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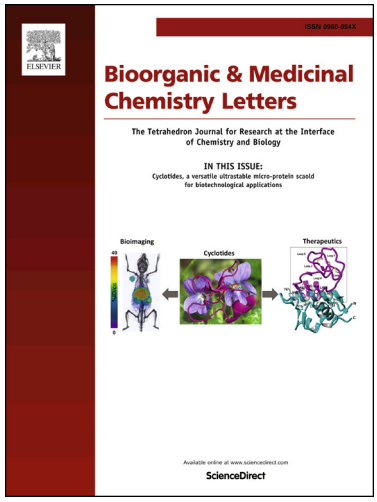

Novel compounds with potent CDK9 inhibitory activity for the treatment of myeloma

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PII: S0960-894X(18)30002-7
DOI: https://doi.org/10.1016/j.bmcl.2018.01.002
Reference: BMCL 25528

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 28 July 2017
Revised Date: 14 December 2017
Accepted Date: 1 January 2018

Please cite this article as: Czudor, Z., Balogh, M., Bánhegyi, P., Boros, S., Breza, N., Dobos, J., Fábián, M., Horváth, Z., Illyés, E., Szántai-Kis, C., Markó, P., Sipos, A., Szokol, B., Őrfi, L., Novel compounds with potent CDK9 inhibitory activity for the treatment of myeloma, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.01.002

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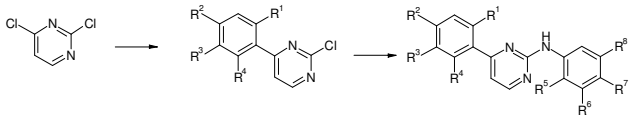

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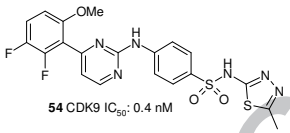
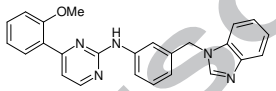


54 CDK9 IC_{50}: 0.4 nM



66 RPMI-8226 IC_{50}: 1.34 uM

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Novel compounds with potent CDK9 inhibitory activity for the treatment of myeloma

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
Cyclin Dependent Kinase inhibitor
2,4-disubstituted pyrimidine
Multiple myeloma
Polo-like Kinase

ABSTRACT

Cyclin-dependent kinases (CDKs) and Polo-like kinases (PLKs) play key role in the regulation of the cell cycle. The aim of our study was originally the further development of our recently discovered polo-like kinase 1 (PLK1) inhibitors. A series of new 2,4-disubstituted pyrimidine derivatives were synthesized around the original hit, but their PLK1 inhibitory activity was very poor. However the novel compounds showed nanomolar CDK9 inhibitory activity and very good antiproliferative effect on multiple myeloma cell lines (RPMI-8226).

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The improper operation of the cell cycle may result in the appearance of cancer cells; therefore, overexpression or amplification of cell cycle regulator kinases (CDKs, PLKs, CHKs, Aurora kinases) play pivotal role in the formation and progression of different tumors. Cyclin-dependent kinases (CDKs) and Polo-like kinases (PLKs), the focus of our research, play an essential role in the regulation of the cell cycle.[1, 2]

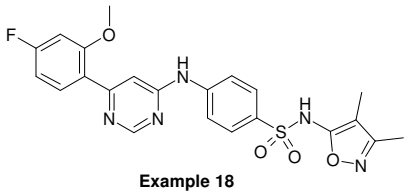
In addition to the regulation of this process, transcription is significantly affected by CDKs.[1] These protein kinases belong to the serine/threonine kinases and their activity depends on cyclin which is a regular subunit.[3] These kinases are activated by the phosphorylation of a threonine residue.[4] CDKs can be divided into subfamilies based on sequence similarity. One of them is the cell cycle related sub-group (CDK1-CDK4 and CDK6), the other is the transcriptional regulator sub-group (CDK3, CDK7, CDK8, CDK9, CDK10) and there are several CDKs with non-cell cycle function (CDK11-CDK20). CDK9 is involved in the regulation of transcription.[1, 5, 6] Through the hexamethylene bisacetamide-inducible protein 1 (HEXIM1), CDK9 can be associated with the development of HIV.[7] Furthermore, inhibition of CDK9 might result in the destruction of cancer cells. This is due to CDK9 being responsible for the synthesis of Mcl-1 and XIAP antiapoptotic proteins that maintain necessary conditions for cancer cells to survive[8, 9], e.g. in chronic lymphocytic leukemia or in breast cancer.[10, 11] Thus inhibitors of CDK9 could be utilized either in the treatment of HIV or cancer.

