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Trisubstituted purine inhibitors of PDGFRα and their antileukemic activity in the human eosinophilic cell line EOL-1

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ABSTRACT

Inhibition of protein kinases is a validated concept for pharmacological intervention in cancers. Many kinase inhibitors have been approved for clinical use, but their practical application is often limited. Here, we describe a collection of 23 novel 2,6,9-trisubstituted purine derivatives with nanomolar inhibitory activities against PDGFRα, a receptor tyrosine kinase often found constitutively activated in various tumours. The compounds demonstrated strong and selective cytotoxicity in the human eosinophilic leukemia cell line EOL-1, whereas several other cell lines were substantially less sensitive. The cytotoxicity in EOL-1, which is known to express the *FIP1L1-PDGFRA* fusion gene encoding an oncogenic kinase, correlated significantly with PDGFRα inhibition. EOL-1 cells treated with the compounds also exhibited dose-dependent inhibition of PDGFRα autophosphorylation and suppression of its downstream signaling pathways with concomitant G_1 phase arrest, confirming the proposed mechanism of action. Our results show that substituted purines can be used as platforms for preparing tyrosine kinase inhibitors with specific activity towards eosinophilic leukemia.

1. Introduction

The development of protein kinase inhibitors has progressed dramatically in the past years: over 30 kinase inhibitors have been approved as drugs, and several others are undergoing advanced clinical evaluation.[1,2] Oncogenic kinases often arise as a result of mutations, overexpression, or chromosomal translocations. Moreover, while chromosomal translocations in hematological malignancies are rarer than in solid tumors, they are significant in cancer therapy as well as in tumor initiation and progression.[3]

Platelet-derived growth factor receptor α (PDGFRA, CD140A) is a type III receptor tyrosine kinase (TK) that regulates cell proliferation, differentiation, adhesion and survival. Ligand binding activates the kinase, stimulating cellular signaling proteins including mitogen-activated protein kinases (MAPKs), signal transducers and activators of transcription (STATs), SRC and phosphatidylinositol-3 kinases. Constitutively activated forms of PDGFRα have been found in various tumors, arising from various mutations in gastrointestinal stromal tumors[4] and melanoma,[5] missense mutations in childhood acute myeloid leukemia,[6] reciprocal translocations in chronic myeloid leukemia, and deletions that give rise to a fusion protein in chronic eosinophilic leukemia.[7] A typical fusion gene encoding a constitutively activated PDGFRα form is *FIP1L1-PDGFRA*, which originates from an 800-kb cryptic interstitial deletion in chromosome 4q12. The fusion gene encodes a ligand-independent and constitutively active TK that confers growth factor-independency to hematopoietic cells and is highly sensitive to the kinase inhibitor imatinib.[8,9] This fusion protein is estimated to occur in 10–20% of eosinophilia cases. The *FIP1L1-PDGFRA* fusion has been found in chronic eosinophilic leukemia, hypereosinophilic syndrome, eosinophilia-associated myeloproliferative disorders, acute myeloid leukemia, and lymphoblastic T-cell non-Hodgkin lymphoma.[3] Other fusion partners include BCR, striatin and ETV6.[3]

Various TK inhibitors have shown activity in *FIP1L1-PDGFRA*-positive cells, including imatinib,[9] sorafenib[10] or ponatinib.[11] These compounds blocked proliferation and induced apoptosis, primarily as a result of direct FIP1L1-PDGFRα inhibition.[9] In clinical settings, however, most patients require life-long treatment with these agents, and resistance has developed in some cases.[8,12] There is

Abbreviations: PDGFRα, platelet derived growth factor receptor alpha; CDK2, cyclin-dependent kinase 2; TK, tyrosine kinase; MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription.

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even a recent report of a patient with imatinib non-responsive chronic eosinophilic leukemia, although in that patient's tumor, PDGFRα was fused with ETV6 rather than FIP1L1.[13] Identification of novel PDGFRα inhibitors or thorough selectivity determination for previously described compounds is therefore still a challenge.

We have previously prepared several series of 2,6,9-trisubstituted purines[14,15] and related purine isosteres such as pyrazolo[4,3-*d*]pyrimidines[16] and pyrazolo[1,5-*a*]pyrimidines[17] that selectively inhibit cyclin-dependent kinases (CDK). Optimization of these compounds yielded other potent CDK inhibitors as well as new compounds with different kinase selectivities. Changes in selectivity were achieved by using different substitution patterns,[18] or more simply, by replacing the 6-benzylamino moiety with a 6-phenylamino group. Independent studies confirmed that this shortening conferred potency against various other kinases, including TKs, while retaining some degree of CDK inhibition. Such compounds (some of them are illustrated in Fig. 1) include the PAK4 inhibitor CGP74514A,[19] the MER receptor inhibitor anilinopurine C52,[20] the SRC and VEGFR2 inhibitor BDBM50451553,[21] and the SRC and BCR-ABL inhibitor AP23464.[22] Importantly, the 6-phenylaminopyrimidine motif present in all these compounds is also present in several TK inhibitors such as erlotinib, gefitinib, and lapatinib (Fig. 1), suggesting that substituted purines may also serve as a source of TK inhibitors.

In this paper we describe potent PDGFRα inhibitors with selective activity against the eosinophilic leukemia cell line EOL-1. Structure-activity analyses and co-crystal structures of CDKs with purine inhibitors indicated that the optimal substituents at positions 2 and 9 are branched alkyl groups with polar substituents (e.g. aminocyclohexylamine) and a small alkyl or cycloalkyl group, respectively.[15,23,24] We expected that 2-(4-aminocyclohexyl) amino-6-anilinopurines with enlarged substituents at position 9 would be weaker CDK inhibitors but more effective inhibitors of TKs, and thus more active towards cell lines with aberrant TK expression.

2. Results

2.1. Synthesis

The three-step synthesis of 2,6,9-trisubstituted purines started from commercially available 2,6-dichloropurine, which was initially alkylated at N^9 with a suitable alcohol under Mitsunobu conditions to obtain a 2,6-dichloro-9-substituted-9*H*-purine with good regioselectivity.[14,15] The crude product was purified by crystallization from methanol or column chromatography (petroleum ether: ethyl acetate, 2:1).

The second step was nucleophilic substitution at the *C6* purine position with a suitable substituted aniline or benzylamine. This reaction was performed in *n*-propanol, using *N*,*N*-diisopropylethylamine as the base. The reaction temperature was kept in the range of 100–120 °C for 3–6 h, depending on aniline reactivity. Crude products were purified by crystallization or column chromatography (petroleum ether: ethyl acetate, 2:1).

The final step was nucleophilic aromatic substitution at the *C2* purine position using a large excess of *trans*-1,4-diaminocyclohexane at 160 °C for a few hours via solution-phase synthesis strategy or via microwave heating. The use of microwave irradiation significantly reduced the time required for this reaction. Final compounds were purified by crystallization or column chromatography on silica (Table 1).

2.2. Biological and biochemical activity

All the prepared compounds were subjected to biochemical assays to determine their activity against CDK2 and PDGFRα, and cytotoxicity assays using 5 cell lines with differing expression of oncogenic TKs. We also performed phenotype screening to evaluate cell cycle changes in treated cells. The results obtained are summarized in Table 2. The previously reported structure–activity relationships for 2,6,9-trisubstituted purines suggest that position 6 can host both phenyl and benzyl groups without significantly altering activity against CDK2 (compare **4u** with **4b**, **4q** with **4a**).[24,25] Further substitution of the phenyl or benzyl ring with chlorine, hydroxy or methoxy groups also did not greatly affect potency. On the other hand, CDK2 inhibition was clearly weakened by increasing the size of the substituent in position 9. The most potent CDK2 inhibitors bear a butyl chain in this position; bulkier substituents (ranging from isopentyl in **4b** to the bulkier benzyl group in **4d** or the longer geranyl unit in **4e**) clearly reduced activity against CDK2.

In contrast to CDK2 inhibitors, the presence of an anilino function is necessary for PDGFRα inhibition; while several anilino derivatives exhibited IC_{50} values below 0.1 μM, its replacement with a homologous benzyl group completely eliminated this activity (compounds **4a** and **4b**). This is consistent with several studies

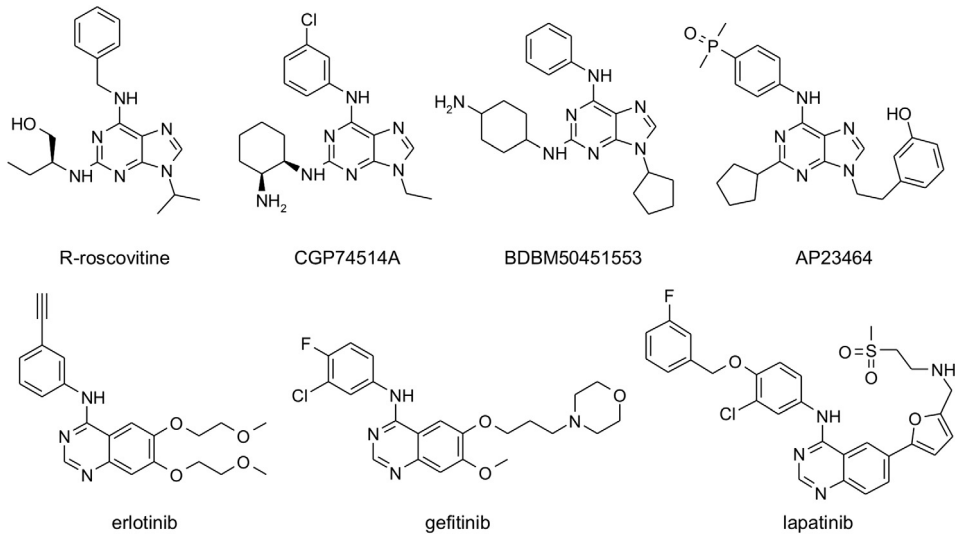
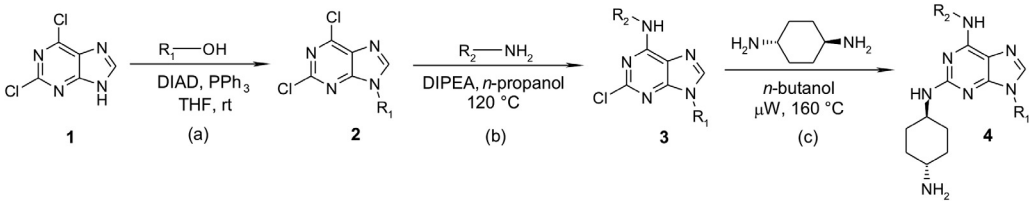


Fig. 1. Some inhibitors of cyclin-dependent kinases and tyrosine kinases.



Scheme 1. General reaction scheme for synthesis of 2,6,9-trisubstituted purine derivatives. (a) The appropriate alcohol R^1-OH (2 equiv), PPh_3 (1.2 equiv), dry THF, DIAD (1.2 equiv), 0°C; (b) the appropriate amine or benzylamine (1.2 equiv), DIPEA (3 equiv), *n*-propanol, 100–120 °C (sealed tube), 3–6 h; (c) *trans*-1,4-diaminocyclohexane (15 equiv), *n*-butanol, 160 °C (sealed tube), 4–10 h.

Table 1
Structures of novel 2,6,9-trisubstituted purine analogues used in this study.

