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Morpholine Derivatives Greatly Enhance the Selectivity of Mammalian Target of Rapamycin (mTOR) Inhibitors

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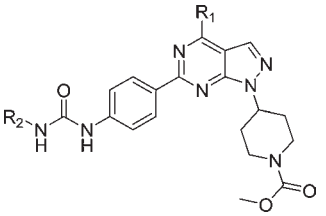
Abstract: Dramatic improvements in mTOR-targeting selectivity were achieved by replacing morpholine in pyrazolopyrimidine inhibitors with bridged morpholines. Analogues with subnanomolar mTOR IC_{50} values and up to 26000-fold selectivity versus PI3Kα were prepared. Chiral morpholines gave inhibitors whose enantiomers had different selectivity and potency profiles. Molecular modeling suggests that a single amino acid difference between PI3K and mTOR (Phe961Leu) accounts for the profound selectivity seen by creating a deeper pocket in mTOR that can accommodate bridged morpholines.

The mammalian target of rapamycin (mTOR[a]) is a member of the phosphoinositide 3-kinase (PI3K) related kinases (PIKKs), a family of unconventional high molecular mass serine/threonine protein kinases. In cancer, mTOR is frequently hyperactivated and is a clinically validated target for therapy.[1] While numerous reports of dual-pan PI3K/mTOR inhibitors have appeared,[2] few studies have identified selective mTOR inhibitors.[1,3−9] The development of specific mTOR inhibitors is particularly challenging because of the extensive conservation of the ATP-binding pockets of the PI3K family. Nevertheless, identification of highly potent and specific ATP-competitive mTOR inhibitors is highly desirable for further validating mTOR as a disease target and exploiting the therapeutic potentials of mTOR-targeting in cancer. Selective mTOR inhibitors may be better tolerated, with the opportunity to achieve a higher therapeutic index for enhanced clinical efficacy.

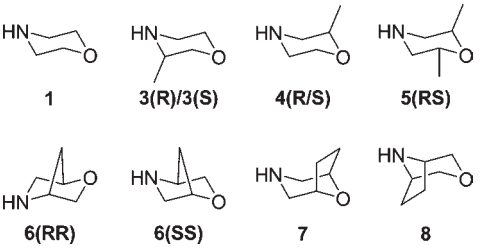
We previously reported that morpholine **1** containing pyrazolopyrimidines (e.g., **2**, Table 1) were potent and selective ATP-competitive inhibitors of mTOR with efficacy in cancer xenograft models in nude mice.[6−8] The binding mode of these inhibitors in a mTOR homology model based on a PI3Kγ X-ray cocrystal structure revealed that morpholine forms the critical kinase hinge-region binding interaction to Val882[10] in mTOR.[7] Morpholine has also been shown or proposed to be the hinge-region binding group in other mTOR/PI3K inhibitors.[9,11,12] We therefore embarked on an investigation of the effects of morpholine substitution on the potency and selectivity of pyrazolopyrimidines incorporating chiral (**3** and **4**) and achiral (**5**) methyl substituted morpholines as well as chiral (**6**) and achiral (**7** and **8**) bridged morpholines. Profound effects on potency and selectivity were achieved with compounds incorporating these morpholine derivatives, including enantiomeric differentiation, leading to analogues with unprecedented potency and selectivity. Enzyme docking studies indicated that a single amino acid difference between mTOR and PI3K in the vicinity of the hinge region was responsible for the mTOR selectivity of bridged morpholine containing inhibitors.

Table 1. 2-Methylmorpholine and Bridged Morpholine Containing Analogues



compd	R_1	R_2	mTOR[a]	PI3Kα[a]	PI3Kα/mTOR
2	**1**	Me	0.46 ± 0.08	100 ± 17	217
24	**7**	Me	0.22 ± 0.01	1803	8013
28	**8**	Me	0.22 ± 0.06	4271	19414
16	**4**	Me	2.3 ± 0.4	268 ± 24	116
19	**1**	Et	0.32 ± 0.06	490 ± 198	1531
25	**7**	Et	0.2 ± 0.02	5333	26665
29	**8**	Et	0.62 ± 0.10	5554	8886
17	**4**	Et	1.9 ± 0.6	721	360
27	**7**	c-propyl	0.62 ± 0.17	4320 ± 2341	6913
20	**1**	3-Pyr	0.20 ± 0.01	35 ± 6	175
26	**7**	3-Pyr	0.11 ± 0.01	613 ± 79	5577
30	**8**	3-Pyr	0.16 ± 0.02	1827	11073
18	**4**	3-Pyr	0.62 ± 0.06	81 ± 9	135

[a] Average IC_{50} ± SEM (nM).



