Bioorganic & Medicinal Chemistry Letters 20 (2010) 1440–1444

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Pyrazolopyrimidines as highly potent and selective, ATP-competitive inhibitors of the mammalian target of rapamycin (mTOR): Optimization of the 1-substituent

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ARTICLE INFO

Article history:
Received 6 October 2009
Revised 18 December 2009
Accepted 22 December 2009
Available online 4 January 2010

Keywords:
mTOR
P13K
Kinase

ABSTRACT

A series of pyrazolopyrimidine mammalian Target Of Rapamycin (mTOR) inhibitors with various substituents at the 1-position have been prepared, resulting in compounds with excellent potency, selectivity and microsomal stability. Combination of a 1-cyclohexyl ketal group with a 2,6-ethylene bridged morpholine in the 4-position and a ureidophenyl group in the 6-positon resulted in compound **8a**, that selectively suppressed key mTOR biomarkers in vivo for at least 8 h following iv administration and showed excellent oral activity in a xenograft tumor model.

Rapamycin and its analogs (e.g., Torisel™) inhibit the serine–threonine protein kinase mammalian Target Of Rapamycin (mTOR) and as such play an important role in the disruption of key cell-signaling pathways in a variety of tumors.[1] mTOR forms two functional complexes in cells: mTORC1 and mTORC2.[2] While rapamycin and its analogs allosterically bind to mTORC1 and inhibit cell signaling mediated by mTORC1, mTORC2 activates Akt by phosphorylation of Ser473, resulting in potentially anti-apoptotic effects.[3,4] Treatment with rapamycin leads to upregulation of Akt signaling in certain cells.[5] Since a direct inhibitor of mTOR would inhibit the function of mTORC1 and mTORC2, a reduction in Akt signaling would be expected. Thus, inhibition of mTOR as opposed to mTORC1 alone may lead to more efficacious therapeutic agents.

We have previously demonstrated that highly potent and selective ATP-competitive inhibitors of mTOR can be prepared from a pyrazolopyrimidine scaffold, equipped at the 4-position with a morpholine derivative, and the 6-position with a ureidophenyl group.[6–9] In this Letter, we describe a detailed overview of the structure–activity relationship (SAR) in the 1-position of the pyrazolopyrimidines culminating in the identification of a compound with excellent in vivo efficacy.

The analogs in Tables 1 and 2 were synthesized as previously described[7,10–12] or as shown in Scheme 1. Cyclization of an appropriate hydrazine with 2,4,6-trichloropyrimidine-5-carbaldehyde **1** afforded the 4,6-dichloro-1*H*-pyrazolo[3,4-*d*]pyrimidine, which was treated with 8-oxa-3-azabicyclo[3.2.1]octane (2,6-ethylene bridged morpholine)[8,13] to give **2**. Suzuki coupling of **2** with the pinacol ester of 4-aminophenylboronic acid gave the corresponding aniline that was converted to the ureidophenyl analog **3** by treatment with triphosgene and an amine. When R_1 is benzylpiperidine, debenzylation of **3** with α-chloroethyl chloroformate was followed by treatment with the appropriate chloroformate to give carbamates **4**.

The compounds in Tables 3 and 4 were prepared according to Schemes 2 and 3. As shown in Scheme 2, 1,4-cyclohexanedione mono-ethyl ketal **5** was treated with *t*-butyl carbazate followed by removal of the *t*-Boc group in refluxing water to give hydrazine **6**. Condensation of hydrazine **6** with trichloroaldehyde **1**, and conversion of resulting **7** to ureas **8** was done as described above.

Scheme 2 also shows the synthesis of the cyclohexyl methyl ether intermediates. The ketal **7** was hydrolyzed to the ketone and reduced with lithium aluminum hydride to yield **9**, as a 3:1 mixture of *trans* to *cis* isomers, respectively, that were separated by silica gel column chromatography. Alkylation with methyl iodide and sodium hydride afforded methyl ethers **10** (*trans*) and **11** (*cis*), that were then converted into the methylureidophenyl compounds (**12** and **13**, respectively).

