DEVELOPMENT OF ANTICANCER AGENTS BY MODIFICATION OF A NOVEL IMMUNOSUPPRESSANT FTY720 AND PDK1 INHIBITOR OSU-03012

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2011

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Abstract

FTY720 is a novel immunomodulator that has been approved by FDA for the treatment of relapsing Multiple sclerosis (MS). It features a unique mechanism of action by reducing circulating levels of naïve lymphocytes by increasing their localization and sequestration in secondary lymphoid organs. It binds several S1P receptors and subsequently induces their internalization and intracellular partial degradation. This blocks the signal that is necessary for egress of lymphocytes from thymus and peripheral lymphoid organs and therefore induces lymphopenia. Recently, FTY720 was found to exhibit anticancer activities in various types of cancers through different signaling pathways independent of S1P1 signaling. For example, our laboratory found that FTY720 induced apoptosis in HCC cells through ROS-PKCδ-caspase-3 signaling axis. Taken together, this prompted us to initiate modifications on FTY720 to develop more potent anticancer agents devoid of immunosuppressive activity.

OSU-2S, one of the FTY720 derivatives shows most potent cell viability suppression activity in four HCC cell lines. It was derived from FTY720 by replacing the pro-(S)-hydroxymethyl of FTY720 with a propyl group, therefore abrogating the immunosuppressive activity. On the other hand, the anticancer activity was increased by one fold compared to FTY720. Mechanistically, OSU-2S induced hepatocellular
carcinoma cells to undergo caspase-3 dependent apoptosis in the same manner as that of FTY720 but at lower concentration.

**OSU-DY-7** is another derivative that shows high potency in lymphocytic leukemia and primacy B cells purified from chronic lymphocytic leukemia patients. Molecular mechanism studies demonstrate that **OSU-DY-7** down regulated the expression level of BIRC5 through transcriptional mechanism by activating MAPK p38. BIRC5 is a cell survival factor and its down regulation may contribute to the cell death.

Over all, two anticancer agents, **OSU-2S** and **OSU-DY-7** were developed from FTY720 and they showed strong anticancer activity in HCC cells and lymphocytic leukemia cells respectively through distinct mechanisms of action.

PAK1 is a serine/threonine kinase that was the first PAK member identified to be downstream effectors of small GTPase Cdc42 and Rac and regulates cell motility and cytoskeleton. PAK1 was also found to be implicated in a wide range of cellular process, such as cell proliferation and apoptosis rendering it an attractive therapeutic target. In this study, we report on the development of a potent and/or selective PAK1 inhibitors based on a novel PDK1 inhibitor OSU-03012, which also inhibits PAK1 directly. The results show that compound **YM-4** and **YM-15** demonstrate stronger PAK1 inhibitory activity in a ATP-competitive manner, and more importantly, are much less effective in inhibiting PDK1 kinase activity, even though they are not specific PAK1 inhibitor.
Dedication

This document is dedicated to my family
Acknowledgments

First of all, I would like to thank my adviser, Dr. Chen for his guidance and encouragement in various aspects. I cannot finish my Ph.D. without his support. His dedication to research; care for everybody in the lab and his elegant gentleman character put great influence on me. Secondly I'd like to thank my parents. They always gave me the greatest support for the years I studied in USA.

I also would like to thank the colleges in our lab. Dr. Wang, a research scientist in our lab helped and tutored me so much in organic synthesis. Navel, who shared with me his abundant experience in chemistry, also cooked delicious Indian food for me, especially Indian sweets. Su-Lin, we discussed extensively on how to do molecular docking and set up a workstation for virtual screen together. He also taught me to do Western blot assay step by step with great patience. I appreciate what he did for me.

I would like to thank Dr. Ringel for giving me a chance to work in his lab to finish up my project. His passion for science impressed me greatly. Dr. Saji, a research professor in Ringel’s laboratory, helped me with variety of biological assay. I would like to say “thank you”. I would like to thank other members in Dr. Rangel’s lab, Samantha, Victoria, Chaojie, Dr. Koo and Dr. Kim. Thanks for accompanying me for the past few months.
And all the other people who helped me and whom I worked with, I would like to say thank you.
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Chapter 1: Introduction

1.1. Sphingosine-1-phosphate receptors

1.1.1. Introduction

Sphingosine-1-phosphate is generated through phosphorylation of sphingosine which is produced by deacylation of ceramide, a backbone component of spingolipid. Sphingosine-1-phosphate exhibits biological activities in extensive biological process such as proliferation, cytoskeleton organization and migration(1). Further studies revealed that many effects of S1P are mediated through the interaction with five G-protein-coupled S1P receptor S1P1-5, which are formerly termed endothelial differentiation gene (EDG) receptor-1,-5-3-6-8, respectively(2). S1P1 receptor protein is expressed primarily in brain followed by lung, spleen, heart and kidney(3). S1P1 is also a dominant receptor on lymphocytes and regulates their egress from secondary lymphatic organs (4). S1P2 and S1P3 are widely and predominantly expressed in cardiovascular system(5). S1P4 and S1P5 are mainly expressed within the lymphoid system and white matter tracts of the central neuron system respectively. In addition, the differential G-protein-coupling of the individual S1P receptor explains their different signaling pathways and the various cellular effects of S1P.

1.1.2 Role of S1P1 receptor in lymphocyte egress from lymphoid organs
Several lines of evidence have demonstrated that S1P1 plays a key role in the migration of lymphocytes in vivo. Allende et al found that mice with conditional deletion of S1P1 from the T-cell linkage showed a block in the egress of mature single-positive thymocytes from the thymus (6). It was also found that S1P1-deficient T-cells or B-cells readily enter lymph nodes, but reduced the recirculation into the efferent lymph and blood (7). This mechanism is exploited by FTY720 that is believed to internalize and degrade membrane S1P1 receptors in lymphocytes to prevent egress from LNs and recirculation to peripheral inflammatory tissues.

1.2. FTY720 is a novel immunosuppressant and anticancer agent

1.2.1. Introduction

FTY720, (2-(4-octylphenethyl)-2-aminopropane-1,3-diol), was chemically modified from myriocin (ISP-1) as shown in Figure 1, a potent immunosuppressive natural product isolated from the culture broth of *isaria sinclarii* (Figure 1), a member of vegetable wasp and plant worm family that has been used as Chinese traditional herbal medicine for hundreds of years (8, 9). Three stages (10) were involved in this chemical modification to furnish FTY720 (Figure 2). Firstly, the carbonyl group and the double bond in the aliphatic chain was found not necessary for the immunosuppressive activity, therefore they were removed to simplify the structure; Secondly, the two hydroxyl groups were removed and the immunosuppressive activity was not affected; Finally, a phenyl group was incorporated into the side chain to obtain FTY720, which displayed remarkable immunosuppressive activity both in vitro and in vivo as well as significant improvement in side effect.
FTY720 is a novel immunomodulator which has proven to be effective in animal models of transplantation and autoimmunity disease (11-16). Recently, results from two Phase III clinical with FTY720, TRANSFORMS (TRial Assessing injectable interferoN vS FTY720 Oral in RrMS) and FREEDOMS (FTY720 Research Evaluating Effects of Daily Oral therapy in Multiple Sclerosis) provided evidence to support the efficacy and safety profile of this first-in-class therapy for multiple sclerosis (MS). MS is a common disorder caused by an autoimmune process in which auto reactive T cells migrate across the blood-brain barrier and attack myelin sheaths, leading to demyelination and axonal damage(17). To date, US Food and Drug Administration (FDA) has approved FTY720 for the oral treatment of multiple sclerosis (MS) at a dosage of 0.5 mg daily, rendering it a first-line treatment for relapsing forms of multiple sclerosis.

Figure 1. Structure of ISP-1.
1.2.2 Mechanism underlying immunosuppressive activity

FTY720 is a novel immunomodulator with unique mechanisms. Unlike the currently used immunosuppressive agents (e.g. the calcineurin inhibitors cyclosporin and tacrolimus), FTY720 does not inhibit T cell activation and proliferation. Moreover, animal studies show it does not impair immunity to viral infection in rodent models (18). Further study demonstrates that administration of a single dose of 10 mg/kg FTY720 to healthy rats reduces the number of peripheral lymphocyte, and recovery of the lymphocytes counts to pretreatment levels take 8 weeks(11). Therefore the long-lasting decrease in lymphocyte may count for its immunosuppressive activity.

1.2.2.1 FTY720 sequesters lymphocytes into secondary lymphoid tissues

As mentioned above, one proposed mechanism for the decrease of lymphocytes is that FTY720 induced circulating lymphocytes to be sequestered in lymph nodes, therefore reducing peripheral lymphocyte counts. FTY720 is an analog of sphingosine and both
of them are able to be phosphorylated by sphingosine kinases to their monophosphorylated forms. It is reported after oral application of FTY720, the blood level of FTY720-P levels rose to four times the level of parent compound (19). Due to the structural similarity with S1P, FTY720-P is capable of interacting with S1P receptors, surprisingly, with even higher binding affinity. S1P1, one of five S1P receptors, is the dominant receptor on lymphocytes. S1P1 was found to be necessary for T- and B-cells to egress from peripheral lymphoid organs and it controls egress of thymocytes from thymus as well (4, 20). Although it is controversial whether FTY720 interacts with sphingosine-1-phosphate receptors as an antagonist, FTY720-P induces internalization and degradation of the S1P1 receptor (Figure 3) that results in prolonged receptor down-regulation (4), thereby creating a temporary pharmacological S1P1-null state in lymphocytes, providing a potential mechanism for FTY720-induced lymphocyte sequestration (21).

Figure 3. Internalization of S1P1 by FTY720-P (17).
1.2.2.2 Apoptosis induced by FTY720 is not a likely mechanism of action

Initially, it was hypothesized that FTY720 caused decrease of lymphocytes by promoting cellular apoptosis based on the observations that FTY720 exerted apoptotic effect in different cell lines. Suzuki S. et al (22) found that FTY720 induced Bcl-2/Bax-associated apoptotic cell death in human MNCs. FTY720 was also found to cause apoptosis by affecting mitochondria functions and thereby releasing cytochrome C from mitochondria in HL-60 cells by Nagahara Y. et al (23). Fujino M. et al (24) reported their findings that FTY720 induced apoptosis in Jurkat cell by activation of caspases in a Bcl-2-dependent manner. However, the higher macro molar concentration level associated with the apoptotic effect exhibited by FTY720 excluded the hypothesis of apoptosis being an underlying mechanism for its immunosuppressive activity. In contrast, FTY720 inactivates the sphingosine-1-phosphate receptor 1 and inhibits the immune response at low nanomolar concentrations which could be reached at a dose range of 0.1-1mg/kg in vivo (11).

1.2.3 FTY720 demonstrated anticancer activity in various cancer cells

Although FTY720 induced apoptosis on activated lymphocytes might not be a likely mechanism of its immunosuppressive activity, this ability may have anticancer potential in cancer cells. Indeed, it has been demonstrated that FTY720 shows anticancer activity in various types of cancer cell lines or tumor models through different molecular pathways (24-37). Herein, we take prostate cancer and hepatocellular carcinoma as examples to demonstrate the anticancer activity of FTY720.
1.2.3.1 In prostate cancer

Prostate cancer is the most common cancer in male and second cause of cancer death. Chua (28) et al found that FTY720 showed anticancer effect on advanced prostate cancer by inhibiting Runx2 expression and its transcriptional activity as well as its downstream events. Runx2 is a transcription factor, which plays an important role in osteoblast differentiation. The role of Runx2 in carcinogenesis in different types of cancer has also been intensively investigated besides prostate cancer. It is reported that the Runx2 might reduce Akt phosphorylation level and suppress prostate cancer cell viability upon treatment of FTY720. In addition, through modulation of Runx2 expression, FTY720 was reported to promote cadherin switching or changes in cadherin expression and suppress cell invasion toward type I collagen which could cause metastasis of advanced prostate cancer.

RhoA-GTPase was another molecular target that was reported to be involved in the invasion ability of androgen-independent prostate cancer cells during the treatment with FTY720. RhoA-GTPase belongs to Rho-GTPase and plays roles in cell motility and cytoskeleton rearrangement. Its activation has been reported responsible for tumor metastasis. Zhou et al (37) found that through the inactivation of Rho-GTPase pathway FTY720 inhibited prostate cancer cell migration and invasion; on the other hand, constitutively active RhoA contributes to the resistance of prostate cancer cells to FTY720 induced migration and invasion. These findings indicate FTY720 might be useful in the treatment of androgen-independent metastatic prostate cancer.

Besides in vitro studies, the anticancer ability of FTY720 on androgen-independent prostate cancer on animal model was also evaluated using a human prostate cancer xenograft CWR22R in nude mice. Chua et al (29) reported their findings that FTY720
is able to suppress androgen independent prostate cancer growth by inducing apoptosis.

1.2.3.2 In hepatocellular carcinoma

FTY720 also shows anticancer activity in human hepatocellular carcinoma both in vitro and in vivo. Lee et al (31) reported that FTY720 could induce G1 arrest and apoptosis in hematoma cells by inhibiting PI3K activity. PI3-K inhibition led to dephosphorylation of Akt at Ser473, which resulted in an increase in the amount of caspase-9 and caspase-3, and consequently, induced apoptosis in cells. For cell cycle arrest, the dephosphorylation of Akt led to decreases in expression of p42/p44 whose activation could cause p27Kip1 degradation (38); on the other hand, the decrease in the level of phosphorylated Akt inactivated GSK-3β and result in the down-regulation of cyclin D1. Therefore the increase of p27Kip1 and decrease of cyclin D1 might account for FTY720-induced G1 arrest. When Hep3B cells were transfected with constitutively active PI3-K enzyme, they showed resistance to apoptosis, and there is no change in p27Kip1 and cyclin D1 level observed in the transfected cells. This further confirmed the above premises.

The activity of suppression of tumor growth by FTY720 was further evaluated in a rat liver tumor model. It was found that by reducing Akt phosphorylation level at Ser 473 and subsequent activating caspase-3, FTY720 induced apoptosis in liver tumor cells. Additionally, in the rat orthotropic liver tumor model, FTY720 showed significant anti-proliferation activity by suppressing the FAK in tumor tissue, which plays an in many cancers (39).
1.2.4. Synthesis of FTY720

As a newly approved unique immunomudulator and a potential anticancer drug, FTY720 has drawn extensive attentions from synthetic chemists. Up to now, a variety of synthetic routes to FTY720 have been reported. Kiuchi et al (40) reported their synthesis as shown in Scheme 1 where the 8-carbon side chains was introduced by acylation of protected 2-phenylethanol followed by reduction with Et3SiH. The alcohol was converted to iodide followed by coupling with diethyl acetamidomalonate. The coupling product underwent reduction, acetylation and hydrolysis to produce FTY720.

Scheme 1. Synthesis of FTY720 (I)

Seidel et al (41) reported a novel coupling method catalyzed by Iron-containing catalyst. As shown in Scheme 2, in the presence of Fe(acac)₃, the uncatalyzed attack of a Grignard reagent onto the ester group does not compete with the iron catalyzed activation of the C-OTf bond, therefore smoothly delivering the desired coupling product.
Scheme 2. Synthesis of FTY720 (II)

(a) NaHSO₄/SiO₂, EtOAc/hexane; (b) TiCl₄, pyridines; (c) C₈H₁₇MgBr, Fe(acac)₃; (d) NaOMe/MeOH;
(e) MeCl, Et₃N, CH₂Cl₂; (f) NaI, 2-butanol; (g) diethylacetamidomalonate, NaOEt, EtOH; (h) LiAlH₄, THF; (i) Ac₂O, pyridines;
(j) 2 N aq LiOH, MeOH.

Scheme 3. Synthesis of FTY720 (III)

(a) CICOCH₃Br,AlCl₃/DCM; (b) diethylacetamidomalonate, NaOEt, EtOH;
(c) Et₃SiH/TiCl₄; (d) LiAlH₄, THF; (e) Ac₂O, pyridine; (f) 2 N aq LiOH, MeOH.

Additionally, Durand et al provided a practical synthetic route to FTY720 with high chemical yield. As shown in Scheme 3, the combination of Et₃SiH/TiCl₄ selectively
reduces the ketone to a methylene group without affecting other functional groups such as ester and amide, facilitating the preparation of FTY720 in large scale.

1.3. PKCδ as a target for treatment of hepatocellular carcinoma

1.3.1 Hepatocellular carcinoma and current therapy

Hepatocellular cancer is one of most common malignancies in the world. Based on the cancer statics 2010, hepatocellular cancer is the fifth leading cause of cancer death in male and ninth in female in the United States (42). So far the best treatment for hepatocellular carcinoma is still surgical resection. However, reoccurrence and metastasis are the main problems associated with this treatment. Recently, the RAF inhibitor sorafenib was approved by Food and Drug Administration for the treatment of metastatic hepatocellular carcinoma (43). However, the efficacy of this kinase inhibitor is not satisfactory as it works for limited patients (44). Therefore, a novel and effective therapy for the treatment of HCC is useful.

1.3.2. Overview of PKC δ

Recently, our group found that FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C δ (44). PKCδ was firstly discovered by Gschwendt et al. in 1986 (45) and is a member of the novel PCK subfamily. The PKCδ structure (Figure 4) contains a C-terminal catalytic domain with two conserved regions, an ATP-binding region (C3), a catalytically active/substrate binding region (C4), an N-terminal regulatory domain with an inhibitory pseudo substrate sequence, and two cysteine-rich zinc-finger-like sequences (Cys1 and Cys2) in the C1 region.
Its physiological functions in different types of cells have been defined by various biochemical approaches. Recently it has been shown that PKCδ plays an important role in mediating apoptosis. Thus it may represent an important target for cancer therapy (44).

Figure 4. The structure of PKCδ.

1.3.2.1 Activation of PKCδ

There are three ways in which PKCδ can be activated: 1) membrane translocation; 2) tyrosine phosphorylation and 3) caspase cleavage activation. PKCδ was firstly identified by the screening of mammalian cDNA libraries and it is activated by diacylglycerol or phorbol ester through the binding of these compounds to the C1 region in its regulatory domain (47). This binding exposes the catalytic domain and confer to the substrates access to this site (46). The subsequent phosphorylation of the conserved serine/threonine residues i.e. Thr-505, Ser-643, and Ser-662 regulates the activity of PKCδ. Thr-505 resides at the activation loop, however, not like in other cPKC isoforms, its phosphorylation is not important for PKCδ activation for the unique acidic residue Glu-500 in the activation loop sequence proposed to partially activate kinase activity (48). Ser-643 is autophosphorylated and Ser 662 is
phosphorylated by upstream kinase probably involving mammalian target of rapamycin (mTOR) (49).

In addition to activation by translocation to cell membrane and binding to diacylglycerol, many tyrosine sites in PKCδ are phosphorylated and thereby regulating kinase activities (46). It is found that PKCδ is tyrosine phosphorylated at Tyr-311, Tyr-332, and Tyr-512 in the hydrogen peroxide treated cells (50). As shown in Figure 4, Tyr-311, Tyr-332 are located in the bridge area connecting regulatory domain and catalytic domain, therefore their phosphorylation might trigger the conformation change and expose the catalytic domain, thereby activating kinase activity. On the other hand, tyrosine phosphorylation of PKCδ could also decrease PKCδ kinase activity (47).

Moreover, PKCδ contains a conserved motif, where caspase-3 cleaves the kinase into a catalytic domain and a regulatory fragment. The dissociation of the PKCδ regulatory subunit from the catalytic subunit results in a constitutively active kinase form. This type of activation has been implicated in apoptosis in many cellular systems. For example, it is found that only expression of catalytic domain of PKCδ is able to induce apoptosis, however, expression of kinase-dead catalytic fragment of PKCδ can not induce apoptosis (46). In addition, recent findings also suggest a feedback activation of caspase-3 by PKCδ (51).

1.3.2.2. Regulation of apoptosis by PKC δ

Earlier studies on the role of PKC in regulation of apoptosis by using phorbol ester as a PKC activator resulted in conflicting findings; phorbol ester induce both anti and pro apoptotic effect depending on cell types (52). This might be due to the fact that
phorbol ester is a non-specific PKC isoform activator. Moreover, phorbol ester could even activate other proteins containing diacylglycerol-responsive C1 domains (53). This makes the identification of the role of PKC\(\delta\) in apoptosis become complicated.

