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Original article

Synthesis and in vitro biological evaluation of 2,6,9-trisubstituted purines targeting multiple cyclin-dependent kinases

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ABSTRACT

Several inhibitors of cyclin-dependent kinases (CDKs), including the 2,6,9-trisubstituted purine derivative roscovitine, are currently being evaluated in clinical trials as potential anticancer drugs. Here, we describe a new series of roscovitine derivatives that show increased potency in vitro. The series was tested for cytotoxicity against six cancer cell lines and for inhibition of CDKs. For series bearing 2-(hydroxyalkylamino) moiety, cytotoxic potency strongly correlated with anti-CDK2 activity. Importantly, structural changes that increase biochemical and anticancer activities of these compounds also increase elimination half-life. The most potent compounds were investigated further to assess their ability to influence cell cycle progression, p53-regulated transcription and apoptosis. All the observed biological effects were consistent with inhibition of CDKs involved in the regulation of cell cycle and transcription.

1. Introduction

The cell division cycle is driven by sequential activation of cyclin-dependent kinases (CDKs), enzymes activated primarily by binding to phase-specific protein cyclins. When complexed with cyclins, CDKs are involved in, e.g., changes in the expression of cell cycle-specific genes in the G1 phase required for cell cycle entry, duplication of chromosomes and centrosomes during the S phase, mitotic spindle formation, nuclear membrane breakout, and chromatin condensation in the M phase [1]. CDKs and cyclins, as well as many other proteins that interact with them, are frequently deregulated in cancer cells, causing the cell cycle control mechanisms to be dismantled and hyperactivation of CDKs [1]. These observations have provided a basis for the development of CDK inhibitors as novel anticancer drugs.

To date, more than 20 of the most potent inhibitors have been registered for clinical trials in cancer patients [2]. These compounds are often classified according to their selectivity towards CDKs. The majority of known inhibitors exhibit activity towards a broad range of CDKs, which may be therapeutically advantageous as genetic studies have shown that CDK 2, 4 and 6 are dispensable for the cycling of most cell types, whereas CDK1 has been shown to be absolutely essential for cell proliferation, at least in mice [3–6].

One of the clinically evaluated compounds is the trisubstituted purine derivative roscovitine, which inhibits CDK 1, 2, 5, 7, and 9 [7,8]. By targeting CDK1 and CDK2, roscovitine arrests the cell cycle, while inhibition of transcriptional CDK7 and CDK9 induces apoptosis in cancer cells [9,10]. Roscovitine is currently being evaluated in patients diagnosed with non-small cell lung cancer and nasopharyngeal cancer [11].

Roscovitine is orally bioavailable but displays a strong first-pass effect and rapid clearance [12]. Moreover, it is a relatively low potency drug in comparison with other clinically evaluated CDK inhibitors, such as AT7519 or dinaciclib [13,14]. Clinical studies suggest that b.i.d. dosing is necessary to maintain therapeutically effective concentrations [15]. The aim of the present work was to prepare novel derivatives of roscovitine that possessed enhanced

Abbreviations: CDK, cyclin-dependent kinase; DIAD, diisopropyldiazadicarboxylate; NMP, *N*-methylpyrrolidone; PARP, poly(ADP-ribose)polymerase; TLC, thin layer chromatography.

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anti-kinase and cytotoxic activities as well as structural features that could increase their elimination half-lives. Replacement of the primary hydroxy group in the side-chain with an amino group, or a secondary or tertiary hydroxy moiety was conducted to prevent formation of the carboxylate, which is a dominant and relatively inactive roscovitine metabolite [16,17]. Furthermore, introduction of a basic moiety was expected to increase the affinity of the compounds for tissues [18–20].

2. Results and discussion

2.1. Synthesis

In this paper, a series of novel 2,6,9-trisubstituted purine CDK inhibitors was successfully synthesized through an improved procedure (see Experimental section 4.3., Scheme 1). The identity of all final compounds was confirmed by ^{1}H NMR, ^{1}H–^{1}H COSY, ^{13}C NMR and ^{1}H–^{13}C HSQC spectrometry, mass spectrometry (ESI-MS) and elemental analysis.

The straightforward three-step synthesis of 2,6,9-trisubstituted purines started from commercially available 2,6-dichloropurine, which was in the first step alkylated by isopropyl alcohol via Mitsunobu alkylation [21] at N9 to give 2,6-dichloro-9-isopropylpurine (**1**). A former conventional method for the preparation of this key intermediate used an isopropyl halide as an alkylating agent [22–24], but the disadvantage of this method was a poor regioselectivity as a considerable amount of by-product, which is N7 − isomer, was also formed. The crude product has to be purified by multiple crystallizations or by column chromatography. Moreover, the method uses a toxic and cancerogenic isopropyl halogenide. For this reason we decided to use the Mitsunobu alkylation reaction, which employs isopropanol as an alkylating agent [21]. The reaction temperature was kept within the range of 20–25 °C to minimize the formation of undesired N7 − isomer, on the contrast to Weibing Lu et al. [21], who carried out the alkylation at 70 °C. We have found that the regioselectivity of the alkylation decreased with an increase of the reaction temperature. This method proceeded very smoothly and was much more regioselective in comparison with the halide method. We also employed a lower excess of DIAD (1.2 eq.) and triphenyl phosphine (1.2 eq.), and shorter reaction time (1–2 h) than Weibing Lu, who used 2.1 eq. of both reactants and longer reaction time (6 h). The crude alkyl derivative was purified from contaminating by-products (triphenyl phosphine oxide and traces of N7 − isomer) by crystallization from lower alcohol or flash chromatography.

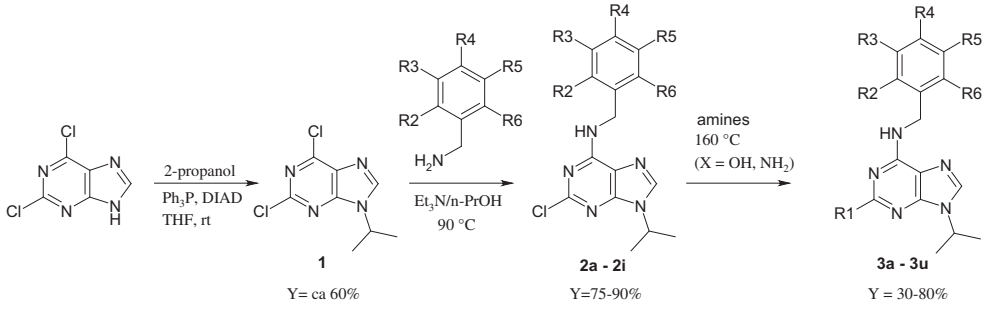
The second step, preparation of 2-chloro-6-(subst. benzylamino)-9-isopropylpurines (**2a**–**2i**, see Experimental section 4.3.2.) was a nucleophilic substitution at C6 purine position with appropriate substituted benzylamine [22,25,26]. The reaction was carried out in *n*-propanol and triethylamine, or *N*,*N*-diisopropyl-*N*-ethyl amine (Hunig's base) was used as an auxiliary base. The reaction temperature was kept within the range of 80–100 °C. The reaction time varied from 3 to 6 h, depending on the reaction temperature and amine reactivity. Crude intermediates were purified by crystallization from isopropanol, if necessary. The yields were satisfactory (70–85%) in all cases.

The final step was accomplished by previously described method [7,22,25,26]. The appropriate aminoalcohol or diamine for the S_N2 substitution at C2 was used in excess (5–20 equiv.) and Hunig's base was employed as an auxiliary base in several cases. When a C2 substituent was a rare sterically hindered amine (4-amino-2-methylbutan-2-ol, 1-amino-2-methylpropan-2-ol, (2*RS*,3*R*)-3-aminopentan-2-ol, (*S*)-3-amino-2-methylbutan-2-ol), only a small excess of amine (5–7 equiv.) was used, in the presence of an auxiliary base (DIPEA), and *N*-methylpyrrolidone was used as a solvent. The reaction temperature was kept at 150–160 °C (sealed tube) and the reaction time varied from several hours in case of reactive amines (all C2 − roscovitine amine derivatives, **3f**, **3n**, **3o**, piperazine derivatives) to 72 h in cases of less reactive sterically hindered aminoalcohols (**3b**–**e**, **3q**, **3r**, **3t**). Yields varied from 60 to 80% in cases of usage of reactive roscovitine type aminoalcohols to 20–40% in cases of sterically hindered secondary and tertiary aminoalcohols.

Crude products were purified by crystallization from diethyl ether and finally re-crystallized from mixture of ethyl acetate and hexanes (1:2). The majority of final compounds were crystallized with exception of **3c**, **3e**, **3n**, **3q**, **3r**, **3t**, which had to be purified by flash chromatography. The reaction conditions including yields were optimized in some cases for the future possibility of a large scale production.

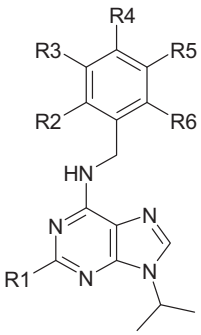
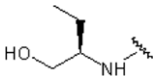
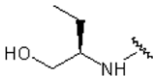
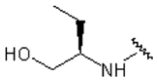
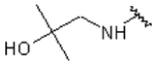
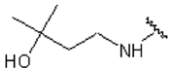
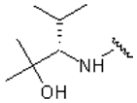
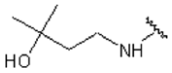
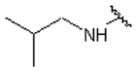
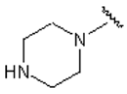
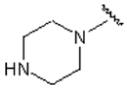
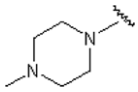
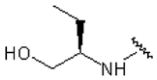
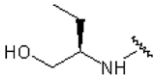
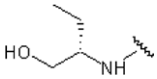
2.2. Structure–activity relationships in biological assays

From a structural point of view, the prepared compounds differ from roscovitine by substitutions at positions 2 and 6. As in roscovitine, all compounds bear an isopropyl substitution at position 9, which was previously shown to be optimal for purine CDK inhibitors [7,27,28]. All newly synthesized purines were tested for cytotoxicity against various cancer cell lines (MCF7, K562, HOS, CEM, HCT-116, and G361) and for CDK2/5/7/9 kinase inhibition, according to established protocols [25,29]. The resulting data are presented in Table 1 and clearly show that most of new compounds are significantly more potent CDK2 inhibitors than the reference compound roscovitine, and about half of them also had higher cytotoxicity. These results confirmed previous data with another library of purine inhibitors where correlation between inhibition of CDK and antiproliferative activity was shown [32]. On the other hand, cytotoxic activities for a number of C2-piperazine derivatives (**3g**–**i**, **3m**) that have not shown reliable anti-kinase activity could be probably explained by additional off-targets as documented e.g. for roscovitine with pyridoxalkinase.



