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The discovery and optimisation of pyrido[2,3-*d*]pyrimidine-2,4-diamines as potent and selective inhibitors of mTOR kinase

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ABSTRACT

We describe a novel series of potent inhibitors of the kinase activity of mTOR. The compounds display good selectivity relative to other PI3K-related kinase family members and, in cellular assays, inhibit both mTORC1 and mTORC2 complexes and exhibit good antiproliferative activity.

The mammalian target of rapamycin (mTOR) is a key target in the development of antitumour therapies.[1] This central regulator of cell growth and proliferation is activated by growth factor/mitogenic stimulation of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway; one of the most frequently dysregulated pathways in cancer.[2] mTOR is a mammalian serine/threonine kinase of approximately 289 kDa in size and a member of the evolutionary conserved eukaryotic PI3K like kinase (PIKK) family of proteins, for example, DNA-PKcs (DNA dependent protein kinase), ATM (Ataxia-telangiectasia mutated).[3–5]

The known mTOR inhibitor rapamycin and its analogues (RAD001, CCI-779, AP23573) bind to the FKBP12/rapamycin complex binding domain (FRB), resulting in suppression of signalling to the downstream targets p70S6K and 4E-BP1 (Fig. 1).[6,7]

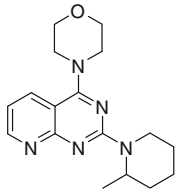
The potent but non-specific inhibitors of PI3K, LY294002 and wortmannin, have also been shown to inhibit the kinase function of mTOR but in this case by targeting the catalytic domain of the protein.[8]

Recently, it has been shown that mTOR exists in two complexes, mTORC1, a rapamycin sensitive complex signalling to p70S6K and 4E-BP1 and mTORC2 a rapamycin insensitive complex that signals to Akt,[9] which provides an explanation for the differences observed in the earlier work of Brunn et al.[8] and Edinger et al.[10]

Therefore, it is proposed that direct targeting of the kinase domain of mTOR would inhibit the signalling through both mTORC1 and mTORC2 and that such a compound would exhibit a different spectrum of pharmacology compared with rapamycin. As such a number of groups including our own have sought to identify selective inhibitors of mTORC1 and mTORC2.[11,12]

Herein we describe the discovery and optimisation of a series of substituted pyrido[2,3-*d*]pyrimidine-2,4-diamines as potent and selective inhibitors of mTOR kinase.

Our programme to develop small molecule mTOR inhibitors began with a high throughput screen which identified the racemate (**1**), which inhibited the kinase activity of mTOR with an IC_{50} of 1.0 μM in an ELISA based assay using immunoprecipitated full-length mTOR with a 4E-BP1 substrate.[13]



(**1**)

Our initial exploration focused on the role of the pyridine nitrogen of the bicyclic core. Synthesis of two structural isomers and the benzenoid analogue of (**1**) was undertaken.[14]

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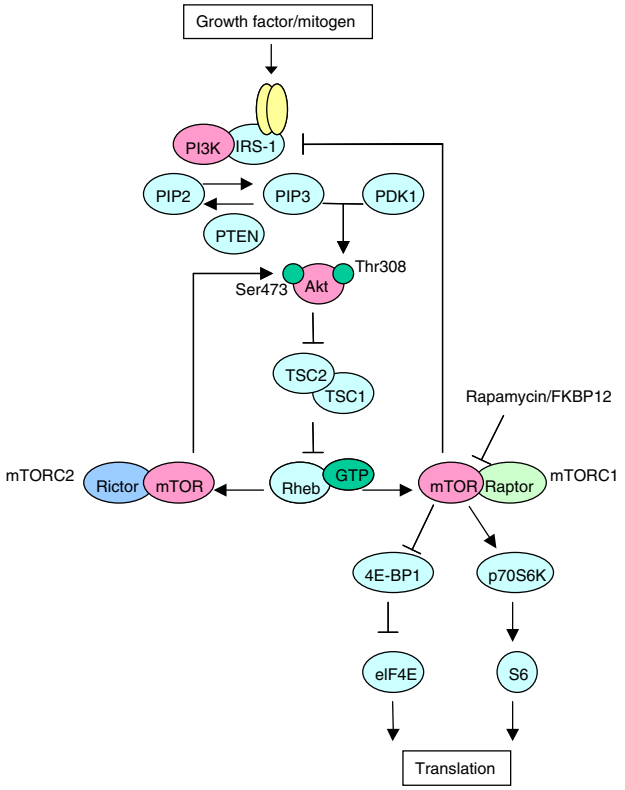


Figure 1. PI3K-Akt-mTOR pathway.

The results, summarized in Table 1, demonstrate that the optimal placement of the pyridine nitrogen is at the 8-position with the other two isomers and the benzenoid analogue possessing much reduced mTOR inhibitory activity.

At this point we decided to determine the preferred absolute stereochemistry of the methyl substituent on the piperidine and investigate the influence of C-2 and C-4 substituents.