Analogues were synthesized by modification of a previously described route,[7,8] substituting a morpholine derivative **3**−**8** for morpholine **1** (Scheme 1). Thus, cyclization of 1-benzyl-4-hydrazinylpiperidine with 2,4,6-trichloropyrimidine-5-carbaldehyde gave an intermediate 4,6-dichloropyrazolopyrimidine **9** which was treated with a morpholine derivative to give **10**. Debenzylation of **10** with α-chloroethyl chloroformate followed by functionalization of the piperidine NH by treatment with a carbamoyl chloride gave the corresponding carbamate **11**. Suzuki coupling of **11** with the pinacol ester of 4-aminophenylboronic acid gave aniline **12** that was

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

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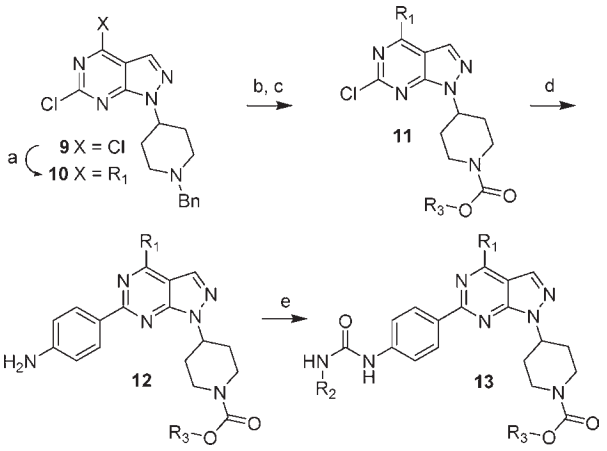
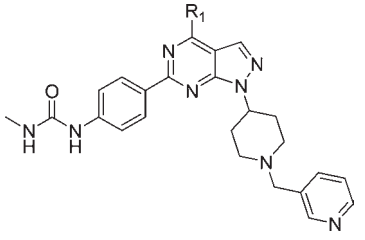
Scheme 1. Synthesis of Pyrazolopyrimidine Analogues[a]



[a] R_1 and R_2 are as defined in the tables. R_3 is Me or Et. Reagents and conditions: (a) **3**–**8**; (b) α-chloroethyl chloroformate; (c) methyl or ethyl chloroformate; (d) 4-aminophenylboronic acid, palladium(0), sodium carbonate; (e) triphosgene, triethylamine, then R_2NH_2.

Table 2. *cis*-2,6-Dimethylmorpholine and Bridged Morpholine Containing Analogues



compd	R_1	mTOR[a]	PI3Kα[a]	PI3Kα/mTOR
14	**1**	0.38 ± 0.05	41 ± 2	108
15	**5**	84 ± 21	3877 ± 971	46
23	**7**	0.48 ± 0.01	677	1410

[a] Average IC_{50} ± SEM (nM).

converted to the corresponding ureidophenyl **13** by treatment with triphosgene and an amine. Trifluoroethyl substituted analogues were prepared according to a literature route.[13]

Replacement of morpholine **1** in the pyrazolopyrimidine mTOR inhibitor **14** with *cis*-2,6-dimethylmorpholine **5** gave **15** that was 90- and 200-fold less potent versus PI3Kα and mTOR, respectively, than **14** (Table 2). Examination of the hinge-region/morpholine interaction in the mTOR homology model showed that the width of the morpholine containing pocket is partially defined by Tyr867 and Cys885 (Figure 1). In **15**, the methyl groups of *cis*-2,6-dimethylmorpholine **5** adopt an equatorial conformation making the morpholine moiety wider than the binding pocket, thus resulting in displacement of the morpholine away from Val882 and accounting for its loss of potency relative to morpholine analogue **14**. An equatorial methyl group giving a wider morpholine group in racemic 2-methylmorpholine **4(R/S)** analogues **16**–**18** may also account for their lower potency versus mTOR and PI3Kα than the corresponding morpholine containing analogues **2**, **19**, and **20** (Table 1). Similarly, replacement of morpholine with (*R*)-3-methylmorpholine **3(R)** (vide infra) gave **21** that was less potent versus mTOR and PI3Kα than the corresponding morpholine **1** containing **22** (Table 3).