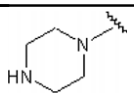
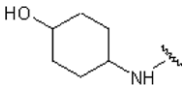
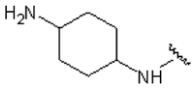
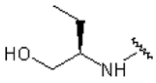
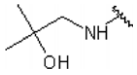
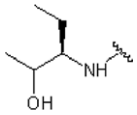
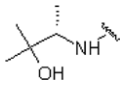
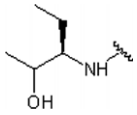
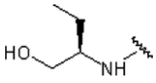
Scheme 1. Synthesis of 2,6,9-trisubstituted purine CDK inhibitors. The numbering of substituents (R1–R6) corresponds to the description of structures given in Table 1.

Table 1
Structures and biological activity of prepared compounds.

General structure	Compound	R1	R2	R3	R4	R5	R6	IC_{50}	
								CDK2 (nM)	Cytotoxicity[a] (μM)
	Roscovitine		H	H	H	H	H	180	22.5
	Olomoucine II		OH	H	H	H	H	50	10.6
	3a		H	H	H_2N	H	H	20	31.9
	3b		NH_2	H	H	H	H	20	17.1
	3c		NH_2	H	H	H	H	790	100
	3d		NH_2	H	H	H	H	70	23.2
	3e		OH	H	H	H	H	260	51.6
	3f		OH	H	H	H	H	220	16.7
	3g		H	H	OH	H	H	340	9.2
	3h		H	H	H_3CO	H	H	890	7.7
	3i		H–	H–	H_3CO–	H–	H–	890	12.6
	3j		H_3CO–	H_3CO–	H–	H–	H–	31	40.5
	3k		OH–	H_3CO–	H–	H–	H–	60	22.2
	3l		OH–	H_3CO–	H–	H–	H–	59	9.0

(*continued on next page*)

Table 1 (*continued*)

General structure	Compound	R1	R2	R3	R4	R5	R6	IC_{50}	
								CDK2 (nM)	Cytotoxicity[a] (μM)
	3m		OH–	H_3CO–	H–	H–	H–	630	20.3
	3n		OH–	H–	H–	Cl–	H–	61	6.0
	3o		OH–	H–	H–	Cl–	H–	25	2.4
	3p		OH–	H–	H–	Cl–	H–	9.4	4.4
	3q		OH–	H–	H–	Cl–	H–	19.0	4.1
	3r		OH––	H–	H–	Cl–	H–	13.1	2.4
	3s		OH–	H–	H–	Cl–	H–	24.3	1.8
	3t		OH–	H–	H–	F–	H–	11.8	2.1
	3u		OH–	H–	H–	F–	H–	19.0	4.6

[a] Cytotoxicity averaged from measurements on a panel of 6 cancer cell lines. Full results available in the Supplementary data.

Several reports in the literature have demonstrated that a hydroxy substituent on the benzyl ring enhances CDK inhibitory activity [25,26,30,31]; an example of such a compound is olomoucine II, which has a 2-hydroxybenzyl side chain [25,32]. Except of derivatives bearing hydroxy group on the benzyl ring (**3e**–**g**) we introduced also compounds with amino group (**3a**–**d**) as well as some derivatives with methoxy group (**3h**, **3i**). CDK activity of compounds bearing this type of substituents at position 2 and different at position 6 (**3c** vs **3e**, R2 = NH_2 vs OH; **3g** vs **3h**, R2 = OH vs OCH_3) demonstrates this positive effect of hydroxy substituent (see Table 1). An addition of methoxy group at meta position (**3j**–**3l**) sustained high anti-CDK2 activity of 2-substituted compounds. A positive effect on CDK inhibition has also been shown for halogenated benzyl derivatives [26,27]. Our results indicate that the combination of both structural motives may be advantageous because substitution of hydrogen in the olomoucine II R5 position with a halide group at R5 further improved inhibitory activity (**3p**, **3u**). Overall, the 2-hydroxy-5-chloro derivatives **3o**–**s** and 2-hydroxy-5-fluoro derivatives **3t** and **3u** were the most potent among the synthesized novel compounds, both in terms of their anti-CDK2 and cytotoxic activity.

Next, we explored changes at the purine position 2 in compounds where the position 6 was occupied by substituted benzylamine. We introduced alkylamino (**3f**), aminoalkylamino (**3g**–**i**, **3m**, **3o**) and hydroxyalkylamino group with primary (**3a**, **3j**–**l**, **3p**, **3u**), secondary (including hydroxycycloalkyl) (**3n**, **3r**, **3t**) or tertiary hydroxyls (**3b**–**e**; **3q**; **3s**) at position 2. A high activity against CDK2 was observed for the structurally diverse 2-hydroxyalkylamino derivatives, which clearly correlated with antiproliferative activity (rank correlation $\rho = 0.72$, $p < 0.01$). While branching of hydroxyalkylamino substituents at position 2 did not have a pronounced effect (**3u** vs. **3t**; **3p** vs. **3r** vs. **3q**), an increase in the distance between the N2 nitrogen and the hydroxy group resulted in a loss of the activity for the compound pairs **3b**–**c** and (to lesser extent) **3p**–**n**. The high activity of **3o** in both the kinase and cytotoxicity assays suggests that not only the terminal hydroxyl (**3n**) but also the terminal amino group (**3o**) may take part in favorable binding interactions within the active site of CDKs. In

contrast, introduction of a piperazine-1-yl group at position 2 of the purine ring (**3g**–**i**, **3m**) led to a marked decrease in activity against CDK2. Since the latter compounds were all highly active against the studied cancer cell lines, their effect is probably mediated by interference with targets other than CDKs. Comparable activities of **3h** and its *N*-methylated derivative **3i** suggest that the terminal amino group acts as a basic center rather than a hydrogen bond donor in this interaction.

We, and others, have previously found that oxidation of the terminal hydroxyl at the purine position 2 to carboxylate is a major route of metabolic inactivation of roscovitine and related inhibitors in vivo [8,16,33], and protection against oxidation contributes to lower metabolic clearance [17]. Overall, the observed structure–activity relationships demonstrate that compounds in which this oxidizable group is replaced by a secondary or tertiary hydroxy or even an amino group, which do not readily convert to carboxylate, retain strong anti-CDK activity. This is in agreement with findings published recently by Wilson et al. [17], who studied similar CDK inhibitors with oxidizable group at position and showed increased stability towards mouse microsomes and promising in vivo PK properties and efficacy in vivo following oral administration for candidate compound αSβR-21 [17] 2. The analogous compounds in our study, **3b** and **3q**–**t**, belong to the most active in the described series of compounds, with IC_{50} values below 20 nM.

Several recent reports have suggested that CDK inhibition in cells causes accumulation of tumor suppressor p53 in an active form [34,35]. A positive relationship between inhibition of CDK and p53-regulated transcription is clear also for this series of compounds; rank correlation between IC_{50} for CDK 2, 5, 9 and concentration inducing p53 in cell reporter system $\rho > 0.71$ ($p < 0.05$). The most potent CDK inhibitors **3o**–**r**, **3u** and **3t** induced p53-regulated transcription at low micromolar concentrations, as assessed by a cellular reporter assay. Molecular mechanisms of induced p53-dependent transcription are not yet fully understood, but our recent studies suggest that inhibition of CDK9 (an essential transcriptional activator) leads to down-regulation of HDM2 (a negative regulator of p53), which in turn stabilizes p53 [36,37]. Therefore, in the present study we investigated the inhibitory activity of the most potent compounds towards CDKs other than CDK2, including CDK5, CDK7 and CDK9. As shown in Table 2, all compounds that potently inhibited CDK2 also exhibited a strong activity towards CDK5 and CDK9, as expected based on the high sequence and structural similarities of their active sites [38]. Interestingly, comparison of the selectivity profiles and effects on p53-regulated transcription (Table 2) suggest that activation of p53 may not be caused only by inhibition of CDK9, but also CDK2, and perhaps other kinases that were not screened. This can be seen by comparing **3r** and **3t** with **3o**, which shows that the latter produces 2-fold lower and 2-fold higher CDK2 and CDK9 inhibition, respectively. Increased potency and selectivity of **3o** towards CDK9 was however not reflected by an increased ability to activate p53.

2.3. Cellular effects of selected compounds

Compound **3r** was selected for this study as the most potent inhibitor among the series of compounds showing over 13-fold higher activity versus roscovitine in terms of CDK inhibition that corresponds with over 9-fold increase in cytotoxicity on a panel of six cancer cell lines (see Table 1). The antiproliferative activity of **3r**, was measured in an asynchronously growing colon carcinoma cell line, HCT-116, and a chronic myeloid leukemia cell line, K562. As shown in Fig. 1, inhibitor **3r** potently arrested cells in late S and G2/M phases. This effect was particularly evident in HCT-116 cells, where about 15% more cells were in the G2/M phase of the cell cycle compared to control cells after the treatment with 5 μM **3r**. Staining treated HCT-116 and K562 cells with 5-bromo-2′-deoxyuridine (BrdU) also revealed a decrease in DNA replication (Fig. 1C) in a dose-dependent manner.

Table 2
Biochemical and cellular activities of selected compounds. CDK inhibitory selectivity was determined in biochemical kinase assays; maximum activation of p53 was determined in a cellular reporter system.