Compound[a]	R^1	R^2
4a	Butyl	3-Chlorobenzyl
4b	Isopentyl	3-Chlorobenzyl
4c	4-Methoxybenzyl	3-Chlorophenyl
4d	Benzyl	3-Chlorophenyl
4e	3,7-Dimethylocta-2,6-dien-1-yl	3-Chlorophenyl
4f	Isopentyl	2,4-Dimethoxyphenyl
4g	Butyl	3,5-Dimethoxyphenyl
4h	Hexyl	3,5-Dimethoxyphenyl
4i	Cyclohexyl	4-Hydroxyphenyl
4j	Cyclohexyl	4-Chlorophenyl
4k	Hexyl	4-Hydroxyphenyl
4l	Isopentyl	3,5-Dimethoxyphenyl
4m	Hexyl	3-Chlorophenyl
4n	3-Methylbut-2-en-1-yl	3-Chlorophenyl
4o	Butyl	Phenyl
4p	Hexyl	4-Chlorophenyl
4q	Butyl	3-Chlorophenyl
4r	Isopentyl	4-Hydroxyphenyl
4s	Isopentyl	Phenyl
4t	Isopentyl	3-Hydroxyphenyl
4u	Isopentyl	3-Chlorophenyl
4v	Butyl	4-Chlorophenyl
4w	Isopentyl	4-Chlorophenyl

[a] See Scheme 1 for position of R^1 and R^2.

on kinase inhibitor selectivity, which reported that the 2,9-disubstituted-6-benzylpurine roscovitine also does not inhibit PDGFRα or other tyrosine kinases (IC_{50} > 10 μM).[23,26,27] Other reports however showed that 6-anilino purines can inhibit tyrosine kinases. For example, AP23464 was identified as a potent inhibitor of SRC and BCR-ABL,[28,22] and compound C52 inhibits MER.[20]

Analogues bearing chlorine- or hydroxyl-functionalized anilino groups at the C6 position did not exhibit appreciably stronger activity towards PDGFRα than the parent compound with a non-functionalized anilino group. However, dimethoxylated anilino groups were usually detrimental to activity: compounds **4h**, **4f**, **4g** exhibited only micromolar inhibition. The activities of **4f** and **4l** suggest that 3,5-dimethoxy substitution is preferred to the 2,4-configuration.

Our results also demonstrate that the substituent at position 9 of the purine should not be too bulky because benzyl derivatives **4c** and **4d** and geranyl derivative **4e** were among the weakest inhibitors, with IC_{50} values above 1 μM. PDGFRα was most sensitive to derivatives with linear or branched chains of 4–5 carbon atoms at the N^9 position.

Cancer cell lines for cytotoxicity assays were selected with respect to their expression of oncogenic kinases. The prepared compounds were active against every cell line included in the panel, but their activities were quite distinct (Table 2). The non-small cell lung cancer cell line HCC827 and the breast cancer cell line BT474 both have activating mutations in EGFR, and responded weakly to the compounds, with mid-micromolar GI_{50} values. Conversely, the breast cancer cell line MCF7 responded strongly, with a single-digit micromolar GI_{50} value, despite having no known tyrosine kinase mutations. The leukemic cell line K562, which expresses the BCR-ABL fusion kinase, was similarly responsive.

The compounds had similar cytotoxicity patterns in all tested cell lines other than the eosinophilic leukemia cell line EOL-1 (Table 2). Most of them were much more active against this line (with submicromolar to mid-nanomolar GI_{50} values) and had completely different relative activity levels, indicating a different mechanism of action in this line. We hypothesized that this may be due to the EOL-1 line's expression of the *FIP1L1-PDGFRA* fusion gene, which encodes an oncogenic variant of PDGFRα. This line has been identified as an *in vitro* model for studying FIP1L1-PDGFRA-positive chronic eosinophilic leukemia and for analysing FIP1L1-PDGFRα inhibitors.[9] Indeed, we found that the compounds' cytotoxicity in EOL-1 correlated strongly with PDGFRα inhibition as determined in a biochemical assay (Pearson coeff. = 0.76, $p < 0.001$, Fig. 2).

To our knowledge, CDK inhibitors usually induce cell cycle arrest preferentially in G_2-M or in both G_1 and G_2-M, whereas receptor tyrosine kinase inhibitors often produce clear G_1 phase arrest. We therefore performed cell cycle measurements in the EOL-1, HCC827 and K562 cell lines after treatment with the new compounds. For each compound, we measured the cell cycle changes after 24 h treatment at a concentration corresponding to the compound's GI_{50}, and determined the relative G_1/G_2-M ratio. Illustrative cell cycle profiles for two potent compounds (**4v** and **4u**) along with lapatinib and CGP74514A as controls (EGFR and CDK inhibitors, respectively) are presented in Fig. 3. As shown in this figure and Table 2, the most sensitive cell line EOL-1 entered G_1 cell cycle arrest (corresponding to a relative G_1/G_2-M ratio above 1) upon treatment, in keeping with this line's dependence on the PDGFRα kinase. Importantly, we found that the compound's PDGFRα inhibiting activity correlated strongly with their relative G_1/G_2-M ratios (Pearson coefficient = −0.73, Fig. 2). Unlike EOL-1, the other tested cell lines exhibited G_2-M arrest. The different effects observed in these cell lines suggest that the compounds may target PDGFRα rather than CDK2 at equitoxic doses, but interact with other kinases if their primary target is absent.

Because **4e** showed some activity against the receptor kinase HER4, which is related to EGFR (preliminary screening, see Supplementary data), we were surprised that the HCC827 and K562 cell lines entered G_2-M arrest when treated with this compound. We therefore expect that the compounds may preferentially target kinases other than receptor tyrosine kinases, potentially including CDKs. The weak correlation between cytotoxicity in HCC827 and CDK2 inhibition may support this hypothesis (Pearson coeff. = 0.55, Fig. 2). While no clear relationship between cytotoxicity and CDK inhibition has been found, targeting CDK inhibition may at least partly contribute to the compounds' mechanism of antiproliferative activity.

Table 2
Activity of novel compounds in cellular and biochemical assays.[a]

Compound	IC_{50} (μM)		GI_{50} (μM)					Relative G_1/G_2-M ratio[b]		
	CDK2	PDGFRα	HCC827	BT474	MCF7	K562	EOL-1	EOL-1	HCC827	K562
CGP74514A	0.017	>20	12.2	5.4	3.3	4.8	0.99	1.28	0.29	0.48
Lapatinib	>20	>20	22.3	0.2	8.1	5.7	0.41	1.17	12.1	0.81
4a	0.207	>20	10.6	4.7	2.2	4.3	0.35	0.99	0.26	1.27
4b	0.306	>20	16.7	6.6	4.7	3.1	1.33	1.77	2.50	1.85
4c	5.504	7.655	18.7	8.2	4.8	2.8	0.53	0.96	1.94	1.43
4d	2.830	5.099	13.7	10.2	3.6	3.0	0.38	0.99	10.37	1.76
4e	7.170	4.739	18.2	4.3	3.6	2.2	0.59	1.13	1.94	1.78
4f	5.967	3.197	32.9	7.9	6.3	5.5	0.63	1.65	23.09	1.00
4g	0.442	1.496	20.1	15.6	4.5	2.4	0.37	1.30	1.35	0.82
4h	0.680	1.412	9.1	3.7	3.5	1.9	0.38	3.78	9.67	1.40
4i	1.441	1.233	32.5	27.3	9.6	8.9	0.22	2.44	9.61	0.95
4j	0.965	1.090	9.1	4.1	2.3	1.7	0.14	2.64	2.68	0.79
4k	0.485	0.452	24.7	7.7	3.5	5.4	0.19	2.50	0.44	0.69
4l	0.980	0.290	11.2	5.9	4.5	2.5	0.14	3.71	1.47	1.60
4m	0.500	0.195	10.2	5.1	2.7	2.3	0.31	1.86	7.37	0.79
4n	0.227	0.187	13.5	7.0	4.2	3.5	0.08	3.10	2.69	0.75
4o	0.134	0.160	9.0	4.9	2.2	3.2	0.02	4.93	0.33	2.29
4p	0.433	0.156	11.7	5.5	4.2	2.1	0.44	4.61	5.20	0.88
4q	0.132	0.154	7.2	5.1	2.0	2.0	0.16	10.75	0.54	0.78
4r	0.490	0.150	14.4	7.7	3.1	4.6	0.03	1.17	2.71	0.73
4s	0.627	0.096	21.7	6.4	5.4	3.5	0.01	1.67	0.39	1.20
4t	0.513	0.066	21.3	14.7	12.6	7.6	0.06	8.94	5.60	1.22
4u	0.275	0.066	10.3	3.8	2.1	1.5	0.06	6.00	1.33	1.10
4v	0.117	0.060	10.8	7.3	2.1	2.7	0.05	6.43	0.28	1.52
4w	0.319	0.059	10.2	5.1	2.7	1.8	0.09	7.80	3.50	0.81

[a] Data are averaged from at least three determinations.
[b] Cell cycle effect at a dose corresponding to the GI_{50} value. Data were normalized against results for untreated controls, so a relative G_1/G_2-M ratio close to 1 means the tested compound had no effect.

Fig. 2. Pearson correlation coefficients for measured parameters. Stars indicate statistical significance ($^{*}P < 0.01$, $^{**}P < 0.001$).

2.3. Mechanism of cellular activity

The most sensitive cell line, EOL-1, bears an oncogenic activation of *PDGFRA* gene, which is consistent with our hypothesis that inhibition of this kinase is linked to the compounds' cellular activity. We therefore used immunoblotting to determine whether the compounds' cytotoxicity and the G_1 phase arrest relate to PDGFRα inhibition. Several compounds with various activity levels were applied to EOL-1 cells at two doses for 1 h, after which the cells were harvested and analyzed for cellular signaling downstream of PDGFRα. We immunoblotted the lysates for STAT3, ERK1/2, and MEK1/2 and their phosphoforms, which are known to be induced by PDGFRα signaling (Fig. 4). There were clear changes in the abundance of the phosphoforms of all three proteins. Two potent compounds, **4t** and **4o**, strongly suppressed the phosphorylation of STAT3 and ERK1/2 at both tested concentrations, whereas the less active **4j** and **4p** only reduced the abundance of these phosphoproteins at the higher concentration, and the least active

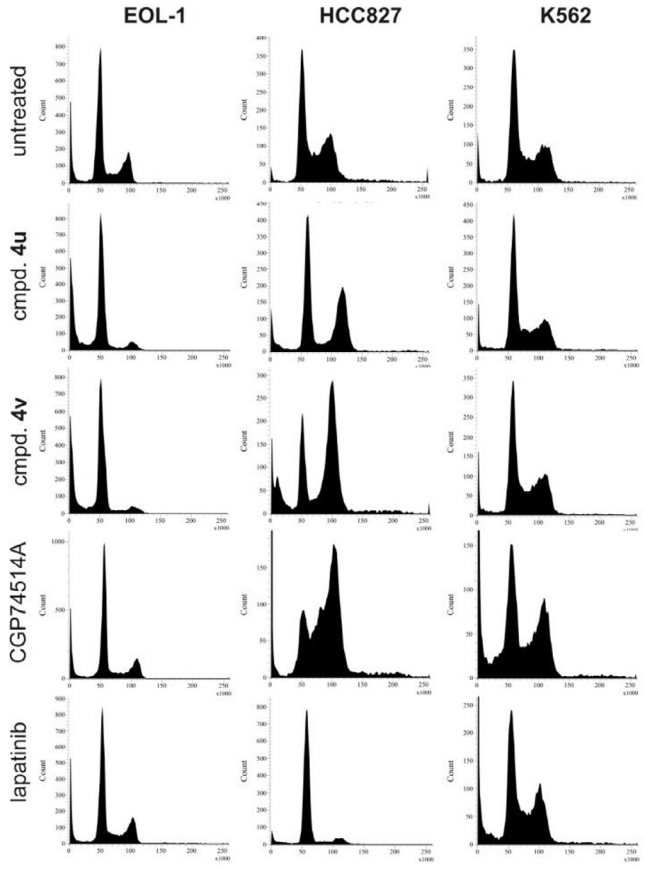


Fig. 3. Cell cycle measurements for EOL-1, HCC827 and K562 treated with **4u** and **4v** for 24 h. CGP74514A and lapatinib (CDK and EGFR inhibitor, respectively) were used as positive controls. The compounds were used at doses corresponding to their GI_{50} values.

