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Original article

Synthesis and biological evaluation of guanidino analogues of roscovitine

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ABSTRACT

A series of 2,9-substituted 6-guanidinopurines, structurally related to the cyclin-dependent kinase (CDK) inhibitors olomoucine and roscovitine, has been synthesized and characterized. A new copper-catalyzed method for the synthesis of 2-substituted 6-guanidino-9-isopropylpurines under mild reaction conditions has been developed. All prepared compounds were screened for their CDK1 and CDK2 inhibitory activities, cytotoxicity and antiproliferative effects in the breast cancer-derived cell line MCF7. The most active derivative **16g** possessed an identical side chain in the C2 position to roscovitine; this compound displayed approximately five fold higher inhibitory activity towards CDK2/cyclin E and more than ten fold increase in cytotoxicity in MCF7 cells. Interestingly and in contrast to previously described findings, (*S*)-6-guanidinopurine derivatives were generally more active than their (*R*)-counterparts. Kinase selectivity profiling of (*R*)- and (*S*)-enantiomers **16e** and **16g**, respectively, revealed that introduction of a guanidino group at the C6 position of the purine moiety decreased selectivity towards protein kinases compared to roscovitine. Nevertheless, increased inhibitory activity and decreased selectivity offer a good starting point for further development of new protein kinase inhibitors.

1. Introduction

Cyclin-dependent kinases (CDKs) are specific serine/threonine protein kinases which play a key role in regulation of the cell cycle, transcription, apoptosis and differentiation [1]. Their activity is regulated on multiple levels such as synthesis or degradation of activating subunit cyclins, phosphorylations, inhibition by specific proteins or intracellular localization. Overactivation of CDKs is a frequent feature of human pathologies associated with abnormal rates of proliferation such as cancer. Although mutations of CDKs have been described in some tumours (CDK4 [2,3], CDK6 [4], CDK9 [5]), more frequent is overexpression of cyclins or inactivation of endogenous CDK inhibitors [4,6]. For these reasons, CDKs have become a promising target in anticancer research and intensive search for small molecule inhibitors of CDKs has been encouraged. Some CDK inhibitors have already been evaluated in clinical trials [6,7].

In spite of large chemical diversity, the vast majority of CDK inhibitors are low molecular weight, flat, hydrophobic heterocyclic molecules that compete with ATP for binding at the kinase ATP-binding site by both hydrophobic interactions and hydrogen bonding [8]. The purine skeleton was the first systematically modified scaffold that led to early discoveries of olomoucine and roscovitine [9–11] (Fig. 1), followed by synthesis of purvalanols [12,13], olomoucine II [14] or CR8 [15], DRF053 [16] and other biaryl derivatives [17,18]. Modification of purine core was another approach to obtain new potent CDK inhibitors such as pyrazolo[1,5-*a*]pyrimidines, pyrazolo[4,3-*d*]pyrimidines or pyrazolo[1,5-*a*]pyridines [19]. Dinaciclib is a pyrazolo[1,5-*a*]pyrimidine which has

Abbreviations: ATP, adenosine 5′-triphosphate; CDK, cyclin-dependent kinase; DABCO, 1,4-diazabicyclo[2.2.2]octane; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_m, Michaelis constant; NMP, *N*-methyl-2-pyrrolidone; PEG, polyethylene glycol; $pH3^{Ser10}$, histone H3 phosphorylated on serine 10; RESP, restrained fit to the electrostatic potential; r.m.s.d., root mean square deviation; RT, room temperature; SAR, structure–activity relationship; TEAB, triethylammoniumbicarbonate; THF, tetrahydrofuran; TMS, tetramethylsilane.

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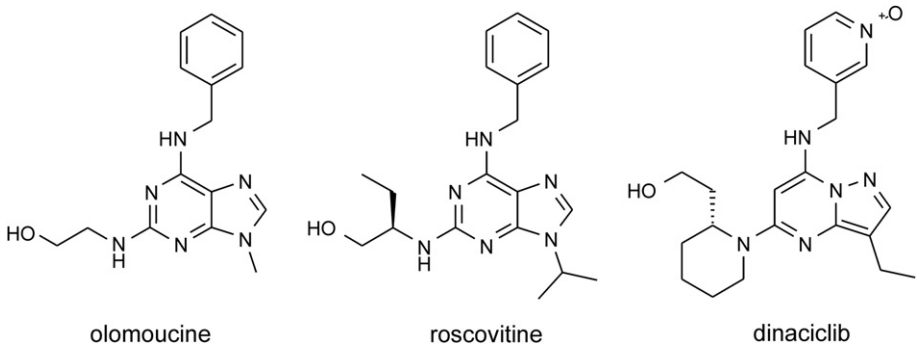


Fig. 1. Some purine and related CDK inhibitors.

already entered phase III clinical trials for the treatment of chronic lymphocytic leukaemia (Fig. 1).

All potent purine and related CDK inhibitors contain an aromatic amine moiety at position 6, such as benzylamino or phenylamino groups, that (i) binds to the hydrophobic pocket near the active site of CDKs (and thus deliver their selectivity) and (ii) forms an essential H-bond to carbonyl of leucine 83 in CDK2 (or to corresponding residues in other CDKs).

Compounds containing the guanidino group such as amino acids or other natural compounds are widespread throughout biological systems with a wide spectrum of biological activities [20]. The ability of the guanidino group to form strong non-covalent interactions at the active site of enzymes is a key factor in their biological activities. For this reason, we decided to explore the influence of the guanidino group at position C6 of the purine and we prepared a library of compounds consisting of 2,9-disubstituted-6-guanidinopurines. These compounds were screened for CDK inhibitory activities and antiproliferative effects in the human breast cancer-derived cell line MCF7.

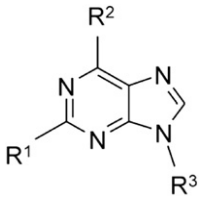
2. Chemistry

Firstly, various substituted 2-amino-6-guanidino-9-(hetero) alkylpurines (Table 1) were synthesized to evaluate the effect of substitution in the N9 position on the activity of CDKs and to confirm whether the introduction of a methyl or isopropyl group to 6-guanidinopurine derivatives is essential for the activity of the compounds, as in the case of olomoucine and roscovitine, respectively [9,10]. 2-Amino-6-guanidinopurine derivatives **1**–**5** bearing the nonpolar aliphatic chains and derivatives **6**–**9** containing the polar functional groups were readily prepared by alkylation of commercially available 2-amino-6-chloropurine with the appropriate alkyl halogenides in the presence of NaH and subsequent guanidinolysis of the obtained intermediates [21]. Compound **10** was prepared by the guanidinolysis of 6-chloro-9-methylpurine. 9-Alkyl-2,6-diaminopurines **11** and **12** were prepared by ammonolysis of appropriate 2-amino-6-chloropurines and compound **13** was isolated as a side product as described by Česnek and co-workers [21].

The SAR study was continued by the derivatization of the active 6-guanidino derivative **3** at the purine position C2. The synthesis of several 2-*O* and 2-*N* derivatives was achieved. The usual synthetic access to substituted 2-aminopurines was based on the treatment of appropriate 2-halogenopurine with corresponding alkyl or arylamine [10,16,22–24]. The coupling reaction of 2-chloro-6-guanidino-9-isopropylpurine with appropriate amines failed. 6-Guanidino-2-iodo-9-isopropylpurine (**15**), as an excellent starting compound for coupling reactions [25,26], was prepared. The most reactive intermediate **15** was prepared by the diazotization reaction of starting 2-amino-6-chloro-9-isopropylpurine in the presence of CuI according to published procedure [27] and subsequent guanidinolysis of obtained 6-chloro-2-iodo-9-isopropylpurine (**14**) in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) and NaH (Scheme 1). A catalyst-free reaction of compound **15** with amines at room temperature (RT) gave no desired products, while at elevated temperature only decomposition of starting material was observed.

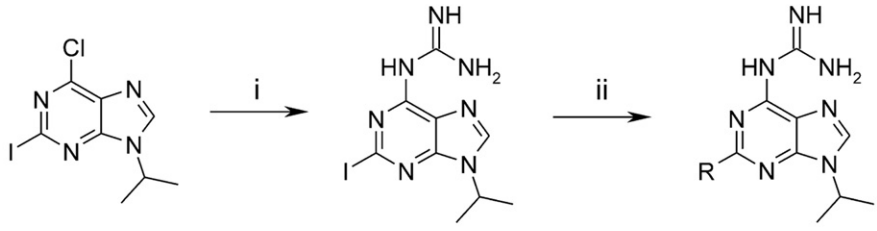
In this way we modified a copper mediated nucleophilic substitution described by Nair and Sells [28] for the introduction of substituted amines into the C2 position of the purine moiety. The

Table 1
Structures of prepared derivatives.



