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Short communication

Regiospecific microwave-assisted synthesis and cytotoxic activity against human breast cancer cells of (*RS*)-6-substituted-7- or 9-(2,3-dihydro-5*H*-1,4-benzodioxepin-3-yl)-7*H*- or -9*H*-purines

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Abstract

Extended studies on the synthesis and pharmacological evaluation of (*RS*)-6-substituted-7 or 9-(2,3-dihydro-5*H*-1,4-benzodioxepin-3-yl)-7*H*- or -9*H*-purines are presented. The microwave-assisted organic synthesis has provided faster access to the target compounds with the advantage of selective obtaining the *N*-7′ or *N*-9′ regioisomers simplifying their isolation. To test the behaviour of the products (including the purine bases) on cellular systems, cytotoxic activity against the MCF-7 human breast cancer cell line was determined, and the three most active compounds were used to study the cell cycle distribution and apoptosis in the MCF-7 cell line.

Keywords: Antitumour compounds; Seven-membered rings; Microwave; MCF-7; 6-Substituted purines

1. Introduction

Cyclin-dependent kinases (CDKs) mediate proliferation and neuronal development, while aberrant CDK activity is associated with cancer and neurodegeneration [1–5]. Consequently, a large number of ATP-competitive CDK inhibitors from a variety of chemical classes have been identified [6–9]. The 2,6,9-trisubstituted purines, which form an ample class of CDK 1, 2, and 5 inhibitors, were developed to combat these pathologies [2]. The CDKs control cell cycle progression in proliferating eukaryotic cells [3]. The activity of CDKs is dependent on the presence of cyclin partners whose levels are sequentially regulated to ensure that the phases of the cell cycle proceed in the correct order. For example, cyclins of the D family complex with CDKs 4 and 6 during G_1 phase, cyclin E with CDK2 in late G_1, cyclin A with CDK2 in S phase, and cyclin B with CDK1 (also known as cdc2) in late G_2/M [4].

The lead guanine-based CDK inhibitor O^6-cyclohexylmethylguanine (NU2058, **1**; Fig. 1) was found to be an inhibitor of both CDK1 and CDK2. Moreover, **1** showed a mean of $IC_{50} = 13 \pm 7$ μM on 57 cell lines of the National Cancer Institute, NCI [10]. On the other hand, the structure of **2** with a naturally occurring base such as uracil, associated with the inhibition of MCF-7 cell proliferation through decreasing cyclin D1 and CDK1, make it a very attractive agent, opening a new strategy in cancer chemotherapy using similar compounds endowed with potent antitumour activities and with null toxicity [11]. Later on, we substituted the pyrimidine base for the purine one (with several substituents at its position 6), with the objective of increasing both the lipophilicity and the structural diversity of the target molecules [12].

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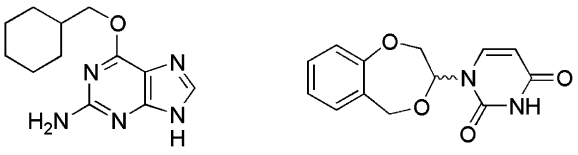


Fig. 1.

Here we propose to extend our studies with the novel 6-substituted-*N*-9′-purines **11**–**17** and the corresponding *N*-7′-purines **18**–**24** (Scheme 1). The use of the cyclohexylmethyloxy group as substituent at position 6 of the purine ring is evident from the previous findings. Moreover other bulky groups such as the phenoxy, phenylthio, *p*-fluorophenylthio, 2,4-dichlorophenylthio ones, and smaller groups such as the allyloxy or the methylthio ones at the 6 position were synthesized in order to demonstrate the importance of the size of the substituent of the purine ring on the biological activity. As we have previously reported, the presence of an amino group on the purine ring is detrimental to the antiproliferative activity [13] and accordingly it was decided to use 6-substituted purines as bases instead of 2-amino-6-substituted purines such as **1**. To test the behaviour of the products (including the purine bases **4**–**10**) on cellular systems, cytotoxic activity against the MCF-7 human breast cancer cell line was determined, and the three most active compounds were used to study the cell cycle distribution and apoptosis in the MCF-7 cell line.