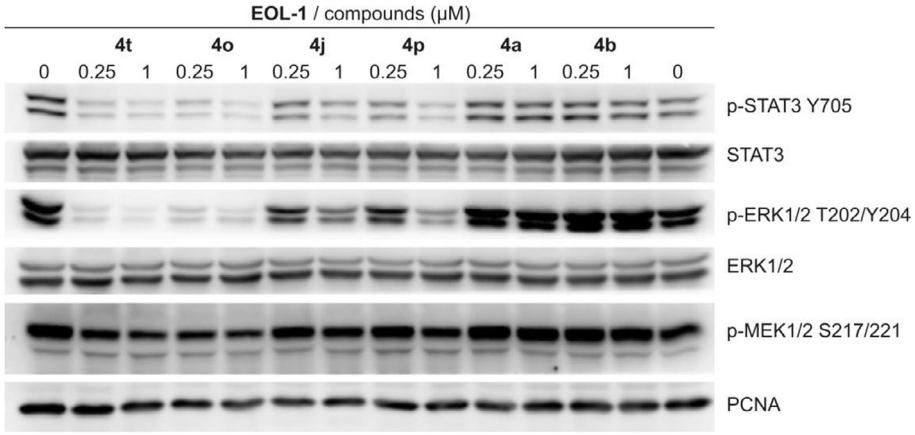


Fig. 4. Inhibition of PDGFRα downstream signaling by compounds with strong (**4t** and **4o**), medium (**4j** and **4p**) and weak (**4a** and **4b**) inhibition of PDGFRα in EOL-1 cells treated for 1 h.

compounds **4a** and **4b** did not affect these phosphoproteins at all. Phosphorylation of MEK1/2 at serines 217/221 decreased slightly only in cells treated with the most potent **4t** and **4o**. These observations correspond well with the kinase inhibition assay results.

In the next experiment, we treated EOL-1 cells for 1 h with one of the most potent compounds, **4u**, to confirm that its mechanism of action is specifically coupled to PDGFRα inhibition. Immunoblotting revealed dose-dependent inhibition of PDGFRα autophosphorylation at tyrosines 754, 849 and 1018 (Fig. 5a). These tyrosines are autophosphorylated upon external stimulation by PDGF, but are constitutively phosphorylated independently of cytokine presence in EOL-1 due to the formation of the FIP1L1-PDGFRα fusion, which interrupts the juxtamembrane domain of PDGFRα.[29] Corresponding downregulations were also seen for the STAT3 and ERK1/2 phosphoforms. When the cells were treated for periods longer than 4 h, they started to degrade FIP1L1-PDGFRα (data not shown), ceasing to proliferate and showing markers of apoptosis after 24 h (Fig. 5b).

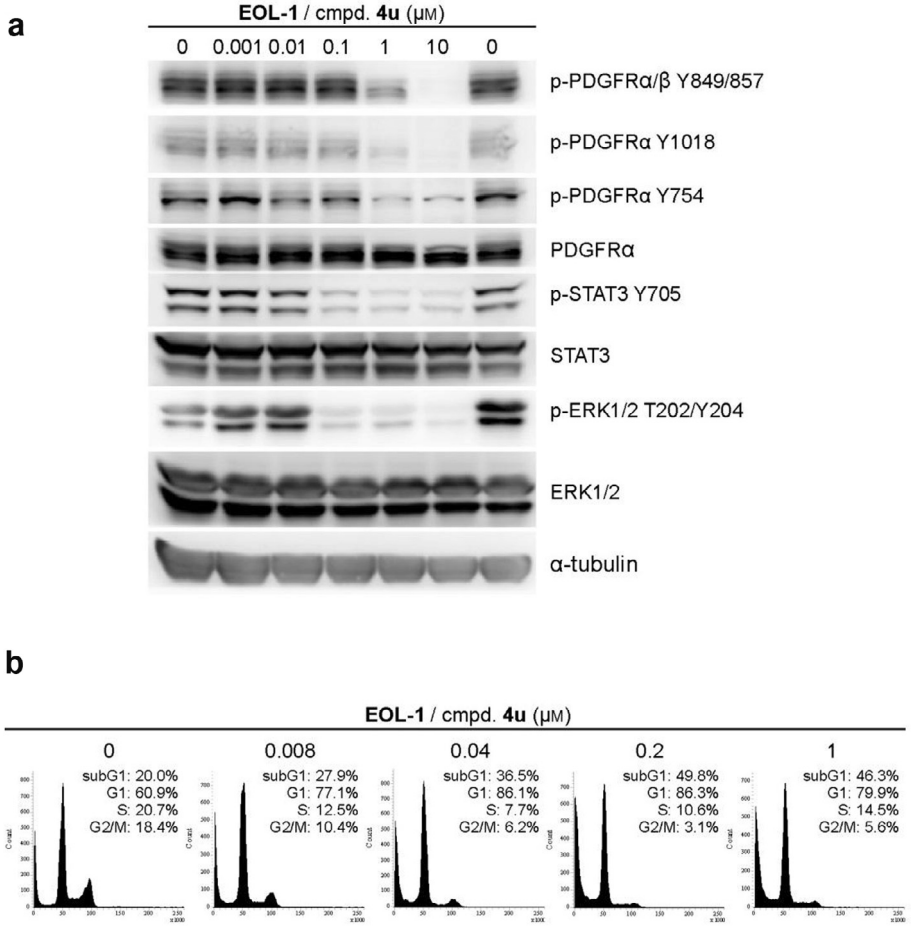


Fig. 5. Cellular effects of compound **4u** in EOL-1. (a) Inhibition of PDGFRα and its downstream signaling pathways after 1 h. (b) Cell cycle in EOL-1 cells treated for 24 h.

3. Conclusion

A collection of 23 novel 2,6,9-trisubstituted purine derivatives with nanomolar inhibitory activities against PDGFRα has been prepared. The compounds demonstrated strong and selective cytotoxicity in the human eosinophilic leukemia cell line EOL-1, which expresses the oncogenic kinase FIP1L1-PDGFRA. Their cytotoxicity in EOL-1 correlated significantly with PDGFRα inhibition. Moreover, they exhibited dose-dependent inhibition of PDGFRα autophosphorylation and suppressed its downstream signaling pathways in treated cells, confirming the proposed cellular mechanism of action. Our results suggest that substituted purines are versatile platforms for preparing tyrosine kinase inhibitors with specific activity towards eosinophilic leukemia and other cancers expressing constitutively activated PDGFRα mutants such as hepatocellular carcinoma, gastrointestinal stromal tumors or melanoma.[4,5,30]

4. Experimental section

4.1. Chemistry and general information

All microwave irradiation experiments were carried out in a dedicated CEM-Discover mono-mode microwave apparatus. The reactor was used in the standard configuration as delivered, including proprietary software. The reactions were carried out in 10 mL glass vials sealed with a silicone/PTFE top, which can be exposed to a maximum of 250 °C and 21 bar internal pressure. The temperature was measured with an IR sensor on the outer surface of the process vial. After the irradiation period, the reaction vessel was cooled to ambient temperature by gas jet cooling. Melting points were determined on Buchi Melting Point B-540 apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol 500 ECA instrument operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C at ambient temperature in DMSO d_6. Chemical shifts are reported in ppm. Coupling constants (J) are reported in Hertz (Hz), and the following abbreviations are used: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), doublet of triplet (dt), quartet (q), quintet (qui), sextet (sex), nonet (n), multiplet (m). Mass spectra were recorded using an LCQ ion trap mass spectrometer (Finnigan MAT). The chromatographic purity of the compounds was determined using HPLC-DAD-MS. An Alliance 2695 separations module (Waters) linked simultaneously to a PDA 996 (Waters) and a Q-Tof micro (Waters) benchtop quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer were used. Samples were dissolved in methanol and diluted to a concentration of 10 μg·mL^{-1} in the mobile phase (initial conditions). Then, 10 μL of the solution were injected on a RP-column (150 mm × 2.1 mm; 3.5 μm; Symmetry C18, Waters). The column was kept in a thermostat at 25 °C. Solvent (A) consisted of 15 mM formic acid adjusted to pH 4.0 with ammonium hydroxide. Methanol was used as the organic modifier (solvent B). At flow rate of 0.2 mL·min^{-1}, the following binary gradient was used: 0 min, 10% B; 0–24 min, a linear gradient to 90% B, followed by 10 min isocratic elution of 90% B. At the end of the gradient, the column was re-equilibrated to the initial conditions for 10 min. The effluent was introduced into the DAD (scanning range 210–400 nm, with 1.2 nm resolution) and an electrospray source was applied (source temperature 110 °C, capillary voltage +3.0 kV, cone voltage +20 V, desolvation temperature 250 °C). Nitrogen was used as both the desolvation gas (500 L·h^{-1}) and the cone gas (50 L·h^{-1}). The mass spectrometer was operated in positive (ESI+) ionization mode, and data were acquired in the 50–1000 m/z range. Elemental analyses were performed using an EA1112 CHN analyser (Thermo Finnigan); the results obtained for C, H, and N were within acceptable limits of the expected values. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The purity of biologically evaluated compounds was >95% as determined by HPLC-DAD-MS and elemental analysis.

4.2. Protocol for preparation of compounds **2a**–**2h**

2,6-Dichloro-9*H*-purine (15.8 mmol), an appropriate alcohol (31.7 mmol), and triphenylphosphine (19.0 mmol) were dissolved in dry tetrahydrofuran (100 mL) and cooled to 0 °C. Diisopropyl azodicarboxylate (19.0 mmol) was added dropwise to the stirred solution under an argon atmosphere, and the temperature was kept between 0 and 20 °C. The reaction mixture was stirred under an argon atmosphere at 20 °C for a further 2–4 h. The reaction was monitored by TLC until completion. The reaction mixture was then evaporated under reduced pressure, and the residue was dissolved in boiling toluene (100 mL). After cooling to room temperature, the solution was inoculated with a small amount of triphenylphosphine oxide and the solution was kept at 4 °C for 24 h. The triphenylphosphine oxide was then filtered off and the filtrate was evaporated under reduced pressure. The residue was crystallized from ethanol or purified by column chromatography on silica (petroleum ether: ethyl acetate, 2:1) to afford the pure product.

4.2.1. 9-Butyl-2,6-dichloro-9H-purine (**2a**)

Yield: 86%; M.p. = 79–80 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.71 (s, 1H), 4.22 (t, J = 7.0, 2H), 1.85–1.70 (m, 2H), 1.32–1.16 (m, 2H), 0.85 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 153.89, 151.34, 150.00, 148.84, 130.88, 44.25, 31.43, 19.72, 13.81. HPLC-MS (ESI+): 245.13 (99.8%). Elemental analysis Calcd. for $C_9H_{10}Cl_2N_4$ (245.11): C, 44.10; H, 4.11; N, 22.86. Found: C, 43.97; H, 4.05; N, 22.53.

4.2.2. 2,6-Dichloro-9-isopentyl-9H-purine (**2b**)

Yield: 83%; M.p. = 80–81 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.73 (s, 1H), 4.22 (t, J = 7.5, 2H), 1.70 (q, J = 7.1, 2H), 1.44 (n, J = 6.5, 1H), 0.88 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 156.68, 153.93, 151.33, 148.95, 130.93, 42.91, 38.14, 25.57, 22.57. HPLC-MS (ESI+): 259.32 (99.8%). Elemental analysis Calcd. for $C_{10}H_{12}Cl_2N_4$ (259.14): C, 46.35; H, 4.67; N, 21.62. Found: C, 46.02; H, 4.58; N, 21.39.

4.2.3. 2,6-Dichloro-9-(3-methylbut-2-en-1-yl)-9H-purine (**2c**)

Yield: 96%; M.p. = 95–96 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.66 (s, 1H), 4.80 (d, J = 7.2, 2H), 1.76 (t, J = 7.2, 1H), 1.77 (s, 3H), 1.68 (s, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 153.71, 151.41, 149.97, 148.68, 138.97, 131.03, 118.34, 42.36, 25.89, 18.42. HPLC-MS (ESI+): 257.63 (96.50%). Elemental analysis Calcd. for $C_{10}H_{10}Cl_2N_4$ (257.12): C, 46.71; H, 3.92; N, 21.79. Found: C, 46.59; H, 3.95; N, 21.63.

