EVALUATION OF A SMALL MOLECULE AGONIST OF EPHA2 RECEPTOR TYROSINE KINASE

AND

COPALIC ACID ANALOGS AS PROSTATE CANCER THERAPEUTICS

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In loving memory of my grandparents who dedicated their lives to education.
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EVALUATION OF A SMALL MOLECULE AGONIST OF EPHA2 RECEPTOR TYROSINE KINASE
AND
COPALIC ACID ANALOGS AS PROSTATE CANCER THERAPEUTICS

NETHRIE IDIPPILY

ABSTRACT

Project I:

Chemotherapeutic drugs have many side effects that are undesirable and are highly toxic. Therefore, there is a growing need for the development of drugs with enhanced efficacy, specificity, and potency to provide cancer patients with a better prognosis. It was discovered that a member of the Receptor Tyrosine Kinase family, EphA2, may prove to be a viable target in developing anti-cancer agents. In the presence of its ligand, EphA2 receptor is responsible for apoptotic and anti-migratory activity. However, in the absence of ligand, EphA2 is able to stimulate cell migration and therefore tumorigenic activity. These conflicting roles of EphA2 and the upregulation of this receptor that is seen in many cancers have provided a novel strategy in designing therapeutic agents. Therefore, small molecules can be used to stimulate ligand-dependent pathways of EphA2 that induce anti-migratory and anti-proliferative effects in cancer cells in order to inhibit metastasis and tumor progression. A small molecule, doxazosin, was identified as an EphA2 agonist in a recent study. It demonstrated positive results in that its actions were similar to those of the natural ligand. Subsequently, a library of compounds was
generated using doxazosin as the lead compound in order to improve its activity. These compounds were tested for their activity in stimulating EphA2 receptor. Two of them showed improved activity compared to doxazosin while mimicking a mechanism similar to the native ligand. The structure activity relationship of these derivatives, the in vitro mechanisms and pharmacokinetic profiles were also analyzed, which provides a basis for further optimization and subsequent in vivo studies of these compounds in the future.

Project II:

Copalic acid which is one of the diterpenoid acids in copaiba oil inhibited the chaperone function of α-crystallin and heat shock protein 27 kDa (HSP27). It also showed potent activity in decreasing the level of an HSP27 client protein, androgen receptor (AR), which makes it useful in prostate cancer treatment. In order to develop potent drug candidates to decrease the AR level in prostate cancer cells, copalic acid analogs were synthesized. Using AR level as a readout 15 copalic acid analogs were screened, which showed that two of those compounds were much more potent than copalic acid. They inhibited AR positive prostate cancer cell growth in a dose-dependent manner. Furthermore, they also inhibited the chaperone activity of α-crystallin.
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ABBREVIATIONS

AF2
Akt
AR
ARE
ASO
BSA
CRPC
DBD
DCC
DHEA
DHT
DMF
DMSO
DRE
DTT
Eck
eIF4G
Eph
FADD
FBS
H
HQC
HRP
HSP27
HSPs

Activation function 2
Protein kinase B
Androgen receptor
Androgen response elements
Antisense oligonucleotides
Bovine serum albumin
Castration resistant prostate cancer
DNA binding domain
Dicyclohexylcarbodiimide
Dehydroepiandosterone
Dihydrotestosterone
Dimethylformamide
Dimethyl sulfoxide
Digital rectal exam
Dithiothreitol
Epithelial cell kinase (EphA2)
Eukaryotic translation initiation factor 4 G
Ephrin
Fas associated-death-domain protein
Fetal bovine serum
Hour(s)
High quality control
Horseradish peroxidase
Heat shock protein 27 kDa
Heat shock proteins
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<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibition coefficient</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>IS</td>
<td>Internal standard</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography Mass spectrometry</td>
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<tr>
<td>LLOQ</td>
<td>Lower limit of quantitation</td>
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<tr>
<td>LQC</td>
<td>Low quality control</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>MAP</td>
<td>Mitogen activated protein kinase pathway</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
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<tr>
<td>Min</td>
<td>Minute</td>
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<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>NTD</td>
<td>N terminal domain</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIA</td>
<td>Proliferative inflammatory atrophy</td>
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<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
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<td>PPT</td>
<td>Protein precipitation</td>
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<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>QC</td>
<td>Quality control</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SC</td>
<td>Subcutaneous</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>TMPRSS2</td>
<td>Transmembrane protease serine 2</td>
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CHAPTER I

INTRODUCTION

1.1 PROJECT I: EVALUATION OF A SMALL MOLECULE AGONIST OF EPHA2 RECEPTOR TYROSINE KINASE

1.1.1 Cancer

Considered the second leading cause of death in the United States, cancer is a disease that is still not fully comprehended today. According to the American Cancer Society, about 1.7 million new cases of cancer are projected to be diagnosed in 2018 and about 600,000 deaths are expected (Figure 1.1) (1). Cancer is a disease that springs from the uncontrollable growth of cells; the normal process of cell division and death is disturbed where the orderly progression is changed (5). There are many types of cancers, such as lung, breast, prostate, colon, bladder, pancreatic, kidney, and brain cancer to name a few (10). There are many causes that give rise to cancer, such as an individual’s inherited genetic background or exposure to carcinogens. While cancer by itself is detrimental, metastasis that can arise poses greater risks. Metastasis is a process where
Figure 1.1: The number of estimated new cases of cancer diagnoses in each state in the US in 2018 (I).
cancer cells detach from the primary position and travel to other locations in the body through the blood circulation or the lymphatic systems and form new tumors (7). This stage of cancer is considered severe since it is not confined to one part of the body. In most cases, the probability of survival is decreased in patients that have metastatic cancer compared to those that have localized tumors (14).

Several types of treatment exist for the different types of localized and metastatic cancers. The choice of treatment depends on factors such as the size and location of the primary cancer, the severity of metastasis, other medications that the patient is taking, and the general health of the patient (9). The most common types of treatment are chemotherapy, hormone therapy, thermal ablation, radiation therapy, and surgery (9). Depending on the severity of the cancer, these treatments are used individually or in combination with each other. Even though the treatment options have grown over the years, the side effects of these therapies are yet undesirable. A few of the most common side effects are nausea, vomiting, loss of appetite, alopecia, thrombocytopenia, diarrhea, constipation, and fatigue. In addition to these, long term effects can also arise within months or years after the treatment is completed (8). These include, but are not limited to, cardiac issues, changes in the endocrine system, lung problems, deterioration of bone and soft tissue, problems in the brain and spinal cord, and emotional problems such as post-traumatic stress disorder (8). Due to these predicaments, the focus of cancer research has always been to improve drug efficacy and potency to provide successful treatments with minimal side effects.
1.1.2 Eph Receptor Tyrosine Kinase

In the Receptor Tyrosine Kinase (RTK) family, erythropoietin-producing hepatocellular (Eph) receptors are the largest group (6). It is composed of fourteen receptor subtypes, EphA1-EphA8, EphA10, EphB1-EphB4, and EphB6, and their ligands, called ephrins that are further categorized into subclasses, ephrinA1-A5 and ephrinB1-B3 (13). Similar to other RTKs, the Eph receptors are involved in signal transduction and cell-cell communication which is important for processes such as embryonic morphogenesis, development and maintaining homeostasis in adult tissue (6).

The Eph receptor is a transmembrane protein with an extracellular region that has the ephrin ligand-binding domain and a region that expands to the cytoplasm which consists of the kinase domain (Figure 1.2) (6). The ephrin ligands that are attached to the surface of cells activate receptors that are expressed in the same cell or are released in order to activate receptors on neighboring cells (15). Upon ligand binding, the Eph receptors undergo endocytosis where they are internalized. Moreover, this binding can cause the oligomerization of both receptor-ligand and receptor-receptor complexes (6). The extent to which this process occurs depends on the cellular context which in turn can depend on the cytoskeleton of the cells (6). These interactions eventually lead to the phosphorylation of tyrosines which then allows the kinase domain to be active (6).

Upon entering the cell, the receptors continue to provide signals for different processes unless they are dephosphorylated, degraded, or directed towards the cell membrane. The Eph receptors regulate many of the same proteins that are involved in the downstream of other RTK members. Proteins such as nonreceptor tyrosine kinases of the
Figure 1.2: Diagram of Eph receptors’ structures with their ligands (6).
Src (proto-oncogene tyrosine-protein kinase) and Abl (tyrosine-protein kinase) groups that have an SH2 domain, and adaptors such as Nck and Crk are employed for inducing signaling (6). In addition, the most important downstream effectors are Akt, Rho, and Ras family members that are involved in the regulation of cell survival and proliferation.

Together with the ephrin-dependent pathways, Eph receptors are also known to have activities that are independent of ligand binding (6). These ephrin-independent pathways are known to have opposite effects compared to the ephrin-dependent pathways. This ligand-independent signaling is observed in many cancers where cell proliferation and migration is induced by an upregulation of the receptors and a decrease in ephrin ligand. One such Eph receptor that is being studied for its role in tumor progression is EphA2 (4). Elucidation of its mechanism may provide the basis for the development of a novel therapeutic target against cancer cells.

1.1.3 EphA2

A member of the Eph receptors, EphA2, which was known as epithelial cell kinase (Eck), is a 130 kDa glycoprotein receptor (4). EphA2 is predominant in the nervous system in the embryonic developmental stage, angiogenesis, and contrary to the other Eph receptors, it can also be found in adult epithelial cells (15). Recent studies have reported that the overexpression of EphA2 in cancer cells lead to metastasis and poor prognosis (10). The seemingly contradictory roles of this receptor have been correlated with tumor progression and malignancy especially in cancers such as glioblastoma, breast, melanoma, and prostate carcinoma (4). Binding of the ligand, ephrin-A1, induces
effects that are suppressive, such as apoptosis and cell migration inhibition \((10)\). This was observed in in vivo studies where administration of ephrin-A1 reduced the invasiveness of carcinoma xenografts \((10)\). In the absence of ligand, however, EphA2 receptor is activated through phosphorylation by Akt which stimulates cell migration. Overexpression of EphA2 is generally associated with a decrease in ephrin-A1 ligand, therefore, the migration stimulatory effects are amplified in oncogenic cells \((11)\).

1.1.4 EphA2 in Cancer

The ligand-independent mechanism of EphA2 mentioned above which is conjectured to be the dominant pathway in tumorigenesis prove that it is a viable candidate for the development of drug therapies against cancer. In a study conducted by Bingcheng Wang et al. it was discovered that EphA2 agonists may provide tumor inhibitory effects \((10)\). Their hypothesis was that in cancer cells where the expression of natural ligand is decreased, a small molecule agonist may be able to activate EphA2 receptor and promote tumor invasion suppressive effects \((10)\). The screening of multiple commercially available compounds revealed that doxazosin activated EphA2 substantially in a manner similar to ephrinA-1 \((10)\). Doxazosin is an FDA approved drug, commonly known as Cardura®, which is being used for the treatment of hypertension and the improvement of urination in men suffering with benign prostate hyperplasia (Figure 1.3) \((2,12)\). Its mechanism of action is to block \(\alpha_{1}\)-adrenoreceptor and thereby induce a decrease in the systemic vascular resistance \((3)\). In cancer cells, however, it was
Figure 1.3: Structure of doxazosin.
confirmed that activation of EphA2 and its inhibitory effects were not due to indirect effects of the suppression of α1-adrenoreceptor by doxazosin (10). Additionally, it was discovered that doxazosin exhibited effects similar to those caused by ligand-binding; Binding of doxazosin resulted in the internalization and degradation of the receptor, and also Akt kinase pathways that are responsible for stimulation of tumor progression were inhibited (10).

1.1.5 Our hypothesis

Chemotherapeutic drugs have many side effects that are undesirable and are highly toxic. Therefore, there is a growing need for the development of drugs with enhanced efficacy, specificity, and potency in order to provide cancer patients with a better prognosis. Because of EphA2’s implications in a number of cancers it is a viable therapeutic target. Taking advantage of EphA2’s tumor suppressor functions that are caused by the binding of ligand provides a path for the establishment of a new therapeutic strategy. Since doxazosin was recently discovered to behave in a similar manner to an EphA2 agonist it can be used as a lead compound to generate more potent analogs. We hypothesize that these new compounds may demonstrate increased agonist activity compared to doxazosin thus leading to tumor suppressive activity.
1.1.6 References


1.2 PROJECT II: COPALIC ACID ANALOGS AS PROSTATE CANCER THERAPEUTICS

1.2.1 Prostate cancer

Prostate cancer is the most common type of cancer among men in the United States. There are about 164,690 new cases and about 29,430 deaths reported in 2018 (21). It occurs in the prostate which is a small walnut-shaped gland that secreted prostate fluid which is a component of semen (3) (Figure 1.4). Among other cancers, prostate cancer is the second leading cause of death in men and occurs more in African-American men compared to Caucasian men (21). Nearly all prostate cancers are adenocarcinomas and the early stages do not present any symptoms (21). Advanced stages may have symptoms such as frequent urination, reduced flow of urine, trouble urinating, pain the pelvic region, blood is semen, and bone pain (14). Typically, prostate cancer grows slowly, however, some cancers may grow and spread rapidly (21).

