Structure-Guided Design and Initial Studies of a Bifunctional MEK/PI3K Inhibitor (ST-168)

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Supporting Information

ABSTRACT: The structure-based design of a new single entity, MEK/PI3K bifunctional inhibitor (7, **ST-168**), which displays improved MEK1 and PI3K isoform inhibition, is described. **ST-168** demonstrated a 2.2-fold improvement in MEK1 inhibition and a 2.8-, 2.7-, 23-, and 2.5-fold improved inhibition toward the PI3K α , PI3K β , PI3K δ , and PI3K γ isoforms, respectively, as compared to a previous lead compound (4; **ST-162**) in *in vitro* enzymatic inhibition assays. **ST-168** demonstrated superior tumoricidal efficacy over **ST-162** in an A375 melanoma spheroid tumor model. **ST-168** was comparatively more effective than **ST-162** in promoting tumor control when administered orally in a tumor therapy study conducted in an A375 melanoma mouse model confirming its bioavailability and efficacy toward combined *in vivo* MEK1/PI3K inhibition.

KEYWORDS: MEK, PI3K, bifunctional inhibitor, PI3K isoforms, targeted therapy, cancer

The MAP kinase (Ras/MEK/ERK) and PI3K/AKT/mTOR oncogenic and survival signaling pathways are key regulators of KRAS-mediated transformation.^{1,2} Phosphoinositide-3-kinases (PI3Ks) belong to a family of signal transducing enzymes that mediate key cellular functions in cancer and immunity and are divided into three classes (I, II, and III) based on substrate specificity, sequence homology, and types of regulatory subunits. Class I PI3Ks are heterodimers formed by a regulatory and a catalytic (p110) subunit, further referred to as PI3K α , PI3K β , PI3K δ , and PI3K γ . They are further subdivided into class IA (PI3K α , PI3K β , PI3K δ) and class IB (PI3K γ), which have different regulatory subunits.³ The mitogen-activated protein kinase (MAPK/MEK/ERK) activates signaling events involved with mediation of signal transduction, proliferation, and survival.⁴ Mutations in the RAS family and/or activation of PI3K and AKT leading to aberrant kinase activity are frequently observed in many types of human cancer including leukemia, melanoma, breast, ovarian, brain, lung, and prostate cancer. Strong evidence suggests that cross-talk and a feedback regulation loop exists between these two pathways, which can contribute to drug resistance mechanisms when targeting a single signaling pathway using monotherapy.^{5–10} Furthermore, synergistic effects in promoting tumor cell death by simultaneous inhibition of both pathways have been demonstrated in *in vitro* as well as *in vivo* studies.^{11–13} Since the Ras/MEK/ERK and PI3K/AKT/mTOR pathways are regulated by different mechanisms, concomitant targeting of these two key complementary signaling pathways could provide an important clinical benefit.^{14–16} In recent years, there has been increasing interest in the drug

industry and academic centers toward developing multifunctional single agent compounds for modulation of multiple biological targets.^{17–20} Single agent multitargeted drugs could provide improved efficacy, simplification of treatment regimen, and reductions in toxicity associated with the combined off-target effects of cocktail drug administration.²¹ As part of this strategy, we recently reported on a series of prototype small molecule MEK/PI3K bifunctional inhibitors for simultaneous targeting of these two dominant KRAS effector pathways.^{21,22} Bifunctional inhibitors were designed by covalent linking of structural analogues of the ATP-competitive pan-PI3K inhibitor ZSTK474 with analogues of the allosteric MEK inhibitors RO5126766 or PD0325901, to provide first generation prototypes including analogues 3 and 4 (Figure 1).^{21,22}

Subsequently, bifunctional inhibitor 4 (**ST-162**) was identified as a potential lead candidate based on its *in vitro* enzymatic inhibition, cellular efficacy against human cancer cell lines (D54, A549), and *in vivo* efficacy following oral administration to D54 glioma and A549 lung tumor bearing mice. **ST-162** displayed an IC₅₀ of enzymatic inhibition of 398 and 191 nM, respectively, for MEK1 and PI3K α ; however, its potency toward the remaining PI3K isoforms (PI3K β , PI3K γ , PI3K δ) was considerably lower (942 < IC₅₀ (nM) < 5803). Significantly, most PI3K inhibitors that have progressed to clinical evaluation as cancer therapeutics are known to display multi-isoform potency. In particular, the

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Figure 1. Structure and *in vitro* enzyme inhibition data for first generation MEK/PI3K bifunctional inhibitors.

