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In vitro apoptotic activity of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones in L5178Y cells

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Abstract

Compounds containing B–N bonds have shown interesting biological activity. One class of such molecules is the 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones (**3a**–**j**), which contain a B–N bond, have an α-amino acid moiety in the heterocycle, and have an exocyclic moiety related to an amino acid. The purpose of this work was to determine the inhibitory effects of **3a**–**j** on the proliferation of murine L5178Y lymphoma cells. A new five-membered heterocyclic nucleus with apoptotic activity was found. The target products showed potent cytotoxicity in the L5178Y cell line. Among them, **3a** exhibited the highest antineoplastic activity in L5178Y cells with an IC_{50} value of 22.5±0.2 μM.

Keywords: Apoptosis; Diphenyloxazaborolidinones; Cytotoxicity; Antineoplastic; L5178Y cell

Introduction

Cell growth and division are highly regulated, although a notable exception is provided by the cancer cell, which arises as a variant that has lost the usual proliferation control pathways. Consequently, there is growing interest in the search for anti-cancer substances with high efficacy, low toxicity, and minimum side effects (Wang et al., 2005). In particular, certain compounds containing B–N bonds possess broad biological activity including insecticidal, fungicidal, herbicidal, antibacterial and calcium channel blockers (Morin, 1994; Dembitsky and Srebnik, 2003; Jabbour et al., 2004; Dobrydneva et al., 2006) and antineoplastic activities. Recently, the proteosome inhibitor PS341, an α-amido-boronic acid, was approved as an effective antineoplastic agent (Adams et al., 1999; Teicher et al., 1999). Several aminoboron compounds have been used in boron neutron capture therapy (BNCT) as well as in chemotherapeutic forms of cancer treatment (Soloway et al., 1998).

Cell growth is regulated by the balance between cell proliferation and apoptosis. Deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression (Evan and Vousden, 2001). It is well known that the regulating mechanisms of apoptosis are extremely complex, but it is also known that the apoptotic process varies, depending on whether the cells involved are tumoral or healthy (Evan and Vousden, 2001; Sanmartín et al., 2005). Apoptosis results from activation of the genes of the cell, which in turn activate preprogrammed biochemical pathways leading to cell death (Fischer, 1994; Wright et al., 1994; Jacobson et al., 1997). These pathways are all closely related to the mitochondria alteration that induces changes in electron transport and loss of mitochondrial transmembrane potential (Ming-Jie et al., 2004; Shakibaei et al., 2005). After such an alteration, there is a redistribution of phosphatidylserine in the extracellular membrane which is an early and common phenomenon in the process of cell apoptosis (Yuan et al., 2006). Apoptotic cells are also characterized by cell shrinkage, skeletal disruption, membrane blebbing, chromatin condensation and ordered cleavage of DNA (Bursh et al., 1992). Another important event is the G_0/G_1 phase arrest, which is a crucial

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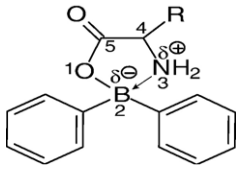


Fig. 1. General structure of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones **3a**–**j**.

DNA damage checkpoint and acts as an important safeguard for genomic stability (Chen et al., 2004; Deng et al., 2004). Apoptotic pathways might be significantly altered in cancer cells with respect to untransformed cells, and these differences may represent a therapeutic window that can be explored for the development of new antineoplastic drugs. There are reports in the literature of the cytoprotector effects of glycine and its nitrogen derivatives, evaluated in different models of damage related to the accumulation of free radicals (Tripathi et al., 2000; Yan-Jun et al., 2000; Ascher et al., 2001; Jacob et al., 2003; Tanonaka et al., 2002). Substantial data supports the existence of a glycine receptor in the membrane of the cell, whose activation reduces the release of reactive oxygen species (ROS), thus protecting the cell from damage by the latter (Yan-Jun et al., 2000; Jacob et al., 2003). This, in turn, maintains the mitochondrial integrity. Recent data indicated that, when either the amino or the carboxylic hydroxyl group in glycine is modified the cytoprotective effect is maintained; whereas, if both groups are modified, the cytoprotective effect is lost. In the latter case, specifically in glioma C6 cells, such compounds induce cell death by apoptosis, as demonstrated by in vitro assays (Trejo-Solís et al., 2005).