2. Results and discussion

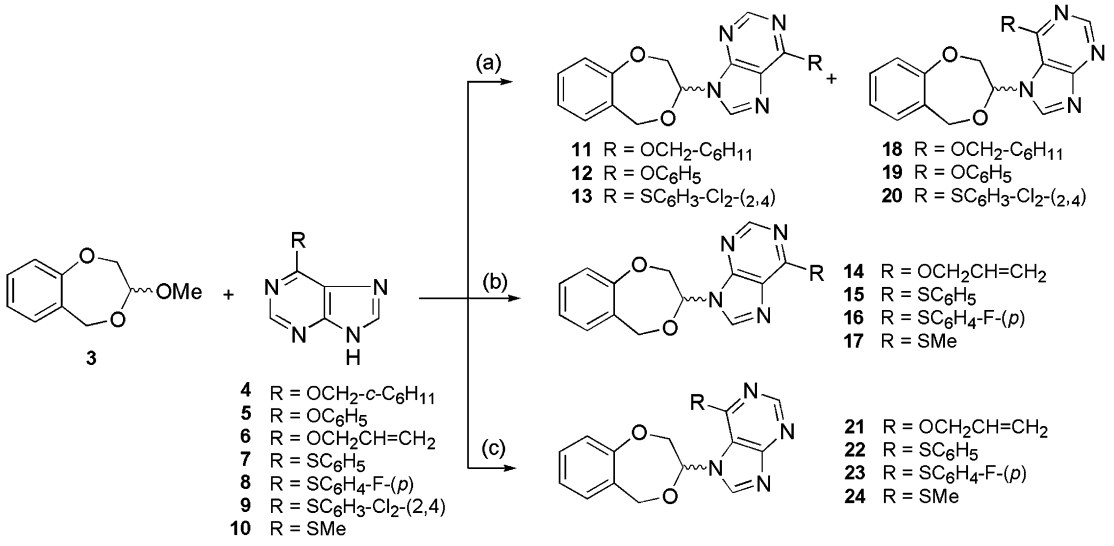
2.1. Chemistry

2.1.1. Synthesis of derivatives

The preparation of the target molecules **11**–**24** was achieved by the Vorbrüggen [14] one-pot condensation of the cyclic acetal **3** [13] and the purine bases **4**–**10** [15,16] using trimethylchlorosilane (TCS), 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and tin(IV) chloride as the Lewis acid in anhydrous acetonitrile. When the reaction was carried out at 45 °C for 24–72 h (Scheme 1, method a), the *N*-9′ and the *N*-7′ cyclic alkylated purine regioisomers (**11**–**13** and **18**–**20**, respectively) were produced and separated by flash chromatography. The isolated single products **11**–**13** and **18**–**20** were obtained with an average yield of 20%.

Modern drug discovery relies on high speed organic synthesis. Microwave-assisted organic synthesis [17–23] is proving to be instrumental for the rapid synthesis of compounds with new and improved biological activities [24,25]. To investigate the Vorbrüggen condensation in microwave-assisted organic synthesis, the reaction mixture was microwave-irradiated at a temperature of 130 °C for 5 min. No starting material was left after the reaction time. The most important feature is that only the *N*-9′ isomer was isolated in the reaction [method (b), **14**–**17**]. The main advantage of the microwave is the quick access to the target molecules as well as the better yield obtained in the only isomer formed making the purification processes much easier. The average yield in the synthesis of **15**–**17** is 37% but the allyloxy-substituted purine derivative **14** was isolated in only 10% yield. It was envisioned that the high reactivity of the allyloxy group would lead to lower yield of the reaction. Trying to improve the yield in the synthesis of **14** we decided to repeat the reaction at a lower temperature. Surprisingly and interestingly, when the microwave reaction was carried out at 100 °C, only the *N*-7′ isomer **21** was isolated (Scheme 1, method c). We then validated method (c) by synthesizing the thio-substituted analogues **22**–**24** [Scheme 1, method (c)] and the *N*-7′ isomers were isolated with an average yield of 26%.

In short we can confirm the regiospecificity of this methodology. Depending on the temperature by microwave activation only one of the isomers was obtained in better yield than the



Scheme 1. Reagents and conditions. Purine base (**4**–**10**), TCS, HMDS, $SnCl_4$, anhydrous MeCN. Method (a), (b) or (c): (a) 45 °C, 24–72 °C; (b) microwave, 130 °C, 5 min; (c) microwave, 100 °C, 5 min.

one produced by conventional heating making the purification process easier. The isolated regioisomers *N*-7′ or *N*-9′ might be prepared in this simple manner. It might be argued that this apparent selectivity was due to the differences in the balance of side products and the ease of isolation of the products, since the isolated yields are below 50%. However, NMR spectra of the crudes supported the selectivity of the processes because neither the *N*-7′ nor the *N*-9′ isomers were detected following methods (b) and (a), respectively.

2.1.2. Spectroscopic characteristics of the N-9′ and N-7′ alkylated purines

As we previously reported, assignments of *N*-9′ *versus* *N*-7′ isomers can be readily made from the ^{13}C NMR signal of the C-4′ peaks ($CDCl_3$) [12,26]: the signal *circa* δ 151 ppm is characteristic of the C-4′ atom for the *N*-9′ regioisomers, whilst the signal *circa* δ 160 ppm is characteristic of the C-4′ atom for the *N*-7′ regioisomers. Besides examining the C-4′ chemical shift trend in **11**–**24** (Table 1) it can be noticed that the presence of the thio substituent in **13**, **15**–**17**, **20**, **22**–**24** as compared to **11**, **12**, **14**, **18**, **19** and **21** causes a small downfield shift of 2 ppm (δ 148 *versus* 151 ppm in the *N*-9′ isomers and δ 159 *versus* 162 ppm in the *N*-7′ isomers).

The influence of the substituent on the chemical shift of C-5′ is more marked; the oxy-substituted purine derivatives were at lower ppm ($\delta_{C\text{-}5'}$ 121 ppm for *N*-9′ regioisomers **11**, **12** and **14**, and 112 ppm for the *N*-7′ regioisomers **18**, **19** and **21**) when compared with the thio-substituted purine analogues ($\delta_{C\text{-}5'}$ 130 ppm for *N*-9′ regioisomers **13** and **15**–**17** and 122 ppm for the *N*-7′ regioisomers **20** and **22**–**24**) which means a downfield shift of 10 ppm for this signal.

