SMALL MOLECULE-BASED DRUG DESIGN OF ANTICANCER AGENTS
THAT TARGET PROTEIN KINASE B / AKT, BCL-xL AND DNA METHYLTRANSFERASES FOR THE TREATMENT OF PROSTATE CANCER

DISSERTATION

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ABSTRACT

Prostate cancer is the most common form of cancer in men and the second leading cause of cancer-related deaths in the United States. So far, several mechanisms have been identified by which androgen-independent prostate cancer can develop, including constitutively active AKT pathway, overexpression of Bcl-xL and hypermethylation of tumor suppressor and caretaker genes, such as RASSF1A and GSTP1. In this dissertation research, we have developed three classes of anticancer agents that target the AKT signaling pathway, Bcl-xL, and DNA methyltransferases based on small molecules as our molecular templates.

In terms of targeting the AKT pathway, we examined the antiproliferative effect of our lead compound doxazosin, our pharmacological study revealed that doxazosin’s apoptotic effect was mediated, in part, through the down-regulation of phospho-AKT. Therefore, a systematic modification of doxazosin was carried out yielding the optimal compounds 33 and 44 which exhibited an order of magnitude improvement in antiproliferative potency.

Development of the Bcl-xL inhibitor arose from our attempt to develop non-nucleoside DNA hypomethylating agents based on procainamide and procaine as molecular templates. However, the antiproliferative effects of these procainamide derivatives in PC-3 cells could not be attributed to the alteration of DNA methylation
status. A search for a molecular target of these agents attributed their antiproliferative
effect in PC-3 cells to the inhibition of Bcl-xL function. Among these derivatives, compound 27 exhibited the greatest inhibitory effect against Bcl-xL activity. Exposure of PC-3 cells to 27 resulted in the release of cytochrome c from mitochondria, followed by the activation of caspase-9 and PARP cleavage.

To continue our research on the development of non-nucleoside DNA hypomethylating agents, (-)-epicatechin gallate (ECG) was selected as our molecular template based on the literature describing its ability to inhibit DNA methyltransferase (DNMT) activity. Screening of some candidates by methylation-specific PCR (MSP) in three cancer cell lines indicated that RASSF1A gene could be consistently demethylated by compound 6A.

Altogether, through this dissertation research, we have successfully developed three classes of anticancer agents that modulate different molecular targets. These findings suggest that small molecule-based drug design can be a powerful tool to develop more structurally diversified anticancer agents.
Dedicated to my parents, Wen-Yi, Brian and Stephen
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CHAPTER 1

QUINAZOLINE-BASED DESIGN OF ANTIPROLIFERATIVE AGENTS THAT BLOCK INTRACELLULAR PROTEIN KINASE B / AKT

1.1 SIGNIFICANCE OF PROTEIN KINASE B / AKT SIGNALING PATHWAY IN CANCER CELLS

1.1.1 REGULATION OF INTRACELLULAR AKT

AKT, also known as protein kinase B (PKB), is a serine / threonine kinase that comprises an amino-terminal pleckstrin-homology (PH) domain, a central catalytic domain and a short carboxy-terminal regulatory domain (1). The PH domain is defined as a lipid-binding module existing in the cytoskeletal protein pleckstrin in several cytoplasmic kinases. AKT can be activated by the action of two regulatory mechanisms (Figure 1.1). The first mechanism is through translocation to the plasma membrane via direct contact of its PH domain with the second messenger, phosphatidylinositol (3, 4, 5) triphosphate (PIP$_3$) synthesized on the inner leaflet of the cytoplasmic membrane from phosphatidylinositol (4,5) diphosphate (PIP$_2$) by phosphatidylinositol-3-kinase (PI3K) (2). The second mechanism is by phosphorylation at Thr308 and Ser473 in the catalytic domain (3, 4). After translocation to the plasma membrane, another serine / threonine kinase named 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates AKT at Thr308. Although maximal activation is achieved after phosphorylation of
Ser473 (5), phosphorylation of Thr308 is necessary and sufficient for AKT activation. Thr473 is phosphorylated by PDK2, a kinase that has been characterized biochemically, but the molecular identity of which remains undetermined (6).

**Figure 1.1** Regulation of intracellular AKT activity (7)

### 1.1.2 ACTIVATION OF AKT LEADS TO CELL SURVIVAL

AKT functions in an anti-apoptotic pathway at several levels. Constitutively active AKT can rescue PTEN-mediated apoptosis (8-11). PTEN protein is the PIP$_3$ phosphatase, which dephosphorylates the 3-position of PIP$_3$ reverting it to the inactive PIP$_2$; thereby, blocking the AKT signaling pathway (Figure 1.2).

AKT also directly phosphorylates BAD to prevent the association between BAD and Bcl-xL (12). BAD is a pro-apoptotic member of the Bcl-2 family of the proteins that promotes cell death by forming a non-functional heterodimer with the survival factor
Bcl-xL. Phosphorylation of BAD at Ser112 by AKT prevents this interaction, restoring Bcl-xL’s anti-apoptotic function. In addition, AKT inactivates the intrinsic catalytic activity of caspase-9 by phosphorylation of the Ser196 residue (13). Bcl-2 family proteins and the caspase-dependent apoptosis pathway will be discussed in the next chapter.

AKT-mediated phosphorylation modulates the function of FKHR, a member of forkhead family of transcription factors, through regulation of its subcellular localization. Under conditions during which AKT is activated, FKHR is phosphorylated and is retained in the cytoplasm. In contrast, when AKT is not activated, FKHR is not phosphorylated and is translocated to the nucleus where it promotes the transcription of pro-apoptotic genes such as BIM and FasL (14).

**Figure 1.2** Biological functions of the PI3K / AKT signaling pathway (7)
AKT can also influence cell survival through indirect effects on two central regulators of cell death: nuclear factor of κB (NF-κB) (15, 16) and p53 (17, 18). NF-κB is a ubiquitous, heterodimeric transcription factor that is released in the cytoplasm by proteins of the IκB family. Upon the phosphorylation of IκB by its upstream kinase (IKK), it is ubiquitylated and undergoes proteasomal degradation. Degradation of IκB releases NF-κB, which permits its nuclear translocation and subsequent activation of its target genes. AKT plays an important role in this pathway by activating IKK via phosphorylation at Thr23 in its regulatory domain resulting in the phosphorylation and degradation of IκB, and activation of NF-κB (15).

The tumor suppressor protein p53 functions to block the cell cycle in response to DNA damage, which provides the cell time to repair DNA. However, if the damage is severe enough, this protein can cause apoptosis thereby eliminating the damaged cell. AKT can affect the activity of p53 protein through phosphorylation of the p53-binding protein, MDM2. Upon the p53-MDM2 dimerization, p53 is ubiquitylated by its E3 ubiquitin ligase and is degraded by the proteasome. Phosphorylation of MDM2 by AKT results in the more efficient translocation of MDM2 into the nucleus, resulting in increased p53 degradation (17, 18).

1.1.3. AKT IS A CELL CYCLE REGULATOR

In addition to the manipulation of cell survival factors, AKT can also influence cell proliferation through signals to the cell-cycle machinery. The cell cycle is divided into four phases (19). In the S (synthesis) phase, the DNA is replicated to produce copies for both daughter cells. G1 and G2 phases represent ‘gap’ periods, during which cells prepare
themselves for the successful completion of the S and M phases. In the G2 phase, new proteins are synthesized and the cell approximately doubles in size. In the M (mitosis) phase, the maternal nuclear envelope breaks down, matching chromosomes is surrounded by a newly formed nuclear envelope, and cytokinesis pinches the cell in half, producing two daughter cells. After passing through mitosis and G1, a cell either continues through another division or ceases to divide, entering a quiescent phase known as G0.

The cell cycle is controlled by the coordinated action of cyclin-cyclin-dependent protein kinase (CDK) complexes and CDK inhibitors (CKIs). Cyclin D1, an important molecule in the G1/S phase transition, is a rather unstable molecule that is transported from the nucleus to the cytoplasm. In the cytoplasm, cyclin D1 is ligated by the SCF ubiquitin ligase for ubiquitination, resulting in proteasomal degradation. AKT has an important role in preventing cyclin D1 degradation by regulating the activity of the cyclin D1 kinase, glycogen synthase kinase-3β (GSK-3β) (20). After phosphorylation of cyclin D1 by GSK-3β, it is targeted for degradation by the proteasome. AKT directly phosphorylates GSK-3β and blocks its kinase activity, thereby allowing cyclin D1 to accumulate and to promote cell proliferation.

1.1.4 MODULATION OF CELL GROWTH BY AKT

Cell proliferation refers to cell division, which results in an increase in cell number; however, cell growth is related to the synthesis of macromolecules, such as lipid, carbohydrate, protein, etc., which leads to increased cell mass or size. mTOR (mammalian target of rapamycin, also known as FRAP1) is a serine/threonine kinase that plays a central role in the regulation of cell growth (29). It acts as a sensor that
controls protein synthesis on the basis of the availability of nutrients. There are two proteins whose phosphorylation states have been shown to be regulated by mTOR. The first targeted protein is p70S6 kinase, a serine/threonine kinase that is activated in response to a broad range of mitogenic stimuli. The second mTOR-dependent phosphorylation targeted protein is the translational-repressor protein PHAS-1 (also termed 4E-BP). Both proteins participate in the regulation of protein synthesis in cells stimulated by either mitogens or hormones. It is now known that mTOR is directly phosphorylated by AKT (30), and its activity can be suppressed by the PI3K inhibitors wortmannin and LY294002 (31). Nevertheless, it is still unclear how or whether phosphorylation of mTOR by AKT is a mechanism for activation (32).

1.1.5 AKT PATHWAY AS A THERAPEUTIC TARGET

The importance of the AKT pathway in various human cancer types is a consequence of its elevated activity in many cancer cell types and the biological effects of its downstream target proteins, many of which promote cell survival, proliferation and growth. Increased AKT activity in cancer cells can occur by a number of mechanisms. A common mechanism is the loss of function of the tumor suppressor gene \textit{PTEN} by mutation, deletion or silencing (33-35). Therefore, the development of small molecules which modulate the AKT signaling pathway has translational potential in cancer therapy. In the following sections, the development of agents that inhibit the proliferation of PC-3 androgen-independent prostate cancer cells, at least in part, through the down-regulation of phospho-AKT will be discussed. How this small molecule-based drug design approach originated from our work on the \textalpha_1-adrenoceptor antagonist, doxasozin, will be
1.2 QUINAZOLINE-BASED α1-ADRENOCEPTOR ANTAGONISTS INDUCE PROSTATE CANCER CELLS APOPTOSIS

1.2.1 α1-ADRENOCEPTOR ANTAGONISTS FOR THE TREATMENT OF BENIGN PROSTATIC HYPERPLASIA (BPH)

Benign prostatic hyperplasia (BPH) is a common disease of older men, which causes urinary obstruction and gradual loss of bladder function due to enlargement of the prostate. So far, the Food and Drug Administration (FDA) has approved finasteride (43) and dutasteride (44) to relieve common symptoms associated with an enlarged prostate. Both of these drugs inhibit the production of the hormone dihydrotestosterone (DHT), which is involved with prostate enlargement. The use of either of these drugs can prevent the progression of prostate growth or can actually shrink the prostate in some men. FDA has also approved terazosin, doxazosin, tamsulosin and alfuzosin for the treatment of BPH (45-48) (Chart 1.1). All four of these drugs act by relaxing the smooth muscle of the prostate and bladder neck to improve urine flow and to reduce bladder outlet obstruction by means of blockade of α1-adrenoceptors. Doxazosin, in particular, has attracted a lot of attention from both clinicians and basic scientists in that it is a first-line antihypertensive agent in addition to a BPH treatment (49).
1.2.2 DOXAZOSIN INDUCES CANCER CELL APOPTOSIS

It has been shown that $\alpha_1$-adrenoceptor antagonists can induce apoptosis in prostate cancer cells. In addition, doxazosin exhibited potential synergistic effects in conjunction with radiation or other chemotherapeutic agents, such as adriamycin and etoposide, against prostate cancer cells. Moreover, the growth and migration of other cell types, such as human vascular smooth muscle cells, was shown to be inhibited by doxazosin (50, 51). However, normal prostatic epithelial cells and bladder and colon cell lines were resistant to doxazosin-induced apoptosis (52).

This apoptotic effect, however, also occurred in cells that lacked $\alpha_1$-adrenoceptors and / or were treated in the presence of excess agonists indicating that apoptosis was induced in an $\alpha_1$-adrenoceptor-independent manner. Moreover, these effects were limited

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**Figure 1.3** Structures of $\alpha_1$ adrenoceptor antagonists
to the quinazoline-based α1-adrenoceptor antagonists, doxazosin and terazosin, and did not extend to the sulfonamide-based antagonist, tamsulosin (53-55).

1.2.3 MECHANISM OF DOXAZOSIN-INDUCED APOPTOSIS IN CANCER CELLS

Caspase-3 was shown to be activated in response to doxazosin, indicating that apoptosis is mediated by the caspase signaling pathway. Since transforming growth factor TGF-β1 (TGF-β1) can activate caspase-3 to induce apoptosis in the prostate cancer cells, it has been proposed that the apoptotic effect of doxazosin on human prostatic stromal cells is mediated through the upregulation of TGF-β1 (56, 57). In support of this hypothesis, microarray-based examination of doxazosin-mediated gene expression showed the rapid upregulation of two TGF-β1-modulated genes, IκBα and p21\textsuperscript{waf-1} (58). Our own study revealed that the antiproliferative effect of doxazosin in PC-3 cells was attributable, at least in part, to the inhibition of intracellular AKT activation.

1.2.4 DOWNREGULATION OF PHOSPHO-AKT AND INHIBITION OF PROLIFERATION IN PC-3 CELLS BY DOXAZOSIN

To shed light on the molecular mechanism of the antiproliferative effect of doxazosin, we investigated the effect of doxazosin on the phosphorylation state of AKT and ERKs, two signaling kinases that play important roles in cell survival, cell proliferation and cell growth in PC-3 cells. Of particular interest was AKT, which can phosphorylate IκBα and p21\textsuperscript{waf-1} which in turn activates the transcription factor NF-κB
and CDKs, respectively. Both IκBα and p21waf-1 have been demonstrated to be upregulated by doxazosin in a previous cDNA microarray study.

The antiproliferative activity of doxazosin was confirmed in PC-3 cells exposed to doxazosin in 1% FBS-supplemented RPMI 1640 medium. Doxazosin caused time- and dose-dependent apoptotic death, as evidenced by the disappearance of the native form of poly(ADP-ribose) polymerase (PARP). However, doxazosin exhibited moderate antiproliferative potency. Although PC-3 cells were susceptible to drug-induced antiproliferative effects at 25 µM and 50 µM, no appreciable effect was observed at 10 µM (Figure 1.4A).

Treatment with doxazosin also resulted in a dose- and time-dependent dephosphorylation of AKT (Figure 1.4B). In contrast, doxazosin, even at 50 µM, did not affect the phosphorylation status of ERKs (Figure 1.4C), indicating a degree of specificity of the drug action on intracellular signaling pathways. It is noteworthy that doxazosin exhibited no inhibitory effects on the kinase activity of immunoprecipitated AKT suggesting that AKT is not a direct target of doxazosin’s actions.
Figure 1.4 The molecular mechanism study of antiproliferative effect induced by doxazosin. (A) Antiproliferative effects of doxazosin in PC-3 cells. Doxazosin reduced cell viability in a time- and dose-dependent manner (left panel), and caused apoptosis as indicated by the induction of poly(ADP-ribose)polymerase (PARP) cleavage. (B) Dose- and time-dependent (upper and lower panels, respectively) effects of doxazosin on AKT phosphorylation. (C) Effect of doxazosin on ERK phosphorylation.
To examine the causal relationship between doxazosin-induced AKT deactivation and inhibition of cell viability, we assessed the protective effect of the enforced expression of constitutively active AKT, AKT$^{T308D/S473D}$, in transiently transfected, doxazosin-treated PC-3 cells. Western blot analysis using antibodies against AKT and the HA tag within the construct confirmed that transient transfection of AKT$^{T308D/S473D}$ led to a several-fold increase in AKT expression (Figure 1.5A). These transient transfectants were exposed to 25 $\mu$M doxazosin in 1% FBS-supplemented medium and their susceptibility to drug-induced cell death was compared to that of cells transfected with an empty pcDNA vector (Figure 1.5B). As shown, AKT$^{T308D/S473D}$ gave partial, yet significant, protection against doxazosin-induced reduction in cell viability. Taken together, these data suggest that the antiproliferative effect of doxazosin in PC-3 cells was mediated, in part, through the inhibition of intracellular AKT activation, and suggest an additional antitumor mechanism in addition to the previously reported upregulation of the TGF-β1 signaling pathway.

1.3 DOXAZOSIN AS A MOLECULAR TEMPLATE TO DEVELOP ANTIPROLIFERATIVE AGENTS THAT BLOCK AKT ACTIVATION

1.3.1 INDUCTION OF APOPTOSIS BY DERIVATIVES OF DOXAZOSIN

To generate derivatives of doxazosin optimized for its antiproliferative activity, we carried out structural modifications in a systematic manner (Figure 1.6). In Strategy A, we replaced the 2,3-dihydrobenzo[1,4]dioxane moiety of doxazosin with different aromatic acyl side chains to produce compounds 1 – 10. In Strategy B, we substituted
the aryl carboxamide function with aryl sulfonamides to generate compounds 11 – 40. In Strategy C, the piperazine moiety of the optimal agents from among compounds 11 - 40 (23 and 33) was replaced by an ethylenediamine linker, generating compounds 41 and 42, respectively. In Strategy D, we modified the methoxy side chains on the quinazoline ring of compound 33 to prepare compounds 43 – 46.

**Figure 1.5** Protective effect of constitutively active AKT on doxazosin-induced apoptotic death in PC-3 cells. (A) Expression of AKT$^{308D/S473D}$ in PC-3 transient transfects. Western blot analysis used antibodies against AKT and the HA tag. (B) Viability of PC-3 cells overexpressing AKT$^{308D/S473D}$ versus cells transfected with empty pcDNA vector (mock) in the presence of 25 µM doxazosin.
Figure 1.6 Overall strategy for the structural modification of doxazosin

1.3.2 ROLE OF THE AROMATIC ACYL SIDE CHAIN

To generate compounds 1-10, the commercially available 2-chloro-6, 7-dimethoxyquinazolin-4-amine (I) was coupled with Cbz-protected piperazine (II) to obtain piperazyl-quinazoline (III). The Cbz-protected piperazine was adopted due to the highly nucleophilic property of piperazine which underwent the coupling with two equivalents of I. Cbz-protected III was then removed by hydrogenolysis to give IV.
Compounds 1-10 were successfully synthesized by the treatment of IV with acyl chlorides in the presence of triethylamine in DMF solution (Figure 1.7).

![Chemical structures](image)

Reagents: (a) n-BuOH, reflux; (b) H₂/ Pd/ C, MeOH; (c) acyl chloride, Et₃N, DMF.

**Figure 1.7 Synthesis of 1-10**

Substitution of the 2,3-dihydro-benzo[1,4]dioxane moiety of doxazosin with different aromatic acyl side chains yielded a series of derivatives with varying antiproliferative potencies (Table 1.1). In general, analogues with hydrophilic side chains, such as the amino group in compound 8, exhibited lower antiproliferative activity, while those with a hydrophobic aromatic system, such as the t-butylphenyl in 9, retained the *in vitro* efficacy (IC₅₀, 47 µM and 45 µM for 9 and doxazosin, respectively). These
findings, however, provided a proof-of-principle that doxazosin was amenable to structural optimization leading to the development of a new class of antiproliferative agents.

\[
\text{MeO} \quad \text{N} \quad \text{O} \\
\text{MeO} \quad \text{N} \quad \text{NH}_2 \\
\text{N} \quad \text{N} \quad \text{MeO} \\
\text{Ar}
\]

Table 1.1 Structures and IC\(_{50}\) values of compounds 1-10

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<tr>
<td>1</td>
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<td>4-cyanophenyl</td>
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<td>4-tert-butylphenyl</td>
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<tr>
<td>10</td>
<td>4-(trifluoromethyl)phenyl</td>
<td>&gt;100</td>
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1.3.3 ARYL SULFONAMIDE DERIVATIVES EXHIBITED HIGH POTENCY IN INHIBITING PROSTATE CANCER CELL PROLIFERATION

Aryl sulfonamide derivatives were synthesized using two combinatorial methods.
Compounds 11-13, 5-19, 21, 22, 24-31, 33 and 34 were prepared by treatment of IV with arylsulfonyl chloride in methanol solution (Figure 1.8, Method A). To our surprise, arylsulfonyl chloride was not as highly active as acyl chloride. Therefore, nucleophilic addition of IV to arylsulfonyl chloride was tolerant in the protic solvent such as methanol. Compounds 14, 20, 23, and 32 were synthesized by an alternative synthetic route (Figure 1.8, Method B). Arylsulfonyl piperazine V was prepared through the coupling of piperazine and arylsulfonyl chloride. In contrast to the previously described preparation of compound III, an excess amount of unprotected piperazine could overcome the problem that generated from the addition of two equivalents of the arylsulfonyl moieties. This result may be due to the discrepant reactivity of sulfonyl chloride in comparison with acyl chloride.

**Method A**

![Method A diagram](image)

Reagents: (a) arylsulfonyl chloride, MeOH; (b) I, n-BuOH, reflux.

**Method B**

![Method B diagram](image)

**Figure 1.8 Synthesis of 11-34**
As shown in the synthetic routes described above, replacement of the carboxamide moiety of 1, 5, 7 and 9 with sulfonamide yielded 11, 17, 30 and 23, respectively. Comparison of these two series of agents permitted investigating the roles of these functional groups in mediating antiproliferative effects. In general, sulfonamides were more potent than carboxamides in terms of suppressing cell viability. For instance, 11 showed 2.6-fold greater activity than its counterpart 1 (IC$_{50}$, 23 and 60 µM, respectively). Likewise, 17, 23 and 30 exhibited 2-, 4.2- and 10-fold increases in potency in comparison to that of 5, 9 and 7, respectively (Table 1.2)

![Chemical structure](image)

<table>
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<tr>
<th>No.</th>
<th>Ar</th>
<th>IC$_{50}$ (µM) in PC-3 cells</th>
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<th>Ar</th>
<th>IC$_{50}$ (µM) in PC-3 cells</th>
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<td>3-carboxy-4,6-dichlorophenyl</td>
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Table 1.2 Structures and IC$_{50}$ values of compounds 11-35
**Figure 1.9** Comparison of chemical and 3D structures of compounds 9 and 23. The 3D structures of small molecules were generated using the software SYBYL 6.9 (Tripos Associate; St. Louis, MO) on Silicon Graphics O2 (Silicon Graphics Inc.; Mountain View, CA). Energy minimization was carried out with default parameters (minimum rms gradient, 0.005 kcal/mol; maximum iterations, 1000; minimum energy change, 0.05 kcal/mol).
To understand the structural basis for this improvement in potency, we compared the energy-minimized structures of 9 and 23 (Figure 1.9). As shown, the core structural component, i.e., the quinazoline base and the adjacent piperazine ring, conferred a high degree of structural rigidity to the molecule. The boat conformation of the piperazine ring oriented the N1 appendage, i.e., carbonyl or sulfonyl, perpendicular to the quinazoline planar structure. We rationalized that the discrepancy in potency was attributable to the transition from a trigonal planar structure of a carboxamide moiety (upper panel) to a tetrahedron-like structure of sulfonamide (lower panel). As a result, the spatial arrangement of the aromatic side arm relative to the neighboring plane of the quinazoline system differed.

