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Synthesis and Biological Evaluation of Cyclic Analogues from Nitrone LQB-278: A New Potential Antileukemia Compound

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Abstract. *Background/Aim: A new set of LQB-nitrones and analogues was synthesized to evaluate anticancer activity based on the substitution of the terpenyl moiety of the antileukemic compound LQB-278 by the conformationally restricted cinnamyl ether. Materials and Methods: A structure-activity relationship study was performed in vitro on Jurkat cells to screen the antileukemic activity of LQB-nitrones and analogues and elucidate the mechanisms of action of the most active derivatives. Results: The cynamyl ramification and its ortho position aldehyde substitution improved the antileukemic activity. Three compounds showed an in vitro antiproliferative action, but only* ***5b*** *induced apoptosis. Analysis of the molecular mechanisms showed increased expression of the cell cycle inhibitor p21CIP1/WAF1/Sdi1, caspase 3, Fas receptor, and Bax/Bcl-2 ratio. Conclusion: The cinnamyl derivative* ***5b*** *(LQB-461) presented higher antileukemic effects than the prototype terpenyl nitrone, inducing Jurkat cell death by activating both extrinsic and intrinsic pathways of apoptosis. Therefore, this compound is a new promising candidate drug against leukemia.*

In a previous work, we reported the effects of *O*-isoprenylated-*N*-methylnitrones, derived from hydroxybenzaldehydes, on different tumor cell lines, and the most effective compound was the *O*-geranylated-*N*-methylnitrone **1** (LQB-278) (1), a conjugated molecule, which presents a great conformational liberty in the terpenyl branch. Constraining the conformation of flexible molecules is a proven strategy to increase potency and selectivity of lead compounds in medicinal chemistry. A representative example of this strategy has been described by Fairlamb *et al*. (2), who suggested that unsaturated cyclic structures, like a benzene ring, could act as a conformationally restricted apolar tail in a farnesyl mimic analogue, for the selective inhibition of the protein farnesyl transferase (PFTase). In this work, we proposed to synthesize and test a new generation of potentially antineoplastic LQB-nitrones, based on the substitution of the terpenyl branch by the conformationally restricted cinnamyl ethers (Figure 1). The presence of the cinnamyl moiety in cinnamaldehyde (3), 2'-hydroxy-cinnamaldehyde (4), cinnamylhydroxyamide (5), and other cinnamyl derivatives (6, 7) has been described to attribute an anti-proliferative and apoptotic activity. Nitrones are transformed into aldehydes and hydroxyamines by cytosolic hydrolases and these compounds may have independent or synergistic biological actions (8, 9). Therefore, we also studied the pharmacologic properties of both the selected nitrones and the corresponding aldehydes. Previous studies have demonstrated the potential of benzaldehyde as an antiproliferative agent (10-12) and the anticancer effects of acetal derivatives of D-glucose or vitamin C (13-15). The antileukemia action of **1** (LQB-278) was related to proliferation inhibition, apoptosis induction, and p21 protein upregulation in T lymphocytic leukemia Jurkat cells (1). Several preclinical and clinical anticancer agents have been shown to exert their antiproliferative and pro-apoptotic effects, at least in part, by enhancing nuclear p21 expression levels, suggesting that this action can be an effective mechanism of anticancer agents (16-19). Apoptosis occurs mainly through two classic pathways, the extrinsic pathway, mediated by the binding of the death ligand (*e.g.*, Fas ligand) to the death receptor (*e.g.,* Fas) and caspase activation (20, 21), while the intrinsic one is mediated by the increased Bax/Bcl-2 ratio, release of cytochrome C, and caspase activation (16, 22). In this study, to explore the structure

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of the antileukemic compound LQB-278 and maximize its antitumor activity, a new set of compounds, derived from cyclization of the terpenyl group of this nitrone and functional groups modifications, was synthesized and a structure-activity relationship study was performed, screening their antitumor action and elucidating the related mechanisms of the most active derivatives.

Materials and Methods

Cell lines. The human tumor cell lines acute T lymphocytic leukemia (Jurkat), chronic myeloid leukemia (K562), lung carcinoma (A549), prostate adenocarcinoma (PC-3), breast adenocarcinoma (MCF-7) and the murine non-tumor fibroblast NIH-3T3 were purchased from the Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil. The cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS; Cultilab, São Paulo, Brazil), penicillin 70 mg/l and streptomycin 100 mg/l (supplemented medium), at 37˚C, and in a 5% CO_2 humidified atmosphere. The cell cultures were expanded twice a week and adherent cells were trypsinized before expansion.

