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Original article

Antineoplastic activity of the thiazolo[5,4-b]quinoline derivative D3CLP in K-562 cells is mediated through effector caspases activation

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ABSTRACT

Thiazolo[5,4-*b*]quinolines are compounds structurally related to m-Amsacrine (m-Amsa), a potent antileukemic drug that intercalates to DNA and inhibits topoisomerase II in vitro inducing cell death. The clinical use of m-Amsa and other neoplastic drugs is limited due to side effects and drug resistance. In the present study we evaluated one thiazolo[5,4-*b*]quinoline derivate, 9-[(3-chloro)phenylamine]-2-[3-(diethylamine)propylamine]thiazolo[5,4-*b*]quinoline (D3CLP), considered isosteric with 9-anilinoacridines, in order to determine its relative cytotoxic activity in tumoral versus non-tumoral cells, as well as the cell death mechanism induced by D3CLP on K-562 human leukemia cells. D3CLP was found to be four times more cytotoxic to tumor cells than Peripheral Blood Monocyte Cells (PBMCs). On the other hand, D3CLP induces cell death without previous cell cycle arrest at any phase, as shown by flow cytometry after 12 h of exposure to this compound. Interestingly, we detected a subdiploid peak 24 h after treatment. Signs of apoptosis were evident, as detected by TUNEL positive cells, chromatin condensation and nuclear fragmentation. Effector caspases activation were assessed with peak activity at 24 h after treatment (as detected by fluorometry assays), at which time a subdiploid peak was found in flow cytometry histograms. All data are consistent with the induction of apoptotic cell death in K-562 cells via effector caspases activation. In conclusion, the significant cytotoxicity of D3CLP together with the cell death type it produces, justifies further experimental and preclinical evaluation of this compound in the effort to find new and highly specific anti-tumor agents against leukemia cells.

1. Introduction

In cancer chemotherapy, both anti-tumor drug resistance and the side effects of currently available drugs have prompted the continuous search for new anti-tumor compounds, which are an important option in the treatment of these diseases. The activity of several anti-tumor drugs is mediated through their interaction with cell division proteins and/or with DNA. Although 9-anilinoacridines, such as m-Amsacrine (m-Amsa, Fig. 1A), are potent DNA intercalators that inhibit DNA topoisomerase II, their clinical use is limited due to side effects. In 1997, Alvarez-Ibarra et al., reported the synthesis and cytotoxic evaluation of thiazolo[5,4-*b*]quinolines, considered isosteric with 9-anilinoacridines, as part of the effort to find better anti-tumor compounds with fewer side effects [1]. Some thiazolo[5,4-*b*]quinolines are cytotoxic to several cancer cell lines as well as being DNA intercalators and inhibitors of human topoisomerase II in vitro [2,3]. According to these studies, the presence of a 2-[3-(diethylamine)ethylamine] substituent and a fluorine atom at position 7 is determinant for their intercalating properties. The thiazolo[5,4-*b*]quinolines that have a 3-(diethylamine)propylamine at position 2, generally were more cytotoxic for several cancer cell lines than analogs bearing a 3-(diethylamine)ethylamine in the same position. D3CLP is a thiazolo[5,4-*b*]quinoline with 3-(diethylamine)propylamine group (Fig. 1B). D3CLP has DNA-intercalating properties and is an inhibitor of topoisomerase II. Moreover, this compound has important cytotoxic activity against several cancer cell lines such as cervical, colorectal and breast cancer, but is particularly active against the human leukemia line of K-562 cells [3]. The aim of the present study was to evaluate D3CLP in terms of the relative cytotoxic activity it produces in tumoral versus non-tumoral cells, and

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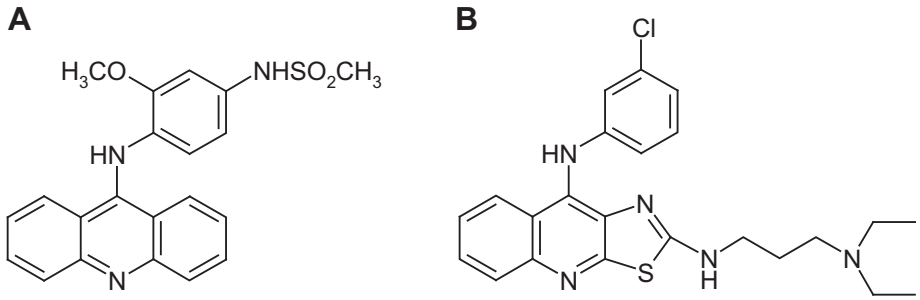