Compound	R^1	R^2	R^3
1	NH_2	Guanidino	Me
2	NH_2	Guanidino	Pr
3	NH_2	Guanidino	^{i}Pr
4	NH_2	Guanidino	^{i}Bu
5	NH_2	Guanidino	Et
6	NH_2	Guanidino	CH_2CF_3
7	NH_2	Guanidino	CH_2CH_2OH
8	NH_2	Guanidino	$CH_2CH_2NH_2$
9	NH_2	Guanidino	$CH_2CH_2N_3$
10	H	Guanidino	Me
11	NH_2	NH_2	^{i}Pr
12	NH_2	NH_2	Et
13	NH_2	$(Me)_2N-$	$CH_2CH_2N_3$
16a	$NH(CH_2)_2OH$	Guanidino	^{i}Pr
16b	$NH(CH_2)_3OH$	Guanidino	^{i}Pr
16c	$NH(CH_2)_6OH$	Guanidino	^{i}Pr
16d	(*R*)-$OCH_2CH(Et)NH_2$	Guanidino	^{i}Pr
16e	(*R*)-$NHCH(Et)CH_2OH$	Guanidino	^{i}Pr
16f	(*S*)-$OCH_2CH(Et)NH_2$	Guanidino	^{i}Pr
16g	(*S*)-$NHCH(Et)CH_2OH$	Guanidino	^{i}Pr
16h	(*R*)-$OCH_2CH(^iPr)NH_2$	Guanidino	^{i}Pr
16i	(*R*)-$NHCH(^iPr)CH_2OH$	Guanidino	^{i}Pr
16j	(*S*)-$OCH_2CH(^iPr)NH_2$	Guanidino	^{i}Pr
16k	(*S*)-$NHCH(^iPr)CH_2OH$	Guanidino	^{i}Pr
16l	Cyclopropylamino	Guanidino	^{i}Pr
16m	(*S*)-*O*-Prolinol	Guanidino	^{i}Pr
16n	(*S*)-*N*-Prolinol	Guanidino	^{i}Pr
16o	(*R*)-*O*-Prolinol	Guanidino	^{i}Pr
16p	(*R*)-*N*-Prolinol	Guanidino	^{i}Pr

Synthesis of compounds **1**–**13** was published by Česnek and co-workers [21].



i) DMF + CH_3CN, DABCO, guanidine solution in DMF
ii) Diisopropylethylamine, CuI, N-methyl-2-pyrrolidone

16a,b,c	R = $NH(CH_2)_xOH$; x=2,3,6
16d,f	R = (*R/S*)-$OCH_2CH(Et)NH_2$
16e,g	R = (*R/S*)-$NHCH(Et)CH_2OH$
16h,j	R = (*R/S*)-$OCH_2CH(iPr)NH_2$
16i,k	R = (*R/S*)-$NHCH(iPr)CH_2OH$
16l	R = cyclopropylamino
16o,m	R = (*R/S*)-*O*-prolinol
16p,n	R = (*R/S*)-*N*-prolinol

Scheme 1. Synthesis of 6-guanidino-9-isopropylpurine derivatives **16a**–**p**.

best results were achieved using 2 equivalents of CuI and 10 equivalents of amine in the presence of *N*,*N*-diisopropylethylamine at RT.

The desired 2-*N*-substituted purine derivatives were obtained in moderate yield because the reaction with hydroxylamines was accompanied by the formation of the corresponding 2-*O*-substituted purine isomers as side-products. The yields were also influenced by the multistep purification process.

Since there seemed to be problem with the solubility of the starting compounds in tetrahydrofuran (THF), the influence of the solvent on the reaction course was studied. Further, preferential formation of undesired *O*-isomer was observed in THF. On the other hand, 2-(*N*,*N*-dimethylamino) derivative was isolated as a major by-product of the reactions run in dimethylformamide (DMF). Finally, *N*-methyl-2-pyrrolidone (NMP) proved to be the most convenient solvent for this type of reaction. Compounds **16a**–**p** were prepared in NMP according to the above mentioned procedure (2 equivalents of CuI and 10 equivalents of amine in the presence of *N*,*N*-diisopropylethylamine at RT).

We also attempted to use 2-chloro-6-guanidino-9-isopropylpurine (**17**) as a starting material for the above mentioned amination reaction both with and without the copper catalyst. This derivative was prepared by the guanidinolysis [21] of known 2,6-dichloro-9-isopropylpurine [29]. The reaction of the compound **17** with amines at RT failed and elevated reaction temperature again caused only the decomposition of the guanidino group, similar to the previously described observations with the amido [25] and guanidinopyrimidine derivative [30].

Transformation of copper halogenide into the soluble complex caused problems with purification of these reaction mixtures. The guanidino group probably makes a complex with a copper ion. Classical silica gel purification was not efficient due to the high polarity of products. The polarity and equal distribution between organic and water phases also did not allow the application of extraction using EDTA or dithisone (diphenylthiocarbazone). Removal of copper from its complex with amines by H_2S gas bubbled into the reaction mixture was left due to formation of by-products. High affinity of the guanidino group on the purine moiety to the Dowex 50 × 8 was used to overcome this problem. After adsorption of neutralized residue on Dowex 50 × 8, the copper residues were removed very efficiently by washing of the column with aqueous HCl:H_2O (1:10). *N*- and *O*-Isomers were subsequently separated on the HPLC column to yield products **16a**–**p**.

3. Pharmacology

CDK inhibitory activity of the prepared compounds was evaluated using purified recombinant enzymes CDK1/cyclin B and CDK2/cyclin E. Cytotoxicity assays were then performed on MCF7 breast cancer cells. Flow cytometry analysis of the cell cycle showed that several compounds arrested cells in G2 + M phases. In an effort to distinguish between G2 and M phase arrest we examined the intracellular level of histone H3 phosphorylated on serine 10 ($pH3^{Ser10}$). This phosphorylation, catalyzed by aurora kinases A and B, is essential for chromatin condensation during mitosis and is often detected as a mitotic marker [31,32]. All the data are summarized in Table 2. CDK inhibitor roscovitine (IC_{50} values: CDK2 = 0.17 μM, CDK1 = 2.4 μM, MCF7 proliferation = 20.2 μM) inducing specifically G2 arrest was used as a positive control throughout the study.

4. Results and discussion

4.1. Structure–activity relationship

Substitution at position 6 is a critical element determining potency and selectivity of purine derivatives towards CDKs [19,33]. Replacement of benzylamine in the roscovitine molecule e.g. with biaryl derivatives improved the biological activity and led to discovery of even more potent compounds such as CR8 [15], DRF053 [16], and others [17,18]. The ability of the guanidino group at position C6 to stabilize binding interaction with the active site of CDKs can be demonstrated by the increased activities of 6-guanidinopurines **3** and **5** (IC_{50} CDK2 < 2 μM, CDK1 < 15 μM) compared to corresponding 6-amino derivatives (**11**, **12**). Similarly, comparison of inactive compound **13** with a weak CDK2 inhibitor **9** showed that the replacement of 6-dimethylamino substituent (**13**) with the guanidino group (**9**) improved CDK inhibitory activity.

As a result, we prepared and evaluated N9-substituted 2-amino-6-guanidinopurines for their biological activity as was previously done with other sets of purine derivatives [10,13,34]. Our results show that the most active compounds of this set in terms of CDK inhibition possess small nonpolar alkyl substituents in sequence from the most active ethyl (**5**) and isopropyl (**3**) as in the roscovitine molecule, then methyl (**1**), propyl (**2**) and the least active was compound **4** with isobutyl. Generally, substitution with polar heteroalkyls led to loss of both CDK inhibitory and cytotoxic activity.

Table 2
Biological activities of prepared compounds.

