Journal of Medicinal Chemistry Letter

J. Med. Chem. **2009,** *52,* 5013–5016 **5013**
DOI: 10.1021/jm900851f

ATP-Competitive Inhibitors of the Mammalian Target of Rapamycin: Design and Synthesis of Highly Potent and Selective Pyrazolopyrimidines†

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Received June 10, 2009

Abstract: The mammalian target of rapamycin (mTOR), a central regulator of growth, survival, and metabolism, is a validated target for cancer therapy. Rapamycin and its analogues, allosteric inhibitors of mTOR, only partially inhibit one mTOR protein complex. ATP-competitive, global inhibitors of mTOR that have the potential for enhanced anticancer efficacy are described. Structural features leading to potency and selectivity were identified and refined leading to compounds with in vivo efficacy in tumor xenograft models.

The mammalian target of rapamycin (mTOR[a]) is a key component of the phosphoinositide 3-kinase (PI3K) pathway aberrant in a large proportion of human cancers and a target of intense inhibitor development efforts.[1−4] mTOR exists as two protein complexes, mTORC1 and mTORC2, of which rapamycin and its analogues selectively target mTORC1 through allosteric binding to the FKBP12-rapamycin binding (FRB) domain. However, selective inhibition of mTORC1 by rapamycin has been shown to cause enhancement of PI3K signaling through a negative feedback mechanism[5] and recent reports indicate that rapamycin does not completely inhibit mTORC1.[6,7] The emerging role of mTORC2 in tumor growth and survival, along with the lack of suppression of this pathway by the rapamycins, makes the discovery of ATP-competitive mTOR inhibitors that target both mTOR complexes highly desirable.

The structural similarity of the ATP-binding sites of mTOR and PI3K has made the search for selective mTOR inhibitors challenging, and reports on the biology of selective mTOR inhibitors have only recently appeared.[8−11] Nonselective inhibitors of PI3K that potently inhibit mTOR and other kinases have previously been reported.[12,13] Selective mTOR inhibitors may be better tolerated, with the opportunity to achieve a higher therapeutic index for enhanced clinical efficacy. In addition, the clinical use of rapamycin analogues in cancer treatment shows selective mTOR inhibition to be a proven strategy. We describe here the first report of detailed structure−activity relationships (SAR) of highly potent and selective ATP-competitive mTOR inhibitors and the structural features that lead to selectivity over PI3K.

High throughput screening followed by hit to lead development produced pyrazolopyrimidine **1**, a potent inhibitor of mTOR (IC_{50} = 9.6 nM) with low selectivity versus PI3Kα (IC_{50} = 47 nM) (Table 1).[14] Phenols characteristically undergo rapid metabolism, as evidenced by the short half-life of **1** in nude mouse microsomes ($T_{1/2}$ = 5 min). Therefore, initial SAR efforts focused on a search for phenol bioisosteres that maintained potent mTOR inhibition while improving selectivity and metabolic stability.

The pyrazolopyrimidine scaffold was constructed by cyclization of 1-benzyl-4-hydrazinylpiperidine with 2,4,6-trichloropyrimidine-5-carbaldehyde (Scheme 1). The resulting 4,6-dichloropyrazolopyrimidine was treated with morpholine to give **2**. Debenzylation of **2** with α-chloroethyl chloroformate gave **3**. The piperidine NH of **3** could be functionalized to **4** by reductive amination to give alkylamines, by acylation to give amides, or by treatment with triphosgene followed by addition of amines or alcohols to give ureas or carbamates, respectively. Suzuki coupling of **4** with the pinacol ester of 4-aminophenylboronic acid gave an aniline that was converted to the corresponding ureidophenyl or carbamoylphenyl analogue **5** by treatment with triphosgene and an amine or alcohol, respectively. Alternatively, **5** could be prepared by protecting the piperidine NH of **3** as a *tert*-butyl carbamate group and converting the chloride to the arylmethylurea as described above. Removal of the *tert*-butyl carbamate group with trifluoroacetic acid gave **6**. The NH group of **6** could be further functionalized to give **5** ($-XR_2$ = $-NHCH_3$) as described above.

Conversion of the 3-phenol group in **1** to the bioisosteric 4-acetamidophenyl group gave compound **7** which exhibited equipotent mTOR activity (IC_{50} = 7 nM) to that of **1** but with improved microsomal stability and an order of magnitude greater selectivity versus PI3Kα (5× vs 58×, respectively) (Table 1). Interestingly, the 3-acetamidophenyl group gave **8** having a marked reduction in mTOR potency (IC_{50} = 2450 nM).[15] The methyl 4-phenylcarbamoyl group gave potent analogue **9** (mTOR IC_{50} = 4.6 nM) with a 3-fold increased selectivity versus PI3Kα (174×) compared with **7**.[16] Preparation of the analogous urea gave **10** with more than an order of magnitude greater enzyme potency (mTOR IC_{50} = 0.38 nM) and cell growth inhibition (LNCap IC_{50} = 3 nM) and comparable selectivity (108×) and microsomal stability.

The structural similarity of the ATP-binding sites of mTOR and the PI3Ks enabled an mTOR homology model to be built based on the X-ray crystal structure of PI3Kγ. Overall sequence similarity between mTOR and PI3Kγ is 25% in the kinase catalytic domain; however, the ATP-binding sites are significantly more conserved with a sequence similarity of 68%. There are two residue differences in the hinge region, namely, Trp2239 (mTOR) is Ile881 in PI3Kγ and Pro2241

†The atomic coordinates and the related experimental data for the X-ray crystal structure of **26** bound to PI3Kγ has been deposited with the Protein Data Bank: PDB code 3IBE.

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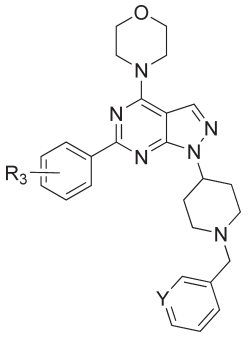
[a] Abbreviations: mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; SAR, structure−activity relationship; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; FRB, FKBP12-rapamycin binding.

Published on Web 07/31/2009

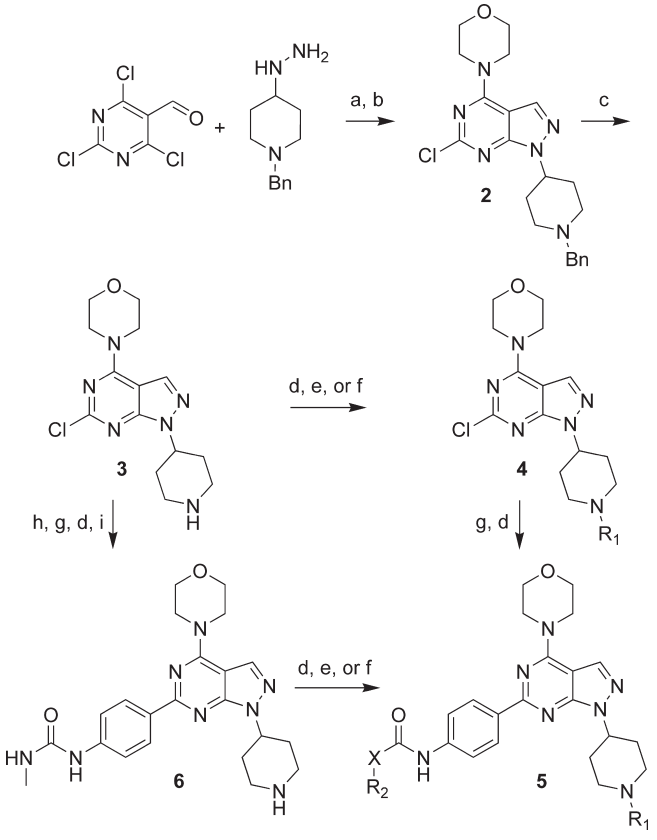
Table 1. Bioisosteres of the Phenol Group



compd	R_3	Y	mTOR[a]	PI3Kα[a]	selectivity[b]	LNCap[a]	micros[c]
1	3−OH	N	9.6 ± 1.5	47 ± 6	5	53 ± 8	5
7	4-$NHCOCH_3$	N	7.0 ± 0.9	404 ± 96	58	66 ± 24	12
8	3-$NHCOCH_3$	CH	2450 ± 450	2005 ± 162			
9	4-$NHCO_2CH_3$	N	4.6 ± 1.1	801 ± 199	174	213 ± 13	14
11	4-$NHCO_2CH_2$-CH_3	CH	46 ± 6	631 ± 22	14	3400 ± 306	
12	4-$NHCO_2CH_2$-CH_2OH	N	1.5 ± 0.1	284 ± 84	189	11 ± 5	6
10	4-$NHCONHCH_3$	N	0.38 ± 0.05	41 ± 2	108	3 ± 2	12

[a] Average IC_{50} (nM) ± SEM. [b] PI3Kα/mTOR. [c] Nude mouse microsomes $T_{1/2}$ (min).

Scheme 1. Synthesis of Pyrazolopyrimidine Analogues[a]



[a] R_1, R_2, and X are as defined in the tables. Reagents and conditions: (a) triethylamine; (b) morpholine; (c) α-chloroethyl chloroformate; (d) triphosgene, then alcohol or amine; (e) acid chloride or chloroformate; (f) aldehyde/sodium triacetoxyborohydride; (g) 4-aminophenylboronic acid or 4-isocyanatophenylboronic acid/amine or alcohol, palladium (0), sodium carbonate; (h) di-*tert*-butyl dicarbonate; (i) trifluoroacetic acid.

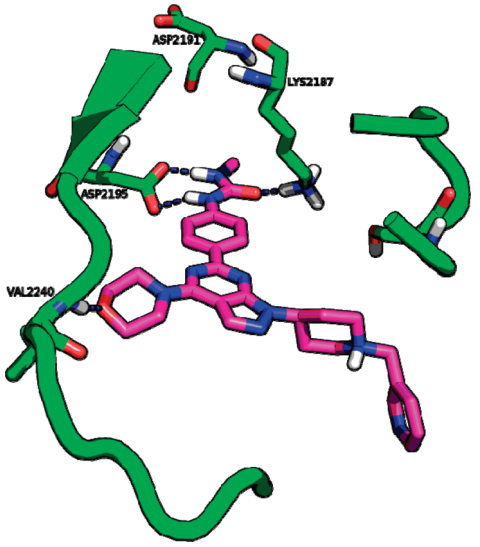


Figure 1. Docking of **10** in an mTOR homology model based on a PI3Kγ crystal structure.

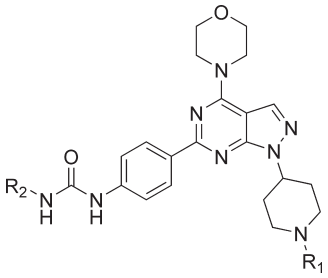
(mTOR) is a lysine in PI3Kγ (Lys883). There are also differences in the so-called specificity surface of the binding site: His2241 and Lys2243 in mTOR are Asp884 and Ala885 in PI3Kγ, respectively. Finally, the "bottom" of the ATP-binding site near the hinge region contains a leucine residue in mTOR (residue 2354), while PI3Kγ has a phenylalanine at this position (Phe961).

The X-ray structures of our inhibitors bound to PI3Kγ (vide infra) were used as the basis for docking studies with the mTOR homology model. Thus, modeling studies of urea **10** with the mTOR homology model show that the urea group makes three hydrogen bonds to the ATP-binding pocket, two between the urea NHs and Asp2195 and one between the urea carbonyl and Lys2187 (Figure 1). In contrast, in the less potent analogue **1**, the analogous phenol group makes only a single hydrogen bond to Asp2195.[14]

SAR of the carbamoylphenyl group revealed that the larger ethyl carbamate in **11** led to lower potency than the methyl carbamate analogue **9** (Table 1).[15] However, conversion of the ethyl carbamate group to a hydroxyethyl carbamate gave **12** that was appreciably more potent than **9** or **11**. Molecular modeling suggests that while the methyl and ethyl carbamate groups have hydrogen bond interactions with Asp2195 and Lys2187, the hydroxyethyl group of **12** can possibly form a third hydrogen bond to Asp2191 which could account for its increased potency.

While **10** was a potent mTOR inhibitor, it suffered from poor metabolic stability in nude mouse microsomes ($T_{1/2}$ = 12 min). Metabolite identification studies showed that the picolylpiperidine group was metabolized by dealkylation of the piperidine ring nitrogen, leading to the less active analogue **6** (Table 2). Therefore, other piperidine ring substituents were explored. Substitution by a benzyl group led to analogue **13** with comparable potency to **10** but lower nude mouse microsomal stability ($T_{1/2}$ = 3 min). An *N*-methyl group gave analogue **14** with decreased potency and stability. Acylation of the piperidine gave compound **15**, with increased stability ($T_{1/2}$ = 27 min) but decreased cellular activity. Similarly, methylurea analogues **16** and **17** had decreased potency. Prolonged microsomal stability ($T_{1/2}$ > 30 min) combined with good potency and selectivity was achieved with the methyl carbamate group (analogue **18**).

