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8-Azapurines as new inhibitors of cyclin-dependent kinases

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Abstract—Purine inhibitors of cyclin-dependent kinases (CDK) seem to be a potential anticancer drug candidate as one of the first representatives, roscovitine, is passing Phase II clinical trials for cancer and glomerulonephritis. In this article, we describe a novel modification of the purine scaffold influencing CDK2 inhibitory activities as well as anticancer properties in cell lines of different histopathological origin. The introduced N at position 8 of the purine ring generally lowered CDK2 inhibitory activity of new 8-azapurines (1,2,3-triazolo[4,5-*d*]pyrimidines) in comparison to the model trisubstituted purines, whereas the antiproliferative potential of some derivatives remained very high, reflecting their ability to activate p53 tumor suppressor.

1. Introduction

All cancers are characterized by an abnormal control of cell proliferation. This is caused by mutation or mis-regulation of cell-cycle regulatory genes and proteins.[1] It has been shown that it deregulates G1/S cyclin-dependent kinase (CDK) activity in tumor cells, while enabling these cells to enter S-phase unchecked, also renders them selectively susceptible to CDK2 inhibition,[2] because CDK2 activity is not only instrumental in initiating S-phase (CDK2/cyclin E), but it is also critically required to terminate E2F-mediated transcriptional activity in S-phase (CDK2/cyclin A). Failure of a cell to terminate this activity in timely fashion constitutes a powerful apoptotic signal.[3] Recent findings also suggest that CDK2 activity—at least in colorectal cancers—may be redundant[4] and that other kinases, possibly CDK3 or AURORA kinases[5], can step in upon CDK2 activation. Even CDK1/cyclin B1 activity, which had traditionally been associated with regulation of M-phase entry, was recently shown to possess S-phase promoting potential.[6] Furthermore, it is now clear that the roles of CDKs are not only confined to cell cycle control, but also function in the regulation of transcription. CDK2, 7, 8, and 9 have been shown to be implicated in transcriptional regulation through activating and deactivating phosphorylations of the C-terminal domain of RNA polymerase II.[7] Despite the facts mentioned above, new CDK-inhibitory pharmacophores and compounds have to be developed and reported. The lack of selectivity as well as incomplete understanding of the selectivity of the CDK inhibitors makes interpretation of their pharmacological effects difficult and the question of which CDK(s) should be inhibited for optimal anticancer activity remains largely unanswered.[8]

The crucial role of CDK within cell division cycle and their frequent deregulations in human tumor and cancer cells have encouraged an active search for chemical CDK inhibitors (CDKI).[9] Systematic screening of the adenine-derived plant hormone cytokinins led to the discovery of 2,6,9-trisubstituted purines, a group of highly active CDK inhibitors.[10] The first specific inhibitor olomoucine,[10] was quickly followed by derivatives with enhanced efficiency, like roscovitine, purvalanol A, olomoucine II and others.[11–15] Several of them have already undergone in vivo efficacy studies, preclinical and clinical evaluation, confirming CDKs as real targets for development of new anticancer drugs.[15–17]

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The pharmacological improvement of the olomoucine structure was till date now mainly based on modifications at positions 2, 6 and 9, respectively.[11–18] In the effort to enhance the affinity of CDKIs, we tried to extend the inhibitor-CDK mutual interactions by introduction of another nitrogen to the purine ring. There are already reports describing rearrangement of the purine heteroatoms.[19,20] One of them characterized successful repositioning of heteroatoms of purine to pyrazolo[4,3-*d*]pyrimidine that lead to improved anti-CDK activity.[19] The shift of heteronitrogen from position 9 to 8 prompted us to further explore and modify the purine ring. Therefore, we prepared several 2,6,9-trisubstituted 8-azapurines as analogs of potent CDK inhibitors. The impact of modification of the purine skeleton on CDK inhibitory activity was consequently verified by p53-transcriptional and anticancer activity testing.

2. Results and discussion

2.1. Chemistry

We used several approaches to synthesize the target compounds. Owing to the low regioselectivity we abandoned the synthesis starting either from 2,6-dichloro-8-azapurine or 5-nitro-2,4,6-trichloropyrimidine, respectively. For example, the reaction of isopropyl amine with 5-nitro-2,4,6-trichloropyrimidine proceeds with all three chlorine atoms simultaneously in contrast to the selective reaction of ammonia.[21] A series of new 2,6,9-trisubstituted-8-azapurines (1,2,3-triazolo[4,5-*d*]pyrimidines) was therefore synthesized using a general synthetic route outlined in Figure 1. In this approach an alkylazide ($R9N_3$) was cyclized with cyanacetamide in the presence of sodium ethoxide to 1-alkyl-4-amino-5-carboxamido-1,2,3-triazole (**I**). Reaction of derivative **I** with diethyl carbonate afforded 9-alkyl-2,6-dihydroxy-8-azapurine (8-azaxanthine derivative **II**). The synthesis of 9-benzyl-2,6-dihydroxy-8-azapurine (**IIa**) followed the cited procedures.[22] This approach also proved to be useful for 9-isopropylderivative **II** despite the lower yields. Chlorination of 8-azaxanthine derivatives **II** was a crucial step in the synthesis which was achieved by the action of $POCl_3$/2,6-lutidine at 120 °C. Decomposition of the evaporated reaction residues by coincident extraction in benzene or chloroform at 0 °C afforded crude dichloroderivatives **III**. While the benzyl derivative **IIIa** was able to be crystallized, the isopropyl analogue **IIIb** seems to be very unstable and was therefore used immediately in subsequent nucleophilic substitution of C1–C6. Furthermore, the yield of 2,6-dichloro-9-isopropyl-8-azapurine was not entirely reproducible as a satisfactory yield (20–25%) was obtained only under the small-mass conditions (up to 2 g). Subsequent regioselective substitution of C1–C6 (**III**→**IV**) was then achieved at 0–20 °C using only one equivalent of $R6\text{-}NH_2$ in the presence of tertiary amine. At room temperature, the highly reactive amines like benzylamines were however, able to substitute C1–C2 to a certain extent. The final substitution reaction of C1–C2 was then performed at elevated temperature (>100 °C, **IV**→**V**). The 2-methyl-8-azaderivative **2** was synthesized by a modified procedure adopted from the synthesis of similar 8-azapurine compounds.[23] Here the cyclization of isopropylderivative **Ib** with ethyl acetate gave rise to 6-hydroxy-9-isopropyl-2-methyl-8-azapurine which after chlorination by Bosshard reagent ($SOCl_2$/DMF) and subsequent nucleophilic substitution at C1–C6 afforded the target compound **2**.

2.2. Biological activity

To verify the effect of an additional nitrogen in position 8 of the active purine inhibitors, new derivatives (summarized in Table 1) were screened for their ability to inhibit recombinant CDK2/cyclin E. The results showed that the introduction of the fifth nitrogen instead of C8H functionality in the purine ring reduces CDK2 inhibitory activity in all cases (30–60-fold). Straightforward structural point of view would consider 8-azapurines to be weaker CDK inhibitors than respective purines; as co-crystals of CDK2 with purine inhibitors show (e.g., purvalanol B or H717),[12,13] hydrogen at C8 of the purine ring interacts with the backbone oxygen of Glu81. In contrast, guanine NU2058 binds to

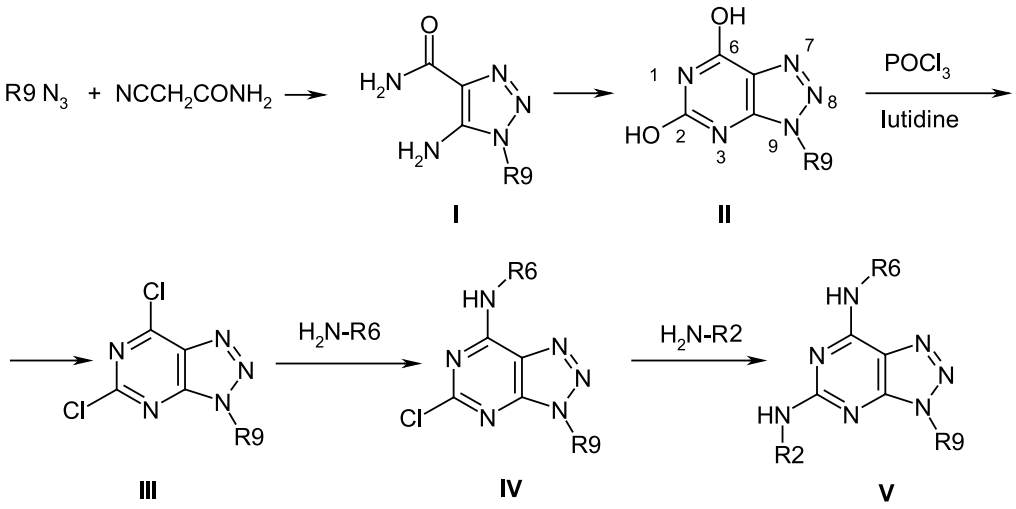

Figure 1. Scheme of synthesis of 2,6,9-trisubstituted-8-azapurines.