Up to now, more than ten PKC isoforms have been identified. Among them, PKC\(\alpha\), PKC\(\epsilon\), PKC\(\tau\) mainly function as antiapoptotic kinases, whereas PKC\(\delta\), PKC\(\theta\), PKC\(\mu\) have been associated with proapoptotic functions. PKC\(\delta\) are activated by a variety of apoptotic such as oxidative stress to induce apoptosis. This mechanism of action has been utilized by etoposide to induce apoptosis in salivary gland acinar cells (54) as well as hydrogen peroxide in rat GH3B6 pituitary adenoma cells (50). Other stimuli inducing apoptosis through PKC includes ceramide (55), TNF-\(\alpha\) (56), Fas ligation (57) and DNA-damaging treatments such as ionizing radiation and so on. On the other hand, the involvement of PKC\(\delta\) in the apoptosis is further confirmed by the fact that inhibition of PKC\(\delta\) could confer cells resistance to apoptosis.

Although PKC\(\delta\) is well established as a proapoptotic kinase, its anti-apoptotic effect has also been studied. For example, it was reported that PKC\(\delta\) played a role in anti-apoptotic effect of TNF\(\alpha\) in human neutrophils (59), fibroblast growth factor in granulosa cells (60), nitric oxide in macrophages (61), and SVN1 viral stimulation in glioma cells (62). Moreover, likewise, these anti-apoptotic effects could be blocked either by PKC\(\delta\) inhibitor rottlerin or a PKC\(\delta\) dominant negative mutant.

1.3.3 FTY720 induces apoptosis in HCC cells through activation of PKC\(\delta\) signaling

Recently, our lab demonstrated that FTY720 demonstrated cell killing effect in three of HCC cell lines through ROS (reactive oxygen species)-PKC\(\delta\) signaling pathway (44). Previous study showed that FTY720 induced apoptosis in HCCs through PI3-K-
mediated Akt dephosphorylation (31). However, this result was contradicted with our finding that over expression of constitutively active Akt could not rescue Huh7 cells from FTY720-mediated cell death. To further clarify the underlying mechanism, we screened many pharmacologic inhibitors for their effects on rescuing Huh7 cell from FTY720-induced cell death. Among these inhibitors, the PKCδ inhibitor rottlerin exhibited the ability to counteract the cell killing activity of 5 μmol/L FTY720; therefore, these findings may suggest that PKCδ activation played a pivotal role in FTY720-mediated apoptosis (44). This hypothesis was confirmed by the observation that shRNA knockdown of PKCδ conferred Huh7 cells resistance to FTY720-mediated apoptotic cell death.

1.3.3.1 PKCδ activation by FTY720 in HCC cells
As discussed above, PKCδ is activated via three different mechanisms. In our study, two of them, phosphorylation and proteolytic cleavage were observed in FTY720-treated Huh7 cells. FTY720 caused a rapid increase in the phosphorylation at Try311, Thr507, and Ser664, without affecting that of Tyr155. Additionally, proteolytic activation of PKCδ by caspase-cleavage was also noted after 12 h of exposure to FTY720 (44).

1.3.3.2 FTY720 activates PKCδ through a ROS-dependent mechanism
A variety of stimuli have been reported to activate PKCδ, including ROS (50, 63), ceramide (55), tumor necrosis factor (56), Fas ligand, and radiation. In our study, we found that FTY720-mediated PKCδ activation was through stimulation of ROS generation in HCC cells (44).
Flow cytometric analysis revealed that FTY720 at 10 μmol/L stimulated an immediate increase in ROS production in Huh7 cells. Meanwhile, inhibition of ROS production by NADPH oxidase inhibitor, DPI (diphenyleneiodonium chloride), was found to be able to suppress the effect of FTY720 on stimulating the phosphorylation and proteolytic cleavage of PKCδ. This indicated that ROS production might be the upstream activator of PKCδ activation. Moreover, suppression of ROS generation by DPI also protected Huh7 cells from FTY720-induced caspase-3 activation and apoptotic death. Taken together, this may suggest that FTY720-induced ROS production and the subsequent PKCδ activation contributes to the cytotoxicity observed in FTY720-treated hepatocellular carcinoma cells (44).

1.4. PAK1 represents a therapeutic target for treatment of cancer

1.4.1 Introduction

In mammalian cells, the Ras-related Rho family of GTPase regulates the cytoskeleton. Of the Rho family, Rho A, Rac1 and Cdc42 are well studied members (64-66). PAK1 was identified as a target protein of Rho GTPase in the search of Rho GTPase binding partners in rat brain cytosol (67). It is activated by GTP-bound forms of Rac1 or Cdc42 but not RhoA and in turn regulates cytoskeleton dynamics and cell motility. Additionally, PAK1 can also be regulated independent of GTPases. For example, receptor tyrosine kinases can activate PAK1 through growth factor receptor-bound protein 2 (GRB2) and NCK which is an SH2/SH3 adaptor protein. The SH3 domain of NCK binds to the Pro-rich motif of PAK1 located near the N-terminus, while the SH2 domain binds the EGF receptor (68). A non-receptor kinase, ETK (epithelial and endothelial tyrosine kinase) can also activate PAK1. Other mediators of PAK1
activation include the phosphatidylinositol 3-kinase (PI3K) pathway (64), sphingosine, and 3-phosphoinositide-dependent kinase-1 (PDK1) (69).

1.4.2. PAK family members

In addition to PAK1, five other PAK isoforms have been identified. PAKs are categorized into two subfamilies based on their sequence and also on the mechanisms by which they are regulated. Group I PAKs (PAK1-3) are activated by binding to the Rho GTPases Cdc42 and Rac, while Group II PAKs (Pak4-6) are active independent of GTPase. The distribution (64) of PAKs are as following: PAK1 is highly expressed in brain, muscles and spleen; PAK2 is expressed in many tissues ubiquitously; PAK3 and PAK5 are specifically expressed in the brain; PAK4 is highly expressed in the prostate testis and colon; PAK6 is primarily expressed in testis and prostate as well as many other tissues.

1.4.3. PAK and cytoskeleton reorganization

PAK1 is an important regulator of cytoskeleton dynamics and cell motility. Actin reorganization determines cell polarity, shape, and motility, by spatial and temporal polymerization and depolymerization (70). PAKs phosphorylate myosin light-chain kinase (MLCK) thereby reducing its ability to phosphorylate myosin light-chain (MLC) (71). MCL in turn regulates the myosin activity and also its interaction with actins, which is critical for actins assembly. On the other hand, PAK1 also stimulates the activity of LIM kinase 1. LIMK increases the phosphorylation of cofilin. This phosphorylation inactivates cofilin, leading to reduced actin filament depolymerization (72). Additionally, PAKs may play a role in intermediate filament and microtubule
organization. For example, vimentin filament is one type of intermediate filaments. PAK1 is able to phosphorylate vimentin and regulate its reorganization (73). Moreover, PAK1 regulates microtubules by phosphorylation of stathmin on Ser16, a microtubule-destabilizing protein (74).

1.4.4. PAK and cancer

Increasing amounts of evidence have implicated PAK1 in multiple aspects of cancer cell behavior. For example, Liu et al found that inhibition of p21-activated kinase-1 suppressed the growth of gastric cancer cells through mediating cyclin B1 (75). By activating ERK, PAK1 was also found to stimulate cell growth and migration/invasion in colon cancer (76) and in colorectal cancer (77). Another MAPK protein kinase, Jun, was reported to be activated by PAK1 to enhance cancer metastasis in hepatocellular carcinoma (78). Moreover, it is reported that the activation of PAK1 may contribute to the invasiveness of human breast cancer (79). In addition, it has been shown that by introducing exogenous p21-binding domain of PAK1, Ras- and Rac-induced transformation of Rat-1 fibroblast cells could be inhibited (80), and that the inducible expression of a constitutively active form of PAK1 rapidly induced breast cancer cell proliferation whereas the expression of a dominant-negative form of PAK1 decreased the metastasis of breast tumor cells (79, 81). PAK1 was found to be implicated in thyroid cancer cell migration and PAK1 expression and pPAK levels are enhanced in the invasive fronts in human thyroid cancer samples. Together, these findings indicate that PAK1 plays important roles in human cancer cells, and suggest that it could serve as a therapeutic target for cancer patients.
1.4.5 Structure of PAK1 and activation

The sequence of PAK1 contains two functional domains: the C-terminal kinase domain and the p21-binding domain near the N-terminus. The region encompassing residues 70-149 (PBD) was found to form the Cdc42/Rac binding domain(67), and the sub region PAK1\textsuperscript{75-87} (CRIB) represents a highly conserved sequence among many Cdc42/Rac binders (82).

The kinase domain of PAK1 comprises the residue sequence 249-545, of which residues 407-432 form the activation loop and 384-394 the catalytic loop. Two very important residues in these loops are Thr423, which is critical for the kinase activity, and Asp389, which is the catalytic base.

In inhibited state, the kinase inhibitory (KI) region is positioned to the outlet of the kinase domain cleft, which prevents ATP from binding and Thr423 from being phosphorylated. Specifically, the activation loop is forced to make a turn by a hydrogen bond formed between the Lys141 in the KI segment and the Asp407 of the activation loop. This results in the formation of two hydrogen bonds between the conserved Glu315 in middle of helix C and the two residues 410 and 414, prevents interaction of the Glu315 with Lys299 in the catalytic site, which would likely be the active conformation.

After the GTPase binding, the IS domain unfolds, thereby moving the KI domain away from the cleft. The key residue Thr423 therefore is exposed and phosphorylated. Phosphorylation of serine residues in the IS domain thereafter prevents the kinase from returning to the autoinhibited conformation.

1.4.6 Current PAK1 inhibitors
Current PAK1 inhibitors fall into two categories, i.e. ATP–competitive inhibitors and allosteric inhibitors. It was reported that CEP-1347, a derivative of staurosporine can selectively inhibit PAK1 activities (83). Compared to staurosporine, CEP-1347 is more bulky in that it has two long side chains at position C3 and C9, which is hypothesized to contribute to the specificity for the fact that PAK1 family kinases appear to have a large ATP-binding pocket and this expanded ATP-binding site can better accommodate CEP-1347 (83). Additional, Maksimoska et al (84) developed a few potent and selective PAK1 inhibitors by using bulky and rigid octahedral ruthenium complexes as structural scaffolds.

A recent study revealed an allosteric small-molecule inhibitor IPA-3 (85) that exhibits a novel inhibitory mechanism of targeting PAK1 autoregulation. As has been discussed, PAK1 possesses an autoinhibitory domain. In the inactive
conformation, PAK1 is autoinhibited by formation of a homodimer in which the auto regulatory region of one monomer binds and inhibits the catalytic domain of its partner and vice versa (86). Thus, the idea that using a small molecule to perturb the conformational changes has been proposed. IPA-3 was discovered to inhibit PAK1 in such a manner. A high-throughput assay measuring ATP hydrolysis as an indicator of PAK1 catalytic activity identified IPA-3 as an inhibitor of PAK1 and a secondary screen in the presence of a much higher concentration of ATP confirmed it as a non-ATP competitive inhibitor(86). Subsequent study showed that IPA-3 directly targets PAK1 and exhibits a selectivity for group I over group II PAKs, which supports the idea that IPA-3 targets the PAK1 autoregulatory mechanism as group II PAKs are not thought to undergo autoinhibition by the same mechanism as group I.

In light of the novel structure associated with anticancer agent/immunosuppressant FTY720 and the therapeutic value of PAK1 in treatment of cancer, Chapter 2 in this dissertation describes the lead optimization of FTY720 to develop potent antitumor agents. In Chapter 3, we initiated lead optimization of OSU-03012 to develop more potent and selective PAK1 inhibitors. Chapter 4 summarized the findings and envisioned the future works.
Chapter 2: FTY720 as a scaffold to develop potent antitumor agents

2.1 Strategies for structural modifications of FTY720

FTY720 has been shown to exert extensive anticancer effect in different types of cancer cells or tumors (17, 33, 87-92). However, the immunosuppressive potential originally associated with FTY720 might impede its therapeutic application as an antitumor agent. Fortunately, none of the reported mechanisms underlying its anticancer activity is S1P1-dependent, therefore allowing us to initiate structural modifications on FTY720 to develop more potent anticancer agents devoid of the immunosuppressive activity. The strategy for modifications on FTY720 (Figure 6) takes into account different considerations from both synthetic and biological perspective. First of all, to simplify the synthesis, an oxygen atom was introduced to the side chain adjacent to the phenyl ring. This reduced the synthesis effort and facilitate further modifications of the side chain for the connection of octyl group (or other alkyl group) to the phenyl ring involves the incontinent cross-coupling of corresponding Grignard reagents with a phenolic triflate (41). Second, the length of the side chain was varied to find the optimal side chain with higher anticancer activity. Third, modification on the linker connecting phenyl ring and the quaternary carbon was carried out as well. Finally, other different alkyl groups were introduced in place of one of the hydroxymethyl group to optimize the activity.
Figure 6. Three sites of FTY720 were modified as shown.

2.2 Synthesis of FTY720 derivatives

The synthesis of compounds FTY3, FTY4, FTY7 was carried out as outlined in Scheme 4. Incorporation of various lengths of side chains to 4-hydroxyphenyl ethanol gave 4-alkoxyxylated phenyl ethanols. The ethanols were converted into iodides, which were condensed with diethyl acetamidomalonate using sodium hydride in DMF to give the coupled intermediate that were reduced with lithium aluminum hydride resulting in crude 1,3-diol compounds. For the reason of purification, the 1,3-diol compounds were acetylated into their triacetylated forms. The following hydrolysis gave rise to FTY3, FTY4 and FTY7.
Compounds **FTY1-2, FTY5-6, FTY11-13** were synthesized using previously reported methods (40). As shown in Scheme 4, iodides were condensed with various 2-substituted diethyl malonates to give diesters. Careful treatment of the diesters with potassium hydroxide in ethanol to hydrolyze only one of the two ester groups. The free carboxylates were treated with ethyl chloroformate followed by sodium azide to generate acyl azides, which were converted to isocyanates by Curtius rearrangement. These isocyanates were heated in methanol yielding methylcarbamates. Reduction with lithium aluminum hydride and hydrolysis with potassium hydroxide gave the target compounds.
FTY8, which has one methylene linkage between phenyl ring and the quaternary carbon was synthesized as depicted in Scheme 5, 4-Hydroxybenzaldehyde was coupled with 1-bromoheptane and the product was condensed with huppuric acid to produce azlactone. Three steps were involved in the successful reduction of the double bond in the azlactone. The quaternary carbon was setup by introducing a propyl group using sodium hydride in DMF. The ring in the intermediate was opened by sodium methoxide in methol to give the methyl ester derivative, which underwent reduction and hydrolysis to yield FTY8.

![Scheme 5. Synthetic procedure for FTY8](image)

(a) EtOH, EtONa, heptyl bromide; (b) Ac₂O, Huppric acid, NaOAc; (c) i, EtOH, NaOH, ii Pd/C, EtOH, iii, DCC/DCM; (d) Iodopropane, NaH, DMF; (e) i, MeOH, NaOMe, ii, LiBH₄, iii, LiOH, MeOH, H₂O

The synthesis of FTY10 and FTY15 is presented Scheme 6. Protection of tris(hydroxymethyl)nitro methane with acetone in the presence of catalytic amounts of
anhydrous p-toluenesulfonic acid (TsOH) gave ketal. Ketal was converted to triflate using triflic anhydride in the presence of pyridine. The triflate was condensed with 4-octylphenol followed by being subject to p-toluenesulfonic acid in methanol to produce $\text{FTY9}$. $\text{FTY10}$ was obtained by reduction of nitro group to amino group by hydrogenation.

Scheme 6. Synthesis of $\text{FTY9}$, 10 and 15
FTY16 was synthesized starting with 2-butyl-propane-1,3-diol in which one of the hydroxyl group was protected and the other one was subject to activation by being transformed into triflate. The following three consecutive reactions, coupling, deprotection and hydrogenation gave FTY16 (Scheme 7). (R)-2, 5-dihydro-3,6-dimethoxy-2-isopropylpyrazine was used as chiral auxiliary to start the synthesis, which underwent subsequent dialkylation to generate quaternary carbon derivatives. The quaternary carbon derivatives were converted to FTY2R, OSU-2S or FTY14 by undergoing hydrolysis and reduction (Scheme 8). The synthesis of OSU-2S phosphate was demonstrated in Scheme 9. Benzyl chloroformate was used to protect the amino group in OSU-2S. The protected OSU-2S was phosphorylated by reaction with dibenzyl diethylamidophosphite, tetrazole and meta-Chloroperoxybenzoic acid. The following hydrogenolysis gave rise to OSU-2S-P.
(a) Butyllithium, alkyl iodide, THF -78°C; (b) Butyllithium, propyl iodide, THF -78°C; (c) TFA, H₂O/CH₃CN; (d) LiAlH₄/THF.

Scheme 8. Asymmetric synthesis of FTY2R, OSU-2S and FTY14
2.3 Structures of FTY720 derivatives and their biological activities

To expedite the screening of antitumor agents, Huh7 cells were used to assess the anti-proliferative activity of FTY720 and its derivatives. Compared to FTY720, **FTY4** (derived from FTY720 by replacing C with O) showed improved potency in suppressing HCC cell viability. Although the improvement in terms of cell killing activity was not dramatically significant; this modification simplified the synthesis and made it easier to incorporate different side chains to the phenyl ring. **FTY3** and **FTY5**, with 5-carbon side chain and 3-carbon side chain respectively, did not show any cell viability suppressing ability (Table 1). This result is consistent with the previous report (40) that the length of the alkyl side chain caused the fluctuation of **OSU-2S Phosphate**

![Chemical structures](image)

(a) Benzyl chloroformate, CHCl₃/NaHCO₃, 99%; (b) PPR, tetrazole, DCM, MCPBA 70%; (c) H₂, Pd/C, MeOH, rt, 90min, quan.

Scheme 9. Synthetic route to **OSU-2S phosphate**
cytotoxicity. Derivatives with longer side chains than 7-carbon were not be synthesized and tested for the concern that hydrophobicity could introduce unfavorable physicochemical properties such as insolubility. Therefore, the 7-carbon side chain was retained for further modifications for its optimal activity.

The subsequent lead optimization of **FTY4** was performed by introducing varying length of aliphatic substituent to the quaternary carbon. This modification yielded a series of racemic compounds (**FTY1**, **OSU-2 FTY11-13**), all of which showed improved anticancer activity (Table 2), suggesting the hydrophobic substituent is superior to hydroxymethyl group in improving anticancer activity.

Modifications on the linker between phenyl ring and the quaternary carbon showed the 2-carbon linker is still the best for the cancer cell killing activity. This phenomena was well demonstrated by comparing the IC50 values between FTY720 and **FTY10; FTY4** and **FTY15; FTY2, FTY16** and **FTY8** (Table 3). For example, FTY720 has the lower IC50 in inhibiting Huh7 cell viability than **FTY10**, and the only difference in their structure is that the linker in FTY720 is ethylene while in **FTY10**, is –OCH2- group.

The dramatic difference between **FTY4** and **FTY15** further proved that 2-carbon linkage is much potent in conferring cell killing activity for **FTY15** which possesses a -OCH2- as linkage almost lost all cell viability suppressing effect. Comparison among **FTY2, FTY16, and FTY8** showed one more time that 2-carbon linkage is better than –OCH2 – and methylene group in suppressing cell viability.

It is noteworthy that among all these derivatives, OSU-2, with a propyl group connected to the quaternary carbon, showed the most potent activity with IC50 1.5 μM. Once again, shortening the length of the side chain decreased the activity to a great extent, as demonstrated by compound **FTY2, FTY6** and **FTY8**. To investigate
the effect of stereochemistry on suppressing HCC cell viability, the activity of the optically active isomers of FTY2 was examined. The result showed that the two enantiomers possessed comparable antiproliferative activity. However, the enantiomer with S configuration demonstrated much less immunosuppressive activity than its counterpart. This is consistent with the study that the Pro-(S) hydroxymethyl group is essential for the immunosuppressive activity of FTY720 (40).