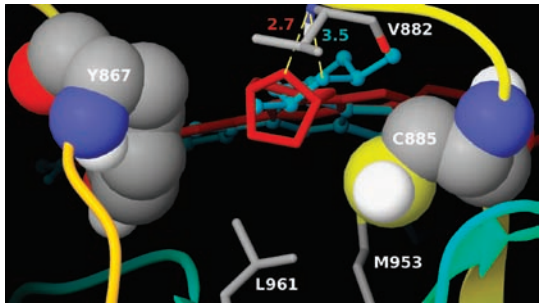
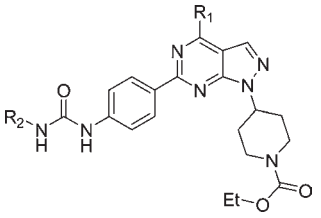


Figure 1. Docking of **15** (cyan ball and stick representation), and **23** (red tube representation) in the mTOR homology model. Tyr867 and Cys885, which define the pocket width, are shown in space filling mode. The backbone is rendered as a ribbon. Yellow lines indicate distances from the hinge NH to the morpholine O (text labels colored the same as the inhibitors are in Å). Residues are numbered according to their positions in PI3Kγ.

The excessive width of the *cis*-2,6-dimethylmorpholine group in **15** causing the morpholine displacement in the enzyme pocket of mTOR and PI3K (vide supra) could be designed out by constraining the equatorial methyl groups in an axial conformation through formation of an ethylene bridge as in 2,6-bridged morpholine **7**. Thus, replacement of *cis*-2,6-dimethylmorpholine in **15** with 2,6-bridged morpholine **7** gave **23** that showed a dramatic increase in mTOR potency relative to **15** with subnanomolar mTOR activity comparable to morpholine **14** (Table 2). Molecular modeling showed that the width of the 2,6-bridged morpholine in **23** is readily accommodated by the mTOR morpholine binding pocket (Figure 1). Remarkably, the PI3Kα activity of **23** was profoundly reduced relative to **14** leading to a 13-fold increase in mTOR selectivity for **23** (PI3Kα/mTOR = 1410). Similarly, replacement of morpholine **1** with **7** in **2**, **19**, and **20** gave the corresponding **24**, **25**, and **26** having comparable or lower subnanomolar mTOR IC_{50} and greatly decreased PI3K activity, leading to marked increases in selectivity (Table 1). For example, **25** has a potent IC_{50} of 0.2 nM versus mTOR and an extremely high selectivity of 26 665 versus PI3Kα. Potent inhibition of cellular proliferation was also retained upon replacement of morpholine with **7**. Thus, bridged morpholine analogue **25** inhibited LNCap cell growth with IC_{50} = 9 nM while the corresponding morpholine containing analogue **19** had IC_{50} = 55 nM.[7] Compounds in this manuscript also showed similar or greater selectivity versus other class I PI3K isoforms. All analogues had IC_{50} values for PI3Kγ that were equal to or greater than their IC_{50} values for PI3Kα. In addition, **27** was shown to be selective for mTOR versus PI3Kδ (IC_{50} = 2629 nM) and PI3Kβ (IC_{50} > 10 000 nM). Selectivity versus PIKKs ATR and hSMG1 has been demonstrated for a related pyrazolopyrimidine containing **7**.[14]

Docking of the bridged morpholine analogues suggests that a single amino acid difference between mTOR and PI3Kα/PI3Kγ causes a difference in the depth of the morpholine binding pockets that is responsible for the increased selectivity observed for these analogues (Figure 2).[10] Modeling indicates that Phe961 of PI3K is too large to comfortably accommodate the ethylene-bridged morpholine of **23**, causing displacement of the morpholine oxygen away from its hydrogen bonding partner, the backbone NH of Val882 (Figure 2A). However, in mTOR, the smaller amino acid substitution leucine (Phe961Leu) creates a deeper pocket, which accommodates the bridged morpholine **7** without