Table 1. Structure–activity relationships of 8-azapurines

Compound	Substitution		IC_{50} (μM)[a]					p53[a]
	R2	R6	CDK2	MCF7	K562	HOS	G361	Arn8
Roscovitine[b]	[1-(Hydroxymethyl)propyl]	Benzyl	0.45	11. 1	40	32	34	++(20)
1	(4-Aminocyclohexyl)	Benzyl	11.3	11.4	19.2	15.6	8.3	++(20)
3	(3-Hydroxypropyl)	Benzyl	100	112	142	159	126	—
4	[1-(Hydroxymethyl)propyl]	Benzyl	4.1	55.3	68.0	123	84.7	+(80)
5	[1-(Hydroxymethyl)-2-methylpropyl]	Benzyl	7.3	32.8	50.0	129	28.2	++(50)
6	[1-(Hydroxymethyl)propyl]	(3-Hydroxy-4-methoxybenzyl)	2.3	31.8	68. 1	32.6	42.6	++(40)
7	(2-Aminocyclohexyl)	(3-Hydroxy-4-methoxybenzyl)	13.7	13.1	30.3	14.3	25.4	++(20)
8	[1-(Hydroxymethyl)propyl]	(2-Hydroxybenzyl)	1.2	22.1	33.1	101.7	49.2	+(80)
9	[1-(Hydroxymethyl)-2-methylpropyl]	(2-Hydroxybenzyl)	0.84	13.6	18.9	35.7	19.1	++(30)
10	(2-Aminocyclohexyl)	(2-Hydroxybenzyl)	4.1	23.4	19.4	69.7	20.5	—
11	(4-Aminocyclohexyl)	(2-Hydroxybenzyl)	16.4	20.2	21.3	33.1	11.8	—
12	(2-Hydroxybenzyl)	(2-Hydroxybenzyl)	>100	>167	>167	>167	>167	—
13	[1-(Hydroxymethyl)-2-methylpropyl]	(3-Hydroxybenzyl)	8.0	22.3	39.8	35.9	41.1	++(30)
14	(4-Aminocyclohexyl)	(3-Hydroxybenzyl)	4.9	26.9	63.2	30.3	77.4	++(30)
15	[1-(Hydroxymethyl)propyl]	(3-Hydroxybenzyl)	10	29.1	54.3	72.2	34.1	++(50)
16	(4-Aminocyclohexyl)	(4-Hydroxybenzyl)	2.8	18.7	24.3	26.7	24.1	++(10)
17	Hexyl	[(2-Hydroxy-1-phenyl)ethyl]	>100	17.8	25.3	31.8	26.9	—
18	[1-(Hydroxymethyl)-2-methylpropyl]	(3-Chlorophenyl)	20	>167	>167	>167	>167	—
19	(4-Aminocyclohexyl)	(3-Chlorophenyl)	1.1	5.5	5.7	8.8	5.5	—
20	(4-Hydroxycyclohexyl)	(3-Chlorophenyl)	>100	>167	>167	>167	>167	—
21	(4-Hydroxycyclohexyl)	Cyclohexylmethyl	>100	>167	>167	>167	>167	—

Biological activities were tested according to methods described in the Section 4.[14] Induction of p53-dependent transcription in Arn8 cells is expressed here as positive (+), strongly positive (++) or negative (−), with the concentration exhibiting maximum activity given in brackets in micromolar.
[a] Values are means of three experiments; SD values did not exceed 15%.
[b] Roscovitine activity was added as a standard compound for comparison.

CDK2 in a different manner, with C8H shifted towards Phe80, unable to H-bond to the protein, but still exhibiting high affinity to CDK2.[24] Similarly, 3,7-disubstituted pyrazolo[4,3-*d*]pyrimidines without C8H also potently inhibit CDKs and probably orient within the active site in a mode of mentioned guanines.[19] The position and presence of heteronitrogens in a purine skeleton thus seems to markedly affect the binding mode of purine-like ligands. In this respect, synthesis and evaluation of 8-azapurines was necessary to elucidate SAR of purine based heterocyclic skeletons.

In summary, the following structure–activity relationships of 8-azapurines have been observed: (1) isopropyl at N9 was the most promising, bulky substituents decreased the inhibitory activity to below detectable level (**20** and **21**); (2) the most significant structural feature was the introduction of 2-hydroxybenzylamino (olomoucine II) or 3-chloroanilinoamino (purvalanol A) substituent at C6 position;[12,14] (3) [1-(hydroxymethyl)propyl]amino (roscovitine),[11] [1-(hydroxymethyl)-2-methylpropyl]amino (purvalanol)[12] and (4-aminocyclohexyl)amino (CGP 74514)[25] C2-side chains were also the most effective with 8-azapurines, like in the case of trisubstituted purines. Although the introduced nitrogen decreased the affinity of 8-azapurines to CDK2, many compounds yet (**6**, **8–10**, **14**, **16**, **19**) retained IC_{50} values below olomoucine (7 μM).

New 8-azapurine derivatives were also examined for their cytotoxic properties against MCF7, HOS, G361 and K562 cancer cell lines. A significant drop in anti-CDK activity, previously shown to correlate with the antiproliferative effect of 2,6,9-trisubstituted purines,[26] was also obvious from the in vitro anticancer tests (Table 1). However, a 50-fold decrease of CDK inhibitory potency of all compounds versus parental trisubstituted purines was unexpectedly not followed by intensive reduction of cytotoxic properties of the respective 8-azapurine derivatives (2–5-fold), active in a micromolar range. Moreover, compound **19** derived from parental purine CGP 74514 surprisingly exerted even slightly stronger cytotoxicity on a panel of 17 cell lines from various human tumor tissues (Table 2).

Table 2. Antiproliferative activity of selected 8-azapurines on cancer cell lines of different origin

Cell line	Compound IC_{50} (μM)[a]			
	8	**11**	**16**	**19**
HOS	101.7	33.1	26.7	8.8
HBL100	123.4	35.3	33.2	9.1
BT474	75.5	21.8	15.6	7.6
MCF7	22.1	20.2	18.7	5.5
HT29	119.2	34.7	34.6	11.3
CEM	16.6	13.6	7.2	5.1
HL60	36.9	21.6	17.6	7.0
K562	33.1	21.3	24.3	5.7
HS913T	110.2	18.9	21.4	7.8
A549	61.5	47.6	157.8	9.7
A431	118.3	32.5	26.2	7.9
SVK14	29.2	27.0	22.9	7.8
G361	49.2	11.8	24.1	5.5
SKUT-1	48.0	33.0	38.6	7.7
HeLa	60.2	31.4	26.8	7.6
T98G	154.6	32.5	114.6	8.5
Arn8	35.2	23.1	25.8	6.1

[a] Values are means of three experiments; SD values did not exceed 15%.

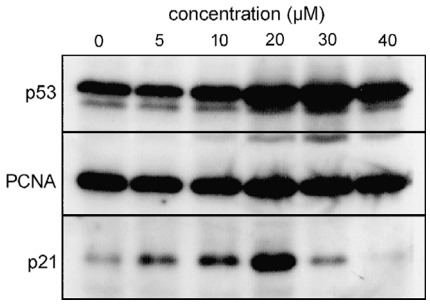

Figure 2. Dose-dependent induction of p53 and $p21^{WAF1}$ proteins in MCF7 cells treated with indicated concentrations (μM) of compound **9** for 24 h. The cells were harvested and analyzed on Western blot with specific antibodies as described in Section 4.