Further examinations of the impact of the aryl sulfonamide function on antiproliferative potency confirmed the preference for bulky, hydrophobic aromatic systems (Table 1.2). Among the 25 derivatives examined, 23, 33 and 35, with the side chains of t-butylphenyl, biphenyl, and phenanthren-9-yl-phenyl, respectively, represented the optimal compounds, with IC50 values in the range of 4 – 5 µM in 10% serum-containing medium at 48 h.

To gain insight into the structure-activity relationships, 33 was further modified by addition of substituents at the 4'-position of the biphenyl ring. 35-40 were prepared in a similar way with the exception of the introduction of Suzuki coupling (Figure 1.10, Method C).
Method C

Reagents: (a) 4-bromobenzenesulfonyl chloride, Et₃N, MeOH; (b) arylboronic acid, Bu₄NBr, Pd(OAc)₂, K₂CO₃, H₂O; (c) H₂, Pd / C, MeOH; (d) I, n-BuOH, reflux.

Figure 1.10 Synthesis of 35-40

Further modifications of the biphenyl ring of 33 by adding alkyl chains such as CH₃ (36, IC₅₀ 3.4 µM), CF₃ (37, IC₅₀ 3.3 µM), or n-C₄H₉ (39, IC₅₀ 3.4 µM) at the 4’ position did not further improve the antiproliferative potency (Table 1.3). However, a significant drop in potency was noted with the 4-methylsulfonyl and bulky t-butyl substitutions (38 and 40, IC₅₀ 7.2 and 10 µM, respectively).
Figure 1.11A shows a dose-dependent induction of apoptosis by compound 33 in 1% FBS-supplemented medium, as evidenced by PARP proteolysis (Figure 1.11B), with an IC$_{50}$ of approximately 2.5 µM at 48 h. Western blot analysis confirmed that this apoptotic effect was attributable, in part, to the inhibition of AKT activation in a dose-dependent manner (Figure 1.11B).

Table 1.3 Structures and IC$_{50}$ values of compounds 36-40

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<td>4-trifluoromethyl</td>
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<td>4-tert-butyl</td>
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<td>4-methylsulfonyl</td>
<td>7.2 ± 0.54</td>
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Figure 1.11 (A) Time- and dose-dependent effects of 33 on the cell viability of PC-3 cells in 1% FBS-supplemented RPMI 1640 medium. (B) Western blot analysis of PARP proteolysis and AKT dephosphorylation in PC-3 cells treated with the indicated concentrations of 33 for 48 hours. (C) Effects of compound 44 on AKT phosphorylation in PC-3 cells treated with the indicated concentrations for 48 hours.

1.3.4 IMPORTANCE OF THE PIPERAZINE RING TO THE ANTIPROLIFERATIVE ACTIVITY

The 4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine moiety of these agents
provide structural rigidity to the molecule, which might play a role in the ligand-protein interactions. To examine this premise, we replaced the piperazine ring of \( 23 \) and \( 33 \) with an ethylenediamine linker, generating \( 41 \) and \( 42 \), respectively (Figure 1.12). This replacement resulted in a 2-fold decrease in apoptosis-inducing potency (\( 41 \) and \( 42 \), IC\(_{50}\), 9.8 and 7.5 \( \mu \)M, respectively), suggesting that this unique structural feature is important for maintaining activity.

\[
R = \text{t-Butyl, Phenyl}
\]

Figure 1.12 Synthesis of \( 41 \) and \( 42 \)

1.3.5 REPLACEMENT OF THE ALKOXY SUBSTITUENT ON THE QUINAZOLINE RING

To further optimize the antiproliferative activity of \( 33 \), we replaced the methoxy side
chains with alkoxy functions with different stereochemical properties. Removal of two methyl groups in I was carried out by treatment with boron tribromide at low to room temperature conditions, resulting in the generation of IX. With compound IX in hand, the addition of alkyl bromide or iodide afforded several kinds of alkoxy side chains in X, followed by the coupling with XI, which yielded 43-46 (Figure 1.13).

Among the four derivatives, 44 represented the optimal compound exhibiting a slight improvement in potency (IC$_{50}$, 2.5 µM in 10% FBS-supplemented medium), while its isopropyl counterpart, 45, displayed a precipitous drop in potency (IC$_{50}$, 24 µM) (Table 25)
These data suggest a very subtle impact of the quinazoline side chain structure on target binding. Again, the antiproliferative effect of 44 was characterized by the dephosphorylation of phospho-Akt in a dose-dependent manner, which was evident at a concentration as low as 1 µM (Figure 1.11C).

![Chemical Structure](image)

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<tr>
<th>No.</th>
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<th>IC_{50} (µM) in PC-3 cells</th>
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**Table 1.4** Structures and IC_{50} values of compounds 43-46
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Table 1.5 *In vitro* testing results of 33 against sixty human tumor cells lines from NCI.

The Growth Inhibitory-50 (GI50) value incorporates a correction for the cell count at time zero (T0); thus, GI50 is the concentration of test drug that inhibits the proliferation of cells by 50% and is defined as 100 · (T - T0)/(C - T0) = 50, where T is the optical
density of the test well after a 48-h period of exposure to test drug, T0 is the optical density at time zero, and C is the control optical density. The GI50 measures the growth inhibitory power of the test agent. The Total Growth Inhibitory (TGI) value is the concentration of test drug where \(100 \cdot \frac{(T - T0)}{(C - T0)} = 0\). Thus, the TGI signifies a cytostatic effect. The Lethal Concentration-50 (LC50), which signifies a cytotoxic effect, is the concentration of drug where \(100 \cdot \frac{(T - T0)}{T0} = -50\). The control optical density is not used in the calculation of LC50.

1.4 CONCLUSION

In this study, we obtained several lines of evidence that doxazosin inhibits cell proliferation, in part, through the down-regulation of AKT signaling in PC-3 cells. We found that the structurally optimized agent 33 exhibited an order-of-magnitude higher potency than the parent compound doxazosin in triggering antiproliferative effects in PC-3 cells. Further structural optimization was accomplished by replacing the methoxy side chains on the quinazoline ring with propoxy functions to obtain 44, which was effective in suppressing the proliferation of different prostate cancer cell lines at low micromolar levels. In addition, both agents were submitted to the Developmental Therapeutic Program (DTP) at the National Cancer Institute (NCI) for screening against sixty human tumor cells lines, representing leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney. All the tested cell lines showed high degrees of sensitivity to the growth inhibitory effects of 33 and 44. The mean GI50 values for 33 and 44 among these sixty cell lines were 2.2 and 1.5 µM, respectively (Table 1.5 &
1.6). These data clearly demonstrate the *in vitro* efficacy of these agents, and their potential application in cancer prevention and/or treatment.

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<th>LC50 (µM)</th>
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**Table 1.6** *In vitro* testing results of 44 against sixty human tumor cells lines from NCI
1.5 EXPERIMENTAL SECTION

Chemical reagents and organic solvents were purchased from Aldrich unless otherwise mentioned. Nuclear magnetic resonance spectra ($^1$H NMR) were measured on Bruker 250 or 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS peak. Electrospray ionization (ESI) mass spectrometry analyses were performed with a 3-tesla Finnigan FTMS-2000 Fourier transform mass spectrometer. Elemental analyses were within ± 0.4% of calculated values. Flash column chromatography was performed with silica gel (230 - 400 mesh).

1.5.1 CHEMICAL PREPARATION

![Chemical Structure](image)

4-(4-Amino-6, 7-dimethoxy-quinazolin-2-yl)-piperazine-1-carboxylic acid benzyl ester (III, also named as 3):

A mixture of 4-amino-2-chloro-6, 7-dimethoxyquinazoline (2.51 g, 10 mmol) and benzyl 1-piperazine-carboxylate (2.24 g, 10 mmol) in 1-butanol (15 mL) was stirred under reflux overnight, and cooled to 80 °C. The crude solid product was collected, washed with cold 1-butanol (2x10 mL), added to methanol (30 mL), and heated under reflux for 1 h. The white solid was filtered, and washed with methanol (2 x 10 mL) to yield compound III. $^1$H-NMR ($d_6$-DMSO) δ 3.59-3.61 (m, 4 H), 3.83-3.89 (m, 4 H), 3.85 (s, 3 H), 3.91 (s, 3 H), 5.14 (s, 2 H), 7.14 (s, 1 H), 7.34-7.88 (m, 5 H), 7.89 (s, 1 H) ppm.
HRMS (M+H)^+ calcd for C_{22}H_{26}N_{5}O_{4} 424.1979, found 424.1989.

6, 7-dimethoxy-2-piperazin-1-yl-quinazolin-4-ylamine (IV)

Compound III (2.12 g, 5.0 mmol) was dissolved in methanol (15 mL), and 10% palladium on charcoal (20 mg, 10% w/w) and triethylamine (0.2 mL) were added. The mixture was treated with hydrogen under atmospheric pressure for 6 h, and filtered. The solvent was evaporated to obtain the intermediate IV without purification. ^1H-NMR (d6-DMSO) δ 3.22 (br s, 4 H), 3.83 (s, 3 H), 3.87 (s, 3 H), 3.98 (br s, 4 H), 7.54 (s, 1 H), 7.69 (s, 1 H) ppm.

[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(4-chloro-phenyl)-meth anone (1):

The intermediate amine IV (0.578 g, 2.0 mmol) was dissolved in dry DMF (10 mL), and triethylamine (0.202 g, 2.0 mmol) was added. The resulting mixture was treated dropwise with 4-chlorobenzoyl chloride (0.35 g, 2.0 mmol) over 15 min, stirred at room temperature for 4 h, and then concentrated. The crude solid product was washed with methanol, filtered, and recrystallized from ethanol to give compound 1. ^1H-NMR (d6-DMSO) δ 3.32-3.34 (m, 4 H), 3.38 (s, 3 H), 3.84 (s, 3 H), 3.77-3.88 (m, 4 H), 7.48-7.57
(m, 5 H), 7.73 (s, 1 H), 8.66 (br s, 1H), 8.88 (br s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{21}$H$_{23}$ClN$_5$O$_3$ 428.1484, found 428.1492.

4-[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine-1-carbonyl]-benzonitrile (2):

Compound 2 was synthesized from the procedure described for compound 1.
$^1$H-NMR ($d_6$-DMSO) δ 3.46-3.47 (m, 4 H), 3.84 (s, 3 H), 3.88 (s, 3 H), 4.03-4.17 (m, 4 H), 7.51 (s, 1 H), 7.66 (d, $J = 8.0$ Hz, 2 H), 7.97 (d, $J = 8.0$ Hz, 2 H) ppm. HRMS (M+H)$^+$ calcd for C$_{22}$H$_{23}$N$_6$O$_3$ 419.1826, found 419.1812.

3-[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine-1-carbonyl]-benzo-nitrile (4):

Compound 4 was synthesized from the procedure described for compound 1.
$^1$H-NMR ($d_6$-DMSO) δ 3.44-3.46 (m, 4 H), 3.77 (s, 3 H), 3.82 (s, 3 H), 3.77-3.99 (m, 4 H), 6.97 (s, 1 H), 7.45 (s, 1 H), 7.60-7.64 (m, 1 H), 7.70-7.72 (m, 1 H), 7.82-7.93 (m, 2 H) ppm. HRMS (M+H)$^+$ calcd for C$_{22}$H$_{23}$N$_6$O$_3$ 419.1826, found 419.1823.
[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(4-nitro-phenyl)-methanone (5):

Compound 5 was synthesized from the procedure described for compound 1. 
$^1$H-NMR (d$_6$-DMSO) $\delta$ 3.21-3.35 (m, 4 H), 3.85(s, 3 H), 3.89 (s, 3 H), 3.93-3.96 (m, 4 H), 7.41 (s, 1 H), 7.73-7.76 (m, 3 H), 7.93-8.39 (m, 2 H), 8.83 (brs, 1 H), 8.90 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{21}$H$_{23}$N$_6$O$_5$ 439.1724, found 439.1718.

[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(3,4-dimethoxy-phenyl)-methanone (6):

Compound 6 was synthesized from the procedure described for compound 1. 
$^1$H-NMR (d$_6$-DMSO) $\delta$ 3.68 (s, 4 H), 3.79(s, 3 H), 3.81 (s, 3 H), 3.84(s, 3 H), 3.88 (s, 3 H), 4.26 (s, 4 H), 7.41 (s, 1 H), 7.00-7.05 (m, 3 H), 7.74 (s, 1 H), 8.54 (brs, 1 H), 8.90 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{23}$H$_{29}$N$_5$O$_5$ 454.2085, found 454.2071.
[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-naphthalen-1-yl-methanone (7):

Compound 7 was synthesized from the procedure described for compound 1. 

\[ \text{H-NMR (} \delta \text{)} 3.18-3.30 \text{ (m, 4 H), 3.67(s, 3 H), 3.78 (s, 3 H), 3.97(brs, 2 H), 4.08 (brs, 2 H), 7.48 (s, 1 H), 7.53-7.59 (m, 1 H), 7.60-7.61 (m, 3 H), 7.65 (s, 1 H), 7.96 (s, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{25}\)H\(_{26}\)N\(_5\)O\(_3\) 444.2030, found 444.2030.} \]

[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(4-amino-phenyl)-methanone (8):

Compound 8 was synthesized from the procedure described for compound 1. 

\[ \text{H-NMR (} \delta \text{)} 3.71(s, 4 H), 3.86(s, 3 H), 3.87 (s, 3 H), 3.92-3.97(m , 4 H), 6.65-6.68 (m, 2 H), 7.13 (d, J = 3.2 Hz, 1 H), 7.25 (d, J = 3.4 Hz, 1 H), 7.27 (d, J = 3.3 Hz, 1 H), 7.62 (d, J = 3.2 Hz, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{21}\)H\(_{25}\)N\(_6\)O\(_3\) 409.1983, found 409.1984.} \]

[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(4-tert-butyl-phenyl)-methanone (9):

Compound 9 was synthesized from the procedure described for compound 1.
\[ \text{H-NMR (}\text{d}_6\text{-DMSO}) \delta 1.32 (s, 9 \text{ H}), 3.66-3.74 (m, 4 \text{ H}), 3.85 (s, 3 \text{ H}), 3.88 (s, 3 \text{ H}), 3.93 (s, 4 \text{ H}), 7.31-7.51 (m, 5 \text{ H}), 7.73 (s, 1 \text{ H}), 8.51 (s, 1 \text{ H}), 8.97 (s, 1 \text{ H}) \text{ ppm. HRMS (M+H)}^+ \text{ calcd for C}_{25}\text{H}_{32}\text{N}_5\text{O}_3 450.2500, \text{ found 450.2485.} \]

\[ \text{[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-} \]
\[ \text{(4-trifluoromethyl-phenyl)-methanone (10):} \]

Compound 10 was synthesized from the procedure described for compound 1.

\[ \text{H-NMR (}\text{d}_6\text{-DMSO}) \delta 3.50 (s, 4 \text{ H}), 3.85 (s, 3 \text{ H}), 3.88 (s, 3 \text{ H}), 4.0 (s, 4 \text{ H}), 7.56 (s, 1 \text{ H}), 7.70 (d, } J = 7.7 \text{ Hz, 1 H}), 7.87 (d, } J = 7.7 \text{ Hz, 2 H}), 8.59 (s, 1 \text{ H}), 8.94 (s, 1 \text{ H}) \text{ ppm. HRMS (M+H)}^+ \text{ calcd for C}_{22}\text{H}_{23}\text{F}_3\text{N}_5\text{O}_3 462.1748, \text{ found 462.1708.} \]

\[ \text{2-[4-(4-Chloro-benzenesulfonyl)-piperazin-1-yl]-} \]
\[ \text{6,7-dimethoxy-quinazolin-4-yl-amine (11):} \]

To a solution of the intermediate amine IV (0.578 g, 2.0 mmol) and triethylamine (0.276 g, 2.0 mmol) in methanol (10 mL), 4-chlorobenzenesulfonyl chloride (0.443 g, 2.1 mmol) was added to the solution. The mixture was stirred at room temperature for 1 h. The resulting solid was filtered, washed with ethyl acetate (2 x 10 mL) to obtain the crude solid product. The crude product was stirred in methanol (10 mL) under reflux for
1 hr, filtered and dried to obtain compound 11. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.27 (s, 4 H), 3.35 (s, 3 H), 3.85 (s, 3 H), 3.99 (s, 4 H), 7.55 (s, 1 H), 7.60-7.80 (m, 5 H), 8.63 (s, 1 H), 8.80 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{20}$H$_{23}$ClN$_5$O$_4$S 464.1154, found 464.1158.

2-[4-(5-Chloro-thiophene-2-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (14):

To a solution of piperazine (0.517g, 6.0 mmol) and 5-chloro-thiophene-2-sulfonyl chloride (0.436 g, 2.0 mmol) in methanol (10 mL), the mixture was stirred at room temperature for 1 h. The solvent was evaporated, and the residue was purified with silica gel chromatography to obtain 1-(5-chloro-thiophene-2-sulfonyl)-piperazine. The intermediate (0.266 g, 1.0 mmol) and 4-amino-2-chloro-6, 7-dimethoxy-quinazoline (0.251 g, 1.0 mmol) in 1-butanol (5 mL) were stirred under reflux overnight, and cooled to 80 $^\circ$C. The collected solid product was washed with ethyl acetate (2 x 10 mL), stirred in methanol (30 mL) under reflux for 1 h, filtered, washed with methanol (2 x 10 ml) to yield compound 14. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.06-3.08 (m, 4 H), 3.80 (s, 3 H), 3.84 (s, 3 H), 3.94 (s, 4 H), 7.36 (s, 1 H), 7.37 (s, 1 H), 7.59 (s, 1 H), 7.60 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{18}$H$_{21}$ClN$_5$O$_4$S$_2$ 470.0718, found 470.0740.
6,7-Dimethoxy-2-[4-(4-phenanthren-9-yl-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-ylamine (35):

To a solution of cbz-protected \(N\)-piperazine (2.24g, 10.0 mmol) and 4-bromobenzenesulfonyl chloride (2.55 g, 10.0 mmol) in methanol (20 mL), triethylamine (1.38g, 10.0 mmol) was added to the solution. The mixture was stirred at room temperature for 2 h, concentrated, and purified by silica gel chromatography to afford 4-(4-bromo-benzenesulfonyl)-piperazine-1-carboxylic acid benzyl ester \(\text{V}\).

Under argon, compound \(\text{V}\) (0.439g, 1.0 mmol), \(K_2\text{CO}_3\) (0.345g, 2.5 mmol), \(\text{Bu}_4\text{NBr}\) (0.322g, 1.0 mmol) and \(\text{Pd(OAc)}_2\) (11 mg, 5 mol%) were added to a stirred solution of 4-phenanthrenylboronic acid (0.243 g, 1.1 mmol) in \(\text{H}_2\text{O}\) (5 mL). The reaction mixture was vigorously stirred at 70 °C for 1 h, cooled to room temperature, and added ethyl acetate (10 mL). The organic layer was dried and concentrated to obtain compound \(\text{V}\).

To a solution of compound \(\text{VI}\) (0.389g, 0.5 mmol) in methanol (5 mL), 10% palladium on charcoal (5 mg, 10% w/w) was added. The mixture was treated with hydrogen under atmospheric pressure for 6 h, and filtered. The solvent was evaporated to yield product \(\text{VII}\). Following the procedure for the synthesis of compound 14, compound 35 was
synthesized. \(^1\)H-NMR \((d_6\)-DMSO\) \(\delta 3.20\) (s, 4 H), 3.81 (s, 3 H), 3.86 (s, 3 H), 3.99 (s, 4 H), 7.28 (s, 1H), 7.60-7.78 (m, 7 H), 7.81 (d, \(J = 8.4\) Hz, 2 H), 8.02 (d, \(J = 8.1\) Hz), 8.4 (s, 1 H), 8.87 (d, \(J = 8.1\) Hz, 1 H), 8.94 (d, \(J = 8.4\) Hz, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{34}\)H\(_{32}\)N\(_5\)O\(_4\)S 606.2169, found 606.2164.

![Compound 12](image1)

2-[4-(4-Bromo-benzenesulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-yl-amine (12):

Compound 12 was synthesized from the procedure described for compound 11.

\(^1\)H-NMR \((d_6\)-DMSO\) \(\delta 3.13-3.14\) (m, 4 H), 3.48-3.49 (m, 4 H), 3.83 (s, 3 H), 3.90 (s, 3 H), 6.99 (s, 1 H), 7.49 (s, 1 H), 7.74 (d, \(J = 8.4\) Hz, 2 H), 7.83 (d, \(J = 8.4\) Hz, 2 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{20}\)H\(_{23}\)BrN\(_5\)O\(_4\)S 508.0649, found 508.0646.

![Compound 13](image2)

2-[4-(4-Iodo-benzenesulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-yl-amine (13):

Compound 13 was synthesized from the procedure described for compound 11.

\(^1\)H-NMR \((d_6\)-DMSO\) \(\delta 2.95\) (s, 4 H), 3.77 (s, 3 H), 3.78-3.82 (m, 4 H), 3.82 (s, 3 H), 7.26-3.9 (m, 2 H), 7.38 (d, \(J = 8.3\) Hz, 2 H), 8.01 (d, \(J = 8.5\) Hz, 2 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{20}\)H\(_{23}\)IN\(_5\)O\(_4\)S 556.0510, found 556.0496.
6,7-Dimethoxy-2-[4-(2-nitro-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (15):

Compound 15 was synthesized from the procedure described for compound 11. 

$^1$H-NMR (d$_6$-DMSO) δ 3.16 (s, 4 H), 3.34 (s, 3 H), 3.43 (s, 3 H), 3.74 (s, 4 H), 7.64 (s, 1 H), 7.95 (t, J = 8.1 Hz, 1 H), 8.21 (d, J = 7.8 Hz, 1 H), 8.41 (s, 1 H), 8.53 (s, 1 H), 8.56 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{20}$H$_{23}$N$_6$O$_6$S 475.1394, found 475.1394.

6,7-Dimethoxy-2-[4-(3-nitro-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (16):

Compound 16 was synthesized from the procedure described for compound 11. 

$^1$H-NMR (d$_6$–DMSO) δ 3.22 (s, 4 H), 3.68 (s, 3 H), 3.77 (s, 3 H), 3.82 (s, 4 H), 6.72 (s, 1 H), 7.19 (brs, 2 H), 7.42 (s, 1 H), 7.82-7.88 (m, 2 H), 7.98-8.07 (m, 2 H) ppm. HRMS (M+H)$^+$ calcd for C$_{20}$H$_{23}$N$_6$O$_6$S 475.1394, found 475.1392.
6,7-Dimethoxy-2-[4-(4-nitro-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (17):

Compound 17 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.00 (s, 4 H), 3.66 (s, 3 H), 3.76 (s, 3 H), 3.83 (s, 4 H), 6.75 (s, 1 H), 7.16 (br s, 2 H), 7.38 (s, 1 H), 8.01 (d, $J$ = 8.6 Hz, 2 H), 8.20 (d, $J$ = 8.5, 2 H) ppm. HRMS (M+H)$^+$ calcd for C$_{20}$H$_{23}$N$_6$O$_6$S 475.1394, found 475.1379.

