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Original article

11a-*N*-Tosyl-5-deoxi-pterocarpan (LQB-223), a promising prototype for targeting MDR leukemia cell lines

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ABSTRACT

Aza-deoxi-pterocarpans (**1**) were synthesized through palladium-catalyzed aza-arylation of dihydronaphtalen, and showed antineoplastic effect on MDR leukemic cell lines (K562, Lucena-1 and FEPS). Compounds **1c**–**d** were prepared to identify the pharmacophoric group responsible for the activity as well as compounds **2a**–**c** were prepared to evaluate the structural requirements in the D-ring. LQB-223 (**1b**) is the most promising antileukemic agent since it was the most active on MDR cells without detectable toxicity to normal immune system cells.

1. Introduction

During the course of our studies aiming for the discovery of new pterocarpan-based antineoplastic compounds [1–6], we reported the synthesis of aza-pterocarpan **1a** and its deoxi-analogue **1b** (Fig. 1) [7].

These compounds presented antineoplastic activity on leukemic cell lines (K562 and HL-60) as well as colon cancer (HCT-8), glioblastoma (SF-295) and melanoma (MDA-MB435) and **1b** was shown to be the most potent [7].

As the development of multidrug resistance (MDR) is a major problem in cancer chemotherapy, we decided to extend the study of **1b** to human leukemia cell lines with MDR phenotype and evaluate its mechanism of action. We also present some structure–activity relationships (SAR) studies aiming to identify the pharmacophoric groups in this new class of compounds. Since sulfonamide moiety is present in the structure of several bioactive compounds [8–13], our first goal was to modify the substituent at the nitrogen atom in the C-ring (compounds **1c**,**d**) to see if this moiety is part of the pharmacophore. In analogues **2a**,**b**, halogen atoms at D-ring were introduced in order to modify the partition coefficient. In addition, halogen bonds occur in many biological systems, being employed by nature for the molecular recognition. These bonds may target diverse set of relevant proteins due to the short halogen···N/O/S contacts in the binding pocket [14]. Compound **2c** was planned to increase the cytotoxicity, since compounds containing the nitro group may generate reactive oxygen species through the action of nitroreductases [15,16].

2. Results and discussion

2.1. Chemistry

2-Iodoanilines **5a**,**b** were prepared by the reaction of 2-iodoaniline **3a** with tosyl chloride **4a** or mesyl chloride **4b**, respectively, in pyridine (condition *i*) while the reaction of **3a** with benzylchloroformate (**4c**) in basic media (condition *ii*) or acetic anhydride (**4d**) in acid media (condition *iii*) led to 2-iodoanilines **5c**,**d** (Table 1). Finally, **5e** was prepared by reaction between **3b** and **4a**. All these products were obtained in good to excellent yields.

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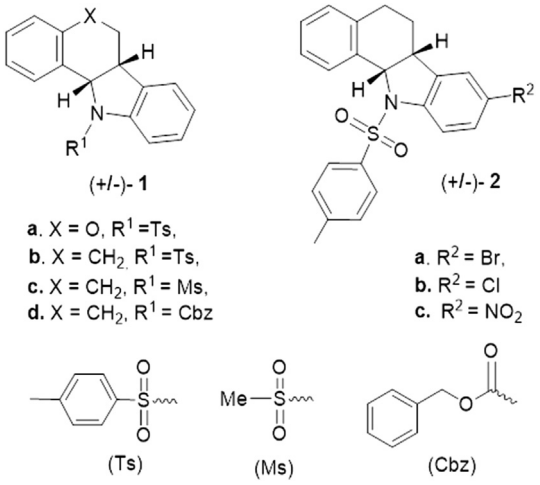
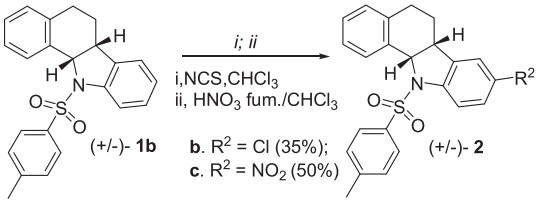


Fig. 1. Aza-deoxi-pterocarpans **1** and **2**.

As previously described [7], compound **1b** was prepared in good yield by a palladium-catalyzed aza-arylation of dihydronaphthalen (**6**) with *N*-tosyl-*o*-iodoanilin (**5a**), as shown in Table 1. Under thermal conditions the reaction took 4 h to completion leading to **1b** in 85% yield. It was also possible to accomplish the aza-arylation of **6** with aniline derivatives **5b**,**c** and **5e**, leading to aza-deoxi-pterocarpans **1c**–**d** and **2a** in reasonable to good yields. In contrast, a low yield of **1e** was obtained when **6** was allowed to react with *N*-acetyl *orto*-iodoanilin (**5d**).

To prepare compounds **2b** and **2c** we took advantage of the great reactivity of the D-ring in **1b** over the A-ring for electrophilic substitution reaction. These compounds were obtained from **1b** by halogenation with NCS in chloroform solution, or by nitration with fuming nitric acid, respectively. As expected, these reactions were chemoselective for D-ring (Scheme 1).



Scheme 1. Halogenation and nitration of **1b**.