Although uncertain, it is speculated that prostate cancer starts as a pre-cancerous condition which is usually revealed with a prostate biopsy (21). In prostatic intraepithelial neoplasia (PIN) the cells are abnormal but do not appear to grow into the other parts of the prostate. This condition can be seen in men in their early 20s, but not all of them develop cancer (21). In the other condition, proliferative inflammatory atrophy (PIA), the cells look smaller than normal and there are signs of inflammation. It is speculated that this may lead to PIN or directly to prostate cancer (21).
Figure 1.4: Prostate gland is a small walnut-shaped gland located below the bladder in males (14).
Several treatment options exist for prostate cancer which include surgery, radiation therapy (external beam therapy and prostate brachytherapy), focal therapy, hormonal therapy (androgen deprivation therapy), chemotherapy, immunotherapy, bone-directed therapy, and the use of radiopharmaceuticals (5). Although these treatments may be successful at times complications such as urinary, erectile, and bowel dysfunction, fatigue, skin irritation, constipation, and loss of fertility, and resistance can occur (16).

1.2.2 Androgen receptor

Androgen receptor (AR) belongs to the steroid and nuclear receptor superfamily and is a key mediator in prostate cancer (6). AR is primarily expressed in tissues of the prostate, skeletal muscle, liver, and the central nervous system (6). The functions of AR are determined by the binding of ligands, known as androgens, which lead to interactions with other proteins or DNA related to male physiology (6, 19). When bound by AR’s native ligands, 5α-dihydrotestosterone (DHT) and testosterone, it leads to male sexual development and differentiation (19).

AR, a 110 kDa protein encoded by the AR gene located in the X chromosome, consists of three domains: N terminal domain (NTD), DNA binding domain (DBD), and the C terminal ligand binding domain (LBD) connected by a small hinge region (Figure 1.5) (19). The NTD, which is encoded by exon 1, has a highly variable sequence and length of polyglutamine (CAG) and polyglycine (GGC) repeats (19). Structurally, the length of the polyglutamine repeats region seems to affect the folding of the NTD and it is essential for the NTD’s modulatory functions such as contacting the transcriptional
**Figure 1.5**: Organization of the AR gene (6).
machinery (11, 19). The DBD is a highly conserved cysteine-rich region in the receptor (19). Each monomer of DBD has two zinc fingers in the core, and each one consists of four cysteine residues that accommodates a zinc ion (19). The α-helix of the N-terminal zinc finger of DBD directly interacts with the nucleotides in the response elements of the DNA (19). Even though many steroidal receptors share a common response element, the target specificity of AR stems from androgen response elements (AREs) which allows for specific AR activation (19).

In the junction between the DBD and the hinge region is the nuclear localization signal (NLS) that is required for importation of AR into the nucleus (19). The large size of AR does not allow passive transport across the nuclear membrane and therefore needs to be actively transported (19). Studies have demonstrated that androgen binding promotes a transformation in the AR that exposes the NLS that allows for the binding to importin-α in order to be imported into the nucleus (19). The hinge region is also essential for localization as well as binding to DNA and the interaction between NTD and LBD (19). The LBD is composed of 11 α-helices and two anti-parallel β-sheets (6). When a ligand binds, it induces a conformational change in the LBD that allows for the formation of an activation function 2 (AF2) regions which is important for the interaction between the amino and carboxyl termini (6).

When free testosterone enters the cell, it is readily converted in to its more active metabolite, DHT, by the enzyme 5α-reductase (Figure 1.6) (19). The binding of DHT to AR results in the release of chaperone proteins, such as heat shock protein 70 and 90 which are responsible for the stability of the inactive AR. Once DHT binds, heat shock
Figure 1.6: AR signaling pathway (19).
protein 27 kDa (HSP27) regulates the stability, shuttling, and transactivation of AR (18). This also causes a conformational change that enables AR to bind to importin-α so that it can be transported into the nucleus (19). Once inside the nucleus, AR dimers bind to AREs in the target genes’ promotor region such as prostate specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2). Subsequently, they recruit coregulatory proteins to promote transcription which lead to outcomes such as growth and survival (11, 19).

Because of its central role in the development and differentiation of male physiology, AR is closely connected to prostate cancer. Similar to the normal prostate cells, cancer cells in the prostate require androgens for growth and survival (19). And the higher than normal proliferation rate is mediated through the AR pathway. Therefore, the rationale for treatment has been to control the latter which is initiated by suppressing androgens by castration, either surgically or chemically (19). However, most patients eventually develop resistance where they no longer respond to androgen deprivation therapy. In castration resistant prostate cancer (CRPC), the tumor cells continue to grow even though the circulating level of androgens is low. Five different mechanisms have been identified to describe what occurs in CRPC. The first possible mechanism is the amplification of AR in order to increase the sensitivity to the low levels of androgens (4). Moreover, mutations in the AR gene itself and/or 5α-reductase (the enzyme that is responsible for the conversion of testosterone into DHT, which is a native ligand of AR) has also shown to contribute to the increased level of AR (4). A second mechanism is the role of co-activators and co-repressors of AR in stimulating transcriptional pathways (4).
The overexpression of these molecules has shown to be associated with tumor progression. CRPC may also emanate in the absence of ligands in what is known as an outlaw pathway (4). Various growth factors, cytokines, and kinase pathways can contribute to the increased signaling of AR thereby causing the cancer to progress (4).

Another pathway is one that results from altered steroidogenesis; for example, androgen precursors produced from the adrenal gland such as dehydroepiandosterone (DHEA) can be converted into DHT through an alternate pathway (4). The fifth mechanism is via variants of AR which are generated typically without the C-terminal ligand binding domain (4). Because of the resistance to the current treatment options there is a great need for the development of a therapeutic agent that has the ability to overcome resistance and provide a better prognosis for CRPC patients.

1.2.3 Heat shock protein 27 kDa (HSP27)

Heat shock protein 27 kDa (HSP27), also known as HspB1, has implications in many cancers, including prostate cancer. It belongs to the highly conserved family of small heat shock proteins (HSPs) (8). HSPs are primarily recognized for their rapid protective response to stress (17). In the latter, the transcription of HSPs increase and post-translational modifications determine their functions that are required for the specific situation. Even though the N-terminal is variable, all small HSPs share a highly conserved C-terminal, α-crystallin domain (20). HSP27 is involved in several cellular protective functions such as refolding of damaged protein, inhibition of apoptosis, protection against oxidative stress, and regulation of the cytoskeleton (Figure 1.7) (8, 20).
Figure 1.7: Functions of HSP27 (20).
During cellular stress, oligomers of non-phosphorylatable HSP27 bind to eIF4G (eukaryotic translation initiation factor 4 G) thereby inhibiting cap-dependent protein translation (17). HSP27 also acts as a chaperone by binding to the misfolded proteins and transferring them to ATP-dependent chaperones or by leading to proteasomal degradation so that aggregation of misfolded proteins decreases (17). In contrast, when HSP27 is phosphorylated it acts as an anti-apoptotic molecule that can interfere with both the intrinsic and extrinsic apoptotic pathways (17). In the intrinsic pathway, following different stimuli such as oxidative stress, hyperthermia, and cytotoxic agents, HSP27 may interact with both upstream and downstream of cytochrome c release by mitochondria (13). HSP27 interacts with phosphatidylinositol 3-kinase (PI3-K) which activates Akt which in turn phosphorylates Bax which prevents the formation of pores in the mitochondria and thereby prevents the release of cytochrome c (8). It is speculated that HSP27 may also interact directly with cytosolic cytochrome c and inhibit the activation of pro-caspase-9 (13). In the extrinsic pathway, apoptosis is initiated from the membrane receptors such as Fas receptor. When the ligand binds, Fas receptor interacts with Fas associated-death-domain protein (FADD) which in turn activates procaspase-8 and other downstream caspases and also regulates the interaction of Bax with mitochondria (8). Furthermore, Fas receptor may also interact with a protein known as Daxx whose translocation from the nucleus to the cytosol can induce apoptosis (8). Phosphorylated HSP27 may interact with Daxx and prevent its translocation which would then inhibit Daxx-dependent apoptotic activity (8).

When exposed to oxidative stress, HSP27 acts as an antioxidant and increases the level of glutathione and iron in the cells in order to decrease the level of reactive oxygen.
species (ROS). It also leads the oxidized proteins to the proteasome so that they can be degraded (2). HSP27 is a downstream effector of the p38 MAP kinase pathway (mitogen activated protein kinase pathway) that regulates the actin filament dynamics (7). Therefore, during stress, HSP27 is able to stabilize actin microfilaments by inhibiting F-actin polymerization (7).

In addition to its involvement in neurodegenerative and cardiovascular diseases, HSP27 was also found to play a role in many types of cancer such as prostate carcinoma, gastric, liver, breast, osteosarcoma, and lung cancer (20). The overexpression of HSP27 leads to the proliferation, differentiation, and invasion of tumor cells (8). Since it is involved in the inhibition of apoptosis, high levels of HSP27 can result in defects in the apoptotic pathway directly or through its client proteins causing tumor progression (8). This indicates that HSP27 is a potential therapeutic target against cancer.

1.2.4 Copaiba oil

Copaiba oil is a popular medicine widely used for its anti-inflammatory, antimicrobial, antileishmanial, larvicidal, wound healing, antineoplastic, and antinoceptive properties. It is obtained from the trunk of the trees of the Copaifera genus which is native to the regions of Latin America and Western Africa (10). Copaiba oil is administered topically or orally to relieve symptoms that are caused by inflammation (10). Even though there are many species, only a few have been studied. Among the many, Copaiba oil from Copaifera multijuga species and its fractions has shown to inhibit tumor growth in mice.
Copaiba oil is a transparent liquid composed of a mixture of sesquiterpenes and diterpenes. Many studies indicate that the sesquiterpenes are the major component (more than 75%) in Copaiba oil. Because of this, the pharmacological activity can be attributed to the sesquiterpenes present. However, a recent study by Faiella et al. showed that some of the diterpenic acids may also contribute to some activity (Figure 1.8). Hardwikiic acid, one of the diterpenes inhibited HSP27 which is an ATP-independent chaperone protein involved in tumorigenesis and thereby acknowledged to be a major therapeutic target in many cancer types. This finding and the similarities in their structures suggest that other diterpenic acids in Copaiba oil may also interact with small chaperone proteins.

1.2.5 Copalic acid

Copalic acid is a diterpenic acid that is present in Copaiba oil (Figure 1.9). Its molecular weight is 304.47 g/mol and is a weakly acidic compound. In addition to being the most abundant diterpenic acid in copaiba oil, copalic acid is also found in fruits (12). Recent studies have demonstrated that copalic acid interferes with small chaperone proteins such as HSP27 and α-crystallin which makes it a potential lead compound for the development of therapeutic agents (9).
Figure 1.7: The main diterpenic acids present in Copaiba oil (10).
Figure 1.9: The structure of copalic acid (9).
1.2.6 Our hypothesis

The development of resistance to treatment in prostate cancer patients demonstrate the need for a better therapeutic agent. Androgen receptor plays a major role in prostate cancer where it can stimulate the growth and survival of cancer cells in the presence or the absence of its ligands. Since HSP27 is involved in the stability of AR it can be speculated that HSP27 has an indirect role in prostate cancer. Hardwickiic acid obtained from copaiba oil has the ability to inhibit HSP27. Analysis of other diterpenic acids in copaiba oil (because of the structural similarities to harwickiic acid) revealed that another diterpenic acid, copalic acid, also showed inhibition of HSP27. Previously, a study was conducted by knocking down HSP27 with antisense oligonucleotides (ASO) or siRNA in order to decrease the level of AR. Since in vitro and in vivo experiments were successful with this siRNA named “OGX-427” a clinical trial was organized (22). Even though the trial seemed successful, the drawback of this strategy is related to drug delivery. We hypothesize that small molecules such as copalic acid may allow for improved drug delivery. And more potent analogs of copalic acid may be able to inhibit HSP27 at a higher level which would lead to decreased AR activity.

1.2.7 References


CHAPTER II

EVALUATION OF A SMALL MOLECULE AGONIST OF EPHA2 RECEPTOR TYROSINE KINASE

2.1 INTRODUCTION

Cancer is a disease that begins when cells grow uncontrollably. The normal process of cell division and death is disturbed, and the orderly progression is changed. According to the American Cancer Society, about 1.7 million new cases of cancer are projected to be diagnosed in 2018 and about 600,000 deaths are expected (I). Even though there are no specific factors that can cause cancer, there are some that are speculated to pose a greater risk, such as genetics, diet, infections, or exposure to carcinogens (I). There are many types of cancer, including breast, prostate, bladder, kidney, and lung cancer to name a few. Cancer may arise in any tissue and travel and spread to other parts of the body in a process known as metastasis. This stage is severe since it is not confined to one part of the body, and therefore it is more difficult to treat at which point the patient’s probability of survival is decreased. There are several treatment options that are available such as chemotherapy, radiation therapy, hormone therapy, or surgery. These treatments are often accompanied by undesirable side effects. Because of
this, there is a need for the development of a treatment with improved efficacy, potency, and minimal side effects.

Recently, it was discovered that a member of RTK family, EphA2, may prove to be a viable target in developing anti-cancer agents. EphA2 belongs to the Eph receptors which is the largest group of RTKs. Eph receptors are involved in signal transduction and cell-cell communication (10). They are transmembrane proteins with an extracellular binding domain and a region that expands into the cytoplasm consisting of a kinase domain (10). Eph receptors have native ligands known as ephrins whose binding causes the internalization of the receptors. The binding can result in the oligomerization of receptor-ligand and receptor-receptor complexes which will depend on the cellular context of the cells (10). These interactions lead to the phosphorylation of tyrosines activating the kinase domain which would then regulate its downstream proteins that are involved in the regulation of cell survival and proliferation (10).

