# ChemInform Abstract: Synthesis of 5-Substituted-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one Analogues and Their Biological Evaluation as Anticancer Agents: mTOR Inhibitors.

**ARTICLE** *in* CHEMINFORM · NOVEMBER 2014

Impact Factor: 0.74 · DOI: 10.1002/chin.201447182

European Journal of Medicinal Chemistry 80 (2014) 201–208

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Preliminary communication

# Synthesis of 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs and their biological evaluation as anticancer agents: mTOR inhibitors

G. Lakshma Reddy [a,d,1], Santosh Kumar Guru [b,1], M. Srinivas [a,d,1], Anup Singh Pathania [b,d,1], Priya Mahajan [c,d,1], Amit Nargotra [c,d,1], Shashi Bhushan [b,d,**,1], Ram A. Vishwakarma [a,d,*,1], Sanghapal D. Sawant [a,d,*,1]

[a] *Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*
[b] *Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*
[c] *Discovery Informatics, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India*
[d] *Academy of Scientific and Innovative Research, India*

ARTICLE INFO

*Article history:*
Received 8 October 2013
Received in revised form
14 April 2014
Accepted 15 April 2014
Available online 18 April 2014

*Keywords:*
Pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one
$K_2S_2O_8$ catalyst
Microwave irradiation
Cytotoxicity
mTOR inhibitor

ABSTRACT

A microwave assisted strategy for synthesis of series of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-ones has been developed and their biological evaluation as anticancer agents is described. The synthetic protocol involves simple procedure by oxidative coupling of 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide with different aldehydes in presence of $K_2S_2O_8$ offering 5-substituted-1*H*-pyrazolo[4,3-*d*] pyrimidin-7(6*H*)-one compounds in excellent yields. The *in vitro* anticancer activity screening against human cancer cell lines HeLa, CAKI-I, PC-3, MiaPaca-2, A549 gave good results. The in detailed mechanistic correlation studies of compound **3m** revealed that the compound shows anticancer activity through apoptosis mechanism and also inhibits mTOR with nonomolar potency. The design was based on docking with mTOR protein. The concentration dependent cell cycle analysis, western blotting experiment and nuclear cell morphology studies have been described.



## 1. Introduction

The pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-ones and their bioisosteres are heterocyclic compounds with important biological functions including antitumor activity and many other activities [1]. 6-cycloalkyl-pyrazolopyrimidinones are reported for CNS disorders [2], GHS-R1a antagonists and inverse agonists for the treatment of obesity is also reported [3]. Recently, imine-pyrazolopyrimidinones are presented as anticancer derivatives [4]. 1-aryl-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ones were identified as inhibitors of cyclin-dependent kinase (CDK) with $IC_{50}$ in the low micromolar range [5] and several other reports are available for various activities for this scaffold. On the basis of biological data reported in literature, pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one class of compounds are very important in the treatment of impotence, as can be evidenced by the top selling PDE5 inhibitory drug in market i.e. sildenafil [6]. Few more to include in pyrazolopyrimidinone class other than sildenafil as PDE5 inhibitors are acetildenafil (hongdenafil), aildenafil (methisosildenafil), sulfoaildenafil (thioaildenafil), etc.

A pyrazolopyrimidine scaffold based molecule i.e. PP242, PP30 and some others [7] (Fig. 1), are reported as highly potent, selective and ATP-competitive mTORC1/mTORC2 inhibitor ($IC_{50}$ = 8 nM for PP242 and 80 nM for PP30). PP242 has >10 folds selectivity over the other PI3K family kinases ($IC_{50}$ 0.102 μM, 0.408 μM, 1.27 μM, 1.96 μM and 2.2 μM for p110γ, DNA-PK, p110δ, p110α and p110β, respectively). PP242 is also reported to exhibits excellent selectivity over 215 other protein kinases. PP242 differentially inhibits insulin-stimulated phosphorylations of cellular proteins both *in vitro* and *in vivo* in a manner distinctly different from that seen in mTORC2-functional knockout $SIN1^{-/-}$ cells or in cultures treated with Rapamycin, which targets only mTORC1, but not mTORC2.

* Corresponding authors. Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180 001, India and Academy of Scientific and Innovative Research, India.
** Corresponding author. Cancer Pharmacology Division and Academy of Scientific and Innovative Research, India.
*E-mail addresses:* sdsawant@iiim.ac.in, sawant.rrl@gmail.com (S.D. Sawant).
[1] IIIM Communication No. IIIM/1610/2013.

http://dx.doi.org/10.1016/j.ejmech.2014.04.051
0223-5234/

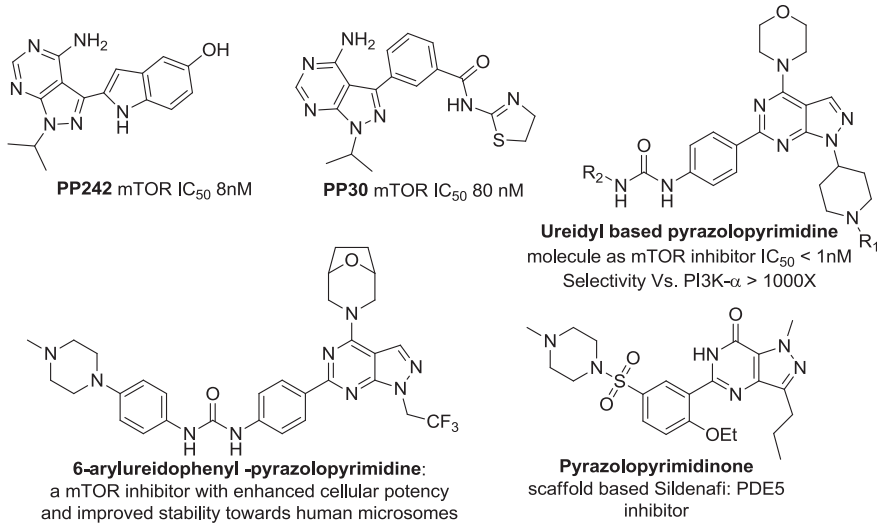



**Fig. 1.** Pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold based potential candidates and drugs.

Moreover, it is reported that PP242 can significantly enhance iPSC generation, which is experimentally confirmed by J.A Menendez et al. that this mTOR inhibitor (PP242) is the most powerful longevity-promoting molecule that enhances iPSC generation [8], and robustly decelerates the cellular senescence imposed by a DDR equivalent to senescence that is caused by pluripotency associated transcription factor expression. However, support for this hypothesis was evidenced by recent findings that well-characterized mTOR inhibitors and autophagy activators (e.g., PP242, rapamycin and resveratrol) notably improve the speed and efficiency of iPSC generation [8].

Synthesis of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one is well exploited and there are various methods already reported for the synthesis of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one class of compounds using traditional as well as microwave mediated approaches [9]. The aim of this study was to synthesize and evaluate the biological potential of pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one analogs for their anticancer potential. In this direction, we initiated our efforts towards its synthesis and biological activity. The detailed chemistry and biological evaluation of these compounds as anticancer agents is explained in the present study.