4.2.4. 2,6-Dichloro-9-hexyl-9H-purine (**2d**)

Yield: 75%; M.p. = 82–84 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.70 (s, 1H), 4.19 (t, J = 7.2, 2H), 1.78 (qui, J = 7.2, 2H), 1.27–0.91 (m, 6H), 0.76 (t, J = 7.1, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 156.69, 153.99, 151.35, 149.03, 130.97, 44.49, 31.09, 29.29, 26.05, 22.44, 14.34. HPLC-MS (ESI+): 273.19 (99.8%). Elemental analysis Calcd. for $C_{11}H_{14}Cl_2N_4$ (273.16): C, 48.37; H, 5.17; N, 20.51. Found: C, 48.21; H, 4.96; N, 20.33.

4.2.5. 2,6-Dichloro-9-cyclohexyl-9H-purine (**2e**)

Yield: 79%; M.p. = 111–112 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.82 (s, 1H), 4.41 (qui, J = 6.3, 1H), 2.04–1.95 (m, 2H), 1.93–1.77 (m, 4H), 1.69–1.65 (m, 1H), 1.49–1.35 (m, 2H), 1.30–1.16 (m, 1H). HPLC-MS (ESI+): 271.17 (99.7%). Elemental analysis

Calcd. for $C_{11}H_{12}Cl_2N_4$ (271.15): C, 48.73; H, 4.46; N, 20.66. Found: C, 48.67; H, 4.39; N, 20.44.

*4.2.6. 2,6-Dichloro-9-(4-methoxybenzyl)-9H-purine (**2f**)*

Yield: 91%; M.p. = 121–123 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.77 (s, 1H), 7.28 (d, J = 8.5, 2H), 6.86 (d, J = 8.5, 2H), 5.36 (s, 2H), 3.67 (s, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.63, 153.75, 151.60, 150.25, 148.78, 131.01, 129.90, 128.02, 114.66, 60.16, 47.05. HPLC-MS (ESI+): 309.74 (95.4%). Elemental analysis Calcd. for $C_{13}H_{10}Cl_2N_4O$ (309.15): C, 50.51; H, 3.26; N, 18.12. Found: C, 50.42; H, 3.35; N, 18.03.

*4.2.7. (E)-2,6-Dichloro-9-(3,7-dimethylocta-2,6-dien-1-yl)-9H-purine (**2g**)*

Yield: 51%; M.p.= 101–102 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.21 (s, 1H), 5.22 (t, J = 6.8, 1H), 4.99–4.86 (m, 3H), 2.05–1.87 (m, 4H), 1.78 (s, 3H), 1.52 (s, 3H), 1.45 (s, 3H). HPLC-MS (ESI+): 325.22 (96.20%). Elemental analysis Calcd. for $C_{15}H_{18}Cl_2N_4$ (325.24): C, 55.39; H, 5.58; N, 17.23 Found: C, 55.32; H, 5.60; N, 17.21.

*4.2.8. 9-Benzyl-2,6-dichloro-9H-purine (**2h**)*

Yield: 93%; M.p. = 148–149 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.81 (s, 1H), 7.39–7.16 (m, 5H), 5.45 (s, 2H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 153.90, 151.66, 150.33, 148.95, 136.16, 131.01, 129.34, 128.63, 128.14, 47.64. HPLC-MS (ESI+): 279.72 (99.8%). Elemental analysis Calcd. for $C_{12}H_8Cl_2N_4$ (279.12): C, 51.64; H, 2.89; N, 20.07. Found: C, 51.56; H, 2.95; N, 19.90.

*4.3. Protocol for preparation of compounds **3a–3w***

To a suspension of 9-substituted-2,6-dichloro-9*H*-purine (6.25 mmol) in a mixture of *n*-propanol (40 mL) and *N*,*N*-diisopropyl-*N*-ethylamine (18.75 mmol), the appropriate amine or benzylamine (7.5 mmol) was added. The suspension was heated with stirring in a sealed tube under an argon atmosphere at 120 °C until completion of the reaction was indicated by TLC. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure and the residue was partitioned between water (50 mL) and dichloromethane (50 mL). In addition, the water phase was extracted with dichloromethane (2 × 30 mL). The combined organic phases were washed with water (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. If necessary, the crude product was purified by column chromatography on silica (petroleum ether: ethyl acetate, 2:1).

*4.3.1. 9-Butyl-2-chloro-N-(3-chlorobenzyl)-9H-purin-6-amine (**3a**)*

Yield: 61%; M.p. = 120–123 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.79 (t, J = 5.9, 1H), 8.15 (s, 1H), 7.35 (s, 1H), 7.32–7.21 (m, 3H), 4.59 (d, J = 6.0, 2H), 4.05 (t, J = 7.0, 2H), 1.74–1.65 (m, 2H), 1.23–1.13 (m, 2H), 0.82 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 155.29, 153.41, 150.55, 142.41, 142.15, 133.43, 130.72, 127.69, 127.32, 126.55, 118.65, 43.34, 43.16, 31.82, 19.72, 13.87. HPLC-MS (ESI+): 350.82 (99.98%). Elemental analysis Calcd. for $C_{16}H_{17}Cl_2N_5$ (350.25): C, 55.19; H, 4.34; N, 20.11. Found: C, 55.02; H, 4.38; N, 19.98.

*4.3.2. 2-Chloro-N-(3-chlorobenzyl)-9-isopentyl-9H-purin-6-amine (**3b**)*

Yield: 63%; M.p. = 120–121 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.80 (s, 1H), 8.17 (s, 1H), 7.48–7.13 (m, 4H), 4.69–4.51 (s (br), 2H), 4.08 (t, J = 6.5, 2H), 1.63 (q, J = 6.5, 2H), 1.42 (n, J = 6.5, 1H), 0.85 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 155.30, 153.40, 150.53, 142.50, 142.10, 133.47, 130.69, 127.73, 127.34, 126.54, 118.70, 43.18, 42.05, 38.63, 25.58, 22.63. HPLC-MS (ESI+): 364.80 (99.53%). Elemental analysis Calcd. for $C_{17}H_{19}Cl_2N_5$ (364.27): C, 56.05; H, 5.26; N, 19.23. Found: C, 56.18; H, 5.23; N, 19.24.

*4.3.3. 2-Chloro-N-(3-chlorophenyl)-9-(4-methoxybenzyl)-9H-purin-6-amine (**3c**)*

Yield: 71%; M.p. = 115–117 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.47 (s, 1H), 8.40 (s, 1H), 8.01 (t, J = 2.5, 1H), 7.79 (d, J = 8.5, 1H), 7.34 (t, J = 8.0, 1H), 7.27 (d, J = 8.5, 2H), 7.10 (d, J = 7.5, 1H), 6.88 (d, J = 8.5, 2H), 5.29 (s, 2H), 3.68 (s, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.50, 152.75, 152.62, 151.38, 143.12, 140.97, 133.37, 130.70, 129.72, 128.88, 123.44, 120.85, 119.85, 114.72, 55.62. Elemental analysis Calcd. for $C_{19}H_{15}Cl_2N_5O$ (400.26): C, 57.01; H, 3.78; N, 17.50. Found: C, 57.06; H, 3.84; N, 17.17. HPLC-MS (ESI+): 400.71 (95.10%).

*4.3.4. 9-Benzyl-2-chloro-N-(3-chlorophenyl)-9H-purin-6-amine (**3d**)*

Yield: 65%; M.p. = 124–125 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.49 (s, 1H), 8.43 (s, 1H), 8.00 (s, 1H), 7.79 (d, J = 8.1, 1H), 7.42–7.21 (m, 6H), 7.09 (d, J = 7.7, 1H), 5.37 (s, 2H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 154.92, 154.57, 153.24, 144.13, 140.79, 137.47, 134.43, 130.17, 128.68, 128.48, 127.74, 122.58, 122.54, 120.89, 120.18, 48.86. HPLC-MS (ESI+): 370.76 (96.26%). Elemental analysis Calcd. for $C_{18}H_{13}Cl_2N_5$ (370.24): C, 58.39; H, 3.54; N, 18.92. Found: C, 58.23, H, 3.59, N, 18.73.

*4.3.5. (E)-2-Chloro-N-(3-chlorophenyl)-9-(3,7-dimethylocta-2,6-dien-1-yl)-9H-purin-6-amine (**3e**)*

Yield: 73%; ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.44 (s, 1H), 8.26 (s, 1H), 8.02 (t, J = 1.5, 1H), 7.80 (d, J = 8.0, 1H), 7.34 (t, J = 8.5, 1H), 7.09 (d, J = 7.5, 1H), 5.37 (t, J = 6.0, 1H), 4.97 (t, J = 6.0, 1H), 4.73 (d, J = 7.0, 2H), 2.07–1.97 (m, 4H), 1.76 (s, 3H), 1.54 (s, 3H), 1.48 (s, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.54, 151.30, 142.80, 141.53, 141.04, 133.37, 131.59, 130.72, 124.23, 123.39, 120.77, 119.77, 119.48, 118.95, 41.56, 39.31, 26.16, 25.94, 18.05, 16.68. HPLC-MS (ESI+): 416.14 (93.30%). Elemental analysis Calcd. for $C_{21}H_{23}Cl_2N_5$ (416.35): C, 60.58; H, 5.57; N, 16.82. Found: C, 60.51; H, 5.63; N, 16.77.

*4.3.6. 2-Chloro-N-(2,4-dimethoxyphenyl)-9-isopentyl-9H-purin-6-amine (**3f**)*

Yield: 71%; M.p. = 144–145 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.01 (s, 1H), 8.22 (s, 1H), 7.51 (d, J = 7.1, 1H), 6.64 (d, J = 2.6, 1H), 6.53 (dd, J = 8.7, J' = 2.6, 1H), 4.11 (t, J = 7.4, 2H), 3.75 (d, J = 4.3, 6H), 1.66 (q, J = 7.0, 2H), 1.45 (n, J = 6.5, 1H), 0.88 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm): 158.53, 153.14, 153.08, 142.55, 129.29, 120.23, 118.95, 104.94, 99.70, 56.31, 55.89, 42.11, 38.60, 25.62, 22.66. HPLC-MS (ESI+): 376.43 (99.98%). Elemental analysis Calcd. for $C_{18}H_{22}ClN_5O_2$ (375.15): C, 57.52; H, 5.90; N, 18.63. Found: C, 57.38; H, 5.72; N, 18.58.

*4.3.7. 9-Butyl-2-chloro-N-(3,5-dimethoxyphenyl)-9H-purin-6-amine (**3g**)*

Yield: 80%; M.p. = 100–102 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.18 (s, 1H), 8.32 (s, 1H), 7.20 (d, J = 2.1, 2H), 6.22 (t, J = 2.2, 1H), 4.12 (qui, J = 6.5, 2H), 3.70 (s, 6H), 1.75 (qui, J = 7.0, 2H), 1.22 (sex, J = 7.0, 2H), 0.87 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.84, 155.29, 153.57, 152.71, 143.05, 141.09, 119.42, 99.82, 95.53, 55.57, 43.52, 31.77, 19.75, 13.90. HPLC-MS (ESI+): 362.32 (96.69%). Elemental analysis Calcd. for $C_{17}H_{20}ClN_5O_2$ (361.83): C, 56.43; H, 5.57; N, 19.36. Found: C: 56.36; H: 5.59; N: 19.17.

*4.3.8. 2-Chloro-N-(3,5-dimethoxyphenyl)-9-hexyl-9H-purin-6-amine (**3h**)*

Yield: 54%; M.p. = 90–101 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.16 (s, 1H), 8.30 (s, 1H), 7.20 (d, J = 2.2, 2H), 6.22 (t, J = 2.2, 1H), 4.10 (t, J = 7.1, 2H), 3.70 (s, 6H), 1.76 (qui, J = 6.5, 2H), 1.29–1.14 (m, 6H), 0.84–0.74 (t, J = 7.0, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.81, 152.69, 152.52, 151.32, 143.00, 141.11, 119.41, 99.79, 95.53, 55.56, 43.77, 31.11, 29.67, 26.10, 22.46, 14.35. HPLC-MS (ESI+): 390.79 (98.27%). Elemental analysis Calcd. for $C_{19}H_{24}ClN_5O_2$ (389.88): C, 58.53; H, 6.20; N, 17.96. Found: C, 56.77; H, 6.18; N, 16.79.