Compound	IC_{50} (nM)				Maximum p53 activation (μM)
	CDK2	CDK5	CDK7	CDK9	
Roscovitine	180	1080	793	2694	24.5
Olomoucine II	51	270	n.a.	815	11.4
3b	20	740	1200	1240	16.8
3c	790	>5000	n.a.	>5000	>100
3d	70	675	290	845	24.0
3e	260	4485	n.a.	>5000	50.0
3f	220	2360	n.a.	3130	>100
3n	61	550	540	910	14.6
3o	25	125	160	39	3.4
3p	9	150	n.a.	285	10.0
3q	19	69	435	n.a.	4.1
3r	13	71	97	165	2.1
3s	24	n.a.	n.a.	n.a.	1.8
3t	11	67	180	100	2.5
3u	19	240	n.a.	106	6.6

n.a. – not available.

We next monitored levels of phosphorylation of RNA polymerase II, which is a substrate of CDK7 and CDK9, in cells treated with **3r**. Immunoblotting analysis revealed a rapid decrease in phosphorylation at serines 2 and 5 (Fig. 2A, B), confirming cellular inhibition of these two kinases. Several recent reports have suggested that inhibition of CDK9 (and inhibition of transcription in general) in cells leads to accumulation of the tumor suppressor p53 in an active form [34,35]. Therefore, we investigated the effect of treating colorectal carcinoma HCT-116 cells with **3r**. We found that **3r** rapidly increased the expression of p53 and p53-regulated $p21^{WAF1}$ at concentrations of 1 μM and higher (Fig. 2C). Accumulation of p53 was accompanied by decreased expression of Mdm-2, which is a negative regulator of p53. We also observed the same effect in a reporter assay using cell line Arn-8. **3r** exerted a dose-dependent effect on p53 transcriptional activation, with the maximum effect obtained at a concentration of 2 μM (Fig. 2D).

The strong cytotoxicity of many of the observed potent CDK inhibitors towards different cancer cell lines prompted us to analyze the mechanism of cell death induced by **3r** in HCT116 and K562 cells. The results of an immunoblotting analysis of several proteins involved in apoptotic cell death are shown in Fig. 3A and B. While expression of PUMA, Bcl-2 and caspase-3 remained largely unchanged in both cell lines treated with **3r**, the level of anti-apoptotic protein Mcl-1 showed a large dose-dependent decrease. Another typical apoptotic marker, an 89 kDa fragment of poly(ADP-ribose)polymerase (PARP), was detected in treated cells. A fluorimetry-based caspase-3/7 activity assay of lysates of HCT-116 and K562 cells treated with **3r** revealed potent dose-dependent activation of the caspase in K562 but only weak activation in HCT-116 cells (Fig. 3C).

3. Conclusions

This study involved the synthesis and modification of the biological activity of novel purine CDK inhibitors derived from roscovitine by changing the moieties at positions 2 and 6. Many of the prepared compounds proved to be more potent at limiting the proliferation of the tested cancer cell lines than roscovitine and olomoucine II. For the 2-hydroxyalkylamino derivatives studied,

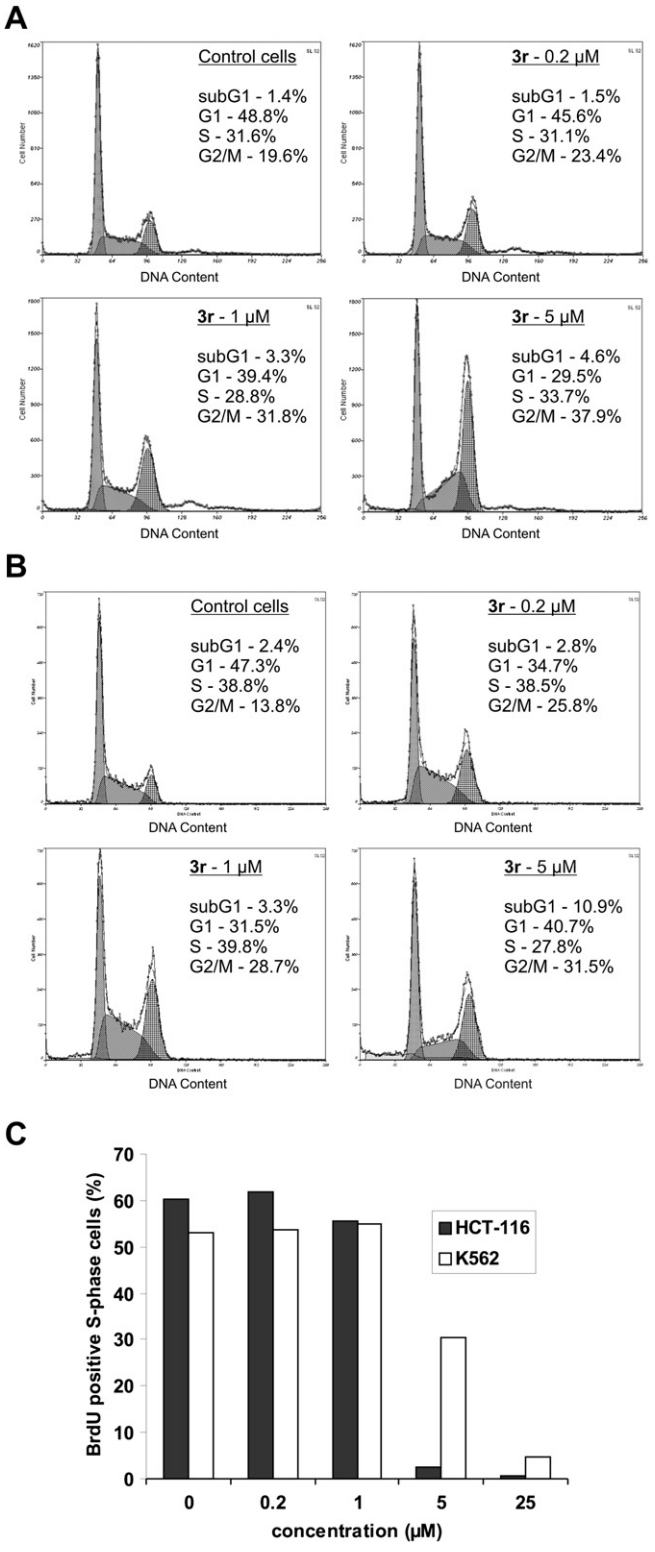


Fig. 1. Effect of **3r** on the cell cycle of HCT-116 and K562 cancer cell lines following 24 h treatment. Flow cytometric analysis of cell cycle after propidium iodide staining in (A) HCT-116 and (B) K562 cell lines, and (C) quantification of actively-replicating BrdU positive S phase cells in both cell lines.

cytotoxic potency strongly correlated with anti-CDK2 activity. However, highly cytotoxic compounds with piperazin-1-yl group at position 2 were only weak CDK2 inhibitors. Importantly, structural changes that were expected to increase the elimination half-life did not abolish biological activity. Example compound **3r** blocked cell cycle progression and induced apoptosis in cells as a result of transcriptional perturbations due to reduced phosphorylation of Ser-2 and Ser-5 in the C-terminal domain of RNAP-II, caused by inhibition of CDK9 and CDK7. Pharmacokinetic studies of selected derivatives are currently underway.

4. Experimental

4.1. General procedures

The elemental contents of the prepared compounds were determined using an EA1108 CHN analyzer (Thermo Finnigan). Their melting points were determined using a Büchi Melting Point B-540 apparatus. Thin layer chromatography (TLC) was performed using silica gel 60 WF_{254} plates (Merck) with a mobile phase ($CHCl_3$:MEOH:conc. NH_4OH, 8:2:0.2, v/v/v). Flash chromatography was performed using a VersaFlash purification station (Supelco) coupled to a 2110 Fraction Collector (Bio-Rad). Compounds were separated on VersaPak Cartridges (25 × 100 mm, Supelco) containing 23 g of spherical silica and eluted with a mobile phase ($CHCl_3$:MeOH, 90:10, v/v). To determine their HPLC purity, samples were dissolved in HPLC mobile phase (initial conditions), applied to an RP-column (150 mm × 4.6 mm, 5 μm, Microsorb C18; Varian) and the separated constituents were eluted with a linear methanolic gradient (10–90% over 30 min, pH adjusted to 4 using ammonium formate) at a flow rate of 0.6 ml/min. Eluting compounds were detected by scanning the UV absorbance of the eluate between 240 and 300 nm. CI+ and EI+ mass spectra were recorded using a Polaris Q (Finnigan) mass spectrometer equipped with a Direct Insertion Probe (DIP). The compounds were heated in an ion source with a 40–450 °C temperature gradient, the mass monitoring interval was 50–1000 am, and spectra were collected using 1.0 s cyclical scans, applying 70 eV electron energy. In the CI+ ionization mode, isobutane was used as a reagent gas at a flow rate of 2 l/h. The mass spectrometer was directly coupled to an Xcalibur data system. NMR spectra were acquired using a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz (^1H). Samples were prepared by dissolving compounds in DMSO-d_6 and tetramethylsilane (TMS) was used as the internal standard.

4.2. Chemicals

2,6-Dichloropurine and 4-aminomethylphenol were obtained from OlChemim. Triphenylphosphine, diisopropyldiazadicarboxylate (DIAD), 2-aminobenzylamine, 4-aminobenzylamine, 4-methoxybenzylamine, *R*,*S*-2-amino-1-butanol, *S*-valinol, 1,4-*trans*-diaminocyclohexane, azacycloheptane, piperazine, *N*-methylpiperazine were purchased from Sigma–Aldrich. Variously substituted benzylamines were prepared via reduction of oximes of corresponding substituted benzaldehydes [39]. Secondary and tertiary aminoalcohols, as (2*RS*, 3*R*)-3-aminopentan-2-ol, (*S*)-3-amino-2-methylbutan-2-ol, 1-amino-2-methylpropan-2-ol, 4-amino-2-methylbutan-2-ol and 2,4-dimethyl-3-aminopentan-2-ol were synthesized according the described procedure [40]. Lach-Ner supplied methanol, 2-propanol, chloroform, diethyl ether, dimethylformamide, ethyl acetate, anhydrous magnesium sulfate. Milli-Q water was used throughout. Solvents and chemicals used were all of standard p.a. quality.