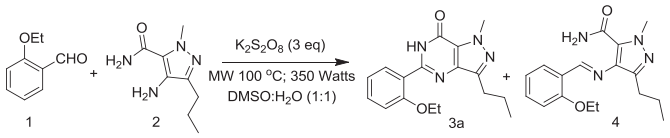
## 2. Result and discussion

### 2.1. Chemistry

#### 2.1.1. Synthesis of 1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one analogs

We intended to synthesize compounds based on 1*H*-pyrazolo [4,3-*d*]pyrimidin-7(6*H*)-one scaffold by using microwave assisted protocol (Scheme 1). In this direction we started the studies for optimization of synthesis of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one **3a**.

The optimization studies were initiated by screening of different oxidizing agents as depicted in Table 1, see Supplementary data using DMSO:Water in 1:1 proportion to see the conversion in desired product. Amongst all oxidants, the best result was observed with $K_2S_2O_8$, in equivalence studies for catalyst, 3 eq. of catalyst has given maximum yields (Table 1, see Supplementary data). Therefore, all reactions were conducted using this condition after optimization of catalyst. However, oxone has also given the product **3a** with minor yields.




**Scheme 1.** Microwave assisted synthesis of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one.

After screening of the catalyst we started study of selectivity for solvent that could affect the formation of 5-(2-ethoxyphenyl)-1-methyl-3-propyl-1*H*-pyrazolo[4,3-d]pyrimidin-7(6*H*)-one **3a**. The solvent screening was carried out to find out the best conversion, the mixture of DMSO:$H_2O$ in 1:1 proportion has given the best results with excellent yields (Table 2, see Supplementary data).

The microwave protocols were optimized for this reaction as mentioned in Table 3, see Supplementary data; the reactions carried under different microwave Watt powers have given varied results. Wherein, entry 3(b) (Table 3, see Supplementary data) was found to be the best condition for maximum conversion.

A series of compounds based on 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold was synthesized using these optimized conditions, wherein, all kind of substrates with diversity around aryl ring were chosen for conversion and in all cases products obtained in good to excellent yields (Table 1).

### 2.2. Biology

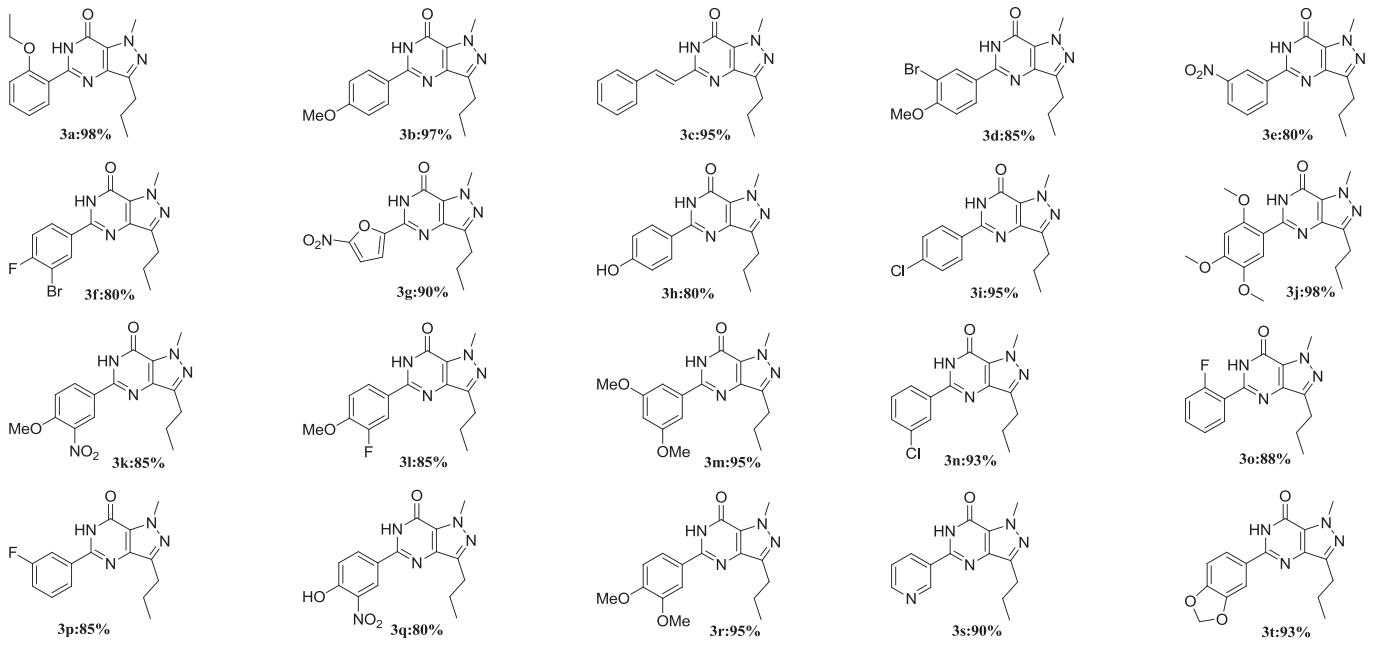
#### 2.2.1. In vitro anticancer activity

These compounds were taken up for *in vitro* cell based cytotoxicity screening against various human cancer cell lines and the results for this screening are mentioned in Table 2.

#### 2.2.2. Compound **3m** inhibits significant cell growth inhibition in different panel of human cancer cell lines

Cytotoxicity assay was performed by using tetrazolium based calorimetric method (MTT assay) against human lung cancer cell lines A549, kidney cancer cell line Caki-1, pancreateic cancer cell line MiaPaCa-2, prostate cancer cell line PC-3 and cervical cancer cell line HeLa. Compound **3m** caused concentration dependent inhibition of cell proliferation in these cell lines in 48 h (Fig. 2). Compound **3m** has $IC_{50}$ value of approximately 14 μM in A549 cell

**Table 1**
Synthesis of compounds based on 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold.[a]




[a] All yields are isolated yields after column purification.

line, 17 μM in Caki-1 cells, 24 μM in MiaPaCa-2 cells, 38 μM in PC-3 cells, in 19 μM HeLa cells after 48 h.

*2.2.3. Compound **3m** altered whole cell and nuclear morphology*

Treatment of human leukemia HL-60 cells with compound **3m** at 10, 30, 50, and 70 μM concentrations caused cell wall deformation, shrinkage of cell size, nuclear condensation and formation of scattered apoptotic bodies as shown by arrows in Fig. 3A and B, while the nuclei of untreated cells are healthy and round in shape. The number of apoptotic bodies increased with increased concentration of compound **3m** in A549 cells. This revealed that compound **3m** induce cell death through induction of apoptosis in A549 cells.

