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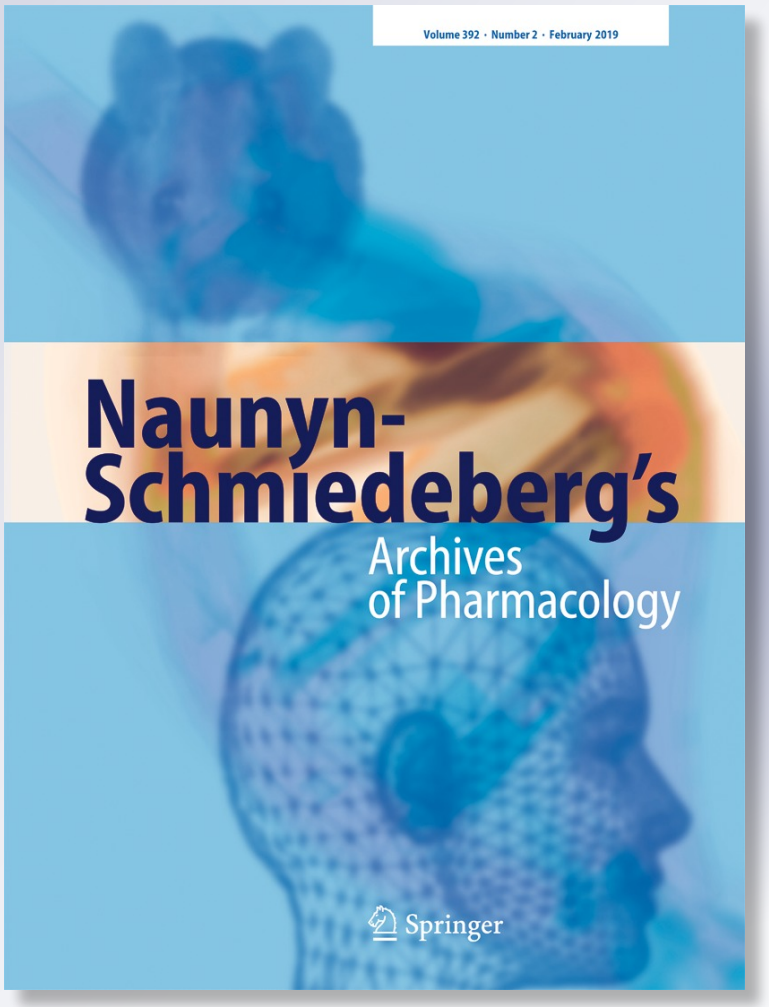

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ORIGINAL ARTICLE

Anti-leukemia activity of 4-amino-2-aryl-6,9-dichlorobenzo[g]pteridines

Antonio J. Ruiz-Alcaraz[1] · Violeta Carmona-Martínez[1] · Antonio Guirado[2] · Jesús Gálvez[3] · María Martínez-Esparza[1] · Pilar García-Peñarrubia[1]

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Abstract

Pteridines are bicyclic heterocyclic compounds with a pyrazino[2,3-d]pyrimidine nucleus that have shown a wide range of therapeutic utilities. Concretely, 4-aminopteridine derivatives have demonstrated both anti-inflammatory and anti-cancer properties, and some of them, such as methotrexate, are profusely used in medical practice. We have recently synthesized and tested the biological activity of a novel series of 4-amino-2-aryl-6,9-dichlorobenzo[g]pteridines, finding that they present anti-inflammatory properties, as they were able to inhibit in vitro the production of pro-inflammatory cytokines TNF-α and IL-6. Now, we have evaluated the anti-tumor potential of these compounds on HL-60 and K562 leukemia cell lines. Cells growing at exponential rate were exposed to decreasing doses of each compound, from 50 to 0.39 μM, for 24, 48, and 72 h. Cell viability was tested by MTT assay and cell death fashion determined by annexin V/propidium iodide assay. The cytotoxicity of the compounds was determined in differentiated macrophage-like HL-60 cells and in human peripheral blood mononuclear cells to evaluate the potential side effects on quiescent tumor cells and normal cells, respectively. Among the series, compounds **1a**, **1b**, **1g**, **1j**, and **1k** showed anti-proliferative activity. Compounds **1j** and **1k** were active against both HL-60 and K562 cells, with a lower IC_{50} against HL-60 cells. Compounds **1a**, **1b**, and **1g** had a great cytotoxic activity against HL-60, but they were far less potent against K562 cells. None had side effects in differentiated tumor cells or in human peripheral blood mononuclear cells. In conclusion, our results demonstrate that some compounds of this series of 4-amino-2-aryl-6,9-dichlorobenzo[g]pteridines have anti-cancer properties in vitro.

