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# VIOLACEIN CYTOTOXICITY AND INDUCTION OF APOPTOSIS IN V79 CELLS

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## SUMMARY

Violacein, a pigment produced by *Chromobacterium violaceum*, is reported to be a potential drug for the treatment of Chagas' disease. Violacein is also effective against leukemia and lymphoma cells in culture ($IC_{50}$ $10^{-8}$ *M*). Changes in the nuclear acid content, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide reduction and neutral red uptake in these cells were used to evaluate the cytotoxicity of violacein in V79 Chinese hamster (M-8) fibroblasts. Violacein was highly cytotoxic to V79 fibroblasts ($IC_{50}$ 5–12 μ*M*). Using the TUNEL method and the Feulgen reaction coupled to image analysis, violacein (5 and 10 μ*M*) was found to trigger apoptosis but not necrosis in V79 cells. The morphological changes seen in the nuclei of these cells included chromatin condensation and a decrease in deoxyribonucleic acid content. These results demonstrating that violacein induces apoptosis in V79 cells strengthen its potential as a therapeutic agent.

*Key words:* neutral red; MTT; fibroblasts; apoptosis.

## INTRODUCTION

The current therapy for Chagas' disease is unsatisfactory and only benznidazole is widely used. However, because of its toxicity, this drug is prescribed only for chronically ill patients. Violacein, a pigment produced by *Chromobacterium violaceum*, is a potential drug for the treatment of Chagas' disease (Haun et al., 1992). However, little is known of the mechanism by which violacein causes cell death. In the study, we examined the cytotoxicity of violacein in fibroblasts and the mechanism that leads to their death.

Numerous in vitro assays have been developed as alternatives to testing for toxicity in vivo. In vitro tests are generally rapid, sensitive, amenable to automation, and economical when compared to the use of animals. Different endpoints have been used to assess cytotoxicity in vitro, including the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT), neutral red uptake (NRU), and nuclear acid content (NAC). These endpoints generally assess different aspects of cellular functions. The reduction of MTT assesses the functional intactness of mitochondria based on the enzymatic reduction of a tetrazolium salt by the mitochondrial dehydrogenase of viable cells (Denizot and Lang, 1986; Loveland et al., 1992). NRU is a measure of lysosomal integrity since it reflects the capacity of viable cells to incorporate vital dye into these organelles (Renzi et al., 1993; Reppetto and Sanz, 1993). Finally, assay of nucleic acid content evaluates the total cellular material as an indication of total cell number (Cingi et al., 1991; Haun et al., 1992).

Cell death may occur by apoptosis or necrosis, depending on the stimulus, its intensity, and cell type. Apoptosis represents the terminal morphological and biochemical events in programmed cell death (Wyllie, 1997) and was first recognized on the basis of the distinctive morphology it produces (Kerr et al., 1972; Wyllie et al., 1981), which differs from the morphological and biochemical changes associated with necrosis. Apoptosis is currently the subject of intense research because of its occurrence during neoplasm in response to cancer chemotherapy and radiation. A complete understanding of the molecular control of this phenomenon should contribute to the development of new therapies for many diseases (Wyllie, 1997), including Chagas' disease as pointed out before.

## MATERIAL AND METHODS

*Compound.* Violacein was isolated and purified from *C. violaceum* as described by Antônio (1994) and Durán et al. (1994). Violacein was dissolved in absolute ethanol, with the final concentration of ethanol not exceeding 0.1% in the cell culture medium.

*Antitumoral activity of violacein.* The antitumor assays were carried out by the National Cancer Institute (NCI) through the European Organization for Research and Treatment of Cancer-New Drug Development Office (EORTC-NDDO Registration no. E95/255, NSC 681000), Amsterdam (The Netherlands).

The acquired immunodeficiency syndrome (AIDS)-related lymphoma (ARL) screen uses parameters similar to those of the antitumor drug screen which has been used for several yr by the National Institute of Health and by NCI. The ARL screen uses five human lymphoma cell lines, which grow in suspension (two of them established from AIDS patients), and a leukemia cell line (CCRF-CEM) which is included in the antitumor screen. The cytotoxicity of violacein was tested by culturing the cells with a maximum concentration of $1 \times 10^{-4}$ *M* of the compound for 48 h. At the end of this period, cell viability was determined indirectly using the fluorescent dye propidium iodide in an assay improved for single cells or a clustered cell suspension (Sasaki et al., 1987; Ellwart and Dörmer, 1990; Seidl et al., 1999)

*NCI cell line panel.* The antitumoral activity of violacein was assayed at the same concentrations as above in 54 tumors: 6 leukemia, 9 non–small cell

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lung cancer, 7 colon cancer, 7 melanoma, 4 ovarian cancer, 7 renal cancer, 2 prostate cancer, and 7 breast cancer cell lines (Hendriks et al., 1993).

