Discovery of an Orally Bioavailable Dual PI3K/mTOR Inhibitor Based on Sulfonyl-Substituted Morpholinopyrimidines

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Supporting Information

ABSTRACT: The discovery and optimization of a series of 2-morpholino-pyrimidine derivatives containing various sulfonyl side chains at the C₄ position led to the identification of compound 26 as a potent dual PI3K/mTOR inhibitor. It exhibited high inhibitory activity against PI3K $\alpha/\beta/\gamma/\delta$ (IC₅₀ = 20/376/204/46 nM) and mTOR (IC₅₀ = 189 nM), potent functional suppression of AKT phosphorylation (IC₅₀ = 196 nM), and excellent antiproliferative effects on a panel of cancer cells. Enzymic data and modeling simulation indicate that a cyclopropyl ring on the C₄ sulfone chain and a fluorine on the C₆ aminopyridyl moiety are responsible for its maintained PI3K activity and enhanced mTOR potency, respectively. Furthermore, compound 26 exhibited higher efficiency in the HT-29 colorectal carcinoma xenograft model at the daily dose of 3.75 and 7.5 mg/kg relative to BKM120 at the dose of 15 and 30 mg/kg.

KEYWORDS: Phosphoinositide-3-kinase, mammalian target of rapamycin, dual PI3K/mTOR inhibitor, morpholino-pyrimidine

The phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) transduction pathway plays a critical role in a diverse set of cellular functions, including cell growth, proliferation, motility, differentiation, and survival, which is often constitutively dysregulated in various human cancers, providing validated therapeutic targets associated with a wide range of human malignancies.¹ PI3Ks can be divided into classes I, II, and III according to their structural characteristics and substrate preferences.² The most studied class I PI3K is a heterodimer protein that is composed of a catalytic subunit (p110 α , p110 β , p110 δ , and p110 γ) and a regulatory subunit (p85, p101, or p87).³ The p110 catalytic subunits are able to convert phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3), which leads to the subsequent activation of serine-threonine kinase AKT in terms of phosphorylation of AKT.⁴ The downstream kinase mTOR, a central regulator of cell growth and proliferation, can be further activated by phosphorylated AKT (pAKT).⁵ Compared with individually targeting PI3K or mTOR, dual inhibition of PI3K and mTOR has recently been proposed to represent a more effective

approach for cancer therapy since this strategy can directly target the most commonly mutated kinase-PI3K (which is generally encoded by the gene *PIK3CA* in the catalytic site of the p110 α subtype⁶) while overcoming multiple mTOR-related negative feedback loops that a selective mTOR inhibitor usually fails to repress.⁷

NVP-BKM120 (BKM, Figure 1), a compound with a pyrimidine scaffold developed by Novartis AG, displayed a

Figure 1. Design of a novel PI3K/mTOR dual inhibitor 26.

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Table 1. Structure–Activity Relationship Studies at the R¹, R², and R³ Groups^a

Compound	R ¹	R ²	R ³	PI3K α IC ₅₀ (μ M)	PC-3 pAKT IC ₅₀ (μ M)	T47D IC ₅₀ (μ M)	MCF-7 IC ₅₀ (μ M)
1		H	CF ₃	0.189	3.01	1.668	1.513
2		H	CF ₃	0.056	2.446	1.155	1.139
3		H	CF ₃	0.025	0.846	0.513	0.408
4		H	CF ₃	0.218	n/d ^b	n/d	n/d
5		H	CF ₃	0.013	0.312	0.058	0.224
6		H	CF ₃	0.013	0.319	0.167	0.283
7		H	CF ₃	0.015	0.248	0.138	0.134
8		H	CF ₃	0.042	0.384	0.218	0.34
9		H	CF ₃	0.321	n/d	n/d	n/d
10		(R)-CH ₃	CF ₃	0.223	n/d	n/d	n/d
11		(S)-CH ₃	CF ₃	0.009	0.546	0.129	0.112
12		(S)-CH ₃	CF ₃	0.006	0.306	0.175	0.214
13		(S)-CH ₃	CF ₃	0.006	0.184	0.093	0.078
14		(S)-CH ₃	CF ₃	0.022	0.674	0.139	0.159
15		H	CF ₃	0.013	0.301	0.225	0.295
16		H	CF ₃	0.040	0.947	0.552	0.512
17		H	CF ₃	0.058	0.43	0.16	0.437
18		H	CF ₃	0.065	1.178	1.063	0.599
19		H	CF ₃	0.026	0.668	0.324	0.513
20		H	CF ₃	0.009	1.548	0.478	0.897
21		H	CF ₃	0.057	1.275	0.619	0.5715
22		H	CF ₃	0.014	0.606	0.242	0.286
23		H	CF ₃	0.007	0.367	0.129	0.184
24		H	F	0.041	0.369	0.062	0.027
25		H	F	0.066	0.215	0.123	0.104
26		(S)-CH ₃	F	0.010	0.196	0.03	0.011
27		(S)-CH ₃	F	0.020	0.121	0.033	0.015
BKM	-	-	-	0.041	0.365	0.286	0.206