*2.2.4. Compound **3m** increases sub-G0 DNA fraction of cell cycle phase in concentration dependent manner*

A549 cells treated with compound **3m** exhibited concentration dependent increase in hypo diploid sub-G0 DNA fraction (<2nDNA) indicative of apoptotic population as analyzed by modfit software (Fig. 4). Control cells showed 1% sub-G0 DNA fraction while A549 cells treated with compound **3m** for 24 h exhibited continuous increase in sub-G0 fraction which may comprise both apoptotic and debris fraction implying together the extent of cell death.

The damage was more apparent with higher concentration of compound **3m** over the period of study. The sub-G0 fraction increased from ~1% of control to ~28% after 24 h of treatment of compound **3m**. There was hardly any significant effect after 24 h of treatment on G1, S and G2/M, which indicated that decrease in DNA fluorescence is not cell cycle selective.

*2.2.5. Compound **3m** inhibits mTOR-p70S6Kinase signaling and induces apoptosis in A549 cells*

Compound **3m** at indicated concentrations inhibits phosphorylated (Serine 2448) and non phosphorylated form of mammalian target of rapamycin (mTOR), its two other subunits rictor and raptor and their two main substrate p70S6K and 4EBP1in A549 cells after 24 h (Fig. 5). Compound **3m** inhibits active phosphorylated form of eIF4E (Serine 209) and p-p70S6Kinase (T389) at all concentrations (Fig. 5).

Compound **3m** also inhibits mTOR kinase in a cell free enzyme assay (K-LISA™ mTOR kit) and $IC_{50}$ value was found to be 203 nM (Fig. 6). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase present downstream of phosphatidylinositol 3-kinase/Akt signaling pathway and it involves in regulating basic cellular functions including cellular growth and proliferation (Wullschleger et al, 2006) [10]. Aberrant activation of the PI3K/Akt/mTOR pathway is found in many types of cancer including small cell lung cancer (Marinov et al. 2009) [11]. AKT can activate mTOR by phosphorylating at serine 2448. Activated mTOR have two well characterized downstream targets, ribosomal protein S6 kinase 1 (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), both of which are involve in the regulation of protein synthesis (Hay and Sonenberg, 2004) [12]. Simultaneously, compound **3m** also alter the expression of key apoptotic proteins like caspas-3 activation and PARP-1 (Poly-ADP-ribose-polymerase) cleavage (Fig. 5).

*2.3. Docking studies*

Based on the docking studies, the molecule **3m** shows one H-bond interaction with Val2240 at a distance of 2.203 Å (Fig. 7). Further, this ligand also shows more stability within the binding pocket due to the hydrophobic cleft formed by Leu 2185, Trp 2239, Met 2345 and Ile 2356 around the ligand. The best dock score and the calculated binding energy of this complex were −6.8 and −80.95 respectively. Based on this evidence and the preliminary biological activity, the in detailed biological activity for **3m** was conducted.

## 3. Conclusion

In present study, 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one scaffold have been constructed using microwave assisted protocol and the biological evaluation results as anticancer agents are promising.