Compound	IC_{50} [μM]			Effects on the cell cycle of MCF7 cells dose 100 μM, 24 h	
	CDK1/cyclin B	CDK2/cyclin E	MCF7	G2 + M[a] (compound/control)	pH3 (Ser10)[b] (compound/control)
1	38.2 ± 5.5	6.8 ± 2.8	>100	1.5 ± 0.7	0.81 ± 0.09
2	55.9 ± 1.5	10.7 ± 4.9	36.5 ± 9.8	4.0 ± 1.0	6.71 ± 2.49
3	14.8 ± 3.3	1.3 ± 0.2	97.2 ± 24.2	2.0 ± 0.8	0.18 ± 0.01
4	>100	66.5 ± 30.0	96.6 ± 38.2	3.5 ± 0.6	7.00 ± 1.00
5	12.6 ± 1.3	1.7 ± 1.0	54.3 ± 25.7	1.6 ± 0.2	0.10 ± 0.03
6	>100	>100	>100	0.8 ± 0.5	ND
7	>100	>100	>100	0.9 ± 0.3	ND
8	>50	>100	>100	1.0 ± 0.6	ND
9	>100	74.9 ± 14.1	>100	1.4 ± 0.8	ND
10	>100	>100	>100	1.0 ± 0.4	ND
11	64.9 ± 17.4	22.2 ± 17.2	>100	0.9 ± 0.4	ND
12	37.5 ± 10.7	7.9 ± 3.7	>100	1.2 ± 0.6	ND
13	>100	>100	>100	1.0 ± 0.4	ND
16a	15.1 ± 9.2	0.82 ± 0.34	12.4 ± 6.4	2.9 ± 1.1	0.15 ± 0.02
16b	4.1 ± 0.3	0.18 ± 0.07	8.8 ± 3.3	2.5 ± 1.2	0.18 ± 0.02
16c	4.2 ± 1.7	1.6 ± 0.1	5.2 ± 3.1	2.0 ± 0.8	0.33 ± 0.01
16d	47.6 ± 11.0	13.3 ± 7.0	>100	0.8 ± 0.4	1.08 ± 0.21
16e	2.1 ± 0.4	0.14 ± 0.06	4.5 ± 1.3	1.8 ± 1.1	0.08 ± 0.06
16f	8.1 ± 5.1	6.2 ± 3.4	16.3 ± 2.9	1.5 ± 0.4	0.07 ± 0.01
16g	0.9 ± 0.5	0.037 ± 0.031	1.6 ± 0.3	1.6 ± 0.8	0.09 ± 0.03
16h	15.6 ± 6.8	8.0 ± 3.3	79.4 ± 22.1	2.1 ± 0.7	0.39 ± 0.06
16i	2.0 ± 1.0	0.14 ± 0.03	10.5 ± 3.8	2.3 ± 1.0	0.13 ± 0.09
16j	1.1 ± 0.3	1.8 ± 0.8	3.4 ± 0.7	1.7 ± 0.4	0.09 ± 0.07
16k	1.1 ± 0.6	0.071 ± 0.046	2.1 ± 0.4	1.8 ± 0.7	0.09 ± 0.08
16l	6.4 ± 1.5	0.50 ± 0.18	5.4 ± 0.2	2.4 ± 0.6	0.17 ± 0.03
16m	>100	25.2 ± 4.8	>100	1.7 ± 0.2	0.80 ± 0.10
16n	1.5 ± 0.1	0.16 ± 0.04	6.2 ± 3.3	1.7 ± 0.8	0.11 ± 0.08
16o	81.5 ± 27.0	23.6 ± 4.9	>100	1.3 ± 0.5	ND
16p	6.2 ± 2.2	0.67 ± 0.44	20.4 ± 16.8	2.0 ± 0.7	0.10 ± 0.07
Roscovitine	2.4 ± 0.94	0.17 ± 0.05	20.2 ± 12.3	1.5 ± 0.1	0.11 ± 0.01

[a] Ratio of G2 + M populations in treated cells and G2 + M populations in control cells.
[b] Ratio of pH3-positive cells treated and pH3-positive control cells.

In spite of CDK inhibitory activities of several N9-substituted 2-amino-6-guanidinopurines, the majority had no significant cytotoxic effect on MCF7 cells (IC_{50} > 100 μM) or only moderate (IC_{50} > 50 μM). Nonetheless, some were still able to accumulate cells in G2 + M phases (compounds **2**–**5**). In comparison with roscovitine, derivatives **3** and **5** were weaker CDK inhibitors with IC_{50} at least 5-times higher and their cytotoxicity was also lower (4× and 2×, respectively). However, these compounds induced increase in the G2 + M population (2× and 1.6×, respectively) and more than 5-times decrease in mitotic marker $pH3^{Ser10}$ in a similar manner to G2-block induced by roscovitine. On the other hand, propyl (**2**) and isobutyl (**4**) derivatives caused an increase in the mitotic cell subpopulation as evidenced by a strong (7-times) accumulation of $pH3^{Ser10}$. It is interesting that such a small change in the structure of the N9 substituent apparently leads to alternative mechanisms of action resulting in different responses in biological systems.

Having confirmed isopropyl as one of the most active substituents in the group of various N9 substituents, we synthesized substituted 6-guanidino-9-isopropylpurines and investigated the effects of the introduction of various substituents related to the olomoucine or roscovitine C2 side chain to position C2. The importance of the C2 substituent was demonstrated by comparison of the biological activities of 2-amino-6-guanidino-9-methylpurine (**1**), which inhibited CDKs in micromolar range, and inactive, C2 unsubstituted 6-guanidino-9-methylpurine (**10**). Compound **16a** containing 2-hydroxyethylamino group, the olomoucine C2 side chain, did not significantly improve the CDK inhibitory activity of the corresponding 2-amino derivative (**3**), but it potentiated its antiproliferative properties towards MCF7 almost 8-times. This observation suggests that the increased potency is not due to inhibition of CDKs. Elongation of olomoucine C2 side chain by a methylene group (**16b**) increased CDK inhibitory activity approximately 4-times, but no significant increase in cytotoxicity was observed (Table 2). Further, elongation by methylene groups (**16c**) did not potentiate the biological activity.

A set of 6-guanidinopurines with various *N*- or *O*-linked C2 substituents was then prepared. Generally, derivatives with the high CDK inhibitory activity tended to be more active against MCF7. This trend was observed for both 2-*N*- and 2-*O*-subgroups. This observation suggests that at least part of the activity is due to CDK inhibition. 2-*O*-Substituted derivatives were several times less potent than their 2-*N*-regioisomers. The only exception was **16j**, one of the most active compounds of our set. Although the 25× less potent CDK2 inhibitor than its *N*-regioisomer **16k**, its effects on CDK1, MCF7 cells and on the cell cycle were surprisingly nearly identical.

Compound **16g** was the most active 6-guanidinopurine bearing the same substituent at position C2 as roscovitine (in addition to the roscovitine N9 substituent). This compound was more active in CDKs (2.7× for CDK1 and 4.6× for CDK2) as well as on MCF7 cells (IC_{50} more than 10× lower) than roscovitine itself. In contrast to roscovitine, which is a more potent CDK inhibitor as an (*R*)-enantiomer [35], (*S*)-enantiomer (**16g**) seems to be more active than its (*R*)-counterpart **16e**. Similarly, the other guanidinopurines in (*S*)-configuration also proved to be more active in CDK inhibition as well as in the antiproliferative effects in MCF7 cells. We found the same with 2-*N*-enantiomers **16e** and **16g**, **16i** and **16k** as well as 2-*O*-enantiomers **16d** and **16f**, **16h** and **16j**.

The presence of a cyclopropylamino group at position C2 (**16l**) improved both CDK inhibitory (about 2.5-times) and cytotoxic activity (almost 20-times) in comparison with the similar 2-amino derivative **3**. (*S*) and (*R*)-Prolinol were other cyclic C2 substituents tested. In a group of various prolinol isomers and enantiomers at

position C2 (**16m**–**p**) we observed that the most active compound **16n** possessed *N*-linked (*S*)-prolinol. The biological activity of this derivative was very similar to that of roscovitine.

4.2. Kinase selectivity

The most active compounds **16e** and **16g** were further evaluated for selectivity on a panel of protein kinases. As selectivity and potency are usually inversely correlated for kinase inhibitors [36] it was not surprising that both these derivatives were found to inhibit a broad range of various kinase targets (Table 3). In comparison, roscovitine has been shown to be more selective, inhibiting only a few kinases besides CDKs as CaM Kinase 2, CK1α, CK1δ, DYRK1A, EPHB2, ERK1, ERK2, FAK and IRAK4 as published previously [35,37]. The reason for this decreased selectivity is probably the removal of aromatic substitution at C6 that was designed as a key determinant of CDK selectivity [33]. In accordance with our other data, (*S*)-enantiomer **16g** inhibited selected kinases more efficiently than its (*R*)-enantiomer **16e**.

4.3. Docking study

In order to acquire insight into the location and molecular interaction of the most active compounds in the binding site, a docking study was performed. When we compared the X-ray structure of CDK2/roscovitine complex (PDB code 2A4L) with the results of docking of two enantiomer pairs **16e**/**16g** and **16i**/**16k**, we found that the position of the purine moiety is conserved as well as the orientation of isopropyl group at position N9 (see Fig. 2A, B). Hydrogen bonding of adenine moiety is also conserved and in comparison to roscovitine is extended by one hydrogen bond donated by a guanidine secondary amino group to the carbonyl oxygen of leucine 83. Interestingly, the docking study of compounds **16e**, **16g**, **16i** and **16k** showed that modification on C2 by [1-(1*S*)-(hydroxymethyl)propyl]amino (compound **16g**) gave a superior binding due to the two hydrogen bonds of the hydroxyl, one donated to the carboxy group of aspartate 145 and one accepted from side chain amino group of lysine 33 (Fig. 3).

