Indian Journal of Chemistry
Vol. 48B, December 2009, pp. 1761-1765

Mammalian target of rapamycin (mTOR) inhibitors with therapeutic potential for colitis

Vijaykumar Deore[1], Mandar R Bhonde[2], Ram A Vishwakarma[1], Nilesh M Dagia[2], Sanjay Kumar[1] & Abhijit Roychowdhury[*1]

[1]Department of Medicinal Chemistry & [2]Department of Pharmacology; Piramal Life Sciences; 1-Nirlon Complex, Off Western Express Highway, Goregaon (E), Mumbai 400 063, India

E-mail : abhijit.roychowdhury@piramal.com

Received and accepted 16 August 2009

Mammalian target of rapamycin (mTOR) is a protein kinase that regulates cell proliferation and protein synthesis in response to mitogens and nutrients. A plethora of recent research has linked increased mTOR activity to heightened inflammatory responses. Many of these findings suggest that mTOR inhibitors may be useful for treatment of ulcerative colitis (UC)[1,2]. The discovery, synthesis and a SAR of new cyano-pyridyl based small molecule inhibitor which suppresses DSS-induced colitis by inhibiting T cell function[3] is discussed. The most potent small molecule inhibitor of mTOR did not inhibit PI3K/Akt phosphorylation and thus turned out to be mTOR selective.

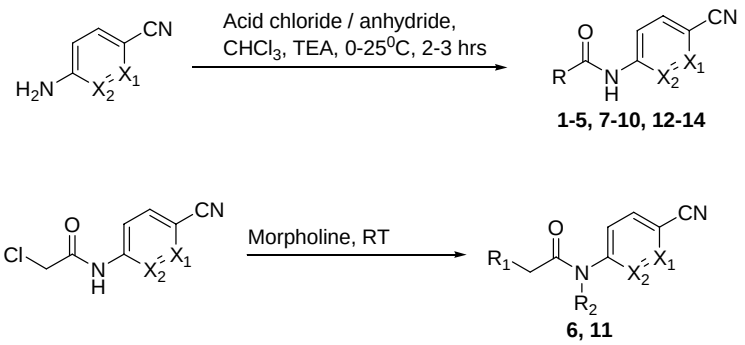
Keywords: Cyanopyridyl, mTOR inhibitor, PI3K, ulcerative colitis, inflammation

Mammalian target of rapamycin (mTOR), protein kinase, is an evolutionary conserved kinase that regulates protein synthesis, cell cycle progression in response to various cues. It's an integral part of the complex downstream signalling network of PI3K pathway and is also responsible for lymphocyte development and function of mature T and B cells. There is a plethora of research relating mTOR to tumorogenesis[1]. Our focus on mTOR was derived from the recent accumulating evidence linking mTOR activity to heightened inflammatory responses. The mTOR pathway regulates the production of nitric oxide[2] and activates STAT1-dependent transcription in macrophages in response to lipopolysaccharide (LPS)[3]. Activation of p70S6K1 as well as that of 4EBP1/PHAS-1[4]; bonafide target proteins of mTOR, has been stimulated by LPS. Interestingly, a recent study showed that rapamycin, a mTOR inhibitor, blunts leukocyte adhesion and extravasation in the gut mucosa leading to suppression of experimental chronic colitis[5]. In another study, treatment with everolimus (another mTOR inhibitor) reduced the number of T-cells in lamina propria and blocked lymphocytic IFN-γ release thereby ameliorating established murine colitis[6]. These findings suggest that mTOR inhibitors may be useful for treatment of ulcerative colitis (UC).

In the quest for finding a small molecule inhibitor of mTOR with implications in colitis, a cyano pyridine based motif is pursued as staring point for medicinal chemistry efforts. The use of pyridine derivative in the field of drug discovery is well known. Recently, several reports have emerged which show the therapeutic qualities of cyanopyridine derivatives. A few examples are cited herein, i) 3-cyano-2,6-dihydropyridine has been reported as potent inhibitor of dihydrouracil dehydrogenase and its co-administration with 1-ethoxymethyl-5-fluoro-uracil has exhibited antitumor effects[7], ii) 2-cyanopyridylureas derivatives have been claimed for their properties in treating hyper-proliferative and angiogenesis disorders[8], iii) pyridothieno- and pyridodithienotriazines are endowed with anti-histaminic and cytotoxic activity[9] and iv) acyclo-3-deazapyrimidine S-nucleosides have anti HIV[10] properties.