![Image of compound 17](image-url)

6,7-Dimethoxy-2-[4-(toluene-4-sulfonyl)-piperazin-1-yl]-quinazolin-4-ylamine (18):

Compound 18 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 2.29 (s, 3 H), 3.03-3.07 (m, 4 H), 3.82 (s, 3 H), 3.85(s, 3 H), 3.98-4.05 (m, 4 H), 7.45 (d, $J$ = 7.6 Hz, 2 H), 7.52 (s, 1 H), 7.65 (d, $J$ = 7.3 Hz, 2 H), 7.73 (s, 1 H), 8.55 (s, 1 H), 8.92 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{21}$H$_{26}$N$_5$O$_4$S 444.1700, found 444.1706.

![Image of compound 18](image-url)

6,7-Dimethoxy-2-[4-(4-trifluoromethyl-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (19):

Compound 19 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.08 (s, 4 H), 3.79 (s, 3 H), 3.84 (s, 3 H), 3.90 (s, 4 H), 7.56 (s, 1
H), 7.80-8.03 (m, 5 H) ppm. HRMS (M+H)+ calcd for C_{21}H_{23}F_{3}N_{5}O_{3}S 498.1417, found 498.1420.

6,7-Dimethoxy-2-[4-(4-methoxy-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (20):

Compound 20 was synthesized from the procedure described for compound 14. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 2.95-2.97 (m, 4 H), 3.73 (s, 3 H), 3.75 (s, 3 H), 3.82 (s, 3 H), 3.73-3.82 (m, 4 H), 6.98 (s, 1 H), 7.06 (d, $J = 21.1$ Hz, 2 H), 7.57 (d, $J = 19.1$ Hz, 2 H) ppm. HRMS (M+H)+ calcd for C_{21}H_{26}N_{5}O_{5}S 460.1649, found 460.1652.

6,7-Dimethoxy-2-[4-(4-trifluoromethoxy-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (21):

Compound 21 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.40 (s, 4 H), 3.66 (s, 3 H), 3.69 (s, 3 H), 3.71 (s, 4 H), 6.68 (s, 1 H), 7.00 (br s, 2 H), 7.23 (s, 1 H), 7.54-755 (m, 2 H), 7.86-7.88 (m, 2 H) ppm. HRMS (M+H)+ calcd for C_{21}H_{23}F_{3}N_{5}O_{5}S 514.1367, found 514.1363.
2-[4-(4-Methanesulfonyl-benzenesulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (22):

Compound 22 was synthesized from the procedure described for compound 11. 

\[ \text{\textsuperscript{1}H-NMR} (d_6^{-}\text{DMSO}) \delta 3.00 \text{ (s, 4 H)}, 3.21 \text{ (s, 3 H)}, 3.76 \text{ (s, 3 H)}, 3.80 \text{ (s, 3 H)}, 3.82 \text{ (s, 4 H)}, 6.69 \text{ (s, 1 H)}, 7.07 \text{ (br s, 2 H)}, 7.26 \text{ (s, 1 H)}, 8.01 \text{ (d, } J = 8.3 \text{ Hz, 2 H)}, 8.16 \text{ (d, } J = 8.4 \text{ Hz, 2 H}) \text{ ppm. HRMS (M+H)\textsuperscript{+} calcd for C}_{21}\text{H}_{26}\text{N}_{5}\text{O}_{6}\text{S}_{2} \text{ 508.1319, found 508.1317.}

2-[4-(4-tert-Butyl-benzenesulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (23):

Compound 23 was synthesized from the procedure described for compound 14.

\[ \text{\textsuperscript{1}H-NMR} (d_6^{-}\text{DMSO}) \delta 1.28 \text{ (s, 9 H)}, 2.90 \text{ (s, 4 H)}, 3.76 \text{ (s, 3 H)}, 3.80 \text{ (s, 3 H)}, 3.90-3.98 \text{ (m, 4 H)}, 6.70 \text{ (s, 3 H)}, 7.17 \text{ (br s, 1 H)}, 7.26 \text{ (s, 1 H)}, 7.45-7.68 \text{ (m, 3 H) ppm. HRMS (M+H)\textsuperscript{+} calcd for C}_{24}\text{H}_{32}\text{N}_{5}\text{O}_{4}\text{S} \text{ 486.2170, found 486.2173.}

3-[4-(4-Amino-6, 7-dimethoxy-quinazolin-2-yl)-piperazine-1-sulfonyl]-benzoic acid
(24):

Compound 24 was synthesized from the procedure described for compound 11. $^1$H-NMR ($d_6$-DMSO) δ 2.99 (s, 4 H), 3.77 (s, 3 H), 3.82 (s, 7 H), 6.78 (s, 1 H), 7.38 (br s, 1 H), 7.44 (s, 1 H), 7.78 (t, $J$ = 7.6 Hz, 1 H), 8.00 (d, $J$ = 7.4 Hz, 1 H), 8.21-8.23 (m, 2 H) ppm. HRMS (M+H)$^+$ calcld for C$_{21}$H$_{24}$N$_5$O$_6$S 474.1442, found 474.1426.

![Structure of Compound 24](image)

[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-(4-trifluoromethyl-phenyl)-methanone (25):

Compound 25 was synthesized from the procedure described for compound 11. $^1$H-NMR ($d_6$-DMSO) δ 3.34 (brs, 4 H), 3.77 (s, 3 H), 3.82 (s, 3 H), 3.95 (s, 4 H), 6.83 (s, 1 H), 7.41-7.51 (brs, 2 H), 7.66-7.71(m, 2 H), 7.85-7.94 (m,2 H), 10.20 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcld for C$_{21}$H$_{24}$N$_5$O$_6$S 474.1442, found 474.1479.

![Structure of Compound 25](image)

2-[4-(2,5-Dichloro-benzenesulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (26):

Compound 26 was synthesized from the procedure described for compound 11. $^1$H-NMR ($d_6$-DMSO) δ 3.33 (brs, 4 H), 3.64 (s, 3 H), 3.78-3.82 (m, 4 H), 3.82 (s, 3 H), 6.74 (s, 1 H), 7.21 (brs, 2 H), 7.43(s, 1 H), 7.69-7.96 (m, 2 H), 7.97 (s, 1 H) ppm. HRMS
(M+H)$^+$ calcd for C$_{20}$H$_{22}$Cl$_2$N$_5$O$_4$S 498.0764, found 498.0768.

4-[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine-1-sulfonyl]-benzene-1,3-diamine (27):

Compound 27 was synthesized from the procedure described for compound 11 following by hydrogenation to get diamine product. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.09 (s, 4 H), 3.34 (s, 3 H), 3.67 (s, 3 H), 3.86-3.89 (m, 4 H), 6.28 (d, $J$ = 8.5 Hz, 1 H), 6.74 (s, 1 H), 7.31 (d, $J$ = 9.0 Hz, 1 H), 7.66 (s, 1 H), 8.23 (s, 1 H), 8.64 (s, 1 H), 8.85 (s, 1 H), 8.99 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{20}$H$_{26}$N$_7$O$_6$S 460.1761, found 460.1758.

5-[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine-1-sulfonyl]-2-chloro-4-fluoro-benzoic acid (28):

Compound 28 was synthesized from the procedure described for compound 11. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.15 (s, 4 H), 3.70 (s, 3 H), 3.76 (s, 3 H), 3.80 (s, 4 H), 6.76 (s, 1 H), 7.37 (brs, 1 H), 7.42 (s, 1 H), 7.82 (s, 1 H), 8.12 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{21}$H$_{22}$ClFN$_5$O$_6$S 526.0958, found 526.0943.
5-[4-(4-Amino-6,7-dimethoxy-quinazolin-2-yl)-piperazine-1-sulfonyl]-2,4-dichloro-benzoic acid (29):

Compound 29 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.37 (brs, 4 H), 3.81 (s, 3 H), 3.85 (s, 3 H), 3.95 (s, 4 H), 7.47 (s, 1 H), 7.71 (s, 2 H), 7.43 (s, 1 H), 8.03 (s, 1 H), 8.33 (s, 1 H), 8.62 (br s, 1 H), 8.86 (br s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{21}$H$_{22}$Cl$_2$N$_5$O$_6$S 542.0662, found 542.0657.

6,7-Dimethoxy-2-[4-(naphthalene-1-sulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (30):

Compound 30 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.08 (s, 4 H), 3.81 (s, 3 H), 3.83 (s, 3 H), 3.95 (s, 4 H), 7.66 (s, 1 H), 7.68-7.78 (m, 4 H), 8.11 (d, $J = 8.0$ Hz, 1 H), 8.18 (d, $J = 7.4$ Hz, 1 H), 8.31 (d, $J = 8.2$ Hz, 1 H), 8.71 (d, $J = 8.6$ Hz, 1 H), 10.29 (brs, 2H) ppm. HRMS (M+H)$^+$ calcd for C$_{24}$H$_{26}$N$_5$O$_4$S 480.1700, found 480.1696.
6,7-Dimethoxy-2-[4-(naphthalene-2-sulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (31):

Compound 31 was synthesized from the procedure described for compound 11. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.00-3.08 (m, 4 H), 3.39 (s, 3 H), 3.43 (s, 3 H), 3.74-3.81 (m, 4 H), 6.67 (s, 1 H), 7.12 (brs, 2 H), 7.36 (s, 1 H), 7.66-7.73 (m, 2 H), 7.77 (d, $J = 8.7$ Hz, 1 H), 8.05 (d, $J = 7.7$ Hz, 1 H), 8.14 (d, $J = 8.7$ Hz, 1 H), 8.20 (d, $J = 7.7$ Hz, 1 H), 8.45 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{24}$H$_{26}$N$_5$O$_4$S 480.1700, found 480.1708.

![Chemical Structure](image)

2-[4-(5-Dimethylamino-naphthalene-1-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (32):

Compound 32 was synthesized from the procedure described for compound 14. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 2.82 (s, 6 H), 3.27-3.29 (m, 4 H), 3.44 (s, 3 H), 3.81 (s, 3 H), 3.85-3.91 (m, 4 H), 7.27 (d, $J = 7.6$ Hz, 1 H), 7.61 (s, 1 H), 7.61-7.70 (m, 3 H), 8.17 (d, $J = 7.4$ Hz, 1 H), 8.35 (d, $J = 8.64$ Hz, 1 H), 8.53 (d, $J = 8.5$ Hz, 1 H), 8.63 (brs, 1 H), 8.85 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{26}$H$_{31}$N$_6$O$_4$S 523.2122, found 523.2153.

![Chemical Structure](image)

2-[4-(Biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (33):

![Chemical Structure](image)
Compound 33 was synthesized from the procedure described for compound 11.

$^1$H-NMR ($d_6$-DMSO) δ 3.02-3.03 (m, 4 H), 3.81 (s, 3 H), 3.86 (s, 3 H), 3.98-3.46 (m, 4 H), 7.38-7.52 (m, 4 H), 7.68-7.74 (m, 3 H), 7.85 (d, $J = 8.2$ Hz, 2 H), 7.94 (d, $J = 8.2$ Hz, 2 H) ppm. HRMS (M+H)$^+$ calcld for C$_{26}$H$_{28}$N$_5$O$_4$S 506.1857, found 506.1840.

![Image of compound 33]

6,7-Dimethoxy-2-[4-(2,4,6-triisopropyl-benzenesulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (34):

Compound 34 was synthesized from the procedure described for compound 11.

$^1$H-NMR ($d_6$-DMSO) δ 1.22 (s, 9 H), 1.24 (s, 9 H), 2.92-2.98 (m, 1 H), 3.23 (s, 4 H), 3.84 (s, 3 H), 3.87 (s, 3 H), 3.94 (s, 4 H), 4.01-4.15 (m, 2 H), 7.32 (s, 2 H), 7.44 (s, 1 H), 7.76 (s, 1 H), 8.69 (brs, 1 H), 8.96 (br s, 1 H) ppm. HRMS (M+H)$^+$ calcld for C$_{29}$H$_{42}$N$_5$O$_4$S 556.2952, found 556.2944.

![Image of compound 34]

7-Dimethoxy-2-[4-(4'-methyl-biphenyl-4-sulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (36):

Compound 36 was synthesized from the procedure described for compound 35.

$^1$H-NMR ($d_4$-MeOH) δ 2.28 (s, 3 H), 3.12 (s, 4 H), 3.78 (s, 3 H), 3.84 (s, 3 H), 3.86 (s, 4
H), 6.88-6.90 (m, 3 H), 7.18 (d, J = 8.1 Hz, 1 H), 7.36 (s, 1 H), 7.38-7.40 (m, 2 H), 7.44 (d, J = 8.1 Hz, 1 H), 7.46-7.78 (m, 2 H) ppm. HRMS (M+H)$^+$ calcd for C$_{27}$H$_{30}$N$_5$O$_4$S 520.2013, found 520.2040.

6,7-Dimethoxy-2-[4-(4'-trifluoromethyl-biphenyl-4-sulfonyl)-piperazin-1-yl]-quinazolin-4-yl-amine (37):

Compound 37 was synthesized from the procedure described for compound 35. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.05 (s, 4 H), 3.78 (s, 3 H), 3.82 (s, 2 H), 3.91 (s, 3 H), 7.53 (s, 1 H), 7.71-7.89 (m, 4 H), 7.94-8.07 (m, 4 H), 10.16 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{27}$H$_{26}$F$_3$N$_5$O$_4$S 574.1730, found 574.1728.

2-[4-(4'-Methanesulfonyl-biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-ylamine (38):

Compound 38 was synthesized from the procedure described for compound 35. $^1$H-NMR ($d_6$-DMSO) $\delta$ 2.91 (s, 4 H), 3.26 (s, 3 H), 3.78 (s, 2 H), 3.83 (s, 3 H), 3.91 (s, 4 H), 6.97 (s, 1 H), 7.55 (s, 1 H), 7.88 (d, J = 8.2 Hz, 2 H), 8.08 (m, 6 H) ppm. HRMS
(M+H)$^+$ calcd for C$_{27}$H$_{30}$N$_5$O$_6$S$_2$ 584.1632, found 584.1658.

2-[4-(4'-Butyl-biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-yl-amine (39):

Compound 39 was synthesized from the procedure described for compound 35. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 0.89 (t, $J$ = 7.3 Hz, 3 H), 1.29-1.34 (m, 2 H), 1.55-1.59 (m, 2 H), 2.60-2.64 (m, 2 H), 3.13 (s, 4 H), 3.81 (s, 3 H), 3.86 (s, 3 H), 3.97 (s, 4 H), 7.31-7.33 (m, 3 H), 7.63-7.69 (m, 3 H), 7.82 (d, $J$ = 8.3 Hz, 2 H), 7.91 (d, $J$ = 8.4 Hz, 2 H), 8.66 (brs, 1 H), 8.79 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{30}$H$_{36}$N$_5$O$_4$S 562.2483, found 562.2458.

2-[4-(4'-tert-Butyl-biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-dimethoxy-quinazolin-4-yl-amine (40):

Compound 40 was synthesized from the procedure described for compound 35. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 1.31 (s, 9 H), 3.14 (s, 4 H), 3.81 (s, 3 H), 3.86 (s, 3 H), 3.95 (s, 4 H), 7.19 (s, 1 H), 7.45-7.53 (m, 2 H), 7.60-7.66 (m, 3 H), 7.82-7.84 (m, 2 H), 7.92 (d, $J$ = 8.3 Hz, 2 H), 8.66 (brs, 1 H), 8.81 (brs, 1 H) ppm. HRMS (M+H)$^+$ calcd for
C_{30}H_{36}N_{5}O_{4}S 562.2483, found 562.2471.

\[ \text{N-[2-(4-Amino-6,7-dimethoxy-quinazolin-2-ylamino)-ethyl]-4-tert-butyl-benzene sulfonamide (41):} \]

A mixture of ethylenediamine (0.36 g, 6.0 mmol) and \( t \)-butylbenzenesulfonyl chloride (0.464 g, 2.0 mmol) in methanol (15 mL) was stirred for 3 h, concentrated, and purified by silica gel chromatography to yield \( \text{N-(2-amino-ethyl)-4-t-butyl-benzenesulfonamide} \). Following the procedure for the synthesis of compound 14, compound 42 was obtained. \(^1\)H-NMR (\( d_6 \)-DMSO) \( \delta \) 1.17 (s, 9 H), 3.11 (s, 2 H), 3.48 (s, 2 H), 3.86 (s, 3 H), 3.93 (s, 3 H), 6.90 (s, 1 H), 7.50 (d, \( J = 8.4 \) Hz, 2 H), 7.55 (s, 1 H), 7.70 (d, \( J = 8.4 \) Hz, 2 H) ppm. HRMS (M+H)\(^+\) calcd for C_{22}H_{30}N_{5}O_{4}S 460.2013, found 460.2010.

\[ \text{N-[2-(4-Amino-6,7-dimethoxy-quinazolin-2-ylamino)-ethyl]-4-biphenylsulfonamide (42):} \]

Compound 42 was synthesized from the procedure described for compound 41. \(^1\)H-NMR (\( d_6 \)-DMSO) \( \delta \) 3.05 (s, 2 H), 3.46 (s, 2 H), 3.80 (s, 3 H), 3.83 (s, 3 H), 6.91(brs, 1 H), 7.40-7.47 (m, 3 H), 7.60-7.64 (m, 3 H), 7.78-7.83 (m, 2 H), 7.86-7.88 (m, 2 H),
8.01 (s, 1 H) ppm. HRMS (M+H)+ calcd for C_{24}H_{26}N_{5}O_{4}S 480.1700, found 480.1687.

6,7-Bis-allyloxy-2-[4-(biphenyl-4-sulfonyl)-piperazin-1-yl]-quinazolin-4-ylamine (43):

A solution of 4-amino-2-chloro-6,7-dimethoxyquinazoline (2.51g, 10.0 mmol) in CH$_2$Cl$_2$ (30 mL) was cooled to –70°C under argon, and added boron tribromide (6.01g, 12.0 mmol). The mixture was allowed to warm up to room temperature over a period of 4 h, cooled to –70°C, added methanol (30 mL), and concentrated. The solid residue was washed with ethyl acetate to obtain 4-amino-2-chloro-6,7-dihydroxyquinazoline. $^1$H-NMR ($d_6$-DMSO) δ 7.04 (s, 1 H), 7.51 (s, 1 H).

A mixture of the first intermediate (0.21 g, 1.0 mmol), allyl bromide (0.432 g, 3.6 mmol) and K$_2$CO$_3$ (0.331 g, 2.4 mmol) in methanol (10 mL) was stirred under reflux for 12 h, concentrated, and purified by silica gel chromatography to afford 4-amino-2-chloro-6,7-diallyloxyquinazoline.$^1$H-NMR ($d_6$-DMSO) δ 4.63 (d, $J = 5.4$ Hz,
2 H), 4.70 (d, J = 1.5 Hz, 2 H), 5.28 (d, J = 1.3 Hz, 2 H), 5.42 (d, J = 1.3 Hz, 1 H), 5.49 (d, J = 1.3 Hz, 1 H), 6.07-6.11 (m, 2 H), 7.06 (s, 1 H), 7.62 (s, 1 H) ppm.

A solution of the second intermediate (0.291 g, 1.0 mmol) and 1-(biphenyl-4-sulfonyl)-piperazine (0.302 g, 1.0 mmol) in 1-butanol (5 mL) was stirred under reflux for 8 h, and concentrated. The solid residue was stirred with methanol under reflux for 30 min, filtered, washed with methanol to yield compound $43$. $^1$H-NMR ($d_6$-DMSO) $\delta$ 3.36 (br s, 4 H), 4.01 (br s, 4 H), 4.60-4.64 (m, 4 H), 5.26-5.35 (m, 4 H), 6.02-6.13 (m, 2 H), 7.30-7.52 (m, 4 H), 7.72-7.75 (m, 3 H), 7.83-7.86 (m, 4 H), 8.63 (s, 1 H), 8.83 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for $C_{30}H_{32}N_5O_4S$ 558.2169, found 558.2169.

2-[4-(Biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-dipropoxy-quinazolin-4-yl-amine (44):

Compound 44 was synthesized from the procedure described for compound 43. $^1$H-NMR ($d_6$-DMSO) $\delta$ 0.99 (t, J = 7.3 Hz, 6 H), 1.71-1.83 (m, 4 H), 3.12 (br s, 4 H), 3.93-4.14 (m, 8 H), 7.33 (s, 1 H), 7.44-7.54 (m, 3 H), 7.65 (s, 1 H), 7.75-7.79 (m, 2 H), 7.86 (d, J = 8.3 Hz, 2 H), 7.94 (d, J = 8.3 Hz, 2 H). HRMS (M+H)$^+$ calcd for $C_{30}H_{36}N_5O_4S$ 562.2483, found 562.2466.
2-[4-(Biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-diisopropyloxy-quinazolin-4-yl-amine (45):

Compound 45 was synthesized from the procedure described for compound 43. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 1.25 (s, 3 H), 1.27 (s, 3 H), 1.33 (s, 3 H), 1.35 (s, 3 H), 3.12 (brs, 4 H), 3.96 (brs, 4 H), 4.41-4.66 (m, 2 H), 7.36 (s, 1 H), 7.41-7.53 (m, 3 H), 7.68-7.84 (m, 3 H), 7.83-7.86 (m, 4 H), 8.59 (brs, 1 H), 8.76 (br s, NH) ppm. HRMS (M+H)$^+$ calcd for C$_{30}$H$_{36}$N$_5$O$_4$S 562.2483, found 562.2478.

![Chemical structure of 2-[4-(Biphenyl-4-sulfonyl)-piperazin-1-yl]-6,7-diisopropyloxy-quinazolin-4-yl-amine (45)](image)

2-[4-(Biphenyl-4-sulfonyl)-piperazin-1-yl]-6, 7-dibutoxy-quinazolin-4-ylamine (46):

Compound 46 was synthesized from the procedure described for compound 43. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 0.934 (t, $J = 7.5$ Hz, 6 H), 1.44 (q, $J = 7.5$ Hz, 4 H), 1.66-1.812 (m, 4 H), 3.13 (brs, 4 H), 3.91 (brs, 4 H), 3.91-4.08 (m, 4 H), 7.21 (s, 1 H), 7.41-7.53 (m, 3 H), 7.64 (s, 1 H), 7.73 (d, $J = 8.0$ Hz, 1 H), 7.74 (s, 1 H), 7.829-7.864 (m, 3 H), 7.94 (d, $J = 8.0$ Hz, 2 H). HRMS (M+H)$^+$ calcd for C$_{32}$H$_{40}$N$_5$O$_4$S 590.2795, found 590.2770.

1.5.2 CELL CULTURE

PC-3 (p53$^{-/-}$) human androgen-nonresponsive prostate cancer cells were purchased from the American Type Tissue Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator containing 5% CO$_2$. 

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1.5.3 CELL VIABILITY ASSAY

Effect of the test agent on cell viability was assessed by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] assay in 96-well, flat-bottomed plates, in which 8,000 PC-3 or DU-145 cells/well were seeded. Cells were exposed to the test agent at the indicated concentrations, in six replicates, in 10% FBS-supplemented RPMI-1640 medium at 37 °C in 5% CO₂ for 48 hr. The medium was removed and replaced by 150 µL of 0.5 mg/mL of MTT in RPMI-1640 medium, 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 with 200 µL/well DMSO. Absorbance was determined on a plate reader at 570 nm.