^aIC₅₀, the mean value of duplicate measurements. ^bn/d, not determined.

potent and selective class I PI3K inhibition over many other related kinases and is undergoing phase III clinical trials for

breast cancer treatment (NCT01572727, NCT01610284, and NCT01633060).^{8,9} The C₆ aminopyridyl moiety on BKM

interacts via hydrogen bonding with Asp836, Asp841, and Tyr867, and the 2-morpholine oxygen forms an important hydrogen bond to the hinge Val882 NH that is considered as an identical group for PI3K potency (Figure 1).⁸ A variety of structural modifications has been done, focused on the replacement of its pyrimidine core scaffold and C₆ aminopyridyl moiety with different bicyclic cores and aromatic urea/indole side chains, respectively, and anilines and aminoheterocycles are predominant substitutions at the core pyrimidine C₄ position that orient toward solvent without any specific hydrogen bonding.^{8,10–12} The discovery of morpholinopyrimidine based selective mTOR inhibitor AZD3147 from Astra Zeneca indicates that the NH groups on C₆ phenylthiourea or indole side chain are critical to engage productive interactions with glutamic acid present in mTOR but not PI3K, while C₄ sulfonyl side chains do not participate any specific interaction (Figure 1).^{13–15} Thus, C₄ positions at the core pyrimidine of both mTOR inhibitors and PI3K inhibitors can be considered as flexible sites for generating new ligands with druggable properties and retained potency, capable of tolerating a range of substituents.

Herein, we describe a hybridization approach to discover novel morpholinopyrimidine based PI3K inhibitors by replacing the C₄ morpholine moiety on the BKM with a sulfonyl portion taken from the selective mTOR inhibitors. Structure–activity relationship (SAR) studies conducted on 27 new analogs led to the identification of compound 26 (Figure 1), a potent dual inhibitor of PI3K and mTOR that exhibits remarkable cellular antiproliferative effects compared to BKM. Enzymic data and modeling simulation fully explain that a cyclopropyl ring on the C₄ sulfone chain and a fluorine on the C₆ aminopyridyl moiety are responsible for its maintained PI3K activity and enhanced mTOR potency, respectively. Furthermore, compound 26 also exhibits encouraging ADMET properties and significant *in vivo* tumor growth inhibitory efficacy in the HT-29 colorectal carcinoma xenograft mouse model compared to BKM.

The structural modifications were focused on the sulfonyl side chains with different substituents on the methylene unit and the terminal area. The detailed synthetic procedures and spectrum data of final products 1–27 are provided in the Supporting Information (Scheme S1). All the new analogs were initially evaluated for *in vitro* activities by testing PI3K α enzymic inhibition, the resulting suppression of pAKT in PC-3 cells and subsequent antiproliferation assays on breast cancer cell lines T47D and MCF-7 carrying a PIK3CA H1047R mutation and E545 K mutation, respectively (Table 1).¹⁶ The exploration of alkylation or fluorination at the methylene unit of the sulfone portion led to an improvement in potency in most cases (entries 1–9), whereas these trends were not observed in isopropyl (4) and cyclohexyl (9) analogs indicating a limit to the size of the substituent. Among these analogs, the IC₅₀ values of the *gem*-dimethyl (5), cyclopropyl (6), and cyclobutyl (7) analogs were lower than 20 nM against PI3K α , accompanied by an improvement in cellular phosphorylation of AKT and antiproliferation in comparison with the reference compound BKM. The replacement of morpholine with (*R*)-3-methylmorpholine (in compound 10) resulted in a 14-fold decrease in activity relative to the parent compound 6. Meanwhile, the (*S*)-3-methylmorpholine analog 11 maintained enzymic and cellular effects comparable to those of 6. Further exploration of the (*S*)-3-methylmorpholine fragment paired with *gem*-dimethyl, cyclobutyl, and cyclopentyl substituents