The *cis* and *trans* cyclohexanols, as well as additional ketals could be prepared from ketone **14**, obtained after hydrolysis of **8a** with HCl (Scheme 3). Treatment of **14** with either L-Selectride or lithium aluminum hydride gave the *cis* isomer **16** or the *trans*

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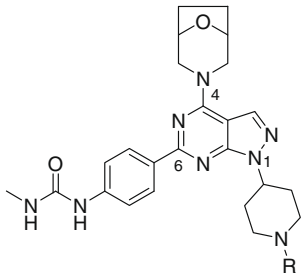
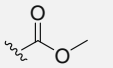
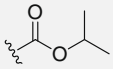
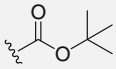
doi:10.1016/j.bmcl.2009.12.086

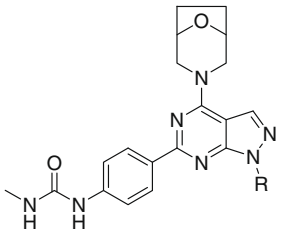
Table 1
1-Piperidine carbamates



Compd	R	mTOR[a]	PI3Kα[a]	Sel.[b]	LNCap[a]	Micros.[c]
4a		0.22	1803	8195	26	>30
4b		0.19	2021	10,636	6	21
4c		0.26	1475	5673	27	11

[a] Average IC_{50} (nM). The average error for IC_{50} determinations was <25%.
[b] PI3Kα/mTOR.
[c] Nude mouse microsomes $T_{1/2}$ (min).

Table 2
1-Alkyl substituted pyrazolopyrimidines



Compd	R	mTOR[a]	PI3Kα[a]	Sel.[b]	LNCap[a]	Micros.[c]
3a	$-CH_3$	1.34	247	184	170	>30
3b	–Et	0.74	270	364	70	>30
3c	–*i*Pr	0.12	234	1950	5	>30
3d	$-CH_2CF_3$	0.50	1616	3232	38	>30
3e	$-CH_2CH_2OH$	2.85	375	132	650	NT
3f	$-CH_2CH_2N(CH_3)_2$	14.5	966	66	400	5

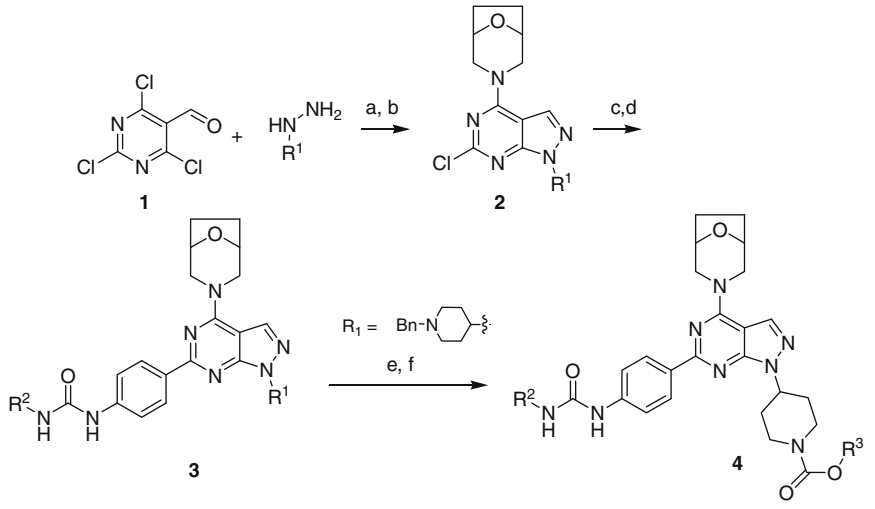
NT = not tested.
[a] Average IC_{50} (nM). The average error for IC_{50} determinations was <25%.
[b] PI3Kα/mTOR.
[c] Nude mouse microsomes $T_{1/2}$ (min).

isomer **15**, respectively. Treatment of ketone **14** with the appropriate alcohol in the presence of trimethyl or tripropyl orthoformate, molecular sieves and p-toluenesulfonic acid afforded the ketals **17** and **18**.

SAR studies of 4-morpholino-pyrazolopyrimidines revealed that a ureidophenyl group was the optimal substituent in the 6-position and resulted in the identification of potent and selective inhibitors with a methylcarbamoyl piperidine in the 1-position.[7,9] The selectivity of these inhibitors could be further enhanced by introduction of a 2,6-ethylene bridged morpholine in the 4-position,[8] leading to compounds such as **4a** (Table 1), which combined high potency and selectivity[14–16] with excellent stability in a nude mouse microsome assay.[17] 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.[8] Compound **4a** was selected as the starting point for further SAR studies, aimed at identifying the optimal substituent in the 1-position. Initial efforts focused on the preparation of other carbamoylpiperidines (e.g., **4b** and **4c**, Table 1). Although the resulting inhibitors remained highly potent and selective, their microsomal stability was significantly reduced. We had previously shown that amides and ureas were also inferior to carbamates in this position.[7]