1.5.4 WESTERN BLOT ANALYSIS

PC-3 cells (1.5 x 10⁶) treated with the test agent at the indicated concentrations in RPMI 1640 medium for 24 h were collected and sonicated. Protein concentrations of the lysates were determined by using a Bradford protein assay kit (Bio-Rad, Hercules, CA); equivalent amounts of proteins from each lysate were resolved in 10% SDS-polyacrylamide gel and then transferred onto Immobilon-nitrocellulose membranes (Millipore, Bellerica, MA) in a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 40 min, the membrane was incubated with the primary antibody (1:1000 dilution) in TBST-1% nonfat milk at 4 °C overnight. After treatment with the primary antibody, the membrane was washed three times with TBST for a total of 15 min, followed by goat anti-rabbit or anti-mouse IgG-horseradish
peroxidase conjugates (diluted 1:3000) for 1 h at room temperature and wash three times with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

1.5.5 TRANSIENT TRANSFECTION

The constitutively active Akt construct HA-PKB-T308D/S473D was kindly provided by Dr. Brain Hemmings (Friedrich Miescher Institute, Basel, Switzerland). PC-3 cells were seeded into T-75 flasks (1.5 x 10^6/flask). Aliquots containing 3 µg of each plasmid or a control pcDNA3.1(+) vector in 750 µl of Opti-MEM medium (Invitrogen-Life Technologies, Inc.,) was incubated with 9 µl of the FuGene 6 reagent (Roche Diagnostics Corp., Indianapolis, IN) for 15 min. Each flask was washed with Opti-MEM medium and then received the plasmid-FuGene 6 mixture and 4 ml of Opti-MEM medium. The flask was placed in a CO_2 incubator for 4 h, and the transfection medium was replaced with 10% FBS-supplemented RPMI 1640. After 24 h, Mock-, and Akt-transfected PC-3 cells were seeded into 96-well plates at 5,000 cells/well in 10% FBS-supplemented RPMI 1640. On the next day, cells were treated in four replicate with the indicated concentrations of doxazosin in 1% FBS-containing medium for 24 h. MTT assay was used to determine the cell viability.
CHAPTER 2

FROM THE DNA HYPMETHYLATING AGENT PROCAINAMIDE TO THE DEVELOPMENT OF A NOVEL CLASS OF OF BCL-xL INHIBITORS

2.1 APOPTOSIS

Apoptosis or programmed cell-death is a process in which a cell actively terminates itself by the destruction of vital cellular components or DNA by means of various molecular signaling pathways. Apoptosis results in a reduction in cell volume, membrane blebbing and fragmentation into membrane-bound apoptotic bodies, which are then cleared by phagocytes of the immune system (59, 60).

Apoptosis is essential for sculpting during the embryonic and fetal growth, removing unnecessary or outdated structures. In the adult organism, apoptosis also maintains tissue homeostasis, shapes the immune responses and restricts the progress of infections. Therefore, apoptosis is important for differentiation, the regulation of cell numbers and the removal of aged, damaged or autoimmune cells. Apoptosis is triggered upon receiving a death stimulus. The stimuli include receptor ligation, growth-factor withdrawal, chemotoxins or even physical damage. During the induction phase, many different internal pathways can come into action depending on the cell types and the specific stimuli. However, all of these internal pathways converge on one or two control points in the execution or commitment phase of the program before the external phagocyte-

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degradation phase becomes apparent (61).

2.2 APOPTOSIS IN CANCER CELLS

The connection between apoptosis and cancer was determined with the identification of B-cell lymphoma 2 (BCL2). Unlike AKT kinase discussed in the previous chapter, the expression of the BCL2 gene has been shown to inhibit cell death, rather than promote proliferation for cell survival (62). This discovery established the concept that impaired apoptosis is a crucial factor in tumorigenesis. The deregulation of apoptosis confers many advantages to neoplastic cells that favor their survival and proliferation. In the face of typically limiting conditions, such as oxygen deprivation and reduced growth factor and cytokine stimulation, defective apoptosis permits cancer cells to survive, to adapt to these conditions and to acquire a more aggressive phenotype. Defective apoptosis also influences the efficacy of chemotherapy (63, 64) by reducing the response to radiation or chemotherapy.

2.3 MECHANISMS OF APOPTOTIC PATHWAY

Apoptosis is mediated by caspases, a family of intracellular cysteine proteases that are originally synthesized as pro-enzymes (65). Once an initiator caspase is activated by proteolysis, it triggers downstream caspases to cleave cellular proteins that are involved in DNA repair, cytoskeletal organization, nuclear integrity and cell survival. So far, two principle apoptotic pathways for activating caspases have been identified in mammalian cells: the death receptor (or extrinsic) pathway and the stress (or intrinsic) pathway. The death receptor pathway is important for immune selection and inflammation, and is
initiated by ligation of plasma membrane trimeric death receptors, including the tumor necrosis factor-β (TNF-β) receptor and CD95 (FAS) (66). Upon activation of the death receptors, they recruit caspase-8 through the adaptor protein FAS-associated death domain (FADD). Activation of caspase-3 by its upstream protease caspase-8 leads to the final stages of the internal apoptotic pathway.

The stress pathway, which is initiated by stimuli such as cytokine deprivation and genotoxic damage, is regulated by Bcl-2 and related proteins and mainly occurs on the mitochondria membrane. Progression through this pathway involves the dysregulation of mitochondria function (67), which involves an increase in mitochondrial membrane permeability, followed by the release of proteins such as SMAC/DIABLO (68) and cytochrome c (69) into the cytoplasm. Interaction between the released cytochrome c and apoptosis protease-activating factor 1 (APAF1) generates a supramolecular caspase-activating complex called the apoptosome that activates caspase-9 which in turn activates effector caspases. In addition to Bcl-2 family proteins, this apoptotic pathway is negatively regulated by the inhibitor of apoptosis proteins (IAPs) through their inhibition of caspase-9, -3 and -7 (70) (Figure 2.1)
Figure 2.1 Apoptotic pathways and their regulators (70)

2.4 BCL-2 FAMILY

B-cell lymphoma 2 (BCL2) was identified originally as the protooncogene involved in the t(14;18) translocation in human follicular lymphoma (62). It was shown that the expression of Bcl-2 oncogene could enhance the cell’s capacity to survive under suboptimal conditions rather than to provide a proliferative advantage to the cell.
At least twenty Bcl-2-related proteins have been identified, including anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A-1, and pro-apoptotic proteins such as Bax, Bak, Bcl-xS, Bad, Bik, and Bim (71). All Bcl-2 family proteins share homology in one to four regions designated the Bcl-2 homology (BH) domains BH1, BH2, BH3 and BH4 (Figure 2.2). While all four homology regions are present in some of the proteins, including Bcl-2 and Bcl-xL, many others lack one or more of these domains. Some pro-apoptotic family members like Bad, Bid and Bim, contain only the BH3 domain.

Figure 2.2 Classification of the Bcl-2 family of proteins based on domain organization
2.4.1 THE ANTI-APOPTOTIC BCL-2 MEMBERS

Bcl-2 and its closest homologues, Bcl-xL and Bcl-w, potentially inhibit apoptosis in response to diverse cytotoxic insults such as γ- and ultraviolet-irradiation, cytokine withdrawal, dexamethasone, staurosporine, and cytotoxic drugs. Their hydrophobic carboxy-terminal transmembrane domain helps target them to the cytoplasmic face of three intracellular membranes: the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the nuclear envelope (72, 73).

2.4.2 HETERODIMERIZATION BETWEEN ANTI-APOPTOTIC AND PRO-APOPTOTIC MEMBERS

The BH1 and BH2 domains are found in all anti-apoptotic Bcl-2 family members but only in one class of pro-apoptotic family members (class I). In both Bcl-2 and Bcl-xL proteins, residues in the BH1 and BH2 domains are essential for their interaction with pro-apoptotic members such as Bak and Bax. The pro- and anti-apoptotic family members can heterodimerize, thereby titrating each other’s function, suggesting that their relative intracellular concentrations may act as a switch for the suicide program (75). Interestingly, although Bak and Bax possess intact BH1 and BH2 domains, it is the BH3 domain region that is involved in heterodimerization with anti-apoptotic members.

The interaction between Bcl-xL and Bak has been characterized by the use of a truncated 16-residue peptide (residues 72 to 87) derived from the BH3 domain in Bak (Figure 2.3). The minimal region of Bak required to bind to Bcl-xL was examined in a fluorescence-based assay which showed that the truncated Bak peptide binds in a hydrophobic cleft formed by the BH1, BH2, and BH3 regions of Bcl-xL.
Figure 2.3 The heterodimerization between Bcl-xL and Bak peptide. (A) Surface representation of the binding pocket of Bcl-xL bound to the Bak peptide. (B) Depiction of the side chains in the binding site of Bcl-xL (75)

2.4.3 PRO-APOPTOTIC Bak AND Bax INDUCE THE RELEASE OF CYTOCHROME C FROM MITOCHONDRIA

The mitochondrion is of importance for the control of life and death of a cell by releasing death-promoting factors into the cytoplasm in addition to the generation of energy. Cytochrome c is a protein that normally shuttles electrons between protein complexes in the inner mitochondrial membrane (76). Once released from mitochondria, cytochrome c interacts with apoptosis protease-activating factor 1 (APAF1) to generate the apoptosome which activates the downstream caspase-9, resulting in the activation of the caspase cascade and ultimately apoptosis.

Release of cytochrome c from the mitochondria is controlled by Bcl-2 family
members. Evidence shows that anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL can prevent cell death by inhibiting the release of cytochrome c, whereas pro-apoptotic Bcl-2 family members like Bak and Bax can promote cell death via the release of cytochrome c (77).

2.4.4 BCL-2 FAMILY AS A TARGET FOR CANCER THERAPY

Overexpression of the anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL have been reported in 70% of breast cancers, 30-60% of prostate cancers, 80% of B-cell lymphomas, 90% of colorectal adenocarcinomas, and many other types of cancer (78). Additionally, numerous studies have demonstrated that overexpression of Bcl-2 and Bcl-xL result in a poor prognosis and resistance to many chemotherapeutic agents and γ-radiation therapy (79-81). Therefore, the Bcl-2 family represents an attractive target for the treatment of many types of cancer.

2.5 DEVELOPMENT OF SMALL-MOLECULE BCL-xL INHIBITORS

2.5.1 CURRENT SMALL-MOLECULE INHIBITORS

Recently, small molecules capable of antagonizing Bcl-2 or Bcl-xL function have been identified or designed by means of high-throughput, structure-based virtual screening approaches. Small molecules offer several potential advantages over peptide-based drugs, because they can be selected and quickly refined for optimal solubility, cell permeability, stability, binding affinity, and activity. HA14-1 (1) was the first small molecule targeting the BH3 binding site in Bcl-2 identified by a computerized structure-based database screening strategy (82). HA14-1 effectively induced apoptosis in
human acute myeloid leukemia cells (HL-60) overexpressing Bcl-2 protein (Figure 2.4).

![Chemical structures](image)

**Antimycin A3 (5, IC50 = 2.0 µM)**

**Figure 2.4** Structures of small-molecule inhibitors of Bcl-2 and Bcl-xL

Compound 2 was also identified as a Bcl-2 inhibitor through a structure-based computer screening strategy (83). Compound 2 was shown to induce apoptosis in a dose-dependent manner in MDA-MB-231 and HL-60 cell lines overexpressing Bcl-2 protein. Compounds 3 and 4 were identified as inhibitors of Bcl-2 or Bcl-xL from the screening of 16,320 chemicals by fluorescence polarization assay (84). In addition, an antibiotic named antimycin A3 was identified as a small-molecule inhibitor of Bcl-2/Bcl-xL, indicating that structurally diverse small molecules are capable of
inhibiting Bcl-2/Bcl-xL function. By interfering with the interactions between Bcl-2/Bcl-xL and pro-apoptotic proteins (peptides) such as Bak, Bad, and Bax, these small molecule agents free the pro-apoptotic proteins from sequestration by Bcl-2 and Bcl-xL, permitting them to trigger the release of cytochrome c from mitochondria which in turn activate the protease cascade and to induce apoptosis (85).

2.5.2 DEVELOPMENT OF A NOVEL CLASS OF BCL-xL INHIBITORS FROM DNA HYPOMETHYLATING AGENT PROCAINAMIDE

Our research into small molecule inhibitors of Bcl-xL originated with our initial interest in the development of non-nucleoside DNA hypomethylating agents for the reversal of silenced expression of hypermethylated tumor suppressor genes in cancer cells. Procainamide and procaine have been shown to exhibit DNA hypomethylating effects on the $GSTP1$ and $RAR\beta$ genes in LNCaP and MCF-7 cells, respectively. However, the molecular mechanism by which procainamide and procaine demethylate DNA is still unclear. To address this issue and to generate derivatives of procainamide and procaine possessing improved DNA demethylating activity, we synthesized a series of compounds for screening. To our surprise, the effects of these derivatives on cell viability and DNA methylation status did not correlate with each other. A subsequent search for other molecular targets crucial to cell survival revealed that Bcl-xL function was inhibited by these procainamide and procaine derivatives as demonstrated through the fluorescence polarization assay.
2.5.3 MONO-SUBSTITUTED PROCAINAMIDE AND PROCANE

DERIVATIVES ARE MODERATE BCL-xL INHIBITORS

As shown below (Figure 2.5), compounds 8-11 were synthesized from procaine (6) or procainamide (7) by the addition of aryl sulfonylchloride / acyl chloride in the presence of one equivalent of triethyl amine under the indicated conditions.

Reagents: a) aryl sulfonylchloride/ acyl chloride, 1.0 eq Et₃N, CH₂Cl₂

**Figure 2.5** Synthesis of compounds 8-11

<table>
<thead>
<tr>
<th>No.</th>
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<th>IC₅₀ (µM)</th>
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<tr>
<td>9</td>
<td><img src="image" alt="R9" /></td>
<td>O</td>
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<td>NH</td>
<td>&gt;20</td>
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**Table 2.1** Inhibition of Bcl-xL activity by 8-11 as determined by the fluorescence polarization assay
Biphenyl carbonyl and biphenyl sulfonyl substituents in 8 and 10 (IC$_{50}$, 17 and 15 µM, respectively) showed moderate potency in inhibiting the interaction of Bcl-xL and Bak BH3 peptide. This finding suggests that the more hydrophobic nature and twist-like structure of the biphenyl moiety are important for interaction with the hydrophobic pocket of the BH3 domain within the Bcl-xL protein (Table 2.1).

2.5.4 BIS-SUBSTITUTED PROCAINAMIDE DERIVATIVES SHOW IMPROVED INHIBITION AGAINST BCL-xL ACTIVITY

To generate more structurally diversified compounds targeting Bcl-xL activity, we further activated the poor nucleophilic carboxamide moiety by treatment with excess triethyl amine and aryl sulfonyl chloride. As a result, compounds 12-16 were synthesized by the addition of two equivalents of aryl sulfonyl moiety on both amino and amido groups of compound 7 (Figure 2.6).

Reagents: (a) aryl sulfonyl chloride , 5.0 eq Et$_3$N, CH$_2$Cl$_2$

Figure 2.6 Synthesis of 12-16 from 7
By incorporation of a 4-bromo benzenesulfonyl moiety on the amido group, 13 (IC$_{50}$, 17 µM) showed more potent inhibitory activity than 11 (IC$_{50}$, >20 µM) (Table 2.2). This improved potency might be attributable to the introduction of the bulky moiety from 4-bromobenzene ring which increased the favorable interaction with the hydrophobic pocket in the Bcl-xL BH3 domain. In contrast, less bulky substituted groups, such as the methyl group (CH$_3$) in compound 12 and methoxy group (OMe) in compound 15, did not improve inhibitory activity against Bcl-xL. However, consistent with the previous data, compound 14 (IC$_{50}$, 12 µM), which possesses a disubstituted biphenyl moiety, exhibited the most potent inhibitory effect, suggesting that a combination of biphenyl groups and 4-amino benzamide could be a rational approach to develop a new class of Bcl-xL inhibitors. Compound 16 containing a 4-methylsulfonylbenzene moiety also showed improved potency with IC$_{50}$ as low as 14 µM.

<table>
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<th>No.</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
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<tr>
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<td>Ph</td>
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<tr>
<td>15</td>
<td>OMe</td>
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<tr>
<td>16</td>
<td>SO$_2$CH$_3$</td>
<td>14 ± 4</td>
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</table>

Table 2.2 Inhibition of Bcl-xL activity by 12-16 as determined by the fluorescence polarization assay

Using 14 as a lead compound for the continued development of Bcl-xL inhibitors, the synthesis of more potent small molecule agents was attempted by further modification
of the bis-biphenyl moiety. Compounds 17 and 18 were synthesized by Suzuki aryl-aryl cross-coupling of the corresponding 3, 5-dimethyl and 4-acetyl derivatives (Figure 2.7).

![Chemical structures]

Reagents: a) aryl boronic acid, Bu$_4$NF, K$_2$CO$_3$, Pd(OAc)$_2$, H$_2$O.

**Figure 2.7 Synthesis of 17 and 18**

To our surprise, both 17 and 18 showed dramatically decreased potency that was even lower than those of the mono-substituted derivatives such as 8 and 10 (Table 2.3). As indicated previously, the biphenyl moiety is essential for the inhibition of Bcl-xL activity because of its hydrophobicity and twist conformation. However, modification with bulkier groups, such as 3,5-dimethyl and 4-acetyl groups, apparently negates its contribution to Bcl-xL inhibitory activity.

<table>
<thead>
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<th>No.</th>
<th>R</th>
<th>IC$_{50}$ (µM)</th>
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<tr>
<td>17</td>
<td>3,5-dimethyl</td>
<td>&gt;20</td>
</tr>
<tr>
<td>18</td>
<td>4-acetyl</td>
<td>19 ± 3</td>
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**Table 2.3** Inhibition of Bcl-xL activity by 17 and 18 as determined by the fluorescence polarization assay
2.5.5 OPTIMIZING INHIBITORY ACTIVITY BY TRUNCATING THE SIDE CHAIN

With the preservation of the bis-biphenyl groups in lead compound 14, further modifications involved generating derivatives with different lengths of side chain based on the scheme shown in Figure 2.8. Overall, synthesis of compounds 22 to 27 was carried out in three steps. Upon the addition of side chains, intermediates were generated, followed by the hydrogenation of the nitro group to obtain 21a-f. Finally, 22-27 were synthesized as described previously with excess amounts of triethyl amine. Thus, addition of the bis-biphenyl-4-sulfonyl group could be carried out despite the presence of bulky-branched side chains such as the isopropyl group in 26.

Reagents: a) RNH₂, THF; b) H₂, Pd/C, MeOH; c) biphenyl-4-sulfonyl chloride, NEt₃, CH₂Cl₂.

Figure 2.8 Synthesis of compounds 22-27

Truncated side chains, such as hydrogen, methyl, ethyl and 2-chloroethyl groups, in 22, 23, 24, and 25 showed comparable or reduced potencies in comparison to 14 (IC₅₀, 70)
12.9, 13.8, 18.6 and 14.7 µM, respectively) as determined by the fluorescence polarization assay (Table 2.4). However, the bulkier isopropyl substituent in 26 (IC\textsubscript{50}, >20µM) abolished the inhibitory effect, suggesting that steric hindrance stemming from the branched side chain accounts for the decreased interaction between Bcl-xL and small molecule inhibitors. It is noteworthy that 27 containing the N-2-(dimethylamino)ethyl substituent exhibited the most promising inhibitory effect (IC\textsubscript{50}, 7.9 µM) merely by reducing two carbons at the terminal position of the amino side chain in 14. As shown above, these findings indicate that alteration of the side chain might influence the binding of small molecule inhibitors targeting anti-apoptotic Bcl-xL. Moreover, 27 was shown to be completely soluble in the RPMI 1640 medium at the 10 µM treatment level, thus overcoming the solubility problem of 14 that was likely due to its hydrophobicity. Taken together, we have synthesized a new class of Bcl-xL inhibitors derived from 4-amino benzamide as a template. The findings indicate the feasibility of constructing potent small molecule anticancer agents that target essential anti-apoptotic proteins such as Bcl-xL in cancer cells.

<table>
<thead>
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<th>No.</th>
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<th>IC\textsubscript{50} (µM)</th>
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<tbody>
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<td>H</td>
<td>12.9 ± 1.5</td>
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<td>23</td>
<td>CH\textsubscript{3}</td>
<td>13.8 ± 3.1</td>
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<tr>
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<td>18.6 ± 4.2</td>
</tr>
<tr>
<td>25</td>
<td>CH\textsubscript{2}CH\textsubscript{2}Cl</td>
<td>14.7 ± 2.7</td>
</tr>
<tr>
<td>26</td>
<td>CH(CH\textsubscript{3})\textsubscript{2}</td>
<td>&gt;20</td>
</tr>
<tr>
<td>27</td>
<td>CH\textsubscript{2}CH\textsubscript{2}N(CH\textsubscript{3})\textsubscript{2}</td>
<td>7.9 ± 1.4</td>
</tr>
</tbody>
</table>

Table 2.4 Inhibition of Bcl-xL activity by 22-27 as determined by the fluorescence polarization assay
2.5.6 SMALL MOLECULE BCL-XL INHIBITOR CAUSES RELEASE OF CYTOCHROME C AND ACTIVATION OF CASPASE-9 LEADING TO APOPTOSIS

It is well established that modulation of apoptosis by anti-apoptotic Bcl-2 members, such as Bcl-xL, involve the sequestration of pro-apoptotic Bcl-2 members, such as Bak, through BH3 domain-mediated heterodimerization, which in turn prevents cytochrome c release from the mitochondria and abrogates apoptosis. For instance, electrophoretic introduction of Bak BH3 domain peptides into PC-3 cells disrupted Bcl-xL-Bak heterodimer formation, resulting in the liberation of Bak and induction of apoptosis via a caspase-dependent pathway. Based on the fluorescence polarization assay, our in vitro data demonstrated that small molecule inhibitors could block the heterodimerization between Bcl-xL and Bak BH3 domain peptide. Consequently, to validate the mode of action of these small molecule inhibitors, we evaluated the most potent and aqueous-soluble inhibitor, compound 27, in a cell-based system using PC-3 prostate cancer cells as our working model. The downstream events of Bcl-xL-regulated apoptotic signaling in PC-3 cells treated with 27 were assessed by Western blot analysis of cytochrome c release, caspase-9 activation and PARP cleavage (Figure 2.9). PC-3 cells treated with 0, 1, 5 and 10 µM compound 27 for 4 hours exhibited a dose-dependent increase in cytoplasmic cytochrome c which was evident at the 1 µM level. Similarly, activation of caspase-9 was also found to be dose-dependent and indicated that 27 mediated cell death through a caspase-dependent pathway. Finally, the cleavage of PARP was observed at 5 µM after the 4-hour treatment. These data are consistent with a mechanism by which 27 inhibits Bcl-xL activity, resulting in the liberation of
pro-apoptotic Bak followed by the release of cytochrome c, activation of caspases, and eventually, cleavage of their cellular substrates, such as PARP.

![Table](image)

**Figure 2.9** Apoptosis induced by 27 in PC-3 cells as indicated by cytochrome c release, cleaved caspase-9, and cleavage of PARP.