Keywords Pteridine derivatives · Anti-tumor drugs · Anti-proliferative agents · Late apoptosis · Necrosis

Introduction

Pteridines are bicyclic compounds based on pyrimido[4,5-b]pyrazine structures. They are naturally produced by many living organisms and are involved in several metabolic pathways in both health and disease conditions (Kośliński et al. 2011). Pteridine derivatives have been widely synthesized and their biological activities have been broadly studied as they have multiple therapeutic potential (Carmona-Martínez et al. 2018). These compounds can act as anti-inflammatory (De Jonghe et al. 2011; Pontiki et al. 2015), antiviral (Ding et al. 2005; El-Sabbagh et al. 2007), and antibacterial (Cheng et al. 2007) agents. They can also reduce tissue damage induced by ischemia/reperfusion (Palanki et al. 2007) and septic shock (Ma et al. 2009), or inhibit several enzymatic activities associated with neurodegenerative diseases (Prins et al. 2009). Many pteridine derivatives have also shown

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✉ Antonio J. Ruiz-Alcaraz
ajruiz@um.es

✉ Pilar García-Peñarrubia
pigarcia@um.es

[1] Departamento de Bioquímica, Biología Molecular (B) e Inmunología, School of Medicine, IMIB and Regional Campus of International Excellence "Campus Mare Nostrum", Universidad de Murcia, 30100 Murcia, Spain

[2] Departamento de Química Orgánica, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain

[3] Departamento de Química Física, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain

anti-tumor potential by inhibiting several enzymes such as carbonic anhydrase (Marques et al. 2010), dihydrofolate reductase (Zhang et al. 2009; Li et al. 2012), and several kinases (Rudolph et al. 2009; Tandon et al. 2015), as well as the monocarboxylate transporter 1 (Wang et al. 2014), among others (Nelson et al. 2004; Mullice et al. 2012).

The biological activity of 4-aminopteridines has been extensively studied. In fact, methotrexate, a 4-aminopteridine also known as MTX, is a dihydrofolate reductase inhibitor that blocks cell proliferation by inhibiting the synthesis of purines and pyrimidines. Thus, MTX has been used for many years as an anti-cancer drug, but also for the treatment of some autoimmune diseases such as rheumatoid arthritis (Abolmaali et al. 2013).

On an earlier study, we discovered that polychlorocyclohexanediones are excellent synthetic equivalents of unavailable chlorinated 1,2-benzoquinones (Guirado et al. 2011). In view of this peculiar chemical behavior, we focused our interest in exploiting these inexpensive and highly attractive starting materials to develop new synthetic methods to produce previously unattainable heterocyclic compounds (Guirado et al. 2011). Some of the compounds obtained by these new synthetic methods, such as certain quinoxaline (Guirado et al. 2012; Tristán-Manzano et al. 2015; Ruiz-Alcaraz et al. 2017) and pteridine derivatives (Guirado et al. 2013), displayed an intensive anti-inflammatory activity. The first synthesis of 4-amino-2-aryl-6,9-dichlorobenzo[g]pteridines was achieved by a route involving a first reaction between 3,3,6,6-tetrachloro-1,2-cyclohexanedione and diaminomaleonitrile to give 5,8-dichloro-2,3-dicyanoquinoxaline, whose reactions with amidines led directly to the targeted products.

In a previous work, we synthesized a series of 4-amino-2-aryl-6,9-dichlorobenzo[g]pteridines that showed a potent anti-inflammatory activity, as they were able to inhibit in vitro the production of pro-inflammatory cytokines TNF-α and IL-6 (Guirado et al. 2013). Now, we have tested in vitro the anti-tumor potential of this series of compounds against the tumor cells lines HL-60 and K562.

Methods

Cell culture

The compounds were tested using the human myeloid leukemia cell line HL-60 (ATCC® CCL-240™), and the K562 cell line (ATCC® CCL-243™) derived from chronic myelogenous leukemia. Cells were incubated in complete culture medium (CCM) consisting on RPMI-1640 (Biowest, Nuaillé, France) with 10% fetal bovine serum (GIBCO Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (GIBCO), at 37 °C with 5% CO_2. All assays based on cell lines were performed after passage number 5 and before passage number 20.

Human peripheral blood mononuclear cells (PBMCs) were cultured in the same conditions, in CCM at 37 °C and 5% CO_2. PBMC were obtained from peripheral blood of healthy donors as described elsewhere (Ruiz-Alcaraz et al. 2016). Briefly, blood was diluted at a 1:1 ratio with sterile PBS and layered in tubes containing Ficoll (Axis-Shield PoC As, Oslo, Norway). The PBMC fractions were collected and washed with RPMI-1640 before being cultured as explained above.