Table 3. 3-Methylmorpholine Containing Analogues



compd	R_1	R_2	mTOR[a]	PI3Kα[a]	PI3Kα/mTOR
22	**1**	Et	0.17 ± 0.05	704 ± 64	4141
47	**3(S)**	Et	1.8 ± 0.1	553	307
21	**3(R)**	Et	0.87 ± 0.02	4362	4985
48	**3(S)**	$-(CH_2)_2F$	1.8	521	289
49	**3(R)**	$-(CH_2)_2F$	1.2 ± 0.3	4546	3666
38	**3(S)**	4-Pyr	0.58 ± 0.1	18 ± 0.5	32
50	**3(R)**	4-Pyr	0.28 ± 0.06	517	1846

[a] Average IC_{50} ± SEM (nM).

causing significant displacement of **23** relative to the corresponding morpholine containing **14** (Figures 1 and 2B). Superposition of the mTOR plus **23** complex on the PI3Kγ crystal structure shows that unfavorable steric contacts with Phe961 would be present if **23** adopted its mTOR binding mode in PI3K (Figure 2C). Sequence alignments of other PIKK family members show that while DNA-PK is most similar to mTOR, with Ile at 961, other PIKKs (e.g., ATM, ATR, and hSMG1) contain a smaller Val at position 961.

The above modeling analysis similarly applies to analogues with 3,5-ethylene bridged morpholine **8** leading to highly potent and selective mTOR inhibitors **28**−**30** (Table 1). For example, replacing the morpholine in **2** with **8** gave **28**, a subnanomolar mTOR inhibitor (IC_{50} = 0.22 nM) with 19414-fold selectivity versus PI3Kα. In an overlay of bridged morpholines **7** and **8** in **23** and **28**, respectively, both bridging ethylene groups are directed toward Leu961 in the deeper mTOR morpholine binding pocket (Supporting Information Figure S1).

Replacement of morpholine with bridged morpholines also greatly increased the selectivity of pyrazolopyrimidine inhibitors with substituents other than piperidine on the pyrazole ring. For example, potent and selective trifluoroethyl analogues were prepared (Table 4). The urea SAR previously reported for morpholine containing pyrazolopyrimidine inhibitors[7,8] was also seen with the bridged morpholine analogues. Thus, larger alkylurea substituents such as 2-fluoroethyl gave increased selectivity (i.e., **31** and **32**) while arylureas gave potent inhibitors at the expense of some selectivity (i.e., **33**−**35**). Replacement of morpholine **1** with bridged morpholines **7** and **8** could convert even an analogue such as **33** having potent PI3Kα activity (IC_{50} = 10 nM) and moderate selectivity (43-fold) into potent mTOR inhibitors (**34** and **35**, respectively) with high (> 1000-fold) selectivity versus PI3Kα.

Intriguingly, the chiral 2,5-methylene bridged morpholines **6(RR)** and **6(SS)** gave pairs of enantiomers that showed differential binding affinity for mTOR and PI3Kα (Table 4). Compounds containing bridged morpholine **6(SS)** were > 14-fold more potent versus mTOR than those with **6(RR)**. In contrast, the difference in potency versus PI3Kα was only 2- to 3-fold. While less potent than the analogous ethylene bridged analogues, the analogues containing **6(SS)** retain single digit mTOR potency and high selectivity versus

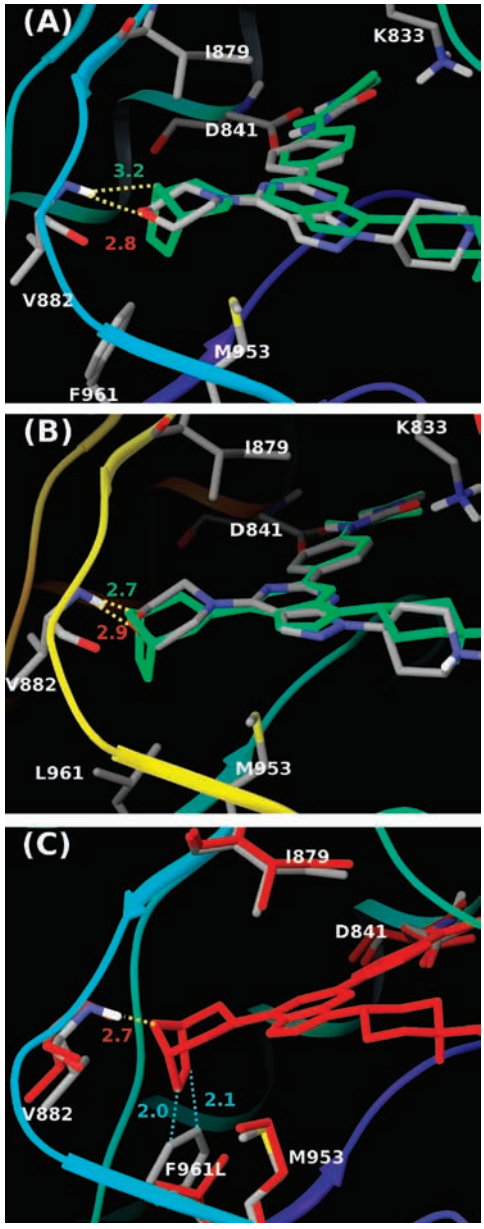
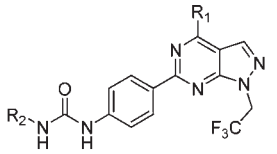