*Cell culture and cytotoxicity assays.* V79 Chinese hamster lung fibroblasts, an M-8 clone generously supplied by Dr. R. Meneghini from the University of São Paulo (São Paulo, SP, Brasil), were routinely grown in Dulbecco modified Eagle medium containing antibiotics (100 U/ml penicillin G; 100 μg/mL streptomycin) and supplemented with 10% fetal calf serum, in a 5% carbon dioxide-humidified atmosphere, at 37° C (Renzi et al., 1993). The cultures were routinely stained with Hoechst 33258, a fluorescent dye, which binds specifically to deoxyribonucleic acid (DNA) (Chen, 1977) to verify possible mycoplasma contamination of cell culture. For the MTT, NAC, and NRU assays, 24-well tissue culture plates were inoculated with $3 \times 10^4$ cells/ml and incubated at 37° C for 48 h. The cells were then incubated for 24 h with 2–30 μ*M* violacein, a concentration range which produced 0–100% cell death (Cingi et al., 1991).

For the NRU assay, V79 fibroblasts ($3 \times 10^4$ cells/ml) were seeded into a 24-well plate and incubated for 48 h. The culture medium was removed and substituted with fresh medium containing violacein (2–30 μ*M*) followed by a further incubation for 24 h. After this period, the culture medium was replaced by fresh medium containing neutral red dye (50 μg/ml). The neutral red solution was incubated alone for 12 h at 37° C and filtered through Millipore membranes (0.22 μm) prior to testing. After 3 h of incubation with neutral red, the cells were washed at 37° C with phosphate-buffered saline containing calcium (PBS-$Ca^{2+}$) in order to eliminate excess dye. One milliliter of 1% glacial acetic acid–50% ethanol was then added to each well to fix the cells and extract the neutral red incorporated into the lysosomes. The plates were shaken for 20 min on a plate shaker and the absorbance measured at 540 nm (Riddell et al., 1986; Renzi et al., 1993).

For the MTT assay, V79 fibroblasts ($3 \times 10^4$ cells/ml) were seeded onto a 24-well plate and incubated for 48 h. The culture medium was removed and substituted by medium containing violacein (2–30 μ*M*) followed by incubation for a further 24 h. This medium was then removed and 1 ml of MTT solution (1 mg MTT/ml in culture medium) was added to each well. After incubation for 5 h at 37° C, the medium was removed and the formazan solubilized in 1 ml of ethanol. The plate was shaken for 20 min on a plate shaker and the absorbance measured at 570 nm (Mosmann, 1983; Denizot and Lang, 1986).

For determining the NAC, V79 fibroblasts ($3 \times 10^4$ cells/ml) were seeded onto a 24-well plate and incubated for 48 h. The culture medium was removed and substituted with new medium containing violacein (2–30 μ*M*) followed by incubation for another 24 h at 37° C. The cells were then washed twice with cold PBS and the soluble nucleotide pool was extracted with cold ethanol. The cell monolayer was solubilized with 1 ml of 0.5 *N* NaOH at 37° C for 1 h. The absorbance of the solution in each well was measured at 260 nm and the results expressed as a percentage of the controls, $A_{260\ nm}$ (Cingi et al., 1991).

*Recovery assay.* For the recovery assay, 24-well plates were inoculated with $2 \times 10^4$ cells/well followed by incubation for 24 h at 37° C in a humidified 5% $CO_2$ atmosphere. After this period, the medium was replaced by one containing different concentrations of violacein for another 24 h. This medium was subsequently replaced by fresh medium and the cells incubated for 24, 48, and 72 h. After two washings with cold PBS, the soluble nucleotide pool was extracted with cold ethanol and the cell monolayer was digested with 0.5 *N* NaOH at 37° C for 1 h. The absorbance of the solution in each well was measured at 260 nm and the results expressed as a percentage of the control ($A_{260\ nm}$) (Cingi et al., 1991). The cells had reached subconfluency at the end of the assay.