With the aim further investigating glycine-related compounds, we studied the apoptotic activity of a set of ten amino acid derivatives, **3a**–**j**. Two important elements were taken into account when devising these experiments: 1) the B–N bond contained in these compounds has shown biological activity and 2) the glycine fragment has activity as an apoptotic inducer when both the amino and carboxylic hydroxyl groups have been modified. Thus, a family of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones (DPOXB) was generated in which both functional groups were modified, forming five member heterocyclic compounds (Fig. 1). These compounds, which all contain a B–N bond, were obtained via the reaction of diphenylborinic acid with the corresponding α-amino acid (Fig. 2) rather than via the previously reported methods (Chremos et al., 1961; Flückiger et al., 1984; Farfán et al., 1993; Trujillo et al., 1998). The compounds (**3a**–**j**) were assayed using several different apoptotic indicator models, such as the inhibitory effect on cell growth measured by the MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide] assay, phosphatidylserine membrane externalization measured by Annexin V, and DNA distribution (sub-G_0/G_1 peak) measured by PI staining, the last two using flow cytometry. We employed DMD with positive control (Kosower et al., 1969).

Materials and methods

*Synthesis of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones (**3a**–**j**)*

In a typical procedure, 0.5 g (6.6 mmol) of glycine was dissolved in 3 mL H_2O and the pH was adjusted to 8 with 5% NaOH. Diphenylborinic acid was prepared from 1.6 g of 2-aminoethyldiphenylborinate this was dissolved in a minimum amount of ethanol, hydrolyzed by addition of 1 M hydrochloric acid with agitation, and the gummy, water-insoluble diphenylborinic acid is extracted with ether. The ethereal solution was added in to the glycine solution. The mixture was refluxed for 4 h, then the solvent was slowly evaporated and the white product was filtered and washed with cold water and hexane to obtain 1.5 g (6.26 mmol) of **3a**. The same methodology was employed to obtain compounds **3b**–**j**, using the appropriate α-amino acid. All the compounds obtained by this new methodology were characterized by the following methods: the ^{1}H-, and ^{13}C-NMR spectra were determined on a JEOL GSX-270 instrument in DMSO-d_6 using TMS as an internal reference, the ^{11}B-NMR spectra were obtained on a JEOL FX90Q spectrometer in DMSO-d_6 using BF3.OEt2 as an external reference. The IR spectra were measured on a MSERIES spectrophotometer using KBr. The physical and spectroscopic constants were found to be equivalent to those in the literature from the derivatives generated by the method previously employed. (Farfán et al., 1993; Trujillo et al., 1998).

Cell line and culture maintenance

For the experiments, 2×10^6 L5178Y tumor cells (ATCC) were implanted into the peritoneal cavity of 8- to 10-week-old BALB/C mice. On day 12 after tumor implantation, the mice

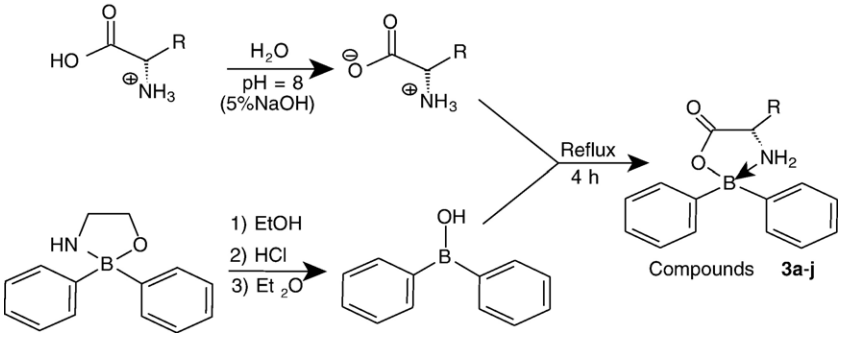
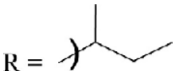
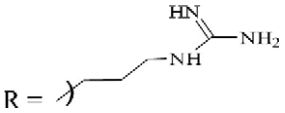
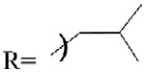
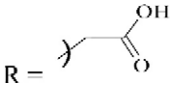
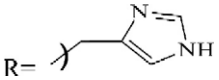
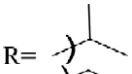
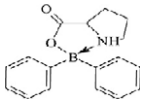
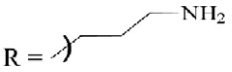


Fig. 2. Synthesis of 2,2-Diphenyl-1,3,2-oxazaborolidin-5-ones amino acid derivatives.