Figure 2. Differences in binding of the bridged morpholine in **23** to mTOR and PI3Kγ. (A) Compounds **14** (color by element) and **23** (green) docked to PI3Kγ. Yellow dotted lines indicate hydrogen bonds, with corresponding distance measurements colored the same as the acceptor oxygen. (B) Compounds **14** (color by element) and **23** (green) docked to mTOR. (C) Superposition of the complex between **23** and mTOR (red) with PI3Kγ (colored by element) with close steric contacts indicated by blue dashed lines. Distances in Angstroms.

PI3K. For example, the 3-pyridylurea **36** has mTOR IC_{50} = 1.2 nM and 482-fold selectivity versus PI3K.

Similarly, (*R*)- and (*S*)-3-methylmorpholines **3(R)** and **3(S)** gave pairs of enantiomers with different binding affinities for mTOR and PI3Kα (Table 3). While **3(R)** containing compounds were approximately 2-fold more potent versus mTOR than the corresponding ones with **3(S)**, **3(S)** containing compounds were > 8-fold more potent versus PI3Kα than those with **3(R)**. The different activity profiles of these enantiomers allow for the design of mTOR inhibitors with high selectivity (e.g., **21**) or dual mTOR/PI3K inhibitors (i.e., **38**) with these morpholine derivatives.

Table 4. Trifluorethyl Substituted Pyrazolopyrimidine Analogues



Cmpd	R_1	R_2	mTOR[a]	PI3Kα[a]	PI3Kα/mTOR
39	**1**	Me	1.0 +/- 0.1	194 +/- 49	194
40	**7**	Me	0.50 +/- 0.13	1616	3232
41	**8**	Me	0.56 +/- 0.03	1367	2419
31	**1**	$-CH_2CH_2F$	0.60 +/- 0.07	314 +/- 50	524
32	**7**	$-CH_2CH_2F$	0.23 +/- 0.01	2746	11939
33	**1**	3-Pyr	0.23 +/- 0.02	10 +/- 3	43
34	**7**	3-Pyr	0.19 +/- 0.01	345 +/- 79	1818
35	**8**	3-Pyr	0.20 +/- 0.02	227	1135
42	**6(SS)**	Me	5.5 +/- 0.5	2092	380
43	**6(RR)**	Me	91 +/- 19	4632	51
44	**6(SS)**	c-propyl	1.9 +/- 0.5	4052 +/- 1957	2170
45	**6(RR)**	c-propyl	53 +/- 5	9500	179
36	**6(SS)**	3-Pyr	1.2 +/- 0.2	602	482
46	**6(RR)**	3-Pyr	17 +/- 3	1935	114

[a] Average $IC_{50} \pm$ SEM (nM).

In summary, morpholine derivatives can profoundly influence the mTOR and PI3K binding affinity of pyrazolopyrimidine analogues containing them, resulting in potent subnanomolar mTOR inhibitors with unprecedented selectivity for mTOR (> 20000-fold). Molecular modeling suggests that increased mTOR selectivity in bridged morpholine containing inhibitors is caused by a leucine for phenylalanine substitution in mTOR versus PI3K that creates a deeper pocket that can better accommodate bridged morpholines. Chiral morpholine derivatives gave inhibitors whose enantiomers had differential binding affinity for mTOR and PI3K resulting in different selectivity and potency profiles. Inclusion of morpholine derivatives in pyrazolopyrimidines and other scaffolds to produce mTOR selective analogues with potent in vivo anticancer efficacy will be reported in due course.

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Supporting Information Available: Experimental, biological, and molecular modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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