PLKs are also classified as serine/threonine kinases and this kinase family has an important function in the cell proliferation.[2, 12] Based on the structural homology, four groups are generally distinguished: PLK1, PLK2 (or Snk), PLK3 (or Fnk) and PLK4 (or Sak). Each of these enzymes regulate the M-phase of mitosis in cell proliferation.[13,14] The overexpression or amplification of PLK1 can also lead to malignant processes. Some studies show correlation between several gastrointestinal cancer types and overexpressed PLK1. Inter alia it might be the cause of esophageal squamous cell carcinoma or colon cancer.[15, 16] In addition, this enzyme may affect the development of acute myeloid leukemia (AML).[17] High prevalence of these types of cancer has been observed in OECD countries, fatal outcomes of the disease can be measured in tens of thousands.[18]

Due to the significant biological role of CDK9 and PLK1 they are promising therapeutic targets therefore numerous inhibitors have been developed against these kinases. Dinaciclib is one of the most known CDK9 inhibitors in Phase II Clinical trials, . Dinaciclib was used as standard in our biological evaluations (CDK9 IC_{50}: 4 nM). It can be used against triple negative breast cancer.[11, 19] Volasertib, a potent PLK inhibitor used against AML, is in Phase II clinical trialalso.[17]

Inhibitors of the kinases mentioned above had already been investigated by our research group demonstrating very favorable behavior in terms of inhibition potency of these enzymes. [20, 21]

Example **18,** shown in Figure 1, has exceptional PLK1 enzyme inhibition potency (IC_{50}: 0.040 μM).[20] In the current study our goal was to explore the relationship between the position of nitrogen atoms on this pyrimidine ring and potency was of particular interest. Novel compounds with 2,4-pyirimidine core structures were synthesized, the synthesis scheme of the associated intermediates is shown in Figure 3-4.



Example 18

Figure 1. Best hit of the patented PLK1 inhibitor series (PLK1 IC_{50}: 0.040 μM).[20]

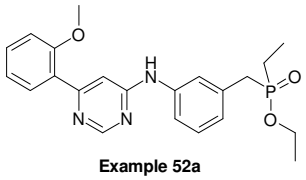
To keep at the sulfisoxazole moiety, several intermediates of **1**-**20** were reacted with sulfisoxazole, yielding compounds **21, 22, 25, 26, 29, 30, 32, 33, 34, 37** (Table 1). These derivatives were found to be less potent inhibitors of PLK1 kinase than 4,6-pyrimidine analogs of the PLK1 Vichem[20], with the best molecule (cpd. **26**) reaching 75% inhibitory activity (10 μM) in PLK1 biochemical assay (data shown in Table 1).

Table 1. Compounds tested in PLK1 biochemical assay

Cpd	R^1	R^2	R^3	R^4	PLK1 inhibition (%)[a]
21	MeO	H	H	H	56
22	MeO	F	H	H	35
25	MeO	F	F	H	37
26	MeO	H	F	F	75
29	MeO	MeO	H	H	34
30	EtO	F	H	H	11
32	EtO	Cl	H	H	5
33	iPrO	F	H	H	13
34	H	H	iPrO	F	65
37	F	F	H	H	55

[a]Compound concentration was 10 μM

Because these molecules did not prove to be particularly efficient PLK1 inhibitors, but the compounds were novel, we sought to identify other kinase target. CDK9 enzyme was chosen due to the fact that several potent 4,6-pyrimidine-based CDK9 inhibitors are well known already, for instance, the structure shown in Figure 2.[21] We hypothesized that in this case it could be meaningful to investigate the role of the positions of the nitrogen atoms in the pyrimidine ring to explore the structure–activity relationship.