In the light of the remarkable role that the fluorinated compounds have played in medicinal chemistry (17, 90, 91, 93-100), 1-trifluoromethylpropyl was asymmetrically introduced to the quaternary carbon in the attempt to achieve an even more potent derivative, leading to FTY14 bearing S configuration. However, FTY14 did not show any improvement in suppressing the growth of Huh7 cells as expected.
Table 1 Two classes of compounds with different length of side chains and their IC$_{50}$ values in inhibiting cell viability for Huh7 and PC-3 cells.
<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>IC 50 for Huh7 (μM)</th>
<th>IC 50 for PC-3 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FTY4</td>
<td>Heptyl</td>
<td>CH₂OH</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>FTY1</td>
<td>Heptyl</td>
<td>Methyl</td>
<td>2</td>
<td>13.2</td>
</tr>
<tr>
<td>8</td>
<td>FTY11</td>
<td>Heptyl</td>
<td>Ethyl</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>FTY2</td>
<td>Heptyl</td>
<td>Propyl</td>
<td>1.5</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>FTY12</td>
<td>Heptyl</td>
<td>Isopropyl</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>FTY13</td>
<td>Heptyl</td>
<td>Butyl</td>
<td>3.8</td>
<td>8.8</td>
</tr>
<tr>
<td>11</td>
<td>FTY14</td>
<td>Heptyl</td>
<td>CH₂CH₂CF₃</td>
<td>1.8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2 Compounds with different substituents at quaternary carbon and their IC₅₀ values in inhibiting cell viability for Huh7 cells and PC 3 cells.
Table 3  Compounds with different linkage between phenyl ring and the quaternary carbon and their IC50 values in inhibiting Huh7 cells and PC-3 cells.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>X</th>
<th>Y</th>
<th>R2</th>
<th>IC 50 for Huh7 (μM)</th>
<th>IC 50 for PC-3 (μM)</th>
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</thead>
<tbody>
<tr>
<td>12</td>
<td>FTY720</td>
<td>CH₂CH₂</td>
<td>CH₂</td>
<td>CH₂OH</td>
<td>3.2</td>
<td>14.1</td>
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<tr>
<td>13</td>
<td>FTY10</td>
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<td>CH₂</td>
<td>CH₂OH</td>
<td>3.5</td>
<td>16</td>
</tr>
<tr>
<td>1</td>
<td>FTY4</td>
<td>CH₂CH₂</td>
<td>O</td>
<td>CH₂OH</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>14</td>
<td>FTY15</td>
<td>OCH₂</td>
<td>O</td>
<td>CH₂OH</td>
<td>&gt;50</td>
<td>N.D.</td>
</tr>
<tr>
<td>4</td>
<td>OSU-2</td>
<td>CH₂CH₂</td>
<td>O</td>
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<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>FTY16</td>
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<td>O</td>
<td>Propyl</td>
<td>&gt;50</td>
<td>N.D.</td>
</tr>
<tr>
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<td>O</td>
<td>Propyl</td>
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<td>N.D.</td>
</tr>
</tbody>
</table>

2.4 Discussion

As a novel immunosuppressive modulator, FTY720 elicits its effect by inhibiting the egress of lymphocytes from secondary lymphoid tissue and thymus. FTY720 is phosphorylated by sphingosine kinase 2 to its biologically active form FTY720-P which can bind to S1P receptors and subsequently regulate lymphocyte migration and trafficking. On the other hand, FTY720 has been shown to induce apoptosis in various human cancer cell lines through several signaling pathway independent of S1P. Mechanistically, the irrelevance of its apoptosis-inducing activity to S1P receptors inhibition provides a rational to develop potent FTY720-derived antitumor agents. In
the light of the concern that the antitumor activity of FTY720 might be compromised
by its original role as a novel immunosuppressant, we carried out lead optimization of
FTY720 to develop potent antitumor agents devoid of immunosuppressive activity.
SAR studies showed that 7-carbon side chain provides optimal activity, as evidenced
by the results that FTY3 and FTY7 demonstrated much weaker potency than FTY4.
This is further validated by the findings that the IC50s are increasing in the order
FTY2, FTY5 and FTY6. Secondly, replacing one of the hydroxymethyl group with an
aliphatic substituent attached to the quaternary carbon increased the potency. For
example, FTY1-2, FTY11-13 showed increased ability to induce cell death relative to
that of FTY4; more importantly, this replacement might attenuate the
immunosuppressive activity for the premise that 1,3 diol amino compound are critical
for conferring the activity. The linker between the aromatic ring and the quaternary
carbon plays certain roles in regulating the antiproliferative activity as well. By
comparing the IC50 of FTY8, FTY16 and FTY2, it is obvious that the 2-carbon linker
is better than the linker with one-carbon in conferring antitumor activity. FTY9, the
precursor of FTY10 with the nitro group not being reduced yet, showed much less
potency compared to its reduced counterpart, which might underscore the necessity of
the presence of an amino group for the reason that the amino group might increase the
potential of drugs to form hydrogen bond(s) with their targets. Finally, the
stereochemistry preference exists with (S)-configuration is preferred.
Among all these derivatives examined, compound OSU-2S represented an optimal
agent with one fold higher potency in killing HCC cancer cells relative to FTY720.
This cytotoxicity stems from its ability to activate PKCδ and subsequent apoptosis, as
evidenced by PARP cleavage. On the other hand, OSU-2S demonstrated much less immunosuppressive activity compared to FTY720(101).

In conclusion, by utilizing FTY720 as a scaffold, OSU-2S was developed and showed increased antitumor activity and decreased immunosuppressive effect. In four HCC cell lines, OSU-2S demonstrated almost one fold higher activity relative to that of FTY720. Consequently, OSU-2S might serve a better candidate for the treatment of hepatocellular carcinoma.

2.5 Discovery of OSU-DY-7 as a potent anticancer drug for blood cells

OSU-DY-7 was synthesized unexpectedly when we were trying to prepare FTY8 during the course of modification on FTY720. Compared to FTY8, OSU-DY-7 is short of a propyl group in structure and is actually a tyrosine derivative. To our surprise, OSU-DY-7 demonstrated strong anticancer activity on HCC cells and most potent activities in leukemia cells among FTY720 derivatives. Further study shows that OSU-DY-7 induces apoptosis in chronic lymphocytic leukemia and lymphoblastic lymphoma through p38 mitogen-activated protein kinase pathway.

The original synthetic route to OSU-DY-7 is pretty much similar to that of FTY8; however, OSU-DY-7 prepared in this way is racemic. Modified synthetic route started with (D)-tyrosine and (L)-tyrosine yielded both enantiomers, as shown in scheme 3. OSU-DY-7, which bearing (R)-configuration, showed more potency than its (S)-enantiomer.
2.5.1 **OSU-DY-7** shows strong anticancer activity in different cancer cells

Initially, the anticancer activity of **OSU-DY-7** was accessed in two of the HCC cell i.e. Heb7 and Huh7 along with several other FTY720 derivatives. As shown in Figure 6, among the derivatives tested, **OSU-DY-7** showed the second most potent antiproliferative activity next to OSU-2S in both Hep3B and Huh7 cells. When tested in blood cell line, **OSU-DY-7** shows the most potent cell killing effect among all the tested derivatives. This may indicate the different mechanisms underlying the anticancer activities of these derivatives in different cancer cell lines. As shown Figure 8(upper pane), the IC₅₀ for **OSU-DY-7** after 24h treatment in Raji cells is about 2 µM, whereas that of **OSU-2S** and FTY720 are 7 µM and 10 µM respectively. The same trend was also observed in MEC-1 cell lines as shown Figure 8(lower panel).
Figure 7. Dose-dependent suppressive effect of FTY8, FTY720, OSU-2S, OSU-DY-7 and FTY14 on the viability of Hep3B and Huh7 cells after 24 h of treatment. Points mean (n = 6).

Figure 8 Dose-dependent suppressive effect of FTY8, FTY720, OSU-2S and OSU-DY-7 on the viability of Raji cells (upper panel) and MEC-1 cells (lower panel) after 24 h of treatment by MTS assay (12h). Columns mean; bars, ±SD (n = 6).
There was a dose-dependent and time-dependent decrease of cell survival for OSU-DY-7 in lymphocytic cell lines and primary B-CLL (Figure 9) cells. Among them, MEC-1 cell line is most sensitive to the OSU-DY-7 induced cytotoxic with IC$_{50}$ less than 2.4 μM and 1.02 μM while neo-697 is the most resistant cell line with IC$_{50}$ around 10.4 μM and 5.21 for 24h and 48h respectively.

![Figure 9 Dose-dependent suppressive effect of OSU-DY-7 on the viability of MEC-1, neo-697, Raji, Ramos and primary B cells after 24 h and 48h of treatment by MTS assay (12h). Columns, mean; bars, ±SD (n = 6).]

2.5.2 Discussion

OSU-DY-7 was synthesized unexpectedly during the course of modification of FTY720 to generate a library of amino alcohol derivatives with quaternary carbon. OSU-DY-7 is much different from the other FTY720 derivatives in that structurally
there is no quaternary carbon to which an amino group and a hydroxymethyl group attaches.

Molecular mechanisms study revealed that OSU-DY-7 induced by activation caspase-3 and PARP cleavage in lymphocytic cells and primary B cells purified from CLL patients by activation MAPK protein p38(102). This mechanism is different from that of FTY720(44) and OSU-2S(101), both of which were found to induce apoptosis in HCC cells by activation PKCδ. Therefore, our findings provided another line of evidence that BIRC5 plays a potential role of in mediating cell apoptosis caused by p38 MAPK activation.

In conclusion, we developed a novel agent, OSU-DY7, which is active for B lymphocytic cell lines representing CLL (MEC-1), acute lymphoblastic leukemia (697 cells), Burkitt lymphoma (Raji and Ramos) and primary B cells from CLL patients. Caspase-3 dependent apoptosis caused by p38 MAPK activation involving down modulation of BIRC5 protein and mRNA reveals OSU-DY-7 as an attractive alternative therapy targeting the p38 MAPK pathway for CLL and other lymphocytic malignancies. Therefore, this study provided useful suggestion for further “on-target” modifications to pursue more effective anticancer agents.
Experimental Section:

Cell Culture. The HCC cell lines were purchased from the following sources: Huh7 (JCRB0403) was obtained from the Health Science Research Resources Bank (Osaka, Japan). Hep3B and PLC5 were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). All cells were cultured at 37°C in a humidified incubator containing 5% CO2. Cells in log-phase growth were harvested by trypsinization for use in various assays and in vivo studies.

Blood from patients with CLL was obtained under a protocol approved by The Ohio State University hospital internal review board. All patients had understood and signed the informed consent in accordance with the Declaration of Helsinki. B-CLL cells were isolated from freshly collected whole blood using Rosette-Sep kit (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions. Human B-lymphocyte cell lines MEC-1, Raji, Ramos, 697 and isolated primary B-CLL cells were incubated using procedures previously described (103). MEC-1 cell line was obtained from the German cell line bank (Braunschweig, Germany) and Raji, 697 and Ramos cell lines were from American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell Viability Assay. Cell viability was assessed by using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in six replicates. In brief, cells were seeded at 4000 cells per well in 96-well flat-bottomed plates; then, 24 to 72 h
later, cells were treated with OSU-A9 and/or SB-203580 at the concentrations indicated in individual figures or with DMSO in serum-free or 5% FBS- supplemented DMEM.

**Chemistry**

Chemical reagents and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Nuclear magnetic resonance spectra (\(^1\)H NMR) were measured on a Bruker DPX 300 model spectrometer. Chemical shifts (\(\delta\)) were reported in parts per million (ppm) relative to the TMS peak. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-Tof II high-resolution electrospray mass spectrometer. Flash column chromatography was performed using silica gel (230-400 mesh).

\[
\begin{align*}
\text{OH} & \quad \text{4-Heptyloxyphenethyl Alcohol (2).} \\
\text{Heptyl bromide (17.9 g, 100 mmol) was added to a solution of 4-hydroxyphenethyl alcohol (13.8 g, 100 mmol) and sodium ethoxide (7.48 g, 110 mmol) in EtOH (400ml). The reaction mixture was refluxed overnight. After evaporating ethanol, the residue was diluted with water and extracted with EtOAc. The extract was washed with 1N NaOH and brine, dried, and evaporated to give 2 (21.0 g, 88%) as yellow oil: }^{1}\text{H NMR (300 MHz, CDCl}_3) \delta 7.13 (d, 2H, } J = 8.7 \text{ Hz), 6.83 (d, 2H, } J = 8.7 \text{ Hz), 3.93 (t, 2H, } J = 6.6 \text{ Hz), 3.82 (t, 2H, } J = 6.3 \text{ Hz), 2.81 (t, 2H, } J = 6.3 \text{ Hz), 1.77 (quint, 2H, } J = 6.6 \text{ Hz), 1.46-1.43 (m, 2H), 1.39-1.24 (m, 6H), 0.89 (t, 3H, } J = 6.9 \text{ Hz).}
\end{align*}
\]
4-Heptyloxyphenethyl Iodide (4). Triphenylphosphine (5.6 g, 21 mmol), imidazole (1.6 g, 24 mmol) and iodine (5.9 g, 23 mmol) were added to the solution of alcohol 49 (4.7 g, 20 mmol) in THF at 0°C. The reaction mixture was then warmed to room temperature and stirring was continued for 2 h. After the reaction was completed, 10% sodium thiosulfate solution was added and the resulting mixture was extracted with ethyl acetate twice. The extracts were combined, washed with brine, dried on sodium sulfate and concentrated. The resulting residue was purified by silica gel chromatograph eluting with hexane to give iodide as colorless oil 4 (6.2 g, 90%):

\[ \text{1H NMR (300 MHz, CDCl}_3\text{)} \delta 7.09 (d, 2H, J = 8.7 \text{ Hz}), 6.84 (d, 2H, J = 8.7 \text{ Hz}), 3.93 (t, 2H, J = 6.6 \text{ Hz}), 3.31 (t, 2H, J = 7.8 \text{ Hz}), 3.11 (t, 2H, J = 7.8 \text{Hz}), 1.77 (quint, 2H, J = 6.9 \text{ Hz}), 1.46-1.24 (m, 8H), 0.89 (t, 3H, J = 6.9 \text{ Hz}); \]

Diethyl 2-Acetamido-2-[2-(4-heptyloxyphenyl)-ethyl]malonate (7). To a solution of NaH (190 mg, 7.9 mmol) in DMF was slowly added Diethyl 2-acetimidomalonate 8 (1.69 g, 7.8 mmol), the resulting mixture was stirred for 30 min to allow the forming of malonate sodium salt. Iodide 7 (900 mg, 2.6 mmol) was then added, and stirring was continued for 1 h at 50-60 °C (bath temperature). Water was added to quench the reaction and the aqueous phase was repeatedly extracted with ethyl acetate. The organic layers were combined,
dried (Na₂SO₄) and concentrated, and the residue was purified on silica gel column (hexanes, then hexanes/EtOAc, 1:1) to give product 7 as a white solid. (927 mg, 82%):

$^1$H NMR (300 MHz, CDCl₃) δ 7.03 (d, 2H, J = 8.4 Hz), 6.86 (d, 3H, J = 8.4 Hz), 4.25 (m, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.63 (t, 2H, J = 7.8 Hz), 2.39 (t, 2H, J = 7.8 Hz), 2.00 (s, 3H), 1.76 (qunint, 2H, J = 6.6 Hz), 1.38-1.20 (m, 14H), 0.89 (t, 3H, J = 6.6 Hz);

2-Amino-2-(4-Heptyloxylphenyl)propane-1,3-diol. A solution of 45 (566 mg, 1.3 mmol) in THF (10 mL) was added dropwise to a suspension of LiAlH₄ (148 mg, 3.9 mmol) in THF (26 mL) at 0 °C. The mixture was stirred at room temperature for 2 h. Saturated Na₂SO₄ (2.5 mL) was then added to quench the reaction. The precipitate was filtered off through Celite and the filtrate was evaporated. The resulting residue was dissolved in pyridine (4 mL) and acetic anhydride (3 mL) and stirred at room temperature for overnight. Water was added to quench the reaction and the resulting mixture was extracted with EtOAc twice. The combined extracts were washed with 1 N HCl, saturated NaHCO₃ and brine, dried over sodium sulfate and concentrated. Purification with Silica gel chromatography gave 2-acetamido-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol diacetate (452mg, 80%) as a white solid. This intermediate (435 mg, 1 mmol) was dissolved in MeOH (5 mL) and 2 N LiOH (4 mL). After refluxing for 2 h, the methanol was removed and the resulting aqueous solution was extracted with EtOAc. The extract was washed with brine, dried, and concentrated. The residue was recrystallized with EtOAc/Hexane to give the white solid FTY4(200 mg, 65%): $^1$H NMR (300 MHz, CDCl₃) δ 7.09 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 3.89 (t, 2H, J = 6.3 Hz), 3.53 (q, 4H, J = 10 Hz), 2.55
(t, 2H, J = 6.9 Hz), 1.77-1.62 (m, 4H), 1.43-1.21 (m, 8H), 0.88 (t, 3H, J = 6.9 Hz); MS (ES) m/z 310 (M^+ + H).

**Diethyl 2-[2-(4-Heptyloxyphenyl)ethyl]-2-propylmalonate (10).** A solution of diethyl propylmalonate (1.8 g, 9 mmol) in DMF (5 mL) was added to a suspension of NaH (0.22 g, 9 mmol) in DMF (10 mL). The mixture was stirred for 30 min at room temperature, 50 (2 g, 5.8 mmol) was then introduced. After being stirred for 3 h at 60°C, the reaction mixture was poured into water, and extracted with EtOAc twice. The extract was washed with brine, dried, and concentrated. The resulting residue was purified by silica gel chromatography with hexanes-EtOAc (9:1) to gave 10 (1.7 g, 70%) as a colorless oil: 1H NMR (300 MHz, CDCl3) δ 7.07 (d, 2H, J = 8.4 Hz), 6.79 (d, 2H, J = 8.4 Hz), 4.19 (q, 4H, J = 7.2 Hz), 3.92 (t, 2H, J = 6.6 Hz), 2.51-2.46 (m, 2H), 2.15-2.10 (m, 2H), 1.94-1.88 (m, 2H), 1.78-1.70 (m, 2H), 1.48-1.26 (m, 16H), 0.98-0.81 (m, 6H).

**2-[2-(4-Heptyloxy-phenyl)-ethyl]-2-methoxycarbonylamino-pentanoic acid ethyl ester (17).** To a solution of KOH (85% pellets, 0.6g, 9.5 mmol) in ethanol was added compound 10 (4g, 9.5 mmol). The mixture was refluxed for overnight. After evaporating ethanol, the residue was acidified with HCl, diluted with water and extracted with EtOAc. The extracts were combined, washed with brine, dried over sodium sulfate and concentrated. The residue
was purified by silica gel chromatography to give the mono ester yellow oil (2.8g, 75% mmol). To a solution of this monoester (2.8, 7.1 mmol) and triethylamine (860mg, 8.5 mmol) in anhydrous THF was added ethyl chloroformate (918mg, 8.5 mmol) at -5 °C. After the mixture was stirred for 30 min at this temperature, a sodium azide (910mg, 14 mmol) solution in water (2ml) was introduced and stirring was continued for 1h at 0°C. Water was then added to quench the reaction and the resulting mixture was extracted with EtOAc. The extract was washed with brine, dried over sodium sulfate and concentrated. To the resulting yellow oil was added benzene and the mixture was refluxed for 2 h. MeOH was then added and refluxing was continued for 7 h. After evaporation, the residue was purified by silica gel chromatography to give 17 (1.2g, 41%) as a yellow oil.

1H NMR (300 MHz, CDCl3) δ 7.01 (d, 2H, J = 8.4 Hz), 6.80 (d, 2H, J = 8.4 Hz), 5.81 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.3 Hz), 3.63 (s, 3H), 2.67-2.57 (m, 2H), 2.35-2.18 (m, 2H), 2.05-1.95 (m, 1H), 1.80-1.62 (m, 3H), 1.46-1.28 (m, 13H), 0.89 (m, 6H);

2-Amino-2-[2-(4-heptyloxy-phenyl)-ethyl]-pentan-1-ol (OSU-2S). LiBH₄ (145 mg, 6.6 mmol) was added slowly to a solution of 63 (1.4 g, 3.32 mmol) in THF (30 mL) and the mixture was refluxed for 3 h. After cooled to 0 °C, the reaction mixture was acidified with 2 N HCl (3 mL), diluted with water, and extracted with EtOAc. The extract was washed with brine, dried, and concentrated. To the resulting residue was added THF (8 mL), MeOH (12 mL), and 5 N KOH (8 mL). The reaction mixture was refluxed for 13 h. The organic solvent was
removed on vacuum and the aqueous mixture was diluted with water followed by extraction with EtOAc. The extract was washed with brine, dried over sodium sulfate, and concentrated. The residue can be recrystallized in EtOAc/Hexane to give 5 as white solid OSU-2S (530mg, 50%):^1\text{H} NMR (300 MHz, CDCl$_3$) $\delta$ 7.09 (d, 2H, $J = 7.5$ Hz), 6.83 (d, 2H, $J = 7.5$ Hz), 3.93 (t, 2H, $J = 6.3$ Hz), 3.36 (s, 2H), 2.55 (t, 2H, $J = 8.4$ Hz), 1.80-1.50(m, 4H), 1.46-1.30 (m, 12H), 0.96-0.86 (m, 6H); MS (ES) $m/z$ 322 (M$^+$+ H).