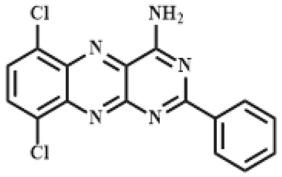
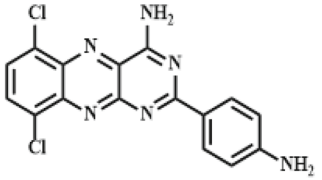
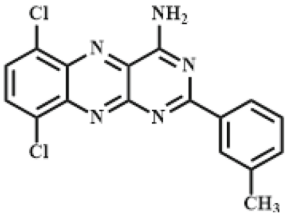
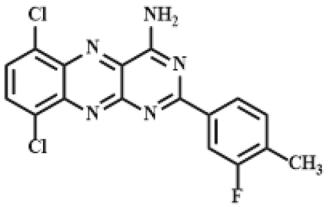
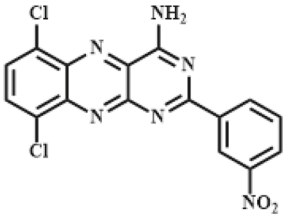
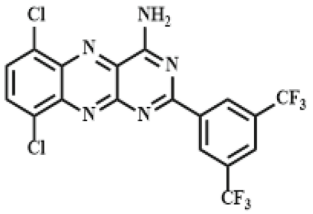
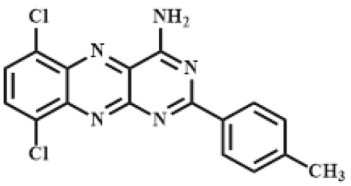
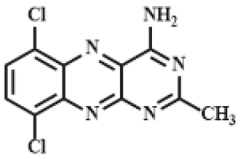
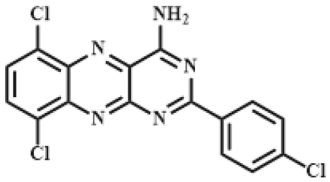
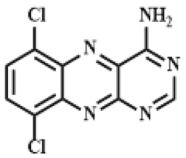
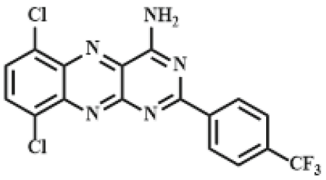
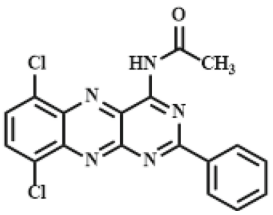
Compound preparation for in vitro assays

Pteridine derivatives, shown in Table 1, were synthesized following the previously described protocol (Guirado et al. 2013). Purity of compounds was equal or superior to 99% as determined by LC-MS and NMR spectral analysis. Compounds for in vitro assays were first dissolved in DMSO as stock solution, at a concentration of 1 mg/mL, and later diluted in CCM to the desired concentrations for assays. The same procedure was done with methotrexate (MTX; Sigma Chemical Co., St. Louis, MO, USA), which was used as positive cytotoxic control. At the highest compounds' doses, the final DMSO dilution varied from 1:150 (6.65 μL/mL, DMSO in MCC) to 1:84 (11.95 μL/mL, DMSO in MCC), with final DMSO concentrations in the range of 0.67 to 1.19%, depending on the molecular weight of the dissolved compound.

Cytotoxicity assays

Anti-tumor activity of the compounds was evaluated by MTT viability assays (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, to purple formazan by cellular mitochondrial enzymes) (Guirado et al. 2012). Briefly, cells were incubated at an exponential growth rate (10^6 HL-60 cells/mL and $0.5 \cdot 10^6$ K562 cells/mL) in 96-well plates containing 200 μL CCM, and exposed to different doses of the tested compounds (0.39 μM, 0.78 μM, 1.56 μM, 3.13 μM, 6.25 μM, 12.5 μM, 25 μM, and 50 μM) for 24, 48, and 72 h. Then, MTT (Alfa Aesar, Thermo Fisher, Karlsruhe, Germany) was added at a final concentration of 483 μM (0.2 mg/mL) and cells were incubated for 2 h at 37 °C and 5% CO_2. Afterwards, an acidified isopropanol solubilization solution containing 0.04 M hydrochloric acid and 0.1% NP-40 detergent was added to each well to dissolve the insoluble purple formazan product, retained inside the cells, giving a colored solution. Finally, the absorbance was measured at 550 nm by a plate-reading spectrophotometer. The percentage of compound cytotoxicity, and thus, the anti-tumor activity was obtained by comparison with the control conditions, in which the cells were exposed to an equivalent dose of vehicle (DMSO) (100% viability, 0% cytotoxicity) and an equivalent

Table 1 Chemical structure of tested pteridine derivatives

Compound	Chemical Structure	Compound	Chemical Structure
1a		1g	
1b		1h	
1c		1i	
1d		1j	
1e		1k	
1f		1l	

Pteridine derivatives were synthesized following the previously reported protocol (Guirado et al. 2013)

dose of MTX as a positive control of cytotoxicity. Each assay was repeated at least three independent times ($n = 3$), using different cell passages (after passage number 5 and before passage number 20).

Alternatively, to test the toxicity of pteridine derivatives in non-proliferative cell assays, human PBMC and HL-60 cells differentiated to macrophage-like cells were also cultured at a ratio of $0.2 \cdot 10^6$ cells/well in 96-well plates and treated with the tested compounds for 24 h. HL-60 cells were differentiated to macrophage-like cells with 16.2 nM (10 ng/mL) phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO, USA) for a period of 24 h, and then, cells rested in CCM without PMA for another 24 h. After differentiation and resting periods (Guirado et al. 2013; Gupta et al. 2014), vehicle (DMSO; MERK, Whitehouse Station, NJ, USA) as negative control, or maximum dose of tested compounds were added to each well, and plates were then incubated at 37 °C with 5% CO_2 for 24 h.

Human PBMCs were obtained from the blood of three healthy donors. Blood was first diluted 1:2 with sterile PBS (Biowest, Nuaillé, France) and layered in tubes containing Ficoll (Axis-Shield PoC As, Oslo, Norway). Lympho/monocyte fractions were collected and washed with CCM.

The ethics committees (Comité Ético de Investigación Clínica del Hospital Universitario Virgen de la Arrixaca, and Comité de Bioética de la Universidad de Murcia) approved the study protocol according to the 1975 Declaration of Helsinki.