4.3. Synthesis of 2,6,9-trisubstituted purines

4.3.1. 2,6-Dichloro-9-isopropylpurine (*1*)

2,6-Dichloropurine (1.89 g; 0.01 mol) was dissolved under a nitrogen atmosphere in a mixture of tetrahydrofuran (40 ml) and

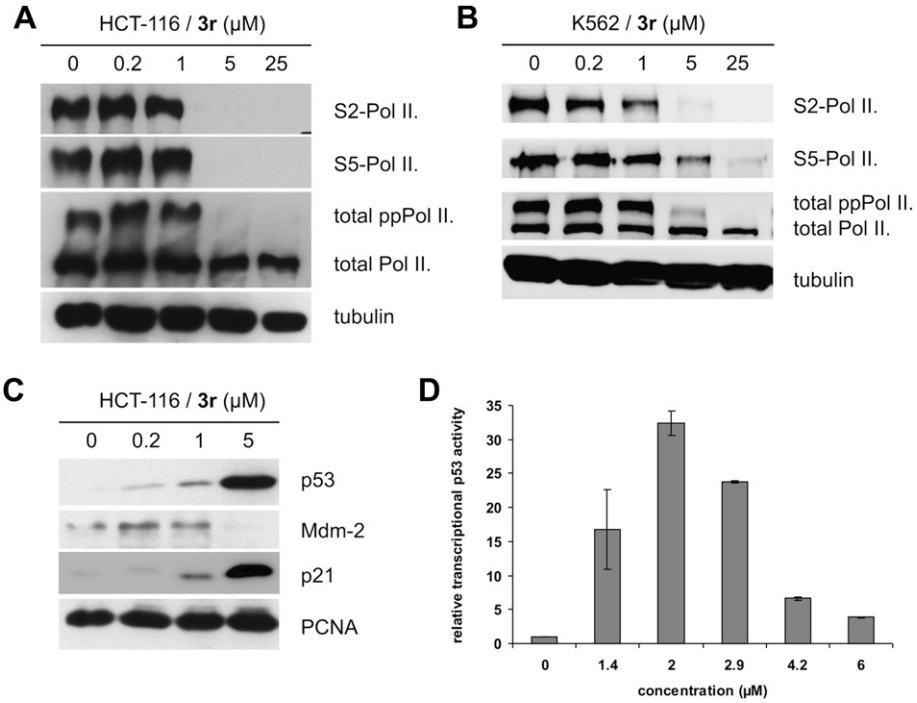


Fig. 2. Effect of **3r** on transcription and activation of p53 in HCT-116 and K562 cell lines following 24 h treatment. (A, B) Immunoblotting analysis of phosphorylation of RNA polymerase II at serines 2 and 5. Tubulin levels were detected to verify equal protein loading. (C) Immunoblotting of p53, $p21^{WAF1}$ and Mdm-2 in HCT-116 cells; PCNA levels were detected to verify equal protein loading. (D) Analysis of relative p53-dependent transcriptional activity by β-galactosidase reporter assay of Arn-8 cell line.

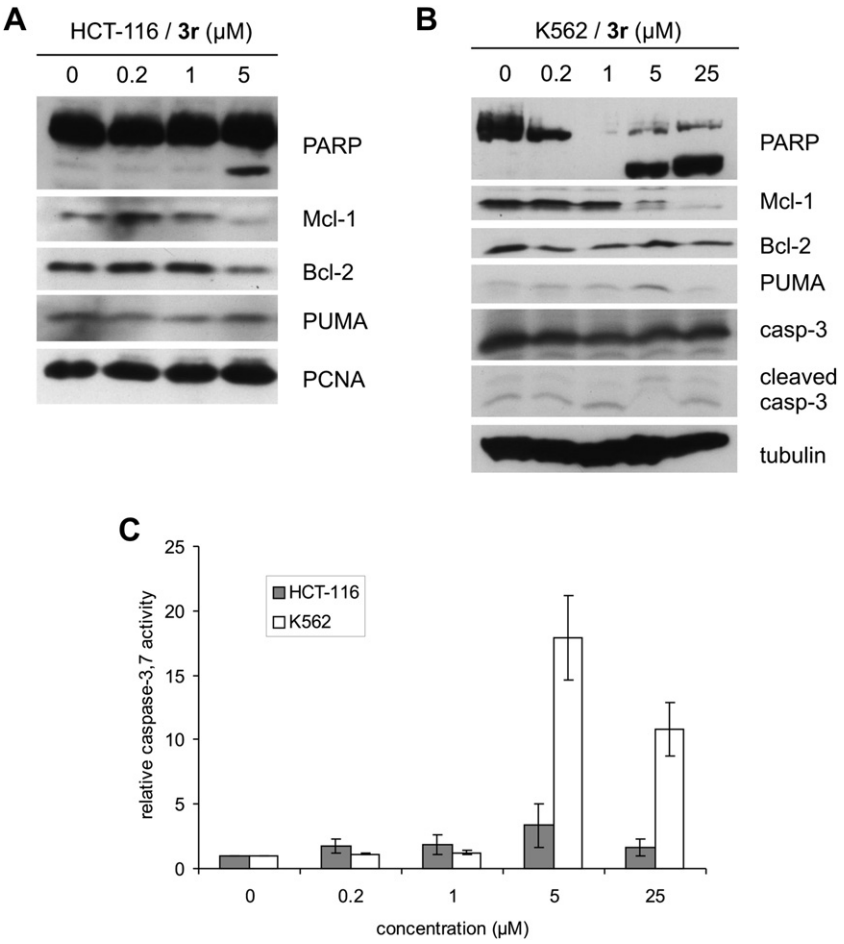


Fig. 3. Effect of **3r** on induction of apoptosis in HCT-116 and K562 cell lines following 24 h treatment. (A, B) Immunoblotting analysis of selected apoptotic markers. PCNA and tubulin levels were detected to verify equal protein loading. (C) Fluorimetric caspase-3,7 activity assay based on the cleavage of a specific Ac-DEVD-AMC peptide substrate.

isopropanol (4 ml; 0.06 mol), and then triphenyl phosphine (3.14 g; 0.012 mol) and DIAD (2.4 ml; 0.012 mol; dropwise) were added. The temperature of reaction mixture was maintained at 20–25 °C during addition of DIAD. The reaction mixture was the stirred for 2 h at 20–25 °C. TLC (mobile phase: ethyl acetate – toluene; 1:1) after 2 h indicated that the reaction had gone to completion. The reaction mixture was then evaporated to a thick yellow residue, which was dissolved in hot (60 °C) methanol (30 ml). After crystallization in a refrigerator (−18 °C) overnight, the crystalline mass was collected by filtration, washed with cold (−10 °C) methanol (2 × 5 ml) and dried in the vacuum drying oven (60 °C) to constant weight. Yield: 1.20 g of almost white crystalline powder (52%). The purity (HPLC): >98%.

M.p. = 149–152 °C. Physico-chemical data including ^{1}H NMR were in accordance with those published [21–24]. The second crop (up to 10%) of product of lower purity could be obtained from mother liquor.

*4.3.2. 2-Chloro-6-(subst. benzylamino)-9-isopropylpurines (**2a**–**2i**)*

2,6-Dichloro-9-isopropylpurine (0.05 mol), appropriate substituted benzylamine (0.05 mol), *n*-propanol (230 ml) and triethylamine (0.15 mol) were placed into a reaction bulb, under nitrogen. The reaction mixture was warmed up to 90 °C, and stirred for a period of 4 h. The contents of the bulb were then evaporated on rotary vacuum evaporator to give a semisolid residue, which was then treated with water (200 ml) for 20 min. The precipitate was filtered off, washed with water (3 × 20 ml) and dried in the vacuum oven to constant weight. Yield: 75–90%, according to the type of benzylamine used. The purity (HPLC): min. 97%. The crude product can be purified by crystallization from isopropyl alcohol if required.

According to this procedure the following compounds were prepared:

*4.3.2.1. (4-Aminobenzyl)-(2-chloro-9-isopropyl-9H-purin-6-yl)-amine (**2a**).* ^{1}H NMR (DMSO-d_6): 1.49 (d, J = 6.6, 6H, CH_3), 4.44 (d, J = 6.7, 2H, CH_2), 4.66 (sep, J = 6.6, 1H, CH), 5.21 (s(br), 2H, NH_2), 6.49 (t, J = 7.0, 1H, ArH), 6.62 (d, J = 7.8, 1H, ArH), 6.94 (t, J = 7.0, 1H, ArH), 7.08 (d, J = 7.8, 1H, ArH), 8.28 (s, 1H, CH), 8.67 (t, J = 6.7, 1H, NH).

*4.3.2.2. (2-Aminobenzyl)-(2-chloro-9-isopropyl-9H-purin-6-yl)-amine (**2b**).* ^{1}H NMR (DMSO-d_6): 1.49 (d, J = 6.6, 6H, CH_3), 4.44 (d, J = 6.7, 2H, CH_2), 4.66 (sep, J = 6.6, 1H, CH), 5.21 (s(br), 2H, NH_2), 6.49 (t, J = 7.0, 1H, ArH), 6.62 (d, J = 7.8, 1H, ArH), 6.94 (t, J = 7.0, 1H, ArH), 7.08 (d, J = 7.8, 1H, ArH), 8.28 (s, 1H, CH), 8.67 (t, J = 6.7, 1H, NH).

*4.3.2.3. 2-[(2-Chloro-9-isopropyl-9H-purin-6-ylamino)-methyl]-phenol (**2c**).* ^{1}H NMR (DMSO-d_6): 1.64 (d, J = 6.8, 6H, CH_3), 4.68 (d, J = 5.3, 2H, CH_2), 4.92 (sep, J = 6.8, 1H, CH), 6.86 (t, J = 7.5, 1H, ArH), 6.97 (d, J = 8.2, 1H, ArH), 7.20 (t, J = 8.2, 1H, ArH), 7.28 (d, J = 7.5, 1H, ArH), 8.44 (t, J = 5.3, 1H, NH), 8.59 (s, 1H, CH), 9.92 (s, 1H, OH).

