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Biological Activities of Violacein, a New Antitumoral Indole Derivative, in an Inclusion Complex with β-Cyclodextrin

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Abstract. Violacein is a poorly water-soluble antitumoral and antibacterial drug. The solubility can be enhanced by complexation with β-cyclodextrin. The inclusion complex was prepared by the co-precipitation method in molar ratios of 1 : 1 and 1 : 2 of violacein/β-cyclodextrin, respectively. The acute toxicity (*E. coli* strain) of violacein did not change up to 400 μM, either in the presence or absence of cyclodextrin. Cytotoxicity (V-79 cell culture) through DNA and MTT assays was significantly decreased in the presence of the 1 : 2 molar ratio complex. Studies on erythrocyte lipid peroxidation by the thiobarbituric acid (TBA) method showed that violacein and violacein/β-CD (1 : 2) at 100 μM cause 50% and 80% inhibition, respectively. At 500 μM the violacein/β-CD complex inhibited lipid peroxidation completely; however, with free violacein only 65% inhibition was reached at that concentration.

Key words: inclusion complex, violacein, β-cyclodextrin, toxicity, cytotoxicity, antioxidant.

1. Introduction

The natural cyclodextrins (CDs) have been extensively studied to improve certain properties of drugs, such as solubility, stability, and/or bioavailability [1–6]. The enhancement of drug activity or the reduction of side effects can be achieