# Synergistic Anticancer Activity of Thiazolo[5,4-*b*]quinoline Derivative D3CLP in Combination with Cisplatin in Human Cervical Cancer Cells

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**Abstract.** *Background: D3CLP (9-[(3-chloro)phenylamine]-2-[3-(diethylamine)propylamine]thiazolo[5,4-b]quinoline) is a potent cytotoxic thiazolo[5,4-b]quinoline synthetic derivative that induces apoptosis of leukemia cells, while it displays low toxicity towards non-tumoral cells. The aim of this study was to determine if D3CLP can enhance the cytotoxicity of other antineoplastic drugs. Materials and Methods: Leukemia, breast and cervical cancer cell lines were exposed to D3CLP-alone or in combination with imatinib, tamoxifen or cisplatin, respectively. Cell viability after treatment was evaluated by the MTT assay, and cell death by the TUNEL assay. The effects of combined treatments were analyzed by combination index and isobolographic analysis. Results: Antiproliferative activity results indicate that D3CLP in combination with antineoplastic drugs induced a synergistic effect, at 3:1 and 1:1 ratios for D3CLP plus imatinib in K-562 leukemia cells, and at a 3:1 ratio for D3CLP with cisplatin in HeLa cells, as determined by their combination index. Furthermore, isobolographic analysis demonstrated a significant synergism for a 3:1 combination ratio of D3CLP with cisplatin in HeLa cells. In addition, TUNEL assay suggests cell death by apoptosis of HeLa cells after treatment with D3CLP and its combination with cisplatin at a 3:1 ratio. Conclusion: Overall the results indicate that D3CLP, in combined preparation with antineoplastic drugs, is a good candidate for pre-clinical studies in the treatment of different carcinoma cell types.*

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Cancer is a major cause of human death worldwide. In 2008, it caused 7.6 million deaths, which represent around 13% of total deaths globally (1). Out of these deaths, 70% occurred in countries with low or middle income. Thus, an increase in the incidence of different types of cancer, such as breast cancer, leukemia, and the high frequency of cervical cancer constitute a growing public health problem.

These types of cancers, are often detected in advanced stages, with a low survival rate in Latin America (2). One of the treatments for advanced cancer stages is chemotherapy. Therefore, the evaluation of new antineoplastic drugs is of great importance in cancer chemotherapy. Anticancer drugs are diverse, and are obtained from both synthetic and natural sources.

It has been demonstrated in several cancer cell lines, that thiazolo[5,4-*b*]quinolines derivatives are cytotoxic compounds. They are structurally related to m-amsacrine (m-Amsa) (3), which is a DNA intercalator and inhibitor of human topoisomerase II *in vitro* (4-6). 9-[(3-chloro)phenylamine]-2-[3-(diethylamine) propylamine] thiazolo[5,4-*b*]quinoline (D3CLP) is a thiazolo[5,4-*b*]quinoline synthetic derivative (Figure 1), which possesses high cytotoxic activity towards several human tumor cell lines (5). Recently, it has been shown that this compound induces significant cell death of leukemia K-562 cells. Treatment of these cells with D3CLP induces apoptosis by activation of effector caspases, without cell-cycle alteration. In addition, D3CLP displays low toxicity towards non-tumoral cells (7).

The use of chemotherapeutic drugs is dose-limiting due to its overall cytotoxicity. The toxicity produced by these drugs towards different organs has led to use it in combination, to achieve better therapeutic outcomes. Combined chemotherapy uses two or more drugs with proven effects against a tumor type (8). Combined treatments with chemotherapeutic drugs may be synergistic, additive or antagonist. If some drugs have a synergistic effect, this provides treatment cost reductions, as well as fewer side