*4.3.2.4. 4-[(2-Chloro-9-isopropyl-9H-purin-6-ylamino)-methyl]-phenol (**2d**).* ^{1}H NMR (DMSO-d_6): 1.46 (d, J = 6.0, 6H, CH_3), 4.49 (d, J = 5.4, 2H, CH_2), 4.57 (sep, J = 6.0, 1H CH), 6.69 (d, J = 7.50, 2H, ArH), 7.13 (d, J = 7.50, 2H, ArH), 7.85 (t, J = 5.4, 1H, NH), 8.15 (s, 1H, CH), 10.15 (s(br), 1H, OH).

*4.3.2.5. (2-Chloro-9-isopropyl-9H-purin-6-yl)-(4-methoxybenzyl)-amine (**2e**).* ^{1}H NMR (DMSO-d_6): 1.49 (d, J = 6.2, 6H, CH_3), 3.72 (s, 3H, CH_3), 4.50 (s(br), 2H, CH_2), 4.65 (sep, J = 6.2, 1H, CH), 6.83 (d, J = 7.75, 2H, ArH), 7.29 (d, J = 7.75, 2H, ArH), 7.80 (s(br), 1H, NH), 7.95 (s, 1H, CH).

*4.3.2.6. (2-Chloro-9-isopropyl-9H-purin-6-yl)-(2,3-dimethoxybenzyl)-amine (**2f**).* ^{1}H NMR (DMSO-d_6): 1.46 (d, J = 6.0, 6H, CH_3), 3.75–3.77 (m, 6H, 2 × CH_3), 4.50 (s(br), 2H, CH_2), 4.58 (sep, J = 6.0, 1H, CH), 6.67 (t, J = 8.2, 1H, ArH), 6.75–6.88 (m, 2H, ArH), 7.50 (s(br), 1H, NH), 7.78 (s, 1H, CH).

*4.3.2.7. 2-[(2-Chloro-9-isopropyl-9H-purin-6-ylamino)-methyl]-6-methoxyphenol (**2g**).* ^{1}H NMR (DMSO-d_6): 1.49 (d, J = 6.6, 6H, CH_3), 3.77 (s, 3H, CH_3), 4.44 (d, J = 6.7, 2H, CH_2), 4.68 (sep, J = 6.6, 1H, CH), 6.68–6.88 (m, 3H, ArH), 8.28 (s, 1H, CH), 8.67 (t, J = 6.7, 1H, NH), 9.32 (s, 1H, OH).

*4.3.2.8. 4-Chloro-2-[(2-chloro-9-isopropyl-9H-purin-6-ylamino)-methyl]-phenol (**2h**).* ^{1}H NMR (DMSO-d_6): 1.47 (d, J = 6.6, 6H, CH_3), 4.53 (d, J = 5.6, 2H, CH_2), 4.62 (sep, J = 6.8, 1H, CH), 6.79 (d, J = 8.5, 1H, ArH), 7.02–7.09 (m, 2H, ArH), 8.28 (s, 1H, CH), 8.61 (t, J = 5.6, 1H, NH), 9.92 (s, 1H, OH).

*4.3.2.9. 2-[(2-Chloro-9-isopropyl-9H-purin-6-ylamino)-methyl]-4-fluorophenol (**2i**).* ^{1}H NMR (DMSO-d_6): 1.50 (d, J = 6.5, 6H, CH_3), 4.56 (d, J = 4.7, 2H, CH_2), 4.68 (sep, J = 6.3, 1H, CH), 6.75–6.92 (m, 3H, ArH), 8.31 (s, 1H, CH), 8.61 (t, J = 4.7, 1H, NH), 9.63 (s, 1H, OH).

*4.3.3. Substitution at C2 (**3a**–**3u**; final compounds)*

2-Chloro-6-(subst. benzyl)amino-9-isopropylpurine (0.01 mol) and appropriate aminoalcohol or diamine (0.1–0.2 mol) were stirred under nitrogen at 160 °C for 3–12 h, the time depending on the reactivity of amine used and on the character of substituent at C6. The course of the reaction was monitored by TLC (mobile phase: chloroform–methanol 85:15 + trace of ammonia). After the reaction had gone to completion, the reaction mixture was evaporated on a rotary vacuum evaporator. The residue was partitioned between water (100 ml) and ethyl acetate (100 ml). The organic layer was separated and the water phase was extracted with ethyl acetate (2 × 50 ml). The combined organic layers were washed with water (30 ml), dried with anhydrous magnesium sulfate, and evaporated to dryness. The sticky residue was treated with diethyl ether (75 ml). The product gradually crystallized. The crude substance was purified by crystallization from ethyl acetate–hexane (1:1). In some cases a purification by flash chromatography of products was necessary. Yield: 30–80%, according to the type of amine used; purity (HPLC: >98%). When an expensive aminoalcohol or diamine were employed, the reaction conditions could be modified by adding a suitable solvent (e.g. NMP) and an auxiliary base (e.g. *N*,*N*-diisopropyl-*N*-ethyl amine).

*4.3.4. (R)-2-[6-(4-Aminobenzylamino)-9-isopropyl-9H-purin-2-ylamino]-butan-1-ol (**3a**)*

The compound was prepared from the intermediate ***2a*** and (*R*)-2-amino-butan-1-ol by heating at 160 °C for 3 h. Yield: 72%; white solid. M.p. = 126–128 °C. ^{1}H NMR (DMSO-d_6): 0.86 (t, J = 6.5, 3H, CH_3), 1.46 (d, J = 6.0, 6H, CH_3), 1.54–1.69 (m, 2H, CH_2), 3.42–3.55 (m, 2H, CH_2), 3.85 (sex, J = 6.2, 1H, CH), 4.40 (s(br), 2H, CH_2), 4.49 (sep, J = 6.0, 1H, CH), 4.57 (t, J = 6.0, 1H, OH), 4.92 (s(br), 2H, NH_2), 5.83 (d, J = 6.2, 1H, NH), 6.47 (d, J = 7.4, 2H, ArH), 7.11 (d, J = 7.4, 2H, ArH), 7.48 (s(br), 1H, NH), 7.78 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 11.50, 22.35, 23.04, 24.43, 48.01, 54.00, 61.13, 111.05, 117.22, 120.37, 128.64, 135.91, 151.37, 154.34, 154.99, 159.39. MS *m*/*z* (ESI) 370.2 $(M + H)^+$. Anal.: Calcd for $C_{19}H_{27}N_7O$: C, 61.77; H, 7.37; N, 26.54. Found: C, 61.39; H, 7.38; N, 26.25.

*4.3.5. 1-[6-(2-Aminobenzylamino)-9-isopropyl-9H-purin-2-ylamino]-2-methylpropan-2-ol (**3b**)*

The compound was prepared from the intermediate ***2b*** and 1-amino-2-methylpropan-2-ol by heating at 160 °C in the presence of *N*,*N*-diisopropyl ethyl amine for 48 h. Yield: 80%; white solid. M.p. = 181–183 °C. ^{1}H NMR (DMSO-d_6): 1.10 (s, 6H, CH_3), 1.45

(d, $J = 6.4$, 6H, CH_3), 3.27 (d, $J = 5.6$, 2H, CH_2), 4.46 (s(br), 2H, CH_2), 4.54 (sep, $J = 6.4$, 1H, CH), 4.84 (s(br), 1H, OH), 5.21 (s(br), 2H, NH_2), 6.09 (t, $J = 5.6$, 1H, NH), 6.45 (t, $J = 7.1$, 1H, ArH), 6.58 (d, $J = 7.1$, 1H, ArH), 6.91 (t, $J = 6.9$, 1H, ArH), 7.12 (d, $J = 7.1$, 1H, ArH), 7.66 (s(br), 1H, NH), 7.80 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 22.07, 27.53, 42.10, 45.63, 52.41, 69.99, 113.61, 114.42, 115.45, 122.86, 127.46, 129.53, 135.04, 146.25, 154.41, 159.47. MS *m*/*z* (ESI) 370.2 $(M + H)^+$. Anal.: Calcd. for $C_{19}H_{27}N_7O$: C, 61.77; H, 7.37; N, 26.54. Found: C, 61.83; H, 7.59; N, 27.02.

4.3.6. 4-[6-(2-Aminobenzylamino)-9-isopropyl-9H-purin-2-ylamino]-2-methylbutan-2-ol (**3c**)

The compound was prepared from ***2b*** and 1-amino-3-methylbutan-3-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 72 h. The crude product was purified by flash chromatography. Yield: 27%; off white amorphous solid. ^{1}H NMR (DMSO-d_6): 1.11 (s, 6H, CH_3), 1.51 (d, $J = 6.5$, 6H, CH_3), 3.35 (q, $J = 6.9$, 2H, CH_2), 4.61 (sep, $J = 6.5$, 1H, CH), 4.68 (s(br), 2H, CH_2), 4.86 (s(br), 1H, OH), 5.21 (s(br), 2H, NH_2), 6.11 (t, $J = 6.9$, 1H, NH), 7.18–7.43 (m, 4H, ArH), 7.67 (s(br), 1H, NH), 7.80 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 22.07, 26.30, 27.53, 42.20, 44.92, 52.50, 70.03, 113.61, 114.42, 115.45, 122.86, 127.46, 129.53, 135.04, 146.25, 154.41, 159.47. MS *m*/*z* (ESI) 384.2 $(M + H)^+$. The compound was transferred into its corresponding dihydrochloride salt. M.p. = 196–199 °C; white solid. Anal.: Calcd for $C_{20}H_{29}N_7O$. 2 HCl: C, 52.63; H, 6.85; N, 21.48. Found: C, 52.41; H, 6.92; N, 21.14.