**Table 2**
*In vitro* cell based screening of 1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one compounds.

| Code | Cell lines | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HeLa (Cervix cancer) | | | CAKI-I (Renal cancer) | | | PC-3 (Prostate Cancer) | | | MiaPaca-2 (Pancreatic Cancer) | | | A549 (Lung cancer) | | |
| | Conc, (μM) | %GI | $IC_{50}$ (μM) | Conc, (μM) | %GI | $IC_{50}$ (μM) | Conc, (μM) | %GI | $IC_{50}$ (μM) | Conc, (μM) | %GI | $IC_{50}$ (μM) | Conc, (μM) | %GI | $IC_{50}$ (μM) |
| **3a** | 10 | 36 | >100 | 10 | 12 | >100 | 10 | 7 | >100 | 10 | 7 | >100 | 10 | 21 | >100 |
| | 30 | 37 | | 30 | 18 | | 30 | 10 | | 30 | 25 | | 30 | 30 | |
| | 100 | 40 | | 100 | 19 | | 100 | 14 | | 100 | 38 | | 100 | 37 | |
| **3b** | 10 | 31 | >100 | 10 | 8 | >100 | 10 | 5 | >100 | 10 | 18 | >100 | 10 | 0 | >100 |
| | 30 | 34 | | 30 | 25 | | 30 | 6 | | 30 | 20 | | 30 | 15 | |
| | 100 | 42 | | 100 | 52 | | 100 | 14 | | 100 | 32 | | 100 | 36 | |
| **3c** | 10 | 22 | 92 | 10 | 26 | >100 | 10 | 33 | 32 | 10 | 11 | >100 | 10 | 30 | 38 |
| | 30 | 43 | | 30 | 33 | | 30 | 48 | | 30 | 20 | | 30 | 45 | |
| | 100 | 59 | | 100 | 46 | | 100 | 98 | | 100 | 44 | | 100 | 80 | |
| **3d** | 10 | 0 | >100 | 10 | 24 | 57 | 10 | 14 | >100 | 10 | 11 | >100 | 10 | 0 | 90 |
| | 30 | 21 | | 30 | 38 | | 30 | 16 | | 30 | 15 | | 30 | 17 | |
| | 100 | 36 | | 100 | 74 | | 100 | 19 | | 100 | 22 | | 100 | 58 | |
| **3e** | 10 | 21 | >100 | 10 | 31 | 30 | 10 | 22 | >100 | 10 | 14 | >100 | 10 | 12 | >100 |
| | 30 | 22 | | 30 | 50 | | 30 | 27 | | 30 | 17 | | 30 | 25 | |
| | 100 | 39 | | 100 | 73 | | 100 | 47 | | 100 | 45 | | 100 | 40 | |
| **3f** | 10 | 34 | 24 | 10 | 30 | 18 | 10 | 32 | 27 | 10 | 34 | 38 | 10 | 20 | 28 |
| | 30 | 69 | | 30 | 68 | | 30 | 62 | | 30 | 45 | | 30 | 57 | |
| | 100 | 81 | | 100 | 78 | | 100 | 74 | | 100 | 98 | | 100 | 98 | |
| **3g** | 10 | 12 | >100 | 10 | 27 | >100 | 10 | 10 | >100 | 10 | 6 | >100 | 10 | 0 | >100 |
| | 30 | 17 | | 30 | 32 | | 30 | 23 | | 30 | 16 | | 30 | 8 | |
| | 100 | 48 | | 100 | 41 | | 100 | 39 | | 100 | 32 | | 100 | 43 | |
| **3h** | 10 | 29 | 34 | 10 | 22 | >100 | 10 | 19 | >100 | 10 | 23 | >100 | 10 | 11 | 90 |
| | 30 | 44 | | 30 | 25 | | 30 | 24 | | 30 | 29 | | 30 | 28 | |
| | 100 | 78 | | 100 | 35 | | 100 | 32 | | 100 | 43 | | 100 | 59 | |
| **3i** | 10 | 12 | >100 | 10 | 34 | 28 | 10 | 22 | >100 | 10 | 11 | >100 | 10 | 19 | 87 |
| | 30 | 19 | | 30 | 56 | | 30 | 31 | | 30 | 22 | | 30 | 39 | |
| | 100 | 14 | | 100 | 66 | | 100 | 47 | | 100 | 43 | | 100 | 58 | |
| **3j** | 10 | 30 | >100 | 10 | 16 | >100 | 10 | 5 | >100 | 10 | 12 | >100 | 10 | 0 | >100 |
| | 30 | 33 | | 30 | 16 | | 30 | 7 | | 30 | 21 | | 30 | 7 | |
| | 100 | 35 | | 100 | 17 | | 100 | 10 | | 100 | 32 | | 7 | 21 | |
| **3k** | 10 | 32 | >100 | 10 | 25 | >100 | 10 | 0 | >100 | 10 | 3 | >100 | 10 | 11 | >100 |
| | 30 | 35 | | 30 | 31 | | 30 | 6 | | 30 | 24 | | 30 | 31 | |
| | 100 | 43 | | 100 | 35 | | 100 | 13 | | 100 | 44 | | 100 | 47 | |
| **3l** | 10 | 49 | 13 | 10 | 35 | 20 | 10 | 33 | 31 | 10 | 38 | 35 | 10 | 26 | 28 |
| | 30 | 66 | | 30 | 65 | | 30 | 78 | | 30 | 57 | | 30 | 57 | |
| | 100 | 87 | | 100 | 87 | | 100 | 97 | | 100 | 87 | | 100 | 90 | |
| **3m** | 10 | 36 | 19 | 10 | 39 | 17 | 10 | 12 | 37 | 10 | 31 | 24 | 10 | 45 | 14 |
| | 30 | 63 | | 30 | 67 | | 30 | 44 | | 30 | 57 | | 30 | 68 | |
| | 100 | 85 | | 100 | 81 | | 100 | 84 | | 100 | 90 | | 100 | 98 | |
| **3n** | 10 | 22 | >100 | 10 | 35 | 29 | 10 | 16 | >100 | 10 | 12 | >100 | 10 | 0 | 87 |
| | 30 | 31 | | 30 | 51 | | 30 | 17 | | 30 | 16 | | 30 | 27 | |
| | 100 | 49 | | 100 | 76 | | 100 | 25 | | 100 | 23 | | 100 | 65 | |
| **3o** | 10 | 11 | 35 | 10 | 24 | >100 | 10 | 17 | >100 | 10 | 11 | >100 | 10 | 0 | >100 |
| | 30 | 43 | | 30 | 26 | | 30 | 23 | | 30 | 20 | | 30 | 8 | |
| | 100 | 65 | | 100 | 49 | | 100 | 43 | | 100 | 43 | | 100 | 23 | |
| **3p** | 10 | 1 | >100 | 10 | 34 | 96 | 10 | 23 | 95 | 10 | 15 | >100 | 10 | 22 | 86 |
| | 30 | 18 | | 30 | 47 | | 30 | 44 | | 30 | 21 | | 30 | 37 | |
| | 100 | 33 | | 100 | 54 | | 100 | 56 | | 100 | 44 | | 100 | 68 | |
| **3q** | 10 | 0 | >100 | 10 | 23 | >100 | 10 | 21 | >100 | 10 | 17 | >100 | 10 | 0 | >100 |
| | 30 | 7 | | 30 | 35 | | 30 | 25 | | 30 | 22 | | 30 | 23 | |
| | 100 | 12 | | 100 | 44 | | 100 | 29 | | 100 | 48 | | 100 | 46 | |
| **3r** | 10 | 13 | >100 | 10 | 21 | >100 | 10 | 11 | >100 | 10 | 21 | 96 | 10 | 9 | >100 |
| | 30 | 21 | | 30 | 28 | | 30 | 14 | | 30 | 32 | | 30 | 14 | |
| | 100 | 44 | | 100 | 36 | | 100 | 27 | | 100 | 55 | | 100 | 48 | |
| **3s** | 10 | 0 | >100 | 10 | 21 | >100 | 10 | 1 | >100 | 10 | 21 | >100 | 10 | 0 | >100 |
| | 30 | 23 | | 30 | 22 | | 30 | 3 | | 30 | 32 | | 30 | 10 | |
| | 100 | 38 | | 100 | 31 | | 100 | 14 | | 100 | 45 | | 100 | 29 | |
| **3t** | 10 | 32 | 88 | 10 | 46 | 27 | 10 | 23 | 98 | 10 | 11 | >100 | 10 | 10 | 54 |
| | 30 | 44 | | 30 | 55 | | 30 | 31 | | 30 | 13 | | 30 | 37 | |
| | 100 | 61 | | 100 | 61 | | 100 | 51 | | 100 | 34 | | 100 | 78 | |

The synthetic protocol can be applied for preparation of analogs of active compound i.e. **3m** which involves a simple procedure to obtain 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one compounds in excellent yields. Moreover, **3m** also acts as mTOR inhibitor and the concentration dependent cell cycle analysis, western blotting experiment and nuclear cell morphology studies suggests that the mechanism through which **3m** acts as anticancer agent is apoptosis. 5-substituted-1*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one is an excellent scaffold and can be exploited for further study in the field of cancer.

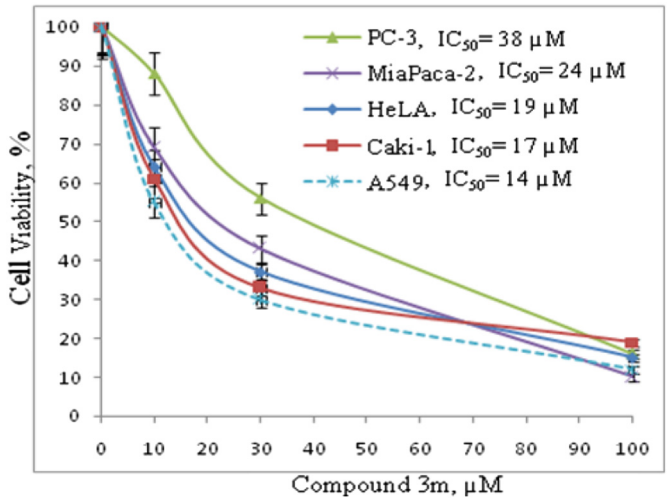



**Fig. 2.** Compound **3m** inhibits cell proliferation in different panel of human cancer cell lines. Cells were grown in 96-well culture plate and when 60–70% confluent was treated with 1, 10, 30, 50, 70 and 100 µM concentration for 48 h. Cells were incubated with MTT and OD measured as described in Materials and Methods. Data are Mean ± SD ($n = 8$ wells), and representative of three similar experiments.