Table 1
Production of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones **3a**–**3j**[a]

DPOXB	α-aa employed	R	m. p. °C	% Yield
3a	Gly	R=H	241–243	94
3b	Ile		221–223	93
3c	Arg		244–246	89
3d	Leu		171–174	90
3e	Asp		273–275	65
3f	His		275–277	92
3g	Val		243–245	80
3h	Ser		259–260	93
3i	Pro		268–269	98
3j	Orn		213–215	90

[a]All the products were characterized by their spectroscopical data (^{1}H NMR, ^{13}C NMR, ^{11}B NMR, EIMS, IR).

were euthanized and the tumors were excised under sterile conditions and washed three times with PBS 1X. The cells were maintained in an RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 1% non-essential amino acids at 37 °C under a 5% CO_2 atmosphere with 95% humidity.

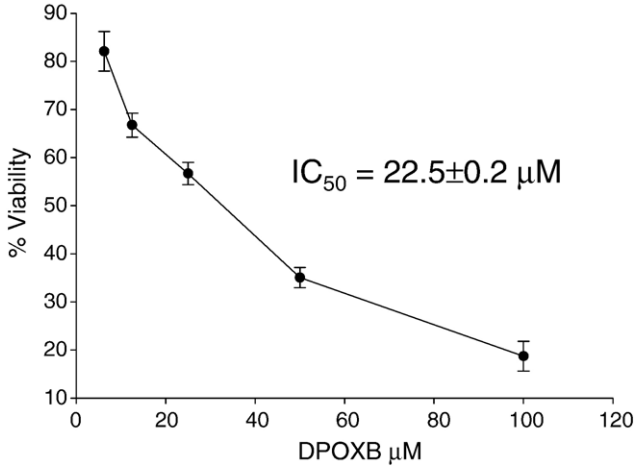


Fig. 3. Effect of compound **3a** on L5178Y viability. The MTT assay was performed at 24 h, as described in the Materials and methods section. Data are represented as the mean±SD, $n=3$.

Table 2
Cytotoxicity assay against murine L5178Y lymphoma cells

DPOXB	IC_{50} (μM)[a] L5178Y
3a	22.5±0.2
3b	42.7±1.8
3c	30.9±1.1
3d	47.8±1.4
3e	102.7±1.9
3f	>200
3g	38.6±0.6
3h	48.7±0.4
3i	75.1±2.5
3j	44.5±0.9

[a] Viable L5178Y cells were determined using the MTT assay, as described previously. The cells were incubated with **3a**–**j** (6.25–100 μM) for 24 h. Control cells were incubated with DMSO. The results are presented as the mean±SD of data from at least three independent experiments.

Determination of cytotoxicity in murine L5178Y lymphoma cells using the MTT assay

Antiproliferative in vitro screening was performed on the murine L5178Y lymphoma cells. Cytotoxicity was assessed by the MTT assay. The L5178Y cells were suspended at a final concentration of 5×10^4 /mL, seeded in 96-well microtiter plates and treated with concentrations ranging from 6.25 to 100 μM of one of the DPOXB **3a**–**j** dissolved in DMSO. The final concentration of solvent was less than 0.1% in cell culture medium. Incubation was carried out at 37 °C for 24 h after exposure to one of the **3a**–**j** compounds, the cells were incubated with MTT (0.5 mg/mL) for 4 h. MTT is reduced by the mitochondrial dehydrogenases of viable cells to a purple formazan product. The MTT/formazan product was dissolved in 100 μL of DMSO and the corresponding absorbance, at 560 nm, was measured with a microplate reader. The concentrations required in order to inhibit cell growth by 50% (IC_{50}) were calculated.