Cell line treatment. For cytotoxicity screening, the tumor cells (1×10^5/ml) were treated (in 96 flat-bottom-well microplates) for 72 h with the compounds at 10 μM. The cytotoxicity of the active compounds was examined at different concentrations to calculate the IC_{50} of the compounds. The selective toxicity of the most active compound to cancer cells was determined by studying their effects on NIH-3T3 non-tumor fibroblasts, under the same culture conditions. Stock solutions of the synthetic substances were prepared with dimethyl sulfoxide 100% (DMSO) and then diluted with RPMI 1640 to 0.01% maximal concentration of DMSO in culture. Cultures treated with DMSO did not present significant difference from cultures without DMSO.

MTT cytotoxicity assay. Cell survival/cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, Sigma Chemical Co., St. Louis, MO, USA] assay (23). Briefly, 10 μl/well of a 5 mg/ml MTT stock solution was added for the last 2 h of culture. Next, 100 μl/well of 10%SDS solution containing 0.01 N HCl was added for formazan solubilization and the absorbance was measured at 570 nm (microplate reader Quant, Bio-Tek Instruments, Inc., Winooski, VT, USA). The cytotoxicity of the substances was determined as a percentage of the control cultures.

Cell proliferation assays. Jurkat cells (1×10^5/ml) were treated or not (control) with **2a**, **5a**, **5b** or Etoposide for 72 h. The viable cells were determined by the trypan blue dye exclusion assay. For the cell cycle analysis, cells (1×10^5/ml) were treated with **2a** (1, 2 and 3 μM), **5a** (1, 3 and 5 μM), **5b** (2, 3 and 5 μM) or Etoposide (0.1 μM) for 72 h in culture flasks. The cells (1×10^5) were then centrifuged at 400 × *g* for 5 min and suspended in DNA staining solution [0.3% Triton X-100 and 50 μg/ml propidium iodide (PI) in 43 mM citrate buffer]. After treatment with 50 μg/ml ribonuclease A (Sigma Chemical Co.), the PI fluorescence was determined (50,000 events per sample in FL2 channel) in a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed by the software Summit v4.3 (Dako Colorado, Inc., Fort Collins, CO, USA).

Annexin V apoptosis assay. Jurkat cells (1×10^5/ml) were treated with **2a** (1, 2 and 3 μM), **5a** (1, 3 and 5 μM), **5b** (2, 3 and 5 μM) or Etoposide (0.1 μM) for 72 h. Apoptosis was determined using Annexin-V-FITC and propidium iodide apoptosis kit (eBioscience, Inc., San Diego, CA, USA), following the manufacturer's instructions. After labeling, the cells were analyzed (50,000 events per sample in FL1 and FL3 channels) in the Gallios flow cytometer (Beckman Coulter). Dot-plot analysis of FL1 *versus* FL3 channels was performed using Summit v4.3 software. Data are presented as mean percentages of Annexin-V^+PI^- cells (early apoptotic cells) and Annexin-V^+PI^+ cells (late apoptotic cells).

Fas-receptor analysis. Jurkat cells (1×10^5/ml) were treated with **5b** (LQB-461) (2, 3 and 5 μM) or etoposide (0.1 μM) for 72 h. Afterwards, cells (5×10^5) were washed and suspended in PBS, labeled with the APC-conjugated anti-human CD95 (Fas) antibody (BioLegend, San Diego, CA, USA) for 30 min on ice. After incubation, cells were washed again, suspended in PBS (500 μl) and analyzed (25,000 per sample) in FL6 channel in the Gallios flow cytometer (Beckman Coulter). Data were analyzed using Summit v4.3 software.

p21, Bax, Bcl-2 and caspase-3 protein expression. Cells (5×10^5) were washed in PBS, treated (10 min) with FACS Lysing buffer (100 μl) at room temperature, washed again with PBS supplemented with 5% FCS and labeled with mouse anti-p21-PE (BD Pharmingen, Inc., San Diego, CA, USA) (1:20), anti-Bax (Biolegend) (1:100) or anti-Bcl-2 antibodies (Biolegend) (1:100) for 30 min on ice, protected from light. After washing in PBS-FCS, the cells were labeled with the APC-Anti-mouse IGg (1:20) for 30 min on ice, protected from light. For Caspase-3 active evaluation, cells were directly labeled with the FITC conjugated rabbit Anti-Active Caspase-3 antibody (BD Pharmingen, Inc.) (1:10). After incubation, the cells were washed, suspended in PBS and analyzed (50,000 events per sample) in the Gallios flow cytometer (Beckman Coulter). Data were analyzed using Summit v4.3 software.