EphA2 receptor is a 130 kDa glycoprotein receptor that is predominant in the nervous system in the embryonic developmental stage, angiogenesis, and adult epithelial cells (5, 13). In addition to the ligand dependent pathway, EphA2 also has a ligand independent pathway (10). In the absence of its ligand, ephrin-A1, the receptor is activated through phosphorylation by Akt, a serine/threonine protein kinase, that induces cell migration. These seemingly contradictory roles of EphA2 receptor have been correlated with tumor progression and malignancy especially in cancers such as glioblastoma, breast, melanoma, and prostate carcinoma (12). In breast epithelial cells, EphA2 was reported to induce morphological transformation, while in prostate cancer and glioma cell lines elevated levels of EphA2 caused increased chemotactic cell
migration and invasion \((10)\). In cancer, the overexpression of EphA2 is associated with a decrease in the level of ephrin-A1. And this amplifies the migratory stimulatory effects in the oncogenic cells. The ligand-independent pathway seems to be dominant in cancer cells \((1)\). Since the ligand-dependent pathway suppresses cell migration this approach has potential to become a novel therapeutic strategy.

Recently, a small molecule, doxazosin, was identified as an EphA2 agonist which activated the receptor in a manner similar to the native ligand, ephrin-A1. Doxazosin is an FDA approved drug, commonly known as Cardura®, which is being used for the treatment of hypertension and the improvement of urination in men suffering with benign prostate hyperplasia \((2, 12)\). It produces anti-hypertensive effects by blocking \(\alpha_1\)-adrenoreceptor and thereby inducing a decrease in the systemic vascular resistance \((3)\). However, it was confirmed that in oncogenic cells activation of EphA2 and its inhibitory effects were not due to indirect effects of the suppression of \(\alpha_1\)-adrenoreceptor by doxazosin \((1)\). Studies showed that doxazosin exhibited effects similar to those caused by ligand-binding; Binding of doxazosin resulted in the internalization and degradation of the receptor, and also the inhibition of the Akt kinase pathways that are responsible for stimulation of tumor progression \((1)\). This provides a basis for the development of more potent compounds using doxazosin as a lead compound.
2.2 RESULTS AND DISCUSSION

2.2.1 Lead optimization to generate doxazosin analogs

From previous studies it was discovered that doxazosin, an $\alpha_1$-adrenoreceptor blocker, behaved in a similar manner to the native ligand of EphA2, ephrin-A1. Although commonly used to treat high blood pressure and urinary retention linked with benign prostate hyperplasia, doxazosin also activated EphA2 to induce the ligand dependent pathway to inhibit breast, glioma, and prostate tumor cell migration \textit{in vitro} and prostate tumor metastasis \textit{in vivo} (10). In this study, we generated 33 compounds using doxazosin as the lead compound using combinatorial chemistry strategy. As shown in Figure 2.1, the moieties A, B, and C were changed systematically, and some symmetric compounds were synthesized as well. The latter were generated subsequently by attaching the best C moiety on both sides of the piperazine ring in order to determine if A and B moieties are necessary. Moreover, in line with this hypothesis of symmetric compounds adapted from a recent study, dimeric molecules were also synthesized to gauge the activity by modifying the B moiety to various substituted amines.

In compounds 1-24, the C moiety contained various aromatic rings and alkyl groups (Figure 2.2). Compounds 25 and 26 were symmetric molecules with the piperazine ring as the core structure synthesized with a C moiety (Figure 2.3). Figure 2.4 shows the synthesis scheme for generating the dimeric compounds, 27 and 28, with various linkers between the piperazine rings of the main compound. In order to explore whether the primary amino group is necessary for the activity, the B moiety was modified into different substituents. Doxazosin and compound 24 were acetylated to generate
compounds 29 and 30, respectively (Figure 2.5). Furthermore, the B moiety in compound 24 was substituted with alkyl groups to synthesize compounds 31 and 32 (Figure 2.6). Finally, the A moiety was changed from a methoxy group to ethoxy group in compound 24 to generate compound 33 (Figure 2.7).

In order to evaluate the activity of these 33 analogs, MDA-MB-231 breast cancer cells line which overexpresses EphA2 receptor (MDA-MB-231-A2) was used. EphA2 receptor activation was measured by calculating the ratio of phosphorylated EphA2 over the total EphA2 protein present after treatment with each analog. Using 50 µM doxazosin as the positive control and 0.5% DMSO as the negative control, the cells were treated with 2, 10, and 50 µM concentrations of the compounds. The EphA2 receptor activation by 50 µM doxazosin was used as the cutoff point to determine the potency of the new compounds. Some compounds had diminished activity while several compounds showed an improvement: Compounds 22, and 24, which has slightly or very bulky electron donating substituents, respectively, showed higher activity compared to doxazosin. Among the dimer structures, compound 27 showed much improved potency compared to compound 28 (Figure 2.8). The latter consists of a shorter linkage compared to compound 27 which suggests that the length of the linker is important for the activity.
Figure 2.1: Proposed modifications to the structure of doxazosin.
**Figure 2.2**: Synthesis of doxazosin analogs with substituted C moiety.
Figure 2.3: Synthesis of symmetric analogs using piperazine ring as core structure.
Figure 2.4: Synthesis of dimers.
Figure 2.5: Synthesis of B moiety acetylated analogs.
Figure 2.6: Synthesis of B moiety modified analogs.
**Figure 2.7**: Synthesis of A moiety modified analogs.
2.2.2 Internalization of EphA2

The overexpression of EphA2 together with a decrease in the level of its native ligand and binding is implicated in tumorigenesis in many cancers (4, 7, 8). Previous in vitro studies have shown that monoclonal antibodies or small molecule ligands can be used to overcome this ligand-independent pathway of EphA2 (4). And it is speculated that these oncogenic effects may be reversed by ligand binding (9). Unlike in the ligand-independent pathway, when the ligand binds to EphA2 receptor, the receptor is internalized by endocytosis and degradation (9, 10). Since doxazosin behaved in a similar manner to ephrin-A1 in activating the kinase, it was important to investigate whether the compounds caused internalization.

In order to investigate this phenomenon, immunofluorescence was used to monitor the localization of EphA2 receptor after the treatment with the compounds. We chose compound 27 for these studies since it had the highest activity in the initial screening and also because of its novel dimeric structure. The breast cancer cell line, MDA-MB-231, was used for this experiment and ephrin-A1-Fc was used as a positive control. As demonstrated in Figure 2.9, majority of EphA2 was internalized after 60 min of treatment with ephrin-A1-Fc. Consistent with its activity, doxazosin was also able to cause internalization at 50 μM. Compound 27 was able to induce internalization even at a low concentration of 0.4 μM, which indicates that it has increased potency compared to the lead compound.
Figure 2.8: Activation of EphA2 by the most potent compounds, 24 and 27.
2.2.3 Docking studies with compounds 24 and 27

The ligand binding domain has been identified through NMR (Nuclear Magnetic Resonance) and other biological assays in previous studies. Since several of the doxazosin analogs showed improved activity compared to doxazosin it was important to investigate the docking of the compounds. This would elucidate the structural components that are important for stronger interactions with the binding domain. In addition to compound 27, compound 24 was also chosen for this study since it was also an active compound. The docking studies were performed with doxazosin, compound 24, and compound 27 with the crystal structure of the EphA2 extracellular domain (The structure with the PDB code 3FL7 was used) (6). The 1st panel in Figure 2.10 shows the interaction of doxazosin with the ligand binding domain where it forms hydrogen bonds with GLY51, SER68, and ARG103. Moreover, the dihydrobenzo-dioxine moiety of doxazosin fits into a hydrophobic pocket. Comparatively, compound 24 (2nd panel) forms more hydrogen bonds: the methoxy group forms a hydrogen bond with GLY51 since the bulkiness of the aromatic group (compared to doxazosin) positions the methoxy group closer, the aromatic amino group forms hydrogen bonds with GLY51 and SER68, and the ketone of the amide bond forms a hydrogen bond with ARG103. Compound 27, illustrated in the 3rd panel, shows a different binding pattern compared to both doxazosin and compound 24. The bulkiness of compound 27 prevents it from fitting into the hydrophobic pocket of the binding domain and is seen to adapt into two grooves of the protein. The length of the linker seems to be critical for this conformation since a shorter linker would not be able to provide enough flexibility to bind to the groove. In addition to the hydrophobic interaction, compound 27 also form 4 hydrogen bonds: two hydrogen
Figure 2.9: Internalization of EphA2 receptor after treatment with doxazosin and compound 27.
bonds are formed by the nitrogen in the quinazoline ring with ARG103, and the other two hydrogen bonds are formed between the ketone and ARG103.

2.2.4 Adrenoreceptor binding

Doxazosin was initially used to treat hypertension and benign prostate hyperplasia due to its $\alpha_1$-adrenoreceptor antagonistic activity. However, when it was discovered that doxazosin also activated EphA2, it was confirmed in previous studies that this activation was independent of $\alpha$-adrenoreceptor activity. When synthesizing the analogs, our goal was to enhance the potency in activating EphA2 but eliminate the $\alpha$-adrenoreceptor binding activity of doxazosin. In order to analyze this, competition experiments for binding $\alpha$-adrenoreceptor were performed with the analogs. Doxazosin was used as a positive control and prazosin, which is also an $\alpha$-adrenoreceptor antagonist, was used as the competitor. The results show that doxazosin displaced a higher percentage of prazosin and competed to bind to $\alpha$-adrenoreceptor more than the analogs, compounds 24 and 27 (Figure 2.11). This is evident in the IC$_{50}$ values: doxazosin showed a value of 0.74 ± 0.30 nM and compounds 24 and 27 had IC$_{50}$ values of 4.28 ± 3.10 nM and 2.50 ± 1.62 nM, respectively. Even though the structure activity relationship about the binding was not elucidated, this is an indication that the modifications to doxazosin reduced the original $\alpha$-adrenoreceptor binding affinity.
Figure 2.10: Docking of doxazosin, and compound 24 and 27 with EphA2 receptor’s ligand binding domain.
2.2.5 Pharmacokinetic studies with compound 27

The *in vitro* studies with compound 27 demonstrated that it was able to activate the EphA2 receptor similar to doxazosin. Compound 27 formed 4 hydrogen bonds in the ligand binding domain compared to the 3 formed by doxazosin and hence showed increased activity. The α-adrenoreceptor targeting effect was also reduced in compound 27 which further validates that its action is through the EphA2 receptor. Since *in vitro* results were promising, next we explored the pharmacokinetic profile of compound 27. Three-month-old female mice were divided into two groups and compound 27 was administered via subcutaneous (SC) or intraperitoneal (IP) injection. Blood samples and brain tissue from these two different sets of mice were collected at different time points. In order to analyze the pharmacokinetic profile of compound 27, an LC-MS/MS method was developed.

First, the parameters of the mass spectrometer were optimized for the detection of compound 27. Prazosin, which has a similar structure to doxazosin was used as the internal standard (IS) (Figure 2.12). Positive ionization mode was selected for the detection based on the structure of compound 27 after introducing 50 ng/mL with 10 ng/mL of the IS in acetonitrile/water (4:1, v/v) through direct infusion. The mass spectra of compound 27 and IS showed molecular ions at [M+2H]+ 387.3 and [M+H]+ 384.1, respectively. The three most abundant ions after the fragmentation of compound 27 were found at m/z 290.1, 247.1, and 231 (Figure 2.13 A) whereas the product ions of IS were m/z 247.2, 231.1, and 95.1 (Figure 2.13 B). The proposed structures of the fragmented ions are shown in Figure 2.14. For quantification, the multiple reaction monitoring
Figure 2.11: Binding of α-adrenoceptor by doxazosin, and compounds 24 and 27.
(MRM) transitions of m/z 387.3 → 290.1 was selected for compound 27, and m/z 384.1 → 247.1 was used for the IS. The other MRM transitions m/z 387.3 → 247.1 and 387.3 → 231 for compound 27 and 384.1 → 231.1 and 384.1 → 95.1 for IS were also monitored for qualitative accuracy. For the chromatographic separation, gradient elution with a water/acetonitrile mobile phase with 0.1 % formic acid was used (Table I). Compound 27 eluted at 4.22 min and the IS eluted at 4.04 min. After optimizing the detection parameters, the LC-MS/MS method was validated for the quantification of compound 27.

The plasma and brain tissue samples were extracted using the liquid-liquid extraction (LLE) method with ethyl acetate since the protein precipitation (PPT) method generated substantial matrix effects. The calibration curve was obtained using blank plasma and brain homogenate supernatant, and 7 non-blank calibration standards whose concentrations were 2, 5, 10, 20, 50, 100, and 200 ng/mL. The peak area ratio of compound 27 to the IS (y) vs. compound 27 concentration (x) was plotted using a weighted (1/x) linear regression. The compound 27 calibration curves in both mouse plasma and brain tissue were highly linear from 2 to 200 ng/mL with the correlation coefficients (r) greater than 0.99 (data not shown).
Figure 2.12: Structure of Prazosin.
Figure 2.13: Product ion spectra of compound 27 (A) and prazosin (B).
Figure 2.14: Proposed structures of the product ions of compound 27 and prazosin.
**Table I**: HPLC gradient program used for the elution of compound 27 and IS\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Minutes</th>
<th>B%</th>
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<tbody>
<tr>
<td>0-1</td>
<td>10 (isocratic)</td>
</tr>
<tr>
<td>1-4</td>
<td>10-90 (linear)</td>
</tr>
<tr>
<td>4-9</td>
<td>90 (isocratic)</td>
</tr>
<tr>
<td>9-10</td>
<td>10 (linear)</td>
</tr>
<tr>
<td>10-17</td>
<td>10 (isocratic)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mobile phase A was 2% acetonitrile with 0.1% formic acid and mobile phase B was 100% acetonitrile with 0.1% formic acid.
The selectivity and sensitivity of this LC-MS/MS method was evaluated by analyzing blank plasma and brain homogenate, and the Lower Limit of Quantitation (LLOQ) sample (1 ng/mL). The MRM chromatograms of blank plasma, brain homogenate, LLOQ sample spiked with IS, mouse plasma collected at 24 h after the administration of compound 27 and spiked with IS, mouse brain homogenate collected at 24 h after the administration of compound 27 and spiked with IS were compared (Figure 2.15). At 4.22 and 4.04 min no endogenous interference was observed for compound 27 and the IS, respectively. The chromatogram of compound 27 at LLOQ level showed a signal-noise ratio above 10.