Flow cytometry analysis for DNA distribution

Cell suspensions (1×10^6) were pelleted, resuspended in 500 μL of phosphate-buffered saline (PBS) and fixed for at least

Table 3
Apoptosis induced by DPOXB **3a**–**j** in L5178Y cells

DPOXB	Percentage of cells Annexin V-FITC/PI$^+$
3a	17.7±1.7*
3b	7.1±2.1
3c	10.8±1.9
3d	8.3±2.6
3e	6.9±1.2
3f	9.0±2.3
3g	12.3±1.1
3h	9.4±1.7
3i	6.4±2.1
3j	10.9±0.8
DMD	38.7±1.8
DMSO Control	6.1±1.3

Cells were treated with 100 μM of DPOXB **3a**–**j** for 24 h. Annexin V-FITC/PI double stained of cells was analyzed by flow cytometry. The percentages of cells were calculated by CELL Quest Pro software. (mean±S.D., $n=3$) * $p\leq0.05$.

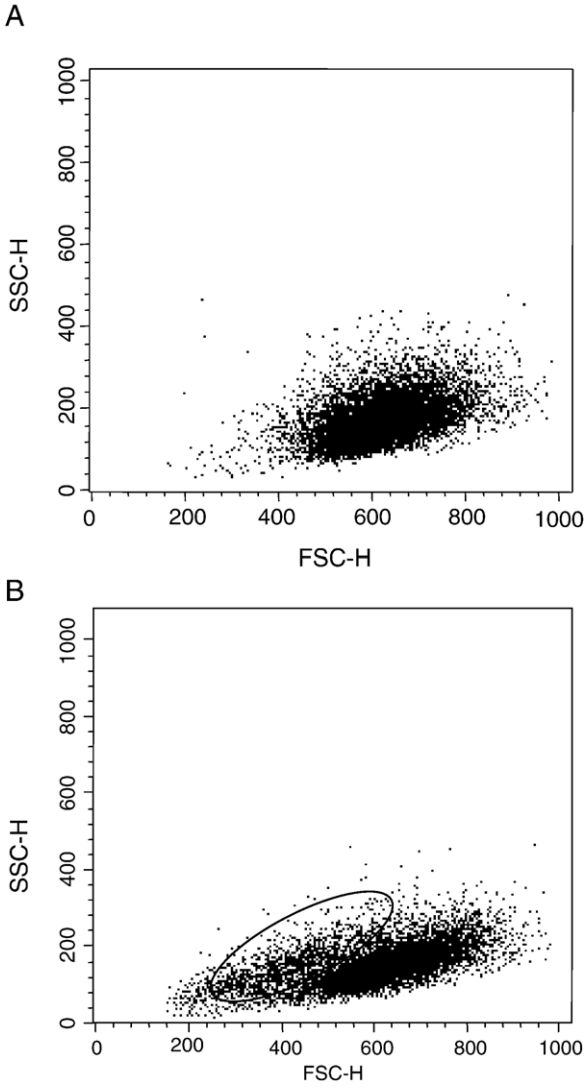


Fig. 4. Effect of **3a** on the cellular volume. FSC vs SSC dot plot of L5178Y cells incubated for 24 h: A) untreated and B) treated with **3a** 100 μM. One representative experiment out of three is shown. The cells indicated in the circle are those with reduced cellular volume.

24 h at −20 °C in 70% ethanol. Fixed cells were washed twice with PBS, stained with 300 μL of 20 μg/mL PI (Sigma), and incubated for at least 30 min at room temperature. The stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) for relative DNA distribution. We employed DMD with positive control (100 μM).

Flow cytometry analysis of apoptosis

Apoptosis was quantified by measuring phosphatidylserine externalization (considered an early marker of apoptosis), using a kit from CALTAG (Laboratories Burlingame, USA) and according to the manufacturer's instructions. Briefly, the cells were treated with 100 μM of one of the DPOXB **3a**–**j** and stained with Annexin-V-FITC. We employed DMD with positive control. Apoptosis was determined in a FACScalibur flow cytometer. At least 10,000 events were acquired and analyzed using CELL Quest Pro software.

Statistical analysis

All the values shown are expressed as the mean ± SD of three independent determinations. The statistical significance of the data was assessed using the Kruskal–Wallis test, and p values ≤ 0.05 were considered significant.

Results

*2,2-diphenyl-1,3,2-oxazaborolidin-5-ones (**3a**–**j**)*

The glycine derivatives were prepared using a new methodology as described in Materials and methods (Table 1). The glycine derivatives with substituents R in the fourth positions were prepared by reacting commercially available α-amino acids and diphenylborinic acid under alkaline conditions. The overall synthetic method is outlined in Fig. 2.