Example 52a

Figure 2. Example of a potential CDK9 inhibitor (G. Nemeth et al. CDK9 IC_{50}: 197 nM).[21]

Our 2,4-pyrimidine derivatives (**21, 22, 25, 26, 29, 30, 32, 33, 34,** and **37**) were tested in biochemical assay and it was concluded that these novel molecules are effective CDK9 enzyme inhibitors; therefore, further analogs were prepared according to the scheme shown in Figure 3-4 (biological data is listed in Table 2-3) and the structure-activity relationship (SAR) was examined.

Altogether fifty-one compounds with 2,4-pyrimidine core structure were prepared. Their structure and inhibition activity of CDK9 enzyme are shown in Table 2-3.

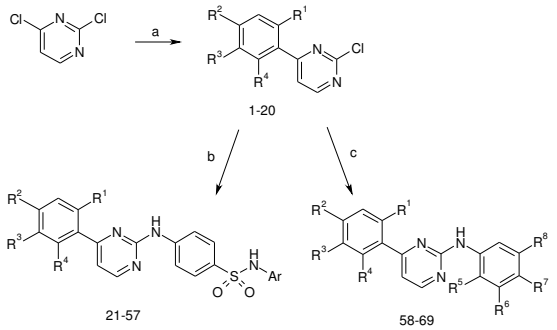


Figure 3. Synthesis of novel 2,4-pyrimidine derivatives. a: substituted phenyl boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, H_2O, DME, inert atmosphere, 4 h, 120 °C, b: corresponding aniline[23], hydrogen chloride solution 4.0 M in dioxane, *tert*-butanol, 5 h, 130 °C, microwave, c: corresponding aniline[24-26], hydrogen chloride solution 4.0 M in dioxane, *tert*-butanol, 5 h, 130 °C, microwave.

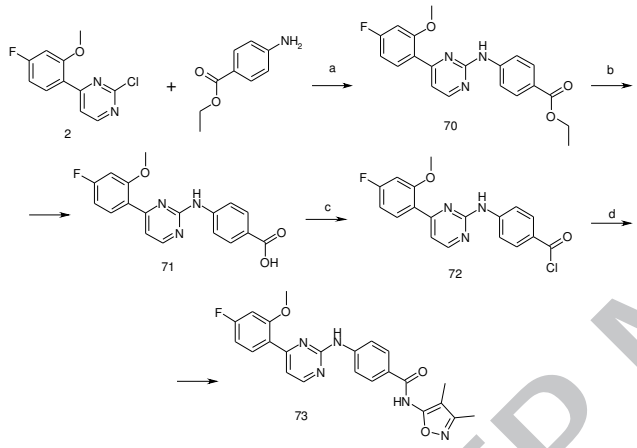


Figure 4. Synthesis of novel 2,4-pyrimidine derivatives. a: cesium carbonate, palladium acetate, BINAP, dioxane, 16 h, 90 °C, b: NaOH, THF, EtOH, H_2O, 4 h, 50 °C, c: thionyl chloride, 3 h, RT, d: 5-Amino-3,4-dimethylisoxazole, pyridine, 4 h, reflux.

Twenty intermediates (cpd **1-20**) were coupled with sulfisoxazole, yielding compounds **21-40**. Several of these structures (cpds **21-26, 29-31, 35, 36**) had an IC_{50} value below 10 nM as shown in Table 2. Seven of these compounds had a methoxy group in R^1-position and were hydrogen- or fluorine-substituted in positions R^2, R^3 and R^4. It was found that the number of fluorine atoms and their position on the ring had no significant effect on the IC_{50} value. Compound **29** had a methoxy substituent in both positions R^1 and R^2 demonstrating the same potency as the former seven inhibitors.