![Structure of OSU-2S](image)

**4-Heptyloxybenzaldehyde (24).** 4-Heptyloxybenzaldehyde was synthesized as described for compound 2: ^1\text{H} NMR (300 MHz, CDCl$_3$) $\delta$ 9.90 (s, 1H), 7.81 (d, 2H, $J = 8.7$ Hz), 6.95 (d, 2H, $J = 8.7$ Hz), 3.93 (t, 2H, $J = 6.6$ Hz), 1.77 (quint, 2H, $J = 6.6$ Hz), 1.46-1.24 (m, 8H), 0.89 (t, 3H, $J = 6.9$ Hz);

![Structure of 4-Heptyloxybenzaldehyde](image)

**4-(4-Heptyloxy-benzylidene)-2-phenyl-4H-oxazol-5-one (25).** To a solution of 4-Heptyloxy-benzaldehyde (2.20 g, 10 mmol) in 50 ml acetic anhydride was added a mixture of huppuric acid (1.8g ,10 mmol) and sodium acetate (0.8g, 10mmol). The reaction mixture was refluxed for 2 h and cooled down to room temperature. The yellow precipitate was collected by filtration and washed with water and ice-cold alcohol to afford the desired product. (2.7 g, 74%): ^1\text{H} NMR (300 MHz, CDCl$_3$) 8.20-8.16 (m, 4H), 7.63-7.50 (m, 3H), 7.27 (s,1H), 6.95 (d,
2H, \( J = 8.7 \text{ Hz} \), 3.93 (t, 2H, \( J = 6.6 \text{ Hz} \)), 1.78 (quint, 2H, \( J = 6.6 \text{ Hz} \)), 1.43-1.20 (m, 8H), 0.89 (t, 3H, \( J = 6.9 \text{ Hz} \));

4-(4-Heptyloxy-benzyl)-2-phenyl-4H-oxazol-5-one (26). The oxazolone (3.6 g, 10 mmol) was dissolved in 30 ml of ethanol and 20 ml of 8% sodium hydroxide was added. The reaction mixture was refluxed for 30 min, cooled down to room temperature and acidified to pH 1 with 6 N HCl. The precipitate was collected by filtration, washed with water and dried to give a yellow solid. The yellow solid was dissolved in 50 ml of ethanol, to which was added 200mg 10% Pd/C. The reaction mixture was shaken under 50 psi H\(_2\) overnight. Pd/C was filtered off through Celite and ethanol was removed on vacuum to give a white solid. The white solid was suspended in 50 ml of dichloromethane and to the stirred mixture DCC (2g, 10mmol) was added. Stirring was continued for 15 h. The precipitate was filtered off and the filtrate was concentrated to give 26 as colorless oil (2.2g, 60%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) 7.90-7.81 (m, 2H), 7.63-7.50 (m, 3H), 7.09 (d, 2H, \( J = 8.7 \text{ Hz} \)), 6.83(d, 2H, \( J = 8.7 \text{ Hz} \)), 4.32(t, 1H, \( J = 6.6 \text{ Hz} \)), 3.93 (t, 2H, \( J = 6.6 \text{ Hz} \)), 3.19-3.06 (m, 2H ), 1.78 (quint, 2H, \( J = 6.6 \text{ Hz} \)), 1.43-1.20 (m, 8H), 0.89 (t, 3H, \( J = 6.9 \text{ Hz} \)).

4-(4-Heptyloxy-benzyl)-2-phenyl-4-propyl-4H-oxazol-5-one (27). To the solution of 26 (776 mg, 2.1 mmol) in DMF (5 ml) was added 52 mg of sodium hydride under Ar. The mixture was stirred for 30 min and
iodopropane (359 mg, 2.1 mmol) was introduced. The stirring was continued for 3h at
50-60°C. Water was then added to quench to reaction and the resulting mixture was
extracted with EtOAc. The extract was washed with brine, dried and concentrated. The
residue was purified on silica gel chromatography to give 27 as colorless oil (610 mg,
75%): $^1$H NMR (300 MHz, CDCl$_3$) 7.98-7.91 (m, 2H), 7.63-7.50 (m, 3H), 7.19 (d, 2H,
$J = 8.7$ Hz), 6.83(d, 2H, $J = 8.7$ Hz), 3.93 (t, 2H, $J = 6.6$ Hz), 3.17-3.02 (m, 2H ), 1.78-
1.60 (m, 4H), 1.43-1.20 (m, 10H), 0.89 (t, 6H, $J = 6.9$ Hz).

2-Benzoylamino-2-(4-heptyloxy-benzyl)-
pentanoic acid methyl ester. Compound 27 (500mg, 1.25 mmol) was dissolved in a
2% solution of sodium methoxide in methoal (10 ml). The solution was stirred at room
temperature until the starting material had disappeared. The solvent was removed and
the residue was purified by silica gel chromatography to gave the methyl eater
(440mg, 80%): $^1$H NMR (300 MHz, CDCl$_3$) 7.80-7.71 (m, 2H), 7.70-7.27 (m, 3H),
6.93 (d, 2H, $J = 8.7$ Hz), 6.83(d, 2H, $J = 8.7$ Hz), 3.93 (t, 2H, $J = 6.6$ Hz), 3.83(s,3H),
3.17-3.02 (m, 1H ), 2.80-2.71(m, 1H), 2.00-1.83(m, 1H), 1.78-1.60 (m, 2H), 1.43-1.20
(m, 10H), 1.23-1.02(m, 1H), 0.89 (t, 6H, $J = 6.9$ Hz);

2-Amino-2-(4-heptyloxy-benzyl)-pentan-1-ol
(FTY8). 2-Benzoylamino-2-(4-heptyloxy-benzyl)-pentanoic acid methyl ester (440mg,
1 mmol) in THF (3 ml) was slowly added to the suspension of LiAlH₄ in anhydrous THF (10 ml) in ice-bath. The reaction mixture was continued for 2 h at this temperature. Saturated sodium sulfate solution was added to quench the reaction and the white precipitate was filtered off. The filtrate was concentrated and diluted with EtOAc, which was washed with brine, dried and evaporated to give yellow oil. The yellow oil was dissolved in methanol (20 ml). 2N solution of LiOH in water (20 ml) was then added and the mixture was refluxed for overnight. Methanol was removed on vacuum and the aqueous phase was extracted with EtOAc. The extract was washed, dried and concentrate. The residue was purified to give fty8 as white solid (180 mg, 60%): ¹H NMR (300 MHz, CDCl₃) δ 7.18 (d, 2H, J = 8.7 Hz), 6.92(d, 2H, J = 8.7 Hz), 3.95 (t, 2H, J = 6.6 Hz), 3.55-3.53(m,2H), 2.94-2.87(m, 2H), 1.78-1.60 (m, 2H), 1.43-1.20 (m, 12H), 0.89 (t, 6H, J = 6.9 Hz).

![Image of (2,2-dimethyl-5-nitro-1,3-dioxan-5-yl)methanol (28).](image)

(2,2-dimethyl-5-nitro-1,3-dioxan-5-yl)methanol (28). 250 mg (1.25 mmol) of p-toluenesulfonic acid was added to a solution of 10g (66.2 mmol) of 2-hydroxymethyl-2-nitro-1,3-propanediol in 100 ml of anhydrous acetone. The reaction mixture was thoroughly stirred for 12h. Acetone was removed on vacuum and water was added to the resulting residue, which was neutralized with sodium carbonate till pH 7.0. The mixture was then extracted with EtOAc. The extract was washed with brine, dried and concentrated. The residue was purified with silica gel chromatography to give the ketal as white solid. (6.3 g, 50%): ¹H NMR (300 MHz, CDCl₃) δ 4.42 (d, 2H, J=12.6 Hz), 4.10 (d, 2H, J=6.3 Hz), 4.08 (d, 2H, J=12.6 Hz), 1.45 (s, 3H), 1.43 (s, 3H).
2,2-Dimethyl-5-nitro-5-(4-octyl-phenoxymethyl)-[1,3] dioxane (29). A solution of triflic anhydride (6.48 g, 23 mmol) in CH₂Cl₂ (10 mL) was added over a period of 1 h to a solution of ketal 4 (2.9 g, 15 mmol) in CH₂Cl₂ (60 mL) and pyridine (4.7 g, 59.5 mmol) at 0 °C. After the addition was completed, stirring was continued for 1 h at ambient temperature. For workup, the reaction was quenched with aqueous saturated NaHCO₃, the aqueous layer was repeatedly extracted with tert-butyl methyl ether, and the combined organic layers were washed with brine, dried over Na₂SO₄ and evaporated. The residue was dried in vacuum to give pure triflate 5 as a colorless oil. The triflate was then added to the solution of 4-octylphenol (3.1 g, 15 mmol) in DMF (20 ml) and K₂CO₃ (6.2 g, 45 mmol), and the resulting mixture was stirred for 3h at 50-60°C. Water was added to quench the reaction and the aqueous phase was repeatedly extracted with EtOAc. The combined organic layers were dried (Na₂SO₄) and evaporated, and the residue was purified by flash chromatography to give product 29 as white solid (4.5 g, 80%): ¹H NMR (300 MHz, CDCl₃) δ 7.10 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.51 (d, 2H, J = 12.6 Hz), 4.40 (s, 2H), 4.19 (d, 2H, J = 12.6 Hz), 2.55 (t, 2H, J = 6.9 Hz), 1.58-1.56 (m, 2H), 1.46 (s, 3H), 1.44 (s, 3H), 1.30-1.10 (m, 10H), 0.88 (t, 3H, J = 6.9 Hz).
2-Nitro-2-(4-octyl-phenoxymethyl)-propane-1,3-diol (FTY9). PTSA (17 mg, 0.0916 mmol) was added to a solution of 35 (345 mg, 0.916 mmol) in MeOH (15 mL) and the resulting solution was stirred at room temperature for 1 h. The reaction was then quenched with saturated aqueous NaHCO$_3$ (5 mL). The organic solvent was removed on vacuum and the aqueous phase was extracted with EtOAc (3 × 20 mL). The extract was washed with brine, dried over sodium sulfate, filtered, and concentrated. Flash chromatography afforded FTY9 (294 mg, 0.870 mmol, 95%) as a white solid: $^1$H NMR (CDCl$_3$, 300 Hz) $\delta$ 7.11 (d, 2H, $J = 8.4$ Hz), 6.82 (d, 2H, $J=8.4$ Hz), 4.41 (s, 2H), 4.32 (d, 2H, $J = 12.6$ Hz), 4.17 (d, 2H, $J =12.6$ Hz), 2.54 (t, 2H, $J =6.9$ Hz), 1.56-1.54 (m, 2H), 1.27-1.18 (m, 10H), 0.88 (t, 3H, $J =6.9$ Hz); MS (ES) m/z 326 (M$^+$ + Na).

2-Amino-2-(4-octyl-phenoxymethyl)-propane-1,3-diol (FTY10). To the solution of 36 (294 mg, 0.870 mmol) in methanol was added 500 mg of 10% Pd/C. The mixture was shaken under 50 psi H$_2$ at room temperature overnight. For work up, Pd/C was filtered off through Celite and the filtrate was concentrated. The residue was purified on silica gel chromatography to afford 37 (188 mg, 70%): $^1$H NMR (CDCl$_3$, 300 Hz) $\delta$ 7.11 (d, 2H, $J = 8.4$ Hz), 6.84 (d, 2H, $J =8.4$ Hz), 3.90 (s, 2H), 3.65 (s, 4H), 2.54 (t, 2H, $J =7.8$ Hz), 1.57-1.55 (m, 2H), 1.29-1.18 (m, 10H), 0.88 (t, 3H, $J =6.9$ Hz); MS (ES) m/z 310 (M$^+$ + H).
2-(benzyloxymethyl)-2-nitropentan-1-ol (32). To the solution of 2-nitro-2-propylpropane-1,3-diol (1.64 g, 10 mmol) in dry THF (20 ml) was added sodium hydride (245 mg, 10 mmol) portionwise. The mixture was stirred for 30 min at room temperature. Benzyl chloride (1.14 ml, 10 mmol) was then added slowly. The reaction mixture was stirred continuously for overnight. The reaction was quenched with water and extracted with ethyl acetate. The extracts were combined, washed with brine and dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified on column chromatography to afford 32 (1.3 g, 51%): \( \delta \) ppm \(^1\)H NMR (CDCl\(_3\), 300 Hz) \( \delta \) ppm 7.20-7.18 (m, 5H), 4.65 (s, 2H), 4.20-3.95 (m, 2H), 3.90-3.79 (m, 2H), 1.83 (t, 2H, J = 6.9 Hz), 1.33 (m, 2H), 0.96 (t, 3H, J = 6.9 Hz).

2-(benzyloxymethyl)-2-nitropentyl trifluoromethanesulfonate (33). To the solution of compound 32 (1.25 g, 5 mmol) (30 ml) and pyridine (0.5 ml, 6.1 mmol) in dry DCM in ice bath was added slowly trifluoromethanesulfonic anhydride (0.875 ml, 5 mmol). The reaction mixture was stirred for 3 h at room temperature. Without being quenched, the mixture was applied to column chromatography and eluted with ethyl acetate. The solvent was removed to afford the colorless oil 33 (1.5 g, 80%).
To the suspension of potassium carbonate 5g in DMF was added compound 33 (1.3g, 3.3 mmol) and 4-(heptyloxy)phenol (0.70g, 3.3 mmol). The mixture was stirred at 50-60°C for 4h. The reaction was quenched with water, extracted with ethyl acetate. The extracts was washed with brine and dried over sodium sulfate. Purification on column chromatography yielded 34 (950 mg, 65%) : δ ppm ¹H NMR (CDCl₃, 300 Hz)  δ 7.20-7.18 (m, 5H), 6.80 (s, 4H), 4.20-3.95 (m, 4H), 3.90-3.79 (m, 2H), 1.83-1.71 (m, 4H), 1.41-1.20 (m, 10H), 0.96 (t, 6H, J =6.9 Hz);

The mixture of compound 34 (440mg, 1mmol) and 50 mg Pd/C was shaken under 50 psi H₂ at room temperature overnight. Pd/C was filtered off through Celite and the filtrate was concentrated. The residue was purified on silica gel chromatography to afford FTY16: ¹H NMR (CDCl₃, 300 Hz) δ 7.22-7.18 (m, 5H), 6.80 (s, 4H), 4.20-3.95 (m, 4H), 3.90-3.79 (m, 2H), 1.83-1.71 (m, 4H), 1.41-1.20 (m, 10H), 0.96 (t, 6H, J =6.9 Hz);

To a solution of (R)-2, 5-dihydro-3,6-dimethoxy-2-isopropylpyrazine (11.1 g, 60 mmol) in dried THF (50 ml)
was added a solution of n-BuLi in hexane (37.5 ml, 60 mmol) at -78°C. The mixture was stirred for 30 min. A solution of iodide (20 g, 60 mmol) in dried THF (50 ml) was added slowly. The stirring was continued at -78°C for 30 min and at 0°C for 1 h. The reaction was quenched with saturated sodium bicarbonate and the resulting mixture was extracted with EtOAc. The extract was washed with brine, dried and concentrated. The residue was purified by silica gel chromatograph to give monoalkylated adduct (17.4 g, 70%): $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.06 (d, $J = 8.7$ Hz, 2H), 6.80 (d, $J = 8.57$Hz, 2H), 3.94-3.86 (m, 4H), 3.61 (s, 6H), 2.55 (m, 2H), 2.27 (m, 1H), 2.05 (m, 1H), 2.03-1.86 (m, 1H), 1.87-1.69 (m, 2H), 1.54-1.17 (m, 9H), 1.05 (d, $J = 6.87$ Hz, 3H), 0.89 (t, $J = 6.54$ Hz, 3H), 0.70 (d, $J = 6.80$ Hz, 3H)

(2S,5S)-2-(4-(heptyloxy)phenethyl)-5-isopropyl-3,6-dimethoxy-2-propyl-2,5-dihydropyrazine (36). Compound 36 was synthesized as described for compound 35.$^1$H NMR (300 MHz, CDCl$_3$) d ppm 7.04 (d, $J = 8.4$ Hz, 1H), 6.79 (d, $J = 8.4$Hz, 1H), 3.91 (t, $J = 6.44$ Hz, 2H), 3.70 (s, 6H), 2.38 (m, 2H), 2.28-2.14 (m, 1H), 2.13-1.98 (m, 1H), 1.93-1.81 (m, 1H), 1.74-1.70 (m, 2H), 1.49-1.20 (m, 9H), 1.11 (d, $J = 6.83$ Hz, 3H), 0.85 (t, $J = 6.54$ Hz, 6H), 0.69 (d, $J = 6.74$ Hz, 3H).

(S)-methyl 2-(4-(heptyloxy)phenethyl)-2-aminopentanoate Compound 36 (2 g, 4.4 mmol) in MeCN (100 mL) and 2M
trifluoroacetic acid in water (50 mL). The reaction mixture was stirred for 72 h. Saturated NaHCO₃ was added to quench the reaction and the resulting mixture was extracted with EtOAc. The extract was washed with brine, dried and concentrated. Purification by silica gel chromatography gave the desired amino acid methyl ester (0.9 g, 59%): ¹H NMR (300 MHz, CDCl₃) δ ppm 7.09 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 3.91 (t, J = 6.6 Hz, 2H), 3.72 (s, 3H), 2.70-2.58 (m, 1H), 2.44-2.40 (m, 1H), 2.06-2.10 (m, 1H), 1.88-1.60 (m, 3H), 1.46-1.31 (m, 8H), 0.95-0.82 (m, 6H).

(S)-2-(4-(heptyloxy)phenethyl)-2-aminopentan-1-ol (OSU-2S). To a suspension of LiAlH₄ (106 mg, 2.8 mmol) in THF (10 mL) was added the methyl ester (700 mg, 2 mmol) in THF (5 mL). The reaction mixture was stirred for 2 h at room temperature and quenched with saturated Na₂SO₄ solution. The precipitate was filtered off and organic solvent was removed on vacuum. The resulting residue was diluted with water and extracted with EtOAc. The extract was washed with brine, dried and concentrated. Purification by silica chromatography yielded 2 (500 mg, 77%): ¹H NMR (300 MHz, CDCl₃) δ ppm 7.11 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.56 Hz, 2H), 3.93 (t, J = 6.45 Hz, 2H), 3.41 (s, 2H), 2.55 (t, J = 8.71 Hz, 2H), 1.81-1.56 (m, 4H), 1.47-1.32 (m, 12H), 0.96-0.86 (m, 6H); MS (ES) m/z 322 (M⁺ + H).
(S)-benzyl 1-(4-(heptyloxy)phenyl)-3-(hydroxymethyl)hexan-3-ylcarbamate (37). Benzyl chloroformate (0.5 ml, 4 mmol) was added to the solution of FTY2S (321 mg, 1 mmol) in chloroform (50 ml) and saturated sodium bicarbonate (25 ml). After the reaction mixture was stirred for 18 h., the organic layer was separated, washed with brine, dried and concentrated. The residue was purified by silica gel chromatography to give compound 37 (414 mg, 91%). 1H NMR (300 MHz, CDCl3) δ 7.28-7.26 (m, 5H), 7.03 (d, 2H, J = 7.5 Hz), 6.88 (d, 2H, J = 7.5 Hz), 5.01 (s, 2H), 3.93 (t, 2H, J = 6.3 Hz), 3.36 (s, 2H), 2.52 (t, 2H, J = 8.4 Hz), 1.78-1.50 (m, 4H), 1.48-1.30 (m, 12H), 0.99-0.88 (m, 6H);

(S)-tert-butyl 3-((bis(benzyloxy)phosphoryloxy)methyl)-1-(4-(heptyloxy)phenyl)hexan-3-ylcarbamate (38). To a solution of compound 37 (0.22 mmol, 100 mg) and tetrazole (recrystallized from toluene, 0.88 mmol, 62 mg) in anhydrous dichloromethane (2 mL) was added dibenzyl diethylamidophosphite (0.33 mmol, 114 mg) solution in DCM (0.5 mL). The reaction mixture was stirred at room temperature under argon for 1 h. meta-Chloroperoxybenzoic acid (0.66 mmol, 147 mg) was then added and stirring was continued for 30 min. Aqueous saturated NaHCO₃ was added to quenched the reaction and the resulting mixture was extracted with dichloromethane (3 ×10 mL). The extract was washed with brine, dried and concentrated. The residue was purified by
chromatography on silica gel eluting with Et₂O--CH₂Cl₂ (1:1) to yield 38 (80 mg, 50%) as a colorless oil: ¹H NMR (300 MHz, CDCl₃) δ 7.32-7.26 (m, 15H), 6.98 (d, 2H, J = 7.5 Hz), 6.76 (d, 2H, J = 7.5 Hz), 5.01 (s, 2H), 4.99 (s, 4H), 4.13 (s, 2H), 3.93 (t, 2H, J = 6.3 Hz), 2.42 (t, 2H, J = 8.4 Hz), 1.99-1.40 (m, 6H), 1.38-1.05 (m, 10H), 0.90-0.88 (m, 6H).