To further examine the effect of 27 on cell viability, PC-3 cells were exposed to several concentrations of 27 in 10% FBS-supplemented RPMI 1640 medium for 24 hours. Compound 27 reduced the viability of PC-3 cells in a dose-dependent manner (Figure 2.10). At 10 µM and above, 27 caused a greater than 80% reduction in the cell viability. These results show that the antiproliferative effect of 27 in PC-3 cells corresponds with its inhibition of the anti-apoptotic Bcl-xL protein and the activation of the caspase-dependent apoptotic pathway.
Figure 2.10 Effect of 27 on the cell viability of PC-3 cells. The cells were incubated with 27 at different concentrations for 24 hours. The cell viability was determined by MTT assay in 96-well plate.

2.6 CONCLUSION

In this study, we have synthesized procainamide and procaine derivatives which induce antiproliferative effects by blocking the activity of anti-apoptotic Bcl-xL rather than by modulating DNA methylation status. This finding led us to develop a novel class of Bcl-xL inhibitors based on the procainamide structure. The bis-biphenyl substituted derivatives were shown to be optimal in inhibiting Bcl-xL activity as determined by fluorescence polarization assay. Moreover, compound 27, in addition to being the most potent inhibitor, was devoid of the solubility problem encountered with the lead
compound 14 that stemmed from the truncation of two methyl groups at the terminal side chain. In cell-based assays, Western blotting data from 27-treated PC-3 cells showed the release of cytochrome c from the mitochondria, which is consistent with the liberation of Bak from heterodimerization with Bcl-xL as was indicated in the fluorescence polarization assay. The activation of caspase-9 was also observed in these treated PC-3 cells indicating that 27-induced apoptosis was mediated through the intrinsic pathway. This novel class of small molecule-based Bcl-xL inhibitors may have value in therapeutic strategies for the treatment of Bcl-xL-overexpressing cancers in the future.

2.7 EXPERIMENTAL SECTION

Chemical reagents and organic solvents were purchased from Aldrich unless otherwise mentioned. Nuclear magnetic resonance spectra (1H NMR) were measured on a Bruker 250-MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to the TMS peak. Electrospray ionization (ESI) mass spectrometry analyses were performed with a 3-T Finnigan FTMS-2000 Fourier transform mass spectrometer. Elemental analyses were within ±0.4% of the calculated values. Flash column chromatography was performed with silica gel (230-400 mesh).
2.7.1 CHEMICAL PREPARATION

2-(Diethylamino)ethyl 4-biphenyl-4-ylcarboxamidobenzoate (8)

To a solution of 6 (2.36 g, 10.0 mmol) in dry CH₂Cl₂, triethyl amine (1.01g, 10.0 mmol) and biphenyl-4-carbonyl chloride (2.37g, 11.0 mmol) were added to the reaction. The mixture was stirred at room temperature overnight. The mixture was washed with saturated NaHCO₃ and H₂O. The organic layer was dried with MgSO₄, concentrated in vacuo to obtain the crude product. The product was purified by flash column chromatography with dichloromethane-hexane (1:1) as eluent to afford 8 as a solid.

¹H-NMR (CDCl₃) δ 0.97-1.03 (m, 6 H), 2.52-2.60 (m, 4 H), 2.75 (t, J = 6.2 Hz, 2 H), 4.26 (t, J = 6.2 Hz, 2 H), 7.41-7.54 (m, 3 H), 7.66 (d, J = 7.4 Hz, 2 H), 7.73-7.81 (m, 4 H), 7.98 (d, J = 8.2 Hz, 2 H), 8.08-8.12 (m, 3 H) ppm. HRMS (M+H)+ calcd for C₂₆H₂₉N₂O₄ 417.2173, found 417.2177.

2-(Diethylamino)ethyl 4-(4-bromophenylsulfonamido)benzoate (9)

Compound 9 was synthesized from the procedure described for compound 8.
\(^1\)H-NMR \((d_6\text{-DMSO}) 0.97 \text{ (t, } J = 7.1 \text{ Hz, 6 H), 2.54-2.57 \text{ (m, 4 H), 2.76-2.78 \text{ (m, 2 H), 4.33 \text{ (t, } J = 5.8 \text{ Hz, 2 H), 7.24 \text{ (s, 1 H), 7.25 \text{ (s, 1 H), 7.77 \text{ (d, } J = 2.5 \text{ Hz, 2 H), 7.91 \text{ (d, } J = 2.5 \text{ Hz, 2 H), 7.99 \text{ (s, 1 H), 8.02 \text{ (s, 1 H) ppm. HRMS (M+H)}^+ \text{ calcd for } C_{19}H_{23}BrN_3O_4S 455.0635, \text{ found 455.0639.}}

\[
\begin{array}{c}
\text{O} \text{S} \text{O} \\
\text{N} \text{H}
\end{array}
\]

2-(Diethylamino)ethyl 4-(biphenyl-4-ylsulfonamido)benzoate (10)

Compound 10 was synthesized from the procedure described for compound 8.

\(^1\)H-NMR \((d_6\text{-Acetone}) \delta 1.01-1.06 \text{ (m, 6 H), 2.55-2.61 \text{ (m, 4 H), 2.80 \text{ (t, } J = 6.2 \text{ Hz, 2 H), 4.32 \text{ (t, } J = 6.1 \text{ Hz, 2 H), 7.17 \text{ (d, } J = 8.6 \text{ Hz, 2 H), 7.24-7.26 \text{ (m, 3 H), 7.53-7.55 \text{ (m, 2 H), 7.64 \text{ (d, } J = 8.2 \text{ Hz, 2 H), 7.83-7.93 \text{ (m, 4 H) ppm. HRMS (M+H)}^+ \text{ calcd for } C_{25}H_{29}N_2O_4S 453.1843, \text{ found 453.1825.}}

\[
\begin{array}{c}
\text{N} \text{H} \\
\text{O} \text{S} \text{O} \\
\text{Br}
\end{array}
\]

4-(4-Bromo-benzenesulfonylamino)-N-(2-diethylamino-ethyl)-benzamide (11)

Compound 11 was synthesized from the procedure described for compound 8.

\(^1\)H-NMR \((d_6\text{-DMSO}) \delta 1.02-1.08 \text{ (m, 6 H), 2.59-2.77 \text{ (m, 6 H), 3.46-3.53 \text{ (m, 2 H), 7.13 \text{ (d, } J = 8.7 \text{ Hz, 2 H), 7.52 \text{ (d, } J = 8.7 \text{ Hz, 2 H), 7.61-7.68 \text{ (m, 4 H) ppm. HRMS (M+H)}^+ \text{ calcd for } C_{25}H_{29}N_2O_4S 453.1843, \text{ found 453.1825.}}

\[
\begin{array}{c}
\text{B} \text{r} \\
\text{N} \text{H} \\
\text{O} \text{S} \text{O} \\
\text{N} \text{H}
\end{array}
\]
calcd for C_{19}H_{25} BrN_{3}O_{3} S 454.0794, found 454.0792.

N-(2-(Diethylamino)ethyl)-4-(4-methylphenylsulfonamido)-N-tosylbenzamide  (12)

To a solution of 7 (2.35 g, 10.0 mmol) in dry CH_{2}Cl_{2}, triethyl amine (5.05 g, 50.0 mmol) and 4-methylbenzene-1-sulfonyl chloride (4.18g, 22.0 mmol) were added to the reaction. The mixture was stirred at room temperature overnight. The mixture was washed with saturated NaHCO_{3} and H_{2}O. The organic layer was dried with MgSO_{4}, concentrated in vacuo to obtain the crude product. The product was purified by flash column chromatography with dichloromethane-hexane (1:1) as eluent to afford 12 as a solid. \(^{1}\)H-NMR (\(d_{6}\)-DMSO) \(\delta\) 1.09-1.26 (m, 6 H), 2.39 (s, 3 H), 2.46 (s, 3 H), 3.17-3.25 (m, 6 H), 3.65 (d, \(J = 5.8\) Hz, 2 H), 7.12 (d, \(J = 8.5\) Hz, 2 H), 7.48-7.51 (m, 4 H), 7.61-7.67 (m, 4 H), 7.95 (d, \(J = 8.5\) Hz, 2 H), 9.04 (s, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C_{27}H_{34}N_{3}O_{5}S_{2} 544.1940, found 544.2325.

4-(4-Bromophenylsulfonamido)-N-(4-bromophenylsulfonyl)-N-(2-(diethylamino)ethyl)benzamide (13)

Compound 13 was synthesized from the procedure described for compound 12. \(^{1}\)H-NMR
(\textit{d}_6-\text{DMSO}) \delta 1.21-1.26 (m, 6 H), 3.20-3.32 (m, 6 H), 3.65-3.71 (m, 2 H), 7.22 (d, \textit{J} = 7.5 Hz, 2 H), 7.75-7.78 (m, 4 H), 7.93-7.96 (m, 6 H), 8.92 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{25}$H$_{28}$Br$_2$N$_3$O$_5$S$_2$ 673.9811, found 673.9781.

\[ \text{4-(Biphenyl-4-ylsulfonamido)-N-(biphenyl-4-ylsulfonyl)-N-(2-(diethylamino)ethyl)benzamide (14)} \]

Compound 14 was synthesized from the procedure described for compound 12. \textsuperscript{1}H-NMR (\textit{d}_6-\text{DMSO}) \delta 0.94-0.99 (m, 6 H), 3.13 (m, 8 H), 7.21 (d, \textit{J} = 8.2 Hz, 2 H), 7.48-7.58 (m, 6 H), 7.79-8.02 (m, 14 H), 8.54 (s, 1 H) ppm. HRMS (M+H)$^+$ calcd for C$_{37}$H$_{38}$N$_3$O$_5$S$_2$ 668.2253, found 668.2245.

\[ \text{N-(2-(Diethylamino)ethyl)-4-(4-methoxyphenylsulfonamido)-N-(4-methoxyphenylsulfonyl)benzamide (15)} \]

Compound 15 was synthesized from the procedure described for compound 12. \textsuperscript{1}H-NMR (\textit{d}_6-\text{DMSO}) \delta 1.19-1.25 (m, 6 H), 3.19-3.24 (m, 6 H), 3.63-3.65 (m, 2 H), 3.90 (s, 6 H), 7.10-7.21(m, 6 H), 7.71-7.75 (m, 4 H), 7.93-7.96 (d, \textit{J} = 7.7 Hz, 2 H), 8.99 (s, 1
H) ppm. HRMS (M+Na)\(^+\) calcd for C\(_{27}H_{33}N_3O_7NaS_2\) 598.1658, found 598.0339.

N-(2-(diethylamino)ethyl)-4-(4-(methylsulfonyl)phenylsulfonamido)-N-(4-(methylsulfonyl)phenylsulfonyl)benzamide (16)

Compound 16 was synthesized from the procedure described for compound 12. ¹H-NMR (\(d_6\)-DMSO) \(\delta\) 0.94-1.01 (m, 6 H), 2.51 (m, 2 H), 3.29-3.32 (m, 6 H), 3.38 (s, 3 H), 3.45 (s, 3 H), 7.25 (d, \(J = 8.4\) Hz, 2 H), 7.90 (d, \(J = 8.4\) Hz, 2 H), 8.11-8.15 (m, 4 H), 8.25-8.28 (m, 4 H), 8.87 (s, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{27}H_{34}N_3O_9S_4\) 672.1178, found 672.1273.

N-(2-(Diethylamino)ethyl)-N-(3',5'-dimethylbiphenyl-3-ylsulfonyl)-4-(3',5'-dimethylbiphenyl-4-ylsulfonamido)benzamide (17)

Under argon, compound 13 (0.673 g, 1.0 mmol), K\(_2\)CO\(_3\) (0.345 g, 2.5 mmol), Bu\(_4\)NBr (0.322 g, 1.0 mmol) and Pd(OAc)\(_2\) (11 mg, 5 mol%) were added to a stirred solution of 4-phenanthrenylboronic acid (0.243 g, 1.1 mmol) in H\(_2\)O (5 mL). The reaction
mixture was vigorously stirred at 70 °C for 1 h, cooled to room temperature, and added ethyl acetate (10 mL). The organic layer was dried and concentrated to obtain the mixture product. The crude product was purified by flash column chromatography with dichloromethane-hexane (1:1) as eluent to afford 17 as a solid. \[^1\text{H}-\text{NMR}\ (d_6-\text{DMSO})\] 0.89-1.01 (m, 6 H), 1.25-1.34 (m, 2 H), 1.56 (m, 2 H), 2.36 (s, 12 H), 2.60 (m, 2 H), 3.12-3.18 (m, 2 H), 7.12 (m, 2 H), 7.20 (d, \(J = 8.3\) Hz, 2 H), 7.39 (m, 4 H), 7.75-7.96 (m, 10 H), 8.61 (s, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{41}\)H\(_{46}\)N\(_3\)O\(_5\)S\(_2\) 724.2879, found 724.2656.

\[
\begin{align*}
\text{N-(4'-Acetylbiphenyl-3-ylsulfonyl)-4-(4'-acetylbiphenyl-4-ylsulfonamido)-N-(2-(dietylamino)ethyl)benzamide (18)}
\end{align*}
\]

Compound 18 was synthesized from the procedure described for compound 17. \[^1\text{H}-\text{NMR}\ (d_6-\text{DMSO})\] 0.93-0.99 (m, 6 H), 2.64 (s, 6 H), 3.28-3.33 (m, 8 H), 7.22 (d, \(J = 8.5\) Hz, 2 H), 7.87 (d, \(J = 8.5\) Hz, 2 H), 7.94-7.97 (m, 8 H), 8.07-8.12 (m, 8 H), 8.54 (s, 1 H) ppm. HRMS (M+H)\(^+\) calcd for C\(_{41}\)H\(_{42}\)N\(_3\)O\(_7\)S\(_2\) 752.2459, found 752.2457.
N-Methyl-4-nitro-benzamide (20b)

To a solution of 4-nitrobenzoyl chloride (1.85 g, 10.0 mmol) in dry 10 mL CH₂Cl₂, methylamine (0.632 g, 20.0 mmol, 40 wt. % solution in H₂O) was added to the reaction. The mixture was stirred at room temperature for 2 hours. The mixture was washed with 1.0 M HCl and H₂O. The organic layer was dried with MgSO₄, concentrated in vacuo to obtain the crude product. The product was purified by flash column chromatography with dichloromethane-hexane (1:4) as eluent to afford 20b as a yellow solid. ¹H-NMR (d₄-MeOH) δ 2.87 (s, 3 H), 7.94 (d, J = 8.8 Hz, 2 H), 8.24 (d, J = 8.8 Hz, 2 H) ppm.

4-Amino-N-methyl-benzamide (21b)

A suspension of compound 20b (0.18 g, 1.0 mmol) in THF (20 mL) was vigorously stirred with palladium hydroxide (10%) under an atmosphere of hydrogen. The reaction mixture was stirred for 12 hours, filtered on Celite, and CH₂Cl₂ was added. The crude product was concentrated in vacuo and purified by flash column chromatography with dichloromethane-hexane (1:2) as eluent to give rise to 21b. ¹H-NMR (CDCl₃) δ 2.95 (d, J = 4.5 Hz, 3 H), 5.95 (brs, 1 H), 6.63 (d, J = 8.5 Hz, 2 H), 7.56 (d, J = 8.5 Hz, 2 H) ppm.
N-(2-Chloro-ethyl)-4-nitro-benzamide (20d)

Compound 20d was synthesized from the procedure described for compound 20b.

$^1$H-NMR (CDCl$_3$) $\delta$ 3.77 (t, $J = 4.7$ Hz, 2 H), 3.84 (t, $J = 4.7$, 2 H), 6.57 (brs, 1 H), 7.94 (d, $J = 8.8$ Hz, 2 H), 8.29 (d, $J = 8.8$ Hz, 2 H) ppm.

4-Amino-N-(2-chloro-ethyl)-benzamide (21d)

Compound 21d was synthesized from the procedure described for compound 21b.

$^1$H-NMR (CDCl$_3$) $\delta$ 3.68 (t, $J = 4.7$ Hz, 2 H), 3.74 (t, $J = 4.7$ Hz, 2 H), 5.45 (brs, 1 H), 6.62 (d, $J = 8.5$ Hz, 2 H), 7.58 (d, $J = 8.5$ Hz, 2 H) ppm.

N-Isopropyl-4-nitro-benzamide (20e)

Compound 20e was synthesized from the procedure described for compound 20b.

$^1$H-NMR (CDCl$_3$) $\delta$ 1.27 (d, $J = 6.5$ Hz, 6 H), 4.24 (m, 1 H), 5.93 (brs, 1 H), 7.89 (d, $J = 8.5$ Hz, 2 H), 8.26 (d, $J = 8.5$ Hz, 2 H) ppm.
4-Amino-N-isopropyl-benzamide (21e)

Compound 21e was synthesized from the procedure described for compound 21b.

$^1$H-NMR (CDCl$_3$) $\delta$ 1.22 (d, $J = 6.5$ Hz, 6 H), 4.22 (m, 1 H), 5.23 (brs, 1 H), 6.65 (d, $J = 8.3$ Hz, 2 H), 7.63 (d, $J = 8.3$ Hz, 2 H) ppm.

N-(2-(dimethylamino)ethyl)-4-nitrobenzamide (20f)

Compound 20f was synthesized from the procedure described for compound 20b.

$^1$H-NMR (CDCl$_3$) $\delta$ 2.26 (s, 6 H), 2.74 (t, $J = 6$ Hz, 2 H), 3.51 (t, $J = 5.2$ Hz, 2 H), 7.03 (brs, 1 H), 7.93 (d, $J = 8.7$ Hz, 2 H), 8.25 (d, $J = 8.7$ Hz, 2 H) ppm.

4-amino-N-(2-(dimethylamino)ethyl)benzamide (21f)

Compound 21f was synthesized from the procedure described for compound 21b.

$^1$H-NMR (CDCl$_3$) $\delta$ 2.25 (s, 6 H), 2.48 (t, $J = 6$ Hz, 2 H), 3.47 (t, $J = 5.5$ Hz, 2 H), 3.93 (brs, 1 H), 6.62 (d, $J = 8.6$ Hz, 2 H), 7.60 (d, $J = 8.6$ Hz, 2 H) ppm.
4-(Biphenyl-4-ylsulfonamido)-N-(biphenyl-4-ylsulfonyl)benzamide (22)

Compound 22 was synthesized from the procedure described for compound 12. 
$^1$H-NMR (acetone-$d_6$) δ 7.22 (s, 1 H), 7.25 (s, 3 H), 7.45-7.58 (m, 6 H), 7.79-7.82 (m, 4 H), 7.82-8.03 (m, 10 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{31}$H$_{24}$N$_2$O$_5$S$_2$Na$^+$ 591.1019, found 591.1002.

N-(Biphenyl-3-ylsulfonyl)-4-(biphenyl-4-ylsulfonamido)-N-methylbenzamide (23)

Compound 23 was synthesized from the procedure described for compound 12. 
$^1$H-NMR ($d_4$-MeOH) δ 7.21-7.25 (m, 2 H), 7.45-7.56 (m, 6 H), 7.74-7.77 (m, 4 H), 7.78-7.87 (m, 10 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{32}$H$_{26}$N$_2$O$_5$S$_2$Na$^+$ 605.1175, found 605.1154.
N-(Biphenyl-3-ylsulfonyl)-4-(biphenyl-4-ylsulfonamido)-N-ethylbenzamide (24)

Compound 24 was synthesized from the procedure described for compound 12. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 1.23 (t, $J = 8.6$ Hz, 3 H), 3.30 (q, $J = 7.1$ Hz, 2 H), 7.19 (s, 1 H), 7.23 (s, 1 H), 7.47-7.59 (m, 6 H), 7.80 (s, 2 H), 7.83 (s, 2 H), 7.88-8.03 (m, 10 H) ppm.

HRMS (M+Na)$^+$ calcd for C$_{33}$H$_{28}$N$_2$O$_5$S$_2$Na$^+$ 619.1332, found 619.1338.

N-(Biphenyl-3-ylsulfonyl)-4-(biphenyl-4-ylsulfonamido)-N-(2-chloroethyl)benzamide (25)

Compound 25 was synthesized from the procedure described for compound 12. 

$^1$H-NMR ($d_6$-DMSO) $\delta$ 3.67-3.76 (m, 2 H), 3.97-4.03 (m, 2 H), 7.21 (s, 1 H), 7.24 (s, 1 H), 7.48-7.57 (m, 6 H), 7.78 (s, 1 H), 7.81 (s, 2 H), 7.88-7.93 (m, 6 H), 7.98-8.14 (m, 4 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{33}$H$_{27}$ClN$_2$O$_5$S$_2$Na$^+$ 653.0948, found 653.0976.
N-(Biphenyl-3-ylsulfonyl)-4-(biphenyl-4-ylsulfonamido)-N-isopropylbenzamide (26)

Compound 26 was synthesized from the procedure described for compound 12. 

$^1$H-NMR ($d_6$-acetone) $\delta$ 1.22 (s, 3 H), 1.25 (s, 3 H), 4.13-4.26 (m, 1 H), 7.18 (s, 1 H), 7.21 (s, 1 H), 7.48-7.58 (m, 6 H), 7.79-7.81 (m, 4 H), 7.79-7.82 (m, 4 H), 7.98-8.14 (m, 6 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{34}$H$_{30}$N$_2$O$_5$S$_2$Na$^+$ 633.1488, found 633.1509.

N-(Biphenyl-3-ylsulfonyl)-4-(biphenyl-4-ylsulfonamido)-N-(2-(dimethylamino)ethyl)benzamide (27)

Compound 27 was synthesized from the procedure described for compound 12. $^1$H-NMR (CDCl$_3$) $\delta$ 2.19 (s, 3 H), 2.22 (s, 3 H), 2.61 (t, $J$ = 5.8 Hz, 2 H), 3.54 (d, $J$ = 5.1 Hz, 1 H), 3.58 (d, $J$ = 5.1 Hz, 1 H), 7.15 (s, 1 H), 7.18 (s, 1 H), 7.40-7.52 (m, 6 H), 7.62 (s, 2 H), 7.65 (s, 2 H), 7.73 (s, 2 H), 7.76 (s, 2 H), 7.82 (s, 1 H), 7.85 (s, 1 H), 7.79 (s, 2 H), 8.01 (s, 2 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{35}$H$_{33}$N$_3$O$_5$S$_2$Na$^+$ 662.1754, found 662.1729.
2.7.2 CELL CULTURE

PC-3 (p53<sup>−/−</sup>) human androgen-nonresponsive prostate cancer cells were purchased from the American Type Tissue Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator containing 5% CO₂.

2.7.3 CELL VIABILTY ANALYSIS

Effect of 27 on cell viability was assessed by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] assay in 96-well, flat-bottomed plates, in which 8,000 PC-3 cells/well were seeded. Cells were exposed to 27 at the indicated concentrations, in six replicates, in 10% FBS-supplemented RPMI-1640 medium at 37 °C in 5% CO₂ for 24 hours. The medium was removed and replaced by 150 µL of 0.5 mg/mL of MTT in RPMI-1640 medium, and cells were incubated in the CO₂ incubator at 37 °C for 2 hrs. Supernatants were removed from the wells and the reduced MTT dye was solubilized with 200 µL/well DMSO. Absorbance was determined on a plate reader at 570 nm.