The CDK inhibitor roscovitine has also been shown to induce nuclear accumulation of active wild-type tumor suppressor protein p53 in human untransformed and tumor-derived cells.[27,28] Thus, the activation of p53-dependent transcription after treatment with different 8-azapurine derivatives was also analyzed in the human melanoma cell line Arn8 expressing β-galactosidase under the control of a p53-responsive promoter.[28] Striking induction of wild-type p53 protein and dramatic enhancement of p53-dependent transcription, was observed after treatment with many 8-azapurines (Table 1). The effect was subsequently verified in 8-azapurine **9** treated MCF7 cells, analyzed on western blots using anti-p53 and anti-$p21^{WAF1}$ antibodies (Fig. 2). Interestingly, the total p53 activity induced by new purine analogs correlates much better with the observed cytotoxicity in different cancer cell lines. These results may highlight the therapeutic potential of purine-like CDK inhibitors as anticancer drugs, especially in tumors retaining a functional wild-type p53 pathway. However, a recent finding that another CDK inhibitor flavopiridol exerts its cytotoxic effect via inhibition of CDK7 and CDK9 suggests that induction of p53 could be a marker and not a cause of cytotoxicity.[29] Despite that, the most active 8-azapurine **19**, that displayed the highest cytotoxicity on a panel of cell lines, did influence neither p53 activity in Arn8 cells (Table 1) nor p53 and $p21^{WAF1}$ levels in MCF7 cells (data not shown).

3. Conclusions

In summary, the prepared trisubstituted 8-azapurines (1,2,3-triazolo[4,5-*d*]pyrimidines) proved the negative action of extra nitrogen in the purine ring on biological activity. Previously reported correlation coefficients between CDK inhibition by roscovitine-like inhibitors and their antiproliferative activity demonstrated an obvious link.[26] On the other hand, the values were not very high, giving us the space to speculate that CDKs, once believed to be specifically inhibited by 2,6,9-trisubstituted purines, seem not to be the one and only targets responsible for the antiproliferative activity of purine CDK inhibitors. Moreover, several other enzymes/proteins were recently identified to interact with purines as well as other types of CDK inhibitors.[30] In the light of the above findings the possibility of targeting other structures besides CDK2 remains the subject of our further work.

4. Experimental

4.1. General experimental procedures

Reagents were from standard commercial sources and of analytical grade. Thin-layer chromatography (TLC) was carried out using aluminium sheets with silica gel F_{254} from Merck. Spots were visualized under UV light (254 nm). Preparative column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400). Melting points were determined on a Kofler block and were uncorrected. The ^{1}H NMR spectra (δ, ppm; J, Hz) were measured on Bruker Avance DRX 500 (500 MHz), Varian VXR-400 (400 MHz) or on Varian Gemini 300 (300 MHz) spectrometers. All spectra were obtained at 25 °C using tetramethylsilane as an internal standard. Mass spectra were measured on a MS Waters/Micromass, ZMD-detector, direct inlet, ESI, coin voltage 20 V. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography.

Synthesis of benzylazide,[31] 4-amino-1-benzyl-5-carbamoyl-1,2,3-triazole (**Ia**)[32] (yield 75%, mp 231–234 °C) and 9-benzyl-2,6-dihydroxy-8-azapurine (**IIa**)[22] (yield 58%, mp 290–291 °C) were prepared according to the indicated references. The isopropylazide was prepared from isopropylbromide according to the common procedure and distilled at 60 °C/850 mbar.[33]

4.2. Prepared compounds

4.2.1. 9-Benzyl-2,6-dichloro-8-azapurine (IIIa). 2,6-Lutidine (0.7 mL) was added to the cooled (−20 °C) mixture of 9-benzyl-2,6-dihydroxy-8-azapurine **IIa** (1.11 mmol) and phosphoryl chloride (3 mL).[22] The reaction mixture was heated for 1 h to reach 110 °C and this temperature was maintained for 9 h. After concentration in vacuo (temperature up to 80 °C) the residue was decomposed by ice–water mixture (0 °C). The product was extracted with chloroform, the solution was decolorized by activated charcoal, evaporated and the residue was crystallized from abs. Et_2O (85%; mp 92–94 °C). ^{1}H NMR (500 MHz, $CDCl_3$): 5.03 (2H, s, J = 6.7, CH_2); 7.34–7.39 (3H, m, Ph); 7.47 (2H, m, Ph).

4.2.2. 4-Amino-5-carbamoyl-1-isopropyl-1,2,3-triazole (I). Isopropylazide (62 g, 0.73 mol) and 2-cyanoacetamide (63 g, 0.75 mol) were added to a solution of sodium ethoxide (from 17.3 g Na^0) in 1 L absolute ethanol. The mixture was heated for 1 h under reflux and gentle reflux was maintained for 20 h. The reaction mixture was concentrated in vacuo (up to 40 °C) and the residue was dissolved in water. The product was extracted with ethyl acetate, the organic layer was evaporated away and the product was crystallized from water and re-crystallized from methanol (yield 36%, 44 g, mp 209–215 °C), MS ESI+170.2 (100, $M+H^+$), 192.1 (30, $M+Na^+$). ^{1}H NMR (500 MHz, D_2O): 1.29 (6H, d, J = 6.7, $(CH_3)_2CH$); 4.29 (1H, sept, J = 6.7, $(CH_3)_2CH$). IR (KBr): 3440 (H_2NCO), 3306

(H_2NCO), 3188 (NH_2), 1666, 1645, 1560, 1304, 1253, 1243, 1079, 1031,1012. Anal. ($C_6H_{11}N_5O$) C, H, N.

4.2.3. 2,6-Dihydroxy-9-isopropyl-8-azapurine (IIb). A mixture of sodium ethoxide (obtained from 12 g Na^0 and absolute 1.4 L EtOH) and 4-amino-5-carbamoyl-1-isopropyl-1,2,3-triazole **Ia** (22 g, 0.12 mol) was warmed to reflux. Diethyl carbonate (22 mL, 0.18 mol) was added over 40 min and it was refluxed for another 4 h. A powerful mechanical stirrer was necessary for appropriate stirring of the viscous mixture. After cooling, 0.4 L of water was added and pH was adjusted to 6. The reaction mixture was then concentrated to volume approximately 0.4 L in vacuo and the precipitated product was filtered. Recrystallization from water and then from methanol afforded the product in 32% yield (7.2 g, mp 303–306 °C), MS ESI+218.1 (100, $M+Na^+$), 413.2 (20, $2\,M+Na^+$), ESI−194.2 (100, $M-H^+$). ^1H NMR (500 MHz, D_2O): 1.33 (6H, d, $J = 6.8$, $(CH_3)_2CH$); 4.56 (1H, sept, $J = 6.8$, $(CH3)_2CH$). IR (KBr): 3412 vb and 3170 sh (HO associates), 1680, 1609, 1571, 1515, 1386, 1274. Anal. ($C_7H_9N_5O_2$) C, H, N.

4.2.4. 2,6-Dichloro-9-isopropyl-8-azapurine (IIIb). 2,6-Lutidine (0.7 mL) was added to the cooled (−20 °C) mixture of 2,6-dihydroxy-9-isopropyl-8-azapurine (**IIb**, 1.11 mmol) and phosphoryl chloride (3 mL). The reaction mixture was heated for 3 h at 120 °C. After evaporation in vacuo (temperature up to 80 °C) the residue was decomposed by ice–water mixture (0 °C) with simultaneous extraction of product in benzene. The solution was immediately dried with $MgSO_4$, evaporated and immediately used in reaction with appropriate amines.

4.2.5. General preparation of 6-alkylamino-2-chloro-9-isopropyl(or benzyl)-8-azapurine (IV). 2,6-Dichloro derivative **IIIa,b** was dissolved in a minimum amount of abs. MeOH (benzylamine or its derivatives) or 1-butanol (3-chloroaniline), 2 eq. of triethylamine were added and the mixture was cooled to 5–10 °C. After addition of 1 alkyl amine eq. the course of the reaction was monitored by TLC chromatography. The reaction with less reactive amines (chloroanilines) was carried out at room temperature. Some products precipitated but all derivatives **IV** were still purified by flash chromatography on Kieselgel. Details are given below, the given yields refer to the weight of crude evaporated residue of dichloroderivative **III**.

4.2.6. 9-Benzyl-2-chloro-6-(cyclohexylmethyl)amino-8-azapurine (IVa). Reaction in MeOH (25 °C, 16 h), 85% yield after column chromatography in chloroform, crystallization from abs. Et_2O, mp 142–147 °C, MS ESI+357.0 (100, $M+H^+$). ^1H NMR (500 MHz, $CDCl_3$): 1.05 (1H, m, C_6H_{11}); 1.24 (4H, m, C_6H_{11}); 1.62–1.87 (6H, m, C_6H_{11}); 3.54 (1.36H, dd, $J = 6.8$, 6.0, $NHCH_2$); 3.97 (0.64H, dd, $J = 6.8$, 6.0, $NHCH_2$); 5.69 (2H, s, CH_2Ph); 6.23 (0.32H, t, $J = 6.6$, NH); 6.94 (0.68H, t, $J = 6.0$, NH); 7.29–7.37 (3H, *meta* + *para* Ph); 7.43 (1.36H, m, *ortho* Ph); 7.45 (0.64H, m, *ortho* Ph). The two compounds (ratio 0.68:0.32) are rotamers (exchange peaks in ROSY between NH and between CH_2 of both species). Anal. ($C_{18}H_{21}N_6Cl$) C, H, N.