Compound 38 (80 mg, 0.11 mmol) was dissolved in MeOH (10 mL) and stirred together with Pd/C (10%, 50 mg) for 2 h under an atmosphere of H₂. Pd/C was filtered off through Celite and the filtrate was dried in vacuo to yield the desired phosphate (25 mg, 56%): ¹H NMR (300 MHz, CDCl₃) δ 7.05 (d, 2H, J = 7.5 Hz), 6.88 (d, 2H, J = 7.5 Hz), 3.93-3.92 (m, 4H), 2.52 (m, 2H), 1.99-1.82 (m, 2H), 1.81-1.65 (m, 4H), 1.39-1.18 (m, 10H), 1.00-0.92 (m, 6H); M⁺+Na 424.2214

![4-Pentyloxyphenethyl Alcohol](image_url)

**4-Pentyloxyphenethyl Alcohol:** ¹H NMR (300 MHz, CDCl₃) δ 7.13 (d, 2H, J = 8.7 Hz), 6.82 (d, 2H, J = 8.7 Hz), 3.93 (t, 2H, J = 6.6 Hz), 3.81 (t, 2H, J = 6.3 Hz), 2.81 (t, 2H, J = 6.3 Hz), 1.77 (quint, 2H, J = 6.6 Hz), 1.46-1.43 (m, 2H), 1.39-1.24 (m, 2H), 0.89 (t, 3H, J = 6.9 Hz).

![4-Propyloxyphenethyl Alcohol](image_url)

**4-Propyloxyphenethyl Alcohol:** ¹H NMR (300 MHz, CDCl₃) δ 7.13 (d, 2H, J = 8.7 Hz), 6.83 (d, 2H, J = 8.7 Hz), 3.91 (t, 2H, J = 6.6 Hz), 3.82 (t, 2H, J = 6.3 Hz), 2.81 (t, 2H, J = 6.3 Hz), 1.77 (quint, 2H, J = 6.6 Hz), 0.89 (t, 3H, J = 6.6 Hz).
4-Pentyloxyphenethyl Iodide: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.11 (d, 2H, J = 8.4 Hz), 6.86 (d, 2H, J = 8.4 Hz), 3.94 (t, 2H, J = 6.6 Hz), 3.34 (t, 2H, J = 7.8 Hz), 3.14 (t, 2H, J = 7.8 Hz), 1.77 (quint, 2H, J = 6.6 Hz), 1.48-1.38 (m, 4H), 0.89 (t, 3H, J = 6.6 Hz).

4-Propyloxyphenethyl Iodide: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.11 (d, 2H, J = 8.4 Hz), 6.86 (d, 2H, J = 8.4 Hz), 3.94 (t, 2H, J = 6.6 Hz), 3.34 (t, 2H, J = 7.8 Hz), 3.14 (t, 2H, J = 7.8 Hz), 1.77 (quint, 2H, J = 6.6 Hz), 0.89 (t, 3H, J = 6.6 Hz).

Diethyl 2-Acetamido-2-[2-(4-pentyloxyphenyl)-ethyl]malonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.03 (d, 2H, J = 8.4 Hz), 6.86 (d, 3H, J = 8.4 Hz), 4.25 (m, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.66 (t, 2H, J = 7.8 Hz), 2.42 (t, 2H, J = 7.8 Hz), 2.00 (s, 3H), 1.76 (quint, 2H, J = 6.6 Hz), 1.42-1.22 (m, 10H), 0.89 (t, 3H, J = 6.6 Hz);

Diethyl 2-Acetamido-2-[2-(4-propyloxyphenyl)-ethyl]malonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.06 (d, 2H, J = 8.4 Hz), 6.86 (d, 2H, J = 8.4 Hz), 4.25 (m, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.66 (t, 2H, J = 7.8 Hz), 2.42 (t, 2H, J = 7.8 Hz), 2.00 (s, 3H), 1.76 (quint, 2H, J = 6.6 Hz), 1.42-1.22 (m, 10H), 0.89 (t, 3H, J = 6.6 Hz);
8.4 Hz), 4.25 (m, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.63 (t, 2H, J = 7.8 Hz), 2.39 (t, 2H, J = 7.8 Hz), 2.00 (s, 3H), 1.30-1.25 (m, 6H), 0.89 (t, 3H, J = 6.6 Hz).

2-Acetamido-2-[2-(4-heptyloxyphenyl)ethyl]propane-1,3-diol diacetate: $^1$H NMR (300 MHz, CDCl$_3$) δ7.09 (d, 2H, J = 8.7 Hz), 6.82 (d, 2H, J = 8.7 Hz), 5.65 (s, 1H), 4.33 (s, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.54 (t, 2H, J = 7.8 Hz), 2.17 (t, 2H, J = 7.8 Hz), 2.08 (s, 6H), 1.95 (s, 3H), 1.76 (quint, 2H, J = 6.6 Hz), 1.43-1.30 (m, 8H), 0.89 (t, 3H, J = 6.6 Hz).

2-Acetamido-2-[2-(4-pentyloxyphenyl)ethyl]propane-1,3-diol diacetate: $^1$H NMR (300 MHz, CDCl$_3$) δ7.08 (d, 2H, J = 8.7 Hz), 6.81 (d, 2H, J = 8.7 Hz), 5.64 (s, 1H), 4.33 (s, 4H), 3.91 (t, 2H, J = 6.6 Hz), 2.51 (t, 2H, J = 7.8 Hz), 2.17 (t, 2H, J = 7.8 Hz), 2.08 (s, 6H), 1.95 (s, 3H), 1.76 (quint, 2H, J = 6.6 Hz), 1.43-1.35 (m, 4H), 0.89 (t, 3H, J = 6.6 Hz).

2-Acetamido-2-[2-(4-propyloxyphenyl)ethyl]propane-1,3-diol diacetate: $^1$H NMR (300 MHz, CDCl$_3$) δ7.07 (d, 2H, J = 8.7 Hz), 6.80 (d, 2H, J = 8.7 Hz), 5.70 (s, 1H), 4.31 (s, 4H), 3.91 (t,
2H, J = 6.6 Hz), 2.54 (t, 2H, J = 7.8 Hz), 2.16 (t, 2H, J = 7.8 Hz), 2.08 (s, 6H), 1.95 (s, 3H), C 0.89 (t, 3H, J = 6.6 Hz).

Diethyl 2-[2-(4-pentyloxyphenyl)ethyl]-2-propylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.18 (q, 4H, J = 7.2 Hz), 3.89 (t, 2H, J = 6.6 Hz), 2.45-2.39 (m, 2H), 2.15-2.10 (m, 2H), 1.94-1.88 (m, 2H), 1.78-1.70 (m, 2H), 1.48-1.22 (m, 12H), 0.98-0.81 (m, 6H).

Diethyl 2-[2-(4-proxyloxyphenyl)ethyl]-2-propylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.18 (q, 4H, J = 7.2 Hz), 3.89 (t, 2H, J = 6.6 Hz), 2.45-2.39 (m, 2H), 2.15-2.10 (m, 2H), 1.94-1.88 (m, 2H), 1.76 (quint, 2H, J = 6.6 Hz), 1.26-1.22 (m, 8H), 0.98-0.81 (m, 6H).

Diethyl 2-[2-(4-heptyloxyphenyl)ethyl]-2-methylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.19 (q, 4H, J = 7.2 Hz), 3.91 (t, 2H, J = 6.6 Hz), 2.52-2.46 (m, 2H), 2.17-
2.11 (m, 2H), 1.76 (quint, 2H, J = 6.6 Hz), 1.48(s,3H), 1.48-1.22  (m, 14H), 0.89 (t, 3H,J = 6.6 Hz).

Diethyl 2-[2-(4-heptyloxyphenyl)ethyl]-2-ethylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.18 (q, 4H, J = 7.2 Hz), 3.91 (t, 2H, J = 6.6 Hz), 2.46-2.40 (m, 2H), 2.17-2.11 (m, 2H), 2.05-1.88 (m, 2H), 1.76 (quint, 2H, J = 6.6 Hz), 1.48-1.22  (m, 14H), 0.98-0.81 (m, 6H).

Diethyl 2-[2-(4-heptyloxyphenyl)ethyl]-2-butylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.18 (q, 4H, J = 7.2 Hz), 3.91 (t, 2H, J = 6.6 Hz), 2.46-2.40 (m, 2H), 2.17-2.11 (m, 2H), 1.98-1.82 (m, 2H), 1.76 (quint, 2H, J = 6.6 Hz), 1.48-1.22 (m, 18H), 0.98-0.81 (m, 6H).

Diethyl 2-[2-(4-heptyloxyphenyl)ethyl]-2-isopropylmalonate: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 4.18 (q, 4H, J = 7.2 Hz), 3.90 (t, 2H, J = 6.6 Hz), 2.49 (t, 2H, J = 6.9),
2.39 (q, 1H, J = 6.9 Hz), 2.16 (t, 2H, J = 6.9 Hz), 1.76 (quint, 2H, J = 6.9 Hz), 1.44-1.27 (m, 14H), 1.00 (d, 6H, J = 6.9 Hz), 0.89 (t, 3H, J = 6.9 Hz).

2-[2-(4-Heptyloxy-phenyl)-ethyl]-2-methoxycarbonylamino-pentanoic acid ethyl ester: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.01 (d, 2H, J = 8.4 Hz), 6.80 (d, 2H, J = 8.4 Hz), 5.81 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.3 Hz), 3.63 (s, 3H), 2.67-2.57 (m, 2H), 2.35-2.18 (m, 2H), 2.05-1.95 (m, 1H), 1.80-1.62 (m, 3H), 1.46-1.28 (m, 13H), 0.89 (m, 6H).

2-[2-(4-Pentyloxy-phenyl)-ethyl]-2-methoxycarbonylamino-pentanoic acid ethyl ester: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.00 (d, 2H, J = 8.4 Hz), 6.76 (d, 2H, J = 8.4 Hz), 5.83 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.62 (s, 3H), 2.55-2.46 (m, 2H), 2.23-2.15 (m, 2H), 2.01-1.96 (m, 1H), 1.72-1.62 (m, 3H), 1.42-1.22 (m, 9H), 0.89 - 0.82 (m, 6H).

2-[2-(4-Propoxy-phenyl)-ethyl]-2-methoxycarbonylamino-pentanoic acid ethyl ester: $^1$H NMR (300 MHz, CDCl$_3$) δ 7.06 (d, 2H, J = 8.4 Hz), 6.82 (d, 2H, J = 8.4 Hz), 5.86 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.67 (s, 3H), 2.58-2.50 (m, 2H), 2.30-2.24 (m, 2H), 2.10-2.00 (m, 6H).
1H), 1.81-1.66 (m, 3H), 1.38-1.22 (m, 5H), 1.06 (t, 3H, J=6.6 Hz), 0.89 (t, 3H, J=6.6 Hz).

**Ethyl 4-(4-heptyloxyphenyl)-2-methoxycarbonyl-amino-2-methylbutyrate:** $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.05 (d, 2H, J = 8.4 Hz), 6.80 (d, 2H, J = 8.4 Hz), 5.67 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.63 (s, 3H), 2.59-2.53 (m, 2H), 2.38-2.33 (m, 1H), 2.14-1.06 (m, 1H), 1.76 (quint, 2H, J = 6.6 Hz), 1.61 (S, 3H), 1.45-1.25 (m, 11H), 0.89 (t, 6H, J = 6.6 Hz).

**Ethyl 4-(4-heptyloxyphenyl)-2-methoxycarbonyl-amino-2-ethylbutyrate:** $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.05 (d, 2H, J = 8.4 Hz), 6.83 (d, 2H, J = 8.4 Hz), 5.67 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.67 (s, 3H), 2.50-2.35 (m, 2H), 2.18-2.14 (m, 2H), 2.95-1.99 (m, 1H), 1.80-1.76 (m, 3H), 1.35-1.28 (m, 11H), 0.90-0.81 (m, 6H).

**Ethyl 2-[2-(4-heptyloxyphenyl)ethyl]-2-methoxycarbonylamino-4-methylpentanoate:** $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.05
(d, 2H, J = 8.4 Hz), 6.80 (d, 2H, J = 8.4 Hz), 5.67 (s, 1H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.63 (s, 3H), 2.70 (m, 1H), 2.59-2.53 (m, 2H), 2.14-1.06 (m, 2H), 1.76 (quint, 2H, J = 6.6 Hz), 1.35-1.21 (m, 11H), 0.90-0.81 (m, 9H).

2-[2-(4-Heptyloxy-phenyl)-ethyl]-2-methoxycarbonylamino-hexanoic acid ethyl ester: $^1$H NMR (300 MHz, CDCl3) δ 7.05 (d, 2H, J = 8.4 Hz), 6.83 (d, 2H, J = 8.4 Hz), 5.67 (s, 1H), 4.17 (m, 2H), 4.17 (m, 2H), 3.91 (t, 2H, J = 6.6 Hz), 3.67 (s, 3H), 2.50-2.35 (m, 3H), 2.18-2.14 (m, 2H), 1.80-1.76 (m, 3H ), 1.35-1.28 (m, 15H), 0.90-0.81 (m, 6H).

2-Amino-2-[2-(4-pentyloxy-phenyl)-ethyl]-pentan-1-ol: $^1$H NMR (300 MHz, CDCl3) δ 7.09 (d, 2H, J = 8.1 Hz), 6.83 (d, 2H, J = 8.1 Hz), 3.91 (t, 2H, J = 6.6 Hz), 3.35 (s, 2H), 2.55 (t, 2H, J = 8.4 Hz), 1.76-1.57(m, 4H), 1.46-1.30 (m, 8H), 0.96-0.86 (m, 6H); MS (ES) m/z 294 (M$^+$ + H).
2-Amino-2-[2-(4-propoxy-phenyl)-ethyl]-pentan-1-ol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.08 (d, 2H, \(J = 8.4\) Hz), 6.83 (d, 2H, \(J = 8.4\) Hz), 3.91 (t, 2H, \(J = 6.6\) Hz), 3.38 (s, 2H), 2.55 (t, 2H, \(J = 8.4\) Hz), 1.80-1.30(m, 8H), 0.96-0.86 (m, 6H)

MS (ES) m/z 254 (M\(^+\) + H).

2-Amino-4-(4-heptyloxy-phenyl)-2-methyl-butan-1-ol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 7.09 (2H, d, \(J=8.4\) Hz), 6.83 (2H, d, \(J=8.4\) Hz), 3.90 (2H, t, \(J=6.6\) Hz), 3.39 (2H, m), 2.49 (2H, m), 1.78-1.64 (4H, m), 1.26-1.38 (8H, m), 1.18 (3H, s), 0.86 (3H, t, \(J=6.8\) Hz).

2-Amino-2-ethyl-4-(4-heptyloxy-phenyl)-butan-1-ol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 7.09 (2H, d, \(J=8.4\) Hz), 6.83 (2H, d, \(J=8.4\) Hz), 3.90 (2H, t, \(J=6.6\) Hz), 3.39 (2H, s), 2.59-2.53 (2H, m), 1.78-1.64 (4H, m), 1.38-1.26 (10H, m), 1.01 (3H, t, \(J=6.8\) Hz), 0.86 (3H, t, \(J=6.8\) Hz).

2-Amino-2-[2-(4-heptyloxy-phenyl)-ethyl]-4-methyl-pentan-1-ol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.11 (d, 2H, \(J = 8.4\)Hz), 6.83 (d, 2H, \(J = 8.4\) Hz), 3.89 (t, 2H, \(J = 6.6\) Hz), 3.57-3.52 (m, 2H), 2.53-2.49 (m, 2H), 2.11-
2.08 (m, 1H), 1.75-1.70 (m, 2H), 1.70-1.63 (m, 2H), 1.38-1.26 (m, 8H), 0.92 (d, 6H, J = 6.6 Hz), 0.85 (t, 3H, J = 6.6 Hz).

2-Amino-2-[2-(4-heptyloxy-phenyl)-ethyl]-hexan-1-ol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.09 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 3.92 (t, 2H, J = 6.6 Hz), 3.36 (s, 2H), 2.55-2.51 (m, 2H), 1.79-1.45 (m, 4H), 1.45-1.30 (m, 14H), 0.95 (t, 3H, J = 6.6 Hz), 0.89 (t, 3H, J = 6.6 Hz.).

2-Amino-2-(4-Heptyloxyphenyl)propane-1,3-diol: \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.09 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 3.89 (t, 2H, J = 6.3 Hz), 3.53 (q, 4H, J = 10 Hz), 2.55 (t, 2H, J = 6.9 Hz), 1.77-1.62 (m, 4H), 1.43-1.21 (m, 8H), 0.88 (t, 3H, J = 6.9 Hz).

2-Amino-2-(4-pentyloxyphenyl)propane-1,3-diol: \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.06 (d, 2H, J = 8.4 Hz), 6.81 (d, 2H, J = 8.4 Hz), 3.89 (t, 3H, J = 6.3 Hz), 3.53 (q, 4H, J = 10 Hz), 2.55-2.53 (m, 2H), 1.77-1.62 (m, 4H), 1.43-1.31 (m, 4H), 0.88 (t, 3H, J = 6.8 Hz); MS (ES) \(m/z\) 304 (M\(^+\) + Na).
2-Amino-2-(4-Propyloxylphenyl)propane-1,3-diol: $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.09 (d, 2H, $J = 8.4$ Hz), 6.83 (d, 2H, $J = 8.4$ Hz), 3.89 (t, 3H, $J = 6.9$ Hz), 3.53 (q, 4H, $J = 10$ Hz), 2.52 (t, 2H, $J = 6.9$ Hz), 1.77-1.57 (m, 4H), 0.88 (t, 3H, $J = 7.2$ Hz); MS (ES) $m/z$ 254 (M$^+$ + H).

(R)-methyl 2-(tert-butoxycarbonyl)-3-(4-(heptyloxy)phenyl)propanoate (2). To the solution of compound 1 (3g, 11mmol) and heptyl bromide (1.75ml, 12mmol) in 100ml ethanol was added sodium ethoxide (440 mg, 20 mol). The reaction mixture was refluxed for overnight. Ethanol was removed under reduced pressure. Water was added and the resulting mixture was extracted with ethyl acetate (3×100ml). The extracts were combined, washed with brine, dried over sodium sulfate and concentrated. Purification on column chromatography provided 2 (3.9g, 92%): $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.03 (d, $J = 8.49$ Hz, 2H), 6.81 (d, $J = 8.68$ Hz, 2H), 3.92 (t, $J = 6.57$ Hz, 2H), 3.40 (t, $J = 6.10$ Hz, 1H), 3.01 (s, 2H), 1.76(q, $J = 6.57$ Hz, 2H), 1.42 (s, 9H), 1.35-1.27 (m, 8H), 0.93 (t, $J = 6.28$ Hz, 3H).

(R)-tert-butyl 3-(4-(heptyloxy)phenyl)-1-hydroxypropan-2-ylcarbamate (3) To the solution of compound 2 (2g, 5mmol) in dry THF (50 ml) was added of lithium borohydride (220mg, 10 mmol). The reaction was refluxed for 3h, cooled down to 0°C
and acidified with 2N HCl. The mixture was extracted with ethyl acetate. The extracts were combined, washed with brine, dried over sodium sulfate and concentrated. Purification on column chromatography provided 3 (1.3g, 70%): ¹H NMR (300 MHz, CDCl₃) δ 7.11 (d, J = 8.31 Hz, 2H), 6.83 (d, J = 8.06 Hz, 2H), 3.93 (t, J = 6.45 Hz, 2H), 3.89-3.73 (m, 1H), 3.72-3.45 (m, 2H), 2.77 (d, J = 6.82 Hz, 2H), 1.77 (q, J = 7.26 Hz, 2H), 1.42 (s, 9H), 1.35-1.27 (m, 8H), 0.93 (t, J = 6.28 Hz, 3H).