Fig. 1. Chemical structures: A) m-Amsacrine, B) 9-[(3-chloro)phenylamine]-2-[3-(diethylamine)propylamine]thiazolo[5,4-b]quinoline (D3CLP).

the mechanism of cell death it induces in a human leukemia cell line. We provide evidence that the thiazolo[5,4-*b*]quinoline derivative D3CLP induced a potent cytotoxic activity mediated by induction of effector caspases in K-562 cells, suggesting that this compound is a good candidate for preclinical anti-tumor evaluation.

2. Results and discussion

2.1. D3CLP is more cytotoxic to leukemia cancer cells than to PBMCs

For a compound to be clinically useful, it must be significantly more toxic to tumor cell lines than to normal cells. PBMCs, which are a commonly used cell culture to test toxicity of a compound on normal cells, were treated with D3CLP at several concentrations and analyzed by the MTT assay to determine the inhibitory concentration for the 50% of cellular growth (IC_{50}). We found that the IC_{50} value for D3CLP on PMBCs was of 28.7 ± 14.2 μM. To assess the cytotoxicity induced by D3CLP on the leukemia cancer cells, we performed a series of experiments to determine the IC_{50} value on K-562 cells. The IC_{50} value obtained was 7.82 ± 0.32 μM. Since the compound was found to have higher potency against K-562 cells than in other tumor cells (see reference [3]), this concentration and these cells were used to study the cell death mechanism.

Cytotoxic analysis showed that the IC_{50} induced by D3CLP was significantly lower, approximately 4 times, against K-562 than PBMCs. The differences were found to be statistically significant ($^*p < 0.05$), indicating that tumor cells are more sensitive to this compound than normal cells (Fig. 2). Interestingly, D3CLP has demonstrated a potency against cancer cell lines significantly higher than that previously reported by other thiazolo[5,4-*b*]quinoline derivatives [3].

2.2. Cell cycle changes induced by D3CLP

To evaluate possible alterations in the cell cycle, K-562 cells were treated with 7.8 μM D3CLP for different times and analyzed by flow cytometry. As shown in Fig. 3, the G2/M peak observed at 6 h after the treatment of K-562 with D3CLP shows a slight increase compared with previous time and vehicle. This could be explained since it has been demonstrated that D3CLP has topoisomerase II inhibitor activity [3], which delays G2 to M transition [4]. After 12 h, the cells showed a subdiploid peak (DNA content < 2n), thus indicating the presence of debris from dead cells. As this peak increased, the number of cells in the other phases decreased. On the other hand, the treatment of K-562 cells with paclitaxel induced a clear arrest of the entire cell population in G2/M, preceding the appearance of the subdiploid peak, and the treatment with vehicle showed no modification in the cell cycle (Fig. 3). After treatment with paclitaxel or D3CLP, there was a clear difference between the changes in the patterns of the various cell cycle stages of K-562 cell populations (Fig. 3). In particular, paclitaxel induced a virtually complete cell arrest at the G2/M phase before the appearance of subdiploid populations, while D3CLP induced an early rise in the subdiploid population without changes in the cell cycle phases. Subdiploid peaks have been associated with DNA fragmentation or cellular debris [5]. Whereas, a similar cell cycle phases pattern was observed after m-Amsa and paclitaxel 24 h treatment to human lymphoblastoid TK6 cells [6].

The statistical analysis of three independent Fluorescence-Activated Cell Sorting (FACs) experiments revealed statistically significant differences when comparing the subdiploid fraction of K-562 cells treated with D3CLP and those treated with the paclitaxel and vehicle, after 12, 24 and 48 h of treatment. Likewise, the cell population fraction in G2/M at 12 h, and the fraction in G0/G1 at 48 h were significantly different between the K-562 cells treated with D3CLP and those treated with paclitaxel or the vehicle. Finally, at all other times cells treated with D3CLP presented only small, statistically insignificant changes in the proportions of cells in G2/M and G0/G1 (data not shown). Therefore, the alterations in the cell cycle dynamics caused by D3CLP, does not lead to a cell cycle arrest previously to cell death, as in the case of paclitaxel (Fig. 3).