2.7.4 WESTERN BLOT ANALYSIS OF CYTOCHROME C RELEASE INTO THE CYTOPLASM

Cytosolic-specific, mitochondria-free lysates were prepared according to an established procedure. In brief, after 27 treatments for 4 hours, both the incubation medium and adherent cells in T-75 flasks were collected and centrifuged at 200 x g for 5 minutes. The pellet fraction was recoverd, placed on ice, and triturated with 300 µL of a
chilled hypotonic lysis solution [50 mmol/L PIPES-KOH (PH 7.4) containing 220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol MgCl₂, 1 mmol DTT, and a mixture of protease inhibitors including 100 μmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 nmol/L aprotinin, 5 μmol/L bestatin, 1.5 μmol/L E-64 protease inhibitor, 2 μmol/L leupeptin, and 1 μmol/L pepstatin A]. After 45-minute incubation on ice, the mixture was centrifuged at 200 × g for 10 minutes. The supernatant was collected in a microcentrifuge tube, and centrifuged at 14,000 rpm for 30 minutes. An equivalent amount of protein (50 μg) from each was resolved in 10% SDS-polyacrylamide gel. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome c antibodies as described below.

2.7.5 IMMUNOBLOTTING

PC-3 cells (1.5 x 10⁶) treated with 27 at the indicated concentrations in 10% FBS-supplemented RPMI 1640 medium for 4 hours were collected and sonicated. Protein concentrations of the lysates were determined by using a Bradford protein assay kit (Bio-Rad, Hercules, CA); equivalent amounts of proteins from each lysate were resolved in 10% SDS-polyacrylamide gel and then transferred onto Immobilon-nitrocellulose membranes (Millipore, Bellerica, MA) in a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 40 min, the membrane was incubated with the primary antibody (1:1000 dilution) in TBST-1% nonfat milk at 4 °C overnight. After treatment with the primary antibody, the membrane was washed three times with TBST for a total of 15 min, followed by goat anti-rabbit or anti-mouse
IgG-horseradish peroxidase conjugates (diluted 1:3000) for 1 h at room temperature and wash three times with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

2.7.6 COMPETITIVE FLUORESCENCE POLARIZATION ASSAY

The binding affinity of the test agent to Bcl-xL was analyzed by a competitive fluorescence polarization assay in which the ability of the agent to displace the binding of a Bak BH3-domain peptide to Bcl-xL was determined. Flu-BakBH3, a Bak-BH3 peptide labeled at the NH$_2$ terminus with fluorescein, was purchased from Genemed Synthesis (San Francisco, CA). COOH-terminal-truncated, His-tagged Bcl-xL was purchased from EMD Bioscences (San Diego, CA). The Kd determination was carried out in a dual-path length quartz cell with readings at $\lambda_{em}$ 480 nm and $\lambda_{ex}$ 530 nm at room temperature using a luminescence spectrometer according to an established procedure (86).

2.7.7 DETERMINATION OF IC$_{50}$ VALUES

Data from fluorescence polarization assay were analyzed by using the CalcuSyn software (Biosoft, Ferguson, MO) to determine IC50 values, in which the calculation was based on the medium-effect equation [i.e., $\log(f_a/f_u) = m\log(D) - m\log(D_m)$], where $f_a$ and $f_u$ denote fraction affected and unaffected, respectively; $m$ represents the Hill-type coefficient signifying the sigmoidicity of the dose-effect curve; and D and Dm are the dose used and IC$_{50}$, respectively] (87).
CHAPTER 3

(-)-EPICATECHIN AS A LEAD FOR THE DEVELOPMENT OF CHEMICALLY STABLE DNA HYPOMETHYLATING AGENTS THAT REVERSE HYPERMETHYLATED TUMOR SUPPRESSOR GENES

3.1 DNA METHYLATION

Carcinogenesis is a process of somatic evolution in that a cell acquires selective advantages through stepwise accumulation of changes in gene function. Every advantageous change can be stably transmitted to the daughter cells and is usually accompanied by a series of clonal expansions. It has been known that many of the cancer-associated gene changes originate from the gain, loss, or mutation of genetic information. However, it is now clear that epigenetic events, such as DNA methylation and histone deacetylation, may also play an important role in many cancers (88-90).

DNA methylation is one of the major epigenetic events for the control of gene expression. It has profound effects on the mammalian genome, including transcriptional repression, modulation of chromatin structure, X chromosome inactivation, genomic imprinting, and mediating detrimental effects of repetitive and parasitic DNA sequences on genome integrity (91-93). Although DNA methylation clearly enhances the ability of cells to regulate and package the genetic information, it also adds an additional burden if it is not well regulated in the cells.
3.1.1 DNA METHYLATION IN CpG ISLANDS

DNA methylation in the human genome occurs almost exclusively at cytosine residues within the regions rich in the CpG symmetric dinucleotide known as CpG islands (94). Methylated cytosine accounts for 0.75~1% of the total DNA bases, and ~70% of all CpG dinucleotides are methylated (95, 96). Methylated cytosines are widely spread throughout the genome while unmethylated cytosines are mainly confined to relatively high densities of CpG islands (97). Under normal conditions, most CpG islands remain free of methylation and are associated with transcriptionally active genes, predominantly the so-called “housekeeping” genes such as *GSTP1*. However, certain CpG islands are methylated, including those associated with imprinted genes and genes on the inactive X chromosome (98).

3.1.2 THE MECHANISM OF DNA METHYLATION

DNA methylation is a covalent bond reaction in which the 5-position of a cytosine residue is methylated by transfer of a methyl group from S-adenosyl-L methionine (SAM). 5-Methylcytosine DNA methyltransferases catalyze the reaction (99) in which the target cytosine of the CpG dinucleotide is extruded from the double helix into the active site cleft of the enzyme where it can be reached by a conserved active site cysteine (Figure 3.1).
3.1.3 DNA METHYLTRANSFERASES IN MAMMALS

So far, five genes encoding DNA methyltransferases (DNMTs) (including potential DNMT-like genes that may not be enzymatically active) have been identified in mammalian cells, DNMT1, 2, 3A, 3B and 3L (100-102). These five genes can be divided into three categories, primarily according to function: the maintenance DNA methyltransferase DNMT1, the de novo DNA methyltransferases DNMT3A and DNMT 3B, and the DNMT-like proteins DNMT2 and DNMT3L (Figure 3.2).
DNMT1 has been shown to have a 10-40 fold preference for hemimethylated DNA and is the most abundant methyltransferase in somatic cells (104, 105). DNMT1 targets to replication loci during S-phase and interacts with the proliferating cell nuclear antigen (PCNA) and the retinoblastoma tumor suppressor protein (Rb) (106-109). This set of features is why DNMT1 is often referred to as the “maintenance” methyltransferase because it is believed to be the primary enzyme responsible for copying methylation patterns after DNA replication (104). The DNA methyltransferases DNMT3A and DNMT3B have an equal preference for hemimethylated and unmethylated DNA.
substrates (110). Therefore, they are classified as “de novo” methyltransferases. Both of these enzymes are essential for the waves of de novo methylation occurring in embryonic cells following implantation. These enzymes also mediate de novo methylation of newly integrated parasitic DNA sequences, such as retroviruses, as part of a host cell ‘genome defense system’. The DNMT-like proteins DNMT2 and DNMT3L possess all or some of the highly conserved methyltransferase catalytic motifs, respectively. However, none of them has been shown to display enzymatic activity in vitro (100).

3.1.4 CpG ISLAND HYPERMETHYLATION AND CANCER

CpG island hypermethylation has been described in every cancer type. Numerous examples exist of aberrant CpG island hypermethylation in the promoters of tumor suppressor genes (such as RassF1A) (111-113), and genes involved in cell adherence (CHD1, CHD13), DNA repair (hMLH1, MGMT and APC) (114), cell cycle (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b} and p14\textsuperscript{ARF}) (115), apoptosis (DARK) (116), detoxification (GSTP1) (117, 118), hormonal response (RAR\beta, ER) (119, 120) and others. However, the mechanisms of aberrant methylation and its selectivity for certain genes are still not understood. Moreover, hypermethylation is not an isolated layer of epigenetic control, but is linked to the action other epigenetic regulatory molecules, such as methyl-binding proteins, histone deacetylases (121) and histone methyltransferases (122).

3.1.5 DNA METHYLATION LEADS TO TRANSCRIPTIONAL GENE SILENCING

The connection between DNA methylation and transcriptional silencing is firmly
established. Currently, the widely accepted explanation of the mechanism for DNA methylation-mediated gene silencing describes the involvement of proteins that bind selectively to methylated DNA. The first such protein, MeCP2, can be divided into two structural domains: a methyl-CpG binding domain (MBD), which recognizes a symmetrically methylated CpG dinucleotide through contacts in the major groove of the double helix, and a transcriptional repression domain (TRD), which interacts with several other regulatory proteins. In addition, a second mechanism by which DNA methylation could inhibit gene transcription is through the sterically restricted access of transcription factors to DNA resulting from the binding of other methyl-CpG binding proteins (MBDs) (123).

3.1.6 RELATIONSHIP BETWEEN DNA METHYLATION AND HISTONE DEACETYLATION

As mentioned previously, DNA methylation leading to transcriptional silencing is not an isolated epigenetic event and is associated with other epigenetic regulatory processes, such as histone deacetylation. The catalytic subunit of the histone deacetylases (HDAC) removes acetyl groups from the core histone tails, resulting in assembly of tightly packed chromatin which renders promoters inaccessible to the transcriptional machinery by increasing the affinity of histones for DNA and ultimately results in transcriptional repression. An association between DNA methylation and histone deacetylation has been shown to be mediated by MBD proteins, such as MeCP2, which binds to methylated DNA and then recruits the corepressor / HDAC protein complex. Furthermore, recent studies have revealed that DNA methylation and histone deacetylation may be more
tightly linked than ever because DNA methyltransferases and histone deacetylases can directly interact (Figure 3.3). As a matter of fact, HDACs have now been found to interact with all of the catalytically active DNMTs, and DNA methylation and histone deacetylation are reported to act synergistically to repress gene transcription (124).

Figure 3.3 DNMT associated proteins (103)

3.1.7 DNA METHYLATION AS A THERAPEUTIC TARGET

Although both genetic and epigenetic mechanisms contribute to the inactivation of tumor suppressor genes during carcinogenesis, there are some differences between the processes that may be significant for anticancer therapy. First of all, genetic mechanisms such as the gain, loss, or mutation of genetic information confer a fixed, irreversible state of gene inactivation, while epigenetic events do not interfere with the information content of the affected genes and are potentially reversible (125-127). Epigenetic silencing of
tumor suppressor genes may be alleviated at two different levels: inhibition of DNA methylation (e.g. 5-aza-2’-deoxycytidine) and inhibition of histone deacetylation (e.g. trichostatin A) as shown in Figure 3.4. The inhibition of DNMTs, especially DNMT1, would block the hypermethylation of the newly synthesized DNA strand, resulting in the reversal of the hypermethylation and the re-expression of the silenced genes.

**Figure 3.4** Targeting epigenetic machinery (128)

### 3.1.8 DNA HYPMETHYLATING AGENTS

Nucleoside analogues, such as 5-azacytidine (5-aza-C) and 5-aza-2’-deoxycytidine (also called 5-aza-dC, or decitabine), were first synthesized by Piskala and Sorm in 1964 and shown to possess DNA hypomethylating activity (Figure 3.5) (129).
Figure 3.5 Structures of nucleoside analogues as DNMT inhibitors

5-Aza-C was originally developed and tested as a nucleoside antimetabolite with clinical specificity for acute myelogenous leukemia (130). Upon phosphorylation, 5-aza-C could be incorporated into both DNA and RNA, resulting in inhibition of DNA, RNA and protein synthesis. 5-Aza-2'-deoxycytidine (decitabine), which is only incorporated into DNA, is at least 10-fold more cytotoxic than 5-aza-C in cultured cells and animals (131). At low doses that do not trigger apoptosis, both 5-aza-C and decitabine are suicide DNMT inhibitors because the enzyme becomes irreversibly bound to 5-aza-C and decitabine residues incorporated into DNA (132, 133). In addition to their DNMT inhibiting activity, 5-aza-C and decitabine may also induce DNA damage through structural instability at the site of incorporation. Although these nucleoside analogues have the promising DNMT inhibitory activity, they also have been associated with side effects such as thrombocytopenia and neutropenia, which limit clinical use (134). Other compounds, such as procainamide and procaine, have been shown to reduce DNA
methylation in human cells, but they do not seem to be specific inhibitors of DNMTs (135). More recently, a small molecule called RG108 has been identified by means of high-throughput screening to be a potent DNMT inhibitor (136). However, more pharmacological and toxicological studies of this agent are required to assess its therapeutic potential. Although the green tea component, (-)-epicatechin, has been shown to inhibit DNMT activity, a drawback is its chemical instability under physiological conditions (137, 138). In this study, the structure of (-)-epicatechin served as a molecular template for the development of more potent and chemically stable DNA demethylating agents.

3.2 GREEN TEA EXTRACTS CATECHINS AS DNA METHYLTRANSFERASE INHIBITORS

3.2.1 BIOLOGICAL ACTIVITIES OF GREEN TEA POLYPHENOLS

Dietary polyphenols are ubiquitous groups of plant metabolites that commonly exist in the human diet (fruits, vegetables, and beverages). Green tea (Camellia sinensis) is one of the most common beverages consumed worldwide, and its possible beneficial health effects have attracted a great deal of attention (139). Several epidemiological studies suggest that green tea has a protective effect against a variety of cancer types, such as lung, prostate, and breast cancers (140-142). The inhibitory action of tea against experimental carcinogenesis has been demonstrated in different animal models of cancer, including those of the skin, oral cavity, esophagus, stomach, intestine, lung, liver, pancreas, mammary gland, urinary bladder, and prostate (143-147). These
chemopreventive and antitumor effects are primarily due to the active constituents from extracts of green tea known as catechins, which include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC). In spite of their promising biological activities, the molecular mechanisms of their inhibitory effects are not fully understood.

EGCG is the most abundant and the most biologically active among the green tea constituents. Much of the mechanistic information on the possible biological activities of tea is derived from studies in cell lines with EGCG. For example, EGCG has been shown to inhibit cell proliferation and induce apoptosis in many cell lines (148-150) which have been to a variety of mechanisms, including inhibitory effects on proteasome activity (151), antiapoptotic Bcl-2 family proteins (152), telomerase activity (153), DNMT activity (137), and activation of transcription factors such as activator protein-1 and nuclear factor-κB (154,155). In addition, EGCG has been reported to modulate cell cycle progression by affecting the activity or expression level of cyclins, cyclin-dependent kinases, Rb, and other regulatory proteins (156-159). In certain cell lines, EGCG and other catechins interfere with ligand binding and subsequent phosphorylation of receptors such as EGFR, PDGFR, laminin receptor, and other receptor protein kinases (160-164). In other cell lines, EGCG inhibited the release of vascular endothelial growth factor, the phosphorylation of vascular endothelial growth factor receptors (VEGFR), and angiogenesis (165). More interestingly, some studies have been shown that EGCG has high affinities in vitro for specific proteins, such as Bcl-xL, Bcl-2, Jurket T cell chymotrypsin-like proteasome and DNMTs.

In summary, these findings indicate that EGCG could be a promising
chemopreventive and/or chemotherapeutic agent. Of the various putative targets mentioned above, DNMT is especially noteworthy considering its pivotal role in epigenetic silencing.

Most *in vitro* studies using EGCG report that concentrations of up to 20 to 100 µM are required to effectively kill cancer cells. These concentrations are much higher than those observed in blood or tissues (usually lower than 1 µM) after ingestion of green tea by animals or humans (166, 167). The high concentrations needed to achieve efficacy *in vitro* and the low levels detected *in vivo* could be attributable to EGCG’s lack of structural stability under alkaline or even neutral conditions that result in chemical degradation (138). In addition, the hydrophilicity of EGCG might reduce its cellular uptake through hydrophobic cell membranes (168). To generate more chemically stable and hydrophobic DNMT inhibitors based on the structure of EGCG, structural modifications were carried out and the stability and DNA hypomethylating activities of the resulting derivatives were assessed.

### 3.2.2 STABILITY STUDY OF EGCG AND ECG BY NON-INVASIVE $^1$H-NMR SPECTROMETRIC ASSAY

Previous studies have assessed the chemical stability of EGCG and showed that it is stable in the acidic pH range of 2.0-5.5. In neutral pH, however, EGCG is easily autooxidized. The EGCG oxidative products have been identified by LC/MS/MS assay (138). To confirm these previous findings, we developed a low cost, non-invasive assay by using $^1$H-NMR spectrometry to track the degradation of EGCG (169). EGCG was incubated in deuterium oxide at 37°C (Figure 3.6).
Figure 3.6 Incubation of EGCG in D$_2$O

The stacked spectrum showed that the peaks H$_6$ and H$_8$ decreased over time and were hardly detectable within 2 hours (Figure 3.7), suggesting that hydrogen-deuterium exchange between EGCG and deuterium oxide was rapidly completed. This finding indicates that H$_6$ and H$_8$ are susceptible to replacement by electrophiles such as a bromo group via a similar mechanism (170).

Figure 3.7 Evidence of hydrogen-deuterium exchange of H6 and H8 in EGCG identified by $^1$H-NMR stacked spectrum
To rationalize the exchange between hydrogen and deuterium on C-6 and C-8 in the aromatic ring, we proposed the keto-enol tautomerization to explain this phenomenon. As shown in Figure 3.8, the phenolic protons were quickly replaced with deuteriums in the deuterium oxide solution. Either C-5 or C-7 was spontaneously transformed into the keto form, followed by the elimination of a proton upon attack by deuterium oxide to regenerate the enol form with replaced deuterium on the C-6 or C-8 position. After about 2 hours of incubation, deuterated C-6 and C-8 were observed based on $^1$H-NMR spectrometric assay.

**Figure 3.8** Exchange of hydrogen into deuterium at C-6 and C-8
The pattern of the $^1$H-NMR stacked spectrum changed in a time-dependent manner, indicating that the structure of EGCG kinetically transformed into other products. The equal amounts of $H_2^-$ and $H_2^+$ turned into a ratio of 2/1 ($H_2^-/H_2^+$) after 48 hours of incubation, suggesting that the dominant degradation may result in a dimer formation through an autooxidative coupling reaction between two EGCG molecules at the C-2’ position. The half-life ($T_{1/2}$) of EGCG is about 12-24 hour as shown in the stacked spectrum (Figure 3.9). This result was in accordance with the previous studies in which EGCG generated two major products when incubated in McCoy’s medium (138). Previous studies identified theasinsenin by LC/MS/MS assay as a degradation product, which mapped the same $^1$H-NMR spectrum pattern observed in this study based on its structural characteristic (Figure 3.10). In addition, appearance of trace peaks might represent another product though there is no mass spectrometry evidence to support our speculation. Overall, we have established an easy, non-invasive method for monitoring the stability of chemicals by $^1$H-NMR spectrometric assay.

![Figure 3.9](image)

**Figure 3.9** Evidence of autooxidation of EGCG leads to dimerization identified by $^1$H-NMR spectrometric assay
Among (-)-epicatechins, EGCG shows the greatest DNMT inhibitory activity in cell-free *in vitro* assays, however its chemical instability in the culture medium or even in neutral deuterium oxide diminishes its feasibility as a scaffold for the development of novel DNMT inhibitors. To identify an appropriate candidate, the stability of ECG was examined since it shows a level of DNMT inhibition that is second only to EGCG. Therefore, ECG was incubated in deuterium oxide at 37°C and monitored at 0, 12, 24, 36 and 48 hours, respectively (Figure 3.11).

**Figure 3.10** Autooxidation of EGCG results in dimerization
Figure 3.11 Incubation of ECG in deuterium oxide at 37°C for 48 hours

As expected, peaks H₆ and H₈ shown in the stacked spectrum decreased dramatically over time, indicating that hydrogen-deuterium exchange occurred between ECG and deuterium oxide. However, the rest of the peak patterns remained similar or even unchanged with the exception of the appearance of a small peak next to H₂, which is assumed to represent the isomerization of ECG at the C-2 position (Figure 3.12). Nevertheless, this finding demonstrated the greater chemical stability of ECG over that of EGCG and indicates it could serve as a molecular template for the synthesis of DNMT inhibitors.
Figure 3.12 Stability study of ECG in terms of $^1$H-NMR spectrometric assay

3.2.3 (-)-EPICATECHIN AS A SCAFFOLD FOR THE SYNTHESIS OF DNA METHYLTRANSFERASE INHIBITORS

To synthesize more chemically stable ECG analogues targeting DNMTs, commercially available (-)-epicatechin (EC) was used as a starting material. In this study, we were focused on the modification of the benzoyl moiety in which three hydroxyl groups were replaced with different substituents (Figure 3.13). As a result, the precursor of the ECG analogues were produced through a coupling reaction between protected EC and aryl chlorides.
Figure 3.13 (-)-Epicatechin as a scaffold for the synthesis of chemically stable DNMT inhibitors

To selectively protect phenolic hydroxyl groups, many protecting groups have been examined. However, some were unlikely to be good protecting groups, such as trimethylsilyl (TMS) and allyl groups, due to instability to weak acid or difficulty in removal. Eventually, the key intermediate 4A served as an optimal candidate since the benzyl groups could be removed under neutral conditions in the final step. Therefore, to differentiate the phenolic and aliphatic hydroxyl groups in EC, the acetylation of all hydroxyl groups in EC was carried out to obtain compound 1, followed by selective deprotection of four phenolic hydroxyl groups with sodium sulfite ($\text{Na}_2\text{SO}_3$) in methanol, resulting in the formation of compound 2. Benzylation of hydroxyl groups in compound 2 by benzyl bromide with $\text{K}_2\text{CO}_3$ as a base yielded compound 3, followed by deacetylation by treatment with sodium methoxide ($\text{NaOMe}$) which generated the key intermediate 4A (Figure 3.14).
Reagents: a) Ac₂O, pyridine; b) Na₂SO₃, MeOH/THF/H₂O; c) BnBr, K₂CO₃, DMF; d) NaOMe, MeOH.