4.2.7. 9-Benzyl-2-chloro-6-(3-chlorophenyl)amino-8-azapurine. Reaction in 1-butanol (25 °C, 3 days), 80% yield after column chromatography (toluene:chloroform, 7:3), amorphous, MS ESI+371.0 (100, $M+H^+$). Anal. ($C_{17}H_{12}N_6Cl_2$) C, H, N.

4.2.8. 6-Benzylamino-2-chloro-9-isopropyl-8-azapurine (IVb). Reaction in 1-butanol (4–8 °C), precipitated in 83% yield, used without column chromatography, mp 184–185 °C, MS ESI+303.2 (100, $M+H^+$). ^1H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): 1.68 (6H, d, $J = 6.7$, $(CH_3)_2CH$); 4.85 (2H, s, CH_2Ph); 5.10 (1H, sept, $J = 5.8$, $CH(CH_3)_2$); 5.33 (1H, s, NH); 7.30–7.45 (5H, m, Ph). Anal. ($C_{14}H_{15}N_6Cl$) C, H, N.

4.2.9. 2-Chloro-6-(3-hydroxy-4-methoxybenzyl)amino-9-isopropyl-8-azapurine (IVc). Reaction in MeOH (4–8 °C), 65% yield after column chromatography (toluene:EtOAc with a trace of NH_4OH, 7:3), crystallization from $CHCl_3/Et_2O$, mp 177–179 °C, MS ESI+349.1 (100, $M+H^+$), ESI−347.1 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): 1.68 (6H, d, $J = 6.7$, $(CH_3)_2CH$); 3.89 (3H, s, OCH_3), 4.74 (2H, s, CH_2Ar); 5.10 (1H, sept, $J = 6.7$, $(CH_3)_2CH$); 5.23 (1H, s, NH); 6.83 (1H, d, $J = 8.0$, Ar); 6.89 (1H, d, $J = 8.2$, Ar); 6.95 (1H, s, Ar); 7.28 (1H, s, OH). Anal. ($C_{15}H_{17}N_6ClO_2$) C, H, N.

4.2.10. 2-Chloro-6-(2-hydroxybenzyl)amino-9-isopropyl-8-azapurine (IVd). Reaction in MeOH (4–8 °C), precipitated in 60% yield, recrystallization from MeOH, mp 198–206 °C, MS ESI+319.2 (100, $M+H^+$), ESI−317.2 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$): 1.75 (6H, d, $J = 6.7$, $(CH_3)_2CH$); 4.85 (2H, d, $J = 6.5$, CH_2Ar); 5.18 (1H, sept, $J = 6.7$, $(CH_3)_2CH$); 6.85 (1H, dd, $J = 7.3$, $J = 7.3$, Ar), 7.00 (1H, d, $J = 8.0$, Ar), 7.24 (1H, dd, $J = 7.3$, $J = 8.6$, Ar); 7.43 (1H, d, $J = 7.5$, Ar); 9.28 (1H, bt, NH); 9.58 (1H, s, OH). Anal. ($C_{14}H_{15}N_6ClO$) C, H, N.

4.2.11. 2-Chloro-6-(3-hydroxybenzyl)amino-9-isopropyl-8-azapurine (IVe). Reaction in MeOH (4–8 °C), 65% yield is after column chromatography (0–1% MeOH in chloroform), crystallization from Et_2O, mp 189–193 °C, MS ESI+319.2 (100, $M+H^+$), ESI−317.2 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): 1.68 (6H, d, $J = 6.6$, $(CH_3)_2CH$); 4.77 (2H, s, CH_2Ar); 5.09 (1H, sept, $J = 6.6$, $(CH_3)_2CH$); 6.77 (1H, dd, $J = 7.0$, $J = 3.0$, Ar); 6.87 (2H, m, Ar); 7.18 (1H, dd, $J = 7.8$, $J = 7.8$, Ar). Anal. ($C_{14}H_{15}N_6ClO$) C, H, N.

4.2.12. 2-Chloro-6-(4-hydroxybenzyl)amino-9-isopropyl-8-azapurine (IVf). Reaction in MeOH (4–8 °C), 70% yield after column chromatography (0–2% MeOH in chloroform), crystallization from $CHCl_3/Et_2O$, mp 206–209 °C, MS ESI+319.2 (100, $M+H^+$), ESI−317.2 (100, $M-H^+$).^1H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): 1.68 (6H, d, $J = 7.2$, $(CH_3)_2CH$); 4.73 (2H, s, CH_2Ar); 5.09 (1H, sept, $J = 7.2$, $(CH_3)_2CH$); 6.81 (2H, d, $J = 7.9$, Ar); 7.27 (2H, d, $J = 7.9$); 7.30 (1H, s, OH). Anal. ($C_{14}H_{15}N_6ClO$) C, H, N.

4.2.13. 2-Chloro-9-isopropyl-6-(1-phenyl-2-hydroxyethyl)amino-8-azapurine (IVg). Reaction in MeOH (4–8 °C), 65% yield after column chromatography (chloroform),

crystallization from Et_2O, mp 180–186 °C, MS ESI+333.2 (100, M+H$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): 1.67 (6H, dd, J = 7.8, J = 7.8, $(CH_3)_2CH$); 4.07 (2H, m, CH_2); 5.08 (1H, sept, J = 7.7, $(CH_3)_2CH$); 5.58 (1H, dd, J = 5.1, J = 5.1 CHPh), 7.28–7.49 (5H, m, Ph). ^{13}C NMR (125 MHz, $CDCl_3$): 22.01q; 50.96d; 56.65d; 66.50t; 123.80s; 126.94d; 128.10d; 128.87d, 138.22s; 149.30s; 158.44s. Anal. ($C_{15}H_{17}N_6ClO$) C, H, N.

4.2.14. 2-Chloro-6-(3-chlorophenyl)amino-9-isopropyl-8-azapurine (IVh). Reaction in 1-butanol (25 °C, 3 days), 80% yield after column chromatography (toluene:chloroform, 7:3), amorphous, MS ESI+323.0 (100, M+H$^+$), 325.0 (63, M+H$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): 1.70 (6H, d, J = 6.8, $(CH_3)_2CH$); 4.95 (1H, sept, J = 6.8, $(CH_3)_2CH$); 5.10 (1H, br s, NH); 7.10 (1H, bd, J = 15.0, Ar); 7.27–7.39 (3H, m, Ar). Anal. ($C_{13}H_{12}N_6Cl_2$) C, H, N.

4.2.15. General procedure for preparation of 2-alkylamino-6-alkylamino-9-isopropyl(or benzyl)-8-azapurines (V). The appropriate 2-chloro derivative (**IVa–h**) was dissolved in 1-butanol or *N*-methyl-2-pyrrolidon (NMP) (usually 0.1 g in 1.5 mL) and 5 eq. of appropriate alkyl amine R2NH$_2$ (unless otherwise stated) were added. Neat amine was used in the case of less reactive 2-amino-1-butanol and amino-3-methyl-1-butanol. After heating (as specified below) the reaction mixture was evaporated in vacuo and the residue was purified by flash chromatography on Kieselgel. Details of individual compounds are given below.

4.2.16. 2-(*trans*-4-Aminocyclohexyl)amino-6-benzylamino-9-isopropyl-8-azapurine (1). Reaction in NMP (100 °C, 1 h), 72% yield after column chromatography (0–10% MeOH in toluene with a trace of NH_4OH), crystallization from $CHCl_3$/Et_2O, mp 143–150 °C, MS ESI+381.3 (100, M+H$^+$). ^{1}H NMR (500 MHz, CD_3OD): 1.29–1.50 (4H, m, C_6H_{11}); 1.61 (6H, d, J = 6.6, $CH_3)_2CH$; 2.00–2.20 (4H, m, C_6H_{11}); 3.00 (1H, bm, $CHNH_2$); 3.81 (1H, bm, $CHNH$); 4.61 (2H, s, CH_2Ph); 7.21–7.39 (5H, m, Ph). Signal of $CH(CH_3)$ is hidden in solvent signal. Anal. ($C_{20}H_{28}N_8$) C, H, N.