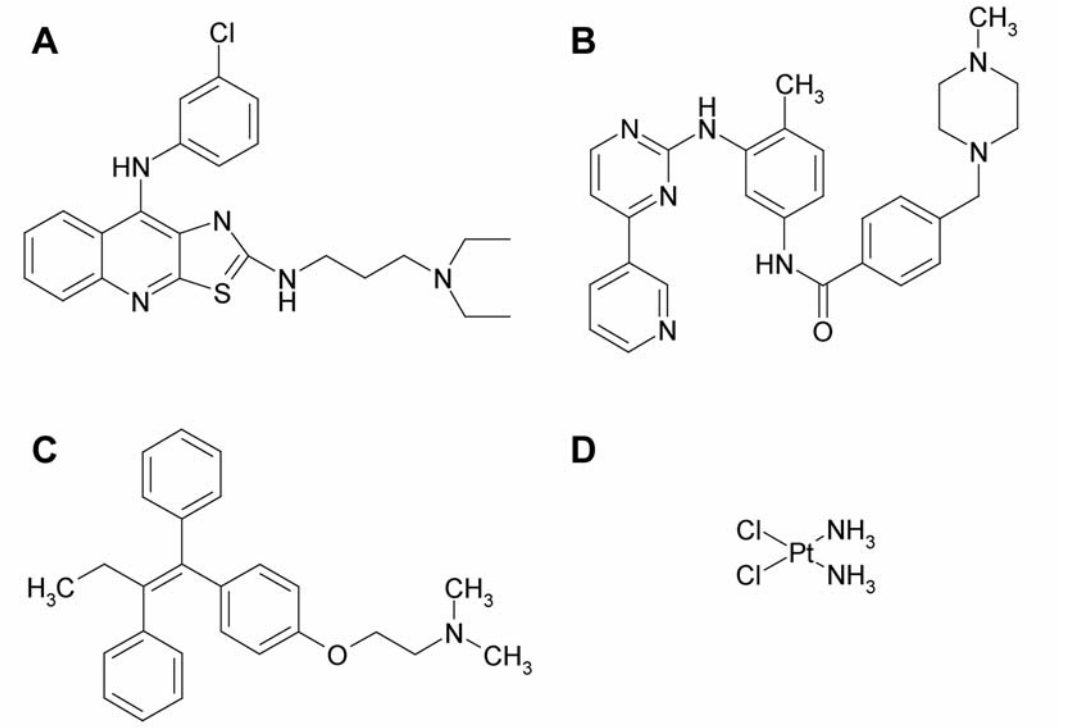


Figure 1. *Chemical structures of cytotoxic compounds used in this study: D3CLP (A), imatinib (B), tamoxifen (C), and cisplatin (D).*

effects. There are two methods widely used to analyze combined effects of drugs: the combination index (CI) (9), and the isobolographic analysis (10). The aim of this work was to evaluate if D3CLP has *in vitro* synergistic effects when combined with antineoplastic drugs currently in clinical use, such as imatinib, tamoxifen and cisplatin (Figure 1). Initially, we evaluated the effects on cell viability of single-agents, and then, the antiproliferative combined effect of D3CLP with the antineoplastic drugs were analyzed by the CI and isobolograms in three cell lines originating from neoplasms, which present high incidence in Mexico: leukemia, breast and cervical cancer.

## Materials and Methods

*Drugs*. Antitumor drugs used were: Cisplatin (Lemery Laboratories, México, D.F., México), tamoxifen (Sigma-Aldrich, St. Louis, MO, USA), imatinib mesylate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). D3CLP was prepared by Dr. Alfonso Lira-Rocha. Its structure, chemical synthesis and characterization have been previously reported (5). Cisplatin was used in isotonic solution, and all other compounds were dissolved in dimethyl sulfoxide (DMSO; J. T. Baker, Phillipsburg, NJ, USA).

*Cell culture*. Different human cancer cells lines, leukemia (K-562), breast (MCF-7) and cervix (HeLa) were obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa and K-562 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, 100 U/ml streptomycin sulfate and 0.25 μg/ml amphotericin B (Invitrogen Carlsbad, CA, USA). Previously to drug exposure, only MCF-7 cells were suspended and plated in DMEM without phenol red, containing 10% charcoal-stripped FBS. The cultures were performed under standard conditions, as previously described (7).

*Cytotoxicity analysis*. Cell viability after treatments was assessed by a modified MTT assay (11, 12), in three independent experiments. K-562, MCF-7 and HeLa cells were seeded at $7\times10^3$ cells/well containing 200 μl of the corresponding medium in 96-well plates. After 24 h, the cells were treated with different concentrations of D3CLP, imatinib, tamoxifen, cisplatin or the vehicle, respectively, which were added to each well in a volume of 50 μl, to give a total volume of 250 μl of the indicated final concentration. Then 48 h later, 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 7.4) was added for cell viability quantification. After 2 h at 37˚C, the supernatants were removed and 0.2 ml of DMSO was added to each well, followed by gentle shaking. Absorbance was measured at 540 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The amount of formazan detected is proportional to the number of living cells, and the corrected cell viability was determined using non-linear regression with OriginPRO® 7.0 software package (OriginLab, Northampton, MA, USA), as reported elsewhere (7). The drug concentrations that induced a cell viability of 50% ($IC_{50}$), as compared with the vehicle-treated cells, were the result of three independent experiments with six replicates each.