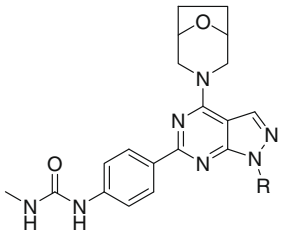
Next, a number of 1-alkyl substituted pyrazolopyrimidines were investigated (Table 2). The presence of alkyl groups at this site generally led to inhibitors with reduced selectivity and moderate cellular activity (e.g., **3a** and **3b**). Two compounds that were highly selective and possessed good cellular activity (isopropyl analog **3c** and trifluoroethyl analog **3d**) were stable in nude mouse microsomes but suffered from low human microsomal stability ($T_{1/2}$ = 12 and 5 min, respectively). Introduction of polar substituents (primary alcohol **3e** or tertiary amine **3f**) reduced both enzyme and cellular potency.

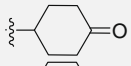
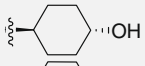
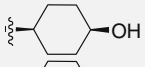
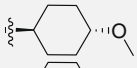
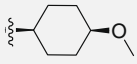
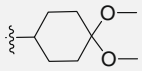
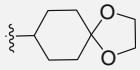
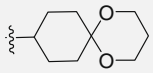
As substitution at the 1-position with acyclic alkyl groups did not result in analogs which met our requirements for cellular



Scheme 1. R^1, R^2, and R^3 are as defined in tables. Reagents and conditions: (a) ethanol, triethylamine; (b) 2,6-ethylene bridged morpholine; (c) 4-aminophenylboronic acid pinacol ester, palladium (0), sodium carbonate, toluene, ethanol; (d) dichloromethane, triethylamine, triphosgene, then R_2NH_2; (e) dichloroethane, α-chloroethyl chloroformate; (f) dichloromethane, R_3OCOCl.

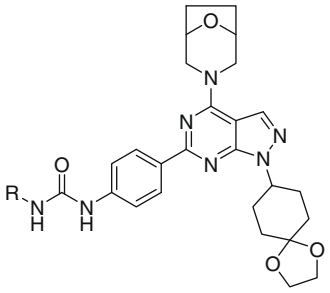
Table 3
1-Cyclohexyl analogs

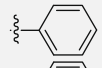
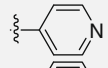
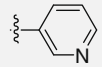


Compd	R	mTOR[a]	PI3Kα[a]	Sel.[b]	LNCap[a]	Micros.[c]	
						Mouse	Human
14		0.22	1301	5913	32	20	10
15		0.24	882	3975	20	>30	>30
16		0.30	227	757	20	>30	>30
12		0.38	1170	3079	2	>30	15
13		0.33	705	2136	1	17	NT
17		0.74	648	875	2	15	30
8a		0.21	1180	5619	2	>30	26
18		1.19	305	305	8	>30	>30

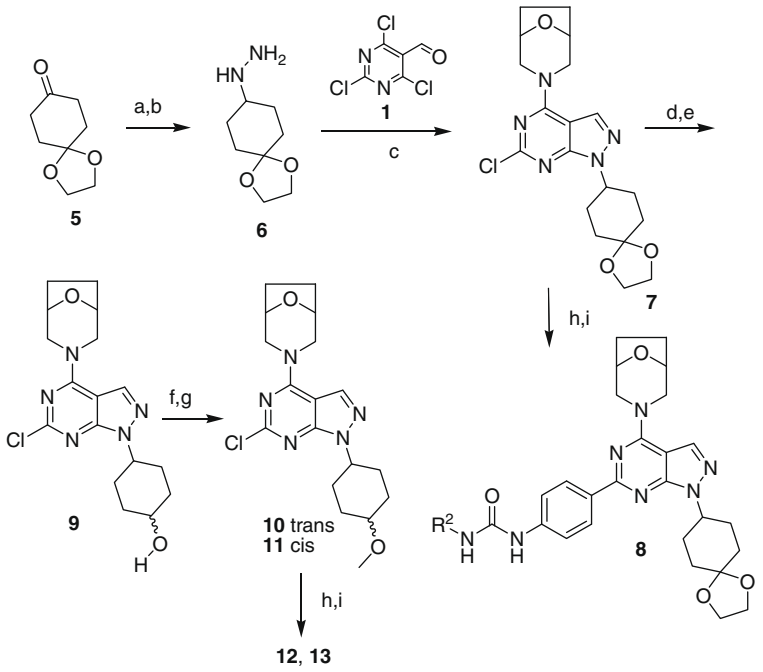
NT = not tested.
[a] Average IC_{50} (nM). The average error for IC_{50} determinations was <25%.
[b] PI3Kα/mTOR.
[c] Nude mouse or human microsomes $T_{1/2}$ (min).