*4.3.9. 4-((2-Chloro-9-cyclohexyl-9H-purin-6-yl)amino)phenol (**3i**)*

Yield: 58%; M.p. = 156–157 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.97 (s, 1H), 9.27 (s, 1H), 8.33 (s, 1H), 7.47 (d, J = 8.5, 2H), 6.71 (d, J = 8.5, 2H), 4.28 (qui, J = 6.6, 1H), 1.99–1.95 (m, 2H), 1.86–1.79 (m, 4H), 1.67–1.65 (m, 1H), 1.48–1.32 (m, 2H), 1.26–1.15 (m, 1H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 154.45, 153.23, 152.78, 150.47, 140.53, 130.59, 124.10, 119.09, 115.47, 54.25, 32.84, 25.55, 25.27. HPLC-MS (ESI+): 344.59 (95.16%). Elemental analysis Calcd. for $C_{17}H_{18}ClN_5O$ (343.82): C, 59.39; H, 5.28; N, 20.37. Found: C, 59.76; H, 5.42; N, 20.16.

*4.3.10. 2-Chloro-N-(4-chlorophenyl)-9-cyclohexyl-9H-purin-6-amine (**3j**)*

Yield: 59%. M.p. = 134–136 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): δ 10.39 (s, 1H), 8.43 (s, 1H), 7.85 (d, J= 7.0, 2H), 7.38 (d, J= 7.0, 2H), 4.38–4.27 (m, 1H), 1.98 (d, J = 8.3, 2H), 1.91–1.77 (m, 4H), 1.67 (d, J = 8.3, 1H), 1.50–1.36 (m, 2H), 1.30–1.16 (m, 2H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.67, 152.39, 150.88, 141.31, 138.42, 128.95, 127.56, 123.10, 119.53, 54.39, 32.81, 25.54, 25.27. HPLC-MS (ESI+): 362.38 (95.45%). Elemental analysis Calcd. for $C_{17}H_{17}Cl_2N_5$ (362.26): C, 56.37; H, 4.73; N, 19.33. Found: C, 56. 20; H, 4.79; N, 19.28.

*4.3.11. 4-((2-Chloro-9-hexyl-9H-purin-6-yl)amino)phenol (**3k**)*

Yield: 63%; M.p. = 195–196 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.96 (s, 1H), 9.25 (s, 1H), 8.23 (s, 1H), 7.47 (d, J = 8.0 Hz, 2H), 6.70 (d, J = 8.0, 2H), 4.08 (t, J = 7.5, 2H), 1.76 (qui, J = 6.5, 2H), 1.15–1.30 (m, 6H), 0.79 (t, J = 6.9, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 154.43, 153.16, 152.98, 150.98, 142.43, 130.56, 124.04, 118.93, 115.47, 43.68, 31.12, 29.71, 26.10, 22.47, 14.37. HPLC-MS (ESI+): 346.42 (95.72%). Elemental analysis Calcd. for $C_{17}H_{20}ClN_5O$ (345.83): C, 59.04; H, 5.83; N, 20.25. Found: C, 58.78; H, 5.79; N, 20.03.

*4.3.12. 2-Chloro-N-(3,5-dimethoxyphenyl)-9-isopentyl-9H-purin-6-amine (**3l**)*

Yield: 68%; M.p. = 103–104 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.18 (s, 1H), 8.35 (s, 1H), 7.20 (d, J = 2.2, 2H), 6.22 (t, J = 2.2, 1H), 4.13 (t, J = 7.0, 2H), 3.70 (s, 6H), 1.69 (q, J = 7.0, 2H), 1.46 (n, J = 6.6, 1H), 0.89 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.82, 152.70, 152.52, 151.31, 142.98, 141.10, 119.41, 99.72, 95.50, 55.56, 42.19, 38.56, 25.62, 22.65. HPLC-MS (ESI+): 376.32 (97.14%). Elemental analysis Calcd. for $C_{18}H_{22}ClN_5O_2$ (375.86): C, 57.52; H, 5.90; N, 18.63. Found: C, 57.21; H, 5.98; N, 18.50.

*4.3.13. 2-Chloro-N-(3-chlorophenyl)-9-hexyl-9H-purin-6-amine (**3m**)*

Yield: 78%; M.p. = 108–110 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.48 (s, 1H), 8.39 (s, 1H), 8.02 (t, J = 2.5, 1H), 7.80 (d, J = 8.5, 1H), 7.35 (t, J = 8.0, 1H), 7.08 (d, J = 8.0, 1H), 4.12 (t, J = 7.0, 2H), 1.78 (qui, J = 6.5 2H), 1.25–1.18 (m, 6H), 0.80 (t, J = 6.9, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.78, 152.25, 151.33, 143.12, 140.90, 133.40, 130.75, 123.49, 120.71, 119.71, 118.59, 44.01, 31.11, 29.58, 26.06, 22.46, 14.36. HPLC-MS (ESI+): 364.48 (96.99%). Elemental analysis Calcd. for $C_{17}H_{19}Cl_2N_5$ (364.27): C, 56.05; H, 5.26; N, 19.23. Found: C, 55.88; H, 5.14; N, 18.97.

*4.3.14. 2-Chloro-N-(3-chlorophenyl)-9-(3-methylbut-2-en-1-yl)-9H-purin-6-amine (**3n**)*

Yield: 72%; M.p. = 115–116 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.43 (s, J = 6.2, 1H), 8.27 (s, 1H), 8.01 (t, J = 2.1, 1H), 7.79 (dd, J = 8.5, J' = 2.1, 1H), 7.33 (t, J = 8.5, 1H), 7.08 (dd, J = 8.5, J' = 2.1, 1H), 5.38–5.34 (m, 1H), 4.71 (d, J = 7.2, 2H), 1.76 (s, 3H), 1.68 (s, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.53, 151.23, 142.83, 140.98, 138.22, 133.35, 130.68, 123.36, 120.74, 119.74, 119.43, 119.05, 41.60, 25.81, 18.44. HPLC-MS (ESI+): 348.30 (97.37%). Elemental analysis Calcd. for $C_{16}H_{15}Cl_2N_5$ (348.22): C, 55.19; H, 4.34; N, 20.11. Found: C, 55.02; H, 4.38; N, 19.98.

*4.3.15. 9-Butyl-2-chloro-N-phenyl-9H-purin-6-amine (**3o**)*

Yield: 83%; M.p. = 133–134 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.25 (s, 1H), 8.31 (s, 1H), 7.80 (d, J = 8.5, 2H), 7.32 (t, J = 7.0, 2H), 7.06 (t, J = 7.5, 1H), 4.13 (t, J = 7.0, 2H), 1.76 (qui, J = 7.0, 2H), 1.24 (sep, J = 7.0, 2H), 0.87 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.89, 152.74, 151.34, 142.92, 139.36, 129.05, 123.95, 121.74, 119.31, 43.51, 31.79, 19.76, 13.91. HPLC-MS(ESI+): 302.31 (98.58%). Elemental analysis Calcd. for $C_{15}H_{16}ClN_5$ (301.78): C, 59.70; H, 5.34; N, 23.21. Found: C, 59.51; H, 5.12; N, 23.14.

*4.3.16. 2-Chloro-N-(4-chlorophenyl)-9-hexyl-9H-purin-6-amine (**3p**)*

Yield: 84%; M.p. = 149–151 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.38 (s, 1H), 8.33 (s, 1H), 7.85 (d, J = 9.0, 2H), 7.37 (d, J = 9.0, 2H), 4.12 (t, J = 7.0, 2H), 1.82–1.72 (m, 2H), 1.29–1.17 (m, 6H), 0.80 (t, J = 6.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 156.55, 152.62, 151.45, 143.18, 138.44, 128.95, 127.56, 123.08, 119.38, 43.79, 31.11, 29.67, 26.09, 22.46, 14.36. HPLC-MS (ESI+): 364.61 (99.49%). Elemental analysis Calcd. for $C_{17}H_{19}Cl_2N_5$ (364.27): C, 56.05; H, 5.26; N, 19.23. Found: C, 55.85; H, 5.41; N, 19.49.

*4.3.17. 9-Butyl-2-chloro-N-(3-chlorophenyl)-9H-purin-6-amine (**3q**)*

Yield: 84%; M.p. = 106–107 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.44 (s, 1H), 8.34 (s, 1H), 8.02 (d, J = 1.7, 1H), 7.81 (d, J = 8.5, 1H), 7.35 (t, J = 8.0, 1H), 7.09 (t, J = 8.0, 1H), 4.13 (t, J = 7.1, 2H), 1.81–1.72 (m, 2H), 1.29–1.18 (m, 2H), 0.86 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.56, 152.50, 151.55, 143.31, 141.03, 133.36, 130.65, 123.37, 120.79, 119.79, 119.48, 43.57, 31.77, 19.76, 13.90. HPLC-MS (ESI+): 336.20 (98.57%). Elemental analysis Calcd. for $C_{15}H_{15}Cl_2N_5$ (336.22): C, 53.59; H, 4.50; N, 20.83. Found: C, 53.84; H, 4.75; N, 21.09.

*4.3.18. 4-((2-Chloro-9-isopentyl-9H-purin-6-yl)amino)phenol (**3r**)*

Yield: 61%; M.p. = 234–235 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.97 (s, 1H), 9.26 (s, 1H), 8.26 (s, 1H), 7.48 (d, J = 8.5, 2H), 6.71 (d, J = 8.5 Hz, 2H), 4.11 (t, J = 7.3, 2H), 1.68 (q, J = 7.0, 2H), 1.44 (non, J = 6.6, 1H), 0.88 (d, J = 6.6 Hz, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 154.43, 153.16, 152.98, 142.34, 130.61, 124.07, 118.96, 115.48, 42.10, 38.61, 25.62, 22.66. HPLC-MS (ESI +): 332.59 (95.45%). Elemental analysis Calcd. for $C_{16}H_{18}ClN_5O$ (331.80): C, 57.92; H, 5.47; N, 21.11. Found: C, 57.70; H, 5.48; N, 20.80.

*4.3.19. 2-Chloro-9-isopentyl-N-phenyl-9H-purin-6-amine (**3s**)*

Yield: 60%; M.p. = 124–126 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.26 (s, 1H), 8.33 (s, 1H), 7.80 (d, J = 7.7, 2H), 7.32 (t, J = 7.5, 2H), 7.06 (t, J = 7.5, 1H), 4.14 (t, J = 7.4, 2H), 1.68 (q, J = 7.0, 2H), 1.47 (n, J = 6.5, 1H), 0.89 (d, J = 6.5, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.90, 152.73, 151.32, 142.88, 139.35, 129.06, 123.96, 121.75, 119.29, 42.18, 38.58, 25.63, 22.67. HPLC-

MS (ESI+): 316.26 (98.37%). Elemental analysis Calcd. for $C_{16}H_{18}ClN_5$ (315.80): C, 60.85; H, 5.75; N, 22.18. Found: C, 60.25; H, 5.79; N, 21.86.

*4.3.20. 3-((2-Chloro-9-isopentyl-9H-purin-6-yl)amino)phenol (**3t**)*

Yield: 84%; M.p. = 156–157 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.11 (s, 1H), 8.34 (s, 1H), 7.30 (t, J = 1.5, 1H), 7.22 (d, J = 8.1, 1H), 7.07 (t, J = 8.1 Hz, 1H), 6.47 (dd, J = 8.1, J' = 2.3, 1H), 4.13 (t, J = 7.0, 2H), 1.68 (q, J = 7.0, 2H), 1.47 (n, J = 6.0, 1H), 0.88 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 157.97, 152.80, 151.19, 142.74, 140.32, 129.64, 119.11, 113.70, 112.60, 111.18, 108.89, 42.22, 38.56, 25.63, 22.67. Elemental analysis Calcd. for $C_{16}H_{18}ClN_5O$ (331.12): C, 57.92; H, 5.47; N, 21.11. Found: C, 57.87; H, 5.50; N, 21.09. HPLC-MS (ESI+): 332.30 (99.16%).