Inhibition of cell growth

The cytotoxic effect of the 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones was first evaluated in the murine L5178Y lymphoma cells. Treatment with 6.25–100 μM of each of the compounds **3a**–**j** caused a concentration-dependent decrease in cell viability. These results demonstrated that **3a**–**j** exhibited cytotoxic effects on the L5178Y cell line tested. The best effect was achieved for compound **3a** with $IC_{50} = 22.5 \pm 0.2$ μM (Fig. 3), while compounds **3b**, **3c**, **3d**, **3g**, **3h**, **3i** and **3j** showed from good to moderate IC_{50} values. Compound **3f** showed a markedly lower activity $IC_{50} > 200$ μM (Table 2) in relation to the other compounds.

Phosphatidylserine externalization

L5178Y cells were exposed to compounds **3a–j** at 100 μM and the pro-apoptotic effect was analyzed in order to evaluate externalization of phosphatidylserine, which is an early event in the apoptotic process. Cells were double stained with Annexin V-FITC and PI and measured by flow cytometry (Table 3). **3a**

Table 4
Effect of 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones on the cell cycle in L5178Y cells at 24 h

DPOXB	% G_0/G_1	% S	%G_2/M	% Sub G_0/G_1
3a	39.85	31.33	7.54	21.27±4.2*
3b	49.38	31.70	8.01	10.92±2.9
3c	52.54	31.93	8.29	7.24±2.4
3d	49.05	32.03	10.93	7.99±2.6
3e	59.87	30.13	6.01	3.99±1.3
3f	55.60	32.52	5.75	6.12±1.1
3g	49.34	34.46	9.10	7.10±2.2
3h	49.26	31.58	9.88	9.28±1.4
3i	60.41	31.83	4.13	3.60±1.2
3j	56.31	30.99	7.12	5.58±1.3
DMD	23.43	27.24	7.30	42.03±2.3
DMSO Control	61.86	30.19	3.60	4.35±2.1

Cells were incubated with 100 μM DFB for 24 h, then fixed and stained with PI as described. The stained cells were analyzed by flow cytometry. The percentages of cells were calculated by CELL Quest Pro software. (mean ± S.D., $n = 3$). Results from one representative experiment representing 10,000 events are shown. *$p \leq 0.05$.

turned out to be the most effective molecule, inducing 17.7± 1.7% apoptotic cells after 24 h of treatment at 100 μM (Fig. 5). The cell shrinkage characteristic of apoptotic cells was also evident in the FSC vs SSC dot plots generated by **3a**. This cell shrinkage was observed as a reduction in the FSC, as seen in Fig. 4A and B.

DNA distribution

The induction of apoptosis was also analyzed in PI-stained cells by flow cytometry. The appearance of the sub-G_0/G_1 phase indicates that the compound tested induced apoptosis in the cells assayed. This technique detects DNA fragmentation, which represents a very late stage of apoptosis. L5178Y cells were treated with 100 μM DPOXB **3a-j** for 24 h, then stained with PI and analyzed by flow cytometry (Table 4) in order to quantify the DNA in the sub-G_0/G_1 cell cycle phase (Fig. 6). The 2,2-diphenyl-1,3,2-oxazaborolidin-5-one obtained from glycine (**3a**) induced the greatest increase in L5178Y cells in the sub-G_0/G_1 cell cycle phase. **3a** induced 21.2±4.2% apoptotic cells while for the control cells only 4.35±2.1% were observed to be apoptotic.