**Figure 3.14** Synthesis of intermediate 4A from (-)-epicatechin

As shown in Figure 3.15, compounds 5A-5H were prepared from 4A through coupling reactions with various aryl chlorides in pyridine solution. Hydrogenolysis and hydrogenation of nitro groups were carried out under high pressure (60 psi) of hydrogen system catalyzed with Pd(OH)₂, resulting in the formation of final products 6A-6H.
To generate more structurally diversified ECG analogues, 7I and 6J were prepared with 1H-tetrazol benzoyl and phenoxy acetyl moieties, respectively (Figure 3.16).
Figure 3.16 Preparation of 6J and 7I

Previously published molecular modeling data predicted that hydrogen bonding is important for the docking of EGCG into the cytosine binding site of DNMT. To validate the significance of this hydrogen bonding for DNMT inhibitory activity, we synthesized compound 7K in which the hydroxyl groups in 6A were replaced with methoxy groups (Figure 3.17). Finally, 6L was prepared from (+)-catechin via a similar synthetic route for the comparison of two different structural conformations of catechin in terms of inhibitory activity against DNMT (Figure 3.18).
**Figure 3.17** Preparation of 7K as a counterpart of 6A

**Figure 3.18** Preparation of 6L from (+)-catechin
3.2.4 INHIBITION OF DNA METHYLTRANSFERASE ACTIVITY

Inhibition of DNMT activity by EGCG, ECG and their derivatives was evaluated using an in vitro assay in which the nuclear extracts from LNCaP cells were incubated for 2 hours at 37°C with a deoxyinosine-deoxycytosine (Sigma) template and ³H-labeled S-adenosylmethionine (Amersham). The reaction was terminated through the loading of samples into Spin-50 Mini-columns (USA Scientific) and the inhibitory effect was determined by scintillation counting. The inhibitory concentrations of the lead compound ECG ranged from about 50 to 100 µM. Therefore, the inhibitory activities of compounds 6A-6H were screened and evaluated at the 100 µM level. The results showed that EGCG exhibited greater inhibition of DNMT activity (63.7% inhibition) than ECG which showed relatively poor activity (47.0% inhibition) (Figure 3.19 and Table 3.1). The discrepancy between EGCG and ECG in inhibiting DNMT might be attributable to the differences in binding affinity as predicted previously. However, 6B and 6C exhibited slightly improved inhibitory activity in comparison to ECG (49.9% and 50.3% inhibition, respectively). In addition, 7K (18% inhibition) was much less active than 6A (41.7% inhibition) indicating that hydrogen bonding involving the four hydroxyl groups contributed significantly to the inhibitory activity most likely by enhancing binding affinity with DNMT. Furthermore, the lower potencies exhibited by 6D-6G suggested that hydrophobic groups, such as chloro (Cl), methoxy (OMe), and phenyl (Ph), were unfavorable for the ligand-protein binding. Compared with its counterpart 6H (37.2% inhibition), 6L (40.7% inhibition) exhibited a comparable potency in inhibiting DNMT activity, which was surprising since it was generated from (+)-catechin. This interesting result indicated that a structure containing the polyphenol skeleton could be an alternative
for the design of DNMT inhibitors.

**Figure 3.19** Inhibition of DNMT by ECG analogues at 100 µM level

<table>
<thead>
<tr>
<th>Entry</th>
<th>(%) Inhibition</th>
<th>Entry</th>
<th>(%) Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>63.7 ± 7.3</td>
<td>6F</td>
<td>17.8 ± 0.3</td>
</tr>
<tr>
<td>ECG</td>
<td>47.0 ± 1.8</td>
<td>6G</td>
<td>12.5 ± 0.5</td>
</tr>
<tr>
<td>6A</td>
<td>41.7 ± 1.5</td>
<td>6H</td>
<td>37.2 ± 0.4</td>
</tr>
<tr>
<td>6B</td>
<td>49.9 ± 2.0</td>
<td>6I</td>
<td>29.6 ± 1.0</td>
</tr>
<tr>
<td>6C</td>
<td>50.3 ± 2.0</td>
<td>7J</td>
<td>29.9 ± 0.9</td>
</tr>
<tr>
<td>6D</td>
<td>17.3 ± 0.4</td>
<td>7K</td>
<td>18.0 ± 1.2</td>
</tr>
<tr>
<td>6E</td>
<td>20.7 ± 0.7</td>
<td>6L</td>
<td>40.7 ± 1.4</td>
</tr>
</tbody>
</table>

**Table 3.1** Inhibition of DNA methyltransferase at 100 µM
3.2.5 CELL-BASED DNA METHYLATION ASSAY

To confirm that the DNMT inhibitory activities determined by the cell-free in vitro assay system corresponds to the demethylation of hypermethylated genes in an intact cell-based system, three methylation-silenced genes (GSTP1, RASSF1A and RARβ) were evaluated in LNCaP cells. Initially, the LNCaP cells were exposed to three analogues (6C, 6H and 6L) at the 20 µM level along with the positive control 5-aza-dC (10 µM level) for 3 days. As shown in Figure 3.20, 6C and 6L demethylated the GSTP1 and RARβ genes, but not the RASSF1A gene, suggesting selectivity for specific genes by the inhibitors. In contrast, 5-aza-dC demethylated the RASSF1A and RARβ genes, but not the GSTP1 gene. However, there was no obvious demethylating effect exhibited by ECG on the tested genes, suggesting that cell-free in vitro DNMT inhibitory activity cannot be correlated with activity determined by the cell-based methylation-specific PCR (MSP) assay, perhaps as a result of factors such as hydrophobicity and permeability into cell membranes (Table 3.2).

![Figure 3.20](image)

**Figure 3.20** Demethylation of GSTP1, RASSF1A and RARβ genes in LNCaP cells by selected DNMT inhibitors as determined by methylation-specific PCR (MSP) assay
Table 3.2 Calculated logP values of EGCG and its analogues by ChemDraw Ultra 9.0

<table>
<thead>
<tr>
<th>No.</th>
<th>LogP</th>
<th>No.</th>
<th>LogP</th>
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<tbody>
<tr>
<td>EGCG</td>
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<td>6F</td>
<td>5.31</td>
</tr>
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<td>ECG</td>
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<td>6E</td>
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<td>6L</td>
<td>1.74</td>
</tr>
</tbody>
</table>

3.2.6 DEMETHYLATION OF HYPERMETHYLATED TUMOR SUPPRESSOR RASSF1A GENE

To determine whether the reversal of methylation-silenced genes by ECG analogues is a general phenomenon that also occurs in other cell lines, we studied the demethylating effect of ECG analogues on the RASSF1A gene in the MCF-7 breast cancer, A2780 ovarian cancer and LNCaP prostate cancer cell lines.

As shown, 5-aza-dC as a positive control exhibited demethylating activity on the RASSF1A gene in the three cell lines at the 5 µM level after 4 days of treatment. Surprisingly, none of three cell lines showed demethylation in response to treatment with EGCG, suggesting that either gene specificity or instability of EGCG resulted in the observed reduction of potency in these cells in spite of EGCG being the most potent of the tested compounds in the cell-free in vitro DNMT activity assay. On the other hand, ECG exhibited demethylating activity in two cell lines. This result might be attributable to its greater chemical stability in comparison to EGCG. Interestingly, among the tested ECG analogues, 6A exhibited consistent RASSF1A gene demethylating activity in all three cell lines at the 10 µM level. This potent demethylating effect of 6A may be a result
of gene specificity and/or intracellular accumulation of the agent over the long-term treatment.

Figure 3.21 Demethylation of RASSF1A gene exhibited by ECG analogues determined by MSP assay. (A) A2780 cell line (B) MCF-7 cell line and (C) LNCaP cell line. Except for 5-aza-dIC (5 µM), all samples were tested at the 10 µM level for 4 days.

To gain a more detailed picture of the RASSF1A gene demethylating effect, 6A was administered over a dose range to MCF-7 cells. As shown in Figure 3.22, the
demethylating effect mediated by 6A could be observed at a concentration as low as 2.5 µM. However, demethylation could not be observed after treatment with either EGCG or ECG. Taken together, the data suggest that chemical stability and hydrophobicity of DNMT inhibitors in this study was a determinant of the efficient and consistent demethylation of the RASSF1A gene.

![Figure 3.22](image)

**Figure 3.22** Demethylation of RASSF1A gene by 6A in a dose-dependent manner in MCF-7 cells

3.2.7 STABILITY STUDY OF 6A AS DETERMINED BY $^1$H-NMR SPECTROMETRIC ASSAY

To determine whether the consistent demethylating activity of 6A on the RASSF1A gene in the cell-based assay is, at least in part, due to its chemically stability, $^1$H-NMR spectrometry was used to assess the stability of 6A over a time course (Figure 3.23).
As shown in Figure 3.24, the hydrogen-deuterium exchange occurred at H₆ and H₈ upon the incubation of 6A in D₂O. The absorption peaks of the other protons remained unchanged, indicating that 6A is chemically stable under the experimental conditions used (37°C, deuterium oxide solution, 48 hour incubation). This result may account for the reversal of hypermethylation of the RASSF1A gene by 6A.

Figure 3.24 Stability study of 6A in terms of ¹H-NMR spectrometric assay

3.3 CONCLUSION

In this study, we have established a non-invasive ¹H-NMR spectrometric assay for the study of chemical stability of EGCG, ECG and EC-derived analogues. In addition, the
EC skeleton within ECG was determined to be an appropriate molecular template for the development of DNMT inhibitors based on the chemical stability demonstrated by ECG. Screening of ECG analogues using the MSP assay revealed that 6A possessed the greatest DNA demethylating activity using the \textit{RASSF1A} gene as a target in three different cell lines. Furthermore, 6A reversed the hypermethylation of the \textit{RASSF1A} gene in MCF-7 cells in a dose-dependent manner. Finally, we have simplified the structure of ECG and demonstrated that these changes yielded a compound with consistent demethylating activity. These ECG analogues warrant further study using other powerful assays to gain insight into their mechanism of DNA methylation.

3.4 EXPERIMENTAL SECTION

3.4.1 CHEMICAL PREPARATION

Chemical reagents and organic solvents were purchased from Sigma-Aldrich unless otherwise mentioned. Nuclear magnetic resonance spectra (\textsuperscript{1}H NMR) were measured on a Bruker 250- or 300-MHz spectrometer. Chemical shift (\(\delta\)) are reported in ppm relative to the d-solvent peaks. Electrospray ionization (ESI) mass spectrometry analyses were performed with a 3-T Finnigan FTMS-2000 Fourier transform mass spectrometer. Flash column chromatography was performed with silica gel (200-400 mesh) or aluminum oxide (neutral, ~150 mesh).
Acetic acid 2-acetoxy-4- (3, 5, 7-triacetoxy-chroman-2-yl)-phenyl ester (1)

To a solution of (-)-epicatechin (5.0 g, 17.2 mmol) in 70 ml pyridine with ice bath, acetic anhydride (10.34 g, 86.2 mmol) was added to the solution. The mixture was stirred for 24 hours. 100 ml 1N HCl(aq) and 70 ml ethyl acetate were added to the solution, the organic layer was collected (make sure no pyridine on TLC plate) and dried with MgSO$_4$, concentrated $in$ $vacuo$ to obtain 1 without any further purifications. $^1$H-NMR (CDCl$_3$) $\delta 1.89$ (s, 1 H), 2.27 (s, 12 H), 2.9 (dd, $J = 15.0$, 1.8 Hz, 1 H), 3.01 (dd, $J = 18.5$, 3.5 Hz, 1 H), 5.13 (s, 1H), HRMS (M+Na)$^+$ cald for C$_{25}$H$_{24}$O$_{11}$Na$^+$ 523.1211; found 523.1217.

Acetic acid 2-(3, 4-dihydroxy-phenyl)-5, 7-dihydroxy-chroman-3-yl ester (2)

To a solution of 1 (8.09 g, 15.48 mmol) in 100 ml of methanol was added a solution of sodium sulfite (3.45g, 27.44 mmol) in 100 ml of water. After stirred at room temperature for 16 hours, the methanol was flash evaporated (45°C) and the remaining aqueous layer extracted with ethyl acetate (3 x 50 ml). Drying (MgSO$_4$) and evaporation of the ethyl acetate gave a product which was purified by flash column chromatography with ethyl acetate-hexane (1:1) as eluent to afford 2 as a solid. $^1$H-NMR ($d_6$- acetone) $\delta$ 1.98 (s, 3 H), 2.87-2.98 (m, 2 H), 5.01 (d, $J = 5.0$ Hz, 1 H), 5.39 (d, $J = 3.7$ Hz 1 H), 5.97
A solution of benzyl bromide (1.66g, 5.0 mmol) was dropwise under Ar to a cooled (0 °C) solution of compound 2 (0.332g, 1.0 mmol) and potassium carbonate (0.69g, 5.0 mmol) in DMF (10 mL). Stirring was maintained for 2 hours at 0 °C and then allowed to come to room temperature over 10 hours. The reaction mixture was diluted in 50 mL of ethyl acetate, washed with 30 mL of water and 30 mL of brine and finally dried over MgSO₄. Evaporation of the solvent furnished an oily product which was purified by flash column chromatography using ethyl acetate: hexane (35: 65) as eluent to give product 3. 

\[ \text{Acetic acid 5, 7-bis-benzyloxy-2-(3, 4-bis-benzyloxy-phenyl)-chroman-3-yl ester (3)} \]

A solution of benzyl bromide (1.66g, 5.0 mmol) was dropwise under Ar to a cooled (0 °C) solution of compound 2 (0.332g, 1.0 mmol) and potassium carbonate (0.69g, 5.0 mmol) in DMF (10 mL). Stirring was maintained for 2 hours at 0 °C and then allowed to come to room temperature over 10 hours. The reaction mixture was diluted in 50 mL of ethyl acetate, washed with 30 mL of water and 30 mL of brine and finally dried over MgSO₄. Evaporation of the solvent furnished an oily product which was purified by flash column chromatography using ethyl acetate: hexane (35: 65) as eluent to give product 3. 

\[ \text{1H-NMR (CDCl}_3\text{):} \delta 1.98 (s, 3 H), 2.97-3.04 (m, 2 H), 4.21 (s, 1 H), 4.96 (s, 1 H), 5.01 (s, 2 H), 5.07 (s, 2 H), 5.16 (s, 2 H), 5.19 (s, 2 H), 6.28 (s, 2 H), 6.96-7.04 (m, 2 H), 7.21 (s, 1 H), 7.22-7.41 (m, 20 H) ppm. \]

\[ \text{(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis(benzyloxy)phenyl)chroman-3-ol (4A)} \]

A solution of MeONa (15 mL prepared from sodium (1.0 g) and methanol (40 mL)) was added dropwise to a solution of compound 3 (692 mg, 1.0 mmol) in 15 mL of
CH₂Cl₂ at room temperature with stirring under argon. The reaction mixture was stirred for an additional 30 min and after removal of the solvent, washed with water (30 mL) and extracted with CH₂Cl₂ (3 x 40 mL). The organic layer was separated, rinsed with brine, dried with MgSO₄ and concentrated to dryness. The crude product was finally purified by flash column chromatography with ethyl acetate: hexane (40: 60) as eluent to obtain compound 4A as a white solid. ¹H-NMR (CDCl₃) δ 2.93-3.00 (m, 2 H), 4.20 (s, 1 H), 4.91 (s, 1 H), 5.01 (s, 4 H), 5.16 (s, 2 H), 5.18 (s, 2 H), 6.26 (s, 2 H), 6.97-7.02 (m, 2 H), 7.19 (s, 1 H), 7.21-7.42 (m, 20 H) ppm.

(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy) phenyl) chroman-3-yl 3-nitro benzoate (5A)

To a solution 4A (0.65 g, 1.0 mmol) in pyridine (10 mL), 3-nitrobenzoyl chloride (0.36 g, 2.0 mmol) and DMAP (0.244 g, 2.0 mmol) were added to the solution. The mixture was stirred at room temperature for 4 hours. The mixture was washed with ethyl acetate (30 mL) and 1N HCl (30 mL) couple times until the pyridine was gone (monitored with TLC). The organic layer was dried with MgSO₄, concentrated in vacuo and purified with silica gel chromatography with eluent of CH₂Cl₂: hexane (1:3) to obtain 5A. ¹H-NMR (CDCl₃) δ 3.23 (d, \( J = 2.9 \) Hz, 2 H), 5.02 (s, 1 H), 5.08 (s, 2 H), 5.12 (s, 2
H), 5.15 (s, 2 H), 5.17 (s, 2 H), 5.75 (s, 1 H), 6.38 (d, J = 2.0 Hz, 1 H), 6.46 (d, J = 2.0 Hz, 1 H), 6.88 (d, J = 3.2 Hz, 1 H), 6.93 (d, J = 3.3 Hz, 1 H), 7.25 (s, 1 H), 7.37-7.51 (m, 20 H), 7.54 (s, 1 H), 8.26 (d, J = 7.8 Hz, 1 H), 8.41 (d, J = 7.8 Hz, 1 H), 8.79 (s, 1 H) ppm.

3-Amino-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-3-yl ester (6A)

A suspension of compound 5A (0.6g, 0.75 mmol) in MeOH (20 mL) and THF (5 mL) was vigorously stirred with palladium hydroxide (10%, 300 mg) under 60 psi of hydrogen. The reaction mixture was stirred for 12 hours, filtered on Celite, and MeOH was added. The product was concentrated in vacuo to give rise to 6A. $^1$H-NMR ($d_4$-MeOH) δ 2.82 (dd, J = 17.5, 2.3 Hz, 1 H), 2.97 (dd, J = 17.5, 2.2 Hz, 1 H), 5.23 (brs, 1 H), 5.89 (s, 2 H), 6.61 (d, J = 8.2 Hz, 1 H), 6.72 (d, J = 8.2 Hz, 1 H), 6.88 (s, 1 H), 7.07-7.27 (m, 3 H), 7.42 (brs, 2 H) ppm. HRMS (M+Na)$^+$ clacld for C$_{23}$H$_{21}$NO$_6$Na$^+$ 432.1053; found: 432.1054.
4-Nitro-benzoic acid 5, 7-bis-benzyloxy-2-(3, 4-bis-benzyloxy-phenyl) -chroman-3-yl ester (5B)

Compound 5B was synthesized from the procedure described for compound 5A. ^1H-NMR (CDCl₃) δ 3.03 (m, 2 H), 4.88 (s, 2 H), 4.91 (s, 2 H), 4.94 (s, 2 H), 4.97 (s, 2 H), 5.05 (d, J = 3.6 Hz, 11 H), 5.67 (s, 1 H), 6.18 (d, J = 6.8 Hz, 2 H), 6.22 (d, J = 6.7 Hz, 1 H), 6.79 (s, 1 H), 6.88 (s, 1 H), 6.98 (s, 1 H), 7.15-7.31 (m, 20 H), 7.93 (d, J = 8.9 Hz, 2 H), 8.07 (d, J = 8.9 Hz, 2 H) ppm.

4-Amino-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-3-yl ester (6B)

Compound 6B was synthesized from the procedure described for compound 6A. ^1H-NMR (d₄-MeOH) δ 2.76 (dd, J = 17.5, 2.7 Hz, 1 H), 2.89 (dd, J = 17.5, 4.4 Hz, 1 H), 4.95 (s, 1 H), 5.39 (brs, 1 H), 5.82 (s, 1 H), 5.84 (s, 1 H), 6.47 (d, J = 8.2 Hz, 2 H), 6.58 (d, J = 8.2 Hz, 1 H), 6.68 (d, J = 8.2 Hz, 1 H), 6.85 (s, 1 H), 7.51 (s, 1 H), 7.54 (s, 1 H) ppm. HRMS (M+Na)^⁺ claed for C₂₃H₂₁NO₆Na⁺ 432.1053; found: 432.1059.
(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy) phenyl) chroman-3-yl 4-chloro-3-nitrobenzoate (5C)

Compound 5C was synthesized from the procedure described for compound 5A.

$^1$H-NMR ($d_4$-MeOH) $\delta$ 3.20 (dd, $J = 20, 4$ Hz, 2 H), 5.03 (s, 1 H), 5.07 (s, 2 H), 5.11 (s, 2 H), 5.15 (s, 2 H), 5.17 (s, 2 H), 5.72 (s, 1 H), 6.37 (d, $J = 6.8$ Hz, 1 H), 6.42 (d, $J = 6.7$ Hz, 1 H), 6.99 (d, $J = 2.5$ Hz, 1 H), 7.05 (d, $J = 2.5$ Hz, 1 H), 7.18 (s, 1 H), 7.37-7.45 (m, 20 H), 7.47 (s, 1 H), 7.98 (d, $J = 8.9$ Hz, 1 H), 8.39 (s, 1 H) ppm.

3-Amino-4-chloro-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5, 7-dihydroxy- chroman-3-yl ester (6C)

Compound 6C was synthesized from the procedure described for compound 6A. $^1$H-NMR ($d_4$-MeOH) $\delta$ 2.84 (dd, $J = 20, 4$ Hz, 2 H), 4.96 (s, 1 H), 5.46 (brs, 1 H), 5.87 (s, 2
H), 6.69 (d, $J = 1.69$ Hz, 1 H), 6.71 (d, $J = 1.65$ Hz, 1 H), 6.86 (s, 1 H), 7.17 (s, 2 H), 7.27 (s, 1 H) ppm. HRMS (M+Na)$^+$ cald for C$_{22}$H$_{18}$ClO$_7$Na$^+$ 466.06695; found 466.08904.

![Chemical structure of 5D]

(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy) phenyl) chroman-3-yl 4-chloro-3-nitrobenzoate (5D)

Compound 5D was synthesized from the procedure described for compound 5A. $^1$H-NMR (CDCl$_3$) $\delta$ 3.13 (dd, $J = 17.5$, 5.2 Hz, 2 H), 4.91 (s, 1 H), 4.98 (s, 2 H), 5.02 (s, 2 H), 5.06 (s, 2 H), 5.09 (s, 2 H), 5.76 (s, 1 H), 6.29 (s, 1 H), 6.31 (s, 1 H), 6.91 (d, $J = 2.5$ Hz, 1 H), 7.09 (d, $J = 2.5$ Hz, 1 H), 7.25 (s, 1 H), 7.27-7.42 (m, 20 H), 7.44 (s, 1 H), 7.68 (d, $J = 2.5$ Hz, 1 H), 7.98 (s, 1 H) ppm.

![Chemical structure of 6D]

3, 4-Dichloro-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5, 7-dihydroxy-chroman-3-yl ester (6D)
Compound 6D was synthesized from the procedure described for compound 6A. 

$^1$H-NMR ($d_6$-acetone) δ 2.91 (dd, $J = 17.6, 4.4$ Hz, 1 H), 3.12 (dd, $J = 17.6, 2.3$ Hz, 1 H), 5.19 (s, 1 H), 5.64 (brs, 1 H), 6.04 (s, 1 H), 6.06 (s, 1 H), 6.75 (d, $J = 8.1$ Hz, 1 H), 6.89 (d, $J = 8.1$ Hz, 1 H), 7.05 (s, 1 H), 7.67 (d, $J = 8.4$ Hz, 1 H), 7.79 (d, $J = 8.4$ Hz, 1 H), 8.18 (s, 1 H) ppm. HRMS (M+Na)$^+$ clacd for C$_{23}$H$_{20}$O$_7$Cl$_2$Na$^+$ 485.0171; found: 485.0490.

\[
\begin{align*}
\text{O} & \quad \text{OBn} \\
\text{OBn} & \quad \text{OBn} \\
\text{OBn} & \quad \text{O} \\
\text{OMe} & \quad \text{OMe} \\
\text{OMe} & \quad \text{OMe}
\end{align*}
\]

3, 4, 5-Trimethoxy-benzoic acid 5, 7-bis-benzyloxy-2-(3,4-bis-benzyloxy-phenyl)-chroman-3-yl ester (5E)

Compound 5E was synthesized from the procedure described for compound 5A. 