After analysis of the effect of the R^1-methoxy group, R^1-ethoxy and R^1-isopropoxy analogs were synthesized. Two of the most potent compounds (**22** and **24**) were tested via replacement of the methoxy group to ethoxy and isopropoxy groups. In position R^1 ethoxy substitution (**30** and **31**) influenced inhibition inconsiderably; on the other hand, substitution by isopropoxy moiety (**33** and **34**) reduced IC_{50} significantly.

Table 2. The synthesized 2,4-pyrimidines (cpd **21-57**) with inhibition activity of CDK9 enzyme.

Cpd	R^1	R^2	R^3	R^4	Ar	CDK9 IC_{50} (nM)[a]
21	MeO	H	H	H	3,4-dimethyl-isoxazole	3
22	MeO	F	H	H	3,4-dimethyl-isoxazole	2
23	MeO	H	F	H	3,4-dimethyl-isoxazole	9
24	MeO	H	H	F	3,4-dimethyl-isoxazole	1
25	MeO	F	F	H	3,4-dimethyl-isoxazole	8
26	MeO	H	F	F	3,4-dimethyl-isoxazole	1
27	MeO	Cl	H	H	3,4-dimethyl-isoxazole	12
28	MeO	H	H	Cl	3,4-dimethyl-isoxazole	25
29	MeO	MeO	H	H	3,4-dimethyl-isoxazole	9
30	EtO	F	H	H	3,4-dimethyl-isoxazole	8
31	EtO	H	H	F	3,4-dimethyl-isoxazole	2
32	EtO	Cl	H	H	3,4-dimethyl-isoxazole	17
33	iPrO	F	H	H	3,4-dimethyl-isoxazole	49
34	H	H	iPrO	F	3,4-dimethyl-isoxazole	458
35	MeS	F	H	H	3,4-dimethyl-isoxazole	13
36	Me	F	H	H	3,4-dimethyl-isoxazole	6
37	F	F	H	H	3,4-dimethyl-isoxazole	11
38	F	MeO	H	F	3,4-dimethyl-isoxazole	21
39	Cl	F	H	H	3,4-dimethyl-isoxazole	55
40	Cl	Cl	H	H	3,4-dimethyl-isoxazole	50
41	MeO	H	H	H	4,5-dimethyl-isoxazole	4
42	MeO	F	H	H	4,5-dimethyl-isoxazole	3
43	MeO	F	H	H	benzo[d]isoxazole	5
44	MeO	H	H	H	thiazole	33
45	MeO	F	H	H	thiazole	122
46	MeO	H	H	F	thiazole	2
47	EtO	F	H	H	thiazole	210
48	EtO	Cl	H	H	thiazole	378
49	MeO	H	H	H	2-methyl-[1,3,4]thiadiazole	12
50	MeO	F	H	H	2-methyl-[1,3,4]thiadiazole	6
51	MeO	H	F	H	2-methyl-[1,3,4]thiadiazole	9
52	MeO	H	H	F	2-methyl-[1,3,4]thiadiazole	2
53	MeO	F	F	H	2-methyl-[1,3,4]thiadiazole	10
54	MeO	H	F	F	2-methyl-[1,3,4]thiadiazole	0.4
55	MeO	MeO	H	H	2-methyl-[1,3,4]thiadiazole	14
56	EtO	F	H	H	2-methyl-[1,3,4]thiadiazole	2
57	EtO	Cl	H	H	2-methyl-[1,3,4]thiadiazole	5

[a]Dinaciclib as reference compound, IC_{50}: 4 nM

Table 3. The synthesized 2,4-pyrimidines (cpd **58-69**) with inhibition activity of CDK9 enzyme.