2.2. Biological activities

The antitumour potential of the target molecules is reported against the MCF-7 human breast cancer cell line including 5-FU as reference drug (Table 2). The purine *O*,*N*-acetals **11**–**24** are more active than their corresponding purine bases **4**–**10**. The differences in the antiproliferative effect of the *N*-7′ and *N*-9′ regioisomers are not significant with the exception of the allyloxy derivatives **14** and **21**. The biological effect is dependent on the substituent present in position 6 of the purine ring although a clear structure–activity relationship between the size of this moiety and the antiproliferative effect of the MCF-7 human breast cancer cell line is not observed. The most active compound (**14**), that presents an allyloxy group as substituent at position 6 of the purine ring, shows an $IC_{50} = 5.04 \pm 1.68$ μM nearly equipotent as 5-FU. The following two more active compounds, **15** and **20**, present bulky substituents as the phenylthio and 2,4-diclorophenylthio ones, respectively. The CDK1 and CDK2 inhibitor NU2058 (**1**) has a low activity as an antiproliferative agent in the MCF-7 human breast cancer cell line.

Table 1
Quaternary carbon signals of the purine rings

Compound	Isomer	R	C-4′	C-5′	C-6′
11	*N*-9′	$OCH_2{-}C_6H_{11}$	151.64	121.53	161.41
12	*N*-9′	OC_6H_5	152.46	121.63	160.52
14	*N*-9′	$OCH_2CH{=}CH_2$	151.72	121.43	160.70
13	*N*-9′	$SC_6H_5{-}Cl_2$-(2,4)	148.90	130.95	159.53
15	*N*-9′	SC_6H_5	148.54	130.79	161.44
16	*N*-9′	$SC_6H_5{-}$F-(*p*)	148.57	130.74	162.69
17	*N*-9′	SMe	147.86	130.62	162.39
18	*N*-7′	$OCH_2{-}C_6H_{11}$	161.55	111.95	157.05
19	*N*-7′	OC_6H_5	162.81	112.04	156.18
21	*N*-7′	$OCH_2CH{=}CH_2$	161.93	112.10	156.51
20	*N*-7′	$SC_6H_5{-}Cl_2$-(2,4)	159.62	122.30	151.36
22	*N*-7′	SC_6H_5	159.39	122.24	Missing
23	*N*-7′	$SC_6H_5{-}$F-(*p*)	159.03	121.74	162.48
24	*N*-7′	SMe	158.13	122.65	154.00

Table 2
Antiproliferative activities against the MCF-7 cell line for 5-FU, for the purine bases (**4**–**10**), and for the seven-membered alkylated purine derivatives (*N*-9′ isomers: **11**–**17**; *N*-7′ isomers: **18**–**24**)

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
5-FU [26]	4.32 ± 0.02	**9**	21.4 ± 0.42	**17**	24.0 ± 1.95
1	24.2 ± 0.67	**10**	23.6 ± 4.11	**18**	13.4 ± 1.94
2 [12]	5.00 ± 0.05	**11**	11.6 ± 0.93	**19**	21.1 ± 2.93
4	32.3 ± 1.42	**12**	24.7 ± 3.82	**20**	8.4 ± 0.91
5	28.4 ± 0.45	**13**	12.0 ± 0.59	**21**	20.9 ± 1.24
6	44.7 ± 0.74	**14**	5.04 ± 1.68	**22**	15.6 ± 3.74
7	31.0 ± 0.16	**15**	7.12 ± 0.46	**23**	14.1 ± 0.67
8	31.6 ± 0.54	**16**	11.2 ± 1.32	**24**	31.8 ± 5.46

To study the mechanisms of the antitumour and antiproliferative activities of the most active compounds (**14**, **15** and **20**), the effects on the cell cycle distribution were analyzed by flow cytometry (Table 3). DMSO-treated cell cultures contained a 58.62 ± 0.74 of the G_0/G_1-phase cells, a 33.82 ± 0.72 of the S-phase cells and a 7.55 ± 1.34 of the G_2/M-phase cells. In contrast, MCF-7 cells treated during 48 h with the IC_{50} concentrations of **14**, **15** and **20** showed important differences in cell cycle progression compared with DMSO-treated control cells. The cell cycle regulatory activities can be divided into the following two groups: (a) the breast cancer cells for **14** showed an accumulation in the S phase, up to 37.00 ± 2.00 of the cells, mainly at the expense of the G_0/G_1-phase population that decreased to a percentage of 55.63 ± 1.57 of the cells; (b) compounds **15** and **20** accumulated the cancerous cells in the G_2/M-phase (11.08 ± 1.01 and 19.16 ± 0.56, respectively) at the expense of the S-phase cells (26.82 ± 1.26 and 22.73 ± 0.37, respectively) (Table 1).

In response to **15** (and **20**), the percentage of apoptotic cells increased, from 0.22 ± 0.31 in control cells to a maximum of 73.37 ± 0.12 (and 65.28 ± 1.92) apoptotic cells at a concentration equal to their IC_{50} against the MCF-7 cell line. This is a remarkable property because the demonstration of apoptosis in MCF-7 breast cancer cells by known apoptosis-inducing agents has proved to be difficult. These compounds are more potent as apoptosis inductors against the MCF-7 human breast cancer cells than paclitaxel (Taxol®), which induced programmed cell death up to 43% of cell population [27].