Statistical analysis. Data variance was evaluated by One-way ANOVA and significant differences compared to control were accessed by the Dunnett's test, with a significance level at $p<0.05$. All statistics analysis was performed using GraphPad Prism 7.0 software (GraphPad Company, San Diego, CA, USA).

Results

Chemistry. All nitrones and analogues studied in this article are described in Figure 1. Nitrones, aldehydes, alcohols, and ester derivatives with cinnamyl side chains were prepared from commercial hydroxyaldehydes to identify pharmacophoric groups and explore the structure-activity relationship with lead compound **1** (LQB-278). The three nitrones, **2b** (LQB-460), **3** (LQB-465), and **4** (LQB-467), and four cinnamyl aldehydes, LQB-461 (**5b**), LQB-464 (**9**), LQB-466 (**6**), and LQB-468 (**7**), have not been previously described in the literature. These compounds, nitrones and aldehydes, are differentiated by the position of the cinnamyl group in the aromatic ring (*ortho*, *meta* and *para*), allowing us to evaluate the pharmacophoric group used and the substitution pattern in the action against tumor cells.

Biology. The results of the cytotoxic effects of **1** (LQB-278) derivatives are shown as IC_{50} (μM) values in Table I. It can be shown that **2a** (LQB-287), **5a** (LQB-292), and **5b** (LQB-461) had significant cytotoxic effects (IC_{50}=1.4 μM, IC_{50}=2.6 μM

and IC_{50}=2.8 μM, respectively) and that these cytotoxicities were specific for Jurkat acute lymphocytic leukemia cells, since chronic myeloid leukemia cells were not so sensitive.

Treatment of Jurkat cells with **2a**, **5a** and **5b** for 72 h also reduced the number of viable cells in a concentration-dependent manner (Figure 2A-C). Otherwise, **2a** and **5a** inhibited tumor cell proliferation without affecting cell cycle (Figure 2D-E, respectively), while **5b** increased the number of cells in Sub-G1 and reduced the number of cells in G1 and G2/M phases (Figure 2F).

Apoptosis was studied by the Annexin V-PI labeling assay. Cinnamylated-nitrone **2a** and the Cinnamylated-aldehyde **5a** did not induce significant apoptosis at any tested concentration (Figure 3A and B), confirming their low cytotoxicities, as shown by the absence of a sub-G1 phase (Figure 2D-E). However, the effect of **5b** on Jurkat cell apoptosis was concentration-dependent. Low concentrations did not induce apoptosis (Figure 3C), whereas 5 μM triggered significant apoptosis. Since only 5b induced apoptosis, we then questioned whether it was related to the augmented levels of p21CIP1/WAF1/Sdi1, Fas receptor protein expression, and caspase 3. Indeed, these effects were confirmed for the highest apoptosis-inducing concentration (Figure 3D-F). Finally, 5b showed increased Bax/Bcl-2 ratio (Table II).

Table I. *Cytotoxicity of target nitrones and analogues shown by the IC_{50} (μM).*

Entry	Compound	Jurkat	K562	NIH 3T3
1	Etoposide	0.03	-	3.6
2	**1** (LQB-278[1])	6.7	19	15.2
3	**2a** (LQB-287)	1.4	>10	9.3
4	**3** (LQB-465)	>10	>10	-
5	**4** (LQB-467)	>10	>10	-
6	LQB-291	>10	>10	-
7	**5a** (LQB-292)	2.6	>10	>10
8	**6** (LQB-466)	>10	>10	-
9	**7** (LQB-468)	>10	>10	-
10	**5b** (LQB-461)	2.8	>10	>10
11	**2b** (LQB-460)	>10	>10	-
12	**8a** (LQB-463)	>10	>10	-
13	**8b** (LQB-462)	>10	>10	-
14	**9** (LQB-464)	>10	>10	-

Cells were treated for 72 h and cytotoxicity was determined by MTT assay. (–) indicates not performed. The human acute T lymphocytic leukemia (Jurkat) and chronic myeloid leukemia (K562) cell line were studied, in addition to the non-tumor NIH-3T3 cell line. Lung carcinoma (A549), breast carcinoma (MCF-7) and prostate adenocarcinoma (PC-3) were also evaluated but showed no sensitivity to cytotoxic effects at the tested concentrations.