![Structure of (R)-2-amino-3-(4-(heptyloxy)phenyl)propan-1-ol (OSU-DY-7).](image)

(R)-2-amino-3-(4-(heptyloxy)phenyl)propan-1-ol (OSU-DY-7). The solution of compound 3 (365mg, 1mmol) in 3N HCl solution in methanol (100ml) was stirred for 4h. MeOH was evaporated in vaccuo and to the resulting residue was added saturated sodium carbonate solution (10 ml). The mixture was extracted with ethyl acetate. The extracts were combined, washed with brine, dried over sodium sulfate and concentrated. Purification on column chromatography provided OSU-DY-7: ¹H NMR (300 MHz, CDCl₃) δ 7.08 (d, J = 8.42 Hz, 2H), 6.84 (d, J = 8.39 Hz, 2H), 3.93 (t, J = 6.48 Hz, 2H), 3.69-3.55 (m, 1H), 3.44-3.28 (m, 1H), 3.18-2.97 (m, 1H), 2.80-2.64 (m, 1H), 2.55-2.38 (m, 1H), 1.77 (q, J = 6.89 Hz, 2H), 1.35 (m, 8H), 0.89 (t, J = 6.89 Hz, 3H).
Chapter 3: Design and Synthesis of OSU-03012-Derived PAK1 Inhibitors

3.1 Modification strategy

Due to the accumulating structural knowledge of PAK1 proteins, it might be promising to design ATP-competitive inhibitors for PAK1 instead of allosteric inhibitors. Aspartate-Phenylalanine-Glycine (DFG) is a much conserved motif in kinase ATP binding pocket and phenylalanine is able to flip out of its original position to form DFG-out conformation for some kinases. Designing allosteric inhibitors requires kinases to adopt a DFG-out conformation, which is common for Tyr kinases but not observed for any of the Ser/Thr protein kinases except p38 MAP kinase. Moreover, the DFG-out conformation for PAK1 has never been reported.
Designing PAK1 inhibitors like IPA-3 requires advanced knowledge about how the activation mechanism is targeted, which is not so sufficient at the time. Therefore, our approach was to design ATP competitive inhibitors for PAK1.

Recently, a novel PDK-1 inhibitor OSU-03012 was developed in our lab and was reported (23) to also inhibit PAK1 activity through competition with ATP binding. In two human cancer cell lines, OSU-03012 inhibited PAK1 phosphorylation at lower concentrations than that required to inhibit PDK-1 activity. These findings prompted us to possibly develop PAK1 inhibitors by structural modification of this drug with the goal of eliminating its PDK1 inhibitory activity while maintaining its PAK1-targeted activity.

There are three sites involved in the modifications Figure 10: firstly, we were interested in the how the geometry change affects the activity of the drug by replacing 2-phenanthrene with 3-phenanthrene; Secondly, CF3 was replaced by groups (i.e. hydroxyl group and amine group) which can form hydrogen bonds; finally, glycine residue in OSU03012 was either taken off or replaced by one methylene longer residue beta-alanine. By combination, 17 compounds was synthesized and biologically evaluated for their cell viability suppression activity using MTT assays and PAK1 inhibitory activity using either western bolt assay or kinase assay.

3.2 Chemistry

The synthesis of YM-7-14 started with either 2-acetophenanthrene or 3-acetophenanthrene. They were condensed with diethyl oxalate to form the 1,3-diketone intermediate 3 and 4 respectively. 3 and 4 reacted with 4-
nitrophenylhydrazine hydrochloride to generate pyrazole derivatives 5 and 6, which underwent reduction by hydrazine in the presence of Pd/C to give 7 and 8. 7 and 8 is coupled with Boc-protected glycine or beta-alanine to intermediates 9-12. The following modifications on the ester group went into two branched: one was reducing the ester group to hydroxymethyl group; the other one was transforming the ester group to amide group. After deportation, compounds YM-7-14 were prepared as shown in Scheme 11.

The synthesis of YM-5, 6, 15, 16 were depicted in Scheme 12 and begun with intermediate 5 and 6. 5 and 6 were reduced by lithium aluminum hydride to afford 21 and 22, or reacted with methylamine to give 23 and 24. The nitro groups in these intermediates were reduced by hydrazine with the catalysis of Pd/C to furniture YM5, 6, 15, and 16.

YM-3 and YM-4 were prepared as shown in Scheme 13. 3-Acetonphenatherene was condensed with trifluoroacetic acetate and the produced 1,3-diketone intermediate reacted with 4-nitrophenylhydrazine hydrochloride to produce 26. 26 were reduced by hydrazine likewise and the reduced products were coupled with Boc-glycine or Boc-alanine to provide 28 and 19, which underwent deprotected to give YM-3 and YM-4.

YM-1, YM-2 and YM-15 were prepared in the similar way to that of YM-7, except that benylamine, ethylamine and isopropylamine instead of methylamine were used respectively.
Scheme 11. Synthesis of YM-7-14
Scheme 12. Synthesis of YM-5, 6, 16 and YM-17

(a) Lithium aluminum hydride / THF; ice bath; (b) MeNH₂ / MeOH; (c) Hydrazine, 10% Pd-C.

Scheme 13. Synthesis of YM-3 and YM-4

(a) trifluoroacetic acid, potassium isobutoxide; (b) 4-Nitrophenylhydrazine hydrochloride; (c) Hydrazine, 10% Pd-C; (d) Boc-Gly or Boc-beta Alanine EDC-HCl; (e) HCl/MeOH, NaHCO₃.
3.3 Biological activity

Figure 11. (A) In vitro screening kinase assay analysis performed by Dr. Saji of the PAK1 inhibitory activities of 17 compounds compared with OSU-03012 at 7.5 μM; (B), dose-response suppression of PAK1 activity by compound YM-1, YM-3, YM-4, YM-10, YM-11, YM-15 and OSU-03012 at indicated concentrations.

Figure 12. Quantification of results for YM-4 and YM-15 from dose-response PAK1 kinase assay. Columns, mean; bars, SD (n = 3)
These seventeen derivatives along with OSU-03012, each at 7.5μM were assessed for their ability to inhibit PAK1 activity by in vitro PAK1 kinase assay as a screening assay (Figure 11A). The PAK1 kinase assay was performed using recombinant active PAK1 from cell signaling, MBP as substrate, 2.5 μM ATP and 7.5 μM of respective inhibitors. The result of this initial screen demonstrated that among the compounds tested, six compounds (YM-1, YM-3, YM-4, YM-10, YM-11, YM-15) exhibited apparent similar potencies in inhibiting the PAK1 activity in this screening assay as indicated by the less intensity of the band relative to OSU-031012 and it was noteworthy that YM-15 demonstrated the highest potency in inhibiting PAK1; on the contrary, it is unexpectedly to note that compound YM-12 may stimulate PAK1 activity in this assay as evidenced by the darker band associated with it than that of DMSO control. The mechanism in its mode of action needs investigation.

Figure 13. Lineweaver-Burk plot of the competition of YM-4 and YM-15 with ATP using PAK1 kinase assay. The activity of PAK1 was determined by varying the concentration of ATP (5–40 μM) at different indicated concentrations of YM-4 and YM-15. The plot shown here is representative of three separate determinations.
To further investigate these six compounds, dose-response studies on PAK1 activity were performed by using PAK1 kinase assay. All six compounds exhibited dose-related suppression of PAK1 activity (Figure 11B), and at higher concentration than 5 μM, PAK1 activity was almost completely inhibited by these compounds except OSU-03012. Moreover, compound YM-15 showed the most potent inhibitory activity, consistent with the screen results. Their IC50 for inhibiting PAK1 were quantified as around 1 μM as shown in Figure 12. We next questioned whether YM-4 and YM-15 might inhibit PAK1 in an ATP competitive manner, similar to their parent compound OSU-03012. The subsequent result for kinetic experiments suggests both of them are ATP competitive inhibitors for PAK1 (Figure 13).

Figure 14. Western blot analysis of the effects of the selected compounds vis-à-vis OSU-03012 on the phosphorylation of Akt(Thr308) and Vimentin(Ser56) relative to that of DMSO in 1% FBS-containing medium after 6h of treatment in BCPAP, FTC133 and TPC1 cells.

To assess the cellular potency of these six compounds against PAK1, the phosphorylation level of vimentin, one of the downstream effectors of PAK1 was
examined by western blots using an antibody against the PAK phosphorylation site (Ser56) in three thyroid cancer cell lines. BCPAP, FTC133, TPC1 cells were treated with the six compounds, each at 7.5 μM concentration, and were harvested after six hours of treatment and blotted. The results are shown in Figure 14. It is not surprising that some of the compounds did not demonstrate the same ability to inhibit PAK1 in cells as in vitro kinase assays. This may be due to their physiochemical properties such as solubility and the ability to cross cell membrane varies, resulting in different bioavailability; Nevertheless, the ability of each compounds to reduce vimentin phosphorylation at Ser56 seems cell specific. For example, YM-3 and compound YM-11 considerably decrease vimentin phosphorylation in BCPAP cells and to lesser extent in TCP1 cells, however in FTC133 cells, they did not demonstrate any inhibitory effect at all; compound YM-1 and compound YM-10 in FTC133 cells showed modest activity in inhibiting PAK1, but no effect associated with them was observed in the other two cell lines. On the other hand, it is interesting to note that YM-4 and YM-15 consistently demonstrated relatively potent PAK1 inhibitory activity across the three thyroid cell lines. Specifically, in BCPAP and FTC 133 cells, OSU-03012 is still the best at decreasing the phosphorylation level of Vimentin. This is different from the result of in vitro kinase assay where YM-15 and YM-4 demonstrated more potency than their parent compound OSU-03012. The contradictory could be explained by the fact that OSU-03012 inhibited both PAK1 and PDK1 which directly phosphorylates and activates PAK1 (69) and therefore the reduction of phosphorylated Vimentin could be the result of the combination of these two inhibitions, although there are the other possible targets both YM-4 and YM-15 may have. In addition, the cell line specific inhibition by these compounds may be related
to the genetic alterations in the different cell lines. Specifically, there is a genetic
alteration i.e. BRAF V600E mutation presented in BCPAP cells and TPC1 cells, TCP1
cells also harbor RET/PTC1 rearrangement; FTC133 cells harbors PTEN mutation. To
confirm this premise will require functional studies and evaluations in more cell lines.
On the other hand, we are also very interested in the PDK1 inhibitory activity of the
compounds at cellular level for all of them are derived from a novel PDK1 inhibitor
OSU-03012. PDK1 is able to phosphorylate Akt and Thr 308, therefore, the
phosphorylation level of Akt at Thr 308 was examined to assess the extent to which
the PDK1 inhibitory activity was inhibited from OSU-03012. As shown figure 14, YM-3
did not demonstrated PDK1 kinase activity to phosphorylate Akt at Thr308, while the inhibition of Akt phosphorylation at Thr308 by YM-10 varied with cell type.
For example, in BCPAP cells YM-10 inhibited Akt phosphorylation to the same extent
as OSU03012, while in TPC1 and FTC133 cells; it did not show inhibitory activity at
all. In contrast, YM-1 and YM-11 inhibited Akt phosphorylation at Thr 308 regardless of cell type. And it is notable that in TPC1 cells, the phosphorylation level
of Akt at Thr 308 was even completely inhibited by YM-11.
In contrast, compounds YM-4 and YM-15 inhibited PAK1 but do not apparently
inhibit PKD1. Thus, they appeared to maintain PAK inhibitory activity without PDK1
effect. Therefore, these are two most promising candidate whose biological activity
needs further exploring. On the other hand, in cell based assay, YM-11 selectively
inhibition Akt phosphorylation over PAK1 phosphorylation, although in vitro kinase
assay demonstrated more potent PAK1 inhibitory activity. This may suggest that YM-
11 itself might be a more potent PDK1 inhibitor than PAK1 inhibitor in cells.
Figure 15. Western analysis of the dose-dependent effect of YM-4 and YM-15 on the phosphorylation of Vimentin, Akt and ERK in BCPAP, FTC133 and TCP1 cells after 6h of drug treatment.

To confirm the screen result, we assessed the dose-dependent effect (104) of YM-4 and YM-15 on the phosphorylation of Vimentin and Akt by western blot analysis. As shown in Figure 15, consistent with the result from screening assay, both these two compounds inhibited PAK1 in a dose-dependent manner as demonstrated by the
decreasing level of PAK-phosphorylated vimentin which is a downstream effector of PAK1; Akt phosphorylation reduction was not observed again after 6h of drug treatment. Because of the crucial role that ERK plays in mediating cell apoptosis in cancer cells, it phosphorylated form was also examined. YM-4 and YM-15 exhibited differential effect on inhibiting ERK phosphorylation. As shown, YM-15 inhibited ERK phosphorylation at concentrations higher than 5 μM in all three cell lines, while YM-4 did not demonstrate any inhibitory activity against ERK phosphorylation.

Table 4. The in vitro inhibition of kinases activities (%) by YM-4 and YM-15.

<table>
<thead>
<tr>
<th>Kinases</th>
<th>YM-4 @ 5 μM</th>
<th>YM-15 @ 5 μM</th>
<th>Kinases</th>
<th>YM-4 @ 5 μM</th>
<th>YM-15 @ 5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKI(h)</td>
<td>65</td>
<td>68</td>
<td>PAK2(h)</td>
<td>70</td>
<td>81</td>
</tr>
<tr>
<td>CDK1/cyclinB(h)</td>
<td>83</td>
<td>95</td>
<td>PAK4(h)</td>
<td>101</td>
<td>105</td>
</tr>
<tr>
<td>CHK1(h)</td>
<td>82</td>
<td>89</td>
<td>PAK5(h)</td>
<td>114</td>
<td>104</td>
</tr>
<tr>
<td>c-RAF(h)</td>
<td>104</td>
<td>108</td>
<td>PAK6(h)</td>
<td>81</td>
<td>101</td>
</tr>
<tr>
<td>EGFR(h)</td>
<td>99</td>
<td>100</td>
<td>PKA(h)</td>
<td>110</td>
<td>119</td>
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<td>FAK(h)</td>
<td>87</td>
<td>96</td>
<td>PKBα(h)</td>
<td>103</td>
<td>99</td>
</tr>
<tr>
<td>FGFR2(h)</td>
<td>92</td>
<td>99</td>
<td>PKBβ1(h)</td>
<td>76</td>
<td>84</td>
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<tr>
<td>GSK3α(h)</td>
<td>133</td>
<td>118</td>
<td>PKBγ(h)</td>
<td>33</td>
<td>56</td>
</tr>
<tr>
<td>GSK3β(h)</td>
<td>170</td>
<td>137</td>
<td>PKCa(h)</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>IGF-1R(h), activated</td>
<td>97</td>
<td>83</td>
<td>PKCδ(h)</td>
<td>85</td>
<td>110</td>
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<tr>
<td>JAK2(h)</td>
<td>152</td>
<td>139</td>
<td>Ret(h)</td>
<td>97</td>
<td>76</td>
</tr>
<tr>
<td>LIMK1(h)</td>
<td>130</td>
<td>117</td>
<td>ROCK-I(h)</td>
<td>93</td>
<td>95</td>
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<tr>
<td>MEK1(h)</td>
<td>106</td>
<td>101</td>
<td>ROCK-II(h)</td>
<td>79</td>
<td>96</td>
</tr>
<tr>
<td>Met(h)</td>
<td>89</td>
<td>77</td>
<td>SGK(h)</td>
<td>27</td>
<td>64</td>
</tr>
<tr>
<td>p70S6K(h)</td>
<td>28</td>
<td>38</td>
<td>Src(T341M)(h)</td>
<td>105</td>
<td>94</td>
</tr>
</tbody>
</table>

*a 32 kinases were selected from different kinase superfamily. The values are average of triplicate with typical variations of less than 15%.

To further probe the selectivity of the YM-4 and YM-15, we submitted them to Millipore’s kinase profiler service for screening against other kinases selected based on kinase tree at of 5 μM concentration. Both YM-4 and YM-15 displayed selectivity for the PAK1 family kinase relative to PDK1. Of a panel of 32 kinases tested, YM-4 at
5 μM displayed greater than 50% inhibition against three SGK(h), p70S6K(h) and PKBγ(h); while only one kinase p70S6K(h) was inhibited by greater than 50% by YM-15 at 5 μM (Table 4). YM-4 and YM-15 also demonstrated selectivity over other PAK members. As shown, the kinase activity of PAK3, PAK4 and PAK5 was not inhibited while the activity of PAK2 and PAK6 was only decreased by 30% and 19% respectively; YM-15 only demonstrated inhibition on PAK2 by 19% and did not exhibit and inhibition on the rest PAK members. We did not test our compounds against PAK1, because it is not included in Millipore’s kinase library. In addition, the apparent selectivity of these compounds is based on the limited number of kinases selected.

Table 5. Potencies for inducing cell death in BCPAP, FTC133 and TPC1 cells of the OSU-03012 derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCPAP</td>
</tr>
<tr>
<td>OSU-03012b</td>
<td>2.93±1.05</td>
</tr>
<tr>
<td>YM-1</td>
<td>2.01±0.59</td>
</tr>
<tr>
<td>YM-2</td>
<td>2.67±0.82</td>
</tr>
<tr>
<td>YM-3</td>
<td>2.34±0.27</td>
</tr>
<tr>
<td>YM-4</td>
<td>2.07±0.06</td>
</tr>
<tr>
<td>YM-5</td>
<td>&gt;10</td>
</tr>
<tr>
<td>YM-6</td>
<td>&gt;10</td>
</tr>
<tr>
<td>YM-7</td>
<td>3.71±1.82</td>
</tr>
<tr>
<td>YM-8</td>
<td>2.90±2.35</td>
</tr>
<tr>
<td>YM-9</td>
<td>4.04±1.38</td>
</tr>
<tr>
<td>YM-10</td>
<td>6.53±2.16</td>
</tr>
<tr>
<td>YM-11</td>
<td>4.50±1.04</td>
</tr>
<tr>
<td>YM-12</td>
<td>3.76±1.20</td>
</tr>
<tr>
<td>YM-13</td>
<td>2.76±1.56</td>
</tr>
<tr>
<td>YM-14</td>
<td>3.32±1.09</td>
</tr>
<tr>
<td>YM-15</td>
<td>1.49±0.68</td>
</tr>
<tr>
<td>YM-16</td>
<td>&gt;10</td>
</tr>
<tr>
<td>YM-17</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

The reported IC50 values are concentrations at which BCPAP, FTC133 and TPC1 cell death measures 50% relative to DMSO control after 72-h exposure in 1% FBS-containing RPMI 1640 or DMEM in 96-well plates and are presented as mean ± standard deviation (n=8). The reported IC50 values are concentrations at which BCPAP, FTC133 and TPC1 cell death measures 50% relative to DMSO control after 72-h exposure in 1% FBS-containing RPMI 1640 or DMEM in 96-well plates and are presented as mean ± standard deviation (n=8).
Figure 16. Inhibitory activity of YM-4 and YM-15 on migration of BCPAP, FTC133 and TPC1 cells for 12 h or 24 h. (A) YM-4 and YM-15 inhibited migration of BCPAP cells at 2.5 μM and 5.0 μM concentrations (*, P < 0.05 versus DMSO) after 12 h treatment. (B) YM-4 and YM-15 inhibited migration of TPC1 cells at 5.0 μM concentrations (*, P < 0.05 versus DMSO) after 24 h treatment. (C) YM-4 and YM-15 did not significantly inhibit migration of FTC133 cells at either 2.5 μM or 5.0 μM concentrations after 12 h treatment.

In the light of the important role PAK1 plays in cytoskeleton rearrangement and cell motility, migration assay were carried out to assess the effect of YM-4 and YM-15 on the cell motility of BCPAP, FTC133 and TPC1 cells using Boyden chambers in which motility was induced by serum gradient. As shown in Figure 16 (A), BCPAP cell motility was inhibited by YM-4 after 12 hours of treatment as evidenced by the decreasing migration of 39.1%, 31.6% and 20.3% of control, 2.5 μM and 5.0 μM respectively. YM-15 even demonstrated more potent inhibitory activity on BCPAP cell migration than YM-4 as demonstrated by the lower migration at either 2.5 μM or 5
μM. To a lesser extent, YM-4 and YM-15 also decreased cell motility in TPC1 cells. As shown, the migration of TPC1 cells was significantly inhibited only at 5.0 μM concentration by either YM-4 or YM-15 after 24 hour treatment. However, FTC133 cell motility was not inhibited by YM-4 or YM-15. The susceptibility of BCPAP and TPC1 cells to the compounds in terms of migration inhibition may be correlated to their genetic pathway features, both of which harbor B-RAF V600E mutation to activate both ERK and PAK; in contrast, FTC133 cells does not harbor such mutation but PTEN mutation.

3.4 Discussion
In the light of important roles of PAK1 in cancer and other diseases, this study was aimed at the pharmacological exploitation of OSU-03012 to develop more potent and/or selective PAK1 inhibitors. SAR analysis indicates that there is a conformational geometry effect on their activity to inhibit PAK1. For example, YM-3 demonstrated slightly more potent activity based on in vitro kinase assay than OSU-03012 and in structure they are identical except conformation, suggesting these two isoforms may adapt different binding modes. This premise is suggested by other pairs of isoforms: for example, YM-10 demonstrated more potent activity in inhibiting PAK1 activity while its isoform YM-9 did not show any improvement in PAK inhibitory activity. This may further suggest that the binding mode of OSU-03012 and YM-10 favors an hydrophilic group at site B, while an hydrophobic group is able to increase the potency of the derivatives whose binding mode is similar to that of YM-3 and compound YM-9. Again, this hypothesis is further supported by the phenomenon that the activity is increasing in the order of YM-15 >YM-2>YM-7, all of which beard the 3-phenathere
and therefore adopt the 2nd conformation, paralleling the bulkiness of the groups at B position. In other words, the preference of functional group at B position shows cooperativity with phenanthere conformation.