adjacent to the sulfone side chain (in compounds 12–14) turned out a consistent 2-fold enhancement in enzymic potency. Moreover, this optimization identified compound 13 with low IC₅₀ values of 93 and 78 nM against T47D and MCF-7 in MTT cell proliferation assays, respectively. Another selection of analogs was made to explore replacement of the terminal methyl group of the sulfone with ethyl, isopropyl, and phenyl groups (entries 15–23). The ethyl and isopropyl terminal groups did not lead to better activity (compounds 15–18), even though they were combined with the favorable cyclopropyl or *gem*-dimethyl sulfonyl moieties. Compound 20 featuring a terminal phenyl group combined with *gem*-dimethyl showed low nanomolar activity against PI3K α , but it was relatively weak in the cellular assessment. Interestingly, cellular potency was significantly enhanced once one extra fluorine atom was located at the ortho-position of phenyl ring adjacent to the sulfone (compound 23). Replacement of the trifluoromethyl substituent on the aminopyridyl moiety with fluorine led to a slightly decrease in potency against PI3K α ; however, the antiproliferative effects of the resulting compounds 24–27 were dramatically elevated, especially in the (*S*)-3-methylmorpholine based analogs 26 and 27, which were found to be 10 times more active than BKM against T47D and MCF-7 cells. Additionally, good corrections were observed between cellular p-AKT inhibition in PC-3 cells and antiproliferation results in T47D ($R^2 = 0.88$) and MCF-7 cells ($R^2 = 0.92$) (Figure S1), which demonstrate that p-AKT inhibition is responsible for the cellular antiproliferative effects even if in different cells. Nevertheless, the 2D plot of biochemical PI3K α activity and cellular p-AKT inhibition does not show a nice linear regression ($R^2 = 0.53$), which may be related to distinct permeability.

Compounds 1, 6, 11, and 26 were selected for further characterization in other three class I PI3K subtypes as well as mTOR. The results shown in Table 2 indicate the cyclopropyl

Table 2. Class I PI3K and mTOR Inhibitory Activities of Compounds 1, 6, 11, and 26^a

compd	enzyme IC ₅₀ (μ M)				
	PI3K α	PI3K β	PI3K γ	PI3K δ	mTOR
1	0.189	n/d ^b	n/d	n/d	13.98
6	0.016	0.314	0.288	0.240	1.680
11	0.016	0.264	0.164	0.197	2.120
26	0.020	0.376	0.204	0.046	0.186
BKM	0.040	0.234	0.372	0.125	1.981

^aIC₅₀, the mean values of duplicate measurements. ^bn/d, not determined.

ring presented on the sulfone chain plays an important role to maintain comparable Class I PI3K relative to BKM. Notably, converting the trifluoromethyl group (11) to fluorine (26) on the C₆ aminopyridyl moiety led to a 11-fold potency increase against mTOR and 4-fold improved potency at PI3K δ subtypes. As the sulfone side chain is also present in inhibitors of Ataxia telangiectasia mutated and RAD3-related (ATR) belonging to phosphatidylinositol 3-kinase-related kinase (PIKK) family,¹⁷ the capability of compounds 1, 6, 11, and 26 to impact the ATR cellular activity in terms of inhibition of phosphorylation of Chk1 Ser-345 were also evaluated in HT-29 cells. Compounds 1, 6, 11, and BKM were found to be inactive up to 10 μ M, while 26 shows slight inhibition at the concentration of 10 μ M (Figure 2).

Figure 2. Effect of compounds **1**, **6**, **11**, and **26** on ATR activity in the presence of DNA damage. HT-29 were pretreated with indicated compounds for 1 h, and DNA damage was induced by exposure to camptothecin. ATR activity was measured by detecting phosphorylation of substrate Chk1 and total Chk1, and β -actin was shown as internal control. KU-60019 (KU) is a specific inhibitor of ATM/ATR and DNA-PK.