Apoptosis is a programmed cell death type invoked by normal cells as a normal physiological process during development and is an important mechanism by which many compounds exert their anti-tumor effect [7–11]. Additionally, apoptosis is also a desirable feature in an anti-tumor mechanism of action, in contrast to cell death by necrosis, because necrosis will elicit inflammatory response mechanisms in the tissue surrounding a tumor, which in turn lead to side effects in the patients [7,12]. There are numerous reports indicating an

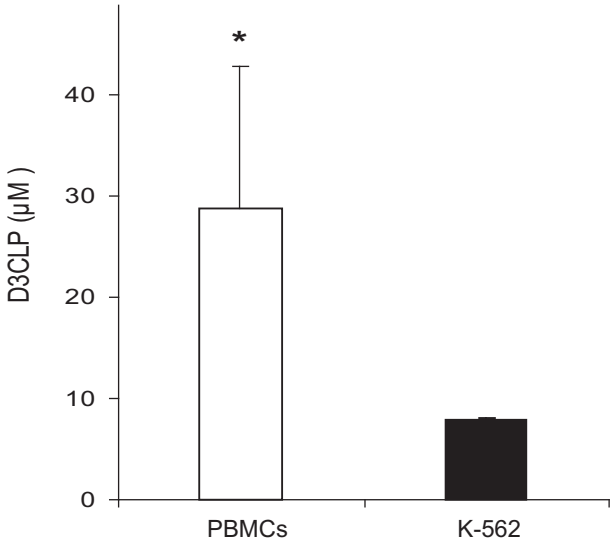

Fig. 2. Cytotoxicity of D3CLP. Cytotoxic activity of D3CLP on PBMCs and K-562 cells. The data indicate the mean of IC_{50} (μM) value ± standard deviation of three independent experiments. Analyzed by Student's *t* test, $^*p \leq 0.05$.