4.2.17. 6-Benzylamino-2-(3-hydroxypropyl)amino-9-isopropyl-8-azapurine (3). Reaction in 1-butanol (100 °C, 1.5 h), 85% yield after column chromatography (0–2% MeOH in chloroform), crystallization from $CHCl_3$/Et_2O, mp 123–125 °C, MS ESI+342.3 (100, M+H$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.65 (6H, d, J = 7.0, $(CH_3)_2CH$); 1.80 (2H, m, $CH_2CH_2CH_2$); 3.65 (2H, m, $CH_2CH_2CH_2$); 4.76 (2H, br s, CH_2Ph); 4.88 (1H, sept, J = 6.6, $CH(CH_3)_2$); 5.27 (1H, br s, NH); 6.30 (1H, br s, NH); 7.30–7.40 (5H, m, Ph). Anal. ($C_{17}H_{23}N_7O$) C, H, N.

4.2.18. 6-Benzylamino-2-[1-(hydroxymethyl)propyl]amino-9-isopropyl-8-azapurine (4). Reaction in neat amine (100 °C, 50 amine eq., 20 h), 64% yield after column chromatography (0–2% MeOH in chloroform), crystallization from $CHCl_3$/Et_2O, mp 122–127 °C, MS ESI+356.3 (100, M+H$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.02 (3H, t, J = 7.4, CH_3CH_2); 1.56 (2H, m, CH_2CH_3); 1.64 (6H, d, J = 6,9, $(CH_3)_2CH$); 3.64(1H, m, $CHNH$); 3.77–4.02 (2H, m, AB, CH_2OH); 4.77 (2H, br s, CH_2Ph); 4.89 (1H, sept, J = 6.9, $CH(CH_3)_2$); 5.14 (1H, br s, NH); 6.54 (1H, br s, NH); 7.25–7.38 (5H, m, Ph). Anal. ($C_{18}H_{25}N_7O$) C, H, N.

4.2.19. 6-Benzylamino-2-[1-(hydroxymethyl)-2-methylpropyl]amino-9-isopropyl-8-azapurine (5). Reaction in NMP (110 °C, 20 h, 10 amine eq.), 64% yield after column chromatography (0–1% MeOH in chloroform), crystallization from abs. Et_2O, mp 137–142 °C, MS ESI+370.2 (100, M+H$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): 1.01 (6H, dd, J = 5.8, J = 5.8, $(CH_3)_2CHCH$); 1.64 (6H, dd, J = 6.7, J = 4.0, $(CH_3)_2CHN$); 1.69 (1H, br s, $(CH_3)_2CHCH$); 1.97 (1H, s, OH); 3.70 (1H, br s, $CHHOH$); 3.86–3.89 (2H, bm, $CHHOH$ + $CHCH_2OH$); 4.77 (2H, bm, CH_2Ph); 4.89 (1H, sept, J = 6.7, $NCH(CH_3)_2$); 5.15 (1H, br s, NH); 6.47 (1H, br s, NH); 7.26–7.38 (5H, m, Ph). Anal. ($C_{19}H_{27}N_7O$) C, H, N.

4.2.20.6-(3-Hydroxy-4-methoxybenzyl)amino-2-[1-(hydroxymethyl)propyl]amino-9-isopropyl-8-azapurine (6). Reaction in 1-butanol (100 °C, 5 amine eq., 2 h), 68% yield after column chromatography (0–2% MeOH in chloroform), crystallization from $CHCl_3$/abs. Et_2O, mp 121–123 °C, MS ESI+402.3 (100, M+H$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.02 (3H, t, J = 7.3 Hz, CH_3CH_2); 1.68 (6H, d, J = 6.8, $(CH_3)_2CH$); 1.57 (2H, m, CH_2CH_3); 3.65 (1H, m $CHHOH$); 3.81 (1H, m $CHHOH$); 3.89 (3H, s, CH_3O); 4.77 (2H, br s, CH_2Ar); 4.92 (1H, sept, J = 6.8, $CH(CH_3)_2$); 5.23 (1H, br s, NH); 6.83–6.89 (2H, m, Ar); 6.95 (1H, s, Ar). Anal. ($C_{19}H_{27}N_7O_3$) C, H, N.

4.2.21. 2-(*cis*-2-Aminocyclohexyl)amino-6-(3-hydroxy-4-methoxybenzyl)amino-9-isopropyl-8-azapurine (7). Reaction in 1-butanol (100 °C, 20 amine eq., 2 h), 56% yield after column chromatography (0–5% MeOH in chloroform with a trace of NH_4OH), crystallization from abs. Et_2O, mp 118–121 °C, MS ESI+427.4 (100, M+H$^+$). ^{1}H NMR (400 MHz, $CDCl_3$): ^{1}H NMR (500 MHz, CD_3OD): 1.45–2.12 (8H, m, C_6H_{11}); 1.61 (3H, d, J = 6.8, $(CH_3)CH$); 1.62 (3H, d, J = 6.8, $CH(CH_3)$); 3.56 (1H, br s, $CHNH_2$); 3.89 (1H, s, CH_3O); 4.45 (1H, br s, $CHNH$); 4.74 (2H, s, CH_2Ar); 4.96 (1H, m, $(CH_3)_2CH$); 6.83 (1H, d, J = 8.0, Ar); 6.89 (1H, d, J = 8.2, Ar); 6.95 (1H, d, J = 8.6, Ar). Anal. ($C_{21}H_{30}N_8O_2$) C, H, N.

4.2.22. 6-(2-Hydroxybenzyl)amino-2-[1-(hydroxymethyl)propyl]amino-9-isopropyl-8-azapurine (8). Reaction in NMP (105 °C, 10 amine eq., 2 h), 73% yield after column chromatography (0–1% MeOH in chloroform), amorphous, MS ESI+372.3 (100, M+H$^+$), ESI−370.2 (100, M−H$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): 1.07 (3H, t, J = 7.3, CH_3CH_2); 1.68 (6H, dd, J = 7.6, J = 7.6, $(CH_3)_2CH$); 1.73 (2H, m, CH_3CH_2,); 3.74 (1H, m, $CHHOH$); 3.94–4.09 (2H, m, $CHHOH$ + CHN); 4.47 (1H, br s, $CHHPh$); 4.58 (1H, br s, $CHHPh$); 4.96 (1H, sept, J = 7.3, $(CH_3)_2CH$); 5.70 (1H, br s, NH); 6.79 (1H, dd, J = 7.3, J = 7.3, Ar); 6.89 (1H, d, J = 8.0, Ar); 7.17 (1H, dd, J = 7.3, J = 7.3, Ar); 7.39 (1H, d, J = 8.0, Ar). Anal. ($C_{18}H_{25}N_7O_2$) C, H, N.

4.2.23. 6-(2-Hydroxybenzyl)amino-2-[1-(hydroxymethyl)-2-methylpropyl]amino-9-isopropyl-8-azapurine (9). Reaction in 1-butanol (120 °C, 5 amine eq., 22 h), 88% yield

after column chromatography (chloroform), crystallization from abs. Et_2O, mp 77–81 °C, MS ESI+386.3 (100, $M+H^+$), ESI−384.2 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$): 1.07 (6H, d, $J = 6.6$, $(CH_3)_2CHCH$); 1.66 (3H, d, $J = 7.6$, $(CH_3)CHN$); 1.67 (3H, d, $J = 7.6$, $CH(CH_3)N$); 2.06 (1H, m, br s, $(CH_3)_2CHCH$); 3.71–3.99 (3H, m AB + m, $CH_2HOH + CHNH$); 4.60 (2H, bd, CH_2Ar); 4.93 (1H, sept, $J = 6.6$, $NCH(CH_3)_2$); 5.52 (1H, br s, OH); 6.81 (1H, t, $J = 7.3$, Ar); 6.91 (1H, d, $J = 8.0$, Ar); 7.19 (1H, t, $J = 7.4$, Ar); 7.36 (1H, d, $J = 8.0$, Ar); 8.50 (1H, br s, $NHCH_2$). The proton 2D-COSY experiments were used for the assignment of signals. Anal. ($C_{19}H_{27}N_7O_2$) C, H, N.