2.2. Evaluation of biological activity

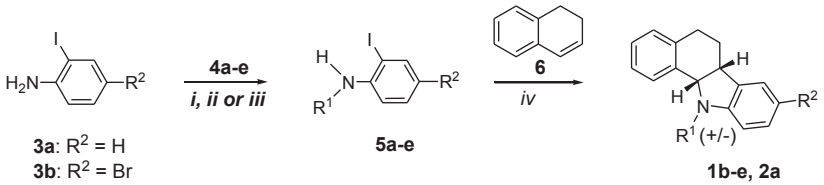
2.2.1. Anticancer activity

The antineoplastic effect of the new aza-deoxi-pterocarpans was evaluated on two human erythroleukemic cell lines with MDR phenotype, Lucena-1 [17] and FEPS [18] and compared with the results obtained with the parental K562. The latter is derived from a patient with chronic myeloid leukemia and known to be resistant against oxidative stress damage due to its high expression of glutathione and catalase [19,4]. Lucena-1 and FEPS, both derived from K562 as described [17,18], among other mechanisms stand out for their higher expression and activity of ABCB1 and ABCC1 proteins, responsible for the MDR phenotype [20]. The cytotoxic effect of the six compounds is summarized on Table 2. The results obtained showed that for K562 and Lucena-1 the presence of the tosyl group at the nitrogen atom is essential for the antineoplastic effect while the substitution at the D-ring led to a decrease in potency, being **1b** 4–25-fold more potent than the other compounds. Despite the fact that FEPS is described as more resistant to chemotherapeutic agents such as vincristine (VCR), daunorubicin (DNR), cisplatin and even imatinib mesylate [18], the IC_{50} obtained for all compounds was consistently lower in this cell line when compared with K562 and Lucena-1. Compound **1b** was also in this case 2–10-fold more potent than the other compounds. These results suggest that the new synthetic aza-deoxi-pterocarpans exert cytotoxic activity independent of the presence of the MDR proteins ABCB1 and ABCC1.

2.2.2. DNA incorporation

Since **1b** was the most active compound evaluated, it was selected for further tests. In order to evaluate if this compound could lead to inhibition of cellular proliferation, we incubated K562, Lucena-1 and FEPS with 1.25, 2.5 and 5.0 μM of this compound for 24–72 h and measured DNA duplication by incorporation of [^{3}H]-thymidine after 6 h. As seen in Fig. 2, both K562 and Lucena-1 showed a dose and time-dependent inhibition in [^{3}H]-thymidine incorporation. After 24 h of exposure to 2.5 μM of **1b** proliferation of all three cell lines was inhibited in nearly 25%, and the sub-toxic

Table 1
Synthesis of compounds **1b-d** and **2a**.



i. TsCl(**4a**) ou MsCl (**4b**), pyridine, 80^{0}C, 12h
ii. ClCbZ (**4c**), NaOH, t.a, 1h
iii. Ac_2O (**4d**), H_2SO_4, t.a, 5 min.
iv. acetone, 10 mol% $Pd(OAc)_2$, 3 equiv. Ag_2CO_3, 70^{0}C, 4 hours

R^1	R^2	Compound	Yield (%)	R^1	R^2	Compound	Yield (%)
Ts	H	**5a**	75	Ts	H	**1b**	85
Ms	H	**5b**	70	Ms	H	**1c**	45
Cbz	H	**5c**	89	CbZ	H	**1d**	67
Ac	H	**5d**	85	Ac	H	**1e**	10
Ts	Br	**5e**	67	Ts	Br	**2a**	75

Table 2
Effect of the aza-deoxi-pterocarpans on the growth of human leukemic cells.

Compound	K562	Lucena-1	FEPS
1b	2.90 ± 0.65	2.49 ± 0.14	2.12 ± 0.73
1c	40.64 ± 16.12	27.03 ± 10.90	18.07 ± 11.98
1d	>50	>50	20.65 ± 5.05
2a	16.60 ± 1.79	14.33 ± 9.93	6.76 ± 3.36
2b	28.65 ± 6.79	34.77 ± 5.50	15.42 ± 6.13
2c	12.91 ± 1.15	23.23 ± 3.68	4.20 ± 1.21

Results are reported as IC_{50} values ± SD (concentration required to inhibit cell growth by 50%) in μM. Data represent means of three independent experiments, with each concentration tested in triplicate. Assays were performed as described in the Experimental Section.

concentration of 1.25 μM was not able to induce the same effect even after 72 h. When cells were incubated with a greater concentration than the IC_{50} inhibition was as high as 50% after 24 h. FEPS cells presented a different pattern, as inhibition after 48 h seems higher than in 72 h. Since the doubling time of these cells is roughly 24 h [18], viable cells at 48 h could still be incorporating [^{3}H]-thymidine after 72 h, leading to this discrepancy. Taken together, data suggests that the mechanism of action exerted by **1b** involves impairment of DNA duplication, at least in K562 and Lucena-1 cells.

2.2.3. Cell cycle analysis

Furthermore, our next step was to find out if this inhibition of nucleotide incorporation would lead to disruption of the cell cycle. To test this hypothesis, we cultured K562, Lucena-1 and FEPS within the same conditions of the previous experiment, and analyzed the percentage of cells in each phase of the cell cycle by flow cytometry. In Fig. 3A, it can be observed that K562 and its MDR counterpart Lucena-1 presented similar behavior after 24 h of incubation with 2.5 μM of **1b**, consistent with the inhibition of cell proliferation previously observed. Both cells were arrested in the G2 phase of the cycle (G2/M K562 = 31.24%; G2/M Lucena-1 = 44.02%) and it was maintained up to 48 h in Lucena-1. As seen in Fig. 3B, FEPS cells exhibited a similar pattern, but in a timely fashion; only after 48 h of incubation cells accumulated in G2 phase (G2/M FEPS = 33.89%). This could be explained by the lengthier cell cycle demonstrated by FEPS when compared to K562 and Lucena-1 [18]. After 72 h massive DNA fragmentation was observed (Fig. 3C–H K562 = 64.73%; H Lucena-1 = 34.79%; H FEPS = 55.69%), indicative of apoptotic cell death. These results corroborate previous findings of our group, when three hybrid pterocarpanquinones bearing similarities with **1b** on the C and D-rings, were described as capable of inducing apoptosis in both K562 and Lucena-1 cells after 72 h [3,21].