The relative matrix effect and extraction efficiency from mouse plasma were evaluated at concentrations of 3, 25, and 160 ng/mL. The relative matrix effect of all the 3 concentrations are approximately 110% which indicates minimal matrix effects (Table II). The relative extraction efficiency for the 3 concentrations ranged from 93% to 102% which indicates that the extraction method is efficient to meet the requirements of quantification. Because of the limited availability of blank brain homogenate, the matrix effect was only evaluated at 3 ng/mL and was found to be in the same range as the plasma matrix effect.

The precision and accuracy of the intra-assay and inter-assay were analyzed at 3 different concentrations of 3, 25, and 160 ng/mL. As shown in Table III, the precision (%CV) and accuracy (%RE) for all the quality control (QC) plasma samples were within
Figure 2.15: Chromatogram of blank plasma and brain homogenate (A), compound 27 and IS in mouse plasma (B), and brain homogenate (C).
**Table II**: Relative matrix effect and extraction efficiency of compound 27 in plasma.

<table>
<thead>
<tr>
<th>Compound 27 Concentration (ng/ml)</th>
<th>Relative matrix effects</th>
<th>Relative extraction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>109.0%</td>
<td>92.6%</td>
</tr>
<tr>
<td>25</td>
<td>111.6%</td>
<td>99.7%</td>
</tr>
<tr>
<td>160</td>
<td>110.1%</td>
<td>101.8%</td>
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15% in both the intra- and inter-assays which indicates that the precision and accuracy of this method fulfills the FDA criteria for the quantitative determination of drug concentration in biological matrices.

The stability of compound 27 in mouse plasma was determined at various storage conditions by measuring the concentrations of the compound in QC samples after storage and comparing the results with the theoretical value. As shown in Table IV, there was no significant degradation of compound 27 in mouse plasma after storing at room temperature for 6 and 24 h, at 20°C for 30 days, and after 3 freeze/thaw cycles. Compound 27 was stable in mouse plasma at room temperature for at least 24 h with a recovery of >90%. The storage stability at 20°C for 30 days was 85.6% and 90.4% at low QC (LQC) and high QC (HQC) levels, respectively. The recovery of compound 27 after 3 freeze/thaw cycles was 87%, and 92.7% at LQC and HQC levels, respectively.

This validated LC-MS/MS method was used to quantify the amount of compound 27 in plasma and brain tissue of mice after dosing at different times. Two sets of plasma and brain tissue samples were collected after IP or SC administration of a single dose of 10 mg/kg compound 27 in phosphate buffered saline (PBS) with 10% Cremophor. The concentration of compound 27 in plasma and brain tissue at different time points was collected for both administration routes. With IP injection, a maximum concentration of 54 ng/mL was reached in plasma at 15 min, whereas with SC injection a maximum concentration of about 150 ng/mL between 30 min and 4 h of administration (Figure 2.16). The diverse pharmacokinetic profiles after the two different types of administration suggest that compound 27 may undergo phase I metabolism at different levels. IP injection is very likely to result in the metabolism of compound 27 in the mouse liver
**Table III:** Intra-assay and Inter-assay for the quantification of compound 27 in mouse plasma.

<table>
<thead>
<tr>
<th>Spiked conc. (ng/ml)</th>
<th>Intra-assay (n=5)</th>
<th>Inter-assay (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Determined conc. (ng/ml)±SD</td>
<td>Precision (% CV)</td>
</tr>
<tr>
<td>3</td>
<td>2.64 ± 0.03</td>
<td>1.1</td>
</tr>
<tr>
<td>25</td>
<td>22.4 ± 1.89</td>
<td>8.4</td>
</tr>
<tr>
<td>160</td>
<td>149 ± 3.74</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Table IV: Analysis of the stability of compound 27 in mouse plasma.

<table>
<thead>
<tr>
<th>Storage Conditions (n= 3)</th>
<th>Spiked conc. (ng/ml)</th>
<th>Determined conc. (ng/ml)±SD</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At room temperature for 4 hr</td>
<td>3 160</td>
<td>2.88 ± 0.16 151 ± 5.6</td>
<td>96 94.3</td>
</tr>
<tr>
<td>At room temperature for 24 hr</td>
<td>3 160</td>
<td>2.76 ± 0.01 151.3 ± 7.8</td>
<td>92.0 94.5</td>
</tr>
<tr>
<td>Three freeze-thaw cycles</td>
<td>3 160</td>
<td>2.62 ± 0.06 148.3 ± 4.7</td>
<td>87.0 92.7</td>
</tr>
<tr>
<td>Long-term stability (at -20°C for 30 days)</td>
<td>3 160</td>
<td>2.57± 0.10 144.7 ± 8.5</td>
<td>85.6 90.4</td>
</tr>
</tbody>
</table>
leading to a lower maximum concentration compared to that which is administered via SC injection. In the brain, the maximum concentration of compound 27 was 5 pg/mL at 5 h with IP injection, and 26 ng/ml at 3 h with SC injection. These results indicate that a portion of compound 27 that is in circulation may pass through the blood brain barrier and accumulate in brain tissue.

The results from the pharmacokinetic studies reveal that compound 27 may be subjected to first pass metabolism. Therefore, further investigation of compound 27’s metabolites may provide valuable information for the optimization of its structure to improve its pharmacokinetic behavior. Compound 27 was also able to pass the blood brain barrier which suggests that it can ultimately be used for the treatment of glioblastoma. Further studies would include evaluation of EphA2 agonist activity and therapeutic efficacy of compound 27 in vivo. Based on those results the structure of compound 27 can be optimized to increase efficacy and potency. Thus far, the results have been promising in generating a novel anti-cancer agent that functions as an EphA2 agonist to inhibit tumor progression.
Figure 2.16: Pharmacokinetic profile of compound 27 in mouse plasma and brain after IP (A) and SC (B) administration.
2.3 MATERIALS AND METHODS

2.3.1 Chemistry

Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in flame-dried glassware. Solvents were distilled before use under argon. Thin-layer chromatography (TLC) was performed on pre-coated silica gel F254 plates (Whatman). Column chromatography was performed using silica gel 60Å (Merck, 230-400 Mesh), and hexane/ethyl acetate was used as the elution solvent. Mass spectra were obtained on the electrospray mass spectrometer at Cleveland State University MS facility Center. The molecular weight of the compounds was examined with LC-MS. All the NMR spectra were recorded on a Bruker 400 MHz in either DMSO-d6 or CDCl3. Chemical shifts (δ) for 1H NMR spectra are reported in parts per million to residual solvent protons.

HPLC grade acetonitrile and ACS grade ethyl acetate were purchased from BDH chemicals (Carle Place, NY) and LC-MS grade formic acid was purchased from Fisher Scientific (Waltham, MA). Deionized water was purified using a Barnstead NANOpure® water purification system from Thermo Scientific (Waltham, MA). Six different lots of Non Swiss Albino Mouse plasma were obtained from Innovative Research (Novi, MI).
2.3.2 Synthesis of doxazosin analogs

Compounds 1-24, 27 and 28 were prepared according to the following procedure.

Dimethoxyquinazolin and piperazine were dissolved in n-butanol (n-BuOH) with 1 to 5 mole ratio, and the mixture was heated to 100°C for 24 h and cooled down to room temperature. The precipitated while solid was collected via filtration and washed with n-BuOH. After it was dried, the solid was mixed with different substituted acetyl chlorides and potassium carbonate (K₂CO₃) in dimethylformamide (DMF). It was stirred until the reaction was completed. The reaction was quenched with sodium carbonate (Na₂CO₃) aqueous solution and stirred overnight, and the precipitated product was collected via filtration and washed with water and dried to give the corresponding compounds.

**Compound 1: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(naphthalen-2-yl) methanone**

White solid, melting point 2226-229°C, 72% yield for the last step; ¹H-NMR (400MHz, DMSO-d₆) δ 8.016 (4H, m), 7.580 (3H, m), 7.440 (1H, s), 7.167 (2H, br), 6.745 (1H, s), 3.795 (12H, m), 3.490 (2H, br). ¹³C-NMR (100MHz, DMSO-d₆) δ 169.642, 161.632, 158.757, 154.699, 149.117, 145.535, 133.709, 132.754, 130.970, 129.745, 128.525, 127.826, 125.353, 105.645, 104.111, 103.460, 67.487, 56.294, 66.889, 25.592. DUIS-MS calculated for C₂₅H₂₅N₅O₄ [M + H]⁺: 444.1, found : 443.5

**Compound 2: [1,1'-biphenyl]-3-yl(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl) methanone**

White solid, melting point 179-182°C, 73% yield for the last step; ¹H-NMR (400MHz, DMSO-d₆) δ 7.735 (4H, m), 7.498 (6H, m), 7.152 (2H, br), 6.740 (1H, s), 3.794 (12H,
m), 3.464 (2H, m). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.475, 161.620, 158.802, 154.687, 149.184, 145.519, 140.765, 139.825, 137.207, 129.509, 128.279, 127.297, 126.522, 125.633, 105.669, 104.085, 103.455, 56.287, 66.879. DUIS-MS calculated for C$_{27}$H$_{27}$N$_5$O$_3$ [M + H]$^+$: 470.1, found : 469.2

**Compound 3:** (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) (benzo [d] [1,3] dioxol-5-yl) methanone

White solid, melting point 267-269°C, 71% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.431 (1H, s), 7.145 (2H, br), 6.981 (3H, m), 6.736 (1H, s), 6.094 (2H, s), 3.791 (10H, m), 3.541 (4H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.133, 161.610, 158.769, 154.682, 149.187, 148.700, 147.605, 145.499, 130.049, 121.990, 108.458, 105.666, 104.094, 103.440, 101.901, 56.503, 56.283, 55.878, 44.074, 19.023. DUIS-MS calculated for C$_{22}$H$_{23}$N$_5$O$_5$ [M + H]$^+$: 438.1, found : 437.5

**Compound 4:** 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)-2-(4-methoxyphenyl) ethanone

White solid, melting point 240-243°C, 65% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.422 (1H, s), 7.168 (4H, m), 6.881 (2H, d, J= 8.8 Hz), 6.734 (1H, s), 3.831 (3H, s), 3.788 (3H, s), 3.729 (3H, s), 3.671 (4H, m), 3.601 (2H, br), 3.532 (4H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.795, 161.584, 158.522, 154.669, 149.169, 145.485, 130.419, 128.163, 114.233, 105.668, 104.061, 103.412, 56.276, 55.662, 45.928, 44.254, 43.886, 41.754. DUIS-MS calculated for C$_{23}$H$_{27}$N$_5$O$_4$ [M + H]$^+$: 438.1, found : 437.5
Compound 5: 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)-2-(3-methoxyphenyl) ethanone

White solid, melting point 198-201°C, 60% yield for the last step; $^1$H-NMR (400MHz, DMSO-$d_6$) $\delta$ 7.423 (1H, s), 7.231 (1H, m), 7.143 (1H, br), 6.817 (3H, m), 6.737 (1H, s), 3.830 (3H, s), 3.788 (3H, s), 3.738 (5H, m), 3.598 (9H, m). $^{13}$C-NMR (100MHz, DMSO-$d_6$) $\delta$ 169.382, 161.590, 159.707, 158.745, 154.676, 149.159, 145.495, 137.832, 129.834, 121.631, 115.114, 112.281, 105.662, 104.060, 103.416, 67.488, 56.275, 55.464, 45.977, 44.237, 43.878, 41.783. DUIS-MS calculated for C$_{23}$H$_{27}$N$_5$O$_4$ [M + H]$^+$: 438.1, found : 437.5

Compound 6: 1- (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)-2-phenylethanone

White solid, melting point 221-223°C, 96% yield for the last step; $^1$H-NMR (400MHz, DMSO-$d_6$) $\delta$ 7.420 (1H, s), 7.272 (5H, m), 7.140 (2H, br), 6.733 (1H, s), 3.829 (3H, s), 3.786 (3H, s), 3.775 (2H, s), 3.607 (8H, m). $^{13}$C-NMR (100MHz, DMSO-$d_6$) $\delta$ 169.500, 161.590, 159.707, 158.745, 154.676, 149.159, 145.495, 137.832, 129.834, 121.631, 115.114, 112.281, 105.662, 104.060, 103.416, 67.488, 56.275, 55.464, 45.977, 44.237, 43.878, 41.783. DUIS-MS calculated for C$_{22}$H$_{25}$N$_5$O$_3$ [M + H]$^+$: 408.1, found : 407.2

Compound 7: 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)-2-(naphthalen-2-yl) ethanone

White solid, melting point 236-238°C, 70% yield for the last step; $^1$H-NMR (400MHz, DMSO-$d_6$) $\delta$ 7.862 (3H, m), 7.774 (1H, s), 7.436 (4H, m), 7.133 (2H, br), 6.725 (1H, s), 3.957 (2H, s), 3.823 (3H, s), 3.782 (3H, s), 3.610 (8H, m). $^{13}$C-NMR (100MHz, CDCl$_3$-$d_6$) $\delta$ 169.688, 160.521, 158.510, 155.158, 149.759, 145.970, 133.577, 132.459, 128.540,