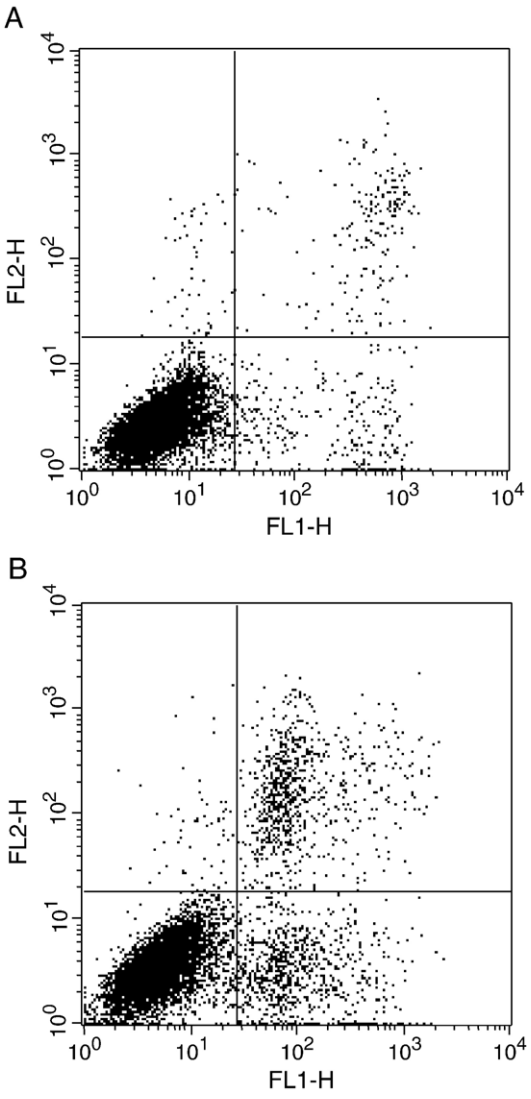


Fig. 5. Apoptotic induction by **3a**. FL1-H vs FL2-H dot-plot of L5178Y cells incubated 24 h and stained with Annexin-FITC and PI after treatment with: A) untreated and B) treated with **3a** 100 μM.

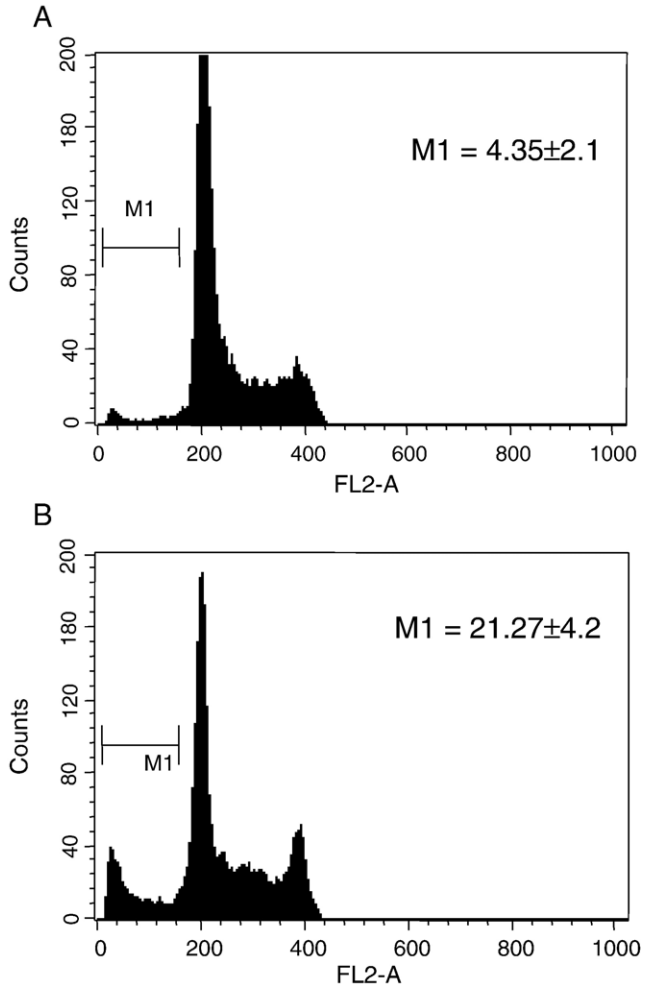


Fig. 6. Effect of **3a** on DNA distribution. DNA histograms of L5178Y cells: A) untreated and B) treated with **3a** 100 μM. Results are expressed as a percentage of the sub G_0/G_1.