Table I. *Cytotoxicity in independent drug treatments. Data represent the mean of three independent experiments±SEM.*

| Drug | Cell line | $IC_{50}$ (μM) |
|---|---|---|
| D3CLP | K-562 | 8.55±1.29 |
| | MCF-7 | 11.22±1.45 |
| | HeLa | 8.81±1.17 |
| Imatinib | K-562 | 2.37±0.12 |
| Tamoxifen | MCF-7 | 22.71± 1.59 |
| Cisplatin | HeLa | 15.62±0.48 |

Table II. *Combination index values for the combination of D3CLP with antineoplastic drugs of current clinical use at differents ratios.*

| Cancer cell lines | D3CLP combination | 3:1 | 1:1 | 1:3 |
|---|---|---|---|---|
| K-562 | Imatinib | 0.69 | 0.86 | 1.20 |
| MCF-7 | Tamoxifen | 1.20 | 1.26 | 1.22 |
| HeLa | Cisplatin | 0.85 | 1.04 | 1.06 |

If the combination index value is close to 1, the interaction is additive; values lower than 1 are an indication of synergistic interaction, and values higher than 1 correspond to antagonistic interaction (see Materials and Methods).

*Combined cytotoxicity assay*. From the $IC_{50}$ data for single-compound treatments, we evaluated new doses to determine the combined effect of D3CLP plus antineoplastic drugs currentl used. For the combination at a 3:1 ratio, the drugs were used as follow: $0.75\times IC_{50A}$ plus $0.25\times IC_{50B}$, where $IC_{50A}$ was the $IC_{50}$ of D3CLP and $IC_{50B}$ was the $IC_{50}$ for the other antineoplastic drug for the same cell line. Two additional higher doses and two lower doses in geometrical proportions were added to cells. Treatments in a similar manner were considered for the combinations at a 1:1 ratio ($0.5\times IC_{50A}$ plus $0.5\times IC_{50B}$) and 1:3 ($0.25\times IC_{50A}$ plus $0.75\times IC_{50B}$). In the combined treatments, the drugs were administered one after the other and the effect on the cell viability was evaluated by the MTT assay after 48 h, and analyzed using both the CI (13), and isobolographic method (14, 15) to determine cytotoxic synergism, additivity or antagonism. The $IC_{50}$ value of the drug combination was the result of three independent experiments with six replicates each.

The CI was determined as CIx=(D1/Dx1)+(D2/Dx2), where CIx represents the CI value for 50% viability (50% effect), Dx1 and Dx2 represent the doses of agents 1 and 2 required to exert 50% effect alone, and D1 and D2 represent the doses of agents 1 and 2 that elicit the same 50% effect in combination with the other agent, respectively. For the CI analysis, we considered that the two drugs under evaluation had different mechanisms of inducing cell death. If the CI value is close to 1, the interaction is additive; values lower than 1 are an indication of synergistic interaction; and values higher than 1 correspond to antagonistic interaction (16).

For the isobolographic method, we constructed isobolograms for a given $IC_{50}$ level of effect, where equipotent doses of D3CLP and the studied antineoplastic drug are plotted on the x- and y-axes, respectively. The line that connects these doses corresponds to the additivity line and represent the theoretical $IC_{50}$ value for all possible combinations of these drugs. The experimental data point, obtained from the dose–response curve of the combination is subsequently represented. The site in the graph where the experimental data point is located determines the type of interaction. If the experimental point falls under the additivity line, and is statistically different from additivity, the effect of the combination is synergistic. If the point falls on the additivity line, there is no interaction between the drugs and the effects are simply additive. In contrast, if the point that represents the $IC_{50}$ for the combination is located above the additivity line, and is statistically different from additivity, therefore antagonism is present (10, 17).