Flow cytometry cell death assays and cell cycle assays

Cells incubated at an exponential rate (10^5 cells/mL, in 6 wells plates) were exposed for 72 h to a 15 μM dose of the previously selected active compounds **1a**, **1b**, **1g**, **1j**, and **1k**, which showed anti-tumor potential in the MTT assays, and 0.5 μM MTX as positive control. The type of cellular death was determined by the annexin V/propidium iodide assay (Annexin V Apoptosis Detection Kit FITC; eBiosciences, Thermo Fisher, Karlsruhe, Germany; PBS and distilled water; Biowest, Nuaillé, France) following manufacturer's instructions. To analyze cell cycle, cells were first suspended in 2 mL of ethanol/PBS (70:30%) and incubated for 30 min at 0 °C. Then, cells were centrifuged for 10 min at 1000 rpm and resuspended in a mix of 800 μL of PBS, 100 μL of RNAse (1 mg/mL) and 100 μL of propidium iodide (400 μg/mL) and incubated for 30 min at 37 °C. Flow cytometry analyses were performed with a FACSCalibur cytometer (BD Medical Technology, USA) using its own software, or the version 2.5.1 of Flowing Software. Ten thousand to 30,000 events were acquired and analyzed.

Statistical analysis

Data are reported as mean ± SEM. Statistical differences were analyzed using the Mann–Whitney *U* test and *p* values lower than 0.05 were considered to indicate statistical significance. Calculations were performed using the SPSS 21.0 software (Chicago, IL, USA).

Results and discussion

Evaluation of the anti-proliferative potential

The anti-proliferative potential of pteridine derivatives against leukemic cell lines HL-60 and K562 was evaluated in vitro. Cell viability was measured by performing MTT assays, and the compound cytotoxicity was calculated in comparison with the control conditions. Sensibility of cells lines to a referential chemotherapeutic agent was tested by using equivalent doses of MTX as positive controls.

The most active group of compounds among the tested series of pteridine derivatives included compounds **1a**, **1b**, **1g**, **1j**, and **1k** (Table 2, Fig. 1), while the rest were considered non-active or poorly active compounds, as the majority did not reach 50% cytotoxicity at any of the tested times (Suppl. Table 1). Both compounds **1a** and **1b** reached 100% cytotoxicity in the HL-60 cell line at the highest dose (50 μM), and similar levels of cytotoxicity (> 93%) at the sub-maximal dose of 25 μM, at all times (24, 48, and 72 h) (Table 3). The most effective compound, **1b** (Fig. 1b), showed the lowest IC_{50} (Table 2), followed closely by compound **1a**, which was the second most potent cytotoxic compound in this cell line (Fig. 1a, Table 2). Nonetheless, the effect of these two compounds against K562 cells was weaker, with compound **1a** showing the highest cytotoxicity level of

Table 2 IC_{50} values of pteridine derivatives obtained from MTT assays

	IC_{50} (μM)					
	HL-60			K562		
Compound	24h	48h	72h	24h	48h	72h
1a	4.8 ± 1.2	5.8 ± 1.3	5.4 ± 1.2	–	–	10.4 ± 1.4
1b	3.2 ± 0.6	3.3 ± 1.1*	3.5 ± 1.0	–	34.1 ± 1.3	18.5 ± 3.1
1g	18.8 ± 4.6$^{*,\#}$	12.5 ± 0.7$^{*,\#}$	10.7 ± 4.9	–	–	6.6 ± 0.5$^{\#}$
1j	6.8 ± 2.3	9.3 ± 1.3$^{*,\#}$	7.5 ± 1.7$^{*,\#}$	37.2 ± 1.42	20.5 ± 0.2$^{\#}$	9.4 ± 1.0
1k	29.0 ± 4.7$^{*,\#}$	10.0 ± 2.2$^{*,\#}$	9.7 ± 1.7$^{*,\#}$	19.0 ± 2.4$^{\$}$	13.7 ± 1.4$^{\#,\$}$	10.9 ± 1.4
MTX	–	0.0013 ± 0.0002	0.0007 ± 0.00006	–	0.0029 ± 0.0009	0.0013 ± 0.0001

Results summarized represent values of IC_{50} (μM) ± SEM following 24-, 48-, and 72-h exposure to different doses of compounds whose cytotoxicity reached at least 75% with the highest dose at any of the studied times in MTT assays, and 50% of cytotoxicity at the corresponding time point. Statistical significance was analyzed by the Mann–Whitney *U* test. $p < 0.05$ for *compound **1a** vs. others; $^{\#}$ compound **1b** vs. others; and, $^{\$}$ compound **1j** vs. others. IC_{50} values of MTX were significantly different to all compounds analyzed at all time points ($n = 3$)