PI3K inhibitors BAY80-6946 (Copanlisib) and GDC0032, which demonstrate subnanomolar PI3K α and PI3K δ isoform inhibition ($IC_{50} < 0.7$ nM), are currently undergoing clinical evaluation for the treatment of advanced solid tumors, non-Hodgkin's lymphoma, and diffuse large-cell lymphoma.²³ We hypothesized that a bifunctional inhibitor analogue with more potent inhibition toward multiple Class I PI3K isoforms as compared to **ST-162** would demonstrate more robust PI3K inhibition *in vivo*. Accordingly, an important aspect of the present study was to improve the class I PI3K isoform inhibition of **ST-162** by suitable compound structure modification. We previously observed that bifunctional inhibitor **3**, wherein the spacer linker bearing the MEK binding motif was attached to the PI3K binding motif via an amide bond, shows PI3K α and PI3K δ inhibition of 172 and 58 nM, respectively (Figure 1). In particular, inclusion of an amide linkage at the PI3K binding motif was also well tolerated for PI3K α inhibition ($54 < IC_{50}$ (nM) < 341) in a series of MEK/PI3K bifunctional inhibitors that incorporate the benzhydroxamate MEK binding motif.²¹ Additionally, analogue **6a** (the *N*-acetyl derivative of **2**; Table 1) was shown to display potent inhibition ($2.9 < IC_{50}$ (nM) < 21) toward all four PI3K isoforms. Accordingly, compound **7** (**ST-168**), which contains an amide bond linker attachment to the PI3K binding motif **2**, was chosen as a candidate for initial development and evaluation.

Prior to undertaking the synthesis of **ST-168**, we conducted an analysis of its binding interactions at the MEK and PI3K kinase selectivity pockets using *in silico* docking studies. Details of the X-ray crystal structure analysis of the murine PI3K δ -ZSTK474 inhibitor complex as well as the MEK-PD318088 inhibitor complex are reported in the literature, and the key interactions contributing toward optimal binding of these prototype inhibitors at their respective kinase targets have been previously reviewed.^{22,24,25} In brief, PD318088 is shown to bind to a unique allosteric hydrophobic pocket that is adjacent to but separate from the Mg-ATP binding site (Figure 2).²⁵ In this class of MEK inhibitors, key hydrogen bond interactions are predicted for both hydroxamate oxygen atoms with Lys97 and the 4-fluorine atom of the A ring with the backbone amide NH of Val211 and Ser212.

Furthermore, the iodine atom on the B ring occupies a hydrophobic pocket involving an electrostatic interaction with the backbone carbonyl of Val127.

Analysis of the ZSTK474 inhibitor interaction at the PI3K ATP-binding pocket suggests that one of the two morpholine oxygen atoms of ZSTK474 hydrogen bonds with a valine residue (Val828) of the PI3K hinge region, while the 1,3,5-triazine group functions as an adenosine mimic.²² Since the second morpholine group is predicted to lack a similar binding interaction, we substituted morpholine with a piperazine group to provide **2** in order to facilitate synthetic attachment of the linker group bearing the MEK inhibitor. Our docking analysis (Figure 3) shows that **ST-168** is predicted to maintain many of the important binding interactions displayed by PD318088 at the MEK allosteric site, including that of both hydroxamate oxygen atoms with Lys97, the A ring 4-fluorine atom with the backbone NH of Val211 and Ser212, and the iodine containing B ring within the hydrophobic pocket. In addition, **ST-168** is also expected to retain the binding interactions demonstrated by ZSTK474 at the PI3K ATP-binding pocket including that of the morpholine group oxygen with the valine backbone amide NH group (Val828) and that of the imidazole nitrogen with the Lys779 side chain amino group.