A bulky group at position C was observed to be associated with higher PAK1 inhibitory potency by comparing the activity of YM-9 vs. YM-11, YM-3 vs. YM-4, YM-8 vs. YM-14 and YM-7 vs. YM-13. The latter compound in each pair bears a beta-Alanine and demonstrated higher potency that its counterpart bearing glycine. However, YM-12 is an exception, which seems to activate PAK1 activity and its mode of action warrant further investigation. Again, YM-5, 6, 16 and 17 without amino acid residue did not show any activity, underscoring the importance of a bulky group at C position regardless of the binding mode.

Among all the derivatives examined, YM-4 and YM-15 represented structurally optimized derivatives, which demonstrated comparable PAK1 inhibitory activity but were abrogated of the PDK1 inhibitory activity. This selectivity might result from the larger bulkiness of YM-4 and YM-15, which prevented them from binding with PDK1; on the other hand, the open ATP-binding site of PAK1 allows for the accommodating of YM-4 and YM-15. This is consistent with the previous report that by enlarging the size of lead compounds led to the achievement of more selective PAK1 inhibitors (83, 84).

We next investigated the inhibitory activity of YM-4 and YM-15 on cell motility. The migration of BCPAP cells and TPC1 cells were inhibited by these two compounds at 5.0 μM after 12 h and 24 h treatment respectively; it is noteworthy that even at 2.5 μM concentration, YM-4 and YM-15 were able to inhibit BCPAP cells motility significantly. However, FTC133 cell motility was not inhibited, indicating a different
mechanism involving in migration signaling pathway in FTC133 cell line than the other two cell lines.

Cell killing activities of YM-4 and YM-15 on BCPAP cells, FTC133 and TPC1 cells were also examined along with the other derivatives (Table 5) and both of them demonstrated lower IC_{50} in suppressing cell viability. In light of the cell viability suppression activity of these compounds, dose-response study of the effect of these two compounds on ERK phosphorylation was performed for its role in mediating cell’s proliferation and apoptosis. Interestingly, YM-15 inhibited phosphor-ERK at higher concentration than 5 μM paralleling its lower IC_{50} in killing cancer cells; while YM-4 did not show such inhibitory activity even at 7.5 μM concentration. Taken together, of the detected biomarkers, ERK, PDK1 and PAK1, YM-4 apparently more specifically inhibit PAK1.

3.5. Conclusion

PAK1 is an emerging therapeutic target for its role in regulating cytoskeleton remodeling and cell motility as well as tumor formation and cell invasiveness in thyroid cancers. Because of certain drawbacks associated with current PAK1 inhibitors, such as moderate potency, less stability and selectivity, it may be useful to develop novel agents for clinical testing. In this study, we have developed new PAK1 inhibitors with unique specificity and perhaps potency versus the parent compound, OSU-03012. The results show that YM-4 and YM-15 were abrogated of the PDK1 inhibitory activity associated with their parent compound OSU-03012, but retained its PAK1 inhibitory activity. Additionally, they also demonstrated comparable cell viability suppression activity. Therefore, in vivo study of these two compounds is
necessary to further evaluate their activities. However, Millipore profiling kinase assay results revealed other kinases these two compounds potentially targets, which necessities further lead modification to pursue more selective PAK1 inhibitors; on the other hand, the role of PAK1 activity in regulating the biological effect of these two compounds also needs investigation.
Experimental Section:

Cell Viability Analysis. The effect of OSU-03012 and its derivatives on cell viability was assessed by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. Eight replicates were performed in each individual experiment, and experiments were repeated at least three separate times. Cells were grown in 96-well plates for 24 h and were exposed to various concentrations of drugs dissolved in DMSO (final concentration 0.1%) in RPMI 1640 or DMEM containing 1% FBS for 72 h. 10 μL of 0.5 mg/ml MTT was added to each well and cells were incubated in the CO2 incubator at 37°C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was solubilized in 100 μL of solution C (12 ml of 1N HCl in 300 mL of isopropanol). Absorbance at 570 nm was determined on a microplate reader.

Kinase assay. Recombinant active PAK 1 (Calbiochem) was incubated with selected derivatives or 0.1% DMSO in the presence or absence of MBP (Sigma) in a 1x kinase buffer (Cell Signaling). The addition of [γ-32P]-ATP (2.5 μCi/reaction) (Perkinelmer, OH) started the reaction, which was incubated at 30°C for 30 min. The reaction was terminated by the addition of EDTA. Results were obtained after visualization of 32P-labeled MBP after separation on 12% polyacrylamide gel electrophoresis and autoradiography. For the Lineweaver-Burk plot analysis, the ratio of radioactive ATP to nonradioactive ATP was not altered. The PAK1 kinase activities are the means of three independent experiments quantified using P81 phosphocellulose paper by a scintillation counter after three washes with 0.75% phosphoric acid.
**Westernbolting.** Cells were washed twice with ice-cold PBS, scraped and resuspended with 500 μl of PBS. After centrifugation at 500 g for 5 min, cells were lysed with M-PER buffer (Fisher Scientific, Pittsburgh, PA) containing 0.3 μM Okadaic acid and 1 μg/ml of aprotinin, pepstatin, leupeptin, and 20 mM of 4-amidino-phenyl methane-sulfonyl fluoride (APMSF). After 10 min incubation with the M-PER buffer on ice, the lysate was centrifuged at 16,000 g for 15 min at 4°C. Supernatant was collected and protein concentrations were measured by BCA protein assay (Fisher Scientific). Twenty-five μg of total lysate were suspended in reducing SDS buffer (Life Technologies Corporation) and boiled for 5 min. The reduced and denaturized lysate was loaded into 4-12% SDS-PAGE, separated by electrophoresis, transferred to nitrocellulose membranes, and immunoblotting was performed. Primary antibodies against Akt(#9272), Phospho-Akt (Thr308) (#2965), p44/42 MAPK (Erk1/2) (#9107), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4377) and GAPDH (#2118) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against Vimentin (V6630) and phospho-Ser55 Vimentin were from Sigma-Aldrich, Inc. (St.Louis, MO) and MBL Co. (Nagoya, Japan), respectively.

**Migration assay.** Cells were grown media containing 10% FBS. After 24 h, the medium was aspirated, and media containing 1% FBS was added for 24 h. The cells were trypsinized for 2 to 5 min, washed, and resuspended in media containing 1% FBS. The cell concentration was calculated by hemocytometer. Four hundred microliters of 1% FBS medium was added into 24-well plates, and a Boyden chamber (8 μm pore) was inserted into each well. Cells were added to each insert, and were
allowed to attach in an incubator at 37°C at 5% CO2 for 2 h. The cells were then exposed to various concentrations of drugs for 3 h. The inserts were switched to a new well containing media containing 10% FBS, and the chamber was incubated for 12 h or 24 h. The cells on and under the Boyden chamber membrane were fixed with 3.7% formaldehyde containing 0.05% crystal violet for 15 min after washing cells with PBS. The chambers were washed with distilled water and the excess water was eliminated. The cells on the top (non-migrated) and bottom (migrated) sides of the membrane were collected by scraping the top and bottom of the chamber with a Q-tip, which was subsequently placed into a 1.5 ml tube. The remainder of the cells remained in the Boyden chamber. The Q-tips containing the scraped cells and the Boyden chamber containing the non-migrated cells were separately incubated in 80% methanol, shaken at 500 g for 30 min, and the extracted dye was measured at 570 nm. Migration was quantified as the ratio of the migrated cells over the total cells (non-migrated plus remaining cells) to calculate migration. Experiments were performed in duplicate on multiple occasions.

Chemistry

Chemical reagents and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Nuclear magnetic resonance spectra (1H NMR) were measured on a Bruker DPX 300 model spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to the TMS peak. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-Tof II high-resolution electrospray mass spectrometer. Flash column chromatography was performed using silica gel (230-400 mesh).
(Z)-Methyl 4-hydroxy-2-oxo-4-(phenanthren-2-yl)but-3-enoate (3). To the mixture of 2-Phenanthere (6.6g, 30mmol) and diethyl oxalate (2ml) in MeOH (100 mL) was added sodium hydride (2.9g, 120 mmol) portion wise within 10 min. The reaction mixture was stirred at room temperature for 4h, cooled down to 0°C and acidified with 2N HCl. The precipitate separated out was collected, washed with cold ethanol and dried to yield yellow crystal 3. (7.3g, 80%). It was used for next step without further purification.¹H NMR (300 MHz, CDCl₃ δ ppm 8.83-8.30 (m, 2H), 8.32-8.29 (m, 1H), 8.07-7.83 (m, 4H), 7.69-7.65 (m, 2H), 3.68 (s, 2H), 3.32 (s, 3H);

Methyl 1-(4-nitrophenyl)-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxylate (5). A suspension of 1, 3-diketone 3 (2.6g, 8.2 mmol) in absolute ethyl alcohol (200ml) was treated with concentrated HCl (1 ml) and 4-nitrophenylhydrazine (1.70g, 0.9mmol). The reaction mixture was left for overnight. The reaction was monitored by TLC. After completion, the precipitate was collected by filtration, washed with ethanol and dried under vacuo to give yellow solid 5.(2.0g, 60%).¹H NMR (300 MHz, CDCl₃ δ ppm 8.65 (d, J = 8.51 Hz, 2H), 8.19 (d, J = 9.01 Hz, 2H), 7.96-7.89 (m, 1H), 7.86 (d, J = 1.39 Hz, 1H), 7.82 (d, J = 8.89 Hz, 1H), 7.68
(t, J = 7.05 Hz, 3H), 7.59 (d, J = 9.02 Hz, 2H), 7.40 (dd, J = 8.62, 1.65 Hz, 1H), 7.21 (s, 1H), 4.51 (q, J = 7.10 Hz, 2H), 1.47 (t, J = 7.12 Hz, 3H);

Methyl 1-(4-aminophenyl)-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxylate

Compound (7). A stirred mixture of compound 5 (0.78g, 1.84 mmol) hydrazine (2 ml, 11 mmol) and Pd/C (60mg) in ethanol (20ml) and THF (20ml) was refluxed for 5h. The reaction mixture was allowed to cool down and filtered through Celite, the filtrate was evaporated to dryness. The solid residue 7 (650 mg, 90%) was collected and used for next step without further purification. 1H NMR (300 MHz, CDCl3 δ ppm 8.59 (dd, J = 14.89, 8.29 Hz, 2H), 7.89 (d, J = 7.36 Hz, 1H), 7.83 (s, 1H), 7.75 (d, J = 8.86 Hz, 1H), 7.72-7.57 (m, 3H), 7.43 (dd, J = 8.58, 1.25 Hz, 1H), 7.23-7.09 (m, 3H), 6.59 (d, J = 8.54 Hz, 2H), 4.48 (q, J = 7.09 Hz, 2H), 1.45 (t, J = 7.09 Hz, 3H)

Methyl 1-(4-(2-(tert-butoxycarbonyl)acetamido)phenyl)-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxylate (9). To solution of compound 7 (200mg, 0.5 mmol) and Boc-Gly (100mg, 0.57 mmol) in dry THF (20 ml) was add EDC (100mg, 0.55 mmol) at once. The
mixture was stirred for overnight under nitrogen. The reaction was monitored by TLC. After stating material disappeared, the reaction was quenched with water, extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate and concentrated. Purification using column chromatography (EA/Hexane=1:2) afforded 9 (178mg, 65%).$^1$H NMR (300 MHz, CD$_3$OD) δ ppm 8.80-8.60 (m, 2H), 7.96-7.84 (m, 2H), 7.83-7.74 (m, 1H), 7.72-7.56 (m, 5H), 7.51-7.40 (m, 1H), 7.39-7.28 (m, 2H), 7.21 (s, 1H), 4.54-4.35 (m, 2H), 3.85 (s, 2H), 1.43 (t, $J = 7.09$ Hz, 12H).

![Chemical structure](image1)

**tert-butyl 2-(4-(3-(hydroxymethyl)-5-phenanthren-2-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate (13).** To a suspension of LiAlH$_4$(300 mg) in dry THF at 0°C was added dropwise a solution of 9 (140mg, 0.25 mmol) in THF(5 ml). The reaction mixture was stirred for 1h and quenched with saturated sodium sulfate solution. The resulting mixture was filtered through Celite and concentrated. The residue was applied to column chromatography to afford 13 (78 mg, 60%). $^1$H NMR (300 MHz, CD$_3$OD) δ ppm 8.69 (d, $J = 8.97$ Hz, 2H), 7.87 (d, $J = 14.21$ Hz, 2H), 7.75 (s, 1H), 7.62 (dd, $J = 11.52$, 5.32 Hz, 5H), 7.54-7.39 (m, 1H), 7.28 (d, $J = 8.45$ Hz, 2H), 6.77 (s, 1H), 4.72 (s, 2H), 3.85 (s, 2H), 1.45 (s, 9H).
tert-Butyl 2-(4-(3-(methylcarbamoyl)-5-(phenanthren-2-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate (17). To a solution of methylamine (33% in methanol) (5mL) was added compound 9 (200mg, 36mmol). The mixture was heated at 100°C in sealed tube for overnight. The reaction was monitored by TCL. After completion, the solvent was removed under reduced pressure to yield desired product 17 (180mg, 91%). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ ppm 8.68 (s, 2H), 7.86 (s, 2H), 7.82-7.72 (m, 1H), 7.72-7.51 (m, 5H), 7.51-7.39 (m, 1H), 7.33 (d, $J = 8.51$ Hz, 2H), 7.11 (s, 1H), 3.79-3.65 (m, 2H), 2.96 (s, 3H), 1.45 (s, 9H)

2-Amino-N-(4-(3-(hydroxymethyl)-5-(phenanthren-2-yl)-1H-pyrazol-1-yl)phenyl)acetamide (YM-10). The mixture of compound 13 (60mg, 0.11mmol) in HCl solution (3N in methanol) (5 mL) was stirred for 3h. 10% sodium carbonate was then added. The stirring was continued for another 3h. The reaction mixture was extracted with ethyl acetate, and the combined organic layers were washed with brine, dried over anhydrous sodium sulfate and concentrated. Purification on column chromatography provide white solid YM-10 (28mg, 60%). $^1$H
NMR (300 MHz, CD$_3$OD δ ppm 10.87-10.80 (m, 1H), 8.87-8.70 (m, 2H),
7.91-7.73 (m, 2H), 7.72-7.55 (m, 5H), 7.45-7.34 (m, 2H), 7.28 (d, J = 8.63 Hz, 2H),
6.76 (s, 1H), 4.54 (s, 2H), 3.88-3.71 (m, 2H); HRMS calcd for C$_{26}$H$_{22}$N$_4$O$_2$ (M+Na)$^+$
455.1640, found (M+Na)$^+$ 455.1647.

1-(4-(2-Aminoacetamido)phenyl)-N-methyl-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxamide (YM-8). YM-8 was prepared from
compound 17 as that of compound 10. $^1$H NMR (300 MHz, CD$_3$OD δ ppm 8.70 (d, J
= 8.48 Hz, 2H), 8.00-7.86 (m, 2H), 7.78 (s, 1H), 7.66 (a, 5H), 7.50-7.42 (m, 1H), 7.38
(d, J = 8.85 Hz, 2H), 7.13 (s, 1H), 3.86 (s, 2H), 2.97 (s, 3H); HRMS calcd for
C$_{27}$H$_{23}$N$_5$O$_2$ (M+Na)$^+$ 472.1749, found (M+Na)$^+$ 472.1731.

Methyl 5-(phenanthren-2-yl)-1-(4-(3-pivalamidopropanamido)phenyl)-1H-pyrazole-3-carboxylate (10).$^1$H NMR (300
MHz, DMSO-$d_6$ δ ppm 8.88-8.69 (m, 2H), 8.11-7.95 (m, 2H), 7.93-7.74 (m, 2H), 7.66
(s, 4H), 7.46-7.35 (m, 1H), 7.30 (s, 2H), 6.93-6.84 (m, 1H), 3.87(s, 3H), 3.14-3.04 (m,
2H), 2.40-2.27 (m, 2H), 1.45 (s, 9H).
3-amino-N-(4-(3-(hydroxymethyl)-5-(phenanthren-2-yl)-1H-pyrazol-1-yl)phenyl)propanamid (14). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 8.86-8.67 (m, 2H), 7.99 (d, $J = 0.98$ Hz, 2H), 7.92-7.73 (m, 2H), 7.59 (d, $J = 8.58$ Hz, 4H), 7.45-7.31 (m, 1H), 7.22 (d, $J = 8.46$ Hz, 2H), 6.74 (s, 1H), 4.55 (s, 2H), 3.25-3.13 (m, 3H), 2.45-2.40 (m, 2H), 1.35 (s, 9H)

3-Amino-N-(4-(3-(hydroxymethyl)-5-(phenanthren-2-yl)-1H-pyrazol-1-yl)phenyl)propanamide hydrochloride (YM-12). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 8.88-8.70 (m, 2H), 7.98 (s, 2H), 7.92-7.73 (m, 2H), 7.61 (d, $J = 8.49$ Hz, 4H), 7.45-7.33 (m, 1H), 7.26 (s, 2H), 6.75 (s, 1H), 4.54 (s, 2H), 3.13-2.98 (m, 2H), 2.71 (s, 2H); HRMS calcd for C$_{27}$H$_{24}$N$_4$O$_2$ (M+Na)$^+$ 459.1797, found (M+Na)$^+$ 459.1794.
N-Methyl-5-(phenanthren-2-yl)-1-(4-(3-pivalamidopropanamido)phenyl)-1H-pyrazole-3-carboxamide (18). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ ppm 8.68 (dd, $J = 12.09, 4.97$ Hz, 2H), 8.00-7.83 (m, 2H), 7.77 (d, $J = 8.94$ Hz, 1H), 7.70-7.55 (m, 5H), 7.44 (dd, $J = 8.67, 1.94$ Hz, 1H), 7.37-7.26 (m, 2H), 7.11 (d, $J = 2.18$ Hz, 1H), 3.41-3.34 (m, 2H), 2.97 (s, 3H), 2.58-2.51 (m, 2H), 1.40 (s, 9H)

1-(4-(3-aminopropanamido)phenyl)-N-methyl-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxamide (YM-14). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 10.47 (s, 1H), 8.77 (s, 2H), 8.03 (s, 2H), 7.92-7.73 (m, 2H), 7.67 (d, $J = 8.42$ Hz, 4H), 7.35 (d, $J = 8.42$ Hz, 3H), 7.15 (s, 1H), 3.06 -3.00 (m, 2H), 2.79 (d, $J = 4.30$ Hz, 3H), 2.74 (d, $J = 6.32$ Hz, 2H); HRMS calcd for C$_{28}$H$_{25}$N$_5$O$_2$ (M+H)$^+$ 464.2087, found (M+H)$^+$ 404.2087.
1-(4-Aminophenyl)-N-methyl-5-(phenanthren-2-yl)-1H-pyrazole-3-carboxamide (YM-5). $^1$H NMR (300 MHz, DMSO $\delta$ ppm 8.78 (t, J = 7.80 Hz, 2H), 8.12-7.95 (m, 2H), 7.82 (dd, J = 31.22, 8.91 Hz, 2H), 7.74-7.60 (m, 2H), 7.50-7.35 (m, 3H), 7.25 (d, J = 8.20 Hz, 2H), 7.17 (s, 1H), 2.79 (d, J = 4.49 Hz, 3H); HRMS calcd for C$_{25}$H$_{22}$N$_4$O (M+H)$^+$ 415.1535, found (M+H)$^+$ 415.1527.

(1-(4-Aminophenyl)-5-(phenanthren-2-yl)-1H-pyrazol-3-yl)methanol (YM-6). $^1$H NMR (300 MHz, DMSO-d$_6$ $\delta$ ppm 8.93-8.65 (m, 2H), 8.00 (s, 2H), 7.94-7.75 (m, 2H), 7.73-7.60 (m, 2H), 7.35 (d, J = 8.64 Hz, 5H), 6.78 (s, 1H), 4.55 (s, 2H); HRMS calcd for C$_{24}$H$_{21}$N$_3$O (M+H)$^+$ 388.1426, found (M+H)$^+$ 388.1427.