Discussion

The change of geranyl ether to cinnamyl in **2a** (LQB-287) increased the anti-proliferative activity and this effect was strongly dependent on the position of this group, and within the cinnamyl series only the *ortho* derivative was active (**2a**, **3** and **4,** entries 3 to 5 of Table I). The *o*-cinnamylnitrone, **2a**, showed high anti-proliferative activity on T lymphocytic leukemia Jurkat cells, with an IC_{50} of 1.4 μM, while **1** had an IC_{50} of 6.7 μM (1). This activity was not significant for the *meta* and *para* isomers **3** (LQB-465) and **4** (LQB-467), respectively [entries 4 and 5 (Table I)].

The substitution pattern of the aromatic ring (*ortho*) has already been described to be crucial for anti-leukemia effects, as demonstrated for geranyl/farnesyl-PBN derivatives (1). The higher cytotoxic activities of **2a** over **1** (LQB-278) on Jurkat cells suggest that the cinnamylated group can use a geranyl cyclic analogue in this carbon skeleton. From entries 6 to 10 in Table I, we evaluated the role of the aldehyde group as a pharmacophore analogue in the projected nitrone. LQB-291 is the aldehyde analogue of the active geranylnitrone, **1** (LQB-278) (1), entry 6, while the aldehyde analogs of the cinnamylated ethers are at entries 7 to 11 (**5a**, **6**, **7**, **5b**, **2b**). The *ortho* position proved also to be fundamental for this activity. While the *meta* and *para* isomers **6** (LQB-466) and **7** (LQB-468), respectively, were completely inactive on Jurkat cells, entries 8 and 9 (Table I), *ortho* aldehyde **5a** (LQB-292), had an IC_{50} of 2.6 μM, entry 7, less active than the nitrone **2a** (LQB-287), with an IC_{50} of 1.4 μM. This trend was not observed for nitrone **1** (LQB-278), where its aldehyde analogue (LQB-291) was inactive, entries 2 and 6. No detectable activity was observed on the other tumor cell lines tested at 10 μM.

The introduction of a nitro substituent at the *para* position in the aromatic ring of the cinnamyl fragment changes the electron density of the branch allowing us to know a little more about the structure-activity relationship of these compounds. Therefore, nitrone **2b** (LQB-460) and its aldehyde analogue **5b** (LQB-460) were evaluated in the tumor cell lines described in Table II, entries 11 and 10. Surprisingly, the nitro nitrone **2b** was inactive, whereas its analogue aldehyde **5b** showed a significant activity against Jurkat cells, without any cytotoxic activity against non-tumor fibroblast cells, entry 11 (Table II). We evaluated the possibility that the benzyl alcohols analogues act as prodrugs of the aldehydes **5a** (LQB-292) and **5b** (LQB-461), entries 12 and 13 in Table I. The importance of the stability of cinnamyl group linkage in **5a** was also studied in its ester analogue **9** (LQB-464), entry 14 (Table I). All these derivatives were inactive, which indicated the important role of the aldehyde functional group and the ether bond in the active compound **5a**. It is important to note that **5a**, **2a,** and **5b** showed high anti-proliferative activities for the Jurkat leukemia cells, and low toxicity for the non-tumor fibroblast cell line NIH-3T3 (Table I, entries 3, 7 and 11), indicating tumor specificity and good therapeutic index. Interestingly, benzaldehyde (BA) and beta-cyclodextrin-benzaldehyde (CDBA) also showed higher cytotoxicity against human tumor cell lines, compared to non-tumor cells (13).