Results and Discussion

A series of 14 cyanopyridyl derivatives were synthesized **(Scheme I)**. 5-amino-2-cyanopyridine or 2-amino-5-cyanopyridine was treated with various acid chlorides or anhydrides using a suitable solvent (e.g. chloroform) and a base (*e.g.* triethylamine (TEA)) at 0-25°C for 2-3 hr to get target molecules **1-14.** For the synthesis of compound **1,** in brief,



Scheme I— Synthetic scheme for cyanopyridyl amides

5-amino-2-cyanopyridine was treated with β-chloro-acetylchloride in presence of TEA to synthesize 3-chloro-*N*-(6-cyanopyridin-3-yl)propanamide. The latter underwent simultaneous one pot β-chloro elimination and resulted in *N*-(6-cyanopyridin-3-yl)acrylamide (compound **1**). Compounds **2-5**, **7-10**, **12-14** were conventionally synthesized using either the acid chlorides of anhydrides of the respective acids[11]. Compound **2** and **9** on treatment with morpholine at RT afforded compounds **6** and **11,** respectively. All newly synthesized compounds **1-14** were characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The purity of synthesized compounds was assessed by high performance liquid chromatography (HPLC).

Compound **1** *N*-(4-cyanophenyl)acrylamide showed only weak mTOR inhibitory activity. Though not a significant inhibition it was encouraging to observe that in enzyme-linked immunoassays (ELISA) assay compound **1** inhibited fetal calf serum (FCS)-induced phosphorylation of p70S6K1 in H460 human non-small cell lung cancer cells by 12% at 10 μM **(Figure 1)**. Playing around with electron-withdrawing groups a chloroacetyl substitution of the amide yielded compound **2** with 31% mTOR inhibitory potency. Going by the same rational a 2-fluorocarboxamide **3** substitution and a 3,3,3-trifluoropropanamide **4** substitution would have given an increase in inhibitory potency. Contrary to our expectations activity dropped to 11% inhibition for compound **3** and compound **4** turned out to be inactive. Replacements of the chloro with other electron-donating, -neutral and -withdrawing groups **5-8** did not result in any improvement in mTOR inhibitory activity. Thus the 2-chloro substitution was optimum at this stage of SAR. While investigating with the 3-acetamido pyridyl system an attempt to also investigate the 2-acetamido pyridyl was also pursued. In this attempt compounds **9-12** were synthesized. As evident from the activity chart none of these compounds showed any residual activity. Reverting back to the 3-acetamido scaffold we synthesized compound **13** with a 2,2 dichloro substitution on the 3-pyridylacetamide scaffold. Surprisingly, this compound did not exhibit any mTOR inhibitory activity. It is postulated that multiple electron withdrawing groups on the acetamide might compromise mTOR inhibition. To investigate this hypothesis, one chlorine atom was substituted with a methyl group to yield compound **14**. In line with the same reasoning, compound **14** exhibited significant mTOR inhibitory activity (70% inhibition at 10 μM; **Figure 1** Ref. 17). With the most potent mTOR inhibitor in hand its activity was cross-examined with western blot analysis. The results of these latter experiments revealed that **14** inhibited serum-induced mTOR activity in H460 human non-small cell lung cancer cells as well as HCT-116 human colon carcinoma cells (**Figure 2**).

Given the latest set of literature implying mTOR for inflammatory disorder compound **14** was subjected for a series of anti-inflammatory assays. It has been reported that everolimus, a mTOR inhibitor, inhibits interferon-γ (IFN- γ) production[6], the effect of **14** on the induced production of IFN- γ was investigated. The compound **14** inhibited concanavalin A (ConA)-induced IFN- γ production in a dose dependent manner[12] (**Figure 3**).