*DNA fragmentation detection assay*. Drug-induced DNA fragmentation was evaluated by the TUNEL (In Situ Cell Death Detection kit, Fluorescein®; Roche GmbH Mannheim, Germany) assay, according to the manufacturer's instructions. Petri dishes (60 mm) with glass coverslips were seeded with $5\times10^5$ HeLa cells and were allowed to adhere and grow on glass coverslips, in standard conditions for 24 h. The cells were then incubated with $IC_{50}$ of D3CLP (8.8 μM), or cisplatin (15.6 μM), or its combination in a 3:1 ratio according to the $IC_{50}$ value (6.6 μM D3CLP plus 3.9 μM cisplatin) for 12, 24 and 48 h. After incubation, 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, according to the manufacturer's instructions. After incubation, the samples were rinsed with PBS three times and then placed on slides using DAKO Fluorescent Mounting Medium (Dako, Carpinteria, CA, USA), and analyzed by fluorescence microscopy.

*Statistical analysis*. Data are reported as the mean±standard error of the mean (SEM) of three independent experiments, with six replicates each. Statistically significant differences between two treatments were analyzed with the Student's *t*-test. A $p\leq0.05$ was considered significant.

## Results

*Single-agent effects on cell viability*. As a first step, before assessing the combined effect of two drugs, it is necessary to determine their own cytotoxicity. Cytotoxicity of imatinib, tamoxifen and cisplatin were assessed in human leukemia, breast and cervical cancer cells, respectively. The results showed that the $IC_{50}$ value for the D3CLP, in these cell lines was approx. 10 μM (Table I). Treatment of these cell lines with antineoplastic drugs currently in clinical use demonstrated $IC_{50}$ values from 2 to 22 μM (Table I). The most sensitive cell line to the effect of D3CLP, was K-562 and the less sensitive one was MCF-7 (Table I).

*Combined antiproliferative effect of D3CLP with chemo-clinically used therapeutic drugs*. The CI used in this study was determined from the cytotoxicity data according to what has been previously described (13), and the experiments