5. Conclusion

In this study we evaluated a library of 2,9-disubstituted-6-guanidinopurines, structurally related to the CDK inhibitors olomoucine and roscovitine. From this series, 2-substituted 6-guanidino-9-isopropylpurines were newly synthesized. SAR analysis proved that small changes in the structure of the N9 substituent yielded compounds with a different mode of action. Although the majority of the compounds arrested cells in the G2 phase of the cell cycle in a similar manner to roscovitine, a few of them induced significant accumulation of mitotic cells. Moreover, in contrast to roscovitine, our compounds were more active in (*S*)-configuration as supported by our docking study. The introduction of the guanidino group at position C6 increased the inhibitory potential towards protein kinase targets several times more than with roscovitine but with a concomitant decrease in selectivity. Nevertheless increased potency and decreased selectivity offer a good starting point for further development of new, more selective protein kinase inhibitors.

Table 3
Selectivity of compounds **16e** and **16g** against a panel of recombinant protein kinases. The compounds were screened at 1 μM concentration in duplicate.

Kinase	Remaining activity [%]		Kinase	Remaining activity [%]	
	16e	**16g**		**16e**	**16g**
ABL	5 ± 0	6 ± 3	MINK1	64 ± 0	75 ± 4
AMPK	29 ± 1	22 ± 1	MKK1	41 ± 0	28 ± 9
ASK1	100 ± 6	81 ± 7	MKK2	53 ± 4	39 ± 8
AURA	39 ± 1	30 ± 4	MKK6	72 ± 12	88 ± 2
AURB	47 ± 12	70 ± 3	MLK1	25 ± 5	31 ± 10
BRK	50 ± 15	44 ± 9	MLK3	37 ± 7	37 ± 14
BRSK1	44 ± 2	45 ± 18	MNK1	173 ± 15	178 ± 10
BRSK2	77 ± 12	67 ± 12	MNK2	119 ± 8	122 ± 7
BTK	53 ± 1	48 ± 11	MPSK1	36 ± 2	28 ± 9
CAMK1	35 ± 6	24 ± 5	MSK1	46 ± 1	38 ± 3
CAMKKb	17 ± 0	13 ± 1	MST2	35 ± 5	43 ± 8
CDK2/cyclin A	5 ± 1	7 ± 1	MST4	42 ± 19	40 ± 18
CK1	15 ± 6	21 ± 2	NEK2a	73 ± 11	57 ± 6
CK2	43 ± 1	33 ± 4	NEK6	95 ± 4	85 ± 4
CLK2	4 ± 2	4 ± 2	NUAK1	8 ± 1	7 ± 3
CSK	58 ± 6	59 ± 0	OSR1	16 ± 2	16 ± 1
DAPK1	54 ± 8	36 ± 5	p38a MAPK	87 ± 14	82 ± 17
DYRK1A	1 ± 0	1 ± 0	p38b MAPK	64 ± 3	66 ± 9
DYRK2	27 ± 3	16 ± 1	p38d MAPK	37 ± 9	23 ± 2
DYRK3	18 ± 2	11 ± 3	p38g MAPK	77 ± 2	71 ± 7
EF2K	94 ± 1	94 ± 6	PAK2	45 ± 1	38 ± 5
EIF2AK3	17 ± 1	11 ± 0	PAK4	15 ± 2	12 ± 0
EPH-A2	22 ± 5	17 ± 8	PAK5	18 ± 1	15 ± 3
EPH-A4	22 ± 6	41 ± 13	PAK6	26 ± 3	17 ± 5
EPH-B1	8 ± 0	7 ± 2	PDK1	68 ± 10	62 ± 18
EPH-B2	2 ± 2	2 ± 1	PHK	14 ± 2	12 ± 0
EPH-B4	6 ± 2	6 ± 4	PIM1	85 ± 10	71 ± 20
ERK1	50 ± 3	28 ± 7	PIM2	88 ± 7	91 ± 5
ERK2	32 ± 4	15 ± 3	PIM3	64 ± 10	44 ± 9
ERK8	16 ± 8	18 ± 6	PKA	90 ± 3	83 ± 6
FGF-R1	4 ± 0	5 ± 0	PKBa	99 ± 8	107 ± 24
GCK	15 ± 2	10 ± 1	PKBb	111 ± 19	103 ± 8
GSK3b	37 ± 1	28 ± 12	PKCa	71 ± 1	68 ± 14
HER4	27 ± 2	29 ± 12	PKCz	68 ± 2	61 ± 10
HIPK1	15 ± 1	11 ± 2	PKCγ	73 ± 1	61 ± 7
HIPK2	8 ± 0	6 ± 1	PKD1	17 ± 1	18 ± 1
HIPK3	38 ± 6	24 ± 0	PLK1	108 ± 4	108 ± 19
CHK1	58 ± 8	46 ± 16	PRAK	76 ± 16	74 ± 23
CHK2	59 ± 8	42 ± 15	PRK2	85 ± 22	84 ± 34
IGF-1R	10 ± 2	5 ± 0	RIPK2	72 ± 5	76 ± 15
IKKb	74 ± 6	76 ± 12	ROCK 2	61 ± 23	65 ± 18
IKKe	66 ± 8	52 ± 14	RSK1	18 ± 1	12 ± 4
IR	5 ± 0	3 ± 0	RSK2	35 ± 7	17 ± 1
IRAK1	26 ± 1	17 ± 6	S6K1	49 ± 2	36 ± 3
IRAK4	21 ± 1	11 ± 0	SGK1	60 ± 1	51 ± 15
IRR	19 ± 2	16 ± 2	SMMLCK	49 ± 3	32 ± 2
JAK2	16 ± 3	16 ± 8	SRC	22 ± 22	6 ± 3
JNK1	88 ± 8	78 ± 7	SRPK1	22 ± 1	14 ± 1
JNK2	85 ± 4	73 ± 19	STK33	11 ± 0	9 ± 3
JNK3	96 ± 20	80 ± 19	SYK	58 ± 6	50 ± 4
LCK	13 ± 2	12 ± 3	TAK1	7 ± 2	13 ± 10
LKB1	13 ± 4	16 ± 9	TAO1	9 ± 2	9 ± 3
MAPKAP-K2	41 ± 9	48 ± 27	TBK1	92 ± 1	90 ± 45
MAPKAP-K3	80 ± 18	78 ± 4	TIE2	39 ± 12	40 ± 14
MARK1	31 ± 7	39 ± 0	TLK1	9 ± 1	9 ± 5
MARK2	45 ± 16	62 ± 24	TRKA	24 ± 5	28 ± 14
MARK3	24 ± 3	17 ± 7	TTK	40 ± 1	32 ± 4
MARK4	38 ± 3	29 ± 8	VEGFR	6 ± 1	4 ± 0
MEKK1	91 ± 12	96 ± 5	YES1	13 ± 2	11 ± 4
MELK	27 ± 2	19 ± 2	ZAP70	86 ± 15	109 ± 54

6. Experimental protocols

6.1. General chemical procedures

Unless otherwise stated, solvents were evaporated at 40 °C/2 kPa, and the compounds were dried over P_2O_5 at 2 kPa. NMR spectra were recorded on Bruker Avance 500 (^{1}H at 500 MHz, ^{13}C at 125.8 MHz) and Bruker Avance 400 (^{1}H at 400 MHz, ^{13}C at 100.6 MHz) spectrometers with TMS as internal standard or referenced to the residual solvent signal. Mass spectra were measured on a ZAB-EQ (VG Analytical) spectrometer. The chemicals were obtained from commercial sources (Sigma–Aldrich) or prepared according to published

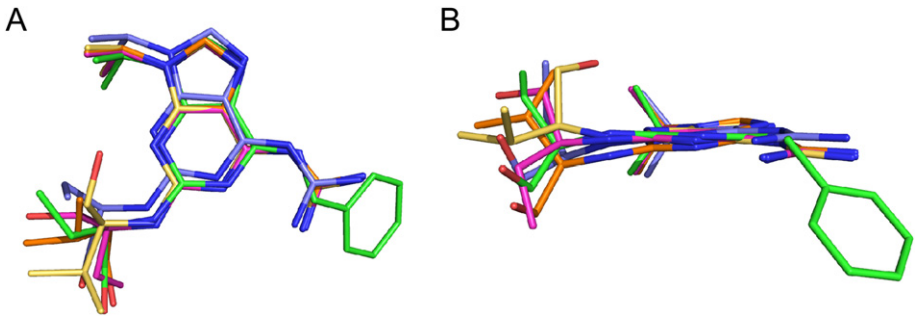


Fig. 2. Overlay of ligands poses and X-ray structure of CDK2–roscovitine complex, based on CDK2 superposition: (A) view perpendicular and (B) parallel to purine plane. Colour coding: carbon atoms green for roscovitine, magenta for **16g**, light-blue for **16e**, orange for **16k** and light-yellow for **16i**; nitrogen atoms blue, oxygen atoms red.