Table 2. Exploration of the Piperidine Ring Substituent



compd	R_1	R_2	mTOR[a]	PI3Kα[a]	selectivity[b]	LNCap[a]	micros[c]
6	−H	$-CH_3$	1.4 ± 0.4	23 ± 10	16	1367 ± 433	> 30
13	−Bn	$-CH_3$	0.52 ± 0.10	14 ± 2	27	1.5 ± 0.3	3
14	$-CH_3$	$-CH_3$	22 ± 7	211 ±40	10	733 ± 133	2
15	$-COCH_3$	$-CH_3$	2.1 ± 0.4	225 ± 18	107	817 ± 17	27
16	$-CONHCH_3$	$-CH_3$	2.0 ± 0.3	149 ± 1	75	2567 ± 186	
17	$-CON(CH_3)_2$	$-CH_3$	1.1 ± 0.1	209 ± 2	190	197 ± 3	> 30
18	$-CO_2CH_3$	$-CH_3$	0.46 ± 0.08	100 ± 17	217	31 ± 5	> 30
26	−CO-3-Pyr	−4-Pyr	0.83 ± 0.29	548 ± 401	660	150 ± 29	22

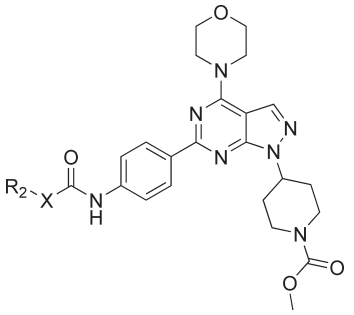
[a] Average IC_{50} (nM) ± SEM. [b] PI3Kα/mTOR. [c] Nude mouse microsomes $T_{1/2}$ (min).

Consistent with expectations of an ATP-competitive, global inhibitor of mTOR, **18** strongly suppressed mTORC1 and mTORC2 biomarkers P-S6K(T389) and P-AKT(S473), respectively, in nude mice bearing PTEN-null PC3MM2 xenograft tumors, upon dosing at 50 mg/kg ip, for at least 4 h. The mTOR selectivity of **18** was evidenced by the lack of appreciable suppression of the PI3K biomarker P-AKT-(T308). In vitro, **18** dose dependently suppressed these mTOR biomarkers in U87MG and PC3MM2 cells and showed selectivity versus the PI3K biomarker (Supporting Information Figure S1). Compound **18** also suppressed tumor growth in U87MG glioma bearing nude mice with 50 mg/kg ip once daily dosing for 5 days with a treated over control (T/C) value of 0.57 ($P < 0.05$) on day 7.

The carbamoyl piperidine group also led to excellent microsomal stability when the methylurea in **18** was replaced with carbamates, giving potent and selective analogues **19** and **20** (Table 3).[16] Analogue **19** completely inhibited mTORC1 biomarker P-S6K(T389) for at least 6 h and substantially inhibited mTORC2 biomarker P-AKT(S473) for 4−6 h in nude mice bearing the PTEN-null PC3MM2 xenograft tumors upon ip dosing at 50 mg/kg.[11] As with **18**, the PI3K biomarker AKT(T308) was not significantly inhibited, showing that **19** selectively inhibited mTOR. In vitro, **19** also dose dependently suppressed these mTOR biomarkers in U87MG and PC3MM2 cells and showed selectivity versus the PI3K biomarker (Supporting Information Figure S1).[11] Dosing at 50 mg/kg twice daily (b.i.d.) and once daily (q.d.) for 5 days in U87MG glioma-bearing nude mice achieved dose dependent suppression of tumor growth with T/C of 0.14 and 0.62 ($P < 0.05$), respectively.[11] Compounds with longer in vivo biomarker suppression and/or greater inhibition of cell growth than **18** and **19** may lead to stronger in vivo efficacy.