*Detection of apoptosis.* V79 cells ($10^5$ cells/ml) were plated on coverslips in Petri dishes (90-mm diameter) and incubated at 37° C for 48 h. After this period, the cells were treated for 24 h with 5 or 10 μ*M* violacein in order to assess whether violacein triggers apoptosis.

*Cell fixation and staining.* Fibroblasts were fixed with ethanol:acetic acid (3:1, v/v) for 1 min, rinsed in 70% ethanol for 3–5 min, and air-dried. The Feulgen reaction was carried out after the hydrolysis of violacein-treated cells for 90 min with 4 *N* HCl as described by Mello (1997). Treated samples were processed by the TUNEL methods of Gravieli et al. (1992) and Rehen et al. (1996). Briefly, treated cells were washed in PBS buffer and then incubated in terminal deoxyribonucleotidyl transferase (TdT) buffer at room temperature for 20 min. This was followed by incubation in TdT buffer containing 0.5 U of TdT/μl, 0.2 nmol of deoxy adenosine triphosphate (dATP)/μl, and 0.013 nmol of biotin-14-dATP/μl for 18 h at 37° C. All materials were from GIBCO BRL. The slides were washed with PBS and stained using the ABC system (Vector). Methyl green was used as a counter stain.

TABLE 1

INHIBITION OF CELL GROWTH IN VITRO BY VIOLACEIN

| Cell origin | Cell line | Concentration(*M*) $GI_{50}$[a] |
|---|---|---|
| Leukemia | MOLT-4 | $5.60 \times 10^{-8}$ |
| | CCRF-CEM | $1.76 \times 10^{-7}$ |
| Lymphoma | RL | $1.20 \times 10^{-7}$ |
| | KD488 | $2.00 \times 10^{-7}$ |
| | AS283 | $3.21 \times 10^{-7}$ |
| | PA682 | $1.38 \times 10^{-7}$ |
| | SU-DHL-7 | $3.13 \times 10^{-7}$ |
| Large cell lung | NCI-H460 | $3.10 \times 10^{-8}$ |
| Colon | KM12 | $6.00 \times 10^{-8}$ |

[a] $GI_{50}$: concentration inhibiting growth by 50% (growth inhibition 50%).

*Image analysis.* Image analysis is a useful tool to detect DNA content and compactness in chromatin areas. Cells in the apoptosis process should show high values of chromatin condensation and lesser DNA content in a smaller nuclear area. To detect such apoptosis alterations, we used the following system. Video image analysis cytometry was carried out using a Global Lab Image System (Data Translation, Inc) and a Zeiss photomicroscope equipped with a Pol-Neofluoar 25/0.60 objective, 1.25 optivar, 1.4 condenser, at an emission wavelength of 546 nm. A 12-V/60-W lamp was used as the light source. The images to be processed were fed from the microscope to the computer via a monochrome charge-coupled device (JVC) video camera. Conversion of pixels to micrometers was done using a micrometer slide. The conversion of gray levels to absorbance or transmittance was done using the Minitab program. Nuclear areas (S), optical densities (OD), and integrated optical densities (IOD or, in this case, Feulgen-DNA values) were chosen from several descriptors provided by the software (Vidal, 1997). Parametric (analysis of variance) and nonparametric (Mann–Whitney) (MW) statistical tests were applied in order to find out the significance of the obtained values.

## Results

Table 1 summarizes some of the data reported by the National Cancer Institute Developmental Therapeutic Program in vitro tests. Table 1 shows that violacein is very cytotoxic to leukemia and lymphoma cells. The most sensitive cells were from a leukemia cell line (Molt-4 $GI_{50}$ $5.6 \times 10^{-8}$ *M*), a non–small cell lung cancer NCI-H460 cell line ($GI_{50}$ $3.1 \times 10^{-8}$ *M*), and a colon cancer cell line (KM12; $GI_{50}$ $6.0 \times 10^{-8}$ *M*). The cytotoxicity of violacein was further characterized in V79 fibroblasts. One assay of cell viability (CV) was based on the NAC of V79 fibroblasts exposed to different concentrations of violacein. The CV test measures the "killing capacity" of a chemical, a 24-h exposure to violacein was sufficient to adversely affect V79 cell viability, causing detachment and loss of cells (Fig. 1). The $IC_{50}$ value was estimated to be 5 μ*M*. Violacein inhibited NRU with an $IC_{50}$ of 12 μ*M* (Fig. 1) twofold greater than the toxicity measured by NAC. MTT reduction decreased progressively with increasing violacein concentration (Fig. 1) with an $IC_{50}$ of 7 μ*M*. This assay is a measure of mitochondrial performance, particularly of succinate dehydrogenase activity (Fry et al., 1995). Incubation of V79 fibroblasts with 2–10 μ*M* violacein for 24–72 h resulted in dose- and time-dependent inhibition of cell growth (Fig. 2). A slight recovery in survival (28%) was observed after 72 h at a violacein concentration of 7 μ*M*. It could be caused by a combination of events including proliferation, necrosis, apoptosis, or just altering morphology (disturbance of cytoskeleton), which are