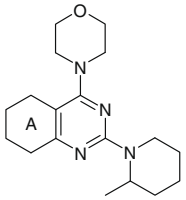
Compounds exemplified in Table 2 were made according to the method shown in Scheme 1.

The nicotinic acid (**5**) was subject to a fusion reaction with potassium cyanate and ammonium chloride to give the dione (**6**). This was chlorinated in the presence of phosphorus oxychloride and diisopropylethylamine to give the dichloro species (**7**). The cyclic amine, for example, morpholine, was selectively introduced to the C4 position (**8**) allowing the final chlorine displacement with selected amines to give compounds of the general structure (**9**).

The *S*-enantiomer (**10**) of compound (**1**) showed marginally improved potency relative to the *R*-enantiomer (**11**) and the racemate, and gave us our first sub-micromolar mTOR inhibitor. Moving the methyl group to the 3-position of the piperidine, compound (**12**), results in a modest loss of activity. In general substitution on the piperidine was quite well tolerated (data not shown) without significant loss of potency. Of particular interest were the analogues (**14**) and (**15**) where the piperidine has been replaced by morpholine, as they offered the possibility of reducing overall lipophilicity of the molecule. For example, *Clog P* compound (**11**) is 2.39 and 1.00 for compound (**14**).[15] The importance of the methyl substituent is evidenced by the much-reduced activity of unsubstituted analogue (**13**).

We next embarked on an exercise to characterize the requirements of the C4 substituent. The morpholine is a well precedented structural motif in inhibitors of PI3K and PI3K-like kinases[16,17] and, at least for the case of the prototypical PI3K inhibitor LY294002, the morpholine ether oxygen has been shown to participate directly in a key hydrogen-bonding interaction in the ATP-binding site of PI3Kγ.[18] By analogy we reasoned that the C4 morpholine ether oxygen of our mTOR inhibitors may be involved in a hydrogen bond interaction with residue Val 133 in the hinge region of the mTOR kinase domain. In order to test this argument we combined a preferred C2 substituent with a small number of changes to the C4 amino substituent.

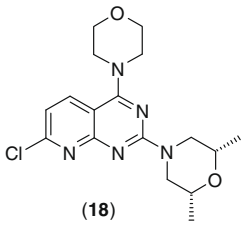
Table 1
Testing the importance of the pyridine nitrogen



Compound	A ring	mTOR inhibition IC_{50}, μM
1		1.0
2		<30% at 10 μM
3		<30% at 10 μM
4		<30% at 10 μM

Replacement of the ether oxygen by a carbon, compound (**16**), gave a significant reduction in activity. The slight change of geometry and increase in bulk associated with the change from morpholine to homomorpholine, as in compound (**17**), did not result in a complete loss of activity but did cause a significant reduction in potency. Both findings are consistent with our view of the involvement of the C4 morpholine oxygen in a key hydrogen binding interaction.

Docking studies using an homology model of mTOR built from an in house crystal structure of PI3Kγ suggested that substitution of the pyridopyrimidine C7 position could access additional binding pockets within the ATP binding site. For these investigations we elected to use the dimethyl-substituted morpholine substituent at C2 as this gave a good balance of potency and lipophilicity. Accordingly, the 7-chloro-substituted intermediate (**18**) was prepared by an analogous route to that described in Scheme 1.

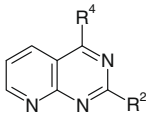


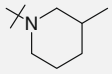
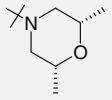
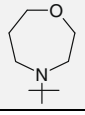
(**18**)

This compound was then subject to either displacement by nitrogen nucleophiles or Buchwald[19] or Suzuki[20] coupling reactions to introduce aromatic substituents. The results for key analogues are summarized in Table 3.

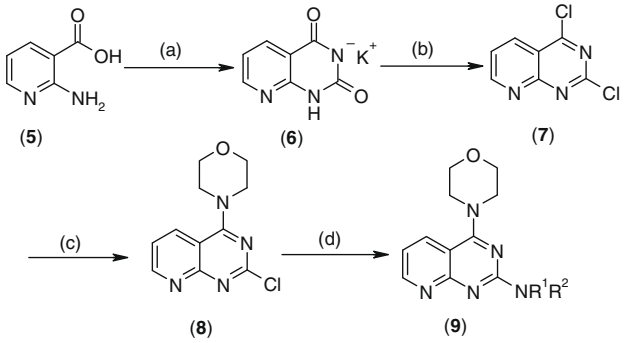
The introduction of aromatic amino substituents, as in compounds (**19**) and (**20**), resulted in potency improvements as did the introduction of directly bonded aromatic and heteroaromatic

Table 2
Modifications to C2 and C4 substituent



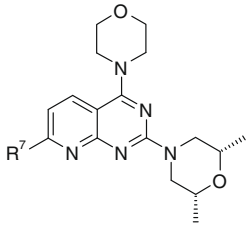
Compound	R^2	R^4	mTOR inhibition IC_{50}, μM
10		4-Morpholino	0.69
11		4-Morpholino	1.2
12		4-Morpholino	3.0
13		4-Morpholino	<30% at 10 μM
14		4-Morpholino	1.8
15		4-Morpholino	1.3
16		1-Piperidino	<30% at 10 μM
17			4.8