2.2.4. Toxicity against normal lymphocytes

For a drug to be successfully used in the clinic toxicity to normal cells should be minimal; hence, selectivity of a newly synthesized compound should be assessed with care. In order to investigate if **1b** could sensitize normal cells, we collected a population of splenocytes from healthy Swiss mice and cultured these cells in presence or absence of 5 μg/mL of ConA, a lectin known for its ability to induce proliferation in immune system cells [22]. As shown in Fig. 4, viability of normal lymphocytes incubated with 10 μM **1b** was higher than 90%, regardless of their activation by ConA (PI+ = 3.67%; PI + ConA = 7.69%). Different types of immune system cells can be obtained from the spleen; mainly T and B lymphocytes, but also macrophages and dendritic cells [23,24]. Thus, the low toxicity against *ex vivo* cultured lymphocytes is indicative of a lower toxicity against the immune system.

3. Conclusions

In conclusion, the newly synthesized aza-deoxi-pterocarpans exhibited antineoplastic effect depending on the pattern of substitution at the nitrogen in C-ring and at the D-ring. Our data suggests the importance of the tosyl group (a sulfonamide), since the cytotoxic effect is dramatically reduced upon removal of tosyl group from the structure (compounds **1c** and **1d**). In other *N*-tosyl derivatives of **1b** substituted at the D-ring (**2a**–**c**), IC_{50} values were lower when electron withdrawing groups such as the halogens chlorine and bromine and a nitro group presented at D ring. The most active compound, **1b**, exerts its cytotoxicity by inhibition of DNA duplication independent of MDR phenotype. Furthermore, **1b** seems to lead the three cell lines to accumulate at the G2 phase of the cycle. Interestingly, FEPS cells are only affected later than K562 and Lucena-1. According to data obtained this arrest precedes the apoptotic cell death, since DNA fragmentation was observed after 72 h. Finally, **1b** demonstrated a selective antitumoral activity, without reducing the viability of immune system cells. Since multidrug resistance is the most important cause of therapeutic failure, our data suggest that **1b** could be a candidate for treatment of unresponsive leukemias with a lower chance of side effects.

4. Experimental section

4.1. Chemistry

Melting points were determined with a Thomas–Hoover apparatus and are uncorrected. Column chromatography was performed on silica gel 230–400 mesh (Aldrich). ^{1}H NMR spectrum was recorded on a Bruker Avance 400 (400.013 MHz) spectrometer at room temperature. All *J* values are given in Hz. Chemical shifts are expressed in parts per million downfield shift from

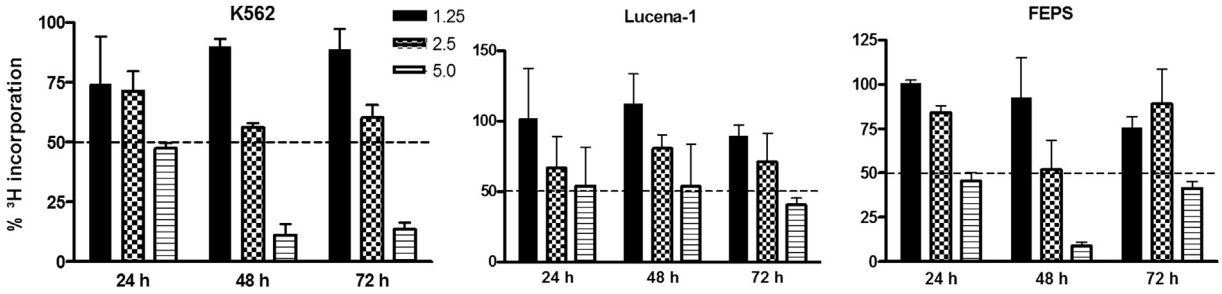


Fig. 2. Inhibitory effect of **1b** on cellular proliferation. Proliferation was measured by incorporation of [^{3}H]-thymidine into the DNA. After 24–72 h cultured with 1.25, 2.5 and 5.0 μM of **1b**, 0.5 μCi of [^{3}H]-thymidine was added to each cell line. Proliferation of control cells was considered as 100%. Bars represent means + SD of three independent experiments, with each concentration tested in triplicate.