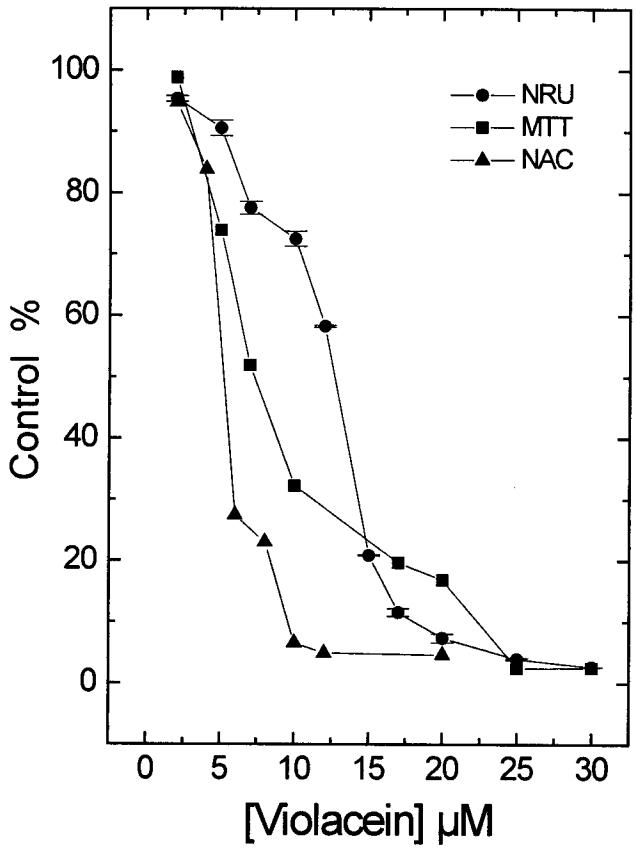


FIG. 1. Inhibitory action of violacein on cell viability based on the NRU assay (NRU, $A_{540\ nm}$), MTT reduction (MTT, $A_{570\ nm}$), and NAC assay (NAC, $A_{260\ nm}$) of V79 cells. The inhibition is expressed relative to the cell viability (100%). The *point* represents the mean ± SD of at least three experiments run in quadruplicate. In some cases, the *error bars* are smaller than the *symbols*.

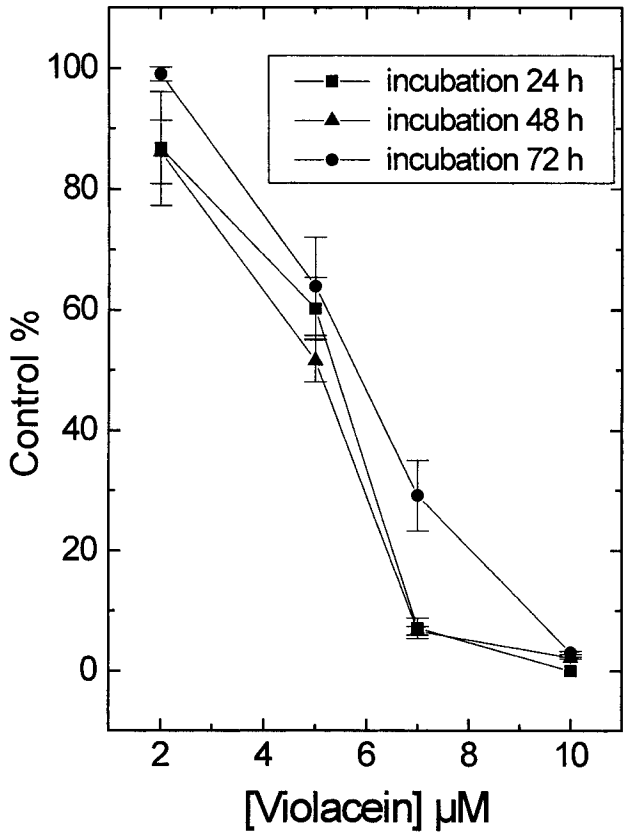


FIG. 2. Antiproliferative action of violacein in V79 fibroblasts. A slight recovery in proliferation was seen after 72 h with 7 μ*M* violacein.