DUIS-MS calculated for C_{26}H_{27}N_{5}O_{3} [M + H]^{+}: 458.1, found : 457.5

**Compound 8: 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)-2-phenylpropan-1-one**

White solid, melting point 207-209°C, 82% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-d$_6$) δ 7.400 (1H, s), 7.306 (5H, m), 7.112 (2H, br), 6.704 (1H, s), 4.155 (1H, q, \(J=6.8\) Hz), 3.816 (3H, s), 3.775 (3H, s), 3.680-3.399 (6H, m), 3.007 (1H, m), 1.316 (3H, d, \(J=6.8\) Hz). \(^{13}\)C-NMR (100MHz, CDCl$_3$-d$_6$) δ 172.254, 160.514, 158.541, 155.142, 149.783, 145.917, 142.085, 128.993, 127.214, 126.849, 105.782, 102.866, 101.177, 56.091, 45.570, 43.777, 42.118, 20.719. DUIS-MS calculated for C_{22}H_{23}N_{5}O_{3} [M + H]^{+}: 422.1, found : 421.5

**Compound 9: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(pyridin-2-yl)methanone**

White solid, melting point 250-252°C, 42% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-d$_6$) δ 8.625 (1H, d, \(J=4.4\) Hz), 7.952 (1H, t, \(J=8\) Hz), 7.615 (1H, d, \(J=8\) Hz), 7.507 (1H, m), 7.432 (1H, s), 7.156 (2H, br), 6.738 (1H, s), 3.831-3.726 (12H, m), 3.469 (2H, m). \(^{13}\)C-NMR (100MHz, DMSO-d$_6$) δ 167.378, 161.623, 158.737, 154.599, 149.175, 148.901, 145.529, 137.864, 125.097, 123.712, 105.685, 104.109, 103.456, 56.299, 55.886, 47.042, 44.409, 43.866, 42.193. DUIS-MS calculated for C_{20}H_{22}N_{6}O_{3} [M + H]^{+}: 395.1, found : 394.4
Compound 10: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) (pyridin-4-yl) methanone

White solid, melting point 235-237°C, 32% yield for the last step; \(^{1}\)H-NMR (400MHz, DMSO-\(d_6\)) \(\delta\) 8.697 (2H, d, \(J= 4.8\) Hz), 7.444 (3H, m), 7.163 (2H, br), 6.735 (1H, s), 3.830-3.710 (14H, m) (Note: it seems that two protons are missing but they may overlap with the peak from water). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 167.422, 161.632, 158.709, 154.711, 150.553, 149.161, 145.559, 143.952, 121.794, 105.669, 104.104, 103.463, 56.301, 55.896, 47.319, 44.215, 43.737, 41.990. DUIS-MS calculated for \(C_{20}H_{22}N_6O_3\) [M + H]\(^{\dagger}\): 395.1, found : 394.4

Compound 11: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) (4-ethylphenyl) methanone

White solid, melting point 240-242°C, 84% yield for the last step; \(^{1}\)H-NMR (400MHz, DMSO-\(d_6\)) \(\delta\) 7.433 (1H, s), 7.370 (2H, d, \(J= 8\) Hz), 7.302 (2H, d, \(J= 8\) Hz), 7.154 (2H, br), 6.735 (1H, s), 3.831-3.445 (14H, m), 2.660 (2H, q, \(J= 7.6\) Hz), 1.214 (3H, t, \(J= 7.6\) Hz). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 169.768, 161.614, 158.774, 154.687, 149.181, 145.696, 133.742, 128.206, 127.749, 105.667, 104.088, 103.453, 56.284, 55.877, 28.458, 15.801. DUIS-MS calculated for \(C_{23}H_{27}N_5O_3\) [M + H]\(^{\dagger}\): 422.1, found : 421.5

Compound 12: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) (4-iodophenyl) methanone

White solid, melting point 254-258°C, 85% yield for the last step; \(^{1}\)H-NMR (400MHz, DMSO-\(d_6\)) \(\delta\) 7.843 (2H, d, \(J= 8\) Hz), 7.423 (1H, s), 7.252 (2H, d, \(J= 8\) Hz), 7.144 (2H, br), 6.735 (1H, s), 3.841-3.716 (14H, m). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 168.865,
161.618, 158.734, 154.704, 149.161, 145.533, 137.730, 135.857, 129.657, 105.670, 104.110, 103.454, 96.865, 56.301, 55.893. DUIS-MS calculated for C_{21}H_{22}IN_5O_3 [M + H]^+: 520.0, found : 519.3

**Compound 13:** (4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) (4-chloro-3-nitrophenyl) methanone

White solid, melting point 268-272°C, 91% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-d\(_6\)) \(\delta\) 8.179 (1H, s), 7.883 (1H, d, \(J= 8\) Hz), 7.792 (1H, d, \(J= 8\) Hz), 7.434 (1H, s), 7.159 (2H, br), 6.740 (1H, s), 3.833-3.401 (14H, m). \(^{13}\)C-NMR (100MHz, DMSO-d\(_6\)) \(\delta\) 166.451, 161.632, 158.722, 154.699, 149.143, 148.029, 145.547, 136.683, 132.589, 126.400, 124.905, 105.664, 104.089, 103.456, 56.293, 55.892. DUIS-MS calculated for C_{21}H_{21}ClN_6O_5 [M + H]^+: 473.0, found : 472.9

**Compound 14:** 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)ethanone

White solid, melting point 244-247°C, 88% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-d\(_6\)) \(\delta\) 7.426 (1H, s), 7.143 (2H, br), 6.745 (1H, s), 3.836-3.684 (10H, m), 3.490 (4H, m), 2.050 (3H, s). \(^{13}\)C-NMR (100MHz, DMSO-d\(_6\)) \(\delta\) 168.845, 161.606, 158.768, 154.682, 149.209, 145.469, 105.675, 104.106, 103.410, 56.289, 55.885, 46.158, 44.261, 43.872, 41.361, 21.803. DUIS-MS calculated for C_{16}H_{21}N_5O_3 [M + H]^+: 332.1, found : 331.4

**Compound 15:** 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)propan-1-one

White solid, melting point 269-272°C, 63% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-d\(_6\)) \(\delta\) 7.428 (1H, s), 7.141 (2H, br), 6.746 (1H, s), 3.837-3.686 (10H, m), 3.507
(4H, m), 2.366 (2H, q, \( J = 7.2 \) Hz), 1.017 (3H, t, \( J = 7.2 \) Hz). \(^{13}\)C-NMR (100MHz, DMSO-d6) \( \delta \) 171.950, 161.602, 158.795, 154.682, 149.227, 145.466, 105.680, 104.110, 103.413, 56.289, 55.879, 45.251, 44.304, 43.930, 41.538, 26.125, 9.897. DUIS-MS calculated for C\(_{17}\)H\(_{23}\)N\(_{5}\)O\(_{3}\) [M + H]\(^{+}\): 346.1, found : 345.4

**Compound 16: 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)butan-1-one**

White solid, melting point 259-262°C, 72% yield for the last step; \(^{1}\)H-NMR (400MHz, DMSO-d6) \( \delta \) 7.426 (1H, s), 7.142 (2H, br), 6.743 (1H, s), 3.836 (3H, s), 3.791 (3H, s), 3.735 (2H, m), 3.683 (2H, m), 3.507 (4H, m), 2.337 (2H, t, \( J = 7.2 \) Hz), 1.555 (2H, m), 0.914 (3H, t, \( J = 7.6 \) Hz). \(^{13}\)C-NMR (100MHz, DMSO-d6) \( \delta \) 171.13, 161.60, 158.78, 154.68, 149.23, 145.47, 105.68, 104.11, 103.41, 67.49, 56.29, 55.86, 45.41, 44.37, 43.96, 41.49, 34.76, 25.59, 18.69, 14.31. DUIS-MS calculated for C\(_{18}\)H\(_{25}\)N\(_{5}\)O\(_{3}\) [M + H]\(^{+}\): 360.1, found : 359.4

**Compound 17: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(cyclohexyl)methanone**

White solid, melting point 220-222°C, 89% yield for the last step; \(^{1}\)H-NMR (400MHz, DMSO-d6) \( \delta \) 7.429 (1H, s), 7.138 (2H, br), 6.740 (1H, s), 3.837 (3H, s), 3.792 (3H, s), 3.703 (4H, m), 3.520 (4H, m), 2.627 (1H, m), 1.701 (5H, m), 1.336 (4H, m), 1.174 (1H, m). \(^{13}\)C-NMR (100MHz, DMSO-d6) \( \delta \) 174.107, 161.596, 158.774, 154.691, 149.220, 145.480, 105.684, 104.138, 103.432, 56.299, 55.879, 45.298, 44.640, 43.992, 41.567, 29.631, 26.081, 25.656, 19.020. DUIS-MS calculated for C\(_{21}\)H\(_{29}\)N\(_{5}\)O\(_{3}\) [M + H]\(^{+}\): 400.2, found : 399.5
Compound 18: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(furan-2-yl)methanone

White solid, melting point 230-233°C, 66% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.869 (1H, d, J= 0.8 Hz), 7.438 (1H, s), 7.162 (2H, br), 7.037 (1H, d, J= 3.2 Hz), 6.752 (1H, s), 6.652 (1H, dd, J= 0.8, 3.2 Hz), 3.795 (14H, m). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 161.633, 158.862, 154.712, 149.213, 147.474, 145.521, 145.205, 116.020, 111.775, 105.700, 104.137, 103.463, 67.487, 56.302, 55.886, 44.153, 25.591. DUIS-MS calculated for C$_{19}$H$_{21}$N$_5$O$_4$ [M + H]$^+$: 384.1, found : 383.4

Compound 19: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(thiophen-2-yl)methanone

White solid, melting point 222-224°C, 56% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.784 (1H, d, J= 4.8 Hz), 7.475 (1H, d, J= 3.2 Hz), 7.438 (1H, s), 7.161 (3H, m), 6.750 (1H, s), 3.811(10H, m), 3.761 (4H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 162.930, 161.637, 158, 732, 154.715, 149.215, 145.523, 137.835, 130.013, 129.650, 127.626, 105.698, 104.143, 103.465, 56.308, 55.892, 44.090. DUIS-MS calculated for C$_{19}$H$_{21}$N$_5$O$_3$S [M + H]$^+$: 400.1, found : 399.5

Compound 20: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(isoazol-5-yl)methanone

White solid, melting point 249-251°C, 43% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 8.782 (1H, s), 7.442 (1H, s), 7.198 (2H, br), 6.984 (1H, s), 6.758 (1H, s), 3.839-3.611 (14H, m). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 161.661, 157.432, 151.435,
Compound 21: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(4-methoxyphenyl) methanone

White solid, melting point 229-232°C, 85% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.420 (3H, m), 7.148 (2H, br), 7.007 (2H, d, $J=8.4$ Hz), 6.738 (1H, s), 3.813 (13H, m), 3.557 (4H, m). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.598, 161.623, 160.689, 158.790, 154.705, 149.211, 145.517, 129.628, 128.371, 114.131, 105.691, 104.134, 103.458, 56.302, 55.803, 44.140. DUIS-MS calculated for C$_{22}$H$_{25}$N$_5$O$_4$ [M + H]$^+$: 424.1, found : 423.5

Compound 22: (4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)(4-bromophenyl) methanone

White solid, melting point 128-130°C, 83% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.665 (2H, d, $J=8.4$ Hz), 7.440 (1H, s), 7.407 (2H, d, $J=8.4$ Hz), 7.164 (2H, br), 6.744 (1H, s), 3.832 (3H, s), 3.793 (3H, s), 3.725 (6H, m), 3.388 (2H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 168.663, 161.624, 158.747, 154.710, 149.178, 145.538, 136.589, 131.933, 129.770, 123.409, 105.679, 104.115, 103.4584, 56.303, 55.895. DUIS-MS calculated for C$_{21}$H$_{22}$N$_5$O$_3$Br [M + H]$^+$: 474.0, found : 472.5

Compound 23: 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl)hexan-1-one

White solid, melting point 170-172°C, 73% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.427 (1H, s), 7.146 (2H, br), 6.743 (1H, s), 3.834 (3H, s), 3.790 (3H, s),
3.731 (2H, m), 3.679 (2H, m), 3.500 (4H, m), 2.335 (2H, t, \(J = 7.2\) Hz), 1.516 (2H, m), 1.287 (4H, m), 0.872 (3H, t, \(J = 6.4\) Hz). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 171.287, 161.603, 158.789, 154.690, 149.226, 145.478, 105.679, 104.123, 103.424, 56.294, 55.878, 45.433, 44.380, 43.959, 41.508, 32.789, 31.497, 24.984, 22.452, 14.356. DUIS-MS calculated for \(\text{C}_{20}\text{H}_{29}\text{N}_5\text{O}_3\) [M + H\(^+\): 388.1, found : 387.5

**Compound 24:** 1-(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl)-2-(4-(benzyloxy)phenyl) ethanone

White solid, melting point 230-234°C, 89% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-\(d_6\)) \(\delta\) 7.381 (6H, m), 7.169 (4H, m), 6.958 (2H, d, \(J = 8.4\) Hz), 6.740 (1H, s), 5.071 (2H, s), 3.830 (3H, s), 3.789 (3H, s), 3.693-3.535 (10H, m). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 169.755, 161.602, 158.781, 157.409, 154.701, 149.206, 145.510, 137.636, 130.455, 128.410, 115.151, 105.703, 104.125, 103.441, 69.643, 67.493, 56.302, 55.879, 45.937, 44.274, 43.903, 41.764, 25.596. DUIS-MS calculated for \(\text{C}_{29}\text{H}_{31}\text{N}_5\text{O}_4\) [M + H\(^+\): 514.2, found : 513.6