*4.3.21. 2-Chloro-N-(3-chlorophenyl)-9-isopentyl-9H-purin-6-amine (**3u**)*

Yield: 81%; M.p. = 86–88 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.43 (s, 1H), 8.36 (s, 1H), 8.01 (t, J = 2.0, 1H), 7.79 (dd, J = 8.5, J' = 2.0, 1H), 7.34 (t, J = 8.1, 1H), 7.12–7.05 (m, 1H), 4.14 (t, J = 6.5, 2H), 1.68 (q, J = 6.5, 2H), 1.46 (n, J = 7.0, 1H), 0.88 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.55, 152.49, 151.52, 143.25, 141.02, 133.36, 130.68, 123.37, 120.79, 119.79, 119.46, 42.24, 38.55, 25.63, 22.66. HPLC-MS (ESI+): 350.39 (97.85%). Elemental analysis Calcd. for $C_{16}H_{17}Cl_2N_5$ (350.25): C, 54.87; H, 4.89; N, 20.00. Found: C, 54.94; H, 4.99; N, 20.13.

*4.3.22. 9-Butyl-2-chloro-N-(4-chlorophenyl)-9H-purin-6-amine (**3v**)*

Yield: 94%; M.p. = 156–157 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.38 (s, 1H), 8.32 (s, 1H), 7.83 (d, J = 8.5, 2H), 7.37 (d, J = 8.5, 2H), 4.12 (t, J = 7.1, 2H), 1.75 (qui, J = 7.0, 2H), 1.22 (sex, J = 7.0, 2H), 0.85 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.63, 152.54, 151.43, 143.23, 138.40, 128.91, 127.55, 122.98, 119.36, 43.54, 31.58, 19.69, 13.96. HPLC-MS (ESI+): 336.50 (95.66%). Elemental analysis Calcd. for $C_{15}H_{15}Cl_2N_5$ (336.22): C, 53.59; H, 4.50; N, 20.83. Found: C, 53.45; H, 4.41; N, 20.78.

*4.3.23. 2-Chloro-N-(4-chlorophenyl)-9-isopentyl-9H-purin-6-amine (**3w**)*

Yield: 81%; M.p. = 156–158 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 10.39 (s, 1H), 8.35 (s, 1H), 7.84 (d, J = 8.5, 2H), 7.36 (d, J = 8.5, 2H), 4.15 (t, J = 7.25, 2H), 1.68 (q, J = 6.5, 2H), 1.46 (n, J = 6.6, 1H), 0.89 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 152.62, 151.43, 143.10, 138.41, 128.94, 127.55, 123.07, 119.36, 42.20, 38.55, 25.61, 22.65. HPLC-MS (ESI+): 350.46 (95.99%). Elemental analysis Calcd. for $C_{16}H_{17}Cl_2N_5$ (350.25): C, 54.87; H, 4.89; N, 20.00. Found: C, 54.68; H, 4.74; N, 19.94.

*4.4. Protocol for preparation of compounds **4a**–**4w***

A 2-chloro-6,9-disubstituted-9*H*-purine (5.0 mmol) and *trans*-1,4-diaminocyclohexane (75.0 mmol) were mixed in *n*-butanol. The reaction mixture was heated in a sealed vial with a Teflon septum using a CEM Discover reactor at 140–170 °C for 0.5–1.5 h (controlled by TLC – chloroform: methanol, 9:1; see Supplementary data) or in a sealed tube under an argon atmosphere at 160 °C for 4–10 h. After cooling to room temperature, water (50 mL) was added to the reaction mixture and the resulting suspension was extracted three times with ethyl acetate (3 × 50 mL). The combined organic phases were washed with water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude product was purified by column chromatography on silica (chloroform: methanol, 19:1 > 4:1) to yield the final compound.

*4.4.1. N^2-(4-Aminocyclohexyl)-9-butyl-N^6-(3-chlorobenzyl)-9H-purine-2,6-diamine (**4a**)*

Yield: 78%; M.p. = 149–150 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 7.87 (s(br), 1H), 7.66 (s, 1H), 7.33 (s, 1H), 7.30–7.25 (m, 2H), 7.24–7.20 (m, 1H), 6.10 (s(br), 1H), 4.60–4.46 (m, 2H), 3.89 (t, J = 5.9, 2H), 3.57–3.46 (m, 1H), 2.63–2.60 (m, 1H), 1.88–1.61 (m, 4H), 1.60–1.57 (m, 2H), 1.45–1.40 (m, 1H), 1.20–1.14 (m, 5H), 0.89–0.80 (m, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.10, 154.78, 144.09, 137.88, 133.29, 130.49, 127.50, 126.91, 126.49, 50.07, 49.87, 33.58, 31.82, 31.43, 29.55, 25.52, 22.73. HPLC-MS (ESI+): 428.23 (99.45%). Elemental analysis Calcd. for $C_{22}H_{30}ClN_7$ (427.98): C, 61.74; H, 7.07; N, 22.91. Found: C, 61.55; H, 7.31; N, 22.73.

*4.4.2. N^2-(4-Aminocyclohexyl)-N^6-(3-chlorobenzyl)-9-isopentyl-9H-purine-2,6-diamine (**4b**)*

Yield: 67%; M.p. = 94–96 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 7.68 (s, 1H), 7.33 (s, 1H), 7.31–7.18 (m, 3H), 6.10 (s(br), 1H), 4.54 (s(br), 2H), 3.99–3.87 (m, 2H), 1.90–1.69 (m, 3H), 1.67–1.55 (m, 2H), 1.43 (non, J = 6.5, 1H), 1.28–1.07 (m, 6H), 0.85 (d, J = 6.5, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.04, 154.76, 144.07, 138.02, 137.82, 133.30, 130.49, 127.50, 126.90, 126.48, 50.06, 49.86, 41.13, 38.85, 33.57, 31.82, 31.42, 29.54, 25.51, 22.72. HPLC-MS (ESI+): 442.17 (97.30%). Elemental analysis Calcd. for $C_{23}H_{32}ClN_7$ (442.01): C, 62.50; H, 7.30; N, 22.18. Found: C, 62.35; H, 7.41; N, 22.11.

*4.4.3. N^2-(4-Aminocyclohexyl)-N^6-(3-chlorophenyl)-9-(4-methoxybenzyl)-9H-purine-2,6-diamine (**4c**)*

Yield: 71%; M.p. = 125–126 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 7.88 (s(br), 1H), 7.65 (s, 1H), 7.38–7.32 (m, 2H), 7.28–7.24 (m, 4H), 7.22–7.19 (m, 2H), 6.08 (s, 1H), 4.53 (s, 2H), 3.89 (s, 3H), 3.58–3.48 (m, 1H), 1.82–1.74 (m, 1H), 1.72–1.62 (m, 4H), 1.16–1.00 (m, 4H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.27, 158.92, 152.27, 142.51, 133.30, 130.34, 129.79, 121.68, 119.60, 118.70, 114.47, 114.13, 65.50, 56.60, 55.63, 50.07, 33.59, 31.47, 24.08, 19.10, 15.63. HPLC-MS (ESI+): 478.54 (99.99%). Elemental analysis Calcd. for $C_{25}H_{28}ClN_7O$ (478.00): C, 62.82; H, 5.90; N, 20.51. Found: C, 62.66; H, 6.01; N, 20.64.

*4.4.4. N^2-(4-Aminocyclohexyl)-9-benzyl-N^6-(3-chlorophenyl)-9H-purine-2,6-diamine (**4d**)*

Yield: 65%; M.p. = 110–111 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.67 (s(br), 1H), 8.30 (s, 1H), 7.93 (s, 1H), 7.84 (s, 1H), 7.42–7.17 (m, 6H), 6.97 (d, J = 7.5, 1H), 6.56 (t, J = 6.75, 1H), 5.19 (s, 2H), 3.70–3.57 (m, 1H), 1.93–1.86 (m, 2H), 1.80–1.71 (m, 2H), 1.26–1.13 (m, 5H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 159.02, 152.28, 142.49, 138.95, 137.85, 133.27, 130.35, 129.12, 128.13, 127.61, 121.71, 119.59, 118.69, 114.01, 50.52, 35.69, 32.36, 31.83. HPLC-MS (ESI+): 448.68 (99.99%). Elemental analysis Calcd. for $C_{24}H_{26}ClN_7$ (447.97): C, 64.35; H, 5.85; N, 21.89. Found: C, 64.15; H, 5.99; N, 21.81.

*4.4.5. (E)-N^2-(4-Aminocyclohexyl)-N^6-(3-chlorophenyl)-9-(3,7-dimethylocta-2,6-dien-1-yl)-9H-purine-2,6-diamine (**4e**)*

Yield: 49%; M.p. = 142–144 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.61 (s(br), 1H), 8.29 (s(br), 1H), 7.77 (s, 1H), 7.24 (t, J = 8.0, 1H), 6.96 (d, J = 7.6, 1H), 6.61–6.48 (m, 1H), 5.33 (t, J = 6.6, 1H), 4.99 (t, J = 6.2, 1H), 4.57 (s, 2H), 3.71–3.60 (m, 1H), 2.68–2.56 (m, 1H), 2.06–1.90 (m, 6H), 1.86–1.71 (m, 5H), 1.55 (s, 3H), 1.48 (s, 3H), 1.35–1.16 (m, 4H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.82, 152.22, 142.57, 140.34, 138.43, 133.28, 131.56, 130.34, 124.26, 121.62, 119.79, 119.53, 118.65, 114.14, 50.26, 34.41, 31.62, 26.25, 25.98, 18.06, 16.63. HPLC-MS (ESI+): 493.89 (98.60%). Elemental analysis Calcd. for $C_{27}H_{36}ClN_7$ (494.08): C, 65.64; H, 7.34; N, 19.84. Found: C, 65.60; H, 7.36; N, 19.79.

*4.4.6. N^2-(4-Aminocyclohexyl)-N^6-(2,4-dimethoxyphenyl)-9-isopentyl-9H-purine-2,6-diamine (**4f**)*

Yield: 52%; M.p. = 108–109 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 8.40 (d, J = 8.8, 1H), 7.77 (s, 1H), 7.74 (s, 1H), 6.63 (d, J = 2.3, 1H), 6.45 (d, J = 8.8, 2H), 3.97 (t, J = 6.5, 2H), 3.89–3.82 (m, 3H), 3.75–3.70 (m, 3H), 3.57 (d, J = 8.3, 1H), 1.94–1.90 (m, 2H), 1.79–1.75 (m, 2H), 1.63 (d, J = 6.7, 2H), 1.44 (t, J = 6.6, 1H), 1.32–1.04 (m, 4H), 0.87 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.99, 158.21, 155.75, 152.19, 150.22, 138.71, 122.44, 113.87, 112.79, 104.62, 99.07, 56.48, 55.86, 50.36, 41.31, 38.80, 35.00, 31.58, 25.50, 22.70. HPLC-MS (ESI+): 454.00 (98.30%). Elemental analysis Calcd. for $C_{24}H_{35}N_7O_2$ (453.59): C, 63.55; H, 7.78; N, 21.62. Found: C, 63.45; H, 7.85; N, 21.56.