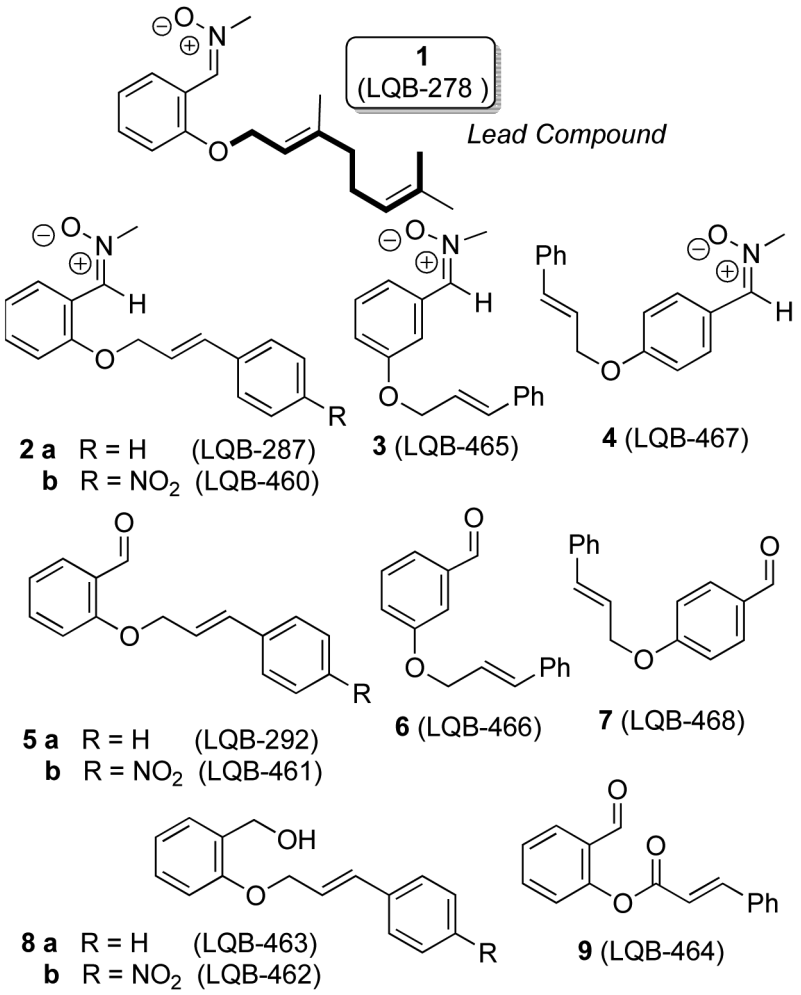

Figure 1. *Lead compound **1** (LQB-278) and compounds synthesized and evaluated as anticancer agents.*

Since the cinnamyl moiety modulates the expression of important genes related to cell proliferation and apoptosis (4-7), these activities were studied here to provide evidence for the pharmacologic value of *o*-cinnamyl-derivatives **2a, 5a,** and **5b**. The effects of **2a** and **5a** compounds on the cell number (Figure 2A-B) and cell cycle (Figure 2D-E), respectively, indicate that they exert antileukemic activity by cytostatic effects, reducing cell proliferation without cytotoxic effects (low level of Sub-G1 cells (Figure 2D-E). However, **5b** has the cinnamyl and benzaldehyde groups, like **5a**, but differently from the latter, the cinnamyl group of **5b** is nitrated and its anti-leukemia effects are concentration-dependent. It did not presented cytotoxicity at low concentrations (Figure 2F), but was cytotoxic at the highest tested concentration (increased sub-G1 phase) (Figure 2F). An anti-proliferative action and/or cytotoxicity have been reported following treatment of tumor cells with cinnamyl derivatives (4-7, 24), nitrone derivatives (1), benzaldehyde (BA), and benzaldehyde derivatives (10-15). No difference in G1, S, or G2 phases was found when tumor cells were treated with BA (15), suggesting that the BA moiety, present in **5a** (LQB-292) and **5b** (LQB-461), may contribute to the non-phase specific effects on the tumor cell cycle. The cytostatic effect can also be related to the BA group present in these compounds, since it shows cytostatic action (25). Furthermore, the apoptosis observed at the highest tested concentration of **5b**, has been described for different cinnamyl derivatives (4-7, 24). Nonetheless, only **5b** induced apoptosis, which can be related to its nitrated-cinnamyl group. Indeed, the antitumor action of the nitrated-s has been described to be different from non-nitrated ones, as demonstrated for the aromatic nitrogen mustard chlorambucil (26). As apoptosis represents an essential property of traditional anticancer compounds (17, 27-29), and only **5b** induced it (Figure 3C), this compound was selected for further studies. In response to stress signals, p53 is able to induce cell cycle arrest and cell death. Since apoptosis can be induced *via* p21CIP1/WAF1/Sdi1 through both p53-dependent and p53-