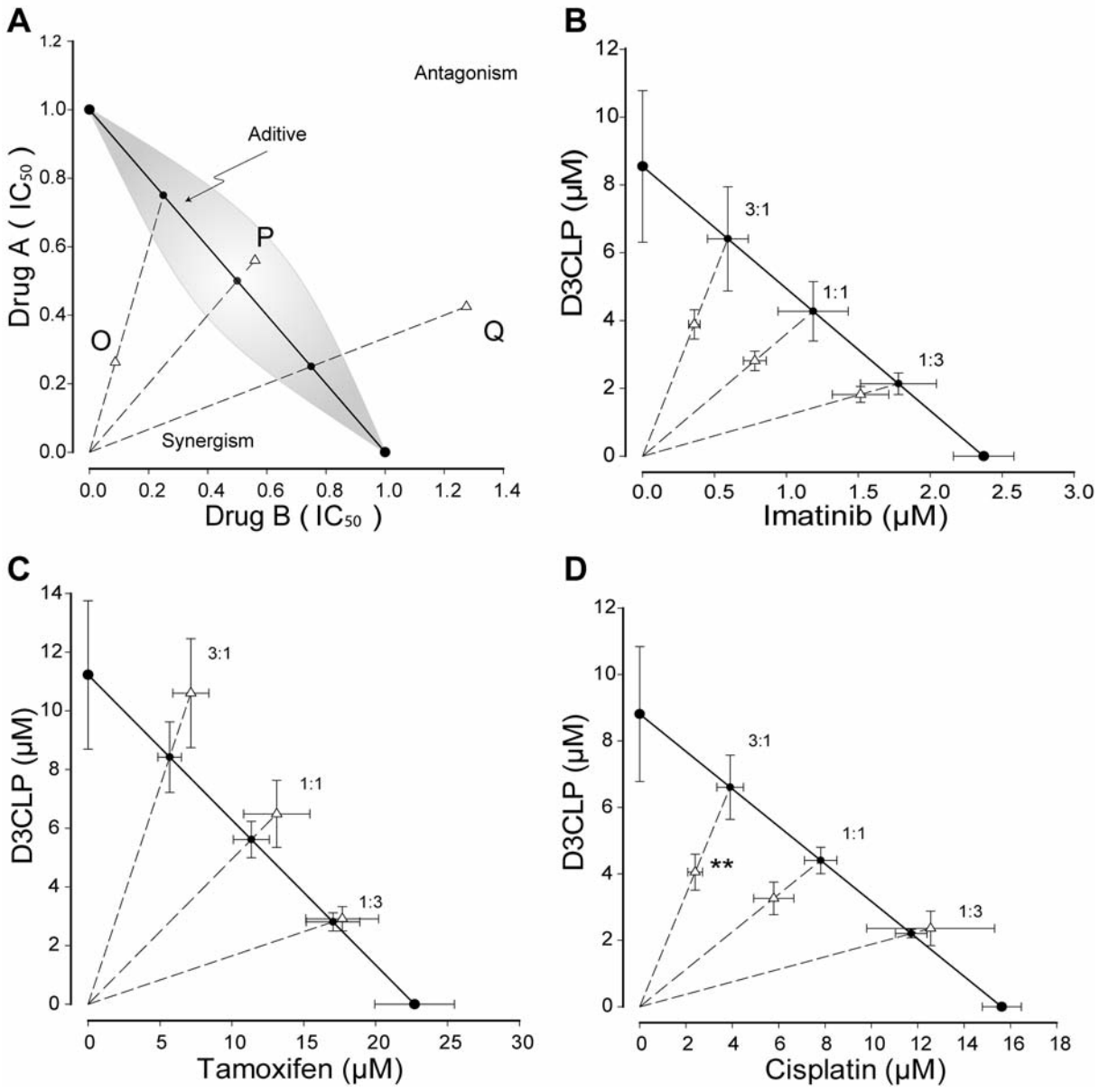


Figure 2. *Isobolograms. Schematic representation of one isobologram; O, P and Q represent synergism, additivity and antagonism, respectively (A), Combined effect of D3CLP plus imatinib in K-562 cells (B), D3CLP plus tamoxifen in MCF-7 cells (C), and D3CLP plus cisplatin on HeLa cells (D). Data represent the mean±SEM. **p<0.05 as compared with the theoretical additive value.*

were performed in several cell lines with different origin. Since D3CLP induces cell death in a diversity of cancer cell types, the most sensitive cells were further assessed as a model to determine the effect of combination treatment (data not shown). The combination of D3CLP with imatinib showed a synergistic antiproliferative effect for a 3:1 ratio, moderate synergism for a 1:1 ratio, and moderate antagonism for a 1:3 ratio, in K-562 cells (Table II). Treatment of MCF-7 breast cancer cells, which express estrogen receptors, with D3CLP plus tamoxifen induced an antagonistic antiproliferative effect at all proportions evaluated. Finally, the antiproliferative effect of D3CLP combined with cisplatin produced a synergistic effect at a 3:1 ratio and an additive effect for the 1:1 and 1:3 ratios, in HeLa cells, by CI (Table II).

Isobolographic analysis of the combination of D3CLP plus imatinib in K-562 cells showed an effect below the additive line for all proportions evaluated (Figure 2B). However, since there was no statistical difference between the experimental and theoretical $IC_{50}$ for the combination of these drugs, this