*4.4.7. N^2-(4-Aminocyclohexyl)-9-butyl-N^6-(3,5-dimethoxyphenyl)-9H-purine-2,6-diamine (**4g**)*

Yield: 72%; M.p. = 87–88 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.20 (s (br), 1H), 7.79 (s, 1H), 7.26 (d, J = 2.0, 2H), 6.39 (s, 1H), 6.10 (s, 1H), 3.95 (t, J = 6.6, 2H), 3.69 (s, 6H), 2.55–2.47 (m, 1H), 1.89 (d, J = 10.9, 2H), 1.77–1.68 (m, 4H), 1.30–1.16 (m, 4H), 1.14–1.07 (m, 2H), 0.85 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.81, 158.92, 152.51, 142.44, 138.74, 116.85, 114.17, 99.05, 94.11, 55.58, 50.41, 50.28, 42.56, 35.44, 31.82, 19.77, 13.93. HPLC-MS (ESI+): 440.00 (99.37%). Elemental analysis Calcd. for $C_{23}H_{33}N_7O_2$ (439.56): C, 62.85; H, 7.57; N, 22.31. Found: C, 62.59; H, 7.69; N, 22.26.

*4.4.8. N^2-(4-Aminocyclohexyl)-N^6-(3,5-dimethoxyphenyl)-9-hexyl-9H-purine-2,6-diamine (**4h**)*

Yield: 69%; M.p. = 108–110 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.19 (s(br), 1H), 7.78 (s, 1H), 7.25 (d, J = 1.8, 2H), 6.38 (s, 1H), 6.12–6.09 (m, 1H), 3.95 (t, J = 7.0, 2H), 3.68 (s, 6H), 1.90–1.88 (m, 2H), 1.71–1.68 (m, 4H), 1.32–1.17 (m, 9H), 1.15–1.04 (m, 1H), 0.79 (t, J = 6.7, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.81, 158.96, 152.50, 142.45, 138.75, 114.20, 99.06, 94.08, 55.59, 50.46, 50.29, 42.91, 35.58, 31.86, 31.18, 29.67, 26.19, 22.47, 14.40. HPLC-MS (ESI+): 468.10 (97.78%). Elemental analysis Calcd. for $C_{25}H_{37}N_7O_2$ (467.62): C, 64.21; H, 7.98; N, 20.97. Found: C, 64.19; H, 8.05; N, 20.83.

*4.4.9. 4-((2-((4-Aminocyclohexyl)amino)-9-cyclohexyl-9H-purin-6-yl)amino)phenol (**4i**)*

Yield: 57%; M.p. = 137–138 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.06 (s, 1H), 7.81 (s, 1H), 7.69 (d, J = 8.8, 2H), 6.63 (dd, J = 8.8, J' = 3.5, 2H), 6.31 (s, 1H), 4.15–4.03 (m, 1H), 3.58–3.50 (m, 1H), 2.53 (t, J = 6.5, 1H), 1.98–1.87 (m, 4H), 1.82–1.75 (m, 4H), 1.64 (d, J = 12.4, 1H), 1.55 (d, J = 10.3, 1H), 1.40–1.27 (m, 3H), 1.26–1.05 (m, 5H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 164.00, 158.72, 152.88, 152.52, 151.72, 136.13, 132.57, 122.22, 115.15, 58.85, 50.46, 35.31, 33.07, 32.77, 31.75, 25.80, 25.41, 18.16. HPLC-MS (ESI+): 422.82 (99.40%). Elemental analysis Calcd. for $C_{23}H_{31}N_7O$ (421.55): C, 65.53; H, 7.41; N, 23.26. Found: C, 65.49; H, 7.45; N, 23.25.

*4.4.10. N^2-(4-Aminocyclohexyl)-N^6-(4-chlorophenyl)-9-cyclohexyl-9H-purine-2,6-diamine (**4j**)*

Yield: 73%; M.p. = 118–120 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.57 (s(br), 1H), 8.05 (d, J = 8.6, 2H), 7.90 (s, 1H), 7.26 (d, J = 8.3, 2H), 6.52 (s, 1H), 4.13 (t, J = 6.5, 1H), 3.62–3.51 (m, 1H), 1.98–1.88 (m, 4H), 1.86–1.75 (m, 4H), 1.69–1.62 (m, 1H) 1.60–1.58 (m, 3H), 1.41–1.06 (m, 6H).^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.62, 152.24, 140.13, 136.89, 128.51, 125.57, 121.89, 121.78, 58.87, 50.48, 35.35, 32.71, 31.73, 31.23, 25.80, 25.42. HPLC-MS (ESI+): 439.90 (99.05%). Elemental analysis Calcd. for $C_{23}H_{30}ClN_7$ (439.99): C, 62.79; H, 6.87; N, 22.28. Found: C, 62.64; H, 7.01; N, 22.23.

*4.4.11. 4-((2-((4-Aminocyclohexyl)amino)-9-hexyl-9H-purin-6-yl)amino)phenol (**4k**)*

Yield: 40%; M.p. = 151–153 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.54 (s(br), 1H), 8.05 (d, J = 8.9, 2H), 7.82 (s, 1H), 7.26 (d, J = 8.9, 2H), 6.51 (s(br), 1H), 3.95 (t, J = 6.1, 1H), 3.64–3.52 (m, 2H), 2.54–2.48 (m, 1H), 1.92 (d, J = 10.5, 2H), 1.80–1.68 (m, 4H), 1.29–1.12 (m, 10H), 0.80 (t, J = 6.8, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.82, 152.21, 140.13, 128.92, 128.67, 128.37, 128.10, 125.55, 121.79, 114.06, 50.61, 42.93, 35.81, 31.84, 31.17, 29.66, 26.18, 22.47, 14.41. HPLC-MS (ESI+): 424.87 (98.95%). Elemental analysis Calcd. for $C_{23}H_{33}N_7O$ (423.57): C, 65.22; H, 7.85; N, 23.15. Found: C, 64.98; H, 7.95; N, 22.89.

*4.4.12. N^2-(4-Aminocyclohexyl)-N^6-(3,5-dimethoxyphenyl)-9-isopentyl-9H-purine-2,6-diamine (**4l**)*

Yield: 81%; M.p. = 118–121 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.19 (s(br), 1H), 7.81 (s, 1H), 7.26 (s, 2H), 6.38 (s, 1H), 6.11–6.09 (m, 1H), 3.98 (t, J = 4.6, 2H), 3.79–3.60 (m, 6H), 1.91–1.88 (m, 2H), 1.74–1.72 (m, 2H), 1.63 (q, J = 6.6 Hz, 2H), 1.45 (n, J = 8.0, 1H), 1.33–1.04 (m, 4H), 0.87 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 160.80, 158.90, 152.50, 142.44, 138.62, 114.94, 99.06, 94.09, 55.58, 50.47, 50.32, 41.24, 38.78, 35.59, 31.79, 25.57, 22.73. HPLC-MS (ESI+): 453.90 (98.73%). Elemental analysis Calcd. for $C_{24}H_{35}N_7O_2$ (453.59): C, 63.28; H, 7.96; N, 19.24. Found: C, 63.45; H, 7.78; N, 19.31.

*4.4.13. N^2-(4-Aminocyclohexyl)-N^6-(3-chlorophenyl)-9-hexyl-9H-purine-2,6-diamine (**4m**)*

Yield: 59%; M.p. = 99–102 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.61 (s(br), 1H), 8.28 (s(br), 1H), 7.84 (s, 1H), 7.24 (t, J = 8.1, 1H), 6.96 (d, J = 8.1, 1H), 6.55 (d, J = 5.6, 1H), 3.95 (t, J = 6.4, 2H), 3.67–3.60 (m, 1H), 2.59–2.50 (m, 1H), 1.94–1.88 (m, 2H), 1.82–1.69 (m, 5H), 1.29–1.10 (m, 12H), 0.80 (t, J = 6.8, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 153.59, 142.57, 139.56, 139.12, 133.29, 131.87, 130.34, 121.62, 119.55, 118.65, 114.12, 50.39, 42.95, 35.18, 31.78, 31.20, 29.67, 26.20, 22.48, 14.40. HPLC-MS (ESI+): 442.16 (99.99%). Elemental analysis Calcd. for $C_{23}H_{32}ClN_7$ (442.01): C, 62.50; H, 7.30; N, 22.18. Found: C, 62.42; H, 7.33; N, 22.12.

*4.4.14. N^2-(4-Aminocyclohexyl)-N^6-(3-chlorophenyl)-9-(3-methylbut-2-en-1-yl)-9H-purine-2,6-diamine (**4n**)*

Yield: 73%; M.p. = 100–101 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.61 (s(br), 1H), 8.28 (s(br), 1H), 7.80 (s, 1H), 7.24 (t, J = 8.1, 1H), 6.96 (d, J = 7.9, 1H), 6.52 (d, J = 8.2 1H), 5.34 (t, J = 7.0, 1H), 4.56 (d, J = 6.5, 2H), 3.69–3.59 (m, 1H), 1.94–1.90 (m, 2H), 1.82–1.72 (m, 5H), 1.68 (s, 3H), 1.32–1.10 (m, 4H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.81, 152.11, 142.48, 138.71, 137.07, 133.37, 130.47, 121.73, 119.98, 119.48, 118.59, 114.03, 50.24, 49.11, 40.73, 34.44, 31.61, 25.85, 18.34. HPLC-MS (ESI+): 426.81 (95.58%). Elemental analysis Calcd. for $C_{22}H_{28}ClN_7$ (425.96): C, 62.03; H, 6.63; N, 23.02. Found: C, 61.90; H, 6.85; N, 22.83.

*4.4.15. N^2-(4-Aminocyclohexyl)-9-butyl-N^6-phenyl-9H-purine-2,6-diamine (**4o**)*

Yield: 68%; M.p. = 183–184 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.34 (s(br), 1H), 7.98 (d, J = 8.5, 2H), 7.79 (s, 1H), 7.22 (t, J = 8.5, 2H), 6.92 (t, J = 7.5, 1H), 6.44 (s(br), 1H), 3.96 (t, J = 5.5, 2H), 3.66–3.55 (m, 1H), 3.38–3.30 (m, 1H), 1.92 (d, J = 10.4, 2H), 1.80–1.64 (m, 4H), 1.29–1.00 (m, 6H), 0.86 (t, J = 7.4, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 221.80, 158.94, 152.56, 141.06, 138.60, 128.74, 122.11, 120.41, 114.06, 65.46, 50.60, 42.59, 36.07, 31.85, 19.78, 15.70, 13.94. HPLC-MS (ESI+): 380.10 (99.67%). Elemental analysis Calcd. for $C_{21}H_{29}N_7$ (379.51): C, 66.46; H, 7.70; N, 25.84. Found: C, 66.26; H, 7.83; N, 25.69.

*4.4.16. N^2-(4-Aminocyclohexyl)-N^6-(4-chlorophenyl)-9-hexyl-9H-purine-2,6-diamine (**4p**)*

Yield: 76%; M.p. = 131–133 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.54 (s(br), 1H), 8.05 (d, J = 8.9, 2H), 7.82 (s, 1H), 7.26 (d, J = 8.9, 2H), 6.51 (s(br), 1H), 3.95 (t, J = 6.1, 1H), 3.64–3.52 (m, 2H), 2.54–2.48 (m, 1H), 1.92 (d, J = 10.5, 2H), 1.80–1.68 (m, 4H), 1.29–1.12 (m, 10H), 0.80 (t, J = 6.8, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.82, 152.21, 140.13, 128.92, 128.67, 128.37, 128.10, 125.55, 121.79, 114.06, 50.61, 42.93, 35.81, 31.84, 31.17, 29.66, 26.18, 22.47, 14.40. HPLC-MS (ESI+): 442.85 (98.39%). Elemental analysis Calcd. for $C_{23}H_{32}ClN_7$ (442.01): C, 62.50; H, 7.30; N, 22.18. Found: C, 62.48; H, 7.33; N, 22.14.