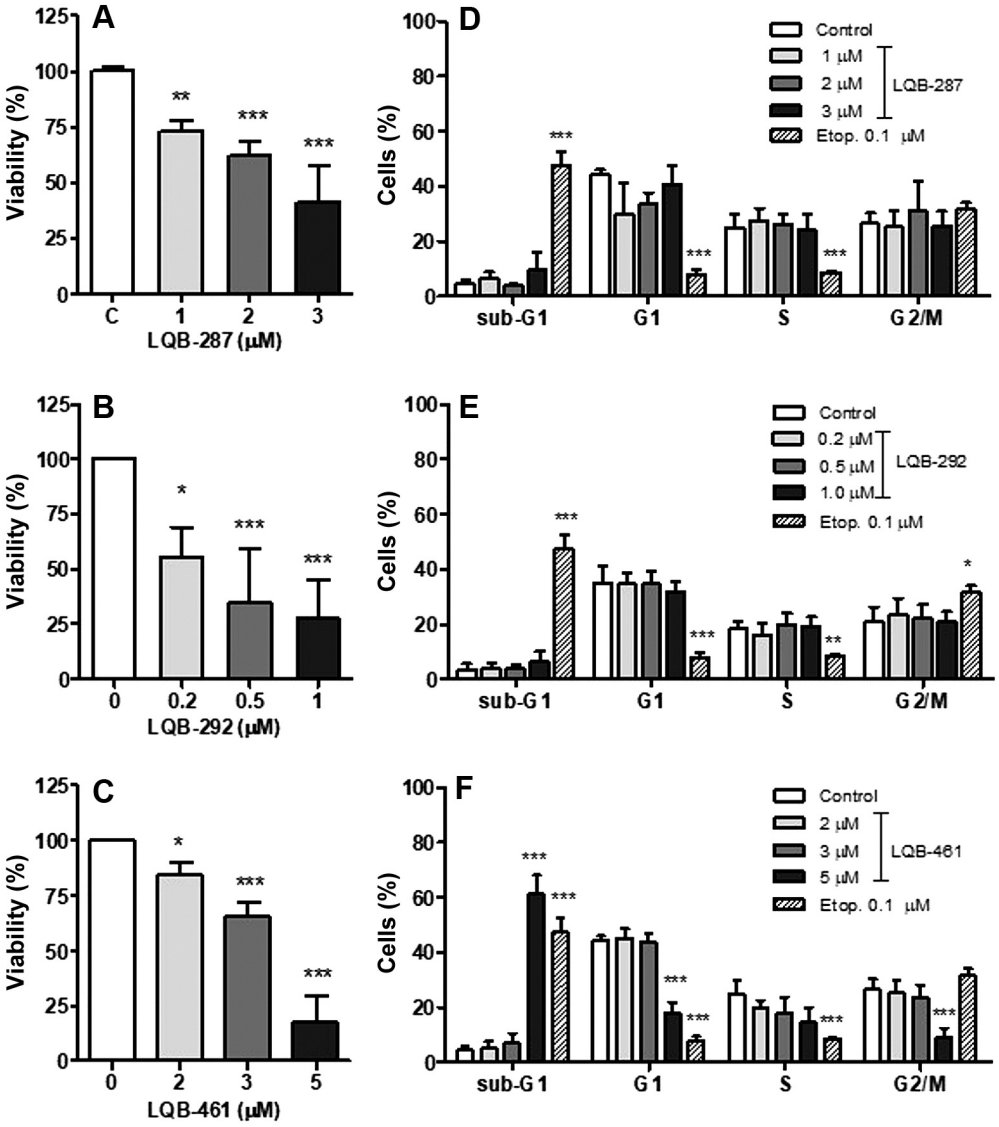

Figure 2. *Effect of **2a**, **5a** and **5b** on Jurkat cell cycle and viability. (A-C) Cell viability analysis. (D-F) Cell cycle analysis. Cells (1×10^5/ml) were treated or not (control) for 72 h with each isomer or Etoposide (Etop) at 0.1 μM. Cell viability (by the Trypan Blue exclusion assay) and cell cycle were analyzed as described in the Methods section. Data represent mean±SD of four independent experiments. *p<0.05, **p<0.01 and ***p<0.001, related to control (One-way ANOVA followed by Dunnett's test).*