**Compound 27:** 1,12-bis(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) dodecane-1,12-dione

White solid, melting point 218-225°C, 68% yield for the last step; \(^1\)H-NMR (400MHz, DMSO-\(d_6\)) \(\delta\) 7.425 (2H, s), 7.145 (4H, br), 6.740 (2H, s), 3.831 (6H, s), 3.787 (6H, s), 3.700 (8H, m), 3.495 (8H, br), 2.331 (4H, t, \(J = 7.2\) Hz), 1.504 (4H, br), 1.267 (12H, br). \(^{13}\)C-NMR (100MHz, DMSO-\(d_6\)) \(\delta\) 171.277, 161.596, 158.786, 154.682, 149.221, 145.467, 105.680, 104.119, 103.417, 56.293, 55.880, 45.427, 44.380, 43.958, 41.504,
32.834, 29.358, 25.306. DUIS-MS calculated for C_{40}H_{56}N_{10}O_{6} [M + H]^+: 774.0, found: 772.9

**Compound 28: 1,6-bis(4-(4-amino-6,7-dimethoxyquinazolin-2-yl) piperazin-1-yl) hexane-1,6-dione**

White solid, melting point 279-280°C, 77% yield for the last step; \(^1^H\)-NMR (400MHz, DMSO-\textit{d}_6) \(\delta\) 7.424 (2H, s), 7.138 (4H, br), 6.743 (2H, s), 2.846-2.507 (28H, m), 2.393 (4H, br), 1.567 (4H, br). \(^1^3^C\)-NMR (100MHz, DMSO-\textit{d}_6) \(\delta\) 171.217, 161.602, 158.789, 154.683, 149.218, 145.471, 105.679, 104.105, 103.413, 56.293, 55.882, 45.433, 44.386, 43.962, 41.521, 32.759, 25.050. DUIS-MS calculated for C_{34}H_{44}N_{10}O_{6} [M + H]^+: 689.1, found: 688.8

**Compounds 25 and 26 were prepared according to the following procedure.**

The substituted acetyl chloride and piperazine were dissolved in DMF and K\textsubscript{2}CO\textsubscript{3} with 2 to 1 mole ratio, and the mixture was stirred at room temperature until the reaction was completed. The reaction was quenched with Na\textsubscript{2}CO\textsubscript{3} aqueous solution and stirred overnight, and the precipitated product was collected via filtration and washed with water and dried to give the corresponding compounds.

**Compound 25: piperazine-1,4-diylbis((4-methoxyphenyl)methanone)**

White solid, melting point 215°C decomposed, 86% yield for the last step; \(^1^H\)-NMR (400MHz, DMSO-\textit{d}_6) \(\delta\) 7.403 (4H, d, \(J= 8.4\) Hz), 6.994 (4H, d, \(J= 8.4\) Hz), 3.799 (6H, s), 3.542 (8H, br). \(^1^3^C\)-NMR (100MHz, DMSO-\textit{d}_6) \(\delta\) 169.643, 160.793, 129.640, 127.996, 114.152, 55.746. DUIS-MS calculated for C_{20}H_{22}N_{2}O_{4} [M + H]^+: 355.1, found: 354.4
Compound 26: piperazine-1,4-diylbis((4-(methylthio)phenyl)methanone)

White solid, melting point 220°C decomposed, 91% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$)$\delta$ 7.378 (4H, d, $J$= 8.4 Hz), 7.315 (4H, d, $J$= 8.4 Hz), 3.355 (6H, s), 3.533 (8H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$)$\delta$ 169.394, 141.119, 132.045, 128.339, 125.694, 14.715. DUIS-MS calculated for C$_{20}$H$_{22}$N$_2$O$_2$S$_2$ [M + H]$^+$: 387.1, found : 386.5

Compounds 29 and 30 were prepared according to the following procedure.

Doxazosin or compound 24 were dissolved in DMF and K$_2$CO$_3$, and acetyl chloride was added to the two reactions, respectively, with 1 to 5 mole ratio to the two compounds. The mixture was stirred until the reaction was completed. The reaction was quenched with Na$_2$CO$_3$ aqueous solution and stirred overnight, and the precipitated product was collected via filtration and washed with water and dried to give the corresponding compounds.

Compound 29: N-(2-(4-(2,3-dihydrobenzo[b][1,4]dioxine-2-carbonyl)piperazin-1-yl)-6,7-dimethoxyquinazolin-4-yl)acetamide

White solid, melting point 269-271°C, 36% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$)$\delta$ 10.464 (1H, s), 7.477 (1H, s), 6.874 (5H, m), 5.296 (1H, dd, $J$= 2.4, 6.4Hz), 4.427 (1H, dd, $J$= 2.4, 11.6 Hz), 4.220 (1H, dd, $J$=6.8, 12 Hz), 3.899 (3H, s), 3.846 (3H, s), 3.713 (8H, m), 2.411 (3H, s). $^{13}$C-NMR (100MHz, DMSO-d$_6$)$\delta$ 171.532, 165.464, 157.725, 156.160, 151.018, 146.535, 143.432, 121.912, 117.443, 105.629, 103.741,
69.985, 65.205, 56.267, 45.373, 44.592, 43.964, 41.858, 25.802. DUIS-MS calculated for C_{25}H_{27}N_{5}O_{6} [M + H]^+: 494.1, found : 493.5

**Compound 30: N-(2-(4-(2-(4-(benzyloxy) phenyl) acetyl) piperazin-1-yl) -6,7-dimethoxyquinazolin-4-yl) acetamide**

White solid, melting point 205-209°C, 29% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) $\delta$ 10.436 (1H, s), 7.382 (6H, m), 7.181 (2H, d, $J=$ 8.4 Hz), 6.958 (2H, d, $J=$ 8.4 Hz), 6.897 (1H, s), 5.076 (2H, s), 3.889 (3H, s), 3.838 (3H, s), 3.704 (6H, m), 3.583 (4H, br), 2.394 (3H, s). $^{13}$C-NMR (100MHz, CDCl$_3$-d$_6$) $\delta$ 170.048, 157.680, 156.151, 154.624, 151.293, 147.091, 136.950, 129.637, 128.582, 127.556, 115.237, 105.657, 100.764, 70.084, 56.270, 45.968, 44.102, 41.715, 40.284, 25.679. DUIS-MS calculated for C$_{31}$H$_{33}$N$_5$O$_5$ [M + H]$^+$: 556.2, found : 555.6

**Compounds 31 and 32 were prepared according to the following procedure.**

The dichloro-dimethoxyquinazolin and piperidine or pyrrolidine were dissolved in n-BuOH with 2 to 1 mole ratio, and the mixture was heated to 70°C for 24 h, and then cooled down to room temperature. The precipitated while solid was collected via filtration and washed with n-BuOH to get the corresponding intermediates. After it was dried, the intermediates and piperazine were dissolved in n-BuOH with 1 to 5 mole ratio, respectively. The two reactions were heated to 100°C for 24 h, then it was cooled down to room temperature. The precipitated while solid was collected via filtration and washed with n-BuOH to get two new intermediates, 6,7-dimethoxy-2-(piperazin-1-yl)-4-(piperidin-1-yl) quinazoline, 6,7-dimethoxy-2-(piperazin-1-yl)-4-(pyrrolidin-1-yl) quinazoline. Last, the two intermediates were mixed with 2-(4-(benzyloxy) phenyl) acetyl
chloride (1:1 mole ratio) and K$_2$CO$_3$ in DMF, respectively. The reactions were stirred until completed and were quenched with Na$_2$CO$_3$ aqueous solution and stirred overnight. The precipitated products were collected via filtration and washed with water and dried to give the corresponding compounds 31, 32.

**Compound 31: 2-(4-(benzyloxy) phenyl)-1-(4-(6,7-dimethoxy-4-(piperidin-1-yl) quinazolin-2-yl) piperazin-1-yl) ethanone**

White solid, melting point 160°C decomposed, 56% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.383 (5H, m), 7.182 (2H, d, J= 8.8 Hz), 6.958 (2H, d, J= 7.2 Hz), 6.848 (1H, s), 5.077 (2H, s), 3.861 (3H, s), 3.813 (3H, s), 3.561 (15H, m), 1.687 (6H, br). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.778, 165.057, 157.608, 154.765, 151.037, 145.215, 137.534, 130.440, 128.408, 115.157, 106.105, 105.426, 104.824, 69.631, 56.021, 55.906, 50.828, 45.820, 44.325, 43.913, 41.647, 25.832, 24.832. DUIS-MS calculated for C$_{34}$H$_{39}$N$_5$O$_4$ [M + H]$^+$: 582.2, found : 581.7

**Compound 32: 2-(4-(benzyloxy) phenyl)-1-(4-(6,7-dimethoxy-4-(pyrrolidin-1-yl) quinazolin-2-yl) piperazin-1-yl) ethanone**

White solid, melting point 137°C decomposed, 60% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.382 (6H, m), 7.179 (2H, d, J= 8.4 Hz), 6.958 (2H, d, J= 8.4 Hz), 6.793 (1H, s), 5.075 (2H, s), 3.815 (10H, m), 3.623 (10H, m), 1.939 (4H, m). $^{13}$C-NMR (100MHz, DMSO-d$_6$) δ 169.772, 159.752, 157.638, 154.027, 150.966, 144.298, 137.636, 130.425, 128.517, 115.166, 106.435, 105.506, 69.638, 56.086, 55.829, 50.454, 25.716. DUIS-MS calculated for C$_{33}$H$_{37}$N$_5$O$_4$ [M + H]$^+$: 568.2, found : 567.7
Compound 33 was prepared according to the following procedure.

Boron tribromide (1:1.2 mole ratio) was added to a solution of 4-amino-2-chloro-6,7-dimethoxyquinazoline in dichloromethane (CH$_2$Cl$_2$) that was cooled to -70 °C under argon (II). The mixture was allowed to warm to room temperature over a period of 4 h and was then cooled to -70 °C; methanol was added to quench the reaction, and the solution was concentrated. The solid residue was washed with ethyl acetate to obtain the intermediate 4-amino-2-chloro-6,7-dihydroxyquinazoline. A mixture of the intermediate, ethyl iodine and K$_2$CO$_3$ in DMF was stirred overnight and purified by silica gel chromatography to afford 4-amino-2-chloro-6,7-diethoxyquinazoline. The diethoxyquinazoline and piperazine were dissolved in n-BuOH with 1 to 5 mole ratio, and the mixture was heated to 100°C for 24 h, and then cooled down to room temperature. The precipitated while solid was collected via filtration and washed with n-BuOH. After it was dried, the solid was mixed with 2-(4-(benzyloxy) phenyl) acetyl chloride (1:1 mole ratio) and K$_2$CO$_3$ in DMF. It was stirred until the reaction was completed. The reaction was quenched with Na$_2$CO$_3$ aqueous solution and stirred overnight, and the precipitated product was collected via filtration and washed with water and dried to give compound 33.

**Compound 33: 1-(4-(4-amino-6,7-diethoxyquinazolin-2-yl) piperazin-1-yl)-2-(4-(benzyloxy) phenyl) ethanone**

White solid, melting point 248-254°C, 69% yield for the last step; $^1$H-NMR (400MHz, DMSO-d$_6$) δ 7.383 (6H, m), 7.177 (2H, d, $J=8.4$ Hz), 7.112 (2H, br), 6.958 (2H, d, $J=8.4$ Hz), 6.714 (1H, s), 5.075 (2H, s), 4.066 (4H, m), 3.590 (6H, m), 1.369 (6H, m). $^{13}$C-NMR (100MHz, CDCl$_3$-d$_6$) δ 170.017, 136.987, 129.646, 128.580, 127.739, 115.192,
DISMS calculated for C\textsubscript{31}H\textsubscript{31}N\textsubscript{5}O\textsubscript{4} [M + H]\textsuperscript{+}: 542.2, found : 541.6

2.3.3 Cell Culture and antibodies.

MDA-MB-231 cells were obtained from ATCC (Rockville, MD). The overexpression EphA2 protein in the cells was generated according to the procedure reported in a previous study \( (10) \). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10\% fetal bovine serum (FBS), 10 mg/ml L-Glutamine, 100 U/mL penicillin-streptomycin, and 0.1 mg/ml streptomycin. EphA2 overexpressed cells were grown in the presence of 1 \( \mu \)g/ml puromycin. Cell cultures were grown at 37 \( ^\circ \)C, in a humidified atmosphere of 5\% CO\textsubscript{2} in a Thermo CO\textsubscript{2} incubator (Grand Island, NY).

Antibodies used included rabbit polyclonal antibodies against pEphA/B (synthesized as described previously) and EphA2 (Santa Cruz, Santa Cruz, CA), as well as mouse monoclonal antibody to EphA2 (clone D7, Millipore, Billerica, MA) \( (8) \). Secondary antibodies used were goat anti-rabbit conjugated to horseradish peroxidase (HRP) (Santa Cruz) and donkey anti-mouse conjugated to Alexa Fluor 488 (Thermo Fisher, Grand Island, NY).

