})

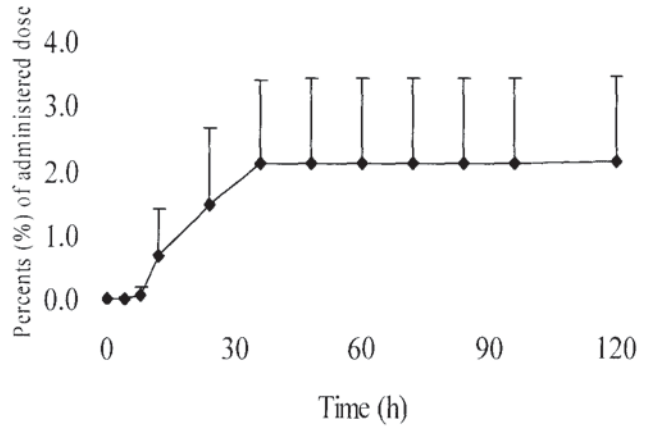


Figure 13 Percents (%) of administered dose profile of excreted into feces in rats after intragastric administration (16.0 mg · kg^{-1})

4.4.2 Excretion of yonkenafil into bile in rats

Total 0.20±0.21% of an intragastric dose of yonkenafil (16.0 mg · kg^{-1}) was excreted into bile after 36 h collection period. The excretion rate of yonkenafil from bile was shown in Table 11. The relative profile was in Fig. 14.

Table 11 Excretion rate of yonkenafil into bile after intragastric administration (16.0 mg · kg^{-1})

Time (h)	△t (h)	Amount of excretion (ng)	Meso-time (h)	Excretion rate (ng/h)
0		0.000		
2	2	1265	1	633
4	2	531.0	3	266
6	2	757.8	5	379
8	2	522.5	7	261
12	4	3332	10	833
24	12	1088	18	90.6
36	12	339.5	30	28.3

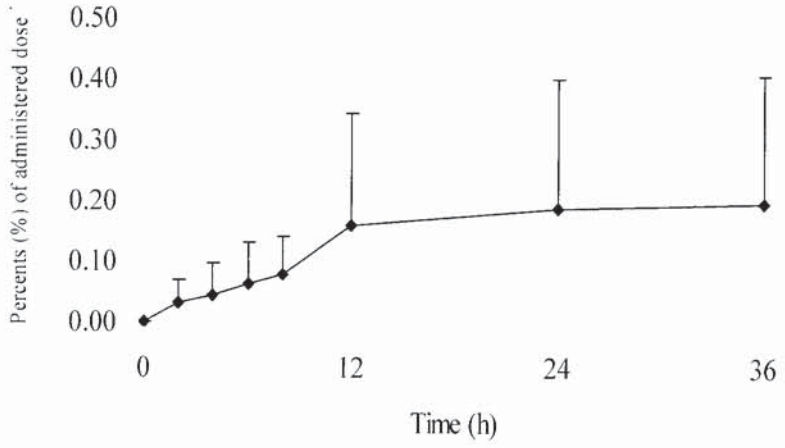


Figure 14 Percents (%) of administered dose profile of excreted into bile in rats after intragastric administration (16.0mg · kg^{-1})

4.4.3 Excretion of major metabolites of yonkenafil

The percent administered dose recovered as the parent drug excreted as metabolites in feces of rats after intragastric administration were shown from Table 12 to Table 14. The percent of administered dose recovered as the parent drug excreted as M3, M4 and M5 over 0-120 h after intragastric administration (16.0mg · kg^{-1}) in rats were 11.0±6.49%, 17.2±11.1%, 24.6±16.9%, respectively.

Table 12 Yonkenafil percents of dose excreted as M3 in feces of rats after intragastric administration (16.0 mg·kg^{-1})

Time (h)	Animals								Mean	S.D.
	female 1	female 2	female 3	female 4	male 5	male 6	male 7	male 8		
0-4 h	0.00	1.87	0.00	0.00	0.01	0.00	0.00	0.07	0.24	0.66
4-8 h	0.00	1.87	0.02	0.00	0.02	0.02	0.00	0.07	0.25	0.65
8-12 h	0.00	1.87	1.46	2.39	2.54	9.85	0.00	4.62	2.84	3.20
12-24 h	10.4	6.96	1.73	19.7	6.09	11.3	0.48	6.14	7.9	6.06
24-36 h	11.5	21.1	5.10	20.2	6.42	11.6	5.13	6.56	10.9	6.50
36-48 h	11.6	21.1	5.11	20.2	6.43	11.6	5.19	6.57	11.0	6.49
48-60 h	11.6	21.1	5.12	20.2	6.43	11.6	5.20	6.57	11.0	6.49
60-72 h	11.6	21.1	5.12	20.2	6.43	11.6	5.20	6.58	11.0	6.49
72-84h	11.6	21.1	5.12	20.2	6.44	11.6	5.21	6.58	11.0	6.49
84-96h	11.6	21.1	5.12	20.2	6.44	11.6	5.21	6.58	11.0	6.49
96-120h	11.6	21.1	5.12	20.2	6.44	11.6	5.22	6.58	11.0	6.49