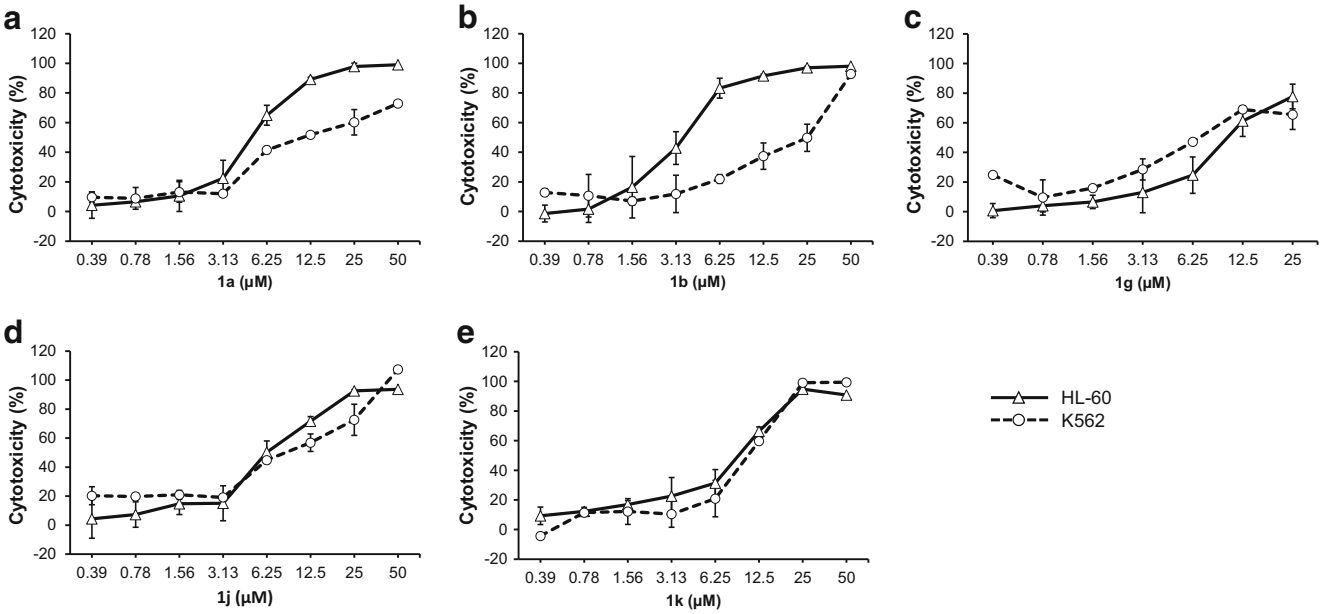


Fig. 1 Cytotoxic potential of pteridine derivatives on leukemia cell lines. Data represent the % of cytotoxicity as mean ± SEM of those compounds that achieved at least 80% of cytotoxicity at the highest dose in both HL-60 or K562 cells after 72-h treatment (HL-60, $n = 3$; K562, $n = 3$). The most active compounds displayed include compounds **1a** (**a**), **1b** (**b**), **1g** (**c**), **1j** (**d**) and **1k** (**e**)

73% at the maximum dose after 72 h of exposure, with an IC_{50} of 10.4 μM, while the most potent agent for HL-60 cells, compound **1b**, reached 93% of cytotoxicity at the highest dose, but with a higher IC_{50} (18.5 μM) after 72 h of exposure, which was actually the highest IC_{50} obtained among the active compounds that showed anti-tumor potential against K562 cells at this time point (Table 2).

The cytotoxicity of compounds **1j** and **1k** exceeded 90% at the highest doses when used against HL-60 and K562 cells (Fig. 1d, e, respectively), with a slightly higher IC_{50} at 72 h in the case of K562 cells (Table 2).

Compound **1g** was the least potent among the active compounds, since its maximum cytotoxicity level in HL-60 cells did not exceed 70% at the highest dose of 50 μM, and its IC_{50} at 72 h was also the highest (10.7 μM). In contrast, in the case of K562 cells, the IC_{50} of compound **1 g** after 72 h was in fact the lowest found with a dose of 6.62 μM, but the maximum cytotoxicity level of this compound did not even reach 70% (Fig. 1c; Table 2).

When we analyzed the kinetics of the cytotoxic activity of these compounds at the different time points studied, we realized that, in general, the HL-60 cells were more sensitive to the anti-proliferative effect of the tested compounds than the K562 cells, which were able to resist more time to this effect. In fact, most of the active compounds did not even reach 50% cytotoxicity on K562 cells after 24 h of treatment, or when they did, as in the case of compounds **1j** and **1k**, the doses needed to reach that cytotoxicity level were very high, with IC_{50} levels at 24 h of 37.2 μM and 19.0 μM, respectively (Table 2). On the contrary, the active compounds were already

Table 3 Cytotoxicity of pteridine derivatives on leukemia cell lines obtained from MTT assays

	Cytotoxicity (%)					
	HL-60			K562		
Compound	24h	48h	72h	24h	48h	72h
1a	93.5 ± 1.1	96.4 ± 2.1	97.9 ± 1.4	19.7 ± 4.2	29.6 ± 11.4	60.2 ± 1.2
1b	90.4 ± 2.3	96.2 ± 2.7	97.1 ± 1.2	35.1 ± 7.7	39.0 ± 15.6	49.8 ± 8.9
1g	65.1 ± 9.5	76.5 ± 3.3	77.8 ± 8.3	7.1 ± 3.8	28.0 ± 4.5	65.5 ± 10.0
1j	77.2 ± 0.2	83.2 ± 1.8	92.6 ± 3.2	13.0 ± 3.2	55.4 ± 0.1	72.6 ± 6.0
1k	80.6 ± 8.0	91.0 ± 3.8	94.7 ± 2.9	91.6 ± 3.5	97.5 ± 0.8	99.1 ± 0.5
MTX	31.2 ± 0.0	64.9 ± 4.2	76.9 ± 7.0	18.9 ± 3.1	54.3 ± 6.2	75.9 ± 2.5