procedures. DMF and acetonitrile were distilled from P_2O_5 and stored over molecular sieves (4 Å). Preparative HPLC purifications were performed on columns packed with 7 μm C18 reversed phase resin (Waters Delta 600 chromatograph column), 17 × 250 mm; in ca. 200 mg batches of mixtures using gradient MeOH/H_2O as eluent or using a 0.1 mol triethylammoniumbicarbonate (TEAB) as a buffer. Deionization was performed on Dowex 50 × 8 (H+-form) columns by the following procedure: after application of crude product the column was washed with water until the UV absorption dropped. Thereafter, the column was eluted with 2.5% aqueous NH_3. Chromatography on Dowex 1 × 2 (acetate form) was as follows: after application of the aqueous solution of the crude product onto the column, it was washed with water until the UV absorption dropped. The column was then eluted with a gradient of dilute acetic or formic acid (0–1 M). All tested compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectrometry. Representative examples of ^{1}H and ^{13}C spectra are provided as supplementary material (Figs. S1–S12).

6.1.1. 6-Guanidino-2-iodo-9-isopropyl-9H-purine (**15**)

A filtered solution of guanidine [38] in NMP (15.5 ml, 15.5 mmol) was added to a mixture of 6-chloro-2-iodo-isopropyl-9*H*-purine [27] (1 g, 3.1 mmol) and DABCO (0.35 g, 3.1 mmol). The reaction mixture was stirred at RT for 4 h. The resulting mixture was neutralized with aqueous HCl, the solids were removed by filtration. The filtrate was evaporated *in vacuo*, co-distilled with DMF (3 × 50 ml) and subsequently with toluene (3 × 50 ml). The residue was dissolved in MeOH (150 ml) and filtered through the Celite pad once again. The filtrate was adsorbed on SiO_2 and purified with column chromatography (HUB III); yield 0.7 g, 65%. FABMS: 345.9 [MH^+] (50). ^{1}H NMR (DMSO-d_6): 1.48 (d, 6H, J(CH_3, CH) = 6.8, CH_3); 4.65 (sept, 1H, J(CH, CH_3) = 6.8, N–CH); 7.40 (br, 4H, NH); 8.13 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.36 (2C, CH_3); 46.45 (N–CH); 118.32 (C-5); 125.28 (C-2); 138.91 (C-8); 150.24 (C-4); 159.16 and 159.74 (N–C and C-6). For $C_9H_{12}IN_7$ (345.14) calcd: C, 31.32; H, 3.50; I, 36.77; N, 28.41. Found: C, 31.55; H, 3.59; I, 36.67; N, 28.46.

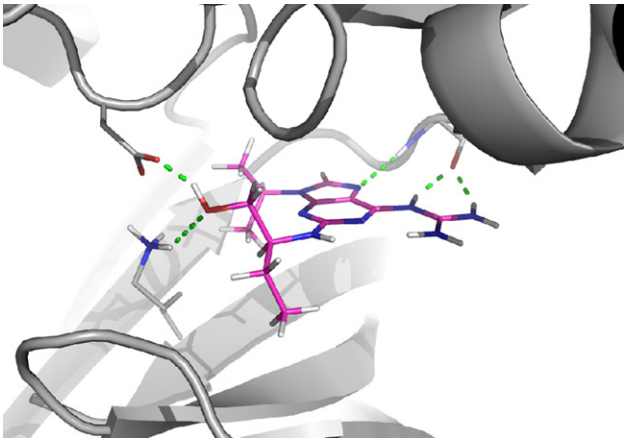


Fig. 3. Complex of CDK2 with compound **16g**, detail of active site. CDK2 shown in cartoon representation, ligand in stick representation. Colour coding: nitrogen atoms blue, oxygen atoms red, hydrogen atoms white, carbon atoms grey for CDK2 and magenta for **16g**. Hydrogen bonds are depicted by green dash-line.

6.1.2. General procedure for preparation of 2-substituted 6-guanidino-9-isopropyl-9H-purines

N,*N*-Diisopropylethylamine (0.48 ml; 2.8 mmol) and appropriate amine (14 mmol) were added to a mixture of compound **15** (0.5 g; 1.4 mmol) and CuI (0.52 g; 2.8 mmol) in NMP (7 ml) under Ar. The reaction mixture was stirred at RT by the time until the starting compound disappeared. The reaction mixture was neutralized with a solution of HCl in DMF and evaporated, co-distilled by DMF and subsequently with toluene. The residue was dissolved in 20% aqueous MeOH (25 ml), applied on column of Dowex (50 × 8) and washed subsequently with 20% aqueous MeOH and water. Impurities (mainly copper salts) were eluted with 10% aqueous HCl and the column was then washed with water to remove the acid. The elution was made with 10% aqueous Et_3N (20% MeOH in H_2O). The collected UV absorbing fractions were evaporated and purified by HPLC column chromatography to give desired products in moderate yields.

6.1.3. 6-Guanidino-2-[(2-hydroxyethyl)amino]-9-isopropyl-9H-purine (**16a**)

General procedure; ethanolamine (1.5 ml, 24 mmol); 2 h, RT; yield 0.41 g, 69%; FABMS: 279.1 [MH^+] (100). ^{1}H NMR (DMSO-d_6): 1.43 (d, 6H, J(CH_3, CH) = 6.7, CH_3); 3.32 (brq, 2H, O–CH_2); 3.55 (t, 2H, J(CH_2, CH_2) = 5.7, N–CH_2); 4.56 (sept, 1H, J(CH, CH_3) = 6.7, N–CH); 4.70 (brt, 1H, OH); 6.60 (brs, 1H, NH) and 7.80 (br, 4H, NH); 7.81 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.21 (2C, CH_3); 44.20 (N–CH_2); 45.75 (N–CH); 60.39 (O–CH_2); 118.90 (C-5); 136.02 (C-8); 151.88 (C-4); 158.09, 2C, (C-6; C-2); 159.25 (N–C). For $C_{11}H_{18}N_8O$·EtOH·HCl (278.31) calcd: C, 43.27; H, 6.98; N, 31.05; Cl, 9.81. Found: C, 43.29; H, 6.75; N, 30.85; Cl, 10.12.

6.1.4. 6-Guanidino-2-[(3-hydroxypropyl)amino]-9-isopropyl-9H-purine (**16b**)

General procedure; 3-aminopropan-1-ol (2.48 ml, 32.6 mmol); 5 h; RT; yield 0.46 g, 57%; FABMS: 293.2 [MH^+] (100). ^{1}H NMR (DMSO-d_6): 1.42 (d, 6H, J(CH_3, CH) = 6.8, CH_3); 1.50 (m, 2H, CH_2-2′); 3.19 (bq, 2H, J(1′–2′) = J(1′–NH) = 6.7 CH_2-1′); 3.32 (m, 2H,

O–CH_2); 4.50 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 4.60 (brs, 1H, OH); 6.40 (br, 1H, NH); 7.31 (vbs, 4H, NH); 7.75 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.25 (2C, CH_3); 29.85 (C-2′); 42.45 (C-1′); 45.60 (N–CH); 60.50 (C-3′); 119.45 (C-5); 135.90 (C-8); 151.80 (C-4); 158.10 (C-2); 159.50 (N–C); 159.70 (C-6). For $C_{12}H_{20}N_8O\cdot 1/3H_2O\cdot 1/3$ HCl (292.34) calcd: C, 46.42; H, 6.82; N, 36.09; Cl 3.81. Found: C, 46.37; H, 6.71; N, 35.92; Cl 4.18.