independent mechanisms (30) and Jurkat cells are known to be deficient in p53 expression (1), the observed up-regulation of p21CIP1/WAF1/Sdi1 expression by **5b** (Figure 3D) represents a p53 independent pathway effect, as seen for **1** (LQB-278) (1). Considering that p53 mutations trigger resistance to chemotherapeutic agents (1), these findings make the cinnamylated derivative **5b** an excellent candidate for future therapeutic approaches in p53 mutated leukemias. As described in the literature, etoposide, used in this work as a positive control, also induces Jurkat cell cytotoxicity (31), apoptosis (32), and p21 upregulation (31). In addition, **5b** increased caspase-3 protein expression (Figure 3F), an important effector protease of both the intrinsic and extrinsic pathways of apoptosis, which is generally activated by chemotherapeutic drugs (20, 21). Increased apoptosis and caspase-3 expression following treatment with cinnamoyl and benzaldehyde derivatives in different types of cancer, including leukemia cells, have been reported (6, 33-35). Finally, increased Fas death receptor expression (Figure 3E) and increased Bax/Bcl-2 ratio (Table II) indicate that **5b** antileukemia effects are related to apoptosis activation by both the extrinsic and intrinsic pathways. Bax and Bcl-2 represent pro- and anti-apoptotic proteins, respectively (16-