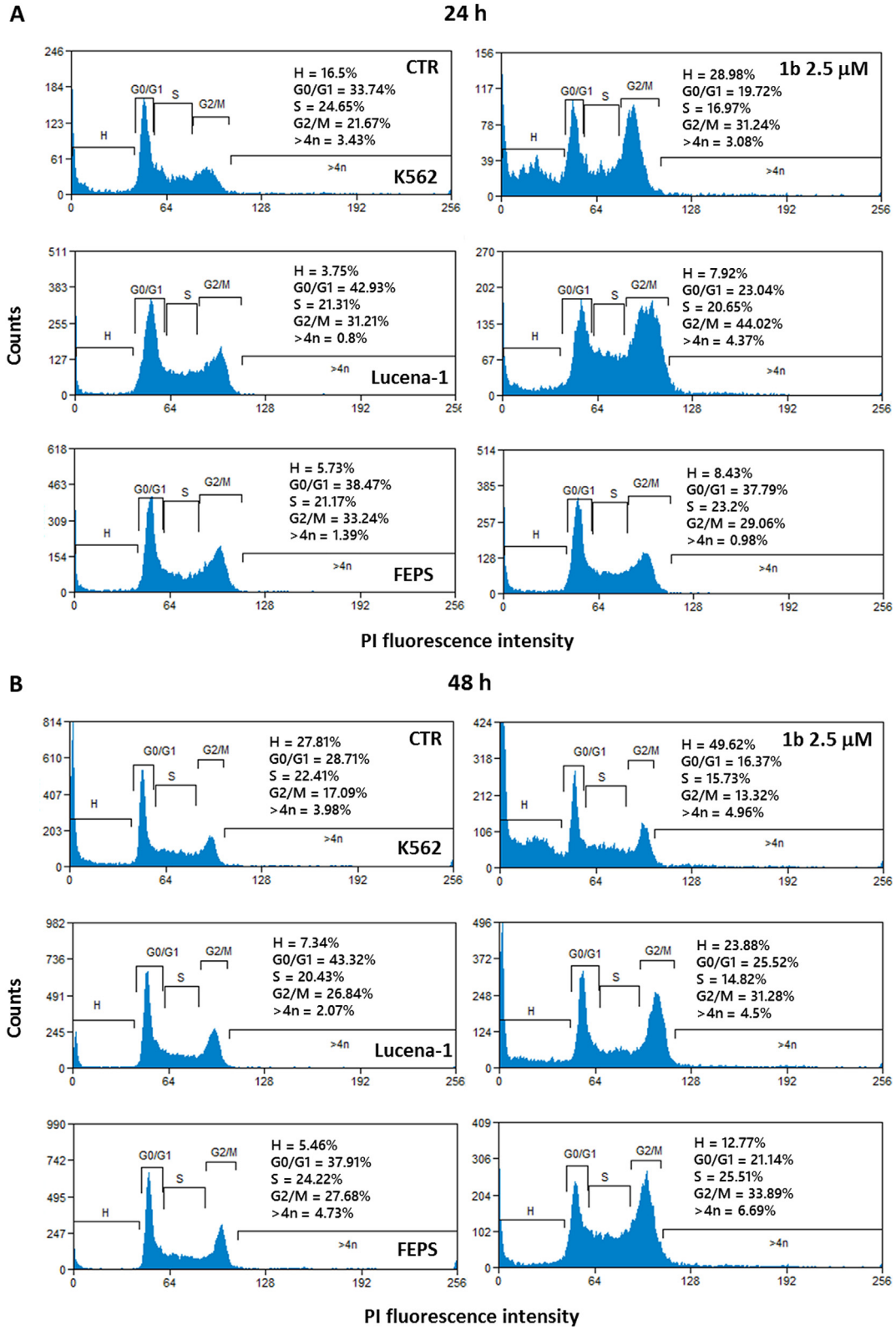


Fig. 3. Effect of compound **1b** on the cell cycle distribution of human leukemic cell lines. Cells were treated with 2.5 μM of **1b** for 24, 48 or 72 h, then stained with PI to analyze DNA content by flow cytometry. (**A**), cells incubated for 24 h; (**B**), incubated for 48 h; (**C**), incubated for 72 h. Histograms representative of three independent experiments; H, hypodiploid cells (apoptotic); G0/G1, S, G2/M, phases of the cell cycle; >4n, cell doublets. Numbers represent the percentage of cellular population in each phase of the cycle.

tetramethylsilane as an internal standard, and reported as position (δ_H) (relative integral, multiplicity (s = singlet, d = doublet, dd = double doublet, dt = double triplet, m = multiplet)), coupling constant (*J* Hz) and assignment. ^{13}C NMR spectrum was recorded on a Bruker Avance 400 (100.003 MHz) spectrometer at room temperature with complete proton decoupling. Data are expressed in parts per million downfield shift from tetramethylsilane as an internal standard and reported as position (δ_C).

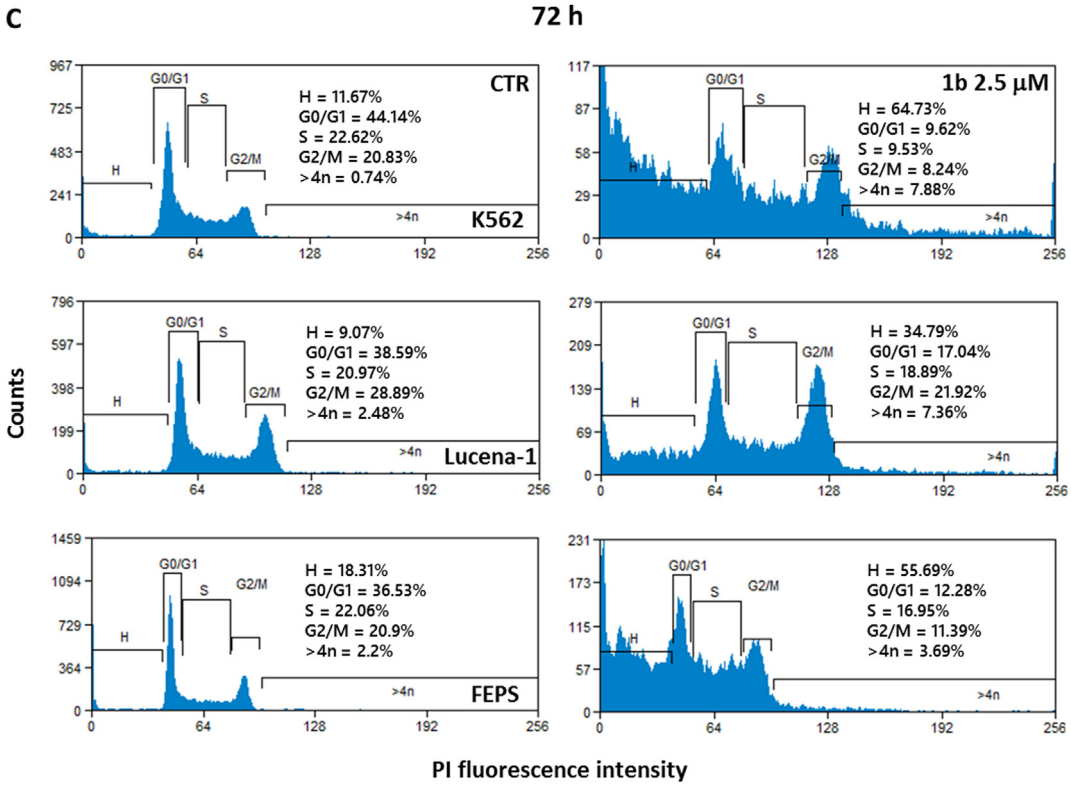


Fig. 3. (*continued*).