Cpd	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	CDK9 IC_{50} (nM)[a]
58	MeO	H	H	H	F	H	MeO	*	1576
59	MeO	F	H	H	F	H	MeO	*	> 12500
60	MeO	H	H	F	F	H	MeO	*	> 12500
61	MeO	F	F	H	F	H	MeO	*	> 12500
62	EtO	F	H	H	F	H	MeO	*	> 12500
63	EtO	Cl	H	H	F	H	MeO	*	> 12500
64	iPrO	F	H	H	F	H	MeO	*	> 12500
65	F	F	H	H	F	H	MeO	*	> 12500
66	MeO	H	H	H	H	**	H	H	3
67	MeO	F	H	H	H	**	H	H	4
68	MeO	H	H	F	H	**	H	H	2
69	MeO	F	H	H	H	***	H	H	> 12500

[a]Dinaciclib as reference compound, IC_{50}: 4 nM

*carboxamide

**1H-benzimidazole-1-yl-methyl

***3,5-dimethyl-isoxazole

The effect of the introduction of other halogens on the benzyl ring on the IC_{50} values was also investigated. Substitution by chlorine decreased the potency by an order of magnitude (cpds **27**, **28**, **32**).

Compound **36,** a sulfisoxazole-linked inhibitor bearing a methyl group in position R^1 (R^2-F, R^3-H and R^4-H), showed low nanomolar IC_{50} value; however, replacement of the methoxy, ethoxy or methyl substituent of position R^1 with other small-size groups such as methylthio groups or halogens decreased CDK9 enzyme inhibition (cpds **35** and **37-40**). Surprisingly, compound **38** showed far lower potency compared to cpds **25** or **26**, whereas the substituents on the ring were the same differing only in their order. This fact supports the importance of R^1-methoxy, -ethoxy or –methyl group.

The inhibitors discussed so far (cpds **21-40**) were coupled with 3,4-dimethylisoxazole via the sulfonamide linker region. Our aim was to investigate how the structure of the isoxazole ring influences CDK9 enzyme inhibition; therefore, 4,5-dimethylisoxazole analogs of two effective inhibitors, cpds **21** and **22,** were prepared. The IC_{50} value of these compounds (**41** and **42**) was found to be practically the same as their 3,4-isoxazole counterparts, thus it can be presumed that the potency of inhibition is independent of the isomerism.

In an effort to better explore the SAR of CDK9 enzyme inhibition, the isoxazole moiety was replaced with benzisoxazole (cpd **43**), and it was concluded that the presence of this bicycle on our molecule caused basically no difference compared to its isoxazole analog, cpd **42.**

The next step in our research was the replacement of isoxazole ring with other five-membered heterocycles, such as thiazole (cpds **44-48**) and 2-methyl-[1,3,4]thiadiazole (cpd **49-57**). In case of molecules containing thiazole, CDK9 enzyme inhibition was less potent (by 1 - 2 orders of magnitude) than their isoxazole-containing analogs. There was an exception: compound **46** (R^1-methoxy, R^2-H, R^3-H, R^4-F substituted similarly to cpd **24**), demonstrating an IC_{50} value of 2 nM.

Inhibition data of **44-48** derivatives (Table 2) reveals that the introduction of the thiazole functional group does not result in more potent inhibitors, in contrary to coupling with thiadiazole ring. Amongst the latter our most potent CDK9 enzyme inhibitor is cpd **54**, with an IC_{50} value measured in subnanomolar region. This inhibitor has similar structure to the isoxazole bearing compound **26** (IC_{50}: 1 nM); both of them are having the same substituents on the benzyl ring (R^1-methoxy, R^2-H, R^3-F, R^4-F). The other thiadiazole analogs were also proved to be potent enzyme inhibitors. This replacement of the original heterocycle provided compounds that were equipotent inhibitors with their isoxazole analogs, with the exception of cpd **54** resulting in an IC_{50} value of 0.4 nM.