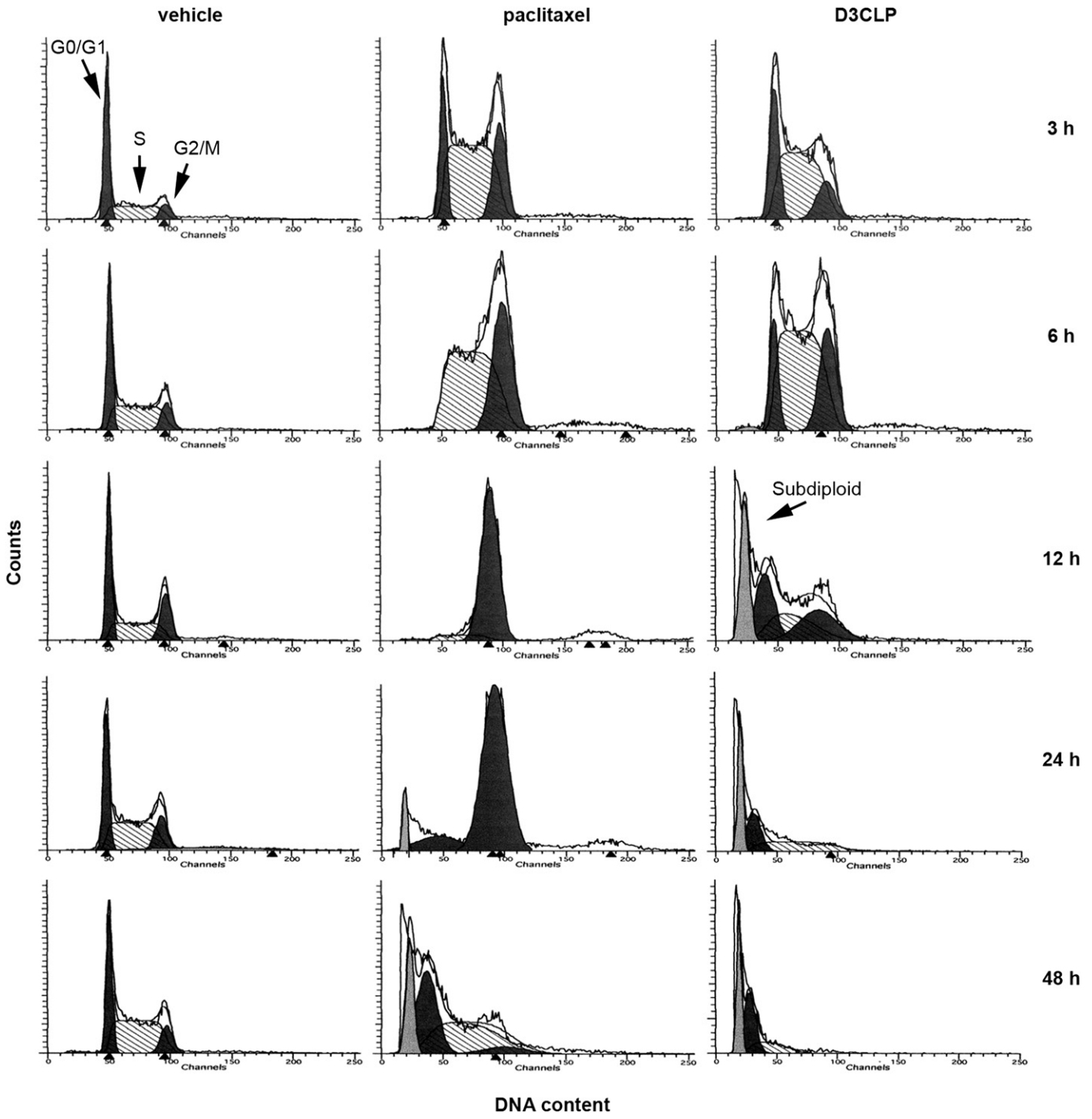

Fig. 3. Flow cytometry histograms for K-562 cells with different treatments. The graphs on each column show the histograms of cells incubated with vehicle (DMSO), paclitaxel or D3CLP, as indicated at the top. The top histogram on the left column indicates the position of peaks corresponding to cell cycle phases. Each row corresponds to a different incubation time, as indicated on the right. No evidence of cell cycle alterations was found in the K-562 cells treated with the vehicle. In D3CLP treatment (right column), at 12 h and thereafter, a subdiploid population was present. An alteration in the proportion of G2/M to G1 populations was observed at 6 h, but the cell cycle was not clearly arrested. Treatment with paclitaxel induces cell cycle arrest in G2/M at 6 h, and a complete arrest of the whole cell population in G2/M is evident at 12 h. Further alterations are observed at 24 and 48 h, including polyploidy and subdiploid peaks. Data show results from only one representative experiment.

apoptotic effect of drugs such as paclitaxel, vinblastine, etoposide, ara-C and adriamycin. Some anti-tumor drugs of natural origin, as well as some acridines induce neoplastic cell death through an apoptotic mechanism, both in vivo and in vitro [11,13–17].

Thiazolo[5,4-*b*]quinolines are compounds structurally related to m-Amsacrine (m-Amsa), a potent antileukemic drug that intercalates DNA and inhibits topoisomerase II in vitro, inducing cell death [18]. The mechanism of cell death induced by m-Amsa, has not been completely elucidated. Therefore, such mechanism for D3CLP has been determined in the present study.

Another chemotherapeutic drug, paclitaxel, is a microtubule stabilizing agent in current clinical use. Since it induces apoptosis in several cancer cell lines, including K-562 cells [19,20], we decided to use it as a positive cell death control.