6.1.5. 6-Guanidino-2-[(6-hydroxyhexyl)amino]-9-isopropyl-9H-purine (**16c**)

General procedure; 6-amino-1-hexanol (1.99 g, 17 mmol); 24 h; not fully converted, RT; yield 0.13 g, 25%; FABMS: 335 [MH^+] (100). ^{1}H NMR (DMSO-d_6): 1.32 (m, 4H, CH_2-3′,4′); 1.42 (m, 2H, CH_2-5′); 1.45 (d, 6H, *J*(CH_3, CH) = 6.8, CH_3); 1.53 (m, 2H, CH_2-2′); 3.20 (bq, 2H, *J*(1′–2′) = *J*(1′–NH) = 6.6 CH_2-1′); 3.37 (m, 2H, CH_2-6′); 4.36 (brs, 1H, OH); 4.54 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 6.46 (brt, 1H, *J*(NH-1′) = 5.3, NH); 7.3 (vbs, 4H, NH); 7.73 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.30 (2C, CH_3); 25.65 (C-4′); 26.84 (C-3′); 29.54 (C-2′); 32.80 (C-5′); 41.36 (C-1′); 45.62 (N–CH); 60.94 (C-6′); 119.62 (C-5); 135.47 (C-8); 151.88 (C-4); 158.17 (C-2); 159.74 (N–C); 159.84 (C-6). For $C_{15}H_{26}N_8O\cdot$MeOH (334.42) calcd: C, 52.44; H, 8.25; N, 30.58. Found: C, 52.84; H, 7.88; N, 30.63.

6.1.6. 2-[2-(2R)-(Aminomethyl)propoxy]-6-guanidino-9-isopropyl-9H-purine (**16d**) and 6-guanidino-2-{[1-(1R)-(hydroxymethyl)propyl]amino}-9-isopropyl-9H-purine (**16e**)

General procedure; *R*-(−)-2-amino-1-butanol (1.32 ml, 14 mmol); 6 h; RT; compound **16d**: yield 0.2 g, 45%; FABMS: 307.0 [MH^+] (20). $[\alpha]_D$ −5.4° (*c* 0.34, methanol); ^{1}H NMR (DMSO-d_6): 0.93 (t, 3H, *J*(4′, 3′) = 7.5, CH_3-4′); 1.28 (m, 1H, CH_2-3′b); 1.48 (d, 6H, *J*(CH_3, CH) = 6.8, CH_3); 1.52 (m, 1H, 3′a); 2.90 (m, 1H, CH-2′); 3.98 (dd, 1H, *J*(1′b–2′) = 6.6, J_{gem} = 10.2, 1′b); 4.07 (dd, 1H, *J*(1′a–2′) = 5.4, J_{gem} = 10.2, 1′a); 4.61 (br, 1H, *J*(CH, CH_3) = 6.7, N–CH); 7.3 (vbs, 4H, NH); 7.96 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 10.60 (C-4′); 22.31 (2C, CH_3); 26.97 (C-3′); 46.11 (N–CH); 51.63 (C-2′); 71.71 (C-1′); 122.07 (C-5); 137.58 (C-8); 151.08 (C-4); 159.93 (N–C); 160.20 (C-2); 160.75 (C-6). For $C_{13}H_{22}N_8O\cdot$MeOH (306.37) calcd: C, 49.69; H, 7.74; N, 33.11. Found: C, 49.82; H, 7.39; N, 33.53.

Compound **16e**: yield 0.15 g, 33%; FABMS: 307.0 [MH^+] (100). $[\alpha]_D$ +32.3° (*c* 0.38, methanol); ^{1}H NMR (DMSO-d_6): 0.89 (t, 3H, *J*(4′, 3′) = 7.4, CH_3-4′); 1.45 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.46 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.46 (m, 1H, 3′b); 1.65 (m, 1H, 3′a); 3.38 (dd, 1H, *J*(1′b–2′) = 5.9, J_{gem} = 10.6, 1′b); 3.49 (dd, 1H, *J*(1′a–2′) = 5.0, J_{gem} = 10.5, 1′a); 4.52 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 4.63 (brs, 1H, OH); 6.12 (brs, 1H, NH); 7.25 (vbs, 4H, NH); 7.71 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 10.87 (C-4′); 22.21 and 22.27 (2C, CH_3); 24.18 (C-3′); 45.63 (N–CH); 54.37 (C-2′); 63.30 (C-1′); 119.84 (C-5); 135.32 (C-8); 151.67 (C-4); 158.06 (C-2); 159.82 (N–C); 160.17 (C-6). For $C_{12}H_{20}N_8O\cdot 2/3$ MeOH (306.37) calcd: C, 50.09; H, 7.59; N, 34.19. Found: C, 49.90; H, 7.29; N, 34.19.

6.1.7. 2-[2-(2S)-(Aminomethyl)propoxy]-6-guanidino-9-isopropyl-9H-purine (**16f**) and 6-guanidino-2–{[1-(1S)-(hydroxymethyl)propyl]amino}-9-isopropyl-9H-purine (**16g**)

General procedure; *S*-(+)-2-amino-1-butanol (1.32 ml, 14 mmol); 6 h; RT; compound **16f**: yield 0.3 g, 44%; FABMS: 307.0 [MH^+] (100). $[\alpha]_D$ +15.6° (*c* 0.39, methanol); ^{1}H NMR (DMSO-d_6): 0.93 (t, 3H, *J*(4′, 3′) = 7.5, CH_2-4′); 1.28 (m, 1H, CH_2-3′b); 1.48 (d, 6H, *J*(CH_3, CH) = 6.8, CH_3); 1.52 (m, 1H, 3′a); 2.90 (m, 1H, CH_2-2′); 3.98 (dd, 1H, *J*(1′b–2′) = 6.6, J_{gem} = 10.2, 1′b); 4.07 (dd, 1H, *J*(1′a–2′) = 5.4, J_{gem} = 10.2, 1′a); 4.61 (br, 1H, *J*(CH, CH_3) = 6.7, N–CH); 7.3 (vbs, 4H, NH); 7.96 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 10.60 (C-4′); 22.31 (2C, CH_3); 26.97 (C-3′); 46.11 (N–CH); 51.63 (C-2′); 71.71 (C-1′); 122.07 (C-5); 137.58 (C-8); 151.08 (C-4); 159.93 (N–C); 160.20 (C-2); 160.75 (C-6).

Compound **16g**: yield 0.2 g, 29%; FABMS: 307 [MH^+] (100). $[\alpha]_D$ −33.3° (*c* 0.21, methanol); ^{1}H NMR (DMSO-d_6): 0.89 (t, 3H, *J*(4′, 3′) = 7.4, CH_3-4′); 1.45 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.46 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.46 (m, 1H, 3′b); 1.65 (m, 1H, 3′a); 3.38 (dd, 1H, *J*(1′b–2′) = 5.9, J_{gem} = 10.6, 1′b); 3.49 (dd, 1H, *J*(1′a–2′) = 5.0, J_{gem} = 10.5, 1′a); 4.52 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 4.63 (brs, 1H, OH); 6.12 (brs, 1H, NH); 7.25 (vbs, 4H, NH); 7.71 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 10.87 (C-4′); 22.21 and 22.27 (2C, CH_3); 24.18 (C-3′); 45.63 (N–CH); 54.37 (C-2′); 63.30 (C-1′); 119.84 (C-5); 135.32 (C-8); 151.67 (C-4); 158.06 (C-2); 159.82 (N–C); 160.17 (C-6). For $C_{12}H_{20}N_8O\cdot$½ HCl (306.37) calcd: C, 48.10; H, 6.99; N, 34.52. Found: C, 48.40; H, 7.01; N, 34.29.

6.1.8. 2-{[(2R)-2-Amino-3-methylbutyl]oxy}-6-guanidino-9-isopropyl-9H-purine (**16h**) and 6-guanidino-2-{[(1R)-1-(hydroxymethyl)-2-methylpropyl]amino}-9-isopropyl-9H-purine (**16i**)

General procedure; (2*R*)-(−)-2-amino-3-methyl-1-butanol (1 g, 10 mmol); 10 h; RT; compound **16h**: yield 0.09 g, 28%; FABMS: 321.0 [MH^+] (40). $[\alpha]_D$ −9.4° (*c* 0.19, methanol); ^{1}H NMR (DMSO-d_6): 0.89 (d, 3H, *J*(4′,3′) = 6.8, CH_3); 0.93 (d, 3H, *J*(4′,3′) = 6.8, CH_3); 1.48 (d, 6H, *J*(CH_3, CH) = 6.8, CH_3); 1.72 (m, 1H, CH-3′); 2.83 (m, 1H, CH-2′); 3.99 (dd, 1H, *J*(1′b–2′) = 7.1, J_{gem} = 10.4, 1′b); 4.19 (dd, 1H, *J*(1′a–2′) = 5.2, J_{gem} = 10.4, 1′a); 4.61 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 7.30 (vbs, 4H, NH); 7.96 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 17.52 and 19.74 (C-4′); 22.29 (2C, CH_3); 30.34 (C-3′); 46.15 (N–CH); 55.07 (C-2′); 70.10 (C-1′); 122.08 (C-5); 137.60 (C-8); 151.08 (C-4); 159.92 (N–C); 160.21 (C-2); 160.75 (C-6). For $C_{16h24}N_8O\cdot 2/3$ MeOH (320.39) calcd: C, 51.54; H, 7.86; N, 32.79. Found: C, 51.35; H, 7.59; N, 33.09.