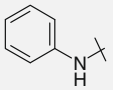
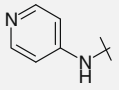
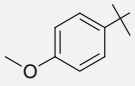
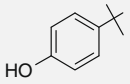
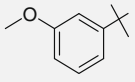
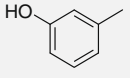
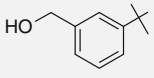
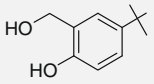
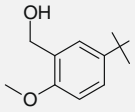
substituents, as in compounds (**21**), (**22**), (**23**) and (**24**). An investigation of substituted analogues of the phenyl compound (**24**) revealed that electron-donating substituents in the *para* position, as in compounds (**25**) and (**26**), were well tolerated. However, a different picture was apparent for *meta* substituents. Here electron-donating character did not seem to be beneficial. Rather the presence of a hydrogen bond donor either as a phenol compound (**28**) or as a primary alcohol as in compound (**29**) was preferable.



Scheme 1. Reagents and conditions: (a) KCNO, NH_4Cl, 200 °C, 76%; (b) $POCl_3$, Hünig's base, toluene, 100 °C, 3 h, 20%; (c) morpholine, Hünig's base, CH_2Cl_2, rt, 40%; (d) selected amine, DMA, 80 °C.

Table 3
Structure–activity of 7-substituted analogues



Compound	R^7	mTOR inhibition IC_{50}, μM
19		0.12
20		0.087
21		0.12
22		0.033
23		0.22
24		0.062
25		0.045
26		0.039
27		0.49
28		0.053
29		0.093
30		0.037
31		0.016

Combining the electron donor at the *para* position with the hydrogen bond donor at the *meta* position resulted in the still more po-

Table 4
Activity of compound (**31**), KU-63794 against PI3K family enzymes

Enzyme	IC_{50}, μM
mTOR (4E-BP1 substrate)	0.016
mTOR (p70S6K peptide substrate)[a]	0.003
PI3Kα[b]	8.9
PI3Kβ	>30
PI3Kγ	>30
PI3Kδ	>5.3
ATR	>10
DNA-PK	>10

[a] Inhibition of mTOR mediated phosphorylation was determined using truncated mTOR expressed in HEK293 and a biotinylated p70S6K peptide substrate. Ref. 21.
[b] PI3K isoform inhibition was measured using PIP2 phosphorylation in an alphascreen competition assay (biotinylated PIP3). Ref. 22.

tent analogue (**31**). These subtle structure–activity relationships at the C7 substituent were not readily explained by the homology model.

Further characterization of compound (**31**), KU-63794, suggests a good degree of selectivity for mTOR relative to other PIKK family members has been achieved (Table 4). In addition compound (**31**) showed no significant activity when tested against a range of 200 non-PI3K related kinases at a concentration of 10 μM.

To determine if the compounds inhibited both mTORC1 and mTORC2 complexes in cellular environment U87MG glioblastoma cells were treated with compound (**31**) for 2 h and phosphorylation of the indirect mTORC1 downstream effector S6 ribosomal protein (Ser235/236) and the direct mTORC2 substrate Akt (Ser473) were measured by Western blot. Both endpoints were inhibited with IC_{50}s of 0.10 and 0.15 μM, respectively. Moreover inhibition of proliferation of a range of cell types was demonstrated. For example, the T47D breast cancer cell line was inhibited with a GI_{50} of 0.35 μM using a sulforhodamine B endpoint.

In conclusion we have discovered a series of highly selective, sub-micromolar potency mTOR kinase inhibitors. The activity of these inhibitors has been confirmed in a cellular environment where they inhibit both mTOR complexes. Thus the compounds warrant further characterization to understand the role of the PI3K-Akt-mTOR pathway in disease and assess their potential as therapeutic agents.

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13. Inhibition of mTOR kinase was assayed using full-length mTOR (extracted from HeLa cytoplasmic extract by immunoprecipitation) with a recombinant 4E-BP1 protein substrate and an ATP concentration of 50 μM. Detection of phosphorylated product through an ELISA format was essentially as described in Ref. 8. Figures quoted are the mean of at least 3 determinations and variability around the mean was <50%.
14. Compounds (**2**), (**3**) and (**4**) were prepared according to the same methods described in Scheme 1 from 3-amino-isonicotinic acid, 3-aminopicolinic acid and anthranilic acid, respectively.
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19. Typical Buchwald conditions: (**18**), relevant amine, tris(dibenzylideneacetone) dipalladium(0), Xantphos, cesium carbonate, dioxane, 150 °C, 40 min.
20. Typical Suzuki conditions: (**18**), relevant boronate ester, tetrakis(PPh_3)$_4$Pd, K_2CO_3, CH_3CN, H_2O, 95 °C, 2 h.
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