Table 3
Cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the three most active compounds

Compound	Cell cycle[a]			Apoptosis[b]
	G_0/G_1	S	G_2/M	
Control	58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
14	55.63 ± 1.57	37.00 ± 2.00	7.37 ± 0.43	44.47 ± 2.98
15	59.10 ± 1.28	26.82 ± 1.26	11.08 ± 0.01	73.37 ± 0.12
20	58.10 ± 0.19	22.73 ± 0.37	19.16 ± 0.56	65.28 ± 1.92

[a] Determined by flow cytometry [29].
[b] Apoptosis was determined using an Annexin V-based assay [30]. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicates and gave similar results. The data are means ± SEM of three independent determinations.

3. Conclusion

In conclusion, a series of (*RS*)-6-substituted-7 or 9-(2,3-dihydro-5*H*-1,4-benzodioxepin-3-yl)-7*H*- or -9*H*-purines were synthesized. The microwave-assisted organic synthesis has provided faster access to the target compounds with the advantage of the selective preparation of the *N*-7′ or *N*-9′ regioisomers, simplifying their isolation. The antitumour effect of the target molecules against the MCF-7 human breast cancer cell line indicates that the biological effect is dependent on the substituent at position 6 of the purine ring. The superiority of chlorine substituents [12] over the substituents used in this study can be highlighted. At present, studies are being carried out to determine the mechanism of action at the molecular level of the most active compounds.

4. Experimental protocols

4.1. Chemistry

Melting points were taken in open capillaries on an Electrothermal melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a 400 MHz ^{1}H and 100 MHz ^{13}C NMR Bruker ARX 400 or 300 MHz ^{1}H and 75 MHz ^{13}C NMR Bruker AMX-300 spectrometers or 400 MHz ^{1}H and 100 MHz ^{13}C NMR Varian NMR-System-TM 400 or 300 MHz ^{1}H and 75 MHz ^{13}C NMR Varian Inova-TM spectrometers at ambient temperature. Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. Signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, double doublet of doublets; dt, doublet of triplets; t, triplet; m, multiplet. The HMBC spectra were measured using a pulse sequence optimized for 10 Hz (inter-pulse delay for the evolution of long-range couplings: 50 ms) and a 5/3/4 gradient combination. In this way, direct responses (^1J couplings) were not completely removed. High-resolution liquid secondary ion mass spectra (HR LSIMS) were carried out on a VG AutoSpec Q high-resolution mass spectrometer (Fisons Instruments). All products had satisfactory C, H and N analyses (within ±0.4%). Small scale microwave-assisted synthesis was carried out in an Initiator 2.0 single-mode microwave instrument producing controlled irradiation at 2.450 GHz (Biotage AB, Uppsala). Reaction time refers to hold time at 130 °C, 100 °C or 60 °C, not to total irradiation time. These parameters were established following the basic principles of microwave-assisted organic synthesis. The temperature was measured with an IR sensor outside the reaction vessel. Anhydrous acetonitrile was purchased from VWR International Eurolab. 6-Substituted purines **4**–**8** were synthesized according to literature procedures [15,16]. 6-Methylthiopurine and NU2058 were purchased from Aldrich.

4.1.1. 6-(2,4-Dichlorophenylthio)purine *9*

A mixture of 6-chloropurine (0.8 g, 5.17 mmol), 2,4-chlorobenzenethiol (1 g, 5.58 mmol) and triethylamine (2.5 mL, 15.5 mmol) in 20 mL of *n*-butanol was refluxed for 24 h. The reaction mixture was cooled to room temperature and the solvent was removed in vacuo. The remaining oil was purified by flash column chromatography on silica gel using CH_2Cl_2/MeOH (9.5/0.5) as eluent to afford **9** as an off-white solid (1.18 g, 77%); mp 262–264 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 8.55 (s, 1H), 8.52 (s, 1H), 7.87 (d, $J = 2.3$ Hz, 1H), 7.82 (d, $J = 8.3$ Hz, 1H), 7.55 (dd, $J = 2.3$, 8.3 Hz, 1H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ (ppm) 157.05, 152.29, 150.77, 144.66, 144.53, 140.58, 139.91, 136.50, 130.50, 128.91, 126.38. HR (LSIMS) calcd for $C_{11}H_6Cl_2N_4S$ $(M+H)^+$ 296.9768, found 296.9768. Anal. for $C_{11}H_6Cl_2N_4S$ (C, H, N, S).

4.1.2. General procedures for the reaction between (*RS*)-3-methoxy-2,3-dihydro-5H-1,4-benzodioxepine *3* and the purine bases *4*–*10*

4.1.2.1. Method (a). A suspension of **3** (200 mg, 1.11 mmol), TCS (142 μL, 1.11 mmol), HMDS (232 μL, 1.11 mmol) and the corresponding purine derivative **4**, **5** or **9** (1.22 mmol) in anhydrous acetonitrile (6 mL) was prepared under argon atmosphere and kept at room temperature for 20 min. The reaction mixture was then cooled at −25 °C and a 1.0 M solution of $SnCl_4$ (1.22 mL, 1.22 mmol) in CH_2Cl_2 was added dropwise. After 48–72 h of stirring at 45 °C, the reaction was quenched by the addition of a concentrated aqueous solution of Na_2CO_3 and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and the solvent removed under vacuum. The residue was purified by flash chromatography under gradient elution conditions using mixtures of hexane/ethyl acetate (1/1 to 1/4) or CH_2Cl_2/MeOH (100/1 to 100/5) to afford **11** and **18**, **12** and **19** and **13** and **20**. Traces of the acyclic alkylated analogues [26] were detected in the reactions.