4.2.24. 2-(*cis*-2-Aminocyclohexyl)amino-6-(2-hydroxybenzyl)amino-9-isopropyl-8-azapurine (10). Reaction in 1-butanol (115 °C, 6 amine eq., 2 h), 72% yield after column chromatography (0–5% MeOH in chloroform with a trace of NH_4OH), amorphous, MS ESI+397.4 (100, $M+H^+$), MS ESI−395.4 (100, $M-H^+$). ^1H NMR (500 MHz, CD_3OD): 1.62 (6H, dd, $J = 6.7$, $J = 2.7$, $(CH_3)_2CH$); 1.45–2.10 (8H, m, C_6H_{10}); 3.55 (1H, br s, $CHNH_2$); 4.44 (1H, br s, CHNH); 4.75 (2H, s, CH_2Ar); 4.96 (1H, m, $(CH_3)_2CH$); 6.81 (2H, m, Ar), 7.11 (1H, dd, $J = 7.6$, $J = 7.6$, Ar); 7.22 (1H, d, $J = 7.2$, Ar). Anal. ($C_{20}H_{28}N_8O$) C, H, N.

4.2.25. 2-(*trans*-4-Aminocyclohexyl)amino-6-(2-hydroxybenzyl)amino-9-isopropyl-8-azapurine (11). Reaction in neat amine (115 °C, 4 h, 35 amine eq.), 84% yield after column chromatography (0–8% MeOH in chloroform with a trace of NH_4OH), crystallization from $CHCl_3$/abs. Et_2O, mp 120–123 °C, MS ESI+397.4 (100, $M+H^+$), MS ESI−395.4 (100, $M-H^+$). ^1H NMR (500 MHz, CD_3OD): 1.29–1.50 (4H, m, C_6H_{10}); 1.61 (6H, d, $J = 6.6$, $CH_3)_2CH$; 2.00–2.25 (4H, m, C_6H_{10}); 3.00 (1H, bt, $CHNH_2$); 3.81 (1H, bm, CHNH); 4.62 (2H, s, CH_2Ar); 4.90 (1H, sept, $J = 6.6$, $(CH_3)_2CH$); 6.81 (2H, m, Ar), 7.11 (1H, dd, $J = 7.6$, $J = 7.6$, Ar); 7.225 (1H, d, $J = 7.2$, Ar). Anal. ($C_{20}H_{28}N_8O$) C, H, N.

4.2.26. 2,6-Bis[(2-Hydroxybenzyl)amino]-9-isopropyl-8-azapurine (12). Reaction in NMP (100 °C, 2 h), 68% yield after column chromatography (chloroform), crystallization from chloroform/abs. Et_2O, mp 215–217 °C, MS ESI+406.3 (100, $M+H^+$), ESI−404.3 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): 1.68 (6H, d, $J = 6.6$, $(CH_3)_2CH$); 4.57 (2H, d, CH_2Ar); 4.62 (2H, s, CH_2Ar); 4.98 (1H, sept, $J = 6.7$, $(CH_3)_2CH$); 6.83–6.92 (4H, m, Ar), 7.19 (2H, dd, $J = 7.4$, $J = 7.4$, Ar); 7.27 (2H, d, $J = 6.8$, Ar). Anal. ($C_{21}H_{23}N_7O_2$) C, H, N.

4.2.27. 6-(3-Hydroxybenzyl)amino-2-[1-(hydroxymethyl)-2-methylpropyl]amino-9-isopropyl-8-azapurine (13). Reaction in 1-butanol (100 °C, 22 h, 10 amine eq.), 67% yield after column chromatography (0–3% MeOH in chloroform), crystallization from abs. EtOAc, mp 150–152 °C, MS ESI+386.2 (100, $M+H^+$), ESI−384.2 (100, $M-H^+$). ^1H NMR (500 MHz, $CDCl_3$): 1.07 (6H, d, $J = 6.6$, $(CH_3)_2CHCH$); 1.66 (3H, d, $J = 7.6$, $(CH_3)CHN$); 1.67 (3H, d, $J = 7.7$, $CH(CH_3)N$); 2.06 (1H, m, br s, $(CH_3)_2CHCH$); 3.70–3.96 (3H, m AB+m, $CH_2HOH + CHNH$); 4.60 (2H, bd, CH_2Ph); 4.93 (1H, sept, $J = 6.6$, $NCH(CH_3)_2$); 5.41 (1H, br s, OH); 6.78 (1H, dd, $J = 8.0$, $J = 3.0$, Ar); 6.88 (2H, m, Ar); 7.18 (1H, m, Ar). Anal. ($C_{19}H_{27}N_7O_2$) C, H, N.

4.2.28. 2-(*trans*-4-Aminocyclohexyl)amino-6-(3-hydroxybenzyl)amino-9-isopropyl-8-azapurine (14). Reaction in 1-butanol (120 °C, 20 h, 6 amine eq.), 77% yield after column chromatography (0–10% MeOH in chloroform with a trace of NH_4OH), crystallization from abs. Et_2O, mp 109–114 °C, MS ESI+397.2 (100, $M+H^+$). ^1H NMR (500 MHz, CD_3OD): 1.25–1.53 (4H, m, C_6H_{10}); 1.60 (6H, d, $J = 6.6$, $(CH_3)_2CH$); 1.97–2.22 (4H, m, C_6H_{10}); 3.00 (1H, m, $CHNH_2$); 3.83 (1H, m, CHNH); 4.69 (2H, s, CH_2Ar); 5.13 (1H, m, $(CH_3)_2CH$); 6.67 (1H, m, Ar); 6.82 (2H, m, Ar); 7.12 (1H, dd, $J = 7.8$, $J = 7.8$, Ar). Anal. ($C_{20}H_{28}N_8O$) C, H, N.

4.2.29. 6-(3-Hydroxybenzyl)amino-2-[1-(hydroxymethyl)propyl]amino-9-isopropyl-8-azapurine (15). Reaction in 1-butanol (100 °C, 8 h, 15 amine eq.), 73% yield after column chromatography (0–3% MeOH in chloroform), crystallization from abs. Et_2O/pentane, mp 105–109 °C, MS ESI+371.2 (100, $M+H^+$). ^1H NMR (300 MHz, $CDCl_3$): 1.03 (3H, t, $J = 7.3$ Hz, CH_3CH_2); 1.56 (2H, bm, CH_2CH_3); 1.64 (6H, d, $J = 6{,}9$ Hz, $(CH_3)_2CH$); 3.65 (1H, m, CHNH); 3.77–4.02 (2H, bm, AB, CH_2OH); 4.77 (2H, br s, CH_2Ar); 4.82 (1H, sept, $J = 6.9$ Hz, $CH(CH_3)_2$); 5.30 (1H, br s, NH); 6.60 (1H, br s, NH); 6.78–6.88 (3H, m, Ar); 7.30 (1H, s, Ar). Anal. ($C_{18}H_{25}N_7O_2$) C, H, N.

4.2.30. 2-(*trans*-4-Aminocyclohexyl)amino-6-(4-hydroxybenzyl)amino-9-isopropyl-8-azapurine (16). Reaction in neat amine (115 °C, 4 h, 35 amine eq.), 84% yield after column chromatography (0–8% MeOH in chloroform with a trace of NH_4OH), crystallization from abs. Et_2O, mp 118–122 °C, MS ESI+397.2 (100, $M+H^+$). ^1H NMR (500 MHz, CD_3OD): 1.25–1.55 (4H, m, C_6H_{10}); 1.62 (6H, d, $J = 6.8$, $(CH_3)_2CH$); 2.00–2.20 (4H, m, C_6H_{10}); 3.08 (1H, m, $CHNH_2$); 3.85 (1H, m, CHNH), 4.64 (2H, s, CH_2Ar); 5.10 (1H, m, $(CH_3)_2CH$); 5.43 (1H, s, NH); 6.750 (2H, d, $J = 8.2$, Ar); 7.210 (2H, d, $J = 8.3$, Ar). Anal. ($C_{20}H_{28}N_8O$) C, H, N.

4.2.31. 2-Hexylamino-9-isopropyl-6-(1-phenyl-2-hydroxyethyl)amino-8-azapurine (17). Reaction in 1-butanol (100 °C, 2 h), 85% yield after column chromatography (chloroform), amorphous, MS ESI+398.4 (100, $M+H^+$). ^1H NMR (400 MHz, $CDCl_3$): 0.90 (3H, t, $J = 6.7$, CH_3CH_2), 1.31 (6H, m, $(CH_2)_3$), 1.63 (2H, m, CH_2CH_2N), 1.67 (6H, dd, $J = 7.8$, $J = 7.8$, $(CH_3)_2CH$); 3.32 (2H, dt, $J = 6.6$, $J = 6.6$, $NHCH_2$); 4.07 (2H, m, CH_2OH); 5.00 (1H, sept, $J = 7.8$, $(CH_3)_2CH$); 5.58 (1H, dd, $J = 5.1$, $J = 5.1$ CHPh); 7.28–7.49 (5H, m, Ph). Anal. ($C_{21}H_{31}N_7O$) C, H, N.