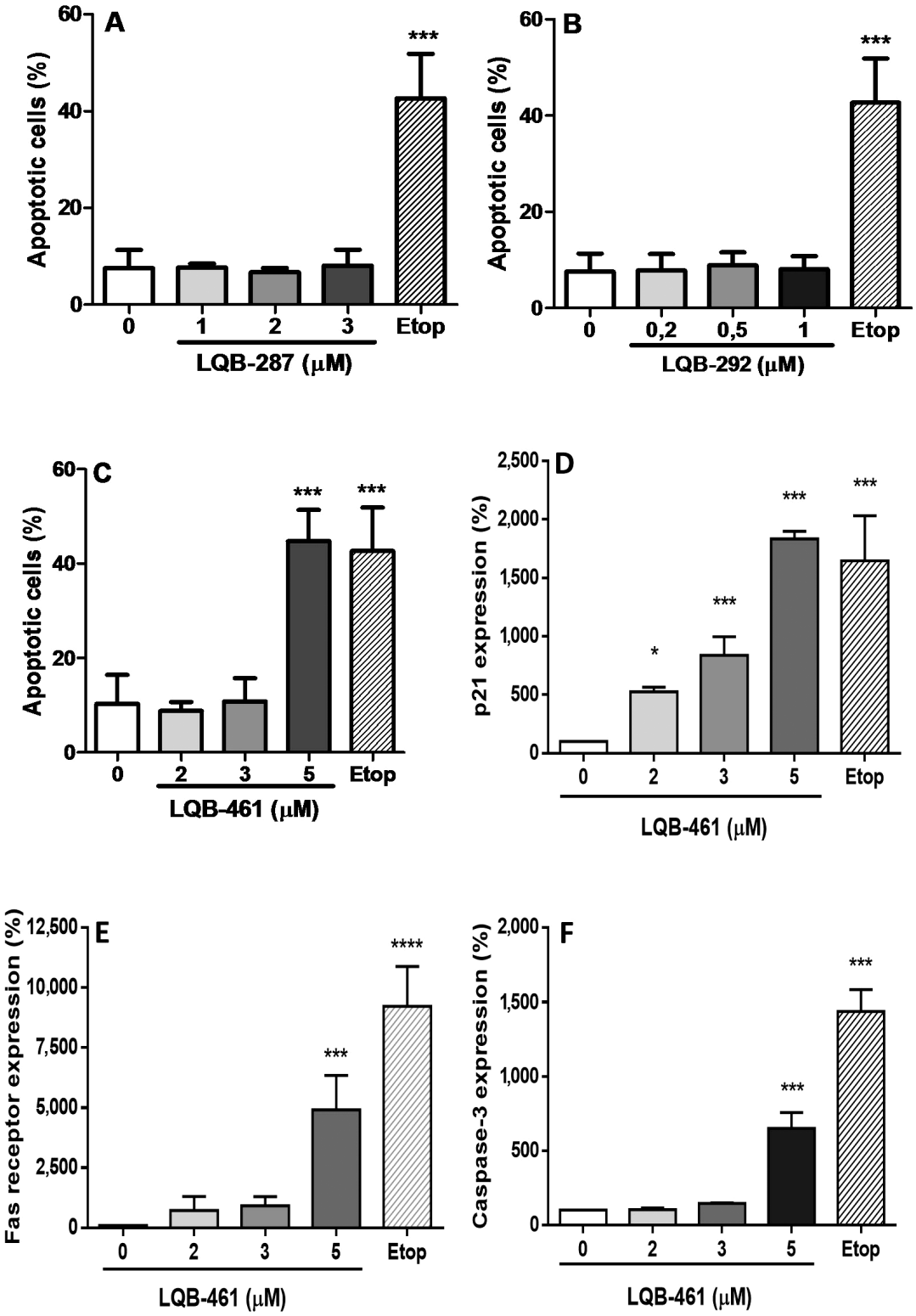

Figure 3. *Effects of **2a**, **5a** and **5b** on apoptosis and of **5b** on p21, caspase-3 and Fas receptor protein expression. (A-C) Apoptosis. (D) p21. (E) caspase-3 and (F) Fas receptor expression. Jurkat cells (1×10^5/ml) were treated or not (control) for 72 h with specific isomer or Etoposide (Etop) at 0.1 μM. Apoptosis was analyzed by the Annexin-V-FITC-PI labeling assay. Cells were labeled with specific antibodies and analyzed by flow cytometry. Data represent the mean±SD of independent experiments. *p<0.05, **p<0.01 and ***p<0.001, related to control (One-way ANOVA followed by Dunnett's test).*

Table II. *Effects of 5b (LQB-461) on the expression ratio of Bax/Bcl-2.*

Samples	$R_{Bax/Bcl-2}$	SD	Significance
Control	0.337	0.240	-
Etoposide (0.1 mM)	0.993	0.012	*
5b (LQB-461) (2 mM)	1.165	0.409	**
5b (LQB-461) (3 mM)	1.577	0.265	***
5b (LQB-461) (5 mM)	0.977	0.025	*

Cells were treated for 72 h and expression of Bax and Bcl-2 was analyzed by flow cytometry using APC-conjugated mouse anti-Bax and anti-Bcl-2 antibodies. Data represent the mean±SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to control (One-way ANOVA followed by Dunnett's test).

19). Interestingly, the benzaldehyde derivative has been shown to induce cancer cell apoptosis by increasing Bax protein expression (35), while cinnamyl derivates have been shown to increase the Bax/Bcl-2 ratio (6, 33, 34).

These results lead to the conclusion that the antileukemic activity was enhanced by cyclization of the geranyl branch in LQB-278 derivatives, and the substitution pattern of the cinnamyl group in the aromatic ring proved to be critical to the observed effects. Finally, the antileukemia actions of **5b** are related to cell proliferation inhibition and cell death induction, triggered by p21 upregulation, and increased Bax/Bcl-2 ratio, Fas-receptor and caspase 3 protein expression, involving both the intrinsic and extrinsic pathways of apoptosis. Therefore, **5b** is a promising candidate for leukemia treatment.

Conflicts of Interest

The Authors declare no conflicts of interest, financial or otherwise, in relation to this study.

Authors' Contributions

Rachell R.C. Thimoteo and Thiago Martino performed cellular and molecular biologic experiments and data analysis. Debora S. S. Costa fabricated the new set of synthetic compounds. Julio C. F. Barcellos and Ayres G. Dias supervised the chemical synthesis experiments of the new set of compounds. Paulo R. R. Costa and Ayres G. Dias contributed to the original idea of the chemical part of the project and participated in the physical and chemical analysis of the new synthetic molecules. Tatiana Simão supervised the molecular biology experiments and data analysis; Marsen G. P. Coelho contributed to the writing of the manuscript, and the design and processing of the figures. Katia C. C. Sabino drafted the manuscript and wrote the biological part of it, with input from all Authors. Ayres G. Dias analysed all the spectra of the different synthetic molecules, securing their identification and purity, and wrote the organic synthesis part of the manuscript. Graça Justo conceived the original idea of the project, supervised the cellular biological experiments of this work, performed data analysis, and contributed to the writing of the manuscript. All Authors discussed the results and contributed to the manuscript.

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