*4.1.2.1.1. (RS)-6-Cyclohexylmethyloxy-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine **11***. White solid (10%); mp 156–158 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.53 (s, 1H), 8.20 (s, 1H), 7.29 (t, $J = 7.6$ Hz, 1H), 7.20 (dd, $J = 2.0$, 7.4 Hz, 1H), 7.13–7.07 (m, 2H), 6.28 (dd, $J = 2.5$, 6.5 Hz, 1H), 4.90 (d, $J = 14.1$ Hz, 1H), 4.85 (d, $J = 14.1$ Hz, 1H), 4.55 (dd, $J = 2.3$, 12.9 Hz, 1H), 4.43 (dd, $J = 6.7$,

12.9 Hz, 1H), 4.39 (d, $J = 6.7$ Hz, 2H), 1.96–1.65 (m, 6H), 1.33–1.07 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 161.41, 158.88, 152.59, 151.64, 140.62, 130.57, 129.95, 129.33, 124.22, 121.53, 120.76, 83.93, 73.80, 72.54, 68.95, 37.31, 29.8 (×2), 26.47, 25.76 (×2). HR (LSIMS) calcd for $C_{21}H_{24}N_4O_3Na$ $(M + Na)^+$ 403.1746, found 403.1748. Anal. for $C_{21}H_{24}N_4O_3$ (C, H, N).

4.1.2.1.2. (RS)-6-Cyclohexylmethyloxy-7-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***18***. White solid (21%); mp 101–104 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.64 (s, 1H), 8.39 (s, 1H), 7.33–7.29 (m, 1H), 7.23 (dd, $J = 1.5$, 7.5 Hz, 1H), 7.13–7.09 (m, 2H), 6.30 (dd, $J = 2.5$, 6.5 Hz, 1H), 4.81 (s, 2H), 4.58 (dd, $J = 2.2$, 12.6 Hz, 1H), 4.44–4.36 (m, 2H), 4.28 (dd, $J = 6.5$, 12.6 Hz, 1H), 1.83–1.76 (m, 6H), 1.26–1.10 (m, 5H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 161.55, 158.88, 157.04, 152.59, 143.97, 130.74, 130.11, 129.38, 124.42, 120.84, 111.95, 86.49, 74.64, 72.51, 68.99, 37.28, 29.86 (×2), 26.40, 25.73 (×2). HR (LSIMS) calcd for $C_{21}H_{24}N_4O_3Na$ $(M + Na)^+$ 403.1746, found 403.1746. Anal. for $C_{21}H_{24}N_4O_3$ (C, H, N).

4.1.2.1.3. (RS)-6-Phenoxy-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***12***. White solid (21%); mp 182–184 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.56 (s, 1H), 8.37 (s, 1H), 7.49–7.46 (m, 2H), 7.35–7.23 (m, 5H), 7.16–7.11 (m, 2H), 6.34 (dd, $J = 2.3$, 6.3 Hz, 1H), 4.94 (d, $J = 14.3$ Hz, 1H), 4.90 (d, $J = 14.3$ Hz, 1H), 4.63 (dd, $J = 2.3$, 13.0 Hz, 1H), 4.50 (dd, $J = 6.3$, 13.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 160.52, 158.87, 152.62, 152.46, 141.76, 130.42, 130.00, 129.71 (×2), 129.34, 125.93, 124.25, 121.91 (×2), 121.63, 120.75, 84.01, 73.72, 69.02, one carbon missing. HR (LSIMS) calcd for $C_{20}H_{17}N_4O_3$ $(M + H)^+$ 361.1301, found 361.1300. Anal. for $C_{20}H_{16}N_4O_3$ (C, H, N).

4.1.2.1.4. (RS)-6-Phenoxy-7-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***19***. White solid (25%); mp 192–193 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.67 (s, 1H), 8.57 (s, 1H), 7.53–7.47 (m, 2H), 7.38–7.23 (m, 5H), 7.18–7.10 (m, 2H), 6.51 (dd, $J = 2.2$, 6.0 Hz, 1H), 4.91 (d, $J = 14.0$ Hz, 1H), 4.85 (d, $J = 14.1$ Hz, 1H), 4.71 (dd, $J = 2.2$, 12.9 Hz, 1H), 4.39 (dd, $J = 6.0$, 12.9 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 162.79, 158.79, 152.68, 151.87, 145.15, 130.52, 130.12, 129.90 (×2), 129.70, 129.37, 126.27, 124.45, 121.91, 121.77 (×2), 120.77, 86.25, 74.46, 68.75. HR (LSIMS) calcd for $C_{20}H_{16}N_4O_3Na$ $(M + Na)^+$ 383.1120, found 383.1121. Anal. for $C_{20}H_{16}N_4O_3$ (C, H, N).