4.3.7. (S)-3-[6-(2-Aminobenzylamino)-9-isopropyl-9H-purin-2-ylamino]-2,4-dimethylpentan-2-ol (**3d**)

The compound was prepared from ***2b*** and 1-amino-2,4-dimethylpentan-2-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 72 h. Yield: 28%; white solid. M.p. = 198–200 °C. ^{1}H NMR (DMSO-d_6): 0.87–0.90 (d, $J = 6.90$, 6H, CH_3), 1.04 (s, 3H, CH_3), 1.18 (s, 3H, CH_3), 1.45 (d, $J = 6.6$, 6H, CH_3), 2.11 (sep, $J = 6.9$, 1H, CH), 3.93–3.97 (d, $J = 6.9$, 1H, CH), 4.33 (s(br), 1H, OH), 4.47 (s(br), 2H, CH_2), 4.53 (sep, $J = 6.6$, 1H, CH), 5.21 (s(br), 2H, NH_2), 5.56 (d, $J = 6.9$, 1H, NH), 6.44 (t, $J = 7.4$, 1H, ArH), 6.59 (d, $J = 7.4$, 1H, ArH), 6.91 (t, $J = 7.4$, 1H, ArH), 7.14 (d, $J = 7.4$, 1H, ArH), 7.57 (s(br), 1H, NH), 7.76 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 17.64, 21.89, 22.79, 28.01, 28.44, 61.27, 67.65, 72.57, 114.35, 115.34, 127.42, 129.73, 134.90, 146.22, 150.60, 154.35, 160.17. MS *m*/*z* (ESI) 412.2 $(M + H)^+$. Anal.: Calcd. for $C_{22}H_{33}N_7O$: C, 64.21; H, 8.08; N, 23.82. Found: C, 63.98; H, 8.11; N, 23.55.

4.3.8. 2-{[2-(3-Hydroxy-3-methylbutylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3e**)

The compound was prepared from the intermediate ***2c*** and 1-amino-3-methylbutan-3-ol by heating at 160 °C for 72 h. The crude product was purified by flash chromatography. Yield: 43%; white solid. M.p. = 72–75 °C. ^{1}H NMR (DMSO-d_6): 1.11 (s, 6H, CH_3), 1.46 (d, $J = 6.7$, 6H, CH_3), 1.65 (t, $J = 6.5$, 2H, CH_2), 3.29 (q, $J = 6.5$, 2H, CH_2), 4.28 (s, 1H, OH), 4.50–4.57 (m, 3H, CH, CH_2), 6.30 (t, $J = 6.5$, 1H, NH), 6.69–6.78 (m, 2H, ArH), 7.05 (t, $J = 7.3$, ArH), 7.14 (d, $J = 7.3$, 1H, ArH), 7.56 (s(br), 1H, NH), 7.79 (s, 1H, CH), 9.99 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 22.02, 29.47, 37.56, 42.53, 45.74, 68.57, 84.60, 115.35, 118.73, 122.30, 126.27, 127.82, 128.85, 135.22, 139.85, 150.47, 155.03, 158.81. MS *m*/*z* (ESI) 385.3 $(M + H)^+$. Anal.: Calcd. for $C_{20}H_{28}N_6O_2$: C, 62.48; H, 7.34; N, 21.86. Found: C, 62.20; H, 7.31; N, 21.55.

4.3.9. 2-[(2-Isobutylamino-9-isopropyl-9H-purin-6-ylamino)-methyl]-phenol (**3f**)

The compound was prepared from ***2c*** and isobutyl amine by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 12 h. Yield: 45%; white solid. M.p. = 83–85 °C. ^{1}H NMR (DMSO-d_6): 0.85 (d, $J = 6.6$, 6H, CH_3), 1.46 (d, $J = 6.7$, 6H, CH_3), 1.84 (sep, $J = 6.6$, 1H, CH), 3.05 (t, $J = 6.3$, 2H, CH_2), 4.47–4.60 (m, 3H, CH_2, CH), 6.39 (t, $J = 6.3$, 1H, NH), 6.71 (t, $J = 7.4$, 1H, ArH), 6.75 (d, $J = 7.4$, 1H, ArH), 7.04 (t, $J = 7.4$, 1H, ArH), 7.14 (d, $J = 7.4$, 1H, ArH), 7.55 (s(br), 1H, NH), 7.79 (s, 1H, CH), 9.94 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 20.26, 21.90, 27.75, 45.62, 48.78, 52.5, 113.41, 114.1, 115.17, 118.60, 126.10, 127.65, 128.66, 135.10, 154.90, 158.92. MS *m*/*z* (ESI) 355.4 $(M + H)^+$. Anal.: Calcd. for $C_{19}H_{26}N_6O$: C, 64.38; H, 7.39; N, 23.71. Found: C, 64.23; H, 7.41; N, 23.55.

4.3.10. 4-[(9-Isopropyl-2-piperazin-1-yl-9H-purin-6-ylamino)-methyl]-phenol (**3g**)

The compound was prepared from the intermediate ***2d*** and piperazine by heating at 160 °C for 20 h. Yield: 51%; white solid. M.p. = 156–158 °C. ^{1}H NMR (DMSO-d_6): 1.46 (d, $J = 6.0$, 6H, CH_3), 2.84–2.95 (m, 4H, CH_2), 3.69–3.80 (m, 4H, CH_2), 4.49 (s(br), 2H, CH_2), 4.57 (sep, $J = 6.0$, 1H, CH), 6.70 (d, $J = 7.5$, 2H, ArH), 7.16 (d, $J = 7.5$, 2H, ArH), 7.85 (s, 1H, CH), 8.15 (s, 1H, NH), 9.50 (s(br), 1H, NH), 10.15 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 21.97, 43.40, 44.07, 45.70, 54.93, 113.41, 114.74, 128.67, 130.76, 132.58, 135.81, 150.37, 154.00, 155.97, 157.91. MS *m*/*z* (ESI) 368.0 $(M + H)^+$. Anal.: Calcd. for $C_{19}H_{25}N_7O$: C, 62.11; H, 6.86; N, 26.68. Found: C, 62.23; H, 6.90; N, 26.55.

4.3.11. (9-Isopropyl-2-piperazin-1-yl-9H-purin-6-yl)-(4-methoxybenzyl)-amine (**3h**)

The compound was prepared from the intermediate ***2e*** and piperazine by heating at 160 °C for 20 h. Yield: 45%; white solid. M.p. = 132–134 °C. ^{1}H NMR (DMSO-d_6): 1.49 (d, $J = 6.2$, 6H, CH_3), 2.64–2.72 (m, 4H, CH_2), 3.56–3.65 (m, 4H, CH_2), 3.72 (s, 3H, OCH_3), 4.50 (s(br), 2H, CH_2), 4.65 (sep, $J = 6.2$, 1H, CH), 6.83 (d, $J = 7.6$, 2H, ArH), 7.29 (d, $J = 7.6$, 2H, ArH), 7.80 (1H, s, CH), 7.95 (s(br), 1H, NH), 10.15 (s(br), 1H, NH). ^{13}C NMR (DMSO-d_6): 21.95, 44.85, 45.25, 45.61, 54.47, 59.65, 64.81, 113.38, 128.66, 132.68, 135.52, 150.54, 156.00, 157.94, 158.33. MS *m*/*z* (ESI) 382.1 $(M + H)^+$. Anal.: Calcd. for $C_{20}H_{27}N_7O$: C, 62.97; H, 7.13; N, 25.70. Found: C, 62.43; H, 7.13; N, 25.11.

4.3.12. [9-Isopropyl-2-(4-methylpiperazin-1-yl)-9H-purin-6-yl]-(4-methoxybenzyl)-amine (**3i**)

The compound was prepared from ***2e*** and *N*-methylpiperazine by heating at 135 °C for 4 h. Yield: 56%; almost white solid. M.p. = 129–130 °C. ^{1}H NMR (DMSO-d_6): 1.46 (d, $J = 6.0$, 6H, CH_3), 2.17 (s, 3H, OCH_3), 2.26–2.37 (m, 4H, CH_2), 3.63–3.72 (m, 6H, CH_2), 4.57 (sep, $J = 6.0$, 1H, CH), 6.85 (d, $J = 7.5$, 2H, ArH), 7.30 (d, $J = 7.5$, 2H, ArH), 7.82 (s, 1H, CH), 7.95 (s(br), 1H, NH), 10.25 (s(br), 1H, H19). ^{13}C NMR (DMSO-d_6): 21.67, 42.45, 43.95, 45.61, 54.84, 64.81, 133.36, 128.64, 132.62, 135.54, 150.48, 153.99, 157.95, 158.20. MS *m*/*z* (ESI) 396.2 $(M + H)^+$. Anal.: Calcd. for $C_{21}H_{29}N_7O$: C, 63.77; H, 7.39; N, 24.79. Found: C, 63.63; H, 7.40; N, 24.55.

4.3.13. (R)-2-[6-(2,3-Dimethoxybenzylamino)-9-isopropyl-9H-purin-2-ylamino]-butan-1-ol (**3j**)

The compound was prepared by heating of ***2f*** in (*R*)-2-amino-butan-1-ol at 160 °C for 3 h. Yield: 65%; white solid. M.p. = 102–105 °C. ^{1}H NMR (DMSO-d_6): 0.81 (t, $J = 7.0$, 3H, CH_3), 1.42–1.45 (m, 1H, CH_2a), 1.46 (d, 6H, $J = 7.0$), 1.47–1.49 (m, 1H, CH_2b), 3.35–3.45 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 4.45–4.54 (m, 2H, 2 × CH), 4.57 (s(br), 2H, CH_2), 5.79 (d, $J = 6.2$, 1H, NH), 6.82 (d, $J = 6.9$, 1H, ArH), 6.85–6.97 (m, 2H, ArH), 7.45 (s(br), 1H, NH), 7.78 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 10.63, 22.00, 22.10, 23.86, 45.65, 54.00, 55.63, 55.68, 62.95, 111.26, 119.64, 124.15, 133.85, 146.00, 152.14, 154.61, 158.99. MS *m*/*z* (ESI) 415.1 $(M + H)^+$. Anal.: Calcd. for $C_{21}H_{30}N_6O_3$: C, 60.85; H, 7.30; N, 20.27. Found: C, 60.61; H, 7.32; N, 20.09.