Results summarized represent mean % of cytotoxicity ± SEM in MTT assays following 24-, 48-, and 72-h exposure to active compounds with the submaximal dose of 25 μM ($n = 3$)

Table 4 Cytotoxicity levels of pteridine derivatives on different cell types after 24-h treatment obtained from MTT assays

Compound	Cytotoxicity (%)			
	PBMC	Macrophage-like	HL-60	K562
1a	0.0 ± 9.9	13.0 ± 9.6	100.0 ± 1.2	39.9 ± 2.3
1b	0.4 ± 4.4	0.2 ± 10.1	98.6 ± 0.6	54.2 ± 3.7
1g	9.0 ± 8.0	15.7 ± 19.3	65.1 ± 9.5	7.1 ± 3.8
1j	0.4 ± 4.2	6.1 ± 7.8	87.6 ± 1.1	88.6 ± 0.8
1k	7.0 ± 4.1	1.8 ± 11.9	60.9 ± 22.9	94.5 ± 3.5
MTX	6.6 ± 4.1	–	49.74 ± 0.1	18.93 ± 0.03

Results summarize cytotoxicity (%) ± SEM of active compounds (**1a**, **1b**, **1g**, **1j**, **1k**) in MTT assays against human PBMC ($n = 3$), HL-60 cells differentiated to macrophage-like cells, and undifferentiated HL-60 and K562 cells after 24 h of exposure to the maximum dose of each compound (50 μM)

effective against HL-60 after 24 h of treatment, among which the most potent were **1a**, **1b,** and **1j**, with levels of IC_{50} at this time point very similar to those obtained at 48 h and at the final time point of 72 h (Table 2).

When we compared the cytotoxicity levels of the new compounds with those of MTX, used as positive cytotoxic control, we observed that, although MTX's IC_{50} levels were significantly lower than those of the pteridine derivatives for both HL-60 and K562 cell lines after 48 and 72 h treatments (< 0.1 μM), the most active pteridine derivatives, compounds **1a**, **1b**, **1g**, **1j,** and **1k**, showed higher levels of cytotoxicity than MTX against HL-60 at the two highest doses assayed (25 μM and 50 μM) for all times (24, 48, 72 h) (Table 3). In the case of K562 cells, only compound **1k** was able to clearly overcome the cytotoxicity of MTX, while the cytotoxicity levels of other active compounds were similar or lower than those of MTX (Table 3). In this regard, the remarkable capacity of most active pteridine derivatives to produce maximum/total levels of cytotoxicity at maximum and sub-maximal doses, as in the case of compounds **1a**, **1b**, **1j**, and **1k** in HL-60, and **1k** in K562 should be noted. This property would be useful to avoid episodes of relapse as well as the appearance o multidrug-resistant (MDR) leukemic cells in long-term low-dose treatments.

Regarding structure–activity relationships, we found that the important structural features for greater anti-proliferative effects corresponded to the presence of the following radicals