The observations that rapamycin[5] and evorilimus[6] (both mTOR inhibitors) are efficacious in animal models of colitis, combined with the findings that blocking IFN- γ production elicits a therapeutic effect in experimental colitis[13], led us to hypothesize that **14** (that inhibits mTOR activation as well as IFN- γ

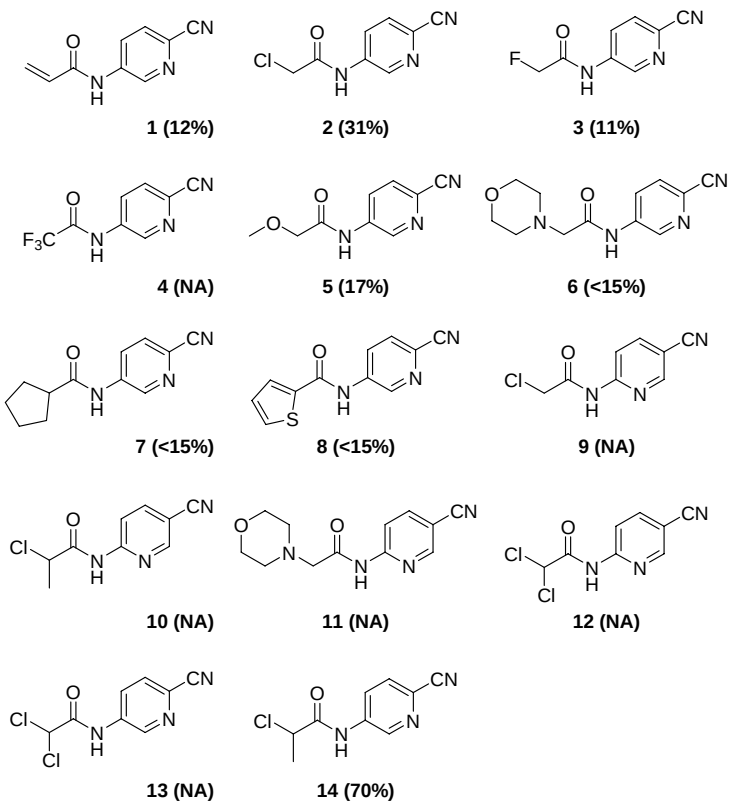


Figure 1 — Structure activity relationship of compounds **1-14**. Numbers in parentheses are % mTOR inhibition at 10μM (*FCS-induced phosphorylation of p70S6K1 in H460 cells was used as a read out in ELISA assays to ascertain the mTOR inhibitory activity of various compounds).

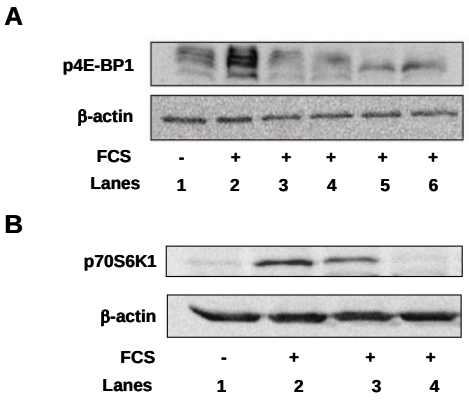


Figure 2 — Compound 14 inhibits mTOR activity[12]**. (A)** Western blots of FCS-stimulated H460 cells treated with **14**, LY2942002, Rapamycin or DMSO. Blots presented are representative of n = 2 experiments. (Lanes 1 and 2: pre-treatment with 0.5% DMSO; Lane 3: pre-treatment with 3 μM **14**; Lane 4: pre-treatment with 10 μM **14**; Lane 5: pre-treatment with 30 μM LY294002 and Lane 6: pre-treatment with 1 μM rapamycin.) **(B)** Western blots of FCS-stimulated HCT-116 cells treated with **14**, LY2942002, or DMSO. Blots presented are representative of n = 2 experiments. (Lanes 1 and 2: pre-treatment with 0.5% DMSO; Lane 3: pre-treatment with 30 μM **14** and Lane 4: pre-treatment with 30 μM LY294002.).

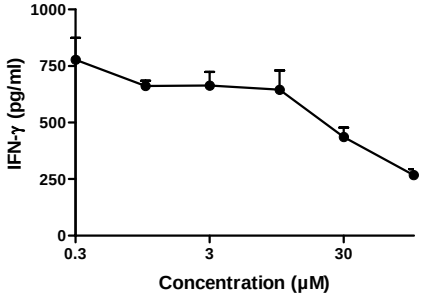


Figure 3 — Compound 14 inhibits Con-A-induced IFN-γ production from human peripheral blood mononuclear cells in a dose-dependent manner [12]. Results presented are representative of n = 3 separate experiments. * indicates $p < 0.05$ compared to DMSO control.