Compound **16i**: yield 0.1 g, 31%; FABMS: 321.0 [MH^+] (100). $[\alpha]_D$ +37.5° (*c* 0.35, methanol); ^{1}H NMR (DMSO-d_6): 0.90 (d, 3H, *J*(4′,3′) = 6.9, CH_3); 0.91 (d, 3H, *J*(4′,3′) = 6.9, CH_3); 1.45 (d, 3H, *J*(CH_3, CH) = 6.9, CH_3); 1.46 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.97 (m, 1H, CH-3′); 3.49 (m, 2H, CH_2-1′); 3.75 (brs, 1H, CH_2-2′); 4.52 (br, *J*(CH–CH_3) = 6.8, CH–N); 4.58 (brs, 1H, OH); 6.13 (br, 1H, NH); 7.4 (vbs, 4H, NH); 7.71 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 18.55 and 19.83 (C-4′); 22.18 and 22.33 (2C, CH_3); 28.82 (C-3′); 45.71 (N–CH); 57.71 (C-2′); 61.67 (C-1′); 119.74 (C-5); 135.38 (C-8); 151.67 (C-4); 158.42 (C-2); 159.76 (N–C); 159.97 (C-6). For $C_{16h24}N_8O\cdot 1/3$ EtOH$\cdot 1/3$ HCl (320.39) calcd: C, 50.63; H, 7.63; N, 32.21. Found: C, 50.61; H, 7.50; N, 32.55.

6.1.9. 2-{[(2S)-2-Amino-3-methylbutyl]oxy}-6-guanidino-9-isopropyl-9H-purine (**16j**) and 6-guanidino-2-{[(1S)-1-(hydroxymethyl)-2-methylpropyl]amino}-9-isopropyl-9H-purine (**16k**)

General procedure; (2*S*)-(−)-2-amino-3-methyl-1-butanol (1 g, 10 mmol); 10 h; RT; compound **16j**: yield 0.12 g, 38%; FABMS: 321.0 [MH^+] (100). $[\alpha]_D$ +12.6° (*c* 0.17, methanol); ^{1}H NMR (DMSO-d_6): 0.89 (d, 3H, *J*(4′,3′) = 6.8, CH_3); 0.93 (d, 3H, *J*(4′,3′) = 6.8, CH_3); 1.48 (d, 6H, *J*(CH_3, CH) = 6.8, CH_3); 1.72 (m, 1H, CH-3′); 2.83 (m, 1H, CH-2′); 3.99 (dd, 1H, *J*(1′b–2′) = 7.1, J_{gem} = 10.4, 1′b); 4.19 (dd, 1H, *J*(1′a–2′) = 5.2, J_{gem} = 10.4, 1′a); 4.61 (br, 1H, *J*(CH, CH_3) = 6.8, N–CH); 7.30 (vbs, 4H, NH); 7.96 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 17.52 and 19.74 (C-4′); 22.29 (2C, CH_3); 30.34 (C-3′); 46.15 (N–CH); 55.07 (C-2′); 70.10 (C-1′); 122.08 (C-5); 137.60 (C-8); 151.08 (C-4); 159.92 (N–C); 160.21 (C-2); 160.75 (C-6).

Compound **16k**: yield 0.12 g, 28%; FABMS: 321.0 [MH^+] (100). $[\alpha]_D$ −35.6° (*c* 0.24, methanol); ^{1}H NMR (DMSO-d_6): 0.90 (d, 3H, *J*(4′,3′) = 6.9, CH_3); 0.91 (d, 3H, *J*(4′,3′) = 6.9, CH_3); 1.45 (d, 3H, *J*(CH_3, CH) = 6.9, CH_3); 1.46 (d, 3H, *J*(CH_3, CH) = 6.8, CH_3); 1.97 (m, 1H, CH-3′); 3.49 (m, 2H, CH_2-1′); 3.75 (brs, 1H, CH_2-2′); 4.52 (br, *J*(CH–CH_3) = 6.8, CH–N); 4.58 (brs, 1H, OH); 6.13 (br, 1H, NH); 7.4 (vbs, 4H, NH); 7.71 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 18.55 and 19.83 (C-4′); 22.18 and 22.33 (2C, CH_3); 28.82 (C-3′); 45.71 (N–CH); 57.71 (C-2′); 61.67 (C-1′); 119.74 (C-5); 135.38 (C-8); 151.67 (C-4); 158.42

(C-2); 159.76 (N–C); 159.97 (C-6). For $C_{16h24}N_8O\cdot 4/5$ MeOH (320.39) calcd: C, 51.37; H, 7.92; N, 32.38. Found: C, 51.23; H, 7.41; N, 32.31.

*6.1.10. 2-Cyclopropylamino-6-guanidino-9-isopropyl-9H-purine (**16l**)*

General procedure; cyclopropylamine (2.3 ml, 32.6 mmol); 4 h, RT; yield 0.25 g, 25%; FABMS: 275.1 [MH^+] (100). ^{1}H NMR (DMSO-d_6): 0.43 and 0.62 (m, 2H, 2× CH_2); 1.48 (d, 6H, $J(CH_3, CH) = 6.7$, CH_3); 2.60 (m, 1H, N–CH); 4.55 (sept, 1H, $J(CH, CH_3) = 6.7$, N–CH); 6.72 (brs, 1H, NH); 7.50 (brs, 4H, NH); 7.77 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 6.68, 2C, (CH_2); 22.27 (2C, CH_3); 24.20 (N–CH); 45.50 (N–CH); 119.79 (C-5); 135.70 (C-8); 151.86 (C-4); 158.75 (C-6); 159.68 and 160.02 (N–C and C-2). For $C_{12}H_{18}N_8\cdot 0.5H_2O\cdot HCl$ (274.32) calcd: C, 45.37; H, 6.26; N, 32.66; Cl 11.09. Found: C, 45.07; H, 6.30; N, 35.04; Cl 10.92.

*6.1.11. 6-Guanidino-9-isopropyl-2-(2S)-(pyrrolidin-2-ylmethoxy)-9H-purine (**16m**) and 6-guanidino-2-[2-(2S)-(hydroxymethyl)pyrrolidin-1-yl]-9-isopropyl-9H-purine (**16n**)*

General procedure; (*S*)-(+)-2-(hydroxymethyl)pyrrolidine (1.43 ml, 14 mmol); 10 h; RT; compound **16m**: yield 0.07 g, 16%; FABMS: 318.0 [MH^+] (10). $[\alpha]_D$ +5.0° (*c* 0.28, methanol); ^{1}H NMR (DMSO-d_6): 1.43 (m, 1H, 3′b); 1.48 (d, 6H, $J(CH_3, CH) = 6.8$, CH_3); 1.65 (m, 2H, CH-4′); 1.82 (m, 1H, 3′a); 2.79 (m, 2H, CH_2-5′); 3.68 (m, 1H, CH-2′); 4.02 (dd, 1H, $J(CH_2–2') = 6.1$, $J_{gem} = 10.3$, O–CH_2b); 4.06 (dd, 1H, $J(CH_2–2') = 6.7$, $J_{gem} = 10.3$, O–CH_2a); 4.61 (br, 1H, J(CH, CH_3) = 6.8, N–CH); 7.40 (vbs, 4H, NH); 7.95 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.27 (2C, CH_3); 25.24 (C-4′); 28.65 (C-3′); 46.09 (N–CH); 46.25 (C-5′); 56.70 (C-2′); 70.32 (O–CH_2); 122.05 (C-5); 137.49 (C-8); 151.06 (C-4); 159.89 (N–C); 160.23 (C-2); 160.72 (C-6).

Compound **16n**: yield 0.22 g, 48%; FABMS: 319 [MH^+] (100). $[\alpha]_D$ −83.4° (*c* 0.33, methanol); ^{1}H NMR (DMSO-d_6): 1.47 (d, 6H, $J(CH_3, CH) = 6.8$, CH_3); 1.83–1.99 (m, 4H, CH_2-3′, 4′); 3.37 (m, 1H, 5′b, CH_2–O); 3.49 (m, 1H, 5′a); 3.66 (bdt, $J(CH_2–OH) = J(CH_2$-2′) = 4.1, $J_{gem} = 10.2$, CH_2–O); 4.04 (brs, CH_2-2′); 4.54 (br, 1H, J(CH, CH_3) = 6.8, N–CH); 4.90 (brs, 1H, OH); 7.3 (vbs, 4H, NH); 7.74 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.01 (2C, CH_3); 23.21 (C-4′); 28.00 (C-3′); 45.85 (N–CH); 47.83 (C-5′); 59.46 (C-2′); 62.48 (O–CH_2); 119.73 (C-5); 135.81 (C-8); 151.64 (C-4); 156.48 (C-2); 159.95 (N–C); 160.09 (C-6). For $C_{12}H_{20}N_8O\cdot 1/3$ MeOH (292.34) calcd: C, 52.32; H, 7.15; N, 34.05. Found: C, 52.45; H, 6.97; N, 34.15.