## 4. Experimental protocols

### 4.1. Chemistry

All reactions were performed in a sealed tube under mentioned microwave irradiation conditions. Analytical thin layer chromatography was performed using TLC pre-coated silica gel 60 $F_{254}$ (20 × 20 cm). TLC plates were visualized by exposing UV light or by iodine vapors or immersion in anisaldehyde charring reagent or in 2,4-dinitrophenyl hydrazine or ninhydrin followed by heating on hot plate. Organic solvent were concentrated by rotary evaporation and dried using high vacuum suction pump. Compounds were purified by column chromatography using normal phase silica-gel (100–200 mesh size). $^1$H NMR spectra were recorded with 400 and 500 MHz NMR instruments. Chemical data for protons are reported in parts per million (ppm, scale) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$: $\delta$ 7.26, DMSO-$d_6$ $\delta$ 2.50 or other solvents as mentioned).

#### 4.1.1. Synthesis of 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one

In a typical procedure, a solution of aromatic aldehyde i.e. 2,4,5-trimethoxybenzaldehyde (0.196 g, 1 eq.) and 4-amino-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (0.191 g, 1.05 eq) in DMSO:$H_2O$ (1:1) add $K_2S_2O_8$ (0.810 g, 3 eq.) was taken in a sealed reaction tube and the reaction mixture was irradiated under microwave conditions for 3 min with a power of 350 Watts at 100 °C. After completion, the reaction mass was diluted with EtOAc (20 mL) and added water (30 mL). Separated the organic layer and extracted with EtOAc (2 × 10 mL). The combined organic layer was then washed with brine solution, concentrated under vacuum and purified on silica-gel (100–200 mesh) column chromatography, affording white solid 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1*H*-pyrazolo[4,3-d] pyrimidin-7(6*H*)-one i.e. compound **3j** in (0.351 g) 98% yield.

#### 4.1.2. Analytical data for a representative compound 3j i.e. 1-methyl-3-propyl-5-(2,4,5-trimethoxyphenyl)-1H-pyrazolo[4,3-d] pyrimidin-7(6H)-one

$^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 10.99 (s, 1H), 8.03 (s, 1H), 6.59 (s, 1H), 4.27 (s, 3H), 4.05 (s, 3H), 3.97 (s, 6H), 2.93 (t, $J = 7.5$ Hz, 2H), 1.93–1.83 (m, 2H), 1.04 (t, $J = 7.4$ Hz, 3H). $^{13}$C NMR (126 MHz, DMSO) $\delta$ 153.61, 152.09, 151.83, 149.06, 144.66, 142.60, 137.96, 123.88, 113.50, 112.81, 97.89, 56.57, 56.15, 55.89, 37.77, 27.07, 21.59, 13.81. HRMS (ESI) calcd for $C_{18}H_{23}N_4O_4$ [M-H$^+$] 359.17193, found 359.17139.

### 4.2. Molecular modeling studies

The computational studies on mTOR were carried out using the Schrodinger suite 2012 molecular modeling software. Crystal structure 4JT5 was taken for docking studies. The coordinates of mTOR protein in complex with the co-crystalized ligand (2-[4-amino-1-(propan-2-yl)-1H-pyrazolo[3,4-d]pyrimidin-3-yl]-1H-indol-5-ol) that was obtained from protein data bank [13]. The protein was prepared for docking using the protein preparation wizard. Hydrogens were added to the protein and water residues were removed beyond 5A° from the heteroatom. Further, only those water residues, having interactions with the protein and heteroatom were kept, and the rest were deleted. Then the ligand was extracted and protein was refined by assigning H-bonds and minimization at OPLS 2005 force field. A grid was generated at active site, identified on the bases of already co-crystalised ligand to the receptor using receptor grid generation module. The docking protocol was standardized using the co-crytalized ligand conformation.

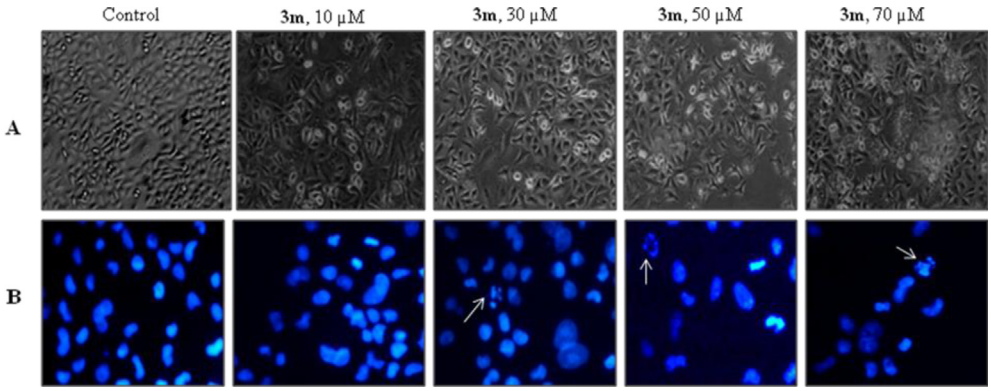
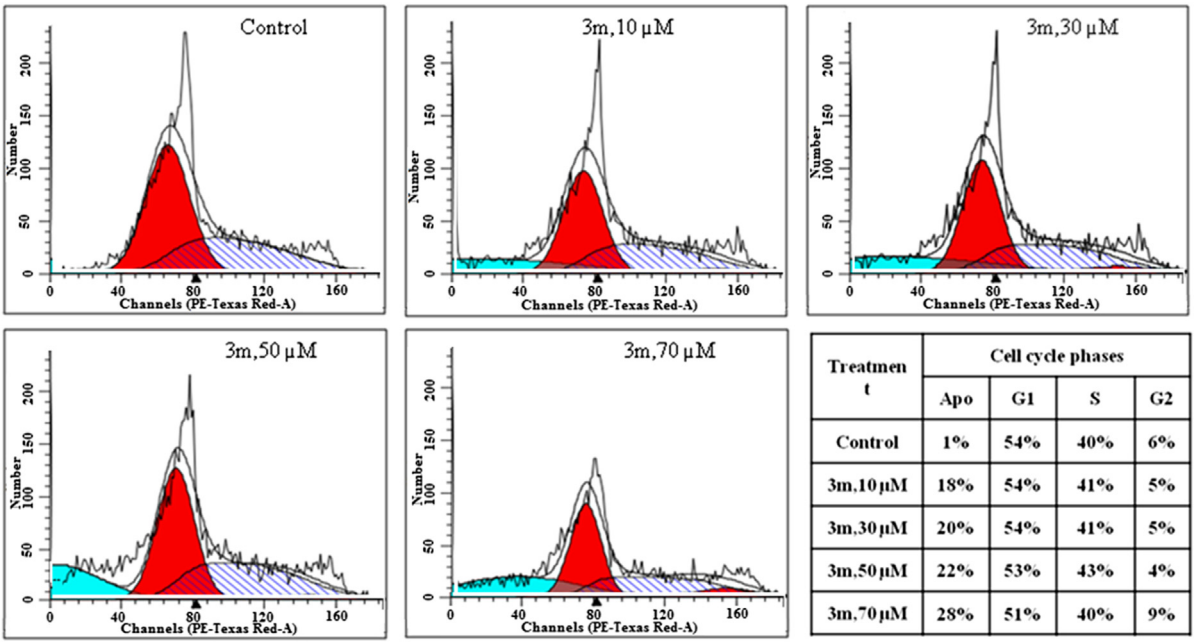