*4.3.14. 2-{[2-((R)-1-Hydroxymethylpropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-6-methoxyphenol (**3k**)*

The compound was prepared by heating of **2g** in (*R*)-2-amino-butan-1-ol (excess) at 160 °C for 3 h. Yield: 43%; almost white solid. M.p. = 100–103 °C. ^{1}H NMR (DMSO-d_6): 0.86 (t, J = 6.9, 3H, CH_3), 1.46 (d, J = 6.5, 6H, CH_3), 1.63 (sex, J = 6.2, 1H, CH), 2.54 (s(br), 1H, OH), 3.39–3.54 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.83 (sep, J = 6.9, 1H, CH), 4.48–4.58 (m, 2H, CH_2), 4.63 (s(br), 2H, CH_2), 5.85 (d, 1H, J = 6.2, NH), 6.68–6.88 (m, 3H, ArH), 7.50 (s(br), 1H, NH), 7.78 (s, 1H, CH), 9.32(s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 10.56, 15.07, 21.86, 21.95, 45.76, 54.01, 55.75, 62.92, 64.83, 110.73, 113.55, 118.43, 120.65, 126.78, 135.21, 144.01, 147.57, 150.55, 154.42, 158.74. MS m/z (ESI) 401.2 $(M + H)^+$. Anal.: Calcd. for $C_{20}H_{28}N_6O_3$: C, 59.98; H, 7.05; N, 20.98. Found: C, 59.63; H, 7.05; N, 20.59.

*4.3.15. 2-{[2-((S)-1-Hydroxymethylpropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-6-methoxyphenol (**3l**)*

The compound was prepared by heating of **2g** in (*S*)-2-amino-butan-1-ol (excess) at 160 °C for 3 h. Yield: 40%; almost white solid. M.p. = 100–102 °C. ^{1}H NMR (DMSO-d_6): 0.86 (t, J = 6.9, 3H, CH_3), 1.46 (d, J = 6.5, 6H, CH_3), 1.63 (sex, J = 6.2, 1H, CH), 2.54 (s(br), 1H, OH), 3.39–3.54 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.83 (sep, 1H, J = 6.9, CH), 4.48–4.58 (m, 2H, CH_2), 4.63 (s(br), 2H, CH_2), 5.85 (d, J = 6.2, 1H, NH), 6.68–6.88 (m, 3H, ArH), 7.50 (s(br), 1H, NH), 7.78 (s, 1H, CH), 9.32 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 10.56, 15.07, 21.86, 21.95, 45.76, 54.01, 55.75, 62.92, 64.83, 110.73, 113.55, 118.43, 120.65, 126.78, 135.21, 144.01, 147.57, 150.55, 154.42, 158.74. MS m/z (ESI) 401.2 $(M + H)^+$. Anal.: Calcd. for $C_{20}H_{28}N_6O_3$: C, 59.98; H, 7.05; N, 20.98. Found: C, 59.63; H, 7.05; N, 20.59.

*4.3.16. 2-[(9-Isopropyl-2-piperazin-1-yl-9H-purin-6-ylamino)-methyl]-6-methoxyphenol (**3m**)*

The compound was prepared by heating of **2g** in molten piperazine (excess) at 160 °C for 3 h. Yield: 65%; almost white solid. M.p. = 158–160 °C. ^{1}H NMR (DMSO-d_6): 1.45 (d, J = 6.90, 6H, CH_3), 2.55–2.73 (m, 4H), 3.45–3.59 (m, 4H), 3.76 (s, 3H, $-OCH_3$), 4.54 (sep, J = 6.90, 1H, CH), 4.61 (s(br), 2H, CH_2), 6.66 (t, J = 7.7, 1H, ArH), 6.75–6.83 (m, 2H, ArH), 7.55 (s(br), 1H, NH), 7.83 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 21.29, 22.58, 45.87, 46.06, 46.27, 56.31, 60.30, 110.96, 114.07, 118.94, 121.03, 127.34, 136.35, 144.38, 147.92, 151.01, 154.76, 158.94. MS m/z (ESI) 398.1 $(M + H)^+$. Anal.: Calcd. for $C_{20}H_{27}N_7O_2$: C, 60.44; H, 6.85; N, 24.67. Found: C, 60.25; H, 6.87; N, 24.73.

*4.3.17. 4-Chloro-2-{[2-(4-hydroxycyclohexylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3n**)*

The compound was prepared by heating of **2h** in excess of 4-aminocyclohexanol at 160 °C for 12 h. The crude product was purified by flash chromatography. Yield: 70%; white solid. M.p. = 201–204 °C. ^{1}H NMR (DMSO-d_6): 1.11–1.18 (m, 4H), 1.24 (d, J = 6.6, 6H, CH_3), 1.46–1.84 (m, 4H), 3.39 (qui, J = 7.0, CH), 3.57 (s(br), 1H, OH), 4.44 (sex, J = 7.7, 1H, CH), 4.48–4.57 (m, 3H, CH_2, CH), 6.06 (d, J = 7.7, 1H, NH), 6.79 (d, J = 8.4, 1H, ArH), 7.04–7.08 (m, 2H, ArH), 7.58 (s(br), 1H, NH), 7.80 (s, 1H, CH), 10.01 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 22.51, 30.93, 34.81, 42.03, 46.13, 49.91, 69.04, 111.12, 114.10, 117.88, 120.60, 126.71, 135.21, 139.82, 147.57, 150.55, 154.42, 158.74. MS m/z (ESI) 431.3 $(M + H)^+$. Anal.: Calcd. for $C_{21}H_{27}ClN_6O_2$: C, 58.53; H, 6.32; N, 19.50. Found: C, 58.22; H, 6.31; N, 19.55.

*4.3.18. 2-{[2-(4-Aminocyclohexylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-4-chlorophenol (**3o**)*

The compound was prepared from **2h** and 1,4-*trans*-diaminocyclohexane (excess) by heating at 160 °C for 4 h. Yield: 38%; white solid. M.p. = 100–103 °C. ^{1}H NMR (DMSO-d_6): 0.97–1.21 (m, 4H), 1.46 (d, J = 6.6, 6H, CH_3), 1.70–1.86 (m, 4H), 2.40 (qui, J = 5.7, 1H, CH), 3.55 (sex, J = 7.1, 1H, CH), 4.05 (s(br), 2H, NH_2), 4.45–4.61 (m, 3H, CH_2, CH), 6.08 (d, J = 7.1, 1H, NH), 6.79 (d, J = 8.5, 1H, ArH), 7.02–7.08 (m, 2H, ArH), 7.62 (s(br), 1H, NH), 7.80 (s, 1H, CH), 10.12(s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 15.71, 22.60, 31.84, 35.82, 46.26, 50.18, 50.52, 65.46, 117.14, 122.34, 127.50, 127.93, 129.38, 135.80, 154.70, 155.00, 158.84. MS m/z (ESI) 430.3 $(M + H)^+$. Anal.: Calcd. $C_{21}H_{28}ClN_7O$: C, 58.67; H, 6.56; N, 22.80. Found: C, 58.29; H, 6.61; N, 22.53.

*4.3.19. 4-Chloro-2-{[2-((R)-1-hydroxymethylpropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3p**)*

The compound was prepared from **2h** and (*R*)-2-amino-butan-1-ol (excess) by heating at 160 °C for 3 h. Yield: 65%; white solid. M.p. = 180–183 °C. ^{1}H NMR (DMSO-d_6): 0.82 (t, J = 7.2, 3H, CH_3), 1.46 (d, J = 6.6, 6H, CH_3), 3.34–3.48 (m, 2H, CH_2), 3.79 (sex, J = 7.3, 1H, CH), 4.46–4.60 (m, 4H, CH, CH_2, OH), 5.89 (d, J = 7.3, 1H, NH), 6.79 (d, J = 8.4, 1H, ArH), 7.05 (d, J = 8.4, 1H, ArH), 7.10 (s, 1H, ArH), 7.65 (s(br), 1H, NH), 7.81 (s, 1H, CH), 10.11 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 11.14, 22.51, 22.61, 24.43, 46.35, 54.62, 63.49, 114.13, 117.22, 122.78, 127.67, 128.21, 129.27, 135.91, 151.37, 154.34, 154.99, 159.39. MS m/z (ESI) 405.1 $(M + H)^+$. Anal.: Calcd for $C_{19}H_{25}ClN_6O_2$: C, 56.36; H, 6.22; N, 20.76. Found: C, 56.29; H, 6.31; N, 20.53.

*4.3.20. 4-Chloro-2-{[2-(2-hydroxy-2-methylpropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3q**)*

The compound was prepared from **2h** and 1-amino-2-methylpropan-2-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 24 h. The crude product was purified by flash chromatography. Yield: 40%; white solid. M.p. = 183–186 °C ^{1}H NMR (DMSO-d_6): 1.06 (s, 6H, CH_3), 1.46 (d, J = 6.6, 6H, CH_3), 3.23 (d, J = 5.7, 2H, CH_2), 4.47–4.58 (m, 3H, CH_2, CH), 4.75 (s(br), 1H, OH), 6.07 (t, J = 5.7, 1H, NH), 6.79 (d, J = 8.3, 1H, ArH), 7.06–7.12 (m, 2H, ArH), 7.75 (s(br), 1H, NH), 7.84 (s, 1H, CH), 10.17 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 17.76, 22.59, 26.88, 28.03, 46.30, 52.91, 70.51, 115.26, 117.25, 122.75, 127.70, 128.20, 136.03, 154.33, 154.37, 159.89. MS m/z (ESI) 405.2 $(M + H)^+$. Anal.: Calcd for $C_{19}H_{25}ClN_6O_2$: C, 56.36; H, 6.22; N, 20.76. Found: C, 56.10; H, 6.28; N, 20.49.

*4.3.21. 4-Chloro-2-{[2-((R)-1-ethyl-2-hydroxypropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3r**)*

The compound was prepared from **2h** and (2*RS*, 3*R*)-3-aminopentan-2-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 72 h. The crude product was purified by flash chromatography. Yield: 29.5%; off white solid. The product is a mixture of diastereoisomers. M.p. = 86–91 °C. ^{1}H NMR (DMSO-d_6): 0.78 (t, J = 6.9, 3H, CH_3), 0.97 (t, J = 5.5, 3H, CH_3), 1.43 (d, J = 6.7, 6H, CH_3), 3.69 (qui, J = 6.9, 2H, CH_2), 4.41–4.72 (m, 4H, CH_2, CH, CH), 5.92 (d, J = 6.6, 1H, NH), 6.76 (d, J = 8.5, 1H, ArH), 7.02–7.08 (m, 2H, ArH), 7.62 (s(br), 1H, NH), 7.77 (s, 1H, CH), 10.12 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 10.78, 20.30, 21.88, 22.53, 45.63, 57.82, 66.94, 68.77, 113.19, 116.53, 122.09, 127.00, 127.57, 128.58, 135.21, 151.25, 153.66, 154.29, 159.22. MS m/z (ESI) 419.2 $(M + H)^+$. Anal.: Calcd for $C_{20}H_{27}ClN_6O_2$: C, 57.34; H, 6.50; N, 20.06. Found: C, 57.31; H, 6.52; N, 19.89.