4.1.2.1.5. (RS)-6-(2,4-Dichlorophenylthio)-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***13***. White solid (21%); mp 72–73 °C melts, 154 °C liquefies. ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 8.64 (s, 1H), 8.33 (s, 1H), 7.68 (d, $J = 8.50$ Hz, 1H), 7.58 (d, $J = 2.0$ Hz, 1H), 7.36–7.21 (m, 3H), 7.14–7.09 (m, 2H), 6.29 (dd, $J = 1.8$, 6.2 Hz, 1H), 4.89 (s, 2H), 4.61 (dd, $J = 1.8$, 12.9 Hz, 1H), 4.44 (dd, $J = 6.2$, 12.9 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 159.53, 158.99, 152.68, 148.90, 142.20, 141.02, 138.75, 137.19, 130.95, 130.60, 130.20, 129.52, 128.08, 125.56, 124.46, 120.94, 84.07, 73.84, 69.21, one carbon missing. HR (LSIMS) calcd for $C_{20}H_{14}N_4O_2NaSCl_2$ $(M + Na)^+$ 467.0112, found 467.0112. Anal. for $C_{20}H_{14}Cl_2N_4O_2S$ (C, H, N, S).

4.1.2.1.6. (RS)-6-(2,4-Dichlorophenylthio)-7-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***20***. White solid (28%); mp 176.7–179.3 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 8.70 (s, 1H), 8.57 (s, 1H), 7.60 (d, $J = 8.4$ Hz, 1H), 7.53 (d, $J = 2.2$ Hz, 1H), 7.32–7.19 (m, 3H), 7.12–7.05 (m, 2H), 6.49 (dd, $J = 2.3$, 5.0 Hz, 1H), 4.79 (d, $J = 13.9$ Hz, 1H), 4.73 (d, $J = 13.9$ Hz, 1H), 4.64 (dd, $J = 2.4$, 13.2 Hz, 1H), 4.49 (dd, $J = 5.0$, 13.1 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 159.62, 158.84, 153,16, 151.36, 146.29, 140.32, 138.54, 137.33, 130.70, 130.59, 130.27, 129.54, 128.15, 125.26, 124.73, 122.30, 120.85, 85.41, 73.96, 67.98. HR (LSIMS) calcd for $C_{20}H_{14}N_4O_2NaSCl_2$ $(M + Na)^+$ 467.0112, found 467.0112. Anal. for $C_{20}H_{14}Cl_2N_4O_2S$ (C, H, N, S).

4.1.2.2. Method (b). In a microwave vial, a suspension of **3** (100 mg, 0.55 mmol), TCS (71 μL, 0.55 mmol), HMDS (116 μL, 0.55 mmol) and the corresponding purine derivative (**6**–**8** and **10**, 0.61 mmol) in anhydrous acetonitrile (3 mL) was prepared under an argon atmosphere and kept at room temperature for 20 min. The reaction mixture was then cooled at −25 °C and a 1.0 M solution of $SnCl_4$ (0.61 mL, 0.61 mmol) in CH_2Cl_2 was added dropwise. The vial was then sealed and after 5 min of microwave irradiation at 130 °C, the reaction was quenched by the addition of a concentrated aqueous solution of Na_2CO_3 and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and the solvent removed under vacuum. The crude product was loaded onto a silica column and purified by flash column chromatography using hexane/ethyl acetate (1/1 to 1/4) as eluent to afford **14**–**17**.

4.1.2.2.1. (RS)-6-Allyloxy-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***14***. White solid (7%); mp 117–119 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.55 (s, 1H), 8.23 (s, 1H), 7.33–7.27 (m, 1H), 7.22 (dd, $J = 1.5$, 7.5 Hz, 1H), 7.13–7.07 (m, 2H), 6.28 (dd, $J = 2.5$, 6.5 Hz, 1H), 6.15 (ddd, $J = 5.6$, 10.5, 17.3 Hz, 1H), 5.48 (dd, $J = 1.4$, 17.3 Hz, 1H), 5.32 (dd, $J = 1.3$, 10.4 Hz, 1H), 5.12 (dt, $J = 1.3$, 1.4, 5.6 Hz, 2H), 4.88 (s, 2H), 4.59 (dd, $J = 2.5$, 13.0 Hz, 1H), 4.45 (dd, $J = 6.5$, 13.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 160.70, 158.90, 152.52, 151.72, 140.85, 132.42, 130.52, 129.96, 129.32, 124.21, 121.53, 120.75, 118.67, 83.91, 73.77, 68.97, 67.79. HR (LSIMS) calcd for $C_{17}H_{17}N_4O_3$ $(M + H)^+$ 325.1301, found 325.1301. Anal. for $C_{17}H_{16}N_4O_3$ (C, H, N).

4.1.2.2.2. (RS)-6-Phenylthio-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***15***. White solid (31%); mp 186–188 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.63 (s, 1H), 8.31 (s, 1H), 7.67–7.65 (m, 2H), 7.48–7.46 (m, 3H), 7.33–7.28 (d, $J = 6.0$ Hz, 1H), 7.13–7.09 (m, 2H), 6.28 (dd, $J = 2.0$, 6.5 Hz, 1H), 4.91 (d, $J = 14.1$ Hz, 1H), 4.87 (d, $J = 14.1$ Hz, 1H), 4.53 (dd, $J = 2.5$, 13.0 Hz, 1H), 4.44 (dd, $J = 6.5$, 13.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 161.45, 158.99, 152.85, 148.54, 141.89, 135.85 (×2), 130.79, 130.58, 130.15, 129.86, 129.55 (×2), 129.49, 127.28, 124.41, 120.91, 84.00, 73.86, 69.17. HR (LSIMS) calcd for

$C_{20}H_{16}N_4O_2NaS$ $(M + Na)^+$ 399.0892, found 399.0892. Anal. for $C_{20}H_{16}N_4O_2S$ (C, H, N).