Table 4
Ureidophenyl analogs of **8a**

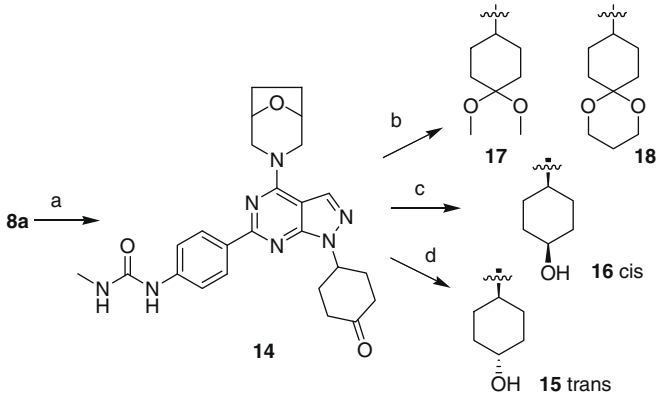


Compd	R	mTOR[a]	P13kα[a]	Sel.[b]	LNCap[a]	Micros.[c]	
						Mouse	Human
8b	–Et	0.26	4577	17,604	1	27	10
8c	$-CH_2CH_2F$	0.22	5129	23,313	1	>30	16
8d		0.22	12,000	54,545	2	25	9
8e		0.16	353	2206	4	>30	13
8f		0.12	220	1833	1	>30	13
8g		0.12	270	2250	1	>30	13

[a] Average IC_{50} (nM). The average error for IC_{50} determinations was <25%.
[b] PI3Kα/mTOR.
[c] Nude mouse or human microsomes $T_{1/2}$ (min).



Scheme 2. R^2 is as defined in tables. Reagents and conditions: (a) hexanes, *t*-butyl carbazate; (b) water, heat; (c) water, ethanol, triethylamine, 2,6-ethylene bridged morpholine; (d) concd hydrochloric acid; (e) tetrahydrofuran, lithium aluminum hydride; (f) chromatography; (g) tetrahydrofuran, sodium hydride, iodomethane; (h) 4-aminophenylboronic acid pinacol ester, palladium (0), sodium carbonate, toluene, ethanol; (i) dichloromethane, triethylamine, triphosgene, then RNH_2.



Scheme 3. Reagents and conditions: (a) concd hydrochloric acid; (b) molecular sieves, *p*-toluenesulfonic acid, corresponding alcohol and alkyl orthoformate; (c) tetrahydrofuran, L-Selectride; (d) tetrahydrofuran, lithium aluminum hydride.

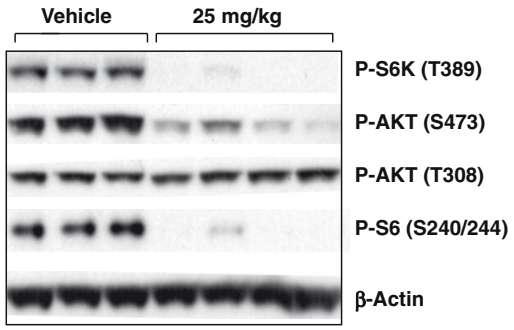


Figure 1. Biomarker inhibition following dosing of compound **8a**. Nude mice bearing MDA361 tumors were dosed intravenously (IV) with vehicle or 25 mg/kg of **8a**. Biomarkers were measured after 8 h. The dosing vehicle was 5% ethanol, 2% tween-80, 5% polyethelene glycol (PEG-400).