*4.3.22. 4-Chloro-2-{[2-((S)-2-hydroxy-1,2-dimethyl-propylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3s**)*

The compound was prepared from **2h** and (*S*)-3-amino-2-methylbutan-2-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 24 h. The crude product was purified by flash chromatography. Yield: 47%; white amorphous solid (foam). ^{1}H NMR (DMSO-d_6): 1.25–1.31 (m, 9H, CH_3), 1.53 (d, J = 6.6, 6H, CH_3), 4.02 (qui, J = 7.0, 1H, CH), 4.50–4.55 (s(br), 2H,

CH_2), 4.59 (sep, $J = 6.9$, 1H, CH), 5.01 (s, 1H, OH), 6.84 (d, $J = 8.4$, 1H, ArH), 6.99 (s(br), 1H, NH), 7.13–7.15 (m, 2H, ArH), 7.52 (s, 1H, CH). ^{13}C NMR (DMSO-d_6): 17.02, 22.53, 24.58, 28.30, 40.91, 46.96, 56.72, 74.10, 114.22, 119.47, 124.25, 126.93, 129.56, 130.62, 135.28, 150.11, 154.24, 154.81, 158.70. MS *m/z* (ESI) 419.3 $(M + H)^+$. Anal.: Calcd for $C_{20}H_{27}ClN_6O_2$: C, 57.34; H, 6.50; N, 20.06. Found: C, 57.10; H, 6.43; N, 19.91.

4.3.23. 2-{[2-((R)-1-Ethyl-2-hydroxypropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-4-fluorophenol (**3t**)

The compound was prepared from ***2i*** and (2*RS*, 3*R*)-3-aminopentan-2-ol by heating at 160 °C in NMP in the presence of *N*,*N*-diisopropyl ethyl amine for 72 h. The crude product was purified by flash chromatography. Yield: 26%; an off white amorphous solid. The product is a mixture of diastereoisomers. The free base was transferred into hydrochloride salt; m.p. = 100–106 °C; yellowish solid. ^{1}H NMR (DMSO-d_6): 0.81 (t, $J = 6.9$, 3H, CH_3), 0.99 (t, $J = 5.5$, 3H, CH_3), 1.46 (d, $J = 6.7$, 6H, CH_3), 3.60–3.72 (m, 2H, CH_2), 4.41–4.65 (m, 4H, CH_2, CH, CH), 5.94 (d, $J = 6.6$, 1H, NH), 6.76 (m, 1H, ArH), 6.83–6.88 (m, 2H, ArH), 7.62 (s(br), 1H, NH), 7.79 (s, 1H, CH), 9.88 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 10.81, 20.00, 22.12, 22.53, 45.63, 55.58, 66.94, 65.25, 112.58, 113.25, 115.00, 115.17, 116.38, 116.48, 122.69, 128.70, 129.12, 129.01, 129.82, 136.00, 151.27, 152.50, 154.42, 155.03, 157.52, 160.00. MS *m/z* (ESI) 403.4 $(M + H)^+$. Anal. Calcd. for $C_{20}H_{27}FN_6O_2$: C, 59.69; H, 6.76; N, 20.88. Found: C, 59.38; H, 6.82; N, 20.63.

4.3.24. 4-Fluoro-2-{[2-((R)-1-hydroxymethylpropylamino)-9-isopropyl-9H-purin-6-ylamino]-methyl}-phenol (**3u**)

The compound was prepared from ***2i*** and (*R*)-2-amino-butan-1-ol (excess) by heating at 160 °C for 3 h. Yield: 82%; white solid. Recrystallized from ethyl acetate. M.p. = 165–167 °C. ^{1}H NMR (DMSO-d_6): 0.84 (t, $J = 6.9$, 3H, CH_3), 1.46 (d, $J = 6.0$, 6H, CH_3), 3.40–3.46 (m, 2H, CH_2), 3.76 (sex, $J = 6.9$, 1H, CH), 4.49–4.58 (m, 4H, CH, CH_2, OH), 5.79 (d, $J = 6.9$, 1H, NH), 6.75–6.93 (m, 3H, ArH), 7.51 (s(br), 1H, NH), 7.79 (s, 1H, CH), 9.75 (s(br), 1H, OH). ^{13}C NMR (DMSO-d_6): 11.13, 22.49, 22.59, 24.43, 46.36, 54.62, 63.50, 113.90, 114.19, 114.85, 115.15, 116.38, 116.48, 122.69, 128.53, 128.73, 128.77, 128.82, 135.90, 151.27, 151.57, 154.42, 155.03, 157.52, 159.40. MS *m/z* (ESI) 389.5 $(M + H)^+$. Anal.: Calcd for $C_{19}H_{25}FN_6O_2$: C, 58.75; H, 6.49; N, 21.63. Found: C, 58.62; H, 6.51; N, 21.44.

4.4. Biological assays

4.4.1. Antibodies

Specific antibodies were purchased from Sigma–Aldrich (anti-α-tubulin, clone DM1A, peroxidase-labeled secondary antibodies), Santa Cruz Biotechnology (anti-Mcl-1, clone S-19; anti-PARP, clone F-2; anti-Mdm-2, clone SMP14; anti-Bcl-2), DAKO Cytomation (anti-caspase-3), Roche Applied Science (anti-5-bromo-2′-deoxyuridine-fluorescein, clone BMC 9318), Jackson ImmunoResearch Laboratory (fluorescein-conjugated Goat Anti-Mouse IgG), Bethyl Laboratories (anti-phospho RNA polymerase II (S5); anti-phospho RNA polymerase II (S2)), Millipore (anti-RNA polymerase II, clone ARNA-3), Cell Signaling (anti-PUMA) or were a generous gift from Dr. B. Vojtěšek (anti-p53, clone DO-1; anti-p21^{waf1}, clone 118; anti-PCNA).

4.4.2. Cell maintenance and cytotoxicity assays

The cytotoxicity of the studied compounds was determined using cell lines of different histological origin as described earlier [29,36]. Briefly, the cells were assayed with compounds using three-fold dilutions in triplicate. Treatment lasted for 72 h, followed by addition of Calcein AM solution, and measurement of the fluorescence of live cells at 485 nm/538 nm (ex/em) with a Fluoroskan Ascent microplate reader (Labsystems). IC_{50} (the drug concentration that reduced the number of viable cells to 50%) values were determined from the dose–response curves.

4.4.3. Kinase inhibition assays

CDK2/Cyclin E kinase was produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column (Qiagen). CDK5/p35, CDK7Cyclin H/MAT1 and CDK9/Cyclin T1 were purchased from ProQinase GmbH. The kinase reactions were assayed with 1 mg/ml histone H1 (for CDK2 and CDK5) or $(YSPTSPS)_2KK$ peptide (for CDK7 and CDK9) in the presence of 15/0.15/1.5/1.5 μM ATP (for CDK2/CDK5/CDK7CDK9), 0.05 μCi [γ-^{33}P] ATP and of the test compound in a final volume of 10 μl, all in a reaction buffer (60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 2.5 μg/50 μl $PEG_{20.000}$). The reactions were stopped by adding 5 μl of 3% aq. H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman), washed 3× with 0.5% aq. H_3PO_4 and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer (Fujifilm). The concentration of the test compounds required to decrease the CDK activity by 50% was determined from dose–response curves and designated as IC_{50} [29,36].

4.4.4. Immunoblotting

Immunoblotting analysis was performed as described earlier [29,36]. Briefly, cellular lysates were prepared by harvesting cells in Laemmli sample buffer. Proteins were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. After blocking, the membranes were incubated with specific primary antibodies overnight, washed and then incubated with peroxidase-conjugated secondary antibodies. Finally, peroxidase activity was detected with ECL+ reagents (AP Biotech) using a CCD camera LAS-4000 (Fujifilm).

4.4.5. Cell cycle analysis

Sub-confluent cells were treated with test compounds at different concentrations for 24 h. The cultures were pulse-labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min at 37 °C prior to harvesting. The cells were then washed in PBS, fixed with 70% ethanol, and denatured in 2 M HCl. Following neutralization, the cells were stained with anti-BrdU fluorescein-labeled antibodies, washed, stained with propidium iodide and analyzed by flow cytometry using a 488 nm laser (Cell Lab Quanta SC, Beckman Coulter) as described previously [29,36].

4.4.6. p53-Dependent transcriptional activity

To measure p53-dependent transcriptional activity, β-galactosidase activity was determined in the human melanoma cell line Arn-8, stably transfected with a p53-responsive reporter construct pRGCΔfoslacZ as described before [29,36]. After 24 h incubation with the inhibitors, the cells were permeabilized with 0.3% Triton X-100 for 15 min, and then 4-methylumbelliferon-ß-D-galactopyranoside was added as a substrate to a final concentration of 80 μM. After 1 h, the fluorescence was measured at 355/460 nm (ex/em) with a Fluoroskan Ascent microplate reader (Labsystems).

4.4.7. Caspase-3/7 assay

The cells were homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, inhibitors of proteases, pH 7.4) on ice for 20 min. The homogenates were clarified by centrifugation at 10,000 × *g* for 30 min at 4 °C, and then the proteins were quantified and diluted to equal concentrations. Lysates were then incubated for 1 h with 100 μM Ac-DEVD-AMC as a substrate in the assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). To serve as negative

controls, the lysates were supplemented with 100 μM Ac-DEVD-CHO, which is a caspase-3/7 inhibitor. The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 355/460 nm (ex/em) as described previously [29].

4.4.8. Statistics

Rank correlation coefficients were calculated in *R*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ejmech.2012.06.036.

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