Table 13 Yonkenafil percents of dose excreted as M4 in feces of rats after intragastric administration (16.0 mg·kg^{-1})

Time (h)	Animals								Mean	S.D.
	female 1	female 2	female 3	female 4	male 5	male 6	male 7	male 8		
0-4 h	0.00	4.55	0.00	0.00	0.02	0.00	0.00	0.03	0.58	1.61
4-8 h	0.00	4.55	0.07	0.00	0.09	0.08	0.01	0.03	0.60	1.60
8-12 h	0.00	4.55	3.64	4.94	6.75	16.18	0.01	4.02	5.01	5.08
12-24 h	12.9	12.5	4.12	31.4	15.4	18.7	0.40	5.72	12.6	9.75
24-36 h	14.7	34.2	10.9	32.2	16.1	19.0	3.78	6.16	17.1	11.1
36-48 h	14.7	34.2	10.9	32.3	16.1	19.1	3.81	6.17	17.2	11.1
48-60 h	14.7	34.2	10.9	32.3	16.1	19.1	3.82	6.17	17.2	11.1
60-72 h	14.7	34.2	10.9	32.3	16.1	19.1	3.82	6.17	17.2	11.1
72-84h	14.7	34.2	10.9	32.3	16.1	19.1	3.82	6.17	17.2	11.1
84-96h	14.7	34.2	10.9	32.3	16.1	19.1	3.82	6.17	17.2	11.1
96-120h	14.7	34.2	10.9	32.3	16.1	19.1	3.83	6.17	17.2	11.1

Table 14 Yonkenafil percents of dose excreted as M5 in feces of rats after intragastric administration (16.0 mg·kg^{-1})

Time (h)	Animals								Mean	S.D.
	female 1	female 2	female 3	female 4	male 5	male 6	male 7	male 8		
0-4 h	0.00	1.20	0.00	0.00	0.00	0.00	0.00	0.18	0.17	0.42
4-8 h	0.00	1.62	0.00	0.00	0.01	0.02	0.00	0.18	0.23	0.56
8-12 h	0.00	1.62	0.78	1.64	5.04	52.1	0.00	20.5	10.2	18.2
12-24 h	12.2	6.49	1.10	23.7	12.9	59.3	1.81	25.9	17.9	19.1
24-36 h	13.2	22.7	4.00	24.2	13.5	60.2	31.1	27.4	24.5	16.9
36-48 h	13.2	22.7	4.01	24.3	13.5	60.2	31.4	27.5	24.6	16.9
48-60 h	13.2	22.7	4.02	24.3	13.6	60.2	31.4	27.5	24.6	16.9
60-72 h	13.2	22.7	4.02	24.3	13.6	60.2	31.4	27.5	24.6	16.9
72-84h	13.2	22.7	4.02	24.3	13.6	60.2	31.4	27.5	24.6	16.9
84-96h	13.2	22.7	4.02	24.3	13.6	60.2	31.4	27.5	24.6	16.9
96-120h	13.2	22.7	4.02	24.3	13.6	60.2	31.4	27.5	24.6	16.9

The results above indicated that yonkenafil changed to a lot of metabolites in rats after intragastric administration. Only a little ratio of yonkenafil excreted as unchanged yonkenafil. The percents of administered dose excreted as M3, M4 and M5 in rats were 52.8% in total.

5 Conclusions

5.1 Absorption experiment

LC/MS/MS method to determine the concentration of yonkenafil was established. The absorption dynamics of yonkenafil in rats and dogs were studied. The rats and dogs were administrated yonkenafil intravenously and orally. Through collection of plasma in different time, the pharmacokinetic parameters were determined. The absolute bioavailability of yonkenafil was 29.5% in rats and was 23.7% in dogs.

5.2 Distribution experiment

LC/MS/MS method to determine the concentration of yonkenafil in different tissues was established. After intragastric administration, yonkenafil dispersed fast in each tissue. T_{max} was between 20 min and 1h. The test substance was not accumulated in any tissues.

5.3 Metabolism experiment

In rat body, yonkenafil was metabolized to six main metabolites. The molecular structures of metabolites were characterized by MS-LC method. The metabolic pathway was supposed in Fig. 10. The metabolites were identified with the control sample we synthesized.

5.4 Excretion experiment

Through the sample collecting and analysis to yonkenafil and its three main metabolites (M3, M4, M5), 52.8% of administrated dosage yonkenafil were excreted out of body as feces.

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