**Fig. 3.** Effect of compound **3m** on the cell wall and nuclear morphology of A549 cells. A) A549 cells were treated with 10, 30, 50 and 70 µM concentrations of compound **3m** for 24 h and visualized under phase contrast inverted microscope (Olympus 1 × 70, 30×). B) Subsequently cells were stained with Hoechst 33258 and visualized for nuclear morphology and apoptotic bodies' formation. Compound **3m** induced the formation of apoptotic bodies as indicated by arrows in concentration dependent manner.Data are representative of one of three similar experiments.




| Treatment | Cell cycle phases | | | |
|---|---|---|---|---|
| | Apo | G1 | S | G2 |
| Control | 1% | 54% | 40% | 6% |
| 3m,10 µM | 18% | 54% | 41% | 5% |
| 3m,30 µM | 20% | 54% | 41% | 5% |
| 3m,50 µM | 22% | 53% | 43% | 4% |
| 3m,70 µM | 28% | 51% | 40% | 9% |

**Fig. 4.** Cell cycle analyses of lung cancer A549 cells through PI staining. Cells were seeded in six well plate and after 60–70% confluence, treated with compounds **3m** for 24 h time period at indicated concentrations. After treatment cells were stained with PI (10 µg/mL) to determine DNA Fluorescence and cell cycle phase distribution as described in Materials and methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. Fraction of cells from apoptotic, G1, S and G2 phases analyzed from FL2-A vs. cell counts is shown in %. Data are representative of one of three similar experiments.

### 4.3. Biological studies

a) Reagents/chemicals: RPMI-1640, streptomycin, kanamycin, penicillin, L-glutamine, phenylmethanesulfonyl fluoride (PMSF), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Propidium iodide (PI), Fetal bovine serum, pyruvic acid were purchased from Sigma–Aldrich (Bangalore, India). Anti-human antibodies to PARP-1, caspase-3 and β-actin were purchased from SantaCruz Biotechnology (SantaCruz, CA). Anti-human antibodies to mTOR and its phosphorylated form (S2448), p-eIF4E (S209), Raptor, Rictor and p-p70S6Kinase (T389) were purchased from Cell signaling technology (Danvers, MA). K-LISA™ mTOR kit (#CBA055) was purchased from Calbiochem, USA. Electrophoresis reagents, Protein estimation kit and protein marker were from Bio-Rad Laboratories (Hercules, CA).

b) Cell culture and treatment: Human lung cancer cell lines A549, kidney cancer cell line Caki-1, pancreatic cancer cell line MIA-PaCa-2, prostate cancer cell line PC3 and cervical cancer cell line HeLa were purchased from ECACC. Cells were grown in RPMI/DMEM/MEM growth medium containing 10% FCS, 100U

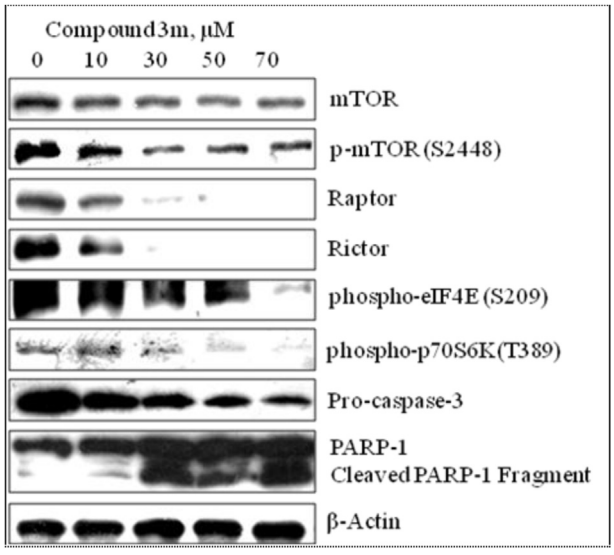



**Fig. 5.** Inhibition of mTOR signaling cascade in lung cancer A549 cells by compounds **3m**. A549 cells treated with compound **3m** at 10, 30, 50 and 70 µM concentrations for 24 h. Total cell lysates were prepared and protein samples (40–80 µg) were loaded on SDS-PAGE gel for western blot analysis, β-actin was used as internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of mTOR, its activated phosphorylated form at serine 2448, Raptor, Rictor along with downstream substrates of mTOR, eIF4E and p70S6kinase. Compound **3m** also induces apoptotic caspase-3 level and PARP cleavage. Immunoblots were representative of one of three similar experiments.

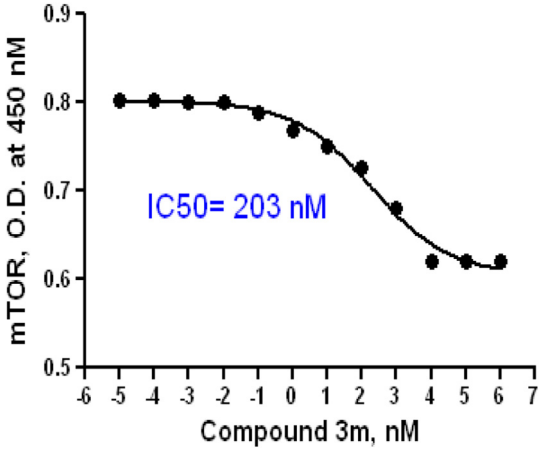



**Fig. 6.** Compound **3m** inhibits mTOR kinase in a cell free enzyme essay (K-LISA™ mTOR kit) showing $IC_{50}$ value as 203 nM.