4.2. Preparation of aryl-sulfonamides

A solution of aryl-sulphonyl chloride (6.5 mmol) and 2-iodo-aniline (5 mmol) in 1 mL of pyridine was stirred overnight at 80 °C. The mixture was concentrated under reduced pressure and the products were purified either by recrystallization from ethanol followed hexane or by column chromatography (hexane/ethyl acetate).

4.2.1. Substance ***5a***

After recrystallization from ethanol, followed by washing several times with hexane, the compound was obtained as a white solid in 70% yield, mp 88–90 °C ^{1}H NMR (400 Mz, $CDCl_3$) δ (ppm): 7.66–7.62 (4H, m); 7.32–7.26 (1H, m); 7.21 (1H, d, $J = 8.1$ Hz); 6.84–6.81 (2H, m) LRMS (EI) *m/z* 218, 91.

4.2.2. Substance ***5b***

After column chromatography using *n*-hexane/ethyl acetate (90:10) as eluent, this compound was obtained as a white solid in 70%, mp 90–92 °C ^{1}H (400 MHz, $CDCl_3$) δ (ppm): 7.82 (1H, d, $J = 9.3$ Hz); 7.63 (2H, d, $J = 6.7$ Hz); 7.38 (1H, d, $J = 7.3$ Hz); 6.93 (1H, t, $J = 7.7$ Hz); 3.02 (3H, m); LRMS (EI) *m/z* 297, 218.

4.2.3. Substance ***5e***

After column chromatography using *n* hexane/ethyl acetate (95:5) as eluant, this compound was obtained as a white solid in

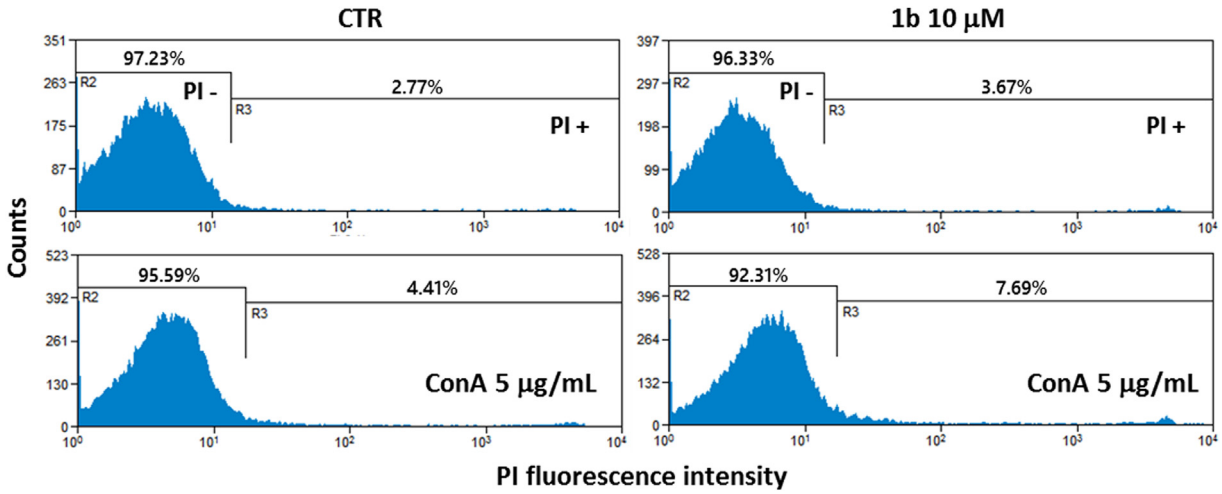


Fig. 4. Effect of compound **1b** on the viability of murine lymphocytes. Splenocytes were exposed to 10 µM of **1b** for 72 h, in presence or absence of 5 µg/mL of ConA. Cells were collected on a flow cytometer, and the region corresponding to the lymphocytes was gated. Fluorescence of PI was measured, and PI-positive lymphocytes were considered non-viable. Histograms representative of three independent experiments. Numbers represent the percentage of viable and non-viable lymphocytes.

67%, mp 92–94 °C ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.78 (1H, d, $J = 2.0$ Hz); 7.62 (2H, d, $J = 8.2$ Hz); 7.53 (1H, d, $J = 8.7$ Hz); 7.43 (1H, dd, $J = 8.7$ and 2.0 Hz); 7.24 (2H, d, $J = 8.2$ Hz); 2.40 (3H, s) LRMS (EI) *m/z* 451, 296.