Thirteen potent compounds (**3**, **5**–**7**, **13**, **16**, **18**, **20**, and **23**–**27**) were used for a metabolic stability study in human and mouse liver microsomes to identify metabolically stable compounds for *in vivo* studies. The results shown in Table S1 indicate that the compounds containing trifluoromethyls exhibit a significantly low metabolic stability relative to BKM (Cl_{int} : human, 10.86 mL/min/kg; mouse, 151.33 mL/min/kg) except the compound **6** (Cl_{int} : human, 9.86 mL/min/kg; mouse, 185.15 mL/min/kg). A cyclobutane ring on the methylene unit, a (*S*)-3-methylmorpholine moiety, and an aromatic terminal group are particularly detrimental to metabolic stability in this series. However, aminofluoropyridines **24**–**27** exhibited comparable or much better liver microsomal stability both in human (Cl_{int} : 5–16 mL/min/kg) and mouse (Cl_{int} : 40–140 mL/min/kg) in comparison with BKM. Given its enhanced mTOR potency and metabolic stability, we were interested in advancing compound **26** into more characterization.

A panel of cell lines was chosen to carry out antiproliferation screening to compare **26** and BKM. The treatment of **26** turned out 2.5-fold to 18.7-fold improvement against different cells (Figure 3A, Table S2), while stronger cytotoxicity against HUVECs (**26**, IC_{50} = 0.108 μ M; BKM, IC_{50} = 0.886 μ M) was also observed without certain cell selectivity. It is known that

BKM shows effects on the destabilization of microtubule that contributes to its therapeutic intervention together with its PI3K inhibition.¹⁸ We thought that this property might transfer to the new hybrid analogs due to their structural similarity. A dose–response cell cycle study in HT-29 cell showed BKM accumulated cells dominantly in G2/M fraction as expected, which leads to mitotic arrest or apoptosis (Figure 3C). In contrast, the treatment of **26** arrested cells in the G1 fraction, which acts the same as cytostatics (Figure 3B). In addition, no obvious effect of **26** at 10 μ M concentration was observed in an off-target test containing 28 different kinases (Table S3), and **26** exhibited comparable results with BKM against the mutant PI3K α E542K, E545K, and H1047R subtype inhibition. Overall, the experimental results elucidate that the elevated mTOR activity is responsible for its dramatically improved cellular antiproliferative effects.

To better understand the interactions with targeted proteins, docking simulations were performed for compound **26** with PI3K α , mTOR, and tubulin (Figure 4). The binding mode of **26** to PI3K α is very similar to that of BKM, which is responsible for similar potency against PI3K α . The morpholine forms a key hydrogen bond with Val851 (Val2240 on mTOR) in the hinge region, the amino group at the 2-position of the pyridine engages in another hydrogen bond with Asp810 (Asp2195 on mTOR), the sulfone side chain occupies the ribose pocket, and the fluorine at the 4-position of the pyridine is involved in an attractive electrostatic interaction with Lys802. At the same position on mTOR, the residue is Glu2190, which causes a repulsive force that twists the binding of compound **26** and thereby enhances the compound's potency at mTOR. Moreover, the overlapped docking poses with tubulin indicate that the cyclopropyl group of **26** is too big to accommodate the tubulin binding site due to the steric hindrance with Lys352, leading to a binding displacement from BKM and weaker binding to tubulin, which may differentiate **26** with BKM.

Further ADMET profiling demonstrates compound **26** does not inhibit the most common cytochrome P₄₅₀ enzymes (CYP) 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 (IC_{50} values >10 μ M, Table S4). *In vitro* evaluation on human hERG channel current indicated a minimal risk of **26** induced long QT syndrome (IC_{50} > 30 μ M, Figure S2). The Caco-2 permeability assay determined **26** as a high permeable compound on both sides (P_{app} > 10×10^{-6} cm/s) with favorable efflux ratio ($P_{app}(B-A)/P_{app}(A-B)$ = 0.66, Table S5), which is supportive for its excellent cellular potency. A pharmacokinetic (PK) profiling was further performed for compound **26** in CD1 mice. As presented in Figure 5 (left), intravenous administration of **26** to mice at 5 mg/kg (dissolved in 15% Captisol) exhibited a similar clearance rate (15.2 mL/min/kg), volume of distribution (1.40 L/kg), and half-life (1.59 h) relative to the corresponding parameters of BKM disclosed in the literature.⁸ Oral administration to mice at 10 mg/kg (dissolved in 15% Captisol) yielded a high C_{max} (2984 ng/mL) and plasma exposure (AUC_{last} = 10379 h·ng/mL) with an excellent oral bioavailability ($F\%$ = 94.6).

With these encouraging *in vitro* effects and preliminary ADMET profiling, compound **26** was tested in *in vivo* efficacy studies in the end. HT-29 colorectal carcinoma xenograft mouse model carrying the PI3KCA P449T mutation was selected for our efficacy studies, as the PI3K/AKT/mTOR pathway is being considered as a potential therapeutic target for the treatment of colorectal cancer.¹⁹ Considering the higher

Figure 3. PI3K α mutant enzymic data. Cancer cell panel screening (A), and cell cycle distribution in HT-29 cells (B–D) of **26**.