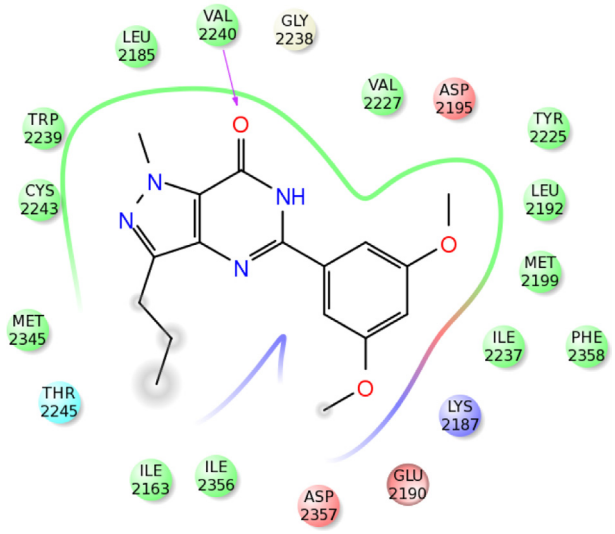



**Fig. 7.** A cartoon showing the interactions of compound **3m** with active site of mTOR using PDB4JT5 (The carbonyl group of molecule showing H-bonding with Val2240 (2.203 Å)).

penicillin and 100 mg/ml streptomycin. Cells were grown in $CO_2$ incubator (Thermocon Electron Corporation, Houston, TX) at 37 °C temperature, 95% humidity and 5% $CO_2$ gas environment. Cells treated with compounds were dissolved in DMSO while the untreated control cultures received only the vehicle (DMSO < 0.2%).

#### 4.3.1. Cell proliferation assay

Cells were seeded in 96 well flat bottom plates. Next day when they attained 60–70% confluency, they were treated with compounds at different concentrations for 24 h and 48 h. MTT dye (2.5 mg/ml in PBS) was added 4 h priors to experiment termination. The supernatant was discarded and the MTT formazan crystals were dissolved in 150 μL of DMSO. The OD measured at 570 nm with reference wavelength of 620 nm (Bhushan et al., 2006) [14]. The most active compound **3m** was taken up for in detailed biological evaluation.

#### 4.3.2. Hoechst staining

Human lung cancer A549 cells were treated with compound **3m** for 24 h at 10, 30, 50 and 70 μM concentrations. After treatment cells were collected, washed twice with PBS and fixed in 400 μl of fixing solution composed of cold acetic acid: methanol (1 + 3, v/v) overnight at 4 °C. Cells were washed with fixing solution and dispensed in 50 μl of fixing solution. Spread cells on a clean cold slide and dried overnight at room temperature. Cells were stained with Hoechst 33258 (5 μg/mL in 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween 20) for 20–30 min at room temperature. After that slides were washed with distilled water followed by in PBS. While wet, pour 40 μl of mounting fluid (PBS: glycerol, 1/1) over the slide and covered with glass cover slip and sealed with nail polished. Cells were observed under microscope for any nuclear morphological changes occur in apoptosis (Saxena et al., 2010) [15].

#### 4.3.3. Cell cycle analysis

Human lung cancer cell line A549 was seeded in 60 mm [2] dishes and after attaining 60–70% confluency, treated with compound **3m** for 24 h at 10, 30, 50 and 70 μM concentrations. Cells were washed with PBS twice after 24 h and fixed overnight in 70% alcohol. Next day cells were washed twice with PBS and thereafter they are subjected to RNase digestion (200 μg/mL) at 37 °C for 1.30 h and then incubated with PI (10 μg/mL) for 30 min. Cells were analyzed immediately on flow cytometer (BD Biosciences, San Jose, CA). The data were collected in list mode on 10,000 events and illustrated in a histogram, where the number of cells (counts) is plotted against the relative fluorescence intensity of PI (FL-2; λem: 585 nm; red fluorescence). Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc., Topsham, ME) for the proportions of cells in $G_0$–$G_1$, S-phase, and $G_2$-M phases of the cell cycle (Chanda et al., 2012) [16].

#### 4.3.4. Cell lysates preparation for western blots analysis

Human lung cancer cell line A549 was treated with compound **3m** for 24 h at 10, 30, 50 and 70 μM concentrations. Cells were collected by centrifugation at 400× g at 4 °C, washed with PBS twice and processed for preparation of whole cell lysates. Cells were lysed with cold Cell lysis buffer (RIPA, Sigma with 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM PMSF and 1% protease inhibitor cocktail) for 40 min. Cells were centrifuged at 12,000× g for 15 min at 4 °C and the supernatant was collected as whole cell lysates for western blot analysis (Bhushan et al., 2007) [17].

#### 4.3.5. Western blot analysis

Protein was measured using Bio-Rad protein assay kit and protein lysates (30–70 μg) were subjected to SDS-PAGE analysis. They are transferred to PVDF membrane for 2 h at 4 °C at 100 V using Bio-Rad electrode assembly. The membrane was blocked by incubation with 3% BSA or 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature to prevent any non-specific binding. After an hour the blots were incubated with respective primary antibodies for 3–4 h at room temperature. They are washed three times with TBST and were incubated with horseradish peroxidase conjugated secondary antibodies for another 1 h. At the end, membranes were washed three times with TBST buffer with 15 min interval and signals detected using ECL plus chemiluminescence's kit on X-ray film (Bhushan et al., 2007) [17].

#### 4.3.6. mTOR kinase essay

mTOR inhibition of compound **3m** was found out by using K-LISA™ mTOR kit from Calbiochem (#CBA055). It is an ELISA-based assay that utilizes a p70S6K-GST fusion protein as a specific mTOR substrate. The assay was carried out according to the manufacturer's protocol. Briefly, 100 μl of recombinant p70S6K-GST fusion protein was pre-incubated at room temperature in the glutathione coated 96-well plate for 1 h after that a mixture of 49 μl of ice-chilled mTOR kinase and 1 μl of test compounds or DMSO was added. The reaction was initiated by the addition of 50 μl of mTOR kinase assay buffer containing 100 μM ATP and 1 μM DTT. The plate was treated first with 100 μl of anti-p70S6K-T389 for 1 h and then with 100 μl of HRP-conjugated antibody for 1 h to detect the T389-phosphorylated p70S6K. Absorbance was measured at 450 nm and 595 nm using microplate spectrophotometer. The $IC_{50}$ values were calculated by analysis non linear regression with variable slope by using GraphPad Prism-5 software.

## Acknowledgment

GLR thanks CSIR-New Delhi for the award of fellowship. The support of funding for carrying present research work from CSIR 12th FYP project BSC0108 is gratefully acknowledged.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.04.051.

## References


[1] (a) M. Chauhan, R. Kumar, Bioorganic & Medicinal Chemistry 21 (2013) 5657–5668;
(b) Y.-L. Li, J.M. Fevig, J. Cacciola, J. Buriak, K.A. Rossi Jr., J. Jona, R.M. Knabb, J.M. Luettgen, P.C. Wong, S.A. Bai, R.R. Wexler, P.Y.S. Lam, Bioorganic & Medicinal Chemistry Letters 16 (2006) 5176–5182;
(c) V.N. Devegowda, J.H. Kim, K.-C. Han, E.G. Yang, H. Choo, A.N. Pae, G. Nama, K.I. Choi, Bioorganic & Medicinal Chemistry Letters 20 (2010) 1630–1633;
(d) H.W. Hamilton, D.F. Ortwine, D.F. Worth, J.A. Bristol, Journal of Medicinal Chemistry 30 (1987) 91–96.