Key intermediates 3,4-difluoro-2-(2-fluoro-4-iodophenylamino)benzoic acid (**1**)²⁶ and 1,3,5-triazine derivative **2**²² were synthesized as previously reported. The synthesis of **7** (Scheme 1) was conducted by initial PyBop mediated coupling of **2** with Boc-aminoxy-PEG3-ol to give intermediate **5** followed by TFA-catalyzed removal of the Boc protecting group to provide the terminal aminoxy derivative **6**. Subsequent PyBop mediated coupling of **6** with the acid derivative **1** provided **7** (**ST-168**) in 32% overall yield.

In vitro MEK1 and PI3K isoform enzyme inhibition assays were conducted using a Kinase-Glo luminescent kinase assay kit (Promega, WI) and fluorescence-based Adapta TR-FRET assay protocol (Life Technologies, Madison, WI), respectively, and these data are shown in Table 1. **ST-168** displays a MEK1 enzymatic inhibition of 182 nM in these assays, which represents a 2.2-fold improvement over that of **ST-162** ($IC_{50} = 398$ nM). The prototype MEK1 inhibitor PD0325901, by comparison, shows an IC_{50} for MEK1 inhibition of 15 nM. The linker attachment in **ST-162** and **ST-168** was designed to retain the key ethoxyhydroxamate structural element critical to MEK1 inhibition contained in the potent MEK1 inhibitor PD0325901. The MEK inhibition shown by inhibitors **ST-162** and **ST-168** thus confirms the effectiveness of our design approach. Analysis of the PI3K isoform enzyme inhibition data for these new compounds when compared to the prototype pan-PI3K inhibitor ZSTK474 reveals some interesting trends. ZSTK474 demonstrates high inhibitory activity toward all four class I PI3K isoforms ($3.9 < IC_{50}$ (nM) < 20.8) while also displaying a 3- to 5-fold greater selectivity toward the PI3K α and PI3K δ isoforms. To facilitate synthetic attachment of the linker to ZSTK474, we replaced one of its morpholine groups with piperazine to give analogue **2**. This modification leads to a 36-fold reduction in inhibition for PI3K α and PI3K δ and a > 70 -fold lower inhibition for PI3K β and PI3K γ . Bifunctional inhibitor **ST-162**, wherein the PEG linker has an alkyl attachment at the piperazine nitrogen, displays a similar degree of PI3K α inhibition as **2**, although its inhibition for the remaining three PI3K isoforms were from 3- and 7-fold lower. Interestingly, analogue **6a** (the *N*-acetyl derivative of **2**) showed potent inhibition ($8 < IC_{50}$ (nM) < 21) toward all PI3K isoforms similar to that of the

Fc1nc2ccccc2n1Nc3nc4nc(N5CCOCC5)c6nc(N7CCOCC7)nc63

IC ₅₀ (nM) ^b							
Compound	X	cLogP ^c	MEK1	PI3K α	PI3K β	PI3K γ	PI3K δ
ZSTK474	O	1.06	n.a	5.0 ± 0.8	15.2 ± 1.4	20.8 ± 0.6	3.9 ± 0.6
<u>2</u>	NH	1.04	n.a.	180 ± 25	1093 ± 168	1873 ± 283	142 ± 7.5
<u>4</u> (ST-162)		5.71	398 ± 1.42 ^d	191 ± 64	4073 ± 290	5803 ± 511	942 ± 120
6a		0.63	n.a.	8.2 ± 0.7	14.3 ± 2.5	21 ± 1	2.9 ± 0.6
6		0.21	n.a.	11.5 ± 0.1	214 ± 49	140 ± 6.6	1.3 ± 0.2
<u>7</u> (ST-168)		5.06	182 ± 1.58	69.2 ± 2.2	1482 ± 377	2293 ± 241	41.7 ± 2.1
PD0325901	n.a.	2.85	15 ± 1.3 ^e	n.a.	n.a.	n.a.	n.a.

^ePD0325901 displays an IC₅₀ = 0.33 nM in cell potency assays (ref 26).