2.3. DNA fragmentation in K-562 cells after D3CLP treatment

The FACs experiments show the early presence of a subdiploid population in D3CLP-treated K-562 cells, indicating a rapid DNA fragmentation of these cells. The TUNEL assay detects DNA fragmentation by labeling free 3′-OH DNA termini. This assay also provides information about other morphological alterations that suggest apoptosis, such as chromatin condensation, nuclear fragmentation and the presence of apoptotic bodies [21–23]. We used the TUNEL assay to further characterize this DNA fragmentation (Fig. 4). K-562 cells treated with 7.8 μM D3CLP, showed an increase in DNA fragmentation and chromatin condensation at 12 and 24 h, which was evident by a significant increase in TUNEL positive cells, suggesting cell death by apoptosis (Fig. 4. panels G and H). In K-562 cells treated with paclitaxel, TUNEL positive cells were observed at 24 and 48 h (Fig. 4. panels E and F), showing typical signs of apoptosis, such as chromatin condensation and appearance of apoptotic bodies. In addition, after 24 h a large number of detached cells were observe by light microscopy (data not shown). In contrast, K-562 cells incubated with the vehicle showed no DNA damage (TUNEL-negative).

In the present study we observed TUNEL positive cells and chromatin condensation after 12 h of treating K-562 cells with D3CLP (Fig. 4), these results are consistent with an apoptotic cell death induction. The fact that the dynamics of the K-562 cell death process induced by D3CLP was clearly different from that observed with paclitaxel, this indicates that these two compounds have distinct cellular targets and mechanisms of action.

Paclitaxel is known to interfere with the cytoskeleton reorganization, particularly at mitosis [20]. On the other hand, given its structure and properties, D3CLP and other thiazolo[5,4-*b*]quinolines should have a different dynamics of cell death. Indeed, the results of flow cytometry in the current contribution (Fig. 3) show that there was an early rise in the subdiploid population, which is consistent with increased DNA fragmentation in K-562 cells and suggests that DNA fragmentation was mediated by the activation of effector caspases.

2.4. Caspases mediated effects of D3CLP on cell death

The analyses of DNA fragmentation indicate that D3CLP induced apoptosis in K-562 cells, probably through an activation of effector caspases. Effector caspases activity was determinated in vitro with the specific synthetic substrates Ac-DEVD-AMC. We observed a marked increase in the activity of effector caspases, in total crude extracts of the soluble proteins from K-562 cells treated with D3CLP, in function of the time, with a maximal response at 24 h (Fig. 5). Beginning 48 h after treatment a decrease in effector caspases activity was detected which correlated with a decrease in cell integrity observed by optical microscopy (data not shown).

Caspases are cystein-asparte proteases and are classified as initiator caspases or effector caspases (executer caspases), according to their role. The activities of effector caspases are responsible for many changes in the cell morphology associated with apoptosis. The activity of effector caspases on the Caspase-Activated DNase Inhibitor (ICAD) lead to the definite DNA fragmentation size caused