[2] H. Niklas, G. Riccardo, 6-Cycloalkyl-pyrazolopyrimidinones for the treatment of CNS disorders, PCT/EP2012/052379.

[3] W. McCoull, P. Barton, A. Broo, A.J.H. Brown, D.S. Clarke, G. Coope, R.D.M. Davies, A.G. Dossetter, E.E. Kelly, L. Knerr, P. MacFaul, J.L. Holmes, N. Martin, J.E. Moore, D. Morgan, C. Newton, K. Österlund, G.R. Robb, E. Rosevere, N. Selmi, S. Stokes, T.S. Svensson, V.B.K. Ullah, E.J. Williams, MedChemComm 4 (2013) 456–462.

[4] A.T. Baviskar, U.C. Banerjee, M. Gupta, R. Singh, S. Kumar, M.K. Gupta, S. Kumar, S.K. Raut, M. Khullar, S. Singh, R. Kumar, Bioorganic & Medicinal Chemistry 21 (2013) 5782–5793.

[5] (a) J.A. Markwalder, M.R. Arnone, P.A. Benfield, M. Boisclair, C.R. Burton, C.-H. Chang, S.S. Cox, P.M. Czerniak, C.L. Dean, D. Doleniak, R. Grafstrom, B.A. Harrison, R.F. Kaltenbach III, D.A. Nugiel, K.A. Rossi, S.R. Sherk, L.M. Sisk, P. Stouten, G.L. Trainor, P. Worland, S.P. Seitz, Journal of Medicinal Chemistry 47 (24) (2004) 5894–5911;
(b) D. Geffken, R. Soliman, F.S.G. Soliman, M.M. Abdel-Khalek, D.A.E. Issa, Medicinal Chemistry Research 20 (2011) 408–420.

[6] E. Peter, T.N. Kenneth, Pyrazolopyrimidinones for the treatment of impotence, WO94/28902.

[7] (a) M.E. Feldman, B. Apsel, A. Uotila, R. Loewith, Z.A. Knight, D. Ruggero, K.M. Shokat, Plos Biology 7 (2) (2009) e38;
(b) M.R. Janes, J.J. Limon, L. So, J. Chen, R.J. Lim, M.A. Chavez, C. Vu, M.B. Lilly, S. Mallya, S.T. Ong, M. Konopleva, M.B. Martin, P. Ren, Y. Liu, C. Rommel, D.A. Fruman, Nature Medicine 16 (2) (2010) 205–213;
(c) J.A. Menendez, L. Vellon, C. Oliveras-Ferraros, S. Cufí, A. Vazquez-Martin, Cell Cycle 10 (21) (2011) 3658–3677;
(d) D.J. Richard, J.C. Verheijen, K. Curran, J. Kaplan, L. Toral-Barza, I. Hollander, J. Lucas, K. Yu, A. Zask, Bioorganic & Medicinal Chemistry Letters 19 (24) (2009) 6830–6835;
(e) A. Zask, J.C. Verheijen, K. Curran, J. Kaplan, D.J. Richard, P. Nowak, D.J. Malwitz, N. Brooijmans, J. Bard, K. Svenson, J. Lucas, L. Toral-Barza, W.-G. Zhang, I. Hollander, J.J. Gibbons, R.T. Abraham, S. Ayral-Kaloustian, T.S. Mansour, K. Yu, Journal of Medicinal Chemistry 52 (16) (2009) 5013–5016;
(f) A. Zask, J. Kaplan, K. Curran, J.C. Verheijen, D.J. Richard, N. Brooijmans, E. Bennett, J. Lucas, L. Toral-Barza, I. Hollander, J.J. Gibbons, R.T. Abraham, S. Ayral-Kaloustian, T.S. Mansour, K. Yu, Molecular Cancer Therapeutics 8 (12) (2009) B145.

[8] T. Chen, L. Shen, J. Yu, H. Wan, A. Guo, J. Chen, Aging Cell 10 (2011) 908–911.

[9] (a) N. Mulakayala, B. Kandagatla, Ismail, R.K. Rapolu, P. Rao, C. Mulakayala, C.S. Kumar, J. Iqbal, S. Oruganti, Bioorganic & Medicinal Chemistry Letters 22 (2012) 5063–5066;
(b) F. Miklos, I. Kanizsai, P. Sohar, G. Stajer, Journal of Molecular Structure 610 (1) (2002) 41–46;
(c) N.R. Reddy, G.M. Reddy, B.S. Reddy, P.P. Reddy, Journal of Heterocyclic Chemistry 42 (2005) 751–754;
(d) K.M. Khan, G.M. Maharvi, M.I. Choudhary, A. Rahman, S. Perveen, Journal of Heterocyclic Chemistry 42 (2005) 1085–1092;
(e) R.J. Abdel-Jalil, M. Khanfar, K. Abu-Safieh, S. Al-Gharabli, M. El-Abadelah, W. Voelter, Monatshefte fuer Chemie 136 (2005) 619–624;
(f) R.J. Abdel-Jalil, M. Khanfar, S. Al-Gharabli, M.M. El-Abadelah, K. Eichele, M.U. Anwar, W. Voelter, Heterocycles 65 (8) (2005) 1821–1827;
(g) H.A. Qi, W. Eric, G. Carla, X. Walter, C. Glen, D. Jean-Mac, Preparation of Pyrimidines as HCV Entry Inhibitors and Antiviral Agents, PCT Int. Appl., 2010, 118367

[10] S. Wullschleger, R. Loewith, M.N. Hall, Cell 124 (2006) 471–484.

[11] M. Marinov, A. Ziogas, O.E. Pardo, L.T. Tan, T. Dhillon, F.A. Mauri, H.A. Lane, N.R. Lemoine, U.Z. Wittke, M.J. Seckl, A. Arcaro, Clinical Cancer Research: An Official Journal of the American Association for Cancer Research 15 (2009) 1277–1287.

[12] N. Hay, N. Sonenberg, Genes & Development 18 (2004) 1926–1945.

[13] H. Yang, D.G. Rudge, J.D. Koos, B. Vaidialingam, H.J. Yang, N.P. Pavletich, Nature 497 (2013) 217–223.

[14] S. Bhushan, J. Singh, J.M. Rao, A.K. Saxena, G.N. Qazi, Nitric Oxide 14 (2006) 72–88.

[15] A. Saxena, A.K. Saxena, J. Singh, S. Bhushan, Chemico-Biological Interactions 188 (2010) 580–590.

[16] D. Chanda, S. Bhushan, S.K. Guru, K. Shanker, Z.A. Wani, B.A. Rah, S. Luqman, D.M. Mondhe, A. Pal, A.S. Negi, European Journal of Pharmaceutical Sciences 47 (2012) 988–995.

[17] S. Bhushan, A. Kumar, F. Malik, S.S. Andotra, V.K. Sethi, I.P. Kaur, S.C. Taneja, G.N. Qazi, J. Singh, Apoptosis 12 (2007) 1911–1926.