Figure 2. Enzyme–inhibitor binding site interactions of (A) PD318088 at allosteric MEK1 binding pocket and (B) ZSTK474 at PI3K δ binding pocket.

prototype PI3K inhibitor ZSTK474. The importance of the amide bond linker attachment at the piperazine nitrogen was further confirmed for the pegylated intermediate **6**, which displayed superior PI3K α and PI3K δ inhibition ($1.3 < \text{IC}_{50}$ (nM) < 11.5) similar to **6a**. Linking of the MEK pharmacophore to **6** to give bifunctional inhibitor **ST-168** does reduce its PI3K isoform inhibition as compared to **6**. Importantly, coupling the PEG linker at the piperazine nitrogen as an amide functionality (**ST-168**) leads to a 2.8- and 23-fold improvement in its PI3K α (IC_{50} = 69.2 nM) and PI3K δ (IC_{50} = 41.7 nM) inhibition, respectively, over the *N*-alkylated PEG derivatized bifunctional inhibitor **ST-162**. Additionally, docking analysis suggests that the amide carbonyl group of the PI3K pharmacophore of **ST-168** is shown to be in close proximity (< 4 Å) to two hydrogen bond donor residues Thr833 and Asn836 (Figure 3B) in multiple docking conformations. This favorable carbonyl group positioning could

Figure 3. Docked structures of **ST-168** at MEK1 allosteric pocket and PI3K δ . (A) Binding mode of **ST-168** within the MEK1 (PDB code: 1S9J) allosteric catalytic site. The PI3K portion of **ST-168** is out toward the solvent (left). (B) Binding mode of compound **ST-168** to PI3K δ (PDB code: 2WXX) catalytic site. MEK1 binding portion is out toward the solvent (right). Atom denotation: gray (carbon); red (oxygen); blue (nitrogen); green (fluorine); white (hydrogen); and purple (iodine). Hydrogen bonds (hashed lines) are highlighted with asterisks (*).

Scheme 1. Synthetic Route for Compound 7 (**ST-168**)^a

^aReagents and conditions: (a) (*t*-Boc)NHO-(CH₂CH₂O)₃CH₂CH₂COOH, PyBop, DIEA, THF:DCM, 4 h, 73%; (b) TFA, DCM, 0–5 °C, 2 h, 82%; (c) **1**, DIEA, PyBop, THF:DCM, 18 h, 54%.

explain the improved *in vitro* binding affinity data demonstrated by **ST-168** compared to **ST-162**. The calculated lipophilicities for inhibitor **ST-168** (cLogP = 5.1) and inhibitor **ST-162** (cLogP = 5.71) were similar and close to the acceptable threshold (cLogP < 5) as a predictor of oral bioavailability.²⁷

In order to compare the functional activity of bifunctional inhibitors **ST-162** and **ST-168** with a prototype MEK inhibitor (PD0325901) and PI3K inhibitor (ZSTK474), cellular MEK1 and PI3K inhibition were measured by monitoring changes in phosphorylation of ERK1/2 and AKT, respectively, in a A375 melanoma cell line. A375 cells have constitutive activation of MEK1 and PI3K pathways. A375 cells were incubated with PD0325901 (10 μ M), ZSTK474 (10 μ M), a combination of PD0325901 and ZSTK474 (10 μ M each), **ST-162** (20 μ M), **ST-168** (20 μ M), or equimolar concentrations of DMSO control for 1 h, and the cell lysates were subjected to immunoblot analysis. As shown in Figure 4A, **ST-162** and **ST-168** displayed complete

inhibition of ERK1/2 phosphorylation resulting in complete modulation of MEK1 kinase enzymatic activity at the 20 μ M dose level within 1 h of oral dosing. Similarly, both inhibitors also showed high potency in significantly inhibiting AKT phosphorylation with substantial modulation of PI3K activity being achieved at this dose level. Taken together, these studies confirm that inhibition of both MEK1 and PI3K activity by analogues **ST-162** and **ST-168** in this cell line correlates well with their relative *in vitro* enzymatic inhibition data. Bifunctional inhibitors **ST-162** and **ST-168** were also evaluated for their cell killing ability in an A375 melanoma spheroid tumor model. Spheroids were treated with 20 μ M **ST-162** or **ST-168** for 72 h followed by staining with a live/dead cell assay and imaging by fluorescence microscopy. As seen from this data (Figure 4B), fluorescence staining for dead cells was significantly higher for **ST-168** as compared to **ST-162** confirming its superior MEK1 and PI3K inhibitory potency.