production) would be efficacious in a murine model of colitis. To pursue these experiments the pharmacokinetic profile of compound **14** was evaluated. These observations reveal that an i.p dose of 100 mg/kg of **14** resulted in maximal concentration of (C_{max}) of 180 μM in plasma[11]. It was also found to be

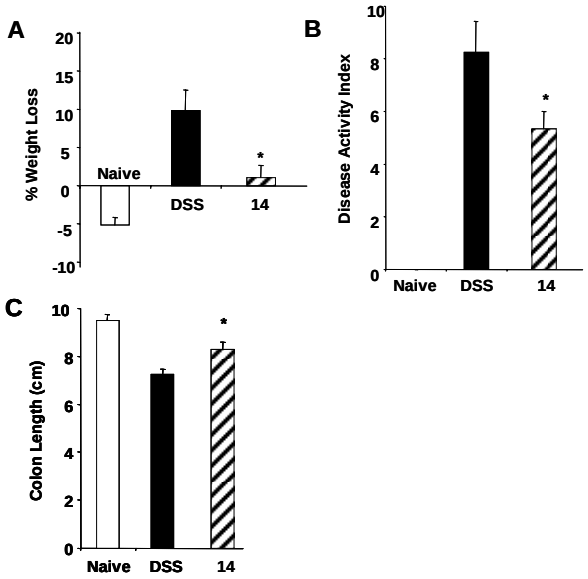


Figure 4 — Compound 14 significantly inhibits DSS-induced colitis [12]. Various groups of mice received DSS daily with some groups receiving daily injections of 15 mg/kg **14** or 0.5% CMC. (A) The percentage weight loss during the study. (B) Disease activity index and (C) the longitudinal length of the colon. All values are averages of 6 mice. Results presented are representative of n = 3 separate experiments. * indicates $p < 0.05$ compared to DSS-treated, CMC administered control group. (Legend: Naïve indicates mice were given regular drinking water from day 0 to day 10. DSS indicates mice were given DSS in drinking water from day 0 to day 10 and administered 0.5% CMC daily from day 0 to day 10, **14** indicates mice were given DSS in drinking water from day 0 to day 10 and administered 15 mg/kg **14**, i.p., daily from day 0 to day 10).

stable in rat, mouse and human plasma. Based on this set of data and some further calculations the effect of **14** on experimental colitis was studied at a dose of 15 mg/kg (i.p.). In the dextran sulphate sodium (DSS)-model of colitis[12], (i) **14** significantly inhibited DSS-induced weight loss, improved rectal bleeding index, decreased disease activity index and reversed DSS-induced shortening of the colon **(Figure 4)**; (ii) **14** distinctly attenuated DSS- induced edema, prominently diminished the leukocyte infiltration in the colonic mucosa and resulted in protection against DSS-induced crypt damage[12] and (iii) **14** blocked DSS-induced activation of mTOR[12]. Collectively, these results provide direct evidence that **14**, a novel mTOR inhibitor suppresses DSS-induced colitis by inhibiting T cell function, and is potential therapeutic for colitis.

It is well known that mTOR falls in the PI3K kinase pathway and its role in tumorogenesis has been well known. Thus compound **14** was also tested for PI3K/ Akt signalling pathway but was found to be almost inactive in inhibiting Akt phosphorylation (18% inhibition – data not shown). This indicates that compound 14 is a selective inhibitor of mTOR. A quick search of the recent literature will present the reader with a plethora of small molecule inhibitors of mTOR. Compounds like NVP-BEZ235 or PI-103 which were originally discovered as PI3K inhibitors, inhibit mTOR by targeting the ATP binding site. While a multi target inhibitor has its own value as an anti-cancer lead molecule but it is quite difficult to use such compounds for mechanistic study for any downstream signalling pathways. As of now it has not been possible to modulate mTOR activity independent of PI3K activity using any of the above-mentioned compounds. It is possible that some of the functions attributed to PI3Ks using the classical inhibitor LY294002 are a consequence of mTOR inhibition, but it is has not been possible to address this issue. This is where compounds like **14** can be utilized for its selective inhibitory profile. To the best of our knowledge this is the first report of a mTOR selective inhibitor which does not inhibit PI3K/Akt phosphorylation.