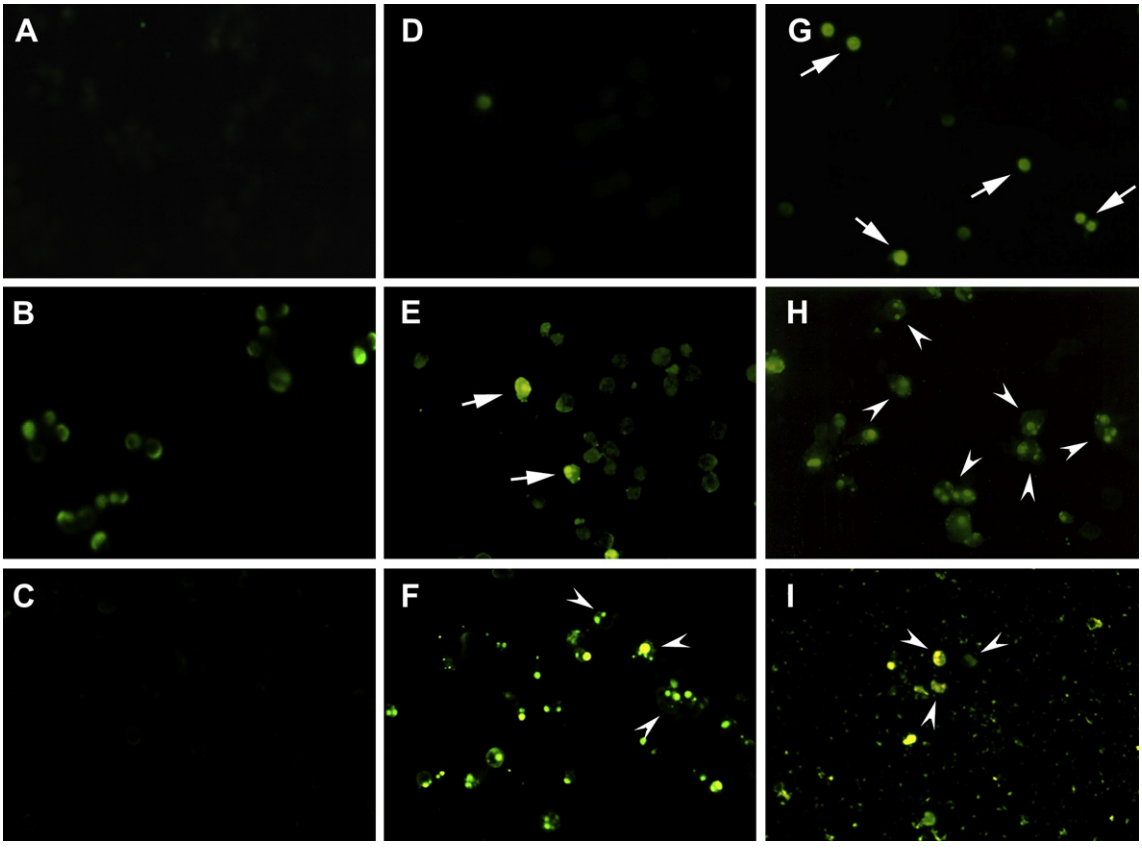

Fig. 4. Determination of DNA fragmentation in K-562 cells treated with D3CLP by TUNEL assay. A) Cells incubated with DMSO for 12 h, prepared with the TUNEL protocol, but in the absence of terminal thymidine transferase (background fluorescence). B) Cells incubated with DMSO for 12 h, and prepared after treatment with DNase I (TUNEL positive control). C) Cells incubated with DMSO for 48 h, and subjected to the TUNEL protocol (vehicle control). D), E) and F) Cells treated with paclitaxel for 12, 24 and 48 h respectively, as the positive control of apoptosis. G), H) and I) Cells treated with D3CLP for 12, 24 and 48 h respectively. D3CLP treatment produced TUNEL positive cells with chromatin condensation (arrows) and fragmented nuclei (arrowheads). All photographs are shown at 20X magnification.

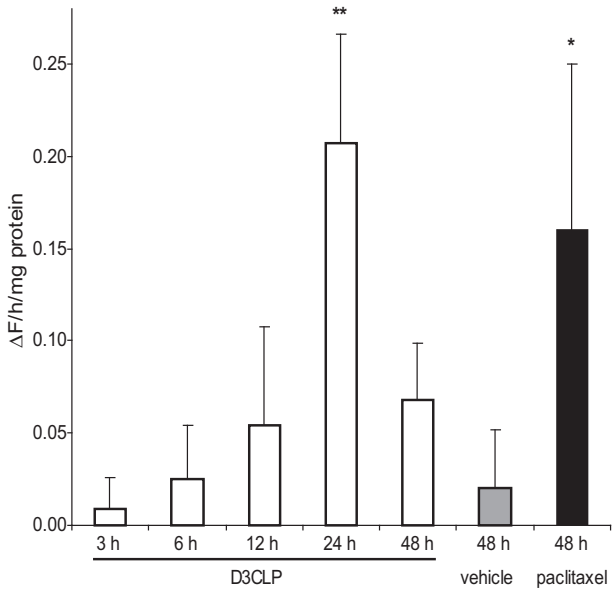

Fig. 5. Effector caspases activity in crude extracts of soluble proteins from K-562 cells treated with D3CLP at different times. Effector caspases specific activity in each sample is expressed as the change in fluorescence intensity per hour per milligram of protein (ΔF/h/mg protein). At 48 h, the activity in crude extracts of soluble proteins from K-562 cells treated with paclitaxel (known to induce apoptosis, see reference [19]) is shown as a reference. Results are the mean ± standard deviation of three independent experiments. $^{**}p < 0.05$ compared with D3CLP treatments at the other times, and with the vehicle, $^{*}p < 0.05$ compared with the vehicle.