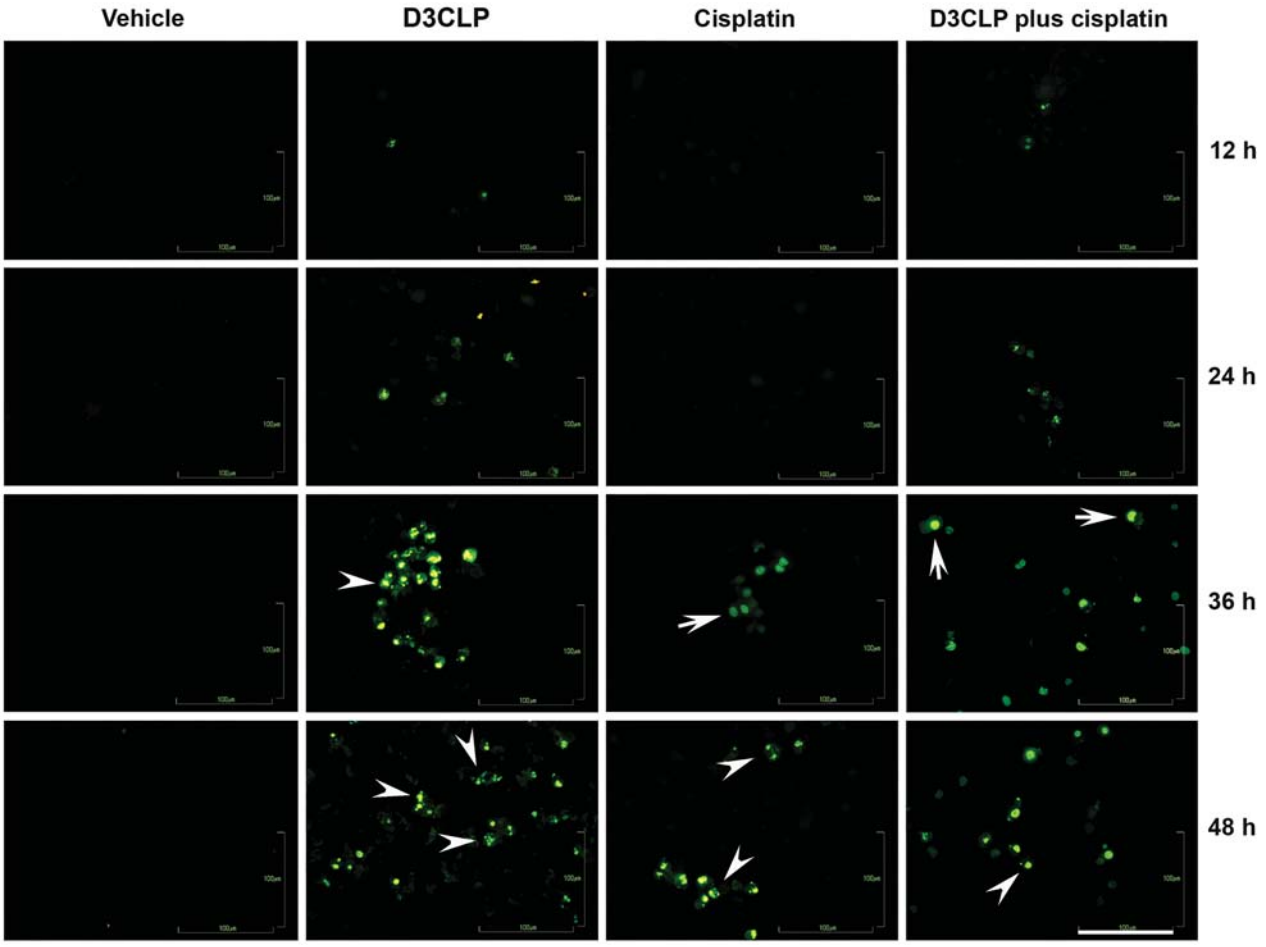


Figure 3. *Drug-induced DNA fragmentation. The treatment of HeLa cells with D3CLP, cisplatin or their combination in a 3:1 ratio produced TUNEL-positive cells with chromatin condensation (arrows) and fragmented nuclei (arrowheads). Scale bar=100 μm.*

indicates that the observed effect for all of the tested proportions was an additive one. In the same manner, treatment of MCF-7 cells with D3CLP plus tamoxifen (Figure 2C) also demonstrated an additive effect. All the evaluated proportions of D3CLP with tamoxifen led to an effect above the additive line, but there was no statistical difference between the experimental and theoretical $IC_{50}$ for each analyzed proportion to indicating an additive effect. In contrast, treatment of HeLa cells with D3CLP plus cisplatin at a 3:1 ratio, showed a significant effect below or in the additive line, indicating a synergistic effect; the effect of the other proportions were below the additive line, thus additive effects were observed (Figure 2D).

*DNA fragmentation after drug exposure of HeLa cells.* To explore the mechanism of cell death in HeLa cells after treatment with D3CLP or its synergistic combination with cisplatin, we assessed DNA fragmentation levels by the TUNEL assay. D3CLP induced early DNA fragmentation in HeLa cells as shown by a significant number of TUNEL-positive cells at 12 h, which was even higher at 36 and 48 h (Figure 3). In contrast, cisplatin treatment induced TUNEL-positive HeLa cells, which were only evident 36 h after treatment. Concerning the combination of D3CLP and cisplatin at 3:1 ratio, positive cells were detected at 12 h, in a similar manner as in the treatment with D3CLP-alone, in the same cell line (Figure 3). In addition to the presence of TUNEL-positive cells, nuclear fragmentation was also observed, which has been widely associated with apoptotic cell death.