Experimental Section

(A) General procedure for synthesis of compounds 1-5, 7-10, 12-14: To a stirring solution of 5-amino-2-cyanopyridine/2-amino-5-cyanopyridine in chloroform, triethylamine (1.5 equivalent) and acid chloride/anhydride (1.1 equivalent) was added drop-wise at 0-5°C. After complete addition of acid chloride/anhydride the reaction-mixture was allowed to come to RT and stirred overnight. After completion of reaction, the reaction-mixture was diluted with chloroform and washed with water. The organic layer was dried over sodium sulfate, concentrated *in vacuuo* and resulting compound was crystallized from chloroform:petroleum ether (1:2).

(B) General procedure for synthesis of compounds 6 and 11: Compounds **2** and **9** was stirred with morpholine at RT for overnight to get compound **6** and **11** respectively. These compounds were purified by column chromatography using ethyl acetate and hexane gradient.

Analytical data for 2-chloro-*N*-(6-cyanopyridin-3-yl)propanamide **14**: ^{1}H NMR (DMSO-d_6 300 MHz) δ: 10.99 (s, 1H), 8.87-8.86 (d, 1H, J = 2.7Hz), 8.29-8.25 (dd, 1H, J = 2.7 & 8.7), 8.01-7.98 (d, 1H, *J* = 8.7Hz), 4.66-4.73 (m, 1H), 1.60-1.62 (d, 3H;. MS: *m/z* 210 (M+1); Calcd for $C_9H_8N_3ClO$ 209.05. HRMS, HPLC 99.42% (acetonitrile:ammonium acetate:riethyl amine *p*H 5.0).

References

1 Easton J B & Houghton P J, *Oncogene*, 25, **2006,** 6436.

2 Weinstein S L, Finn A J, Dave S H, Meng F, Lowell C A, Sanghera J S & DeFranco A L, *J Leukoc Biol,* 67, **2000,** 405.

3 Kristof A S, Marks-Konczalik J, Billings E & Moss J, *J Biol Chem*, 278, **2003**, 33637.

4 Dos Santos S, Delattre A I, De Longueville F, Bult H, & Raes M, *Ann N Y Acad Sci*, 1096, **2007,** 70.

5 Farkas S, Hornung M, Sattler C, Guba M, Steinbauer M, Anthuber M, Herfarth H, Schlitt H J & Geissler E K, *Int J Colorectal Dis,* 21, **2006**, 747.

6 Matsuda C, Ito T, Song J, Mizushima T, Tamagawa H, Kai Y, Hamanaka Y, Inoue M, Nishida T, Matsuda H & Sawa Y, *Clin Exp Immunol,* 148, **2007**, 348.

7 Tatsumi K, Yamauchi T, Kiyono K, Kishi K, Yanagihara Y, Imaoka T, Kawaguchi T & Kubo M, *J Biochem,* 114, **1993**, 912.

8 Scott W J, Boyer S, Lee W, Chen Y, Phillips B, Verma S, Chen J C Z, Fan J, Raudenbush B, Reman A, Yi L, Zhu Q & Adnane L, US *Pat* Application 20040235829, **2004**.

9 Quintela J M, Peinador C, Veiga C, Gonzalez L, Botana L M, Alfonso A & Riguera R, *Bioorg Med Chem* , 6, **1998**, 1911.

10 Liu M C, Luo M Z, Mozdziesz D E, Lin T S, Dutschman G E, Gullen E A, Cheng Y C & Sartorelli, A C, *Nucleosides, Nucleotides, Nucleic Acids*, 19, **2000**, 603.

11 Deore V, Yewalkar N, Bhatia D, Desai N, Gupte R D, Dadarkar S S, Jadhav M G, Tannu A A, Bhatt P, Nemmani K V, Vishwakarma R A, Sharma S, Roychowdhury A, Dagia N M, Bhonde M R & Kumar S, *Bioorg Med Chem Lett*, 19, **2009**, 2949.

12 Bhonde M R, Gupte R D, Dadarkar S D, Jadhav M G, Tannu A A, Bhatt P, Bhatia D R, Desai N K, Deore V, Yewalkar N, Vishwakarma A, Sharma S, Kumar S & Dagia N M, *Am J Physiol Gastrointest Liver Physiol,* 295, **2008**, G1237.

13 Fiorucci S, Antonelli E, Distrutti E, Del Soldato P, Flower R J, Clark M J, Morelli A, Perretti M & Ignarro L J, *Proc Natl Acad Sci USA,* 99, **2002**, 15770.