Discussion

The induction of apoptosis, or programmed cell death, is thought to be one of the most interesting therapeutic strategies to specifically target cancer cells. Until now chemo- and radiotherapy treatments have not been selective for tumor cells, making the development of new therapeutic agents necessary. Apoptosis is a programmed cell death that involves genetically controlled morphological and biochemical events, including phosphatidylserine externalization, cytochrome *c* leakage from the mitochondria, caspase activation, reduction of cellular and nuclear volume, chromatin condensation and internucleosomal DNA fragmentation (Schultz and Harrington, 2003). Apoptotic cells exhibit some morphological modifications that are readily detected by flow cytometry according to their light scattering properties (FSC/SCC) (Lecouer et al., 1997). Thus by using this

technique, a series of ten 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones was evaluated in murine L5178Y lymphoma cells. Our results are consistent with those reported previously by Trejo-Solís et al. (2005), who modified both the amino and carboxylic hydroxyl groups of glycine, which was then used to treat glioma C6 cells and resulted in cell death by apoptosis.

The cytotoxic effect induced in the murine L5178Y lymphoma cells by compound **3a** (2,2-diphenyl-1,3,2-oxazaborolidin-5-one) is highly potent, when compared with the other amino acid derivatives, as measured by the MTT assay (Table 2). According to our hypothesis, the heterocyclic ring is the active moiety in inducing apoptosis when both the amino and carboxylic hydroxyl groups are modified. The results of this study show that the modification of the fourth position of this ring, by inserting a heterocyclic indole ring or any of the R-groups (Table 1), clearly decreased the observed effect in comparison with the lead compound (**3a**) (Table 2).

In spite of the fact that **3a** was more active than the other nine amino acid derivatives (Fig. 3), the latter showed a significant concentration dependent growth inhibition of L5178Y cells. In order to find out if the cells died due to apoptosis or necrosis, the pro-apoptotic effect of DPOXB was evaluated in L5178Y cells exposed to **3a**–**j** at 100 μM. Apoptosis was quantified by flow cytometry using Annexin V to evaluate the flip-flop of phosphatidylserine (an indicator that the cell is entering the apoptotic process), which showed that cell death caused by **3a** was by apoptosis and not by necrosis (Fig. 5B). Phosphatidylserine externalization was exhibited by 6.1 ± 1.3% of the control cells, whereas 17.7 ± 1.7% ($p \leq 0.05$) of cells treated with **3a** showed the same event in the apoptotic process (Table 3). Cell shrinkage characteristic of apoptotic cells was also evident in the FSC vs SSC dot plots generated by **3a**. This cell shrinkage was observed as a reduction in the FSC (Fig. 4B). To confirm that the induction of cell death was by apoptosis, the DNA distribution was evaluated in cells treated with each of the tested compounds at a concentration of 100 μM for 24 h and stained with PI as described in the Materials and methods section. This technique detects the fragmentation of DNA, which occurs in the later part of the apoptotic process. The presence of the sub-G_0/G_1 peak in the histogram of the DNA distribution indicates that a population of cells is dying by apoptosis (Fig. 5B). Particularly important is that 21.2 ± 4.2% ($p \leq 0.018$) of the cells died by apoptosis when treated with **3a** as opposed to 4.35 ± 2.1% when the cells were untreated. When the cells were incubated with compounds **3b**–**j**, the difference in the percentage of apoptosis with respect to the control was not significant ($p \geq 0.05$, Table 4), whereas with **3a** the difference was clearly significant (Table 4). A reduction in cell volume was also observed with this technique, and it was possible to establish that the cells with a reduced FSC were the ones that appear in the sub-G_0/G_1 peak (Fig. 6B). In conclusion, as indicated in the literature, glycine and its N-derivatives show cyto-protective effects. Our results confirm that the modification of both the amino and carboxylic hydroxyl groups gives rise to apoptotic inducing compounds, unlike the effect of glycine, which acts as an apoptotic protector. Our study points out the importance of glycine derivatives as apoptotic inducers, inhibiting the growth of murine L5178Y lymphoma cells (as measured by the MTT assay), externalizing phosphatidylserine (as measured by Annexin V), and inducing the sub-G_0/G_1 peak (as measured by PI staining). The mechanism of action of this compound is now being evaluated in our lab and we will report them in detail in an coming paper. At the moment we can say that this kind of compounds induced transmembrane potential reduction, caspase-3 activation and DNA fragmentation evaluated by TUNEL and DNA electrophoresis. The results described above indicate that this 2,2-diphenyl-1,3,2-oxazaborolidin-5-ones activate the intrinsic apoptotic pathway.

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