Compounds **58-65** are examples of analogs where the five-membered heteroaromatic ring was omitted; instead, functional groups containing heteroatoms were substituted on the ring beside the linker nitrogen (the sulfonamide linker region was also omitted whose role was also investigated, see the end of this section). This modification resulted in remarkable decrease in CDK9 enzyme inhibition.

Based on our previous research, 4,6-pyrimidine analogues of compounds **66-68** (benzimidazole substitution through methylene connection) are potent proliferation inhibitors on many cell lines. We were particularly interested in how this methylene-benzimidazole structure on the 2,4-pyrimidine core affects CDK9 enzyme inhibition potency. Analogs of cpds **21, 22** and **24** were synthesized and as Table 3 shows, each of the three compounds (**66-68**) showed a remarkably decreased IC_{50} value.

Building on the SAR above, our goal was to understand the role of the sulfonamide linker region; therefore, cpds **69** and **73** were synthesized. The structure of cpd **69** lacks the sulfonamide or acid amide group between isoxazole and the benzene ring. This molecule was not capable to inhibit the activation of CDK9 enzyme. Cpd **73** is the analog of cpd **22**, differing in the carboxyl motif only which was changed from sulfonamide to amide group. Comparison of the inhibition potency of these two structures shows that the sulfonamide bearing compound had IC_{50} value two magnitudes lower, cpd **73** showing an IC_{50} value of 135 nM. The synthesis of cpd **73** was carried out through intermediate cpd **71** via chlorination by thionyl chloride followed by acylation in pyrimidine. Other coupling methods were ineffective. This intermediate compound was also measured and surprisingly it was found to be an equipotent inhibitor to some sulfisoxazole-coupled derivatives, with an IC_{50} value of 66 nM.

Due to the therapeutic importance of CDK2 and CDK7 [27-29], the selectivity of our most potent 2,4-pyrimidine CDK9 inhibitors were tested on these cyclin dependent kinases (Table 4). Additional measurement was also carried out for PLK1 inhibition (IC_{50} values are included in Table 4). Experimental data suggests that our derivatives inhibit CDK9 selectively.

Table 4. Cyclin dependent kinase and PLK1 inhibition activity.

Cpd	CDK2 inhibition (%)[a]	CDK7 inhibition (%)[a]	PLK1 IC_{50} (μM)
22	43	3	>12.5
24	44	1	12.35
26	83	2	5.91
31	14	0	>12.5
42	0	0	>12.5
46	46	0	>12.5
52	29	2	>12.5
54	50	7	>12.5
66	9	0	>12.5
68	44	0	>12.5

[a]Compound concentration was 1 μM.

Based on previous reports [30-32], our novel 2,4-pyrimidine derivatives were tested in multiple myeloma cell lines (RPMI-8226). Two of our CDK9 inhibitors showed considerable proliferation inhibitory effect: cpds **66** and **68**. As shown in Table 3, both of these molecules are substituted with a benzimidazole ring. The IC_{50} values are as follows: cpd **66**: 1.34 μM, cpd **68**: 1.78 μM; in case of other derivatives, which are not shown in Table 5, they were over 30 μM (Table 5).

Table 5. Multiple myeloma inhibition activity.

Cpd	RPMI-8226 IC_{50} (μM)	Cpd	RPMI-8226IC_{50} (μM)
21	29.15	54	11.66
24	26.58	58	21.52
41	12.32	60	20.46
43	15.51	66	1.34
44	24.68	68	1.78

In summary, in this paper we disclosed the synthesis and biological evaluation of novel 2,4-pyrimidine derivatives which inhibit CDK9 enzyme in low nanomolar range.

Introducing the sulfisoxazole motif markedly improved efficacy; nevertheless, the most effective proved to be cpd **66** on RPMI-8226 cell line bearing 1H-benzimidazole moiety.

Supplementary Material

Supplementary data (detailed synthetic methods, analytical data, biological protocols) associated with this article can be found in the online version.

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