Compound **19** was selective for mTOR versus a panel of 24 kinases (Supporting Information Table 1) with $IC_{50} > 50\ \mu M$ against these enzymes. It also showed high selectivity (1469×) versus PI3Kγ (IC_{50} = 6315 ± 1371 nM). Compound **19** (3 μmol/L) also did not block VP-16-induced PIKK substrate phosphorylation of P-p53(S15) and P-Chk1(S345), indicating that the underlying PIKKs were not inhibited.[11]

Table 3. SAR of the Urea and Carbamate Groups



compd	R_2	X	mTOR[a]	PI3Kα[a]	selectivity[b]	LNCap[a]	micros[c]
19	$-CH_3$	O	4.3 ± 0.6	1026 ± 434	239	355 ± 45	> 30
20	$-CH_2CH_2OH$	O	1.0 ± 0.2	681 ± 124	681	28 ± 1	> 30
21	−Et	NH	0.32 ± 0.06	490 ± 198	1153	55	> 30
22	$-CH_2CH_2F$	NH	0.61 ± 0.03	505 ± 225	828	52 ± 13	> 30
23	$-CH(CH_2)_2$	NH	0.45 ± 0.04	661 ± 156	1469	42 ± 3	> 30
24	−3-Pyr	NH	0.20 ± 0.01	35 ± 6	175	31 ± 4	24
25	−4-Pyr	NH	0.30 ± 0.02	18	60	60 ± 15	9
27	-Ph-4-Pip-CH_3	NH	0.34 ± 0.02	15 ± 2	44	< 1	> 30
28	−Ph-4-CH_2OH	NH	0.08 ± 0.01	6.0	75	< 1	13

[a] Average IC_{50} (nM) ± SEM. [b] PI3Kα/mTOR. [c] Nude mouse microsomes $T_{1/2}$ (min).

Figure 2. Movement of protein loops (green to purple) in PI3Kγ to accommodate arylurea group of **26**, leading to solvent exposed 4-position of pyridine ring.

The mTOR potency and selectivity of the urea analogues could be tuned by modification of the urea substituent. While alkyl groups increased selectivity, aryl groups increased potency. Thus, conversion of the methylurea moiety in **18** to larger alkyl groups such as ethyl, 2-fluoroethyl, or cyclopropyl led to **21**, **22**, or **23**, respectively, with ∼1000× selectivity versus PI3Kα (Table 3). The pyridylureas **24** and **25** and other arylureas (vide infra) maintained or improved enzyme potency and cellular potency; however, selectivity versus PI3K was generally lower than for the alkylureas.

An X-ray crystal structure of pyridylurea analogue **26** bound to PI3Kγ showed that peptide chains within the enzyme shifted to accommodate the arylurea group and allowed binding of arylurea analogues (Figure 2). This movement within the enzyme exposes the 4-position of the aryl ring to solvent. Water solubilizing groups are often employed in drug development to enhance physicochemical properties and are typically added to regions of the molecule not involved in binding. The solvent accessibility of the 4-position of the aryl group was therefore utilized to incorporate groups that could

further improve the compound properties. Thus, incorporation of an *N*-methylpiperazine group at the 4-position of a phenylurea led to an analogue (**27**) with increased water solubility at pH 3 (> 100 μg/mL) and greatly increased cellular potency (LNCap IC_{50} < 1 nM) (Table 3). Similar increases in cell potency combined with potent mTOR inhibition were seen in **28** bearing a 4-hydroxymethylphenyl group.

In summary, lead optimization led to the discovery of analogues with potent, subnanomolar ATP-competitive mTOR kinase inhibition and > 1000× selectivity versus PI3Kα. Through the exploration of different phenol bioisosteres, urea and hydroxyethyl carbamate groups were found to provide excellent potency because of their formation of three hydrogen bonds to the enzyme. Selectivity versus PI3K could be controlled through variation of the substituents on the ureidophenyl moiety. Metabolite identification was used to identify and remove metabolically labile groups, thus leading to analogues with prolonged in vivo suppression of mTOR biomarkers and efficacy in a nude mouse xenograft model. Further refinement of analogues is ongoing and will be reported in due course.

Acknowledgment. The authors thank Dr. Joseph Marini and Angela Bretz for nude mouse microsome assays and Drs. Inder Chaudhary and Jack Wang for metabolite identification determinations.

Supporting Information Available: Synthetic procedures and characterization data for all compounds, biological methods, mTOR homology model construction, X-ray crystallographic study information on **26** bound to PI3Kγ, cellular biomarker inhibition data for **18** and **19**, and kinase selectivity panel testing data for **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) SAR reveals that benzyl and 3-picolyl groups on the piperidine ring give compounds with similar potency and selectivity.
(16) Compounds **9** and **19** are WYE-687 and WYE-354, respectively, in ref 11.