by the endonuclease CAD [24,25]. On the other hand, chromatin condensation, as well as changes in the cell membrane and nuclei follow the very early activation of effector caspases, particularly caspase-3 in K-562 [19]. Active caspase-3 can cleave the inhibitor ICAD, releasing the CAD endonuclease that cuts DNA [24,25], thus producing populations with fragmented DNA and causing the cell DNA content to fall to subdiploid levels [26]. The patterns in DNA content observed by flow cytometry (Fig. 3) after treatment with D3CLP in the present study are consistent with an increased activity by effector caspases (Fig. 5). The diminished activity of effector caspases at 24 h of treatment is possibly due to the activation of other proteases released at the late stages of cell death.

Since it has been demonstrated that the thiazolo[5,4-*b*]quinoline derivative D3CLP can be inserted in human DNA and inhibit topoisomerase II, these effects contributes in part to its cytotoxic mechanism [3]. In addition to its properties of intercalation into DNA and inhibition of topoisomerase II, in order to induce cell death D3CLP must produce cell damage by another mechanism. All data were consistent with the induction of apoptotic cell death in K-562 cells via effector caspases activation. The knowledge of the molecular mechanism by which a drug exerts its biological effect is of prime importance for antineoplastic therapy, since two drugs may present antagonistic effects when co-administrated as suggested by the simultaneous administration of the combination of tamoxifen and doxorubicin or paclitaxel and vinblastine [27,28].

3. Conclusion

In conclusion, the thiazolo[5,4-*b*]quinoline derivative D3CLP, induced apoptosis through the activation of effector caspases in the human K-562 leukemia cell line. It showed a low IC_{50}, without involving cell cycle perturbation. Furthermore, this compound displays DNA-intercalating properties and inhibitory activity of topoisomerase II [3], which probably contributed to apoptotic cell death in K-562 cells. The significant cytotoxicity together with the cell death type produced by D3CLP indicate that this compound should be of great interest for preclinical evaluation, as part of the efforts to find new and highly specific anti-tumor agents against leukemia cells.

4. Experimental protocols

4.1. Drugs

Paclitaxel was purchase from Sigma (Sigma–Aldrich, St. Louis, MO, USA). D3CLP (9-[(3-chloro)phenylamine]-2-[3-(diethylamine) propylamine]thiazolo[5,4-*b*]quinoline) was prepared by Dr. Alfonso Lira-Rocha. Its structure, chemical synthesis and characterization have been previously reported [3]. Both compounds were dissolved in dimethyl sulfoxide (DMSO, J. T. Baker, USA).

4.2. Isolation of peripheral blood mononuclear cells

With appropriate informed consent, mononuclear cells were obtained from peripheral blood of five healthy donors between 18 and 28 years old. Using a density gradient with Ficoll-Hypaque (Histopaque 1077, Sigma, St Louis, MO, USA), these cells were centrifuged at 400 g for 30 min at room temperature, then, washed twice with RPMI 1640 and centrifuged at 300 g for 10 min. The initial viability of mononuclear cells evaluated by trypan blue exclusion assay, and was 95–98%.

4.3. Cell culture

The human leukemia K-562 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (D-MEM). Peripheral blood mononuclear cells (PBMCs) were cultured in Roswell Park Memorial Institute Medium (RPMI 1640). Both mediums were supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin G, 100 U/ml streptomycin sulfate and 0.25 amphotericin B (Invitrogene Carlsbad, CA, USA). The cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