Figure 4. Target activity of bifunctional inhibitors **ST-162** and **ST-168** in cells and 3D melanoma spheroids. (A) Two-dimensional assessment of **ST-162** and **ST-168**. Immunoblot analysis of A375 melanoma cells treated for 1 h with PD0325901 (10 μ M), ZSTK474 (10 μ M), a combination of PD0325901 and ZSTK474 (10 μ M each), **ST-162** (20 μ M), or **ST-168** (20 μ M) and compared to equimolar concentrations of DMSO control. Phosphorylation of ERK1/2 and AKT at serine 473 was used to define activities of MEK1 and PI3K, respectively. (B) Assessment of dead cells in 3D melanoma spheroids post-treatment with **ST-162** and **ST-168**. A375 melanoma spheroids were treated for 72 h with 20 μ M each of **ST-162** or **ST-168** before staining with Calcein AM and ethidium homodimer-1 for fluorescent microscopy (live/dead viability assay, Molecular Probes). Green fluorescence indicates live cells, and red fluorescence indicates dead cells. Images were obtained with an Olympus IX70 fluorescent microscope using the SPOT advance program. Experiments were performed in replicates, and representative images are shown.

Encouraged by these *in vitro* studies, we investigated the *in vivo* therapeutic efficacy of **ST-162** and **ST-168** in an A375 melanoma tumor xenograft mouse model. Athymic nude mice bearing flank A375 tumors were treated daily for 42 days with vehicle (OraPlus) or 400 mg/kg of **ST-162** or **ST-168** by oral gavage, and tumor growth was assessed by MRI imaging twice per week. Both **ST-162** and **ST-168** demonstrated potent antitumor efficacy against A375 tumors as shown in Figure 5. Tumor growth reduction was more pronounced for the **ST-168** treatment group compared to the **ST-162** group particularly at >35 days post-treatment ($p \leq 0.05$). It is possible that the

Figure 5. Tumor growth inhibition by **ST-162** and **ST-168** in a melanoma xenograft model. Tumor implantation consisted of inoculation of 5×10^6 A375 cells suspended in 100 μ L of media into the flank of nude mice. Treatment was initiated once tumors reached >100 mm³. Mice were randomized into two treatment groups and treated once daily with either vehicle (200 μ L of OraPlus) or 400 mg/kg each of **ST-162** or **ST-168** by oral gavage until sacrifice (42 days). Changes in tumor growth of A375 xenografts were assessed by conducting MRI imaging twice per week. Tumor volume changes between **ST-162** and **ST-168** treatment were determined to be statistically significant ($p \leq 0.05$) using an unpaired Student's *t* test at the last time point. Experimental design: $n = 4$ –6 tumors/treatment group. This data demonstrates the superior *in vivo* activity of bifunctional inhibitor **ST-168** as compared to **ST-162** for suppression of MEK1/PI3K kinase activities *in vivo* in solid tumors.

generation of active metabolites from **ST-168** amide bond hydrolysis *in vivo* could also lead to MEK and PI3K inhibition. Accordingly, additional studies to evaluate the metabolism and *in vivo* pharmacokinetics of these compounds to further validate the efficacy differences observed in Figure 5 are warranted.

In summary, a new MEK/PI3K bifunctional inhibitor **ST-168**, displaying improved *in vitro* enzymatic class I PI3K subtype affinity and improved cellular MEK/PI3K inhibition as compared to a previous lead derivative **ST-162**, is reported. Based upon the strength of the cumulative data on **ST-168**, future studies will be directed toward evaluation of its bioavailability, toxicology, and treatment efficacy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.7b00111.

Experimental procedures for synthesis of compound 7, analytical data (NMR, HPLC), and biological assays (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors B.D.R. and M.E.V.D. are inventors on patents filed by the University of Michigan for the compounds reported, which were licensed to Sarisa Therapeutics, LLC, in which B.D.R. has a financial interest.

■ ABBREVIATIONS

MEK, allosteric mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; br s, broad signal; cLogP, calculated log P; CH₃OH, methanol; CH₃CN, acetonitrile; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DIEA, *N,N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; Et₃N, trimethylamine; rt, room temperature; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; PyBop, (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate

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