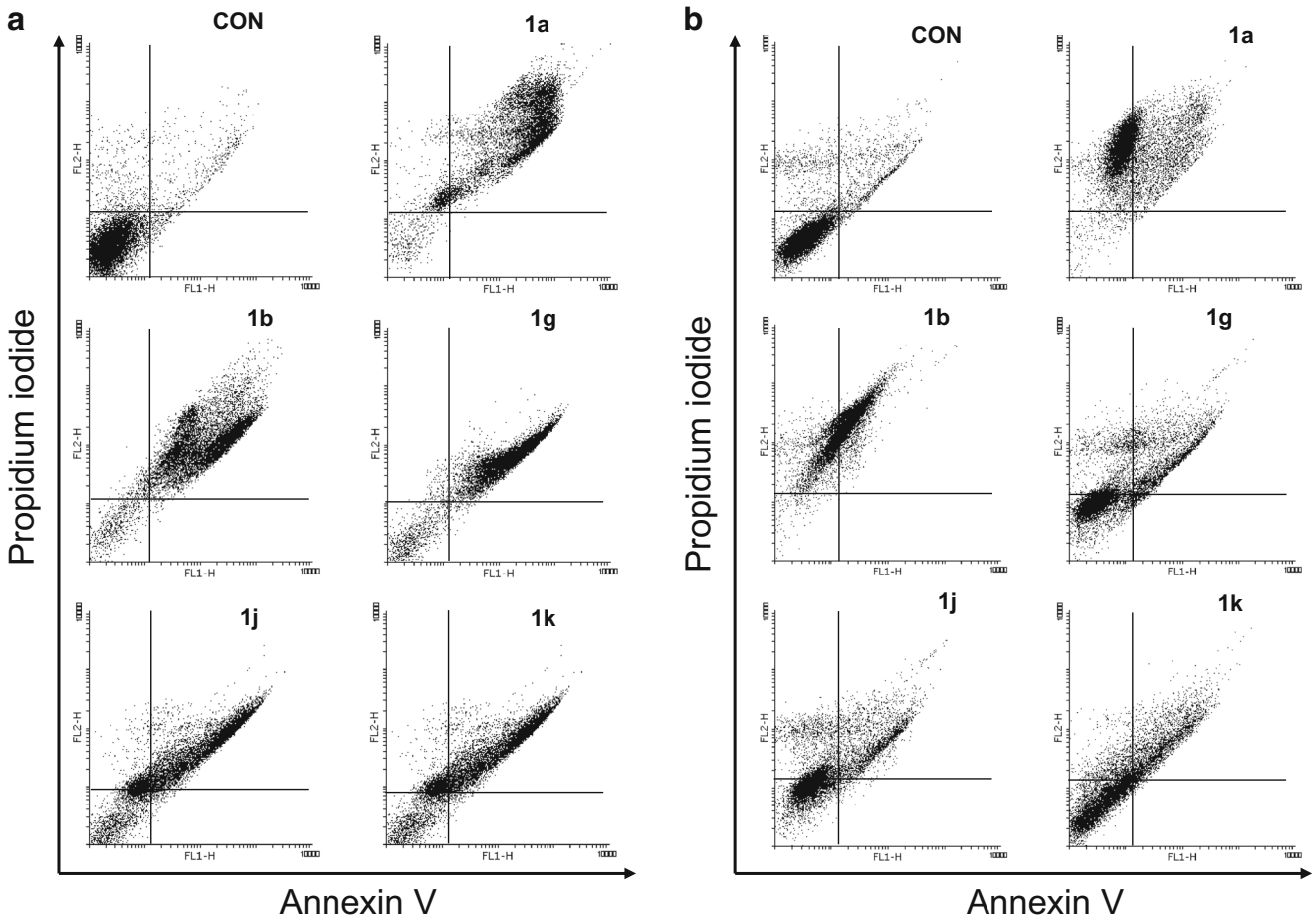


Fig. 2 Cell death analysis. Representative dot-plots of annexin V/propidium iodide assays of HL-60 (**a**) and K562 cells (**b**) under control conditions (CON), and the corresponding cells exposed to 15 μM of active compounds (**1a**, **1b**, **1g**, **1j**, and **1k**) for 72 h