4.2.4. Substance **5c**

To 2-iodoaniline (756 mg, 7.46 mmol) was added an aqueous sodium hydroxide solution (3.5 mL, 186 mmol). The resulting suspension was stirred vigorously as benzylchloroformate (0.75 mL, 3.47 mmol) was added slowly from an addition funnel at room temperature. The progress of the reaction was followed by loss of starring by TLC. When 2-iodoaniline was completely consumed (30 min), the reaction mixture was poured into ethyl acetate. The ethyl acetate layer was separated and the aqueous layers were extracted two times with ethyl acetate (50 mL). The organic layer was combined, dried over Na_2SO_4, filtered, and concentrated under pressure. After column chromatography using *n*-hexane/ethyl acetate (95:5) as eluent, this compound was obtained as a white solid in 92%, mp 58–60 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.07 (1H, d, $J = 8.2$ Hz); 7.75 (1H, dd, $J = 7.9$ and 1.5 Hz); 7.44–7.31 (6H, m); 6.80 (1H, dt, $J = 7.9$ and 1.5 Hz) LRMS (EI) *m/z* 353, 91.

4.2.5. Substance **5d**

One drop of concentrated H_2SO_4 was added to a stirred solution of 2-iodoaniline (100 mg; 0.46 mmol) in acetic anhydride (100 mL). The resulting mixture was stirred at room temperature for 5 min and then quenched with water and extracted with ethyl acetate (3×5 mL). The combined organic layers were washed with water, brine and dried with anhydrous sodium sulfate. The solvent was removed and the crude product was crystallized from ethanol to give the *N*-acetyl derivative as a crystalline solid in 85% yield, mp: 102–104 °C. ^{1}H RMN ($CDCl_3$, 400 MHz): δ (ppm) 8.21 (1H, d, $J = 8.2$ Hz); 7.78 (1H, d, $J = 7.9$); 7.34 (1H, t, $J = 7.9$ Hz); 6.84 (1H, t, $J = 8.2$ Hz); 2.23 (3H,s) LRMS (EI) *m/z* 219, 134.

4.3. Aza-arylation reactions: synthesis of **1b–d** and **2a**

To a stirred solution of **6** (0.5 mmol), tosyl-2-iodoaniline **5a** or **5e** (0.75 mmol), mesyl-2-iodo-aniline **5b** (0.75 mmol), Cbz-2-iodoaniline **5c** (0.75 mmol) or Acetyl-2iodoaniline **5d** (0.75 mmol), in acetone (5 mL), silver-carbonate (1.5 mmol) and $Pd(OAc)_2$ (0.005 mmol) were added. The reaction mixture was refluxed for 4 h and filtered in celite with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The crude product was washed in *n*-hexane and purified by flash chromatography on silica.

4.3.1. Substance **1b**

After column chromatography using n-hexane/ethyl acetate (1:99) as eluant, the compound was obtained as a yellow solid in 85% yield; mp 160–165 °C; IR (neat) 3060, 2923, 1349, 1165 cm^{-1} ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm): 8.0 (1H, d, $J = 7.8$ Hz); 7.61 (1H, d, $J = 7.9$ Hz); 7.51 (1H, d, $J = 8.1$ Hz); 7.29–7.09 (7H, m); 7.04 (1H, d, $J = 7.0$ Hz); 6.93 (1H, d, $J = 7.5$ Hz); 5.42 (1H, d, $J = 8.5$ Hz); 3.13–3.11 (1H, m); 2.54–2.45 (2H, m); 2.37 (3H, m); 2.13–2.09 (1H, m); 2.06–1.98(1H,m); ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm): 143.8 (C); 142.1(C); 137.6 (C); 136.2 (C); 135.6 (C); 134.4 (C); 30.5 (CH); 129.6 (CH); 129.6 (CH); 127.9 (CH); 127.8 (CH); 127.3(CH); 127.1(CH); 127.1 (CH); 126.8 (CH); 125.7 (CH); 123.4 (CH); 119.9 (CH); 64.0 (CH); 39.5 (CH); 24.8 (CH_2); 23.6 (CH_2); 21.5 (CH_3). HRMS calc for $C_{23}H_{21}NO_2S$ $[M+Na]^{+:}$ 398.1185 Found 396.1191.

4.3.2. Substance **1c**

After column chromatography using *n*-hexane/ethyl acetate (95:5) as eluent, the compound was obtained as a white solid in 40%, mp 136–138 °C; IR (neat) 2953, 2928, 1341, 1155 cm^{-1} ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.84 (1H, d, $J = 7.7$ Hz); 7.41 (1H, d, $J = 7.2$ Hz); 7.25–7.13 (5H, m), 6.98 (1H, d, $J = 7.4$ Hz), 5.55 (1H, d, $J = 8.9$ Hz), 4.09–4.05 (1H, m), 2.83 (3H, s), 2.66–2.53 (2H, m), 2.34–2.30 (1H, m), 2.25–2.19 (1H, m) ^{13}C NMR (100 MHz, $CDCl_3$): 141.98 (C); 138.07 (C); 135.03 (C); 134.33 (C); 130.50 (CH); 128.24 (CH); 128.11 (CH); 127.54 (CH); 126.87 (CH); 125.47 (CH); 123.96 (CH); 118.21 (CH); 64.36 (CH); 40.32 (CH); 37.36 (CH_3); 25.24 (CH_2); 24.69 (CH_2) HRMS calc for $C_{17}H_{17}NSO_2$ $[M+Na]^+$: 322.0872 Found: 322.0876.