4.2.32. 6-(3-Chlorophenyl)amino-2-[1-(hydroxymethyl)-2-methylpropyl]amino-9-isopropyl-8-azapurine (18). Reaction in 1-butanol (100 °C, 22 h, 10 amine eq.), yield 56% after column chromatography in chloroform, crystallization from abs. Et_2O, mp 160–163 °C, MS ESI+390.2 (100, $M+H^+$). ^1H NMR (300 MHz, $CDCl_3$): 1.03 (3H, d, $J = 7.0$, $(CH_3)_2CHCH$); 1.05 (3H, d, $J = 7.0$,

($C\mathit{H}_3$)$_2$CHCH); 1.64 (6H, d, J = 6.7, ($C\mathit{H}_3$)$_2$CHN); 1.78 (1H, br s, OH); 2.01 (1H, m, J = 7.0, CHCH(CH_3)$_2$); 3.68–4.02 (3H, m, HO$C\mathit{H}_2$CH); 4.87 (1H, sept, J = 6.7, (CH_3)$_2$CHN); 5.30 (1H, br s, NH); 7.09 (1H, d, J = 7.7, Ar); 7.23–7.25 (2H, m, Ar); 7.48 (1H, m, Ar); 8.00 (1H, br s, NH). Anal. ($C_{18}H_{24}N_7ClO$) C, H, N.

4.2.33. 2-(*trans*-4-Aminocyclohexyl)amino-6-(3-chlorophenyl)amino-9-isopropyl-8-azapurine (19). Reaction in neat amine (115 °C, 4 h, 35 amine eq.), yield 89% after column chromatography (0–8% MeOH in chloroform with a trace of NH_4OH), crystallization from abs. EtOAc/ abs. Et_2O, mp 221–223 °C, MS ESI+401.3 (100, M+H$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.20–1.36 (4H, m, C_6H_{10}); 1.66d (6H, d, J = 6.7, ($C\mathit{H}_3$)$_2$CH); 1.93 (2H, m, C_6H_{10}); 2.20 (2H, m, C_6H_{10}); 2.72 (1H, m, CHNH$_2$); 3.85 (1H, m, CHNH); 4.93 (1H, sept, J = 6.7, (CH_3)$_2$CH); 5.08 (1H, d, NH); 7.09 (1H, d, J = 7.8, Ar); 7.23–7.31 (2H, m, Ar); 7.48 (1H, m, Ar). Anal. ($C_{19}H_{25}N_8Cl$) C, H, N.

4.2.34. 9-Benzyl-6-(3-chlorophenyl)amino-2-(*trans*-4-hydroxycyclohexyl)amino-8-azapurine (20). Reaction in NMP (120 °C, 22 h), yield 66% after column chromatography (0–2% MeOH in chloroform), crystallization from EtOH, mp 209 °C, MS ESI+450.3 (100, M+H$^+$). ^{1}H NMR (500 MHz, $CDCl_3$ + 20% CD_3OD): [1.21–1.40 (2H, m); 1.43–1.60 (2H, bs); 2.00–2.10 (2H, bd); 2.14–2.23 (2H, bd); C_6H_{10}]; 3.65 (1H, m, CHOH); 4.80 (1H, m, CHNH); 5.58 (2H, s, CH_2Ar); 7.10 (1H, d, J = 7.5, Ar), 7.28–7.40 (7H, m, Ar), 7.53 (1H, bm, Ar). Anal. ($C_{23}H_{24}N_7Cl$) C, H, N.

4.2.35. 9-Benzyl-6-(cyclohexylmethyl)amino-2-(*trans*-4-hydroxycyclohexyl)amino-8-azapurine (21). Reaction in NMP (120 °C, 22 h), yield 65% after column chromatography (0–2% MeOH in chloroform), crystallization from chloroform, mp 154–156 °C, MS ESI+436.3 (100, M+H$^+$). ^{1}H NMR: (500 MHz, $CDCl_3$ + 20% CD_3OD): [0.98–1.10 (2H, m); 113–1.30 (6H, m); 1.37–1.50 (2H, m); 1.60–1.85 (4H, m); 1.94–2.20 (5H, m) C_6H_{10}, C_6H_{11}]; 3.39 (2H, s, C$\mathit{H}_2C_6H_{11}$); 3.64 (1H, m, CHOH); 3.82 (1H, m, CHNH); 5.53 (2H, s, CH_2Ph); 7.26–7.40 (5H, m, Ph). Anal. ($C_{24}H_{33}N_7O$) C, H, N.

4.2.36. 2-Methyl-6-hydroxy-9-isopropyl-8-azapurine. Isopropylazide (13 mmol, 1.1 g,) and 2-cyanoacetamide (10 mmol, 0.85 g) were added to a solution of sodium ethoxide (from 0.28 g Na^0, 24 mmol) in 1 L abs. EtOH. The mixture was warmed for 1 h under reflux and gentle reflux was maintained for 2 h. Anhydrous EtOAc was added and the reflux was continued for other 9 h. The reaction mixture was then concentrated in vacuo (up to 40 °C), the residue was dissolved in water and was adjusted to pH 6. The product was extracted with chloroform, solution was dried and concentrated in vacuo. Crystallization from methanol, yield 31% (0.59 g), mp 205–213 °C, MS ESI−192.2 (100, M−H$^+$). Anal. ($C_8H_{11}N_5O$) C, H, N.

4.2.37. 6-Benzylamino-9-isopropyl-2-methyl-8-azapurine (2). Thionylchloride (10 mmol, 0.8 mL), abs. *N*,*N*-dimethylformamide (1.5 mmol, 0.12 mL) and 6-hydroxy-9-isopropyl-2-methyl-8-azapurine (1.2 mmol, 0.23 g) were suspended in chloroform (1.3 mL) and heated under reflux for 90 min (the mixture became clear nearly immediately). After concentration in vacuo (temperature up to 80 °C) the residue was decomposed by ice–water mixture (0 °C) with simultaneous extraction of the product into benzene. The benzene solution was dried with $MgSO_4$, evaporated and dissolved in 1-butanol (3 mL). Benzylamine (3 mmol, 0.3 mL) was added and the reaction mixture was heated to 100 °C for 90 min and then concentrated in vacuo. Product **2** was obtained after flash chromatography on Kieselgel (1% MeOH in chloroform), yield 29%, crystallization from Et_2O, mp 133–139 °C, MS 283.1 (100, M+H$^+$). ^{1}H NMR 1.69 (6H, d, J = 6.6, ($C\mathit{H}_3$)$_2$CH); 2.63 (3H, s, CH_3); 4.88 (2H, br s, CH_2Ph); 5.12 (1H, sept, J = 6.6, CH(CH_3)$_2$); 7.30–7.43 m (5H, Ph). Anal. ($C_{15}H_{18}N_6$) C, H, N.

4.3. Kinase inhibition assay

Human 6× His-tagged cyclin E/Cdk2 complex was produced in Sf9 insect cells co-infected with appropriate baculoviral constructs. The cells were harvested 70 h post infection in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1% Tween 20, protease inhibitors) for 30 min on ice and the soluble fraction was recovered by centrifugation at 14.000*g* for 10 min. The kinase was purified on Ni–NTA column (Qiagen) according to manufacturer's instructions, stored at 4 °C and used within a week. To carry out experiments on kinetics under linear conditions, the final point test system for kinase activity measurement was used. The assay mixture contained 1 mg/mL histone (Sigma Type III-S), 15 μM ATP, 0,2 μCi [γ-^{33}P]ATP and tested compound in a final volume of 10 μL, all in reaction buffer: 50 mM Hepes 7,4 pH, 10 mM $MgCl_2$, 5 mM EGTA, 10 mM 2-glycerolphosphate, 1 mM NaF, 1 mM DTT and protease inhibitors. After 10 min, the incubations were stopped by adding 5% H_3PO_4 and spotted on P81 phosphocellulose paper (Whatman). After washing in 5% H_3PO_4, the kinase activity was measured by digital imaging analyzer BAS-1800 (Fujifilm). The kinase activity was expressed as a percentage of maximum activity, the IC_{50} values were determined by graphic analysis.