2.3.4 Activation of EphA2

MDA-MB-231 EphA2-overexpressing cells were plated in 12-well dishes at a density of 100,000 cells/well and grown for 24 h prior to stimulation with appropriate compounds
and ephrins for 30 min and 10 min, respectively. Compounds were prepared in DMSO at 200 times the final concentrations and 0.5% dimethyl sulfoxide (DMSO) was used as vehicle control. Following treatment, cells were washed and lysed in modified RIPA Buffer (20 mM Tris-HCl pH 7.4, 20 mM NaF, 150 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% DCA, 2 mM EDTA, 1% Triton X-100, 2 mM Na$_3$VO$_4$, and protease inhibitors, including 1 mM phenylmethylsulphonyl fluoride, and 2 µg/ml each of aprotinin and leupeptin) for 20 min, followed by immunoprecipitation. For immunoprecipitation, lysates were combined with 10 µg of ephrin-A1-Fc and 10-15 µl of Gamma-Bind Protein G beads (GE Healthcare Bioscience, Pittsburgh, PA) and rotated for 3 h at 4 ºC. 10 µg of human unconjugated Fc was used as a negative control. Beads were pelleted at 9,000 rpm for 2 min at 4 ºC and washed twice with 1 ml of IP Wash Buffer (20 mM Tris, pH 7.4, 10% glycerol, 50 mM NaCl, 0.2% Triton X-100, 0.5 mM Na$_3$VO$_4$, 0.5 mM PMSF). All wash buffer was removed and beads were resuspended in 25 µl of 4X LDS Loading Buffer (Thermo Fisher, Grand Island, NY) with 4 µl of 10X Bolt Sample Reducing Agent (Thermo Fisher), followed by Western Blotting. Samples were boiled 5 min and run on 4-12% Bis-Tris Plus gels (Thermo Fisher), followed by transfer to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked 1 h at room temperature in 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) followed by overnight incubation with pEphA/B (1:500) and EphA2 (Santa Cruz) (1:1000) primary antibodies. Membranes were washed in TBS-T and incubated with goat anti-rabbit-HRP (1:5000) secondary antibody 1 h at room temperature, followed by washing and developing with Luminol Reagent (Santa
Cruz). Band intensities were quantified using Quantity One software (Bio-Rad, Hercules, CA).

2.3.5 Internalization of EphA2.

MDA-MB-231 cells were plated in 24-well plates on 10 mm square coverslips coated with 10 µg/ml fibronectin and incubated 24-48 h before treatment with 50 µM doxazosin and various doses of compound 27 (0.4, 2, 10, 50 µM) in 0.5% DMSO for 1 h. As another positive control, cells were treated with 2 µg/ml ephrin-A1-Fc. Cells were then washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) on ice for 10 min, followed by washing and permeabilization with 0.4% NP-40 for 10 min on ice. Non-specific binding sites were blocked with 50 mM NH₄Cl on ice for 10 min, followed by washing with PBS and 0.1% BSA in PBS. Cells were then incubated in primary mouse EphA2 antibody (D7, 1:100) in 0.1% BSA/PBS for 1 h at room temperature, washed in PBS and BSA/PBS, followed by incubation in secondary donkey anti-mouse Alexa Fluor 488 antibody (1:300) in BSA/PBS for 30 min at room temperature. Cells were washed in PBS and BSA/PBS, followed by mounting in DAPI Mounting Medium (Electron Microscopy Sciences). Images were taken with a Leica microscope.

2.3.6 Docking investigation of compounds 24 and 27

Docking studies were done to gain insight into the possible mode of interaction between the compounds and EphA2. The structures were drawn with Marvin Sketch.
(www.ChemAxon.com) and energy minimized in MOE 2010 (www.chemcomp.com) using the MMFF94s force field. The database of compounds was then used for the docking studies. The protein crystal structure of 3FL7.pdb was prepared for docking by adjusting the pH of the system to a pH of 7.4 (6). The binding site in EphA2 was delineated by doxazosin. After docking, only the top docked pose of each compound was retained for analysis.

2.3.7 Adrenoceptor binding activity of compounds 24 and 27

Analysis of doxazosin and analog binding to rat α1a adrenergic receptor was performed by Eurofins Panlabs Taiwan, Ltd. (Taiwan). [3H]-prazosin at 0.25 nM was used as the radioligand. Compounds 24, 27, and doxazosin at 5 different doses ranging from 0.08 nM-3 µM in 0.1 % DMSO were incubated with [3H]-prazosin in a final volume of 1 ml of assay buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA) for 1 h at 25°C. Non-specific binding was defined by the use of 10 µM phentolamine. Incubations were terminated by vacuum filtration over 0.1% PEI pretreated glass fiber filters and filters were washed for 10 s with ice cold 0.1M NaCl and the radioactivity retained on the filters was determined by liquid scintillation spectrometry. The IC\textsubscript{50} values were determined by a non-linear regression analysis using GraphPad Prism v.5 (GraphPad).
2.3.8 Pharmacokinetic studies with compound 27

2.3.8.1 Preparation of stock and working solutions

The stock solution of compound 27 and Prazosin (internal standard) were prepared by dissolving the corresponding compounds in DMSO to reach the concentration of 1 mg/mL. One set of compound 27 working solutions at 20, 50, 100, 200, 500, 1000 and 2000 ng/mL, was prepared by serial dilution of the stock solution with acetonitrile, and then used for preparing the calibration standards. Another set of compound 27 working solution at 10, 30, 250 and 1600 ng/mL was made in a similar way, and used for preparation of QC samples. The Prazosin working solution was prepared by diluting 1 mg/mL stock solution to 100 ng/mL with acetonitrile.

2.3.8.2 Preparation of calibration standards and quality control (QC) samples

The calibration standards were prepared by spiking 5 µL of compound 27 working solution into 45 µL blank mouse plasma to give the final concentration of compound 27 at 2, 5, 10, 20, 50, 100 and 200 ng/mL. The QC samples were prepared in a similar manner at 1, 3, 25 and 160 ng/mL, representing LLOQ, LQC, MQC, and HQC of compound 27 in mouse plasma, respectively. The same method was used for the mouse brain homogenate. All calibration standards and QC samples were further treated according to the sample preparation procedure.
2.3.8.3 Preparation of sample for analysis

For unknown study samples, 45 μL of sample (serum or brain homogenate) was used and 5 μL of acetonitrile was added before extraction. Aliquots of 50 μL mouse plasma or brain homogenate samples from the calibration standards, QC samples and pharmacokinetics study samples were mixed with 5 μL internal standard working solution (100 ng/mL) and then vortexed for 10 s. 500 μL of ethyl acetate was added to the samples, followed by vortexing for 60 s and then centrifuging at 12,000 rpm for 15 min. Then 400 μL of supernatant was separated and blow-dried using nitrogen blow down dry evaporator. The dry residue was reconstituted with 200 μL acetonitrile/water (4:1, v/v) and then subjected to LC-MS/MS analysis.

2.3.8.4 LC-MS/MS analysis

The Shimadzu UPLC system (Columbia, MD) used consists of Prominence DGU-20A3R inline degasser, two LC-30AD pumps, a SIL-30AC auto sampler, and a CBM-20A controller. The chromatographic separation was performed on a Kinetex C18 column (50 x 2.1mm, 1.3μm) with a SecurityGuard ULTRA Cartridge and UPLC in-line filter (Phenomenex, CA). An optimized gradient of mobile phase A: water with 2% acetonitrile and 0.1% formic acid and mobile phase B: acetonitrile with 0.1% formic acid at the flow rate of 0.25 ml/min was used (Table II).

The detection of compound 27 was performed on an AB Sciex Qtrap 5500 mass spectrometer (Toronto, Canada) with positive electrospray ionization mode. The MRM function was used for quantification with the transitions setting at m/z 387.3→290.1 for compound 27 and m/z 384.1→247.1 for Prazosin. The optimized ion source parameters
were set as follows: ion spray voltage, 5000 V; temperature, 450°C; heating gas, nebulization gas, curtain gas, 40psi. Compound parameters were as follows: declustering potential, 141 V; entrance potential, 10 V; collision energy, 37 V for compound 27, 39 V for IS; collision exit potential, 24V for compound 27, 18V for internal standard. Data acquisition and quantification were performed using analyst software (version 1.6.2).

### 2.3.8.5 Analytical method validation

#### 2.3.8.5.1 Calibration curve, sensitivity, and selectivity

Calibration curves were constructed using the peak area ratio of compound 27 to IS Prazosin (y) vs. the concentration of compound 27 (x) in the calibration standards. The weighted linear regression was generated using 1/x as weighting factor. The sensitivity of the method was evaluated by whether the LLOQ, the lowest concentration in a calibration curve, can be quantified with accuracy and precision within 20%. The selectivity was evaluated by testing the presence of the interfering peak in blank plasma samples from six different sources and blank brain tissue with untreated mice.

#### 2.3.8.5.2 Matrix effect and extraction efficiency

The relative matrix effect and extraction efficiency were studied at three QC levels: 3, 25, and 160 ng/ml. The relative matrix effect was determined by comparing the peak area ratio of compound 27 and internal standard spiked in the blank plasma post-extraction solution with that in the neat solution (acetonitrile/water (4:1, v/v)). The post-extraction solution was prepared by blank plasma extraction and reconstitution of dry extracts with acetonitrile/water using the procedures described above. The relative extraction efficiency was determined by comparing the peak area ratio of compound 27 and internal
standard spiked in the plasma before extraction with that spiked in blank plasma post-extraction solution. The same procedure was repeated for brain homogenate.

2.3.8.5.3 Precision and accuracy

Intra- and inter-assay precision and accuracy were assessed using QC samples at three different concentrations: 3, 25, and 160 ng/ml. Intra-assays were carried out with five replicates (n=5) for each concentration in the same day, while inter-assay were performed for each concentration in five replicates in five days. The experimental determined concentrations of compound 27 in QC samples were compared to the theoretical spiked values.

2.3.8.5.4 Stability study

The stability of compound 27 in mouse plasma was evaluated by subjecting QC samples (3 and 160 ng/ml) to the following conditions: sitting at room temperature for 6 and 24h, three freeze/thaw cycles, -20°C for 2 months. The freeze/thaw cycle comprises freezing for 24h at -20°C and then thawing at room temperature for 30 min.

2.3.8.6 Pharmacokinetic studies of compound 27 in mouse plasma and brain tissue

The animal study was approved by Case Western Reserve University animal care and use committee and adheres to the Guide for the Care and Use of Laboratory Animals, 8th edition (NIH). Three-month-old female mice (Charles River) were randomly divided to 14 groups (5 mice per group). The mice were treated with the compounds or vehicle via subcutaneous injection or intraperitoneal injection (two sets of different treatment). At different time points ranging from 30 mins to 24 h, the mice were anesthetized to perform
the heart perfusion with saline, and then euthanized. 200 μL of blood sample was collected from each mice and brain tissue was collected as well. The plasma was immediately separated by centrifugation at 5000 rpm for 5 min at room temperature and stored frozen at −20 °C until analysis. The brain tissue was homogenized and centrifuged at 10,000 rpm to collect the supernatant, and then stored frozen at −20 °C until analysis. The feasibility of the developed quantitative method was tested through a pharmacokinetic study of compound 27 in mouse plasma and brain tissue.

2.4 ACKNOWLEDGEMENTS

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2.5 REFERENCES


CHAPTER III

COPALIC ACID ANALOGS AS PROSTATE CANCER THERAPEUTICS

3.1 INTRODUCTION

Prostate cancer is the most common type of cancer among men and the second leading cause of cancer related deaths in the US (11). The cancer (uncontrollable growth of cells) begins in the prostate gland and may eventually metastasize (spread throughout the body). Some of the risk factors that are associated with prostate cancer are age, ethnicity, family history, and geography (9). There are also other factors that may play a role such as diet, smoking, exposure to chemicals, obesity, inflammation of the prostate, and sexually transmitted infections, however it is unclear if they are significant (9). There are different stages of prostate cancer that are categorized based on the size of the primary tumor, whether the cancer has spread to the lymph nodes, whether the cancer has metastasized, PSA level in the bloodstream, and the Gleason score which is a measure of the probability of how likely it is for the cancer to grow rapidly (9).

There are screening tests for early detection, such as the PSA test where elevated PSA levels suggests the development of prostate cancer, and digital rectal exam (DRE)
Currently, several treatment options are available: radiation therapy, cryotherapy, chemotherapy, and bone-directed therapy are some common treatment procedures used for any type of cancer, whereas surgery, and hormone therapy are based on lowering the levels of androgens and androgen receptor, which play a key role in the functioning of the prostate (9). Although they may be successful, most patients eventually develop resistance to these treatments. Because of this, there is a great need for a new therapeutic agent that can overcome resistance and provide patients with a better prognosis.

Androgen receptor belongs to the steroid and nuclear receptor superfamily and is involved in processes that are responsible for male sexual differentiation (3). The ligands that bind to AR are known as androgens, which can be steroidal or nonsteroidal and agonists or antagonists based on their structure and the ability to promote or inhibit gene transcription, respectively (3). The most common ones with the highest affinity for AR are testosterone and 5α-dihydrotestosterone. AR mainly exists in the cytoplasm stabilized by various heat shock proteins (3). When a ligand binds, the heat shock proteins dissociate, and AR is transported to the nucleus by importin-α which is a nuclear transport receptor. The DNA binding domain of AR binds to the promotor region of the DNA and recruits transcriptional factors and other co-regulators (whether they are co-activators or co-repressors) in order to begin transcription (7).

Because of its involvement in male physiological differentiation and functions, AR also has implications in prostate cancer. Elevated androgens and AR receptor levels have shown to promote prostate cancer cell survival and proliferation. Accordingly, many therapeutic agents and/or procedures’ purpose is to decrease the level of androgens. Even though these treatments are partially successful, patients ultimately develop resistance.
which has become a major concern. Especially in CRPC, although the level of androgens is low, AR is able to continue its functions in an androgen independent manner (2). This suggests that there is another mechanism by which AR is able to remain stable and function.