enough to loosen or detach cells without killing them. Nevertheless, this assay measures a change in cell mass (total NAC). The capacity of growing implies survival; therefore, this assay does not measure growth or survival per se, since the total NAC analyzes equivalent parameters to growth and survival. Based on the results in Fig. 2, violacein concentration of 5 and 10 μ*M* were used to examine the effect of this compound on chromatin condensation and typical morphology of cells in the process of dying. At both concentrations, the fibroblast nuclei showed morphological changes similar to those seen during apoptosis, when compared to morphology of control cells and after Feulgen reaction (Fig. 3*a–d*). This was confirmed by TdT-labeling (Fig. 3*e*). Table 2 summarizes the results of image analyses and shows that violacein-treated fibroblasts had a high level of chromatin condensation and a lower DNA content than nontreated cells. These events were accompanied by a decrease in nuclear area.

## DISCUSSION

Several factors can interfere with chemical cytotoxicity including concentration of chemical agent, length of exposure to chemical agent, cell density and type. Duration of exposure (*T*) and drug concentration (*C*) are related, although $C \times T$ is not always a constant. Longer exposures can increase sensitivity beyond that predicted by $C \times T$ due to cell cycle effects and cumulative effect from the agents. Its is especially reasonable if the mechanism of toxicity is slow (apoptotic) rather than immediate (acute necrosis). The tumoral cells in the NCI cell line panel were carried out in different conditions (e.g., 48 h of drug contact; cell viability was measured indirectly by propidium iodide technique) when compared to those in V79 cell cultures where the cytotoxicity was measured (e.g., 24 h of drug contact; DNA content was measured by absorption at 260 nm and also cytotoxicity was followed by NRU and MTT endpoint techniques). The only comparable parameter was the concentration range that was used. The high cytotoxicity observed in this study indicated that violacein would be a potential antitumoral drug, NCI in vitro screening showed that the potency of violacein was of an intermediate value. In general, a $GI_{50} < 3 \times 10^{-8}$ *M* is of sufficient potency for referral (values below $10^{-9}$ *M*). A balance between the therapeutic versus toxicological effects of a compound is an important parameter when verifying its applicability as a trypanocide and/or as an antitumoral drug. Cell culture can be used to evaluate basal cytotoxicity (Ekwall and Ekwall, 1988) and target organ toxicity (Balls and Fentem, 1992) and in some cases, may provide information on the lethal dose in vivo (Shrivastava et al., 1991). We used V79 fibroblasts because this cell line is well characterized and commonly used in mutagenicity and toxicity studies (Cingi et al., 1991). The NRU assay indicates lysosomal integrity and reflects the ability of viable cells to concentrate dye in their