*4.4.17. N^2-(4-Aminocyclohexyl)-9-butyl-N^6-(3-chlorophenyl)-9H-purine-2,6-diamine (**4q**)*

Yield: 41%; M.p. = 128–130 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.61 (s(br), 1H), 8.29 (s(br), 1H) 7.83 (s, 1H), 7.25 (t, J = 8.5, 1H), 6.95 (d, J = 7.5, 1H), 6.54 (s, J = 7.0, 1H), 3.95 (t, J = 6.4, 2H), 3.67–3.56 (m, 1H), 2.57–2.47 (m, 1H), 1.92–1.87 (m, 2H), 1.81–1.66 (m, 4H), 1.22 (m, 6H), 0.89–0.82 (m, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.82, 152.78, 152.23, 142.57, 139.13, 133.30, 130.34, 127.05, 121.63, 119.57, 118.66, 114.15, 50.31, 50.10, 42.66, 33.57, 31.82, 19.80, 13.95. HPLC-MS (ESI+): 413.90 (96.68%). Elemental analysis Calcd. for $C_{21}H_{28}ClN_7$ (413.95): C, 60.93; H, 6.82; N, 23.69. Found: C, 60.85; H, 6.88; N, 23.57.

*4.4.18. 4-((2-((4-Aminocyclohexyl)amino)-9-isopentyl-9H-purin-6-yl)amino)phenol (**4r**)*

Yield: 81%; M.p. = 123–125 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.05 (s(br), 1H), 7.76 (s, 1H), 7.70 (d, J = 8.8, 2H), 6.64 (d, J = 8.8, 2H), 6.28 (s, 1H), 3.97 (t, J = 6.5, 2H), 3.62–3.50 (m, 1H), 1.92–1.88 (m, 2H), 1.78–1.73 (m, 2H), 1.67–1.59 (m, 2H), 1.45 (n, J = 6.5, 1H), 1.27–0.97 (m, 4H), 0.88 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.95, 152.90, 150.97, 138.04, 132.56, 122.27, 115.18, 113.78, 50.55, 41.20, 38.83, 35.79, 31.83, 25.57, 22.75. HPLC-MS (ESI+): 410.00 (95.83%). Elemental analysis Calcd. for $C_{22}H_{31}N_7O$ (409.54): C, 64.52; H, 7.63; N, 23.94. Found: C, 64.33; H, 7.75; N, 23.86.

*4.4.19. N^2-(4-Aminocyclohexyl)-9-isopentyl-N^6-phenyl-9H-purine-2,6-diamine (**4s**)*

Yield: 81%; M.p. = 150–152 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.33 (s(br), 1H), 7.98 (d, J = 7.8, 2H), 7.81 (s, 1H), 7.22 (t, J = 7.8, 2H), 6.91 (t, J = 7.3, 1H), 6.44 (s, 1H), 4.01–3.93 (m, 2H), 3.65–3.53 (m, 1H), 2.55–2.51 (m, 1H), 1.93 (d, J = 11.0, 2H), 1.77 (d, J = 11.7, 2H), 1.63 (sex, J = 6.5, 2H), 1.44 (non, J = 6.5, 1H), 1.32–1.05 (m, 4H), 0.87 (d, J = 6.6, 6H).^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.86, 152.48, 141.01, 138.50, 128.74, 122.10, 120.40, 114.03, 50.45, 41.24, 38.79, 35.38, 32.54, 31.73, 25.54, 22.73. HPLC-MS (ESI+): 394.00 (98.82%). Elemental analysis Calcd. for $C_{22}H_{31}N_7$ (393.54): C, 67.15; H, 7.94; N, 24.91. Found: C, 66.98; H, 8.01; N, 24.83.

*4.4.20. 3-((2-((4-Aminocyclohexyl)amino)-9-isopentyl-9H-purin-6-yl)amino)phenol (**4t**)*

Yield: 86%; M.p. = 144–145 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.17 (s(br), 1H), 7.81 (s, 1H), 7.40 (d, J = 7.8, 1H), 6.97 (t, J = 8.1, 1H), 6.34 (d, J = 7.8, 1H), 4.02–3.91 (m, 2H), 3.65–3.55 (m, 2H), 2.59 (s, 1H), 1.94–1.90 (m, 2H), 1.76 (d, J = 9.5, 2H), 1.62 (d, J = 6.7, 2H), 1.44 (non, J = 6.6, 1H), 1.30–1.07 (m, 6H), 0.87 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): δ 158.83, 157.88, 152.44, 152.44, 141.90, 138.50, 129.33, 114.01, 111.51, 109.52, 107.71, 57.12, 50.13, 33.65, 31.41, 29.53, 25.56, 22.73. HPLC-MS (ESI+): 410.00 (98.80%). Elemental analysis Calcd. for $C_{22}H_{31}N_7O$ (409.54): C, 64.52; H, 7.63; N, 23.94. Found: C, 64.47; H, 7.68; N, 23.92.

*4.4.21. N^2-(4-Aminocyclohexyl)-N^6-(3-chlorophenyl)-9-isopentyl-9H-purine-2,6-diamine (**4u**)*

Yield: 79%; M.p. = 115–117 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.59 (s(br), 1H), 8.27 (s(br), 1H), 7.84 (s, 2H), 7.23 (t, J = 8.1, 1H), 6.94 (d, J = 7.8, 1H), 6.52 (d, J = 7.4, 1H), 4.01–3.93 (m, 2H), 3.66–3.57 (m, 1H), 2.54 (t, J = 10.0, 1H), 1.91 (d, J = 10.8, 2H), 1.76 (d, J = 10.3, 2H), 1.64–1.60 (m, 2H), 1.44 (non, J = 6.5, 1H), 1.31–1.13 (m, 4H), 0.86 (d, J = 6.6, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.80, 157.84, 152.68, 152.20, 142.55, 138.93, 133.26, 130.31, 121.60, 119.53, 118.64, 114.09, 50.37, 41.28, 38.73, 35.05, 31.72, 25.61, 22.72. HPLC-MS (ESI+): 428.00 (97.00%). Elemental analysis Calcd. for $C_{22}H_{30}ClN_7$ (427.98): C, 61.74; H, 7.07; N, 22.91. Found: C, 61.55; H, 7.12; N, 22.83.

*4.4.22. N^2-(4-Aminocyclohexyl)-9-butyl-N^6-(4-chlorophenyl)-9H-purine-2,6-diamine (**4v**)*

Yield: 61%; M.p. = 95–96 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.57 (s(br), 1H), 8.05 (d, J = 8.8, 2H), 7.82 (s, 1H), 7.27 (d, J = 7.4, 2H), 6.54 (s(br), 1H), 3.96 (t, J = 6.5, 2H), 3.58 (s(br), 2H), 2.58–2.53 (m, 1H), 1.94–1.92 (m, 2H), 1.85–1.64 (m, 4H), 1.30–1.10 (m, 6H), 0.86 (t, J = 7.2, 3H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.81, 152.72, 152.22, 140.12, 138.92, 128.52, 125.58, 121.78, 114.05, 50.28, 42.60, 34.70, 31.82, 31.62, 19.78, 13.94. HPLC-MS (ESI+): 414.00 (98.17%). Elemental analysis Calcd. for $C_{21}H_{29}N_7$ (413.95): C, 60.93; H, 6.82; N, 23.69. Found: C, 60.85; H, 7.01; N, 23.57.

*4.4.23. N^2-(4-Aminocyclohexyl)-N^6-(4-chlorophenyl)-9-isopentyl-9H-purine-2,6-diamine (**4w**)*

Yield: 83%; M.p. = 94–97 °C. ^{1}H NMR (500 MHz, DMSO d_6) δ (ppm): 9.56 (s(br), 1H), 8.05 (d, J = 8.0, 2H), 7.82 (s, 1H), 7.25 (d, J = 8.0, 2H), 6.51 (s(br), 1H), 4.11–3.87 (m, 2H), 2.58–2.54 (m, 1H), 1.94 (m, 2H), 1.79 (m, 2H), 1.62 (m, 2H), 1.43 (n, J = 6.5, 1H), 1.31–1.08 (m, 5H), 0.86 (d, J = 6.5, 6H). ^{13}C NMR (126 MHz, DMSO d_6) δ (ppm): 158.79, 152.60, 152.22, 140.12, 138.74, 128.52, 125.56, 121.79, 114.05, 50.47, 41.27, 38.79, 35.39, 31.73, 25.56, 22.73. HPLC-MS (ESI+): 427.90 (98.20%). Elemental analysis Calcd. for $C_{22}H_{30}ClN_7$ (427.23): C, 61.74; H, 7.07; N, 22.91. Found: C, 61.55; H, 7.19; N, 22.73.

4.5. Cancer cell lines and cytotoxicity assay

Human cancer cell lines were obtained from the American Type Culture Collection or the German Collection of Microorganisms and Cell Cultures, and were cultivated according to the provider's instructions. Briefly, MCF-7, BT474 and K562 cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). HCC827 and EOL-1 cell lines were maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cell lines were cultivated at 37 °C in 5% CO_2. The cell lines display various alterations in tyrosine kinase genes: HCC827 has an acquired mutation in the EGFR tyrosine kinase domain (E746-A750 deletion), BT474 has an amplification of EGFR, and EOL-1 and K562 express the fusion kinases FIP1L1-PDGFRα and BCR-ABL, respectively. For the cytotoxicity assays, cells were treated in triplicate with six different doses of each compound for 72 h. After treatment, Calcein AM solution was added for 1 h, and the fluorescence from live cells was measured at 485 nm/538 nm (excitation/emission) using a Fluoroskan Ascent microplate reader (Labsystems). The GI_{50} value, the drug concentration lethal to 50% of the cells, was calculated from the dose-response curves that resulted from the assays.

4.6. Protein kinase inhibition

CDK2/Cyclin E was produced in Sf9 insect cells via baculoviral infection and purified on a NiNTA column (Qiagen). PDGFRα was purchased from ProQinase. The kinase reactions were assayed with suitable substrates (1 mg/mL AGLT (poly(Ala,Glu,Lys,Tyr) 6:2:5:1 hydrobromide) for PDGFRα and 1 mg/mL histone H1 for CDK2) in the presence of 1 and 15 μM ATP for PDGFRα and CDK2, respectively, 0.05 μCi [γ-^{33}P]ATP, and 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. The concentration of the test compounds required to reduce the kinase activity by 50% was determined from dose-response curves and recorded as their IC_{50}. The protein kinase selectivity of compound **4e** was preliminarily evaluated at a single concentration (1 μM) by screening against 50 enzymes as described previously.[31]

4.7. Flow cytometry

Asynchronous cells were seeded into a 96 well plate and then, after a preincubation period, treated with the tested compounds for 24 h. Adherent cells were first washed with PBS, trypsinized, and finally treated with a solution of trypsin inhibitor (0.1%). After incubation, 5× staining solution (17 mM trisodium citrate dihydrate, 0.5% IGEPAL® CA-630, 7.5 mM spermine tetrahydrochloride, 2.5 mM Tris; pH 7.6 containing 50 μg/ml propidium iodide) was added. Leukemic cells were stained directly with the 5× staining solution (i.e. without trypsinization). The cells' DNA content was analyzed by flow cytometry using a 488 nm laser (BD FACS Verse with software BD FACSuite™, version 1.0.6.). Cell cycle distribution was analyzed using ModFit LT (Verity Software House, version 4.1.7). To correlate cell cycle changes with other parameters, the G_1/G_2-M ratio for each combination of cell line and compound was calculated (at a dose corresponding to the compound's GI_{50} value) and divided by the G_1/G_2-M ratio for untreated cells. These values were recorded as relative G_1/G_2-M ratios.

4.8. Immunoblotting

Cell 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). Specific anti-PDGFRα (D1E1E) and anti-phospho-PDGFRα/β Y849/857 (C43E9), anti-phospho PDGFRα Y1018, anti-phospho-PDGFRα Y754 (23B2), anti-STAT3 (79D7), anti-phospho-STAT3 Y705 (D3A7), anti-ERK1/2, and anti-phospho-ERK1/2 T202/Y204, anti-phospho-MEK1/2 S217/221 (41G9) antibodies were purchased from Cell signaling, anti-α-tubulin (DM1A) was purchased from Sigma Aldrich, and anti-PCNA (clone PC-10) was generously gifted by Dr. B. Vojtěšek.

4.9. Statistics

All measurements were performed at least in triplicate. For statistical analysis, Pearson correlation coefficients were calculated from log-transformed values. $P < 0.01$ and $P < 0.001$ were considered statistically significant.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.10.032.

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