Methyl 1-(4-aminophenyl)-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxylate (8). $^1$H NMR (300 MHz, CDCl$_3$ $\delta$ ppm 8.62 (s, 1H), 8.48-8.39 (m, 1H), 7.96-7.86 (m,
Methyl 1-(4-(2-(tert-butoxycarbonyl)acetamido)phenyl)-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxylate (11). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ ppm 8.62 (s, 1H), 8.48-8.38 (m, 1H), 7.91-7.85 (m, 1H), 7.82-7.74 (m, 2H), 7.70 (s, 1H), 7.68-7.66 (m, 2H), 7.51 (d, $J = 8.91$ Hz, 2H), 7.33 (d, $J = 8.96$ Hz, 3H), 7.22 (s, 1H), 3.90 (d, $J = 5.89$ Hz, 2H), 4.00 (s, 3H), 3.79 (s, 2H), 1.47 (s, 9H).

tert-Butyl 2-(4-(3-(hydroxymethyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate (15). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ ppm 8.68-8.56 (m, 1H), 8.49-8.36 (m, 1H), 7.99-7.84 (m, 2H), 7.83-7.73 (m, 2H), 7.67 (d, $J = 3.34$ Hz, 4H), 7.57-7.48 (m, 1H), 7.45-7.32 (m, 2H), 6.88 (s, 1H), 4.78 (s, 2H), 3.79 (s, 2H), 1.47 (s, 9H).
2-Amino-N-(4-(3-(hydroxymethyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenyl)acetamide (YM-9). \(^1\)H NMR (300 MHz, DMSO-\(d_6\) \(\delta\) ppm 8.77-8.66 (m, 1H), 8.64-8.54 (m, 1H), 8.03-7.75 (m, 4H), 7.71-7.55 (m, 4H), 7.44-7.34 (m, 1H), 7.34-7.27 (m, 2H), 6.89-6.79 (m, 1H), 4.56 (s, 2H), 3.79 (s, 2H); HRMS calcd for C\(_{26}\)H\(_{22}\)N\(_4\)O\(_2\) (M+H)+ 445.1640, found (M+H)+ 445.1647.

tert-butyl 2-(4-(3-(methylcarbamoyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate (19). \(^1\)H NMR (300 MHz, CD\(_3\)OD \(\delta\) ppm 8.61 (s, 1H), 8.47-8.34 (m, 1H), 7.95-7.84 (m, 2H), 7.77 (d, \(J = 9.02\) Hz, 2H), 7.69-7.59 (m, 4H), 7.52-7.44 (m, 1H), 7.37 (d, \(J = 8.59\) Hz, 2H), 7.17 (s, 1H), 3.84 (s, 2H), 2.79 (s, 3H), 1.46 (s, 9H).
1-(4-(2-aminoacetamido)phenyl)-N-methyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (YM-7). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 11.15-10.83 (m, 1H), 8.78 (s, 1H), 8.69-8.58 (m, 1H), 8.07-7.76 (m, 4H), 7.76-7.59 (m, 4H), 7.42 (d, $J = 7.20$ Hz, 3H), 7.26 (s, 1H), 3.84 (s, 2H), 2.79 (s, 3H); HRMS calcd for C$_{27}$H$_{23}$N$_5$O$_2$ (M+H)$^+$ 472.1749, found (M+H)$^+$ 472.1769.

Methyl 5-(phenanthren-3-yl)-1-(4-(3-pivalamidopropanamido)phenyl)-1H-pyrazole-3-carboxylate (12). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ ppm 8.62 (s, 1H), 8.53-8.38 (m, 1H), 7.99-7.84 (m, 1H), 7.81-7.74 (m, 2H), 7.70 (s, 1H), 7.66-7.60 (m, 2H), 7.56 (d, $J = 8.72$ Hz, 2H), 7.34 (d, $J = 8.73$ Hz, 3H), 7.22 (s, 1H), 4.00 (s, 3H), 3.47 (d, $J = 5.73$ Hz, 2H), 2.66-2.52 (m, 2H), 1.39 (s, 9H).
tert-butyl (3-((4-(3-(hydroxymethyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenyl)amino)-3-oxopropyl)carbamate (16). $^1$H NMR (300 MHz, CD$_3$OD $\delta$ ppm 8.66-8.51 (m, 1H), 8.46-8.33 (m, 1H), 7.95-7.68 (m, 4H), 7.75-7.60 (m, 4H), 7.53-7.45 (m, 1H), 7.35-7.24 (m, 2H), 6.90-6.80 (m, 1H), 4.74 (s, 2H), 3.44-3.33 (m, 2H), 2.62-2.47 (m, 2H), 1.38 (s, 9H).

3-Amino-N-(4-(3-(hydroxymethyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenyl)propanamide (YM-11). $^1$H NMR (300 MHz, DMSO-$d_6$ $\delta$ ppm 8.86-8.69 (m, 1H), 8.66-8.47 (m, 1H), 7.92 (s, 4H), 7.75-7.58 (m, 4H), 7.53-7.33 (m, 1H), 7.29-7.15 (m, 2H), 6.83 (s, 1H), 4.55 (s, 2H), 3.12-2.98 (m, 2H), 2.86-2.63 (m, 2H); HRMS calcd for C$_{27}$H$_{24}$N$_4$O$_2$ (M+H)$^+$ 437.1978, found (M+H)$^+$ 437.1956.
N-Methyl-5-(phenanthren-3-yl)-1-(4-(3-pivalamidopropanamido)phenyl)-1H-pyrazole-3-carboxamide (20).

$^1$H NMR (300 MHz, CD$_3$OD $\delta$ ppm 8.71-8.60 (m, 1H), 8.52-8.38 (m, 1H), 8.06-7.86 (m, 2H), 7.87-7.74 (m, 2H), 7.68 (s, 4H), 7.56-7.46 (m, 1H), 7.46-7.33 (m, 2H), 7.24-7.12 (m, 1H), 3.48-3.39 (m, 2H), 3.03 (s, 3H), 2.64-2.49 (m, 2H), 1.44 (s, 9H).

1-(4-(3-Aminopropanamido)phenyl)-N-methyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (YM-13).

$^1$H NMR (300 MHz, DMSO-$d_6$ $\delta$ ppm 8.84-8.72 (m, 1H), 8.69-8.58 (m, 1H), 7.95-7.75 (m, 4H), 7.67 (s, 4H), 7.38-7.30 (m, 3H), 7.25 (s, 1H), 3.10-2.95 (m, 2H), 2.76 (s, 3H), 2.76-2.71 (m, 2H); HRMS calcd for C$_{28}$H$_{25}$N$_5$O$_2$ (M+Na)$^+$ 486.1906, found 486.1922.

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(1-(4-Aminophenyl)-5-(phenanthren-3-yl)-1H-pyrazol-3-yl)methanol (YM-16). ¹H NMR (300 MHz, DMSO-d₆ δ ppm 8.72-8.61 (m, 1H), 8.58-8.48 (m, 1H), 8.00-7.94 (m, 1H), 7.92-7.87 (m, 1H), 7.87-7.75 (m, 2H), 7.69-7.61 (m, 2H), 7.46-7.41 (m, 1H), 7.00-6.93 (m, 2H), 6.56-6.48 (m, 2H), 6.80-6.75 (m, 1H), 4.58-4.44 (m, 2H); HRMS calcd for C₂₄H₂₁N₃O (M+Na)+ 388.1426, found 388.1428.

1-(4-Aminophenyl)-N-methyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (YM-17). ¹H NMR (300 MHz, DMSO-d₆ δ ppm 8.70-8.63 (m, 1H), 8.58-8.48 (m, 1H), 8.00-7.93 (m, 1H), 7.93-7.87 (m, 1H), 7.87-7.75 (m, 2H), 7.69-7.59 (m, 2H), 7.48-7.42 (m, 1H), 7.19-7.15 (m, 1H), 7.07-6.99 (m, 2H), 6.60-6.51 (m, 2H), 2.82-2.74 (m, 3H); HRMS calcd for C₂₅H₂₂N₄O (M+Na)+ 415.1535, found 415.1550.
(Z)-1,1,1-trifluoro-4-hydroxy-4-(phenanthren-3-yl)but-3-en-2-one (25). \(^1\)H NMR (300 MHz, CD\(_3\)OD) δ ppm 9.51-9.47 (m, 1H), 8.99-8.89 (m, 1H), 8.27-8.18 (m, 1H), 8.09 (s, 1H), 7.97 (d, \(J = 9.12\) Hz, 2H), 7.87 (s, 1H), 7.82-7.74 (m, 1H), 7.75-7.65 (m, 1H), 7.14-7.06 (m, 1H)

1-(4-nitrophenyl)-5-(phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazole (26). \(^1\)H NMR (300 MHz, DMSO-d\(_6\)) δ ppm 8.97 (d, \(J = 0.61\) Hz, 1H), 8.83-8.66 (m, 1H), 8.28 (d, \(J = 8.87\) Hz, 2H), 8.04-7.95 (m, 2H), 7.89 (d, \(J = 13.45\) Hz, 2H), 7.69 (d, \(J = 8.41\) Hz, 4H), 7.53 (s, 1H), 7.48-7.39 (m, 1H)

4-(5-(Phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)benzenamine (27). \(^1\)H NMR (300 MHz, DMSO-d\(_6\)) δ ppm 8.79 (s, 1H), 8.70-8.57 (m, 1H), 7.89 7.75 (m, 4H), 7.73-7.59 (m, 2H), 7.47 (s, 1H), 7.38 (s, 1H), 7.06 (d, \(J = 8.52\) Hz, 2H), 6.55 (d, \(J = 8.56\) Hz, 2H).
tert-butyl 2-oxo-2-(4-(5-(phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)phenylamino)ethylcarbamate (28). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 8.84 (s, 1H), 8.75-8.65 (m, 1H), 8.07-7.74 (m, 4H), 7.70-7.60 (m, 4H), 7.46-7.26 (m, 4H), 3.86-3.69 (m, 2H), 1.34 (s, 9H)

tert-butyl 3-(2-oxo-2-(4-(5-(phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)phenylamino)ethylamino)propanoate (29). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 8.84 (s, 1H), 8.73-8.60 (m, 1H), 8.07-7.74 (m, 4H), 7.70-7.60 (m, 4H), 7.46-7.26 (m, 4H), 3.19 (d, $J = 6.37$ Hz, 2H), 2.46 (t, $J = 6.37$ Hz, 2H), 1.34 (s, 9H)
2-Amino-N-(4-(5-(phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)phenyl)acetamide (YM-3). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 11.02-10.80 (m, 1H), 8.90-8.80 (m, 1H), 8.70-8.59 (m, 1H), 8.06-7.75 (m, 4H), 7.67-7.60 (m, 4H), 7.44 -7.25(m, 4H), 3.86-3.69 (m, 2H); HRMS calcd for C$_{33}$H$_{27}$N$_5$O$_2$ (M+Na)$^+$ 483.1409, found 483.1404.

3-amino-N-(4-(5-(phenanthren-3-yl)-3-(trifluoromethyl)-1H-pyrazol-1-yl)phenyl)propanamide (YM-4). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 10.66-10.47 (m, 1H), 8.92-8.80 (m, 1H), 8.77-8.58 (m, 1H), 7.96-7.78 (m, 4H), 7.69-7.60 (m, 4H), 7.51-7.31 (m, 4H), 3.16-2.96 (m, 2H), 2.74 (s, 2H); HRMS calcd for C$_{33}$H$_{27}$N$_5$O$_2$ (M+Na)$^+$ 497.1565, found 497.1564.
tert-butyl 2-(4-(3-(benzylcarbamoyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate. $^1$H NMR (300 MHz, DMSO-$d_6$ $\delta$ ppm 8.68-8.56 (m, 1H), 8.48-8.35 (m, 1H), 7.99-7.85 (m, 2H), 7.84-7.69 (m, 2H), 7.69-7.56 (m, 4H), 7.53-7.25 (m, 8H), 7.24-7.19 (m, 1H), 4.68-4.55 (m, 2H), 3.86-3.75 (m, 2H), 1.47 (s, 9H)

1-(4-(2-Aminoacetamido)phenyl)-N-benzyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (YM-1). $^1$H NMR (300 MHz, DMSO-$d_6$ $\delta$ ppm 11.07-10.75 (m, 1H), 9.01-8.88 (m, 1H), 8.85-8.75 (m, 1H), 8.72-8.58 (m, 1H), 8.07-7.76 (m, 4H), 7.76-7.60 (m, 3H), 7.49-7.28 (m, 9H), 4.58 (s, 2H), 3.91 (s, 2H); HRMS calcd for C$_{33}$H$_{27}$N$_5$O$_2$ (M+Na)$^+$ 548.2062, found 548.2081.
**tert-butyl 2-(4-(3-(ethylcarbamoyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate.** $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 8.67-8.58 (m, 1H), 8.50-8.37 (m, 1H), 7.97-7.85 (m, 2H), 7.85-7.72 (m, 2H), 7.70-7.57 (m, 4H), 7.55-7.47 (m, 1H), 7.44-7.35 (m, 2H), 7.23-7.15 (m, 1H), 3.91-3.81 (m, 2H), 3.53-3.43 (m, 2H), 1.47 (s, 9H), 1.27 (t, $J = 7.15$ Hz, 3H)

**1-(4-(2-aminoacetamido)phenyl)-N-ethyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (2).** $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 10.97-10.77 (m, 1H), 8.83-8.72 (m, 1H), 8.69-8.57 (m, 1H), 8.44-8.30 (m, 1H), 8.03-7.76 (m, 4H), 7.66-7.60 (m, 3H), 7.42-7.35 (m, 3H), 7.30 (s, 1H), 3.85-3.70 (m, 2H), 3.53-3.43 (m, 2H), 1.13 (t, $J = 7.21$ Hz, 3H); HRMS calcd for C$_{33}$H$_{27}$N$_5$O$_2$ (M+Na)$^+$ 486.1906, found 486.1924.
tert-butyl 2-(4-(3-(isopropylcarbamoyl)-5-(phenanthren-3-yl)-1H-pyrazol-1-yl)phenylamino)-2-oxoethylcarbamate. $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 8.68-8.58 (m, 1H), 8.49-8.36 (m, 1H), 7.99-7.86 (m, 2H), 7.86-7.72 (m, 2H), 7.72-7.56 (m, 4H), 7.55-7.47 (m, 1H), 7.44-7.35 (m, 2H), 7.33-7.28 (m, 1H), 4.01-3.96 (m, 1H), 3.88-3.79 (m, 2H), 1.47 (s, 9H), 1.19 (d, $J = 6.07$ Hz, 6H)

1-(4-(2-Aminoacetamido)phenyl)-N-isopropyl-5-(phenanthren-3-yl)-1H-pyrazole-3-carboxamide (15). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ ppm 10.94-10.60 (m, 1H), 8.83-8.69 (m, 1H), 8.67-8.52 (m, 1H), 8.06-7.75 (m, 5H), 7.66 (dd, $J = 4.69$, 2.94 Hz, 3H), 7.43-7.40 (m, 3H), 7.30-7.23 (m, 1H), 4.21-4.07 (m, 1H), 3.83-3.70 (m, 2H), 1.19 (d, $J = 6.07$ Hz, 6H); HRMS calcd for C$_{33}$H$_{27}$N$_5$O$_2$ (M+Na)$^+$ 500.2026, found 500.2087.
Chapter 4: Conclusion and Future Work

4.1. Conclusion

Cancer is the second leading cause of death in the United States among all kinds of disease. Therefore it is urgent to develop anticancer agents for various types of cancers. This dissertation describes the findings of four anticancer agents OSU-2S, OSU-DY-7, YM-4 and YM-15. The first two agents were derived from a novel immunosuppressant FTY720 and the second two are analogues of a novel PKD1 inhibitor OSU-03012. They all demonstrated improved activity comparing to their parent compounds.

FTY720 is an immunosuppressant and elicits its ability through causing T lymphocyte homing by targeting on sphingosine-1-phosphate receptors. It also demonstrates potent anticancer activity by inducing cancer cell undergoing apoptosis in an S1P1 dependent manner. From mechanistic perspective the dissociation of these two mechanisms provides a molecular basis to derive FTY720 toward a novel class of anticancer agent devoid of immunosuppressive activity.

Among the derivatives of FTY720, OSU-2S exhibited higher in vitro anti-proliferative efficacy relative to FTY720 against HCC cells without cytotoxicity in normal hepatocytes. And more importantly, it was abrogated of the immunosuppressive activity. In structure, OSU-2S was prepared by replacing the hydroxymethyl group at pro-(S) position with a propyl group. This replacement prevents OSU-2S from being phosphorylated and therefore abrogated its ability to bind with sphingosine-1-
phosphate receptors. This finding was consistent with the premise that the hydroxymethyl group at pro-(S) is essential for the immunosuppressive activity of FTY720. On the other hand, the increased ability of OSU-2S to kill cancer cell were attributable to sphingosine kinase 2 mediated phosphorylation of FTY720, which represents a metabolic inactivation of its antitumor activity. Mechanistic study demonstrated that OSU-2S induces apoptosis in HCC cells, in part, through the reactive species oxygen (ROS)-dependent activation of PKCδ. Take together, these findings suggest that OSU-2S may have therapeutic application in clinic for treatment of hepatocellular carcinoma and warrants further investigation. Additionally, the ability of OSU-2S to activate PKCδ is noteworthy in that it could be used as pharmacologic tools to further identify the functions of PKCδ.

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the western world, with an incidence of around 3.5 cases per 100 000 people per year. OSU-DY-7 was the other derivative of FTY720 which exhibits unique ability to activate p38 MAPK in a variety of lymphoid malignancies including primary B CLL cells. Further studies demonstrate that OSU-DY7 induces cell death in lymphocytic cell Lines and primary B-CLL cells, in part, via p38 MAPK dependent down-regulation of BIRC5 expression. Structurally, compared to FTY720 and OSU-2S, OSU-DY-7 does not possess a quaternary carbon and this may explain its unique signaling pathway target from FTY720 and OSU-2S; additionally, this unique structure may attenuate its immunosuppressive activity although we have not confirmed that. On the other hand, short of quaternary carbon make it metabolically unstable, which may become a big challenge for its delivery and studies to increase its bioavailability is undergoing.
The second part of the dissertation has described the development of PAK1 inhibitors by modification of OSU-03012. PAK1 plays an important role in cell motility and metastasis. It is also involved in cell survival, proliferation and cell apoptosis. Therefore it has become an attractive therapeutic target for treatment of cancer. For the certain drawbacks associated with current PAK1 inhibitors such as selectivity and stability, we carried out lead optimization of OSU-03012 to develop more potent or specific PAK1 inhibitors. OSU-03012 is a novel PDK1 inhibitor and also demonstrated PAK1 inhibitory activity as well. Our aim in this project is to abrogate its PDK1 inhibitory activity but retain or even strengthen the potency to inhibit PAK1 through modification of OSU-03012. Among all the derivatives of OSU-03012, YM-4 and YM-15 are the two such analogues, which demonstrated comparable PAK1 inhibitory activities as OSU-03012 while were devoid of the ability to phosphorylate Akt at Threonine 308, which is a PDK1 phosphorylation site. In other words, by structure modification, we successfully separated the two pharmacologic activities and retained the desired activity. Additionally, either of these two compounds is able to significantly inhibit the motility of BCPAP cells and TPC1 cells at concentrations as low as 2.5 μM and 5 μM, respectively. Moreover, these two compounds also demonstrated increased activity to suppress the viability of thyroid cancer cells, suggesting the potential of their therapeutic application in clinic. However, these compounds are not PAK-specific as noted in the kinase profiling data. Development of more selective and potent inhibitors is scientifically desirable, but compounds with multiple targets may also have advantages.
In conclusion, a few novel anticancer agents were developed by modification of lead compounds FTY720 or OSU-03012. These new agents are abrogated of the main functions but keep or strengthen the desired “side activities” associated with their parent compounds. Therefore, the process once again is a demonstration of the application of “off-target” orientated drug discovery.

4.2. Future work

FTY720 is a novel immunosuppressant with unique mechanism of action by targeting on S1P1 receptors. Its S1P1-independent anticancer activity inspired up to develop OSU-2S. However, synthetically, the introduction of a propyl group in the place of pro-(S) hydroxymethyl group to synthesize OSU-2S generates a chiral molecule and makes the preparation and purification become difficult based on current synthetic strategy. Therefore for the future work, more efficient synthetic method needs to be developed to meet the need of the compound in large scale. Additionally, since OSU-2S demonstrated satisfactory anticancer profile in hepatocellular carcinoma cells and PC-3 prostate cancer cells, its anticancer activity in other types of cancer cells deserve further exploration.

The main drawback associated with the second derivatives of FTY720, OSU-DY-7, is the metabolic stability. Therefore, new drug delivery strategy needs to develop to release the drug to the target site before it is metabolically converted.

For PAK1 inhibitors, although YM-4 and YM-15 are devoid of PDK1 activity and maintained the PAK1 activity, their selectivity against other kinases was also evaluated and the results showed that these two compounds also inhibited a few other kinases. Therefore further study would be to carry out further modification toward specific
PAK1 inhibitor; on the other hand, the activity of these two agents \textit{in vivo} needs further investigation and the side-effect of them could also be explored to develop other agents being of interesting applications.
Bibliography