4.4. Cytotoxicity analysis in non-tumoral cells and K-562 cell line

The cytotoxicity of D3CLP on PBMCs and on K-562 cells was assessed by a modified MTT assay [29,30], in three independent experiments. PBMCs were seeded on a 96 well plates at 6×10^3 cells/well containing 20 μg/ml phytohemagglutinin, and K-562 cells at 7×10^3 cells/well containing 200 μl of the corresponding medium. After 24 h, the cells were treated with different concentrations of D3CLP or the vehicle, which were added to each well in a volume of 50 μl, to give a total volume of 250 μl with the indicated final concentration. Cell viability was determined 48 h later, at which time the medium was removed and 20 μl of 2.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) in PBS pH 7.2 were added. After 2 h, 0.2 ml of DMSO were added to each well, followed by gentle shaking. The absorbance was measured at 540 nm using a Synergy® 2 Multi-Mode Microplate Reader, BioTek® (BioTek, Winooski, VT, USA). The amount of formazan detected is proportional to the number of living cells, and the cell growth inhibition was determined by the formula:

$$\text{Cell growth inhibition}(\%) = (1 - \text{absorbance of treated cells}/\text{absorbance of untreated cells}) \times 100$$

The data presented are the result of three independent experiments with six replicates each. The inhibitory concentration values

for the 50% of cellular growth (IC_{50}) were determined from concentration/viability inhibition curves using a non-linear regression with OriginPRO 7.0®, software package (OriginLab, Northampton, MA, USA).

4.5. Cell cycle analysis

Cellular DNA content was assessed by flow cytometry. Briefly, 1×10^6 K-562 cells were seeded on 100 mm Petri dishes and were allowed to adhere and grow in standard conditions for 24 h. The cells were exposed to D3CLP (7.8 μM), paclitaxel (0.25 μM) and the vehicle (0.28% DMSO) for 3, 6, 12, 24 and 48 h. These concentrations were maintained for all subsequent experiments. After treatment, the cells were detached and rinsed twice with PBS pH 7.4, then fixed with 70% ethanol. Before to FACs, the cells were incubated with RNase and propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO, USA) and more than 20,000 cells were analyzed with FACScan® (BD, New Jersey, NJ, USA). Cell cycle distribution was analyzed using ModFit LT® (BD, New Jersey, NJ, USA).

4.6. Evaluation of DNA fragmentation in situ

The in situ DNA fragmentation was detected by TUNEL (In situ cell death detection kit, fluorescein®, Roche GmbH Mannheim, Germany) assay according to the manufacturer's instructions. On a 60 mm Petri dishes were seeded 5×10^5 K-562 cells and were allowed to adhere and grow on glass coverslips, in standard conditions for 24 h, and were incubated with D3CLP, paclitaxel and the vehicle for 12, 24 and 48 h, at the concentrations above indicated. After incubations, the cells were fixed with 4% paraformaldehyde in PBS for 1 h, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and then rinsed twice with PBS. The DNA nick-labeling reaction was performed at 37 °C for 60 min using 50 μl of TUNEL reaction mixture, which included 45 μl of labeled nucleotide mix and 5 μl of enzyme solution. The samples were rinsed with PBS for three times and analyzed by fluorescence microscopy.

4.7. Effector caspases activity assay

The activity of effector caspases was assessed by fluorometry. Briefly, 1×10^6 K-562 cells were seeded on a 100 mm Petri dish and were allowed to adhere and grow in standard conditions for 24 h, then the cells were exposed to D3CLP for 3, 6, 12, 24 and 48 h, paclitaxel and vehicle for 48 h (as reference). After the respective incubation, the cells were collected by centrifugation. The cellular pellet was resuspended in lysis buffer containing 100 mM Hepes, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 10 mM DTT, 1 mM EDTA, 20 μM EGTA, and Complete Protease Inhibitor Cocktail Tablets® (Roche, Mannheim, Germany). The tetrapeptide acetyl-Asp-Glu-Val-Asp-a-(4-methylcoumaryl- 7-amide) (Ac-DEVD-AMC) was used to evaluate the activity of effector caspases [31,32]. The increase in fluorescence was recorded every 2 s for 10 min, at an excitation wavelength of 330 nm and emission of 440 nm in a luminescence spectrometer (Spectronic Instruments, SLM Aminco-Bowman®, Rochester, NY, USA).

4.8. Statistical analysis

Data are reported as the mean ± standard deviation of three independent experiments. The statistically significant difference between two treatments was analyzed with the Student's *t* test. For multiple treatments, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test with GraphPad Prism 5® for Windows® (GraphPad Software, Inc., La Jolla, CA, USA). A $p \leq 0.05$ was considered significant.

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