4.1.2.2.3. (RS)-6-p-Fluorophenylthio-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***16***. White solid (35%); mp 197–199 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.63 (s, 1H), 8.31 (s, 1H), 7.65–7.61 (m, 2H), 7.32–7.29 (m, 1H), 7.22–7.09 (m, 5H), 6.28 (dd, $J = 2.5$, 6.5 Hz, 1H), 4.89 (s, 2H), 4.59 (dd, $J = 2.5$, 13.0 Hz, 1H), 4.44 (dd, $J = 6.5$, 13.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 165.18, 162.69, 158.97, 152.79, 148.57, 141.99, 138.07, 137.98, 130.10, 130.74, 130.53, 130.17, 129.49, 124.42, 120.91, 116.96, 116.74, 84.02, 73.84, 69.18. HR (LSIMS) calcd for $C_{20}H_{16}FN_4O_2S$ $(M + H)^+$ 395.0978, found 395.0979. Anal. for $C_{20}H_{15}FN_4O_2S$ (C, H, N).

4.1.2.2.4. (RS)-6-Methylthio-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-9H-purine ***17***. White solid (45%); mp 109–111 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.75 (s, 1H), 8.26 (s, 1H), 7.35–7.27 (m, 1H), 7.21 (dd, $J = 1.5$, 7.5 Hz, 1H), 7.06–7.15 (m, 2H), 6.28 (dd, $J = 2.3$, 6.7 Hz, 1H), 4.91 (d, $J = 14.1$ Hz, 1H), 4.86 (d, $J = 14.1$ Hz, 1H), 4.60 (dd, $J = 2.4$ and 12.9 Hz, 1H), 4.44 (dd, $J = 6.7$ and 12.9 Hz, 1H), 2.73 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 162.36, 158.99, 152.50, 147.86, 141.38, 131.40, 130.62, 130.13, 129.49, 124.40, 120.91, 83.93, 73.89, 69.14, 12.04. HR (LSIMS) calcd for $C_{15}H_{14}N_4O_2NaS$ $(M + Na)^+$ 337.0735, found 337.0735. Anal. for $C_{15}H_{14}N_4O_2S$ (C, H, N).

4.1.2.3. Method (c). In a microwave vial, a suspension of **3** (100 mg, 0.55 mmol), TCS (71 μL, 0.55 mmol), HMDS (116 mL, 0.55 mmol) and the corresponding purine derivative (**6–8** and **10**, 0.61 mmol) in anhydrous acetonitrile (3 mL) was prepared under argon atmosphere and kept at room temperature for 20 min. The reaction mixture was then cooled at −25 °C and a 1.0 M solution of $SnCl_4$ (0.61 mL, 0.61 mmol) in CH_2Cl_2 was added dropwise. The vial was then sealed and after 5 min of microwave irradiation at 100 °C the reaction was quenched by the addition of a concentrated aqueous solution of Na_2CO_3 and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and the solvent removed under vacuum. The crude product was loaded onto a silica column and purified by flash column chromatography using hexane/ethyl acetate (1/1 to 1/4) as eluent to afford **21–24**. Traces of the acyclic alkylated analogues [26] were detected in the synthesis of **22–24**. The use of 60 °C as the reaction temperature did not prevent the formation of these acyclic derivatives.

4.1.2.3.1. (RS)-6-Allyloxy-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***21***. Viscous oil (30% at 100 °C). ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 8.68 (s, 1H), 8.45 (s, 1H), 7.33 (t, $J = 7.6$ Hz, 1H), 7.24 (d, $J = 7.0$ Hz, 1H), 7.16–7.11 (m, 2H), 6.37 (d, $J = 4.5$ Hz, 1H), 6.08 (ddd, $J = 5.1$, 10.5, 17.0 Hz, 1H), 5.48 (d, $J = 17.0$ Hz, 1H), 5.32 (d, $J = 10.5$ Hz, 1H), 5.12 (d, $J = 5.1$ Hz, 2H), 4.87 (d, $J = 14.1$ Hz, 1H), 4.82 (d, $J = 14.1$ Hz, 1H), 4.62 (dd, $J = 2.0$, 13.0 Hz, 1H), 4.33 (dd, $J = 6.5$, 13.0 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 161.93, 159.00, 156.51, 152.74, 144.41, 132.14, 130.90, 130.27, 129.56, 124.60, 120.99, 119.05, 112.10, 86.41, 74.69, 68.95, 67.88. HR (LSIMS) calcd for $C_{17}H_{16}N_4O_3Na$ $(M + Na)^+$ 347.1120, found 347.1120. Anal. for $C_{17}H_{16}N_4O_3$ (C, H, N).