## Discussion

It has been well-established that anticancer drugs such as imatinib, which is a specific inhibitor of the BCR/ABL fusion tyrosine kinase (18-21), is an agent specifically active againts

leukemia, and was used in the present study against K-562 leukemia cells. On the other hand, the antiestrogen tamoxifen was used on MCF-7 mammary cancer cells, sensitive to antiestrogen therapy (22-24); and finally, we used the inducer of DNA intra-strand adducts, cisplatin which exerts important wide antineoplastic activity (25, 26), towards HeLa cells (27). In the present study, these drugs were used for combination assays with D3CLP. The results demonstrate that selected combinations exert substantive synergistic effects. Indeed, the combination analysis of D3CLP and these antineoplastic drugs indicate a synergistic effect, as demonstrated by CI for D3CLP plus imatinib on K-562 leukemia cells at 3:1 and 1:1 proportions, and for D3CLP with cisplatin at 3:1 ratio. In contrast, other tested combinations of D3CLP were from additive to sub-additive (antagonistic) (Table II). To further evaluate the anticancer drug activity of our combinations, we used an isobolographic approach, and the results confirmed a synergism for the combination of D3CLP plus imatinib at 3:1 ratio, which was in agreement with its CI for the same ratio. In spite of this effect not being statistically significant, these observations support the idea that these compounds may exert enhanced anticancer activity when combined at other ratios.

On the other hand, the combination of D3CLP with tamoxifen induced a sub-additive activity and, apparently, it was ineffective in various combinations used in this study, thus suggesting that the anti-hormonal activity of tamoxifen and D3CLP combination, may not be of potential clinical anticancer use.

Interestingly in our study, the treatment of HeLa cells with D3CLP plus cisplatin at 3:1 ratio exhibited a super-additive and statistically significant effect, indicating that this combination was synergistic. In addition, as has been shown, D3CLP is a compound that may induce cell death with an $IC_{50}$ at the range of concentrations used as a chemotherapeutic agent, at micromolar doses (28). Furthermore, it displays low toxicity towards control non-tumoral cells (5, 7), thus indicating that this compound may be a good candidate for different combination of anticancer drug studies in animal models of cervical cancer.

Synergistic effects of D3CLP observed in this study are in line with previous reports that indicate that synergistic and additive effects may occur when imatinib (29-31), tamoxifen (32-34), and cisplatin (13, 35-36), are administered in combination with other drugs in different cell lines.

The results of the DNA fragmentation assays indicate that treatment of HeLa cells with D3CLP induces morphological and biochemical changes suggestive of apoptosis. After 12 h of treatment, we observed the appearence of TUNEL-positive cells and a marked increase of apoptotic cells at 36 h for the combined treatment, compared with the cisplatin treatment-alone. This result is in line with our previous report which demonstrated that D3CLP was capable of inducing significant apoptotic cell death in K-562 cells (7). Moreover, it has been demonstrated that treatment of HeLa cells with cisplatin induced DNA fragmentation through active effector caspases, and apoptosis (37, 38). The observation that D3CLP and cisplatin differ in the time of cell death induction suggests a different target by which D3CLP induces cell death, and further validates the use of the equation for the mixture of mutually exclusive drugs in the CI, described by Chou and Talalay (9), as was the case for the combination of cisplatin and D3CLP. Additionally, for the combination of D3CLP with cisplatin at the 3:1 ratio, similar TUNEL staining and histomorphological characteristics have been reported for D3CLP in K-562 cells (7). Our data suggest that treatment of HeLa cells with D3CLP can induce apoptotic death and its combination with cisplatin enhances a similar type of cell death.

## Conclusion

This study demonstrated the *in vitro* cytotoxic synergism of D3CLP and cisplatin towards HeLa cells at a 3:1 ratio, based on the $IC_{50}$, confirmed by both CI and isobolographic analysis. The overall results indicate that D3CLP in combined preparation is a good candidate for preclinical studies and could be useful in the treatment of different carcinomas, including cervical carcinoma.

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