4.4. Anticancer activity in vitro

The tumor cells (purchased from The American Type Culture Collection) were grown in DMEM medium (Gibco-BRL) supplemented with 10% (v/v) fetal bovine serum and L-glutamine (0.3 g/L) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. For anticancer cytotoxicity estimation, 10^4 cells were seeded into each well of a 96-well plate, allowed to stabilize for at least 2 h and the tested inhibitors were added at various concentrations in triplicate. Tested compounds were dissolved in DMSO to achieve 100 mM solution and concentration series were added to cells. Maximum concentration of DMSO in the assay never exceeded 0.1%. Three days after drugs, a Calcein AM (Molecular Probes) solution was added and allowed to enter the

cells for 1 h. Fluorescence of viable cells was quantified on Fluoroskan Ascent (Microsystems) and cytotoxic effective concentrations were expressed as GI_{50}'s subtracted from dose-response curves.

4.5. p53-dependent transcriptional activity

In order to measure p53-dependent transcriptional activity, β-galactosidase activity was determined for the human melanoma cell line Arn8, which was established using stable transfection of cell line A375 with p53-responsive reporter construct pRGCΔfoslacZ.[28] After 24 h incubation with compounds in a 96-well microtitre plate, the cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde, washed with PBS and developed in X-gal solution (0.2 mg/mL in PBS) overnight. Positive cells were scored under a light microscope.

4.6. SDS–polyacrylamide gel electrophoresis and immunoblotting

For direct immunoblotting, total cellular protein lysates were prepared by harvesting cells in hot electrophoresis sample buffer. Proteins were then separated by SDS–polyacrylamide gel electrophoresis on a 12.5% gel and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% low fat milk and 0.1% Tween 20 in PBS for 2 h and probed overnight with monoclonal antibodies against p53 (clone DO-1), PCNA (clone PC-10) and $p21^{WAF1/CIP1}$ (clone 118). After washing three times in PBS plus 0.1% Tween 20, peroxidase conjugated rabbit antimouse immunoglobulin antiserum was applied as the secondary antibody. To visualize peroxidase activity, ECL+ (Amersham Biosciences) reagents were used according to the manufacturer's instructions.

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References and notes

1. Sausville, E. A. *Trends Mol. Med.* **2002**, *8*, S32.
2. Chen, Y.-N. P.; Sharma, S. K.; Ramsey, T. M.; Jiang, L.; Martin, M. S.; Baker, K.; Adams, P. D.; Bair, K. W.; Kaelin, W. G. *Proc. Natl. Acad. Sci. U.S.A* **1999**, *96*, 4325.
3. Lees, J. A.; Weinberg, R. A. *Proc. Natl. Acad. Sci. U.S.A* **1999**, *96*, 4421.
4. Tetsu, O.; McCormick, F. *Cancer Cell* **2003**, *3*, 233.
5. Hinds, P. W. *Cancer Cell* **2003**, *3*, 305.
6. Moore, J. D.; Kirk, J. A.; Hunt, T. *Science* **2003**, *300*, 987.
7. Napolitano, G.; Majello, B.; Lania, L. *Int. J. Oncol.* **2002**, *21*, 171.
8. Fischer, P. M. *Curr. Opin. Drug Discov. Dev.* **2001**, *4*, 623.
9. Sielecki, T. M.; Boyan, J. F.; Benfield, P. A.; Trainor, G. L. *J. Med. Chem.* **2000**, *43*, 1.
10. Vesely, J.; Havlicek, L.; Strnad, M.; Blow, J. J.; Donella-Deana, A.; Pinna, L.; Letham, D. S.; Kato, J.; Detivaud, L.; Leclerc, S.; Meijer, L. *Eur. J. Biochem.* **1994**, *224*, 771.
11. Havlicek, L.; Hanus, J.; Vesely, J.; Leclerc, S.; Meijer, L.; Shaw, G.; Strnad, M. *J. Med. Chem.* **1997**, *40*, 408.
12. Gray, N. S.; Wodicka, L.; Thunnissen, A. M.; Norman, T. C.; Kwon, S.; Espinoza, F. H.; Morgan, D. O.; Barnes, G.; LeClerc, S.; Meijer, L.; Kim, S. H.; Lockhart, D. J.; Schultz, P. G. *Science* **1998**, *281*, 533.
13. Dreyer, M. K.; Borcherding, D. R.; Dumont, J. A.; Peet, N. P.; Tsay, J. T.; Wright, P. S.; Bitonti, A. J.; Shen, J.; Kim, S. H. *J. Med. Chem.* **2001**, *44*, 524.
14. Krystof, V.; Lenobel, R.; Havlicek, L.; Kuzma, M.; Strnad, M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3283.
15. Shum, P. W.; Peet, N. P.; Weintraub, P. M.; Le, T. B.; Zhao, Z.; Barbone, F.; Cashman, B.; Tsay, J.; Dwyer, S.; Loos, P. C.; Powers, E. A.; Kropp, K.; Wright, P. S.; Bitonti, A.; Dumont, J.; Borcherding, D. R. *Nucleos. Nucleot. Nucl. Acids* **2001**, *20*, 1067.
16. O'Connor, D. S.; Wall, N. R.; Porter, A. C.; Altieri, D. C. *Cancer Cell* **2002**, *2*, 43.
17. McClue, S. J.; Blake, D.; Clarke, R.; Cowan, A.; Cummings, L.; Fischer, P. M.; MacKenzie, M.; Melville, J.; Stewart, K.; Wang, S.; Zhelev, N.; Zheleva, D.; Lane, D. P. *Int. J. Cancer* **2002**, *102*, 463.
18. Gibson, A. E.; Arris, C. E.; Bentley, J.; Boyle, F. T.; Curtin, N. J.; Davies, T. G.; Endicott, J. A.; Golding, B. T.; Grant, S.; Griffin, R. J.; Jewsbury, P.; Johnson, L. N.; Mesguiche, V.; Newell, D. R.; Noble, M. E.; Tucker, J. A.; Whitfield, H. J. *J. Med. Chem.* **2002**, *45*, 3381.
19. Moravcova, D.; Krystof, V.; Havlicek, L.; Moravec, J.; Lenobel, R.; Strnad, M. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2989.
20. Capek, P.; Otmar, M.; Masojidkova, M.; Votruba, I.; Holy, A. *Collect. Czech. Chem. Commun.* **2003**, *68*, 779.
21. Robins, R. K.; Dille, K. L.; Christensen, B. E. *J. Org. Chem.* **1954**, *19*, 930.
22. Bredereck, H.; Baumann, W. *Liebigs Ann. Chem.* **1967**, *701*, 143.
23. Biagi, G.; Giorgi, I.; Livi, O.; Scartoni, V.; Breschi, C.; Martiny, C.; Scatizzi, R. *Farmaco* **1995**, *50*, 659.
24. Arris, C. E.; Boyle, F. T.; Calvert, A. H.; Curtin, N. J.; Endicott, J. A.; Garman, E. F.; Gibson, A. E.; Golding, B. T.; Grant, S.; Griffin, R. J.; Jewsbury, P.; Johnson, L. N.; Lawrie, A. M.; Newell, D. R.; Noble, M. E. M.; Sausville, E. A.; Schultz, R.; Yu, W. *J. Med. Chem.* **2000**, *43*, 2797.
25. Imbach, P.; Carparo, H. G.; Furet, P.; Mett, H.; Meyer, T.; Zimmerman, J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 91.
26. Vermeulen, K.; Strnad, M.; Krystof, V.; Havlicek, L.; Van der Aa, A.; Lenjou, M.; Nijs, G.; Rodrigus, I.; Stockman, B.; Van Onckelen, H.; Van Bockstaele, D. R.; Berneman, Z. N. *Leukemia* **2002**, *16*, 299.
27. David-Pfeuty, T. *Oncogene* **1999**, *18*, 7409.
28. Kotala, V.; Uldrijan, S.; Horky, M.; Trbusek, M.; Strnad, M.; Vojtesek, B. *Cell. Mol. Life Sci.* **2001**, *58*, 1333.
29. Demidenko, Z. N.; Blagosklonny, M. V. *Cancer Res.* **2004**, *64*, 3653.
30. Knockaert, M.; Meijer, L. *Biochem. Pharmacol.* **2002**, *64*, 819.
31. Curtius, T.; Ehrhart, G. *Chem. Ber.* **1922**, *55*, 1565.
32. Hoover, J. R. E.; Day, A. R. *J. Am. Chem. Soc.* **1956**, *78*, 5832.
33. Boyer, J. H.; Hamer, J. *J. Am. Chem. Soc.* **1955**, *77*, 951.