$^1$H-NMR (CDCl$_3$) δ 3.18 (d, $J = 3.1$ Hz, 2 H), 3.74 (s, 1 H), 3.85 (s, 3 H), 3.87 (s, 3 H), 3.91(s, 3 H), 5.07 (s, 2 H), 5.09 (s, 2 H), 5.17 (brs, 4 H), 5.69 (s, 1 H), 6.36 (d, $J = 2.1$ Hz, 1 H), 6.41 (d, $J = 2.0$ Hz, 1 H), 6.97 (s, 1 H), 6.98-7.06 (m, 2 H), 7.07 (s, 2 H), 7.28-7.51 (m, 20 H) ppm.
3, 4, 5-Trimethoxy-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5, 7-dihydroxy-chroman-3-yl ester (6E)

Compound 6E was synthesized from the procedure described for compound 6A.

\[ ^1H\text{-NMR (}d_4\text{-MeOH)} \delta 2.88 (dd, J = 17.5, 2.7 \text{ Hz }, 1 \text{ H}), 2.92 (dd, J = 17.5, 2.5 \text{ Hz}, 1 \text{ H}), 3.61 (s, 3 \text{ H}), 3.62 (s, 6 \text{ H}), 5.03 (s, 1 \text{ H}), 5.43 (brs, 1 \text{ H}), 5.88 (s, 1 \text{ H}), 5.89 (s, 1 \text{ H}), 6.63 (d, J = 8.2 \text{ Hz}, 1 \text{ H}), 6.73 (d, J = 8.2 \text{ Hz}, 1 \text{ H}), 6.91 (s, 1 \text{ H}), 6.98-7.06 (m, 2 \text{ H}) \text{ ppm.} \]

HRMS (M+Na)\(^+\) cald for C\(_{26}\)H\(_{26}\)O\(_{10}\)Na\(^+\) 507.1267; found 507.1272.

(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy) phenyl)chroman-3-yl biphenyl-4-carboxylate (5F)

Compound 5F was synthesized from the procedure described for compound 5A.

\[ ^1H\text{-NMR (CDCl}_3\text{)} \delta 2.02 (dd, J = 15, 2.3 \text{ Hz}, 2 \text{ H}), 4.86 (s, 1 \text{ H}), 5.08 (s, 2 \text{ H}), 5.12 (s, 2 \text{ H}), 5.16 (s, 2 \text{ H}), 5.23 (s, 2 \text{ H}), 5.84 (s, 1 \text{ H}), 6.35 (s, 1 \text{ H}), 6.38 (s, 1 \text{ H}), 7.09-7.33 (m, 3 \text{ H}), 7.28-7.46 (m, 20 \text{ H}), 7.51-7.67 (m, 9 \text{ H}) \text{ ppm.} \]
Biphenyl-4-carboxylic acid 2-(3,4-dihydroxy-phenyl)-5,7-dihydroxy-chroman -3-yl ester (6F)

Compound 6F was synthesized from the procedure described for compound 6A. 

$^1$H-NMR ($d_4$-MeOH) $\delta$ 2.51 (dd, $J$ = 16.5, 2.3 Hz, 1 H), 2.62 (dd, $J$ = 16.5, 2.3 Hz, 1 H), 4.57 (s, 1 H), 5.36 (brs, 1 H), 6.46-6.58 (m, 4 H), 6.76 (s, 1 H), 6.77 (s, 1 H), 7.09-7.33 (m, 3 H), 7.32 (d, $J$ = 1.8 Hz, 2 H), 7.33 (d, $J$ = 1.8 Hz, 2 H), 7.63 (d, $J$ = 8.5 Hz, 1 H), 7.66 (d, $J$ = 8.5 Hz, 1 H) ppm. HRMS (M+Na)$^+$ calc for C$_{28}$H$_{22}$O$_7$Na$^+$ 493.1258; found: 493.1261.

3-Formyl-benzoic acid 5, 7-bis-benzyloxy-2-(3, 4-bis-benzyloxy-phenyl)-chroman -3-yl ester (5G)

Compound 5G was synthesized from the procedure described for compound 5A.

$^1$H-NMR (CDCl$_3$) $\delta$ 3.15 (s, 2 H), 4.92 (s, 1 H), 4.99 (s, 2 H), 5.05 (s, 2 H), 5.11(s, 4 H), 5.71 (s, 1 H), 6.31 (d, $J$ = 2.1 Hz, 1 H), 6.37 (s, 1 H), 6.92 (d, $J$ = 5.6 Hz, 1 H), 7.02 (d, $J$
= 5.6 Hz, 1 H), 7.17 (s, 1 H), 7.29-7.43 (m, 20 H), 7.53 (s, 1 H), 8.01 (d, $J = 7.7$ Hz, 1 H), 8.41 (d, $J = 7.7$ Hz, 1 H), 8.41 (s, 1 H), 9.98 (s, 1 H) ppm.

**3-Hydroxymethyl-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5, 7-dihydroxy-chroman-3-yl ester (6G)**

Compound **6G** was synthesized from the procedure described for compound **6A**. \[^1\]H-NMR ($d_4$-MeOH) $\delta$ 2.93 (dd, $J = 17.4$, 2.0 Hz, 1 H), 2.97 (dd, $J = 17.5$, 4.3 Hz, 1 H), 3.28 (s, 2H), 4.99 (s, 1 H), 5.45 (brs, 1 H), 5.91 (s, 1 H), 5.92 (s, 1 H), 6.61 (d, $J = 8.1$ Hz, 1 H), 6.65 (d, $J = 8.1$ Hz, 1 H), 6.73 (s, 1 H), 7.14-7.27 (m, 2 H), 7.57 (d, $J = 6.7$ Hz, 1 H). HRMS (M+Na)$^+$ clacd for C$_{23}$H$_{20}$O$_8$Na$^+$ 447.1050; found: 447.1069.

**2R, 3R-5, 7-bis (benzyloxy)2-(3, 4-bis(benzyloxy) phenyl) chroman-3-yl-4-acetamido- 3-nitrobenzoate (5H)**

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Compound 5H was synthesized from the procedure described for compound 5A. 

$^1$H-NMR (CDCl$_3$) δ 2.32 (s, 3 H), 3.11 (s, 2 H), 4.89 (s, 1 H), 4.94 (s, 2 H), 5.99 (s, 2 H), 5.04 (s, 2 H), 5.09 (s, 2 H), 5.62 (s, 1 H), 6.28 (s, 1 H), 6.34 (s, 1 H), 6.89 (d, $J$ = 8.3 Hz, 1 H), 6.97 (d, $J$ = 8.3 Hz, 1 H), 7.11 (s, 1 H), 7.27-7.45 (m, 20 H), 8.09 (d, $J$ = 7.1 Hz, 1 H), 8.72 (s, 1 H), 8.82 (d, $J$ = 7.1 Hz, 1 H), 10.43 (s, 1 H) ppm.

![Structure of 5H](image)

4-Acetylamino-3-amino-benzoic acid 2-(3, 4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-3-yl ester (6H)

Compound 6H was synthesized from the procedure described for compound 6A. 

$^1$H-NMR ($d_4$-MeOH) δ 2.06 (s, 3H), 2.9-2.98 (m, 2 H), 3.23 (s, 1 H), 4.89 (s, 1 H), 5.46 (1, 1H), 5.88 (s, 2H), 6.69 (d, $J$ = 1.69 Hz, 1 H), 6.71 (d, $J$ = 1.65 Hz, 1 H), 6.86 (s, 1 H), 7.17 (s, 2H), 7.27 (s, 1 H) ppm. HRMS (M+Na)$^+$ calcd for C$_{24}$H$_{22}$N$_2$O$_8$Na$^+$ 489.1268; found 489.1245.

![Structure of 6H](image)
(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy) phenyl) chroman-3-yl 3-cyanobenzoate (5I)

Compound 5I was synthesized from the procedure described for compound 5A.

$^1$H-NMR (CDCl$_3$) $\delta$ 3.15 (dd, $J$ = 18.3, 4.8 Hz, 2 H), 4.70 (s, 1 H), 4.92 (s, 2 H), 4.99 (s, 2 H), 5.04 (s, 2 H), 5.05 (s, 2 H), 5.65 (s, 1 H), 6.28 (d, $J$ = 2.1 Hz, 1 H), 6.33 (d, $J$ = 2.1 Hz, 1 H), 6.88 (d, $J$ = 3.2 Hz, 1 H), 6.96 (d, $J$ = 3.3 Hz, 1 H), 7.08 (s, 1 H), 7.27-7.46 (m, 20 H), 7.72 (d, $J$ = 4.2 Hz, 1 H), 8.07 (d, $J$ = 4.2 Hz, 1 H), 8.16 (s, 1 H) ppm.

(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis (benzyloxy)phenyl)chroman-3-yl 3-(1H-tetrazol-5-yl)benzoate (6I)

To a solution of NaN$_3$ (325 mg, 5.0 mmol) in 10 mL EtOH, compound 5I (779 mg, 1.0 mmol) was added to the solution. The mixture was stirred at reflux for 12 hours. Evaporation of the solvent furnished an oily product which was diluted in 50 mL of ethyl acetate, washed with 10 mL of water and 10 mL of brine and finally dried over MgSO$_4$. The crude product was purified by flash column chromatography using ethyl acetate: hexane (30: 70) as eluent to give compound 6I. $^1$H-NMR (CDCl$_3$) $\delta$ 2.86-3.01 (m, 2 H), 4.19 (s, 1 H), 4.88 (s, 1 H), 4.99 (s, 2 H), 5.07 (s, 2 H), 5.14 (s, 2 H), 5.16 (s, 2 H), 6.26 (s, 2 H), 6.93 (s, 2 H), 7.14 (s, 1 H), 7.31-7.41 (m, 20 H), 7.49 (s, 1 H), 7.77 (d, $J$ = 7.5 Hz, 1 H).
(2R, 3R)-2-(3, 4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl 3-(1H-tetrazol-5-yl)benzoate (7I)

Compound 7I was synthesized from the procedure described for compound 6A. H-NMR (d$_4$-MeOH) δ 2.85-2.98 (m, 2 H), 5.07 (s, 1 H), 5.61 (s, 1 H), 5.93 (d, $J = 2.3$ Hz, 1 H), 5.97 (d, $J = 2.3$ Hz, 1 H), 6.65 (d, $J = 10$ Hz, 1 H), 6.78 (d, $J = 7.5$ Hz, 1 H), 6.97 (s, 1 H), 7.57 (t, $J = 10$ Hz, 1 H), 7.79 (d, $J = 5.2$ Hz, 1 H), 8.16 (d, $J = 7.5$ Hz, 1 H), 8.51 (s, 1 H) ppm. HRMS (M+Na)$^+$ cald for C$_{23}$H$_{18}$N$_4$O$_7$Na$^+$ 485.10677; found 485.10813.

(2R, 3R)-5, 7-bis (benzyloxy)-2-(3, 4-bis(benzyloxy) phenyl) chroman-3-yl 2-phenoxy acetate (5J)

Compound 5J was synthesized from the procedure described for compound 5A.
\(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.94-3.01 (m, 2 H), 4.86 (s, 1 H), 5.01 (s, 2 H), 5.02 (s, 2 H), 5.05 (s, 2 H), 5.10 (s, 2 H), 5.13 (s, 2 H), 6.28 (s, 2 H), 6.61 (s, 1 H), 6.65 (s, 1 H), 6.92-7.14 (m, 5 H), 7.28-7.42 (m, 20 H) ppm.

(2R, 3R)-2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxychroman-3-yl 2-phenoxyacetate (6J)

Compound 6J was synthesized from the procedure described for compound 6A. \(^1\)H-NMR (\(d_6\)-acetone) 3.00-3.07 (m, 2 H), 4.49 (d, \(J = 15\) Hz, 1 H), 4.66 (d, \(J = 15\) Hz, 1 H), 5.09 (s, 1 H), 5.57 (brs, 1 H), 5.99 (s, 1 H), 6.15 (s, 1 H), 6.63 (s, 1 H), 6.67 (s, 1 H), 6.85-6.90 (m, 2 H), 7.06 (s, 1 H), 7.17-7.28 (m, 3 H) ppm. HRMS (M+Na)\(^+\) clacd for C\(_{23}\)H\(_{20}\)O\(_8\)Na\(^+\) 447.1050; found: 447.1081.

((2R, 3R)-2-(3, 4-dimethoxyphenyl)-5, 7-dimethoxychroman-3-yl acetate (4B)

A solution of methyl iodide (710 mg, 5.0 mmol) was dropwise under Ar to a cooled (0 °C) solution of compound 2 (332 mg, 1.0 mmol) and potassium carbonate (690 mg, 5.0 mmol) in DMF (10 mL). Stirring was maintained for 2 hours at 0 °C and then allowed to
come to room temperature over 10 hours. The reaction mixture was diluted in 50 mL of ethyl acetate, washed with 30 mL of water and 30 mL of brine and finally dried over MgSO₄. Evaporation of the solvent furnished an oily product which was purified by flash column chromatography using ethyl acetate: hexane (35: 65) as eluent to give compound 4B. ¹H-NMR (CDCl₃) δ 1.92 (s, 3 H), 2.93 (s, 2 H), 3.77 (s, 3 H), 3.78 (s, 3 H), 3.88 (s, 3 H), 3.89 (s, 3 H), 5.01 (s, 1 H), 5.47 (s, 1 H), 6.11 (s, 1 H), 6.21 (s, 1 H), 6.85 (d, J = 7.5 Hz, 1 H), 6.97 (d, J = 7.5 Hz, 1 H), 7.03 (s, 1 H) ppm.

(2R, 3R)-2-(3, 4-dimethoxyphenyl)-5, 7-dimethoxychroman-3-ol (5K)

A solution of MeONa (15 mL prepared from sodium (1.0 g) and methanol (40 mL)) was added dropwise to a solution of compound 4B (386 mg, 1.0 mmol) in 15 mL of CH₂Cl₂ at room temperature with stirring under argon. The reaction mixture was stirred for an additional 30 min and after removal of the solvent, washed with water (30 mL) and extracted with CH₂Cl₂ (3 x 40 mL). The organic layer was separated, rinsed with brine, dried with MgSO₄ and concentrated to dryness. The crude product was finally purified by flash column chromatography with ethyl acetate: hexane (40: 60) as eluent to obtain compound 5K as a white solid. ¹H-NMR (CDCl₃) δ 2.82-2.96 (m, 2 H), 3.75 (s, 3 H), 3.78 (s, 3 H), 3.88 (s, 3 H), 3.90 (s, 3 H), 4.26 (brs, 1 H), 4.95 (s, 1 H), 6.09 (d, J = 2.2 Hz, 1 H), 6.18 (d, J = 2.1 Hz, 1 H), 6.89 (d, J = 8.1 Hz, 1 H), 7.04 (d, J = 10 Hz, 1 H), 7.06 (s, 1 H) ppm.
(2R, 3R)-2-(3, 4-dimethoxyphenyl)-5, 7-dimethoxychroman-3-yl 3-nitrobenzoate (6K)

Compound 6K was synthesized from the procedure described for compound 5A.\(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 3.06-3.11 (m, 2 H), 3.74 (s, 3 H), 3.78 (s, 3 H), 3.81 (s, 3 H), 3.82 (s, 3 H), 5.13 (s, 1 H), 5.65 (s, 1 H), 6.09 (d, \(J = 2.2\) Hz, 1 H), 6.24(d, \(J = 2.1\) Hz, 1 H), 6.82 (s, 1 H), 6.98-7.04 (m, 2 H), 7.53 (t, \(J = 8\) Hz, 1 H), 8.19 (d, \(J = 7.5\) Hz, 1 H), 8.32 (d, \(J = 7.5\) Hz, 1 H), 8.68 (s, 1 H) ppm.

(2R, 3R)-2-(3, 4-dimethoxyphenyl)-5, 7-dimethoxychroman-3-yl 3-amino benzoate (7K)

A suspension of compound 6K (200 mg, 0.4 mmol) in THF (15 mL) was stirred with activated Pd-C (10%, 70 mg) under hydrogen for 4 hours, filtered on Celite and eluted with EtOH (30 mL) and CH\(_2\)Cl\(_2\) (30 mL). The filtrate was concentrated under vacuum and the resulting product 7K was obtained as a white solid.\(^1\)H-NMR (CDCl\(_3\))
3.02 (s, 2 H), 3.79 (s, 3 H), 3.83 (s, 3 H), 4.01 (s, 3 H), 4.13 (s, 3 H), 5.09 (s, 1 H), 5.65 (s, 1 H), 6.11 (s, 1 H), 6.24 (s, 1 H), 6.77-6.79 (m, 2 H), 6.95-7.35 (m, 5 H) ppm. HRMS (M+Na)^+ clacd for C_{28}H_{27}NO_{7}Na^+ 488.1679; found: 488.1674.

(2R, 3S)-5, 7-bis (benzyloxy)-2-(3, 4-bis(benzyloxy)phenyl)chroman-3-ol (4C)

A solution of benzyl bromide (1.66g, 5.0 mmol) was dropwise under Ar to a cooled (0 °C) solution of (+)-catechin (290 mg, 1.0 mmol) and potassium carbonate (0.69g, 5.0 mmol) in DMF (20 mL). Stirring was maintained for 2 hours at 0 °C and then allowed to come to room temperature over 12 hours. The reaction mixture was diluted in 50 mL of ethyl acetate, washed with 30 mL of water and 30 mL of brine and finally dried over MgSO_4. Evaporation of the solvent furnished an oily product which was purified by flash column chromatography using ethyl acetate: hexane (30: 70) as eluent to give rise to compound 4C. $^1$H-NMR (CDCl_3) δ 2.62 (dd, $J = 10, 9.2$ Hz, 1 H), 3.07 (dd, $J = 10, 9.2$ Hz, 1 H), 3.96 (brs,1 H), 4.59 (d, $J = 7.5$ Hz, 1 H), 4.97 (s, 2 H), 5.01 (s, 2 H), 5.15 (s, 4 H), 6.19 (s, 1 H), 6.25 (s, 1 H), 6.93 (s, 2 H), 7.01 (s, 1 H), 7.34-7.38 (m, 20 H) ppm.
(2R, 3S)-5, 7-bis (benzyl oxy)-2-(3, 4-bis (benzyl oxy) phenyl) chroman-3-yl 4-acetamido-3-nitrobenzoate (5L)

Compound 5L was synthesized from the procedure described for compound 5A. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.33 (s, 3 H), 2.88 (dd, \(J = 10, 7.5\) Hz, 1 H), 3.12 (dd, \(J = 10, 7.5\) Hz, 1 H), 4.13 (s, 1 H), 5.03 (s, 2 H), 5.05 (s, 2 H), 5.12 (s, 2 H), 5.14 (s, 2 H), 5.52 (m, 1 H), 6.31 (s, 1 H), 6.33 (s, 1 H), 6.78-82 (m, 2 H), 7.01 (s, 1 H), 7.28-7.46 (m, 20 H), 8.12 (dd, \(J = 5, 2.4\) Hz, 1 H), 8.75 (dd, \(J = 2.5, 1.3\) Hz, 1H), 8.88 (dd, \(J = 5, 2.4\) Hz, 1 H) ppm.

(2R, 3S)-2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxychroman-3-yl 4-acetamido-3-aminobenzoate (6L)

Compound 6L was synthesized from the procedure described for compound 6A. \(^1\)H-NMR (\(d_4\)-MeOH) \(\delta\) 2.02 (s, 3H), 2.9-2.98 (m, 2 H), 3.23 (s, 1 H), 4.89 (s, 1 H), 5.46 (1, 1H), 5.88 (s, 2H), 6.69 (d, \(J = 1.69\) Hz, 1 H), 6.71 (d, \(J = 1.65\) Hz, 1 H), 6.86 (s, 1 H), 7.17 (s, 2H), 7.27 (s, 1 H) ppm. HRMS (M+Na)\(^+\) cald for C\(_{24}\)H\(_{22}\)N\(_2\)O\(_8\)Na\(^+\) 489.1268; found 489.1251.
3.4.2 STABILITY STUDY

EGCG and ECG were purchased from Sigma (St. Louis, MO, USA). Tested samples were prepared with concentration of 1.0 μg/μL in D₂O solution. For ECG and 6A, the sample solution was added trace amount of d-methanol. The sample solution (3 mL) was loaded into NMR tube and incubated at 37°C water base at 0, 12, 24, 36 and 48 hour for detection. Nuclear magnetic resonance spectra (¹H NMR) were measured on a Bruker 250- or 400-MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to the d-solvent peaks.

3.4.3 CELL CULTURE

LNCaP prostate cancer cells, A2780 ovarian cancer cells and MCF-7 breast cancer cells were purchased from the American Type Tissue Collection (Manassas, VA). LNCaP and A2780 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator containing 5% CO₂. MCF-7 were cultured in DMEM F-12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator containing 5% CO₂.

3.4.4 DNA METHYLTRANSFERASE ASSAY

Cultured LNCaP cells were harvested, and nuclear extracts were prepared with the nuclear extraction reagent (Pierce, Rockford, IL). The DNMT assay was performed according to published methods. The nuclear extracts (1.28 μg of protein) were incubated for 3 hours at 37°C with 0.5 μM of poly(dI-dC) · Poly(dI-dC) (Sigma, St. Louis, MO) and
10 μM of S-adenosyl-L-[methyl-3H]methionine (1 μCi; Amersham, Piscataway, NJ) in a total volume of 20 μL of a DNMT buffer solution (New England, Biolabs Inc.). All of the incubations contained 0.02% DMSO, which was used to dissolve EGCG and other inhibitors. Reactions were initiated by the addition of S-adenosyl-L-[methyl-3H] methionine and stopped by loading into mini-columns (Spin-50, USA Scientific) and centrifuged for 3 min. The radioactivity of resulting solutions was counted in a scintillation counter. All of the assays were performed in duplicate. Background levels were determined in incubations without the nuclear extracts.

### 3.4.5 BISULFITE MODIFICATION AND METHYLATION-SPECIFIC PCR

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<tr>
<th>Gene</th>
<th>5'- primer</th>
<th>3'-primer</th>
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<td>146 bp</td>
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<td>62°C</td>
<td>100 bp</td>
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</table>

Genomic DNA was isolated using the QIAamp Tissue Kit (Qiagen)
Run 2% agarose gel and stain with EtBr

The 5 x 10⁵ LNCaP cells were seeded in 10 cm dish for two days. On the day 3, the cells were treated with DMSO, 10 μM 5'-Aza-deoxycytosine, 20 μM ECG, 20 μM 6C, 20 μM 6h, and 20 μM 6l in RPMI 1640 medium with 2% FCS. For the first 4 days, the
medium was changed to fresh medium and drugs every two days. The medium was not changed in the late 4 days. The genomic DNA was isolated using QIAamp DNA mini KIT (Qiagen). The 2 µg of genomic DNA was treated with sodium bisulfite as mentioned by the manual (Zymo research). The bisulphite-treated DNA was eluted in 40 µl of elution buffer. 1 µl of DNA was amplified with specific primers listed in the above Table. 94°C for 10 min, 94°C 30 sec, annealing temp 62 °C 30 sec and elongation temp 72 °C 45 sec for 45 cycles, and additional 72 °C 10 min was performed. The PCR products were electrophoresized on 2 % of agarose gel.
BIBLIOGRAPHY


10. Sato, S, Fujita, N, and Tsuruo, T Modulation of Akt kinase activity by binding to


58. Zhao, H, Lai, F, Nonn, L, Brooks, JD, and Peehl, DM. Molecular targets of


72. Akao, Y, Otsuki, Y, Kataoka, S, Ito, Y, and Tsujimoto, Y Multiple subcellular


3(2): 173-182.


110. Okano, M, Xie, S, and Li, E Cloning and characterization of a family of novel


122. Tamaru, H and Selker, EU A histone H3 methyltransferase controls DNA


146. Hiura, A, Tsutsumi, M, and Satake, K Inhibitory effect of green tea extract on the process of pancreatic carcinogenesis induced by N-nitrosobis-(2-oxypropyl)amine (BOP) and on tumor promotion after transplantation of


157. Liang, YC, Lin-Shiau, SY, Chen, CF, and Lin, JK Inhibition of cyclin-dependent


168. Kajiya, K, Kumazawa, S, and Nakayama, T Effects of external factors on the