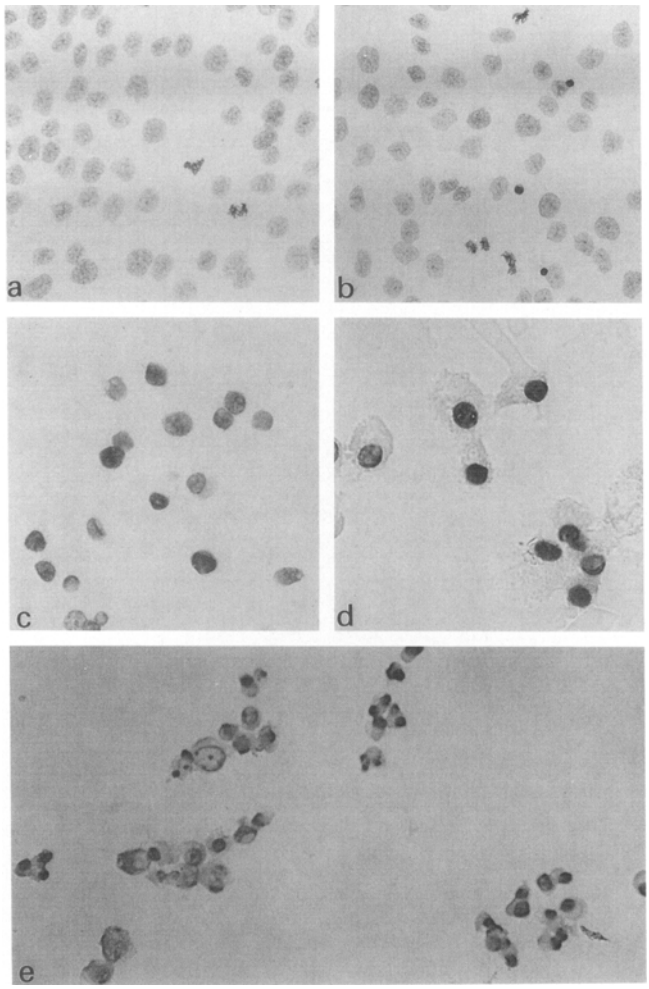


FIG. 3. V79 cells submitted to Feulgen reaction (*a–d*) and TUNEL method (*e*). (*a*) Control cells, (*b*) cells treated with 0.1% methanol, (*c*) and (*d*) 5 and 10 μ*M* violacein-treated cells, respectively, and TdT-labeling in V79 cells treated with 5 μ*M* violacein. Magnification: *a*, *b*, *e*, ×350; *c*, *d*, ×570.

TABLE 2

RESULTS OF THE IMAGE ANALYSES OF V79 FIBROBLASTS IN THE ABSENCE AND IN THE PRESENCE OF VIOLACEIN. NUMBER OF CELLS ANALYZED, 113. IMAGE ANALYSES PARAMETERS ARE EXPRESSED IN MEAN ± SD

| Descriptor | | Mean ± SD | Median | Test |
|---|---|---|---|---|
| IOD | Control | 19.6 ± 6.1 | 17.2 | ANOVA |
| | Violacein | 16.1 ± 6.6 | 15.9 | ANOVA |
| OD | Control | 0.23 ± 0.05 | 0.2 | MW |
| | Violacein | 0.083 ± 0.37 | 0.7 | MW |
| Nuclear area | Control | 84.9 ± 17.9 | 83.3 | ANOVA |
| | Violacein | 22.0 ± 10.8 | 20.8 | ANOVA |

SD, standard deviation; AVOVA, one-way analysis of variance; MW, Mann–Whitney Text; IOD, integrated optical density (Feulgen-DNA); OD, optical density.

lysosomes (Babich and Borefreund, 1990). There is a linear correlation between the amount of neutral red extracted from cultured cells and the number of viable cells (Borefreund and Puerner, 1984; Reppeto and Sanz, 1993). Alterations in cell proliferation can therefore influence the results of this assay. The uptake of neutral red by viable cells is modified by alterations in the cell surface or lysosomal membrane, thus making it possible to distinguish between viable, damaged, or dead cells, based on their specific lysosomal capacity for dye uptake. This weakly cationic dye penetrates cell membranes by nonionic passive diffusion, and concentrates in the lysosomes, where it binds by eletrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix (Reppeto and Sanz, 1993; Sousa et al., 1996). This technique has been extensively used in drug analysis because it is sensitive, simple, economic, and reproducible. Violacein had an $IC_{50}$ value of 12 μ*M*, which is close to that of recently described amphiphilic trypanocide (De Conti et al., 1998). NAC has also been widely used as an effective endpoint for cytotoxicity. The $IC_{50}$ of violacein in this test (5 μ*M*) was half of that obtained for 2-propene-1-amine ($IC_{50}$ 10.8 μ*M*) (De Conti et al., 1998).

Incubation of V79 fibroblasts with violacein resulted in apoptosis. Image analysis revealed that the cytometric descriptors were different for violacein-treated cells when compared to the control cells. The onset of apoptosis in V79 fibroblasts was independent of drug concentration since a positive immunocytochemical reaction was obtained with 5 and 10 μ*M* violacein. The apoptotic process was represented by chromatin condensation, loss of DNA content, and decrease in cell size. Since the TUNEL method used to detect apoptosis is relatively nonspecific (Labat-Moleur et al., 1998), the results of this test were complemented with image analysis after the Feulgen reaction. In particular, image analysis was able to show important changes in chromatin supraorganization and in amounts of DNA during violacein-induced apoptosis. No necrosis was seen at the highest concentration of violacein tested even through this concentration was greater than the $IC_{50}$ for V79 fibroblasts.

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