4.3.3. Substance **1d**

After column chromatography using n-hexane/ethyl acetate (95:5) as eluant, the compound was obtained as a white solid in 63%, mp 112–115 °C; IR (neat) 3038, 1709 cm^{-1} ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.45–7.43 (2H, m); 7.39–7.33 (5H, m); 7.17–7.13 (5H, m); 7.02–6.97 (2H, m); 5.73 (1H, s broad); 5.34 (2H, s); 3.96–3.94 (1H, m); 2.69–2.61 (1H, m); 2.54 (1H, t, $J = 4.3$ Hz); 2.50 (1H, t, $J = 4.3$ Hz); 2.20–2.15 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 154.0 (C); 138.8 (C); 138.6 (C); 136.1 (C); 135.6 (C); 134.0 (C); 128.6 (CH); 128.6 (CH); 128.6 (CH); 128.4 (CH); 128.2 (CH); 128.1 (CH); 127.6 (CH); 127.2 (CH); 126.5 (CH); 123.5 (CH); 123.4 (CH); 67.7 (CH_2); 61.3 (CH); 39.7 (CH); 38.5 (CH); 25.9 (CH_2); 24.8 (CH_2) HRMS calc for $C_{24}H_{21}NO_2$: 378.1465 $[M+Na]^+$ Found: 378.1450.

4.3.4. Substance **2a**

After column chromatography using *n*-hexane/ethyl acetate (98:2) as eluant, the compound was obtained as a white solid in 79%, mp 186–188 °C ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.95 (1H, d, $J = 7.8$ Hz); 7.52 (2H, d, $J = 8.0$ Hz); 7.48 (1H, d, $J = 8.5$ Hz); 7.32–7.27 (2H, m); 7.20 (2H, d, $J = 7.9$ Hz); 7.17–7.13 (2H, m); 6.94 (1H, d, $J = 7.5$ Hz); 5.41 (1H, d, $J = 8.6$ Hz); 3.11–3.07 (1H, m); 2.57–2.46 (2H, m); 2.39 (3H, s); 2.09–1.97 (2H, m) ^{13}C NMR (100 MHz, $CDCl_3$): 144.3(C), 141.5(C), 138.7(C), 137.3(C), 135.5(C), 133.9 (C), 130.9 (CH); 130.4 (CH), 129.8 (CH), 128.1 (CH), 127.6 (CH), 127.1 (CH), 126.9 (CH), 126.6 (CH), 64.3 (CH), 39.7 (CH), 24.8 (CH_2), 23.7 (CH_2), 21.7 (CH_3) HRMS calc for $C_{23}H_{20}BrNO_2S$: 476.0290 $[M+Na]^+$ Found:476.0275.

4.3.5. Substance **2b**

After column chromatography using *n*-hexane/ethyl acetate (99:1) as eluent, the compound was obtained as a white solid in 35%, mp 176–180 °C ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.96 (1H, d, $J = 7.8$ Hz); 7.54–7.51 (3H, m); 7.29–7.27 (1H, m); 7.20–7.13 (5H, m); 6.94 (1H, d, $J = 7.5$ Hz); 5.41 (1H, d, $J = 8.6$ Hz); 3.11–3.07 (1H, m); 2.56–2.46 (2H, m); 2.38 (3H, s); 2.09–1.96 (2H, m) ^{13}C NMR (100 MHz, $CDCl_3$): 144.2(C), 140.9(C), 138.3(C), 137.3(C), 135.3(C), 133.9 (C), 131.2 (C); 130.4 (CH), 129.7 (CH), 128.1 (CH), 128.0(CH); 127.6 (CH), 127.1 (CH), 126.9 (CH), 123.7 (CH), 120.9 (CH); 64.3 (CH), 39.5 (CH), 24.9 (CH_2), 23.2 (CH_2), 21.7 (CH_3) HRMS calc for C_{23} H_{20} ClN O_2S: $[M+Na]^+$ 432.0795 Found: 432.0788.

4.3.6. Substance **2c**

After filtration in celite with ethyl acetate, the product was obtained in 50% yield as a yellow solid, mp 190–192 °C ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.12 (1H, dd, $J = 8.8$ and 2.3 Hz); 7.96 (1H, d, $J = 7.8$ Hz); 7.92 (1H, s); 7.70 (1H, d, $J = 8.8$ Hz); 7.55 (1H, d, $J = 8.2$ Hz); 7.31–7.16 (5H, m); 6.97 (1H, d, $J = 7.6$ Hz); 5.56 (1H, d, $J = 8.9$ Hz); 3.34–3.28 (1H, m); 2.55–2.53 (2H, m); 2.39 (3H, s); 2.13–2.09 (1H, m); ^{13}C NMR (100 MHz, $CDCl_3$): 147.9(C); 145.5(C); 144,8(C); 137.6(C), 137.3(C); 135.5(C); 133.2(C); 130.4(CH); 129.9(CH); 128.3(CH); 128.0(CH); 127.2(CH); 126.8(CH); 124.6(CH); 119.5(CH); 118.8(CH); 65.2(CH); 39.6(CH); 24.9(CH_2); 23.8(CH_2); 21.5(CH_3) HRMS calc for $C_{22}H_{19}N_2NaSO_4$: 443.1036 $[M+Na^+]$ Found: 443.1034.

4.4. Cell lines

The human erythroleukemic cell lines K562 and its multidrug resistant (MDR) variants Lucena-1 and FEPS were all maintained in RPMI-1640 medium, supplemented with 50 μM β-mercaptoethanol, 25 mM HEPES, pH adjusted to 7.4 with NaOH, 60 mg/L penicillin, 100 mg/L streptomycin (all obtained from Sigma Chemical Co., Saint Louis, USA) and 10% fetal bovine serum (FBS) (Cultilab, São Paulo, Brazil), inactivated at 56 °C for 1 h. β-Mercaptoethanol was added due to its capacity of reducing reactive oxygen species and protective effect against toxic metabolites produced by cultured cells, thus improving the microenvironment and promoting *in vitro* cell growth [28]. Lucena-1 and FEPS cells were developed in our laboratory by continuous exposure of K562 cells to increasing concentrations of the cytotoxic drugs vincristine sulfate (VCR) (Sigma) and daunorubicin hydrochloride (DNR) (Sigma), as described before [17,18]. 60 nM VCR and 500 nM DNR were added to Lucena-1 and FEPS cultures respectively in order to maintain the MDR phenotype. All cells were passaged at a concentration of 2×10^4 cells/mL every three days and kept at 37 °C in a 5% CO_2 humidified environment. Before the experiments, vincristine and daunorubicin were removed from the cultures and cell lines were resuspended in medium with fetal bovine serum. The cell lines Lucena-1 and FEPS were kindly donated by Dr. Vivian M. Rumjanek.