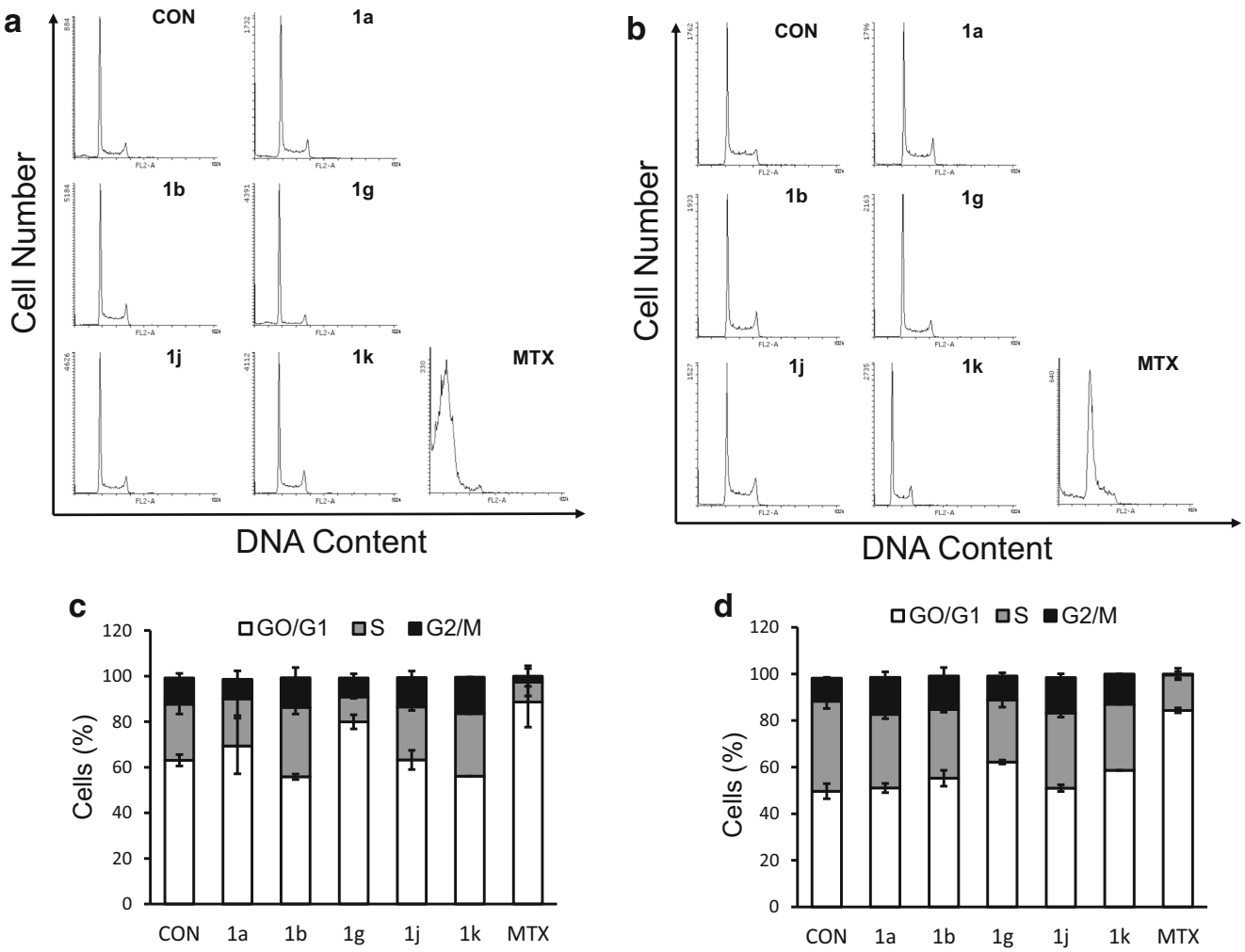


Fig. 3 Cell cycle analysis. Representative histograms of cell cycle assays of HL-60 (**a**) and K562 cells (**b**) under control conditions (CON), after treatment with 15 μM of active compounds (**1a**, **1b**, **1g**, **1j**, and **1k**), and after treatment with 0.5 μM methotrexate (MTX) as positive control for 72 h. Pile up bars represent frequencies (%) of HL-60 (**c**) and K562 cells (**d**) as mean ± SEM included in each cell cycle phase (G0/G1, S and G2/M) (HL-60, $n = 3$; K562, $n = 3$)

in position 2: phenyl (**1a**), 3-methylphenyl (**1b**), 4-aminophenyl (**1g**), and methyl (**1j**), or the absence of any radical as in compound **1k**. In contrast, the presence of halogen atoms such as chlorine and fluorine, as well as the trifluoromethyl group, corresponded to inactive compounds (i.e., compounds **1e**, **1f**, **1h**, **1i**). Something similar happened with the acetylation of the 4-amino group of **1a** to obtain compound **1l**, which attenuated the potent activity shown by the original structure (**1a**). Other modifications entailing inactivity included 3-nitrophenyl (**1c**) and 4-methylphenyl (**1d**) substituents.

Evaluation of cytotoxicity in differentiated cells

In order to test whether the pteridine derivatives could act as specific anti-proliferative agents, we tested the effect of these compounds on a non-proliferative cell model. To do that, we used a model of differentiated HL-60 cells to macrophage-like cells. As shown in Table 4, all active anti-proliferative compounds (**1a**, **1b**, **1g**, **1j**, and **1k**) did not show significant levels of cytotoxicity at 24 h compared to the effect of those compounds on undifferentiated HL-60 and K562 cells growing at an exponential rate, where the cytotoxic effect is quite significant, except for the compound **1g** on K562 cells, which is actually active in longer treatments (72 h). Therefore, these active compounds function as anti-proliferative agents capable of inhibiting active growth without affecting the viability of differentiated cells.

Evaluation of cytotoxicity in human peripheral blood mononuclear cells

To test their potential usefulness as therapeutic drugs, we also evaluated the toxicity of the pteridine derivatives in human PBMC from three different healthy donors. As shown in Table 4, all of the potent anti-proliferative compounds

indicated above (**1a**, **1b**, **1g**, **1j**, and **1k**) did not show significant levels of cytotoxicity after 24-h exposure in human PBMC. Our data suggest that these compounds may be useful as potent anti-tumor agents without presenting collateral toxicity in normal human cells.

Cell death and cell cycle analysis

To gain insight on how the tested compounds killed the tumor cells, annexin V/propidium iodide cell death assays were performed after 72 h of treatment with a 15 μM dose of the active compounds **1a**, **1b**, **1g**, **1j**, and **1k**. The results of these assays demonstrated that the tested compounds induced late apoptosis, also considered as necrosis, of both HL-60 and K562 cells in all cases. Figure 2 displays representative dot-plots of these results, showing how both cell types were double-stained with annexin V and propidium iodide, which means cell death via late apoptosis/necrosis. Our results are in part similar to those of other authors who have shown the same type of cell death with different pteridine derivatives (Chiu et al. 2014; Sun et al. 2016), although, differently to those studies, in which tumor cells were arrested either in phase G2/M (Chiu et al. 2014) or in phase G0/G1 (Sun et al. 2016), and differently to reference compound MTX, which arrests HL-60 and K562 cells in phases subG0 and G0/G1, this new series of compounds does not significantly affect the cell cycle of these two leukemia cell lines (Fig. 3).

Conclusions

Our results have demonstrated the anti-tumor potential of five out of the 12 pteridine derivatives analyzed herein, compounds **1a**, **1b**, **1g**, **1j,** and **1k**, which present a remarkable cytotoxic activity against undifferentiated HL-60 and K562 cells growing at an exponential rate, without affecting the viability of human peripheral blood mononuclear cells or differentiated cells. Some of these anti-proliferative compounds were able to achieve 100% cytotoxicity even at sub-maximal doses, and in all cases the active compounds showed low IC_{50} values at the longer treatment of 72 h. Finally, we also demonstrated that these anti-proliferative pteridine derivatives caused cell death by late apoptosis/necrosis with no significant effect on the cell cycle.

Author contributions AJRA conceived and designed the experimental plan, performed experiments, analyzed the data, and wrote the manuscript. VCM performed experiments, analyzed the data and wrote the manuscript. AG synthesized and provided the new organic compounds and wrote the manuscript. JG analyzed data. MME wrote the manuscript. PGP provided the necessary facilities and wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards The ethics committees (Comité Ético de Investigación Clínica del Hospital Universitario Virgen de la Arrixaca, and Comité de Bioética de la Universidad de Murcia) approved the study protocol according to the 1975 Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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