4.1.2.3.2. (RS)-6-Phenylthio-7-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***22***. White solid (19% at 60 °C and 17% at 100 °C); mp 148–151 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) 8.71 (s, 1H), 8.52 (s, 1H), 7.56 (m, 2H), 7.41 (m, 4H), 7.30–7.04 (m, 3H), 6.53 (dd, $J = 2.2$, 5.3 Hz, 1H), 4.81 (d, $J = 14.1$ Hz, 1H), 4.75 (d, $J = 14.1$ Hz, 1H), 4.64 (dd, $J = 2.6$, 13.2 Hz, 1H), 4.41 (dd, $J = 5.3$, 12.8 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm) 159.39, 159.00, 153.40, 146.15, 135.66, 130.84, 130.38, 130.13, 129.78, 129.68, 127.01, 124.82, 122.24, 121.01, 85.70, 74.31, 68.35, one carbon missing. HR (LSIMS) calcd for $C_{20}H_{16}N_4O_2NaS$ $(M + Na)^+$ 399.0892, found 399.0891. Anal. for $C_{20}H_{16}N_4O_2S$ (C, H, N, S).

4.1.2.3.3. (RS)-6-p-Fluorophenylthio-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***23***. White solid (35% at 60 °C); mp 82–84 °C melts, 135–137 °C liquefies. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.81 (s, 1H), 8.70 (s, 1H), 7.69–7.63 (m, 2H), 7.41–7.35 (m, 1H), 7.31–7.16 (m, 5H), 6.60 (dd, $J = 2.2$, 4.8 Hz, 1H), 4.91 (d, $J = 14.1$ Hz, 1H), 4.84 (d, $J = 14.1$ Hz, 1H), 4.74 (dd, $J = 2.2$, 13.2 Hz, 1H), 4.44 (dd, $J = 5.2$, 13.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 165.81, 162.48, 159.03, 158.99, 153.20, 146.27, 138.15, 138.04, 130.77, 130.45, 129.70, 124.90, 121.74, 121.02, 117.28, 116.99, 104.99, 85.69, 73.23, 68.27. HR (LSIMS) calcd for $C_{20}H_{15}FN_4O_2NaS$ $(M + Na)^+$ 417.0797, found 417.0799. Anal. for $C_{20}H_{15}FN_4O_2S$ (C, H, N, S).

4.1.2.3.4. (RS)-6-Methylthio-9-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl)-7H-purine ***24***. White solid (20% at 100 °C, 25% at 60 °C); mp 69 °C melts, 95 °C foam, 124 °C liquefies. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 8.95 (s, 1H), 8.58 (s, 1H), 7.39–7.34 (m, 1H), 7.29–7.26 (m, 1H), 7.20–7.14 (m, 2H), 6.49 (dd, $J = 2.4$, 5.5 Hz, 1H), 4.84 (s, 2H), 4.68 (dd, $J = 2.4$, 13.0 Hz, 1H), 4.46 (dd, $J = 5.5$, 13.0 Hz, 1H), 2.82 (s, 3H, SMe). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 158.98, 158.13, 154.51, 152.91, 145.49, 130.81, 130.38, 129.67, 124.80, 122.65, 121.00, 85.70, 74.43, 68.38, 12.83. HR (LSIMS) calcd for $C_{15}H_{14}N_4O_2NaS$ $(M + Na)^+$ 337.0735, found 337.0736. Anal. for $C_{15}H_{14}N_4O_2S$ (C, H, N, S).

4.2. Biology

4.2.1. Cell culture and drug treatments

The human breast cancer MCF-7 cells were grown at 37 °C in an atmosphere containing 5% CO_2, with Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2% L-glutamine, 2.7% sodium bicarbonate, 1% Hepes buffer, 40 mg/L gentamicin and 500 mg/L ampicillin. After the synthesis and purification of the compounds stock solutions were prepared as previously described [28]. Briefly, the drugs were dissolved in DMSO and stored at −20 °C. For each experiment, the stock solutions were further diluted in medium to obtain the desired concentrations. The final solvent concentration in cell culture was ≤0.5% v/v of DMSO, a concentration without effect on cell replication.

Parallel cultures of MCF-7 cells in medium with DMSO were used as controls.

4.2.2. Cytotoxicity assays in vitro

The effect of anticancer drugs on cell viability was assessed using the sulforhodamine-B (SRB) colorimetric assay. Aliquots of MCF-7 cell suspension (30×10^3 cells/well) were seeded onto 24-well plates and incubated for 24 h. The cells were then treated with different concentrations of drugs in the culture medium. After three days the wells were aspirated, fresh medium and further drug were added, and cells were maintained for 3 more days. Thereafter, the cells were processed as described previously [29], using a Titertek Multiscan apparatus (Flow, Irvine, California) at 492 nm. We evaluated the linearity of the SRB assay with cell number for each MCF-7 cell stock before each cell growth experiment. The IC_{50} values were calculated from semilogarithmic dose–response curves by linear interpolation. All of the experiments were plated in triplicate wells and were carried out at least twice.

4.2.3. Cell cycle distribution analysis

Cells at 70% confluence were treated with either DMSO alone or with concentrations of the compounds determined by their IC_{50} values. Fluorescence-activated cell sorting (FACS) analysis was performed at 48 h of treatment as described [30]. All experiments were performed in duplicates and yielded similar results.

4.2.4. Apoptosis detection by staining with annexin V-FITC and propidium iodide

The Annexin V-FITC Apoptosis Detection kit I (Pharmingen, San Diego, CA, USA) was used to detect apoptosis by flow cytometry according to Ref. [30]. All experiments were performed in duplicates and yielded similar results.

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