potency and microsomal stability, we examined cyclic analogs which were more structurally related to piperidines. To this end, a series of cyclohexyl analogs were prepared (Table 3). Ketone **14** was potent and selective in the enzyme assays, but was only moderately stable in nude mouse and human microsomes. Alcohols **15** and **16** exhibited sub-nanomolar activity in the enzyme assay and were stable in both nude mouse and human microsomes ($T_{1/2}$ >30 min) and potent in the LNCap cell assay (IC_{50} = 20 nM). Introduction of a methyl ether, as in **12** and **13**, resulted in compounds with improved cellular activity relative to alcohols **15** and **16**. However, microsomal stability remained modest. The inclusion of cyclohexyl ketals at the 1-position (**8a**, **17** and **18**) gave mTOR inhibitors with excellent cellular potency. Analog **8a**[18] also displayed greater than 5000-fold selectivity over PI3Kα and good stability in both mouse and human microsomes. Biomarker studies with **8a** in a nude mouse MDA361 xenograft model (Fig. 1) showed complete suppression of the mTORC1 biomarker P-S6K (T389) and sustained suppression of the mTORC2 biomarker P-AKT (S473), as well as complete inhibition of the downstream biomarker P-S6 (S240/244) for at least 8 h after an intravenous dose of 25 mg/kg.[18] Excellent mTOR selectivity was shown by lack of suppression of the PI3Kα biomarker P-AKT (T308).

The anti-tumor efficacy of analog **8a** in a nude mouse xenograft model with MDA361 tumor cells is shown in Figure 2.[18] A dose of 10 mg/kg given once daily resulted in near complete tumor stasis, whereas a daily dose of 20 mg/kg led to complete inhibition of tumor growth. No significant body weight changes were observed in the mice.

As **8a** contains a ketal functional group which could be potentially labile in vivo, PK studies in the nude mouse were conducted to verify the structure of the active compound. These studies revealed that ketone **14** was not present at a detectible level. Alcohols **15** and **16** were detected at low levels (less than 10% based upon AUC).

Due to the promising properties exhibited by ketal **8a**, a number of analogs were prepared which contained modification at the

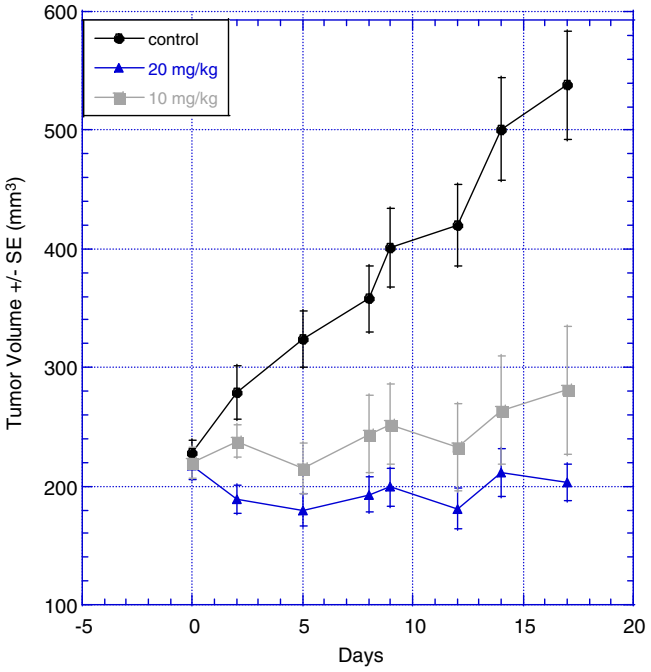


Figure 2. In vivo efficacy study: Nude mouse xenograft model using MDA361 tumors. Compound **8a** was dosed PO qd × 5 per cycle, (ends day 17). The dosing vehicle was 5% ethanol, 2% tween-80, 5% polyethelene glycol (PEG-400).

ureidophenyl moiety (Table 4). A variety of alkyl and aryl ureas displayed excellent potency and selectivity. Although these inhibitors were only moderately stable in human microsomes, they displayed unprecedented selectivity over PI3Kα (cf. **8d**: >50,000-fold selective).

In summary, a thorough examination of substituents at the 1-position of pyrazolopyrimidine mTOR inhibitors led to the discovery of cyclohexylketal derivatives. The combination of this group with a 2,6-ethylene bridged morpholine in the 4-position resulted in compound **8a** which exhibited improved potency, selectivity, and microsomal stability. Administration of this compound to nude mice resulted in in vivo biomarker suppression that was sustained for at least 8 h. Excellent in vivo efficacy was obtained with **8a** in a nude mouse xenograft model using MDA361 tumor cells. Ureidophenyl analogs of **8a** resulted in unprecedented selectivity (>50,000-fold) over PI3Kα. The availability of such highly selective mTOR inhibitors makes these compounds valuable pharmacological tools to help facilitate further elucidation of the mTOR/PI3K pathway.

Acknowledgments

The authors thank Dr. Li Di and Susan Li for human microsome assays, Dr. Joe Marini and Angela Bretz for mouse microsome assays, and Wei-Guo Zhang for mTOR assay development.

References and notes

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