4.5. Cell treatment

Leukemic cell lines were exposed to the different azapterocarpans in culture for 72 h. Stock solutions of 25 mM dissolved in DMSO were prepared, and further dilutions were made in RPMI-1640 medium. Briefly, 2×10^4 cells/mL in 200 μL were seeded in 96-well microtiter plates in drug-free medium or in medium containing different concentrations of each compound and maintained for 72 h at 37 °C in an atmosphere of 5% CO_2, and cell viability was then measured.

4.6. Cell viability

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [25–27]. MTT can be reduced by dehydrogenases present in active mitochondria of living cells. After incubation in presence or absence of the compounds being tested, 20 μL of MTT (5 mg/mL) (Sigma) was added to each well. Plates were then kept at 37 °C in 5% CO_2 for 3 h. After centrifugation at 200 × *g*, 200 μL of DMSO (Sigma) was added to all wells in order to dissolve the dark blue crystals formed by MTT reduction. Absorbance was measured with a Tecan Sunrise ELISA reader at 490 nm (Tecan Group, Switzerland). Absorbance was directly proportional to the amount of formazan (reduction product) present, indicating the percentage of living cells. The IC_{50} values were obtained by nonlinear regression on the GraphPad Prism v4.0 program (GraphPad Software, San Diego, USA).

4.7. Cell proliferation assay

Cell proliferation was assessed by incorporation of [^{3}H]-thymidine into the DNA of cells incubated with **1b**. After different incubation periods (24–72 h) with **1b**, 0.5 μCi of [^{3}H]-thymidine diluted in RPMI-1640 was added to the cell cultures and the radioactivity was measured in a Perkin–Elmer Tri-Carb 2810TR liquid scintillation counter (Perkin–Elmer Inc., Waltham, USA) after 6 h.

4.8. Cell cycle assay

The cell cycle assay was performed by quantifying cells in each phase of the cycle after 24, 48 and 72 h in presence or absence of **1b**. Cells were maintained in culture at a concentration of 2×10^4 cells/mL in 24-well plates with RPMI-1640 supplemented with 10% FCS and kept in 5% CO_2 atmosphere at 37 °C, for periods aforementioned. Cell concentrations were adjusted to 10^5 cells per well, washed with Hank's balanced salt solution (HBSS) (Sigma), and resuspended in 250 μL of HBSS and 50 μL of a 5× concentrated cell cycle solution containing 50 μg/mL propidium iodide (PI), 1 mg/mL RNAse and 0.2% Triton X-100 (all obtained from Sigma). Subsequently, cells were incubated at room temperature for 15 min and analyzed by flow cytometry. This assay was performed to analyze cell cycle profile and assess DNA fragmentation, which was quantified by the percentage of cells in sub-G0/G1 phase.

4.9. Animals

Swiss mice were bred in the animal facilities at the Instituto de Bioquímica Médica Leopoldo de Meis from UFRJ (IBqM-UFRJ), and housed under standard laboratory conditions (20–25 °C, 12-h light regimen) with free access to water and standard chow (*ad libitum*). Two-month female mice were used in experiments. Procedures were approved by the Centro de Ciências da Saúde Ethics Comitee for Animal Use (CEUA-CCS, UFRJ) under the protocol number IBQM082.

4.10. Isolation of splenocytes

Mice were anaesthetized with ethyl ether (Reagen, Rio de Janeiro, Brazil) and euthanized by cervical dislocation. Spleens were surgically removed and macerated with the help of a sterile rubber pressed through a nylon mesh, and fresh cell suspension was homogenized in 3 mL cold RPMI-1640 medium supplemented with 5% FCS. Afterwards, cell suspension was washed and further diluted with cold PBS, incubated with 0.08% Trypan Blue dye (Sigma) and counted in an optical microscope.

4.11. Ex vivo viability assessment of splenocytes

Viability of the normal splenocytes was measured by quantifying the percentage of PI-positive cells after 72 h exposure to **1b**. Cells were maintained in culture at a concentration of 5×10^5 viable cells/mL in 24-well plates with RPMI-1640 supplemented with 10% FCS and kept in 5% CO_2 atmosphere at 37 °C. 5 μg/mL concanavalin A (ConA) (Sigma) was added to cultures in order to stimulate cell proliferation. Cell concentration was adjusted to 10^5 cells per well, washed with PBS supplemented with 5% FCS (Sigma), and resuspended in 300 μL of PBS containing 50 μg/mL propidium iodide (PI). Subsequently, cells were incubated at room temperature for 15 min and analyzed by flow cytometry.

4.12. Flow cytometry analysis

Ten thousand cells were examined under each condition using a FACSCalibur flow cytometer (Becton, Dickinson and Company, San Jose, USA). Cells were acquired based on forward and side scatter parameters, representative of cell size and granulosity. The region corresponding to the lymphocytes was gated, and fluorescence of PI was measured. PI-positive lymphocytes were considered non-viable. All analysis was performed using the Summit v4.3 software (Dako Colorado, Inc., Fort Collins, USA).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.039. These data include MOL files and InChiKeys of the most important compounds described in this article.

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