Figure 4. Docking poses of **26** (orange) with PI3K α (A, PDB entry 4l23), mTOR (B, PDB entry 4jt6), and tubulin (C, **26**, cyan; **BKM**, yellow; entry 5m7e).

Figure 5. PK parameters and efficacy studies of **26**. (left) Male CD1 mice ($n = 18$) were assigned to two groups: nine animals were administered via oral gavage (10 mg/kg), and nine animals were administered via tail vein injection (5 mg/kg). Blood samples were collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h post dose ($n = 3$ mice per time point). (middle) Balb/c *nu/nu* mice bearing HT-29 xenograft tumors were treated with vehicle, **BKM** (15 or 30 mg/kg), or **26** (3.75 or 7.5 mg/kg) by daily oral gavage for 27 days when the mean tumor size was around 240 mm³. The estimated tumor volume was plotted versus time. The tumor growth inhibition (TGI) was calculated after 27-day treatment (**26**, 3.75 mpk, TGI = 54.4%, $*p = 0.02$; **26**, 7.5 mpk, TGI = 72.9%, $*p = 0.02$; **BKM**, 15 mpk, TGI = 39.4%, $*p = 0.02$; **BKM**, 30 mpk, TGI = 55.7%, $*p = 0.04$). (right) Body weight change (BWC) was measured, calculated, and plotted versus time. All data are presented as the mean $\pm$ SEM ($n = 6$). Dunnett's multiple comparison test, $* < 0.05$.

cytotoxicity and stronger antiproliferative effects of **26**, 4-fold lower doses (3.75 and 7.5 mg/kg) were selected in comparison with **BKM** at the doses of 15 and 30 mg/kg, respectively (Figure 5, middle). **BKM** only produced 39.4% tumor growth inhibition (TGI) with the treatment of 15 mg/kg, while better single agent efficacy (TGI = 54.4%) was observed with **26** at daily oral doses of 3.75 mg/kg for 27 days in a well-tolerated manner, which was comparable to the result with **BKM** at the dose of 30 mg/kg (TGI = 55.7%). Interestingly, the proliferation assay result of **BKM** ($IC_{50} = 1.186 \mu\text{M}$) in HT-29 cell was also about 7.3-fold higher than **26** ($IC_{50} = 0.163 \mu\text{M}$) (Table S2), which suggests a hint of correlation between *in vivo* tumor suppression and *in vitro* antiproliferation activity, although drug exposure in tumor tissue and *in vivo* target modulation have not been determined. The 7.5 mg/kg group of **26** displayed more significant TGI (72.9%). All animals survived after 27-day treatment, whereas 15% weight loss was observed in **26**, 7.5 mg/kg group (Figure 5, right). Notably, 60 mg/kg **BKM** was also enrolled at the beginning of in this study, which was terminated after 10-day treatment due to serious toxicity (weight loss > 25%, Figure S3).

In summary, we have designed and synthesized a new series of 6-aminopyridyl, 4-sulfonyl, 2-morpholino-pyrimidine core analogs as novel PI3K inhibitors. Through the SAR profiling of different substituents at the methylene unit and the terminal region of sulfonyl side chains, as well as the combination with different morpholino and aminopyridyl moieties, we successfully addressed the identification of orally bioavailable compound **26**, which exhibited potent enzymic activity against Class I PI3K and PI3K α mutant isoforms, improved mTOR

potency, good suppression of pAKT in cellular assays, and promising pharmacokinetic parameters. Significantly, compound **26** was found to be much more effective both in *in vitro* and *in vivo* studies compared to **BKM**.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00167.

Supplementary figures and table, details of the synthetic chemistry, docking studies, and biological assays (PDF)

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■ ABBREVIATIONS

PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; PIP2, phosphatidylinositol diphosphate; PIP3, phosphatidylinositol triphosphate; pAKT, phosphorylated AKT; ADMET, absorption, distribution, metabolism, excretion, and toxicity; SAR, structure–activity relationship; PK, pharmacokinetics; AUC, area under curve; CYP, cytochrome P450; hERG, human *ether-a-go-go*-related gene; i.v., intravenous administration; p.o., oral administration; TGI, tumor growth inhibition

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