Heat shock protein 27 kDa is another protein that has implications in prostate cancer. And in this project, we focus on this protein mainly because AR is a client protein of HSP27 where it is involved in stabilizing and chaperoning activities. HSP27 is a small chaperone protein that is involved in stress induced cellular functions. Mainly known for their chaperoning function, HSP27 is also involved in the inhibition of apoptosis, protection against oxidative stress, and regulation of the cytoskeleton (12). Although HSP27’s ability to refold denatured proteins remain doubtful, it is involved in leading misfolded proteins to proteasomal degradation (1). This process of managing the level of denatured protein may be one way that HSP27 regulates cell death (3). Following stress, HSP27 is speculated to inhibit apoptosis by interfering in both the upstream and downstream mechanisms of cytochrome c release from the mitochondria (8). HSP27 is also involved in regulating the cytoskeleton by promoting actin polymerization. Moreover, it is able to lower the level of reactive oxygen species during oxidative stress (4).

The overexpression of AR seen in CRPC helps promote the cancer cells’ growth and survival even when the level of androgens is low and eventually leads to resistance. Antagonists of AR may not be an effective therapeutic strategy since resistance may be developed due to mutations in AR at a genomic and/or protein level. Therefore,
degradation of AR indirectly through the inhibition of HSP27 is a viable approach to obstructing cancer cell growth because of HSP27’s involvement in AR’s stability.

Copalic acid, which is a diterpenic acid in copaiba oil, has recently been discovered to inhibit HSP27. Copaiba oil which is native to South America and Africa has been a folk medicine since the 19th century (10). It has anti-inflammatory, antimicrobial, and antinociceptive properties (6). Copaiba oil which comes from the *Copeifera multijuga* species has shown to inhibit tumor growth as well. This activity is due the diterpenic acids that are present in copaiba oil, such as copalic acid (6). The association of HSP27 with AR’s stability and the inhibition of HSP27 by copalic acid suggest that it can be used as a therapeutic agent for the treatment of prostate cancer. Therefore, in this project we generate new compounds using copalic acid as a lead compound in order to increase potency in inhibiting HSP27 and in turn reduce the level of AR in prostate cancer cells.

3.2 RESULTS AND DISCUSSION

3.2.1 Several copalic acid analogs showed better activity in degrading AR

In prostate cancer, AR plays a major role because of its involvement in cell differentiation and proliferation. In the cytoplasm, AR is stabilized by small chaperone proteins, such as HSP27, which has also been known to have implications in prostate cancer. In this study, we speculate that our compounds may inhibit HSP27 which would cause the down regulation of AR. We synthesized 15 analogs of copalic acid with
Figure 3.1: Synthesis and structures of the copalic acid analogs.
different moieties at the carboxyl group (Figure 3.1). These were then evaluated for their activity to down-regulate AR in LNCaP cells by western blot. The results demonstrated that, in general, modifying the carboxylic acid moiety in the copalic acid increased the activity of the analogs. At 20 μM, compounds 3, 5, 12, and 13 displayed moderate activity in decreasing the level of AR which is comparatively better than copalic acid (3.2 A). On the other hand, compounds 4 and 7 seem to eradicate all the AR in the LNCaP cells at 20 μM. Considering the structures, these data suggest that the aromatic moiety that was substituted in place of the carboxylic acid group may be responsible for this activity. The carboxylic acid moiety is ionized into carboxylate ion in the cell culture medium and this generated negative charge limits the ability of copalic acid to penetrate the cell membrane. Therefore, it is possible that converting the carboxylic moiety may enhance permeation of the compounds into the cells. It is also likely that changes in hydrophobicity contributed to the increased activity since it would have the ability to pass through the hydrophobic cell membrane more easily. Comparing compounds 4 and 15, which have the same 4-methoxybenzyl group but a different linking bond, it may be speculated that the nitrogen atom in the amide group of compound 4 which does not allow rotation may be preferred over the ether bond of compound 15 which allows rotation.

Even though compound 4 and 7 showed improved activity, their aromatic structure is not sufficient to provide information to summarize what type of substituents on the phenyl ring is capable of improving activity. Different moieties result in different interactions when binding to the active site, such as hydrogen bonding, Van der Waals
**Figure 3.2:** (A) Western blot analysis of all 15 copalic acid analogs; (B) Dose-dependent activity of compound 4; (C) Dose-dependent activity of compound 7
forces, or ionic bonding, which may lead to stronger or weaker interactions compared to
the lead compound. In order to determine which compound’s moiety results in stronger
interactions and therefore has higher activity a dose-dependent experiment was carried
out to further examine the two analogs. In the cell viability assay, compound 4 showed
the most activity in dramatically decreasing the level of AR in LNCaP cells at 5 μM
(Figure 3.2 B). Compound 7 only showed a decrease in the level of AR starting at 20 μM
(Figure 3.2 C). Compound 4 has the 4-methoxybenzyl group and compound 7 consists of
benzonitrile group which are both electron donating groups. However, more compounds
need to be synthesized and analyzed in order to summarize the structure activity
relationship. The IC\textsubscript{50} values of compounds 4 and 7 were calculated to be 1.73 ± 0.72 μM
and 4.81 ± 0.92 μM, respectively, which suggests that both of these have the potential to
be the hit compounds for further development of therapeutic agents to treat prostate
cancer (Figure 3.3).

3.3.2 Inhibition of chaperone function

Consequently, the chaperone inhibition function of the compounds was
investigated with an \textit{in vitro} chaperone assay. HSP27 is a protein that is involved in
many cellular protective functions, the most prevalent being chaperone function. In the
prostate cells, HSP27 is involved in stabilizing AR in the cytoplasm. Compounds 4 and 7
were evaluated for their potency to inhibit chaperone function. Dithiothreitol (DTT)
induced insulin aggregation can be stabilized by α-crystallin. The latter is a protein that
shares a common functional feature, α-crystallin domain, with HSP27 which is
Figure 3.3: LNCaP cell growth inhibition by compounds 4 and 7.
responsible for the mediation of the assembly of oligomers and the chaperone functions of both \( \alpha \)-crystallin and HSP27. Furthermore, the three-dimensional structures of \( \alpha \)-crystallin and HSP27 overlap which suggests that both proteins may be inhibited by similar inhibitors. The \( \alpha \)-crystallin stabilized DTT induced insulin precipitation chaperone function was inhibited by copalic acid in our previous study. The kinetics of this phenomenon was monitored in the absence of \( \alpha \)-crystallin, or in the presence of \( \alpha \)-crystallin with or without the analogs. Similar to copalic acid, compounds 4 and 7 also demonstrated inhibition of the \( \alpha \)-crystallin chaperone function. Both compounds showed the same level of inhibition at a concentration of 10 \( \mu \)M. These results suggest that these two compounds are small chaperone inhibitors as well (Figure 3.4). Both showed better potency in down regulating AR and inhibiting prostate cancer cell growth compared to the lead compound copalic acid.

3.3 MATERIALS AND METHODS

3.3.1 Chemistry

Crude copaiba oil was obtained from Natural Joint Solutions Inc. (FL). Chemicals and solvents were used as received without further purification. Thin-layer chromatography was performed on silica gel TLC plates with fluorescence indicator 254 nm (Fluka). Flash column chromatography was performed using silica gel 60Å (BDH, 40-63 \( \mu \)M). Mass spectra were obtained from the Bruker ion-trap mass spectrometer at Cleveland State University MS facility center. All NMR spectra were recorded on a
Figure 3.4: α-Crystallin lost the activity to prevent DTT induced insulin aggregation in the presence of compound 4 or compound 7.
Bruker 400 MHz (13C NMR at 100 MHz) using DMSO-$d_6$ as the solvent. Chemical shift ($\delta$) for 1H NMR spectra were reported in parts per million to residual solvent protons.

3.3.2 Chemical Synthesis of copalic acid analogs

The following summarized procedure was followed for the isolation of copalic acid (Detailed procedure described in previous study). Crude copaiba oil that was manufactured by Natural Joint Solutions, Inc. was used for the extraction of copalic acid. The aqueous layer, after extracting the crude copaiba oil in ethyl ether with aqueous potassium hydroxide, was neutralized with hydrochloric acid. After extracting the organic layer with ethyl ether, it was washed with water, dried with anhydrous sodium sulfate, and concentrated under vacuum. This fraction was subjected to column chromatography using gradient elution with different ratios of hexane and/or ethyl acetate to give seven sub fractions. The sub fraction that contained copalic acid was stirred at room temperature with 4-methoxyphenol and dicyclohexyl carbodiimide in dichloromethane. After filtration, the filtrate was concentrated under vacuum and purified by column chromatography. The 4-methoxyphenol ester of copalic acid that was obtained was neutralized by hydrochloric acid and extracted by ethyl acetate. The organic layer that was concentrated under vacuum followed by column chromatography purification produced about 500 mg of copalic acid (5).

Following the extraction of copalic acid, the carboxylic acid moiety was modified into either an amide or ester analog via a one-step reaction. Generally, the reactions were carried out at room temperature using dichloromethane (DCM) as the solvent. About 30
mg (0.1 mmol) of copalic acid was stirred with 62 mg (0.3 mmol) of dicyclohexylcarbodiimide (DCC) and 0.15 mmol of the aromatic amine or the phenol until copalic acid was entirely consumed. Then the insoluble solid was filtered and washed with CH$_2$Cl$_2$, followed by concentration under vacuum and purification by column chromatography to yield the product. Chemical synthesis was carried out to generate 15 copalic acid analogs and their structures and purity were verified through NMR analysis and LC-MS, respectively.

3.3.3 Cell Culture and antibodies

Prostate cancer cell line, LNCaP, was obtained from ATCC (Rockville, MD). Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 2% penicillin-streptomycin, and ciprofloxacin. FBS was heat inactivated for 30 min in a 56°C water bath before use. Cell culture was grown at 37°C, in a humidified atmosphere at 5% CO$_2$ in a VWR CO$_2$ incubator (Bridgeport, NJ).

Antibodies used included rabbit polyclonal antibody against androgen receptor and β-actin (Cell Signaling). Secondary antibody used was anti-rabbit conjugated to HRP (Cell Signaling).
3.3.4 Western Blot

LNCaP cells were treated with DMSO (as the negative control), copalic acid (as the positive control), and the 15 analogs for 48 h. The concentration of copalic acid used was 200 μM since 20μM copalic acid did not show any activity. The cells were lysed, briefly sonicated and centrifuged at 16,000 x g for 50 min. Then 30 μg of protein was boiled with 2X loading buffer for 5 min, electrophoresed on a 10% SDS-polyacrylimide gel, and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h with 5% nonfat milk in PBST followed by incubation with AR primary antibody. After washing, the membrane was incubated with horseradish-conjugated secondary antibody. The bands were visualized by chemiluminescence with ECL reagent (Thermo Scientific).

3.3.5 Cell viability analysis

The effects of select copalic acid analogs on LNCaP cell proliferation were analyzed using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). A 96-well, flat bottomed plate was seeded with 6000 LNCaP cells per well using RPMI 1641 medium for 24 h. Next, the cells were treated with varying concentrations of the compounds dissolved in DMSO (final concentration ≤0.1%) in medium for 72 h. Controls received DMSO at a concentration equal to that of the cells treated with the compounds. The medium was removed and replaced with 120 μL of 0.5 mg/mL MTT reagent in fresh medium and cells were incubated in the CO₂ incubator at 37°C for 1h. The supernatant was removed from the wells and replaced with 200 μL of
DMSO in order to solubilize the reduced MTT dye. Absorbance was measured at 570 nm using Spectramax plate reader.

3.3.6 Anti-Chaperone Assay

3.3.6.1 Endpoint measurement

A mixture of 0.24 mg/mL human recombinant insulin, 150 μg/mL α-crystallin, and compounds 4 and 7 in 98 μL of sodium phosphate buffer, pH 7.4, was incubated at 37°C for 5 min. Then 2 μL of 1M DTT in water was added to initiate insulin aggregation. The absorbance (A) at 400 nm was recorded after 45 min incubation at 37°C in a plate reader. A mixture of 0.24 mg/mL insulin in the absence or presence of 150 μg/mL α-crystallin with DMSO was used as a control. For the evaluation of the effects of the compounds 4 and 7 on DTT-induced insulin aggregation, a mixture of 0.24 mg/mL insulin and 20 mM DTT in the presence of DMSO or compound 4 or compound 7 was incubated for 45 min at 37°C followed by the measurement of the absorbance at 400 nm.

3.3.6.2 Kinetic measurement

A mixture of 0.24 mg/mL human recombinant insulin, 150 μg/mL α-crystallin, and compounds 4 and 7 in 98 μL of sodium phosphate buffer, pH 7.4, was incubated at 37°C for 5 min. Then 2 μL of 1M DTT in water was added to initiate insulin aggregation. The absorbance at 400 nm was recorded after 45 min incubation at 37°C in a plate reader. A mixture of 0.24 mg/mL insulin in the absence or presence of 150 μg/mL α-crystallin with DMSO was used as a control. The α-crystallin inhibition potency (%) of compounds
at 45 min was determined by $A_{(\alpha\text{-crystallin+compound+DTT})} - A_{(\alpha\text{-crystallin+DTT})} / A_{DTT} - A_{(\alpha\text{-crystallin+DTT})}$, where $A_{(\alpha\text{-crystallin+compound+DTT})} - A_{(\alpha\text{-crystallin+DTT})}$ represents an increase of insulin aggregation level in the presence of $\alpha$-crystallin without the compounds while $A_{DTT} - A_{(\alpha\text{-crystallin+DTT})}$ represents a decrease in insulin aggregation level used by $\alpha$-crystallin.

For the evaluation of HSP27 inhibition potency of compounds 4 and 7 the same procedure was followed with the exception of using HSP27 instead of $\alpha$-crystallin. The final concentration of HSP27 in the assay was 15 $\mu$g/mL.

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3.5 REFERENCES