*6.1.12. 6-Guanidino-9-isopropyl-2-(2R)-(pyrrolidin-2-ylmethoxy)-9H-purine (**16o**) and 6-guanidino-2-[2-(2R)-(hydroxymethyl)pyrrolidin-1-yl]-9-isopropyl-9H-purine (**16p**)*

General procedure; (*R*)-(−)-2-(hydroxymethyl)pyrrolidine (1.43 ml, 14 mmol), 10 h; RT; compound **16o**: yield 0.07 g, 15%; FABMS: 319.0 [MH^+] (40). $[\alpha]_D$ −8.6° (*c* 0.21, methanol); ^{1}H NMR (DMSO-d_6): 1.43 (m, 1H, 3′b); 1.48 (d, 6H, $J(CH_3, CH) = 6.8$, CH_3); 1.65 (m, 2H, CH-4′); 1.82 (m, 1H, 3′a); 2.79 (m, 2H, CH_2-5′); 3.68 (m, 1H, CH-2′); 4.02 (dd, 1H, $J(CH_2–2') = 6.1$, $J_{gem} = 10.3$, O–CH_2b); 4.06 (dd, 1H, $J(CH_2–2') = 6.7$, $J_{gem} = 10.3$, O–CH_2a); 4.61 (br, 1H, J(CH, CH_3) = 6.8, N–CH); 7.40 (vbs, 4H, NH); 7.95 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.27 (2C, CH_3); 25.24 (C-4′); 28.65 (C-3′); 46.09 (N–CH); 46.25 (C-5′); 56.70 (C-2′); 70.32 (O–CH_2); 122.05 (C-5); 137.49 (C-8); 151.06 (C-4); 159.89 (N–C); 160.23 (C-2); 160.72 (C-6).

Compound **16p**: yield 0.23 g, 50%; FABMS: 319.0 [MH^+] (50). $[\alpha]_D$ +94.3° (*c* 0.53, methanol); ^{1}H NMR (DMSO-d_6): 1.47 (d, 6H, $J(CH_3, CH) = 6.8$, CH_3); 1.83–1.99 (m, 4H, CH_2-3′, 4′); 3.37 (m, 1H, 5′b, CH_2–O); 3.49 (m, 1H, 5′a); 3.66 (bdt, $J(CH_2–OH) = J(CH_2$-2′) = 4.1, $J_{gem} = 10.2$, CH_2–O); 4.04 (brs, CH_2-2′); 4.54 (br, 1H, J(CH, CH_3) = 6.8, N–CH); 4.90 (brs, 1H, OH); 7.3 (vbs, 4H, NH); 7.74 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.01 (2C, CH_3); 23.21 (C-4′); 28.00 (C-3′); 45.85 (N–CH); 47.83 (C-5′); 59.46 (C-2′); 62.48 (O–CH_2); 119.73 (C-5); 135.81 (C-8); 151.64 (C-4); 156.48 (C-2); 159.95 (N–C); 160.09 (C-6). For $C_{16h22}N_8O\cdot$ ½ EtOH (318.38) calcd: C, 52.77; H, 7.38; N, 32.82. Found: C, 52.75; H, 7.06; N, 32.98.

*6.1.13. 2-Chloro-6-guanidino-9-isopropyl-9H-purine (**17**)*

2,6-Dichloro-9-isopropyl-9*H*-purine [29] was treated with a solution of guanidine [21] at RT. The precipitate was filtered and filtrate was purified by column chromatography (5–15% ethanol:EtOAc followed by EtOAc:acetone:EtOH:H_2O, 6:1:1:0.5). Yield 3.21 g, 75%. FABMS: 254.0 [MH^+] (100). ^{1}H NMR (DMSO-d_6): 1.49 (d, 6H, $J(CH_3, CH) = 6.8$, CH_3); 4.67 (sept, 1H, $J(CH, CH_3) = 6.8$, N–CH); 7.40 (br, 4H, NH); 8.19 (s, 1H, H-8). ^{13}C NMR (DMSO-d_6): 22.30 (2C, CH_3); 46.56 (N–CH); 124.50 (C-5); 139.36 (C-8); 150.57 (C-2); 150.87 (C-4); 160.16 (C-6); 160.34 (N–C). For $C_9H_{12}ClN_7\cdot 1/4H_2O$ (253.69) calcd: C, 41.87; H, 4.88; N, 37.97; Cl, 13.73. Found: C, 42.16; H, 4.68; N, 37.82 Cl, 14.05.

6.2. Enzyme inhibition assay

CDK1/cyclin B and CDK2/cyclin E kinases were produced using the baculoviral expression system and purified on a Ni^{2+}-NTA column (Qiagen). The kinase reaction was performed with 1 mg/ml histone H1 in the presence of 15 μM ATP, 0.05 μCi [γ-^{33}P]ATP, and with a test compound in a final volume of 10 μl, all in the reaction buffer (60 mM HEPES–NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM sodium orthovanadate, 1.2 mM DTT, 2.5 μg/50 μl $PEG_{20.000}$). The reaction was stopped by adding 5 μl of 3% aqueous H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose paper (Whatman), washed 3× with 0.5% aqueous H_3PO_4, and finally air-dried. Kinase inhibition was quantified using digital image analyzer FLA-7000 (Fujifilm). The IC_{50} values were determined from dose–response curves.

Kinase selectivity profiling was carried out under the conditions used as described previously [37]. Compounds **16e** and **16g** were screened at a concentration of 1 μM in duplicate. The assays were initiated with ATP (800 cpm/pmol [γ-^{33}P]ATP at 5, 20 or 50 μM in order to be at or below the K_m for ATP for each enzyme), stopped by the addition of phosphoric acid and spotted onto P81 filter plates. Inhibition was expressed as residual kinase activity.

6.3. Cytotoxicity assay

The cytotoxicity of the studied compounds was determined on MCF7 breast cancer cell line. The cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin and maintained at 37 °C, in a humidified environment with 5% CO_2. Approximately 10,000 cells were seeded into a well of a 96-well microtiter plate. After 4 h stabilization, a tested compound in various concentrations was added in triplicates. The final concentration of DMSO never exceeded 0.5%. After 72 h incubation, calcein AM solution (Molecular Probes) was added to the final concentration of 1 μg/ml for 1 h and the fluorescence of free calcein was measured at 485/538 nm (excitation/emission) using Fluoroskan Ascent reader (Labsystem). The viability was expressed as a percentage of the fluorescence of the control cells and the IC_{50} values (the concentration of an inhibitor that is required for 50% decrease of the intracellular esterase activity) were determined by graphical analysis from dose–response curves. The IC_{50} values represent averages of at least three independent experiments.

6.4. Cell cycle analysis

Approximately million cells were fixed with 90% methanol. After rehydration the cells were incubated with primary antibody

against pH3^{Ser10} and then with secondary antibody conjugated with Alexa Fluor 488. DNA was stained with propidium iodide and the fluorescence signal was measured at 488/575 nm (ex/em) for propidium iodide and 488/525 nm (ex/em) for Alexa Fluor 488 using Cell Lab Quanta^{TM} SC flow cytometer (Beckman Coulter). The data were analyzed using the program Multicycle AV for Windows.

6.5. Docking study

The human CDK2, roscovitine and inhibitors **16e**/**16g** and **16i**/**16k** were prepared for docking in the YASARA modelling package [39] on the basis of deposited structure 2A4L [33]. H atoms were added to the protein to mimic neutral pH and their positions were optimized. The glycerol and water molecules were removed from the model. The parameter set used for the protein was AMBER ff03 [40]. The ligand was optimized in a vacuum and partial charges on its atoms were obtained by a restrained fit to the electrostatic potential (RESP) at the AM1BCC level [41]. The ligand was then docked to the protein using the AutoDock program [42]. 1000 poses were obtained using a local search protocol, these were subsequently clustered based on similarity (r.m.s.d. < 5 Å) and then scored.

Contributions

ID, MC, MD, VK and ZJ conceived and designed the experiments. MC performed organic syntheses. ID performed biological assays and ID, VK and JV analyzed biological data set. ID, MC, VK, JV and ZJ wrote the paper. JB performed the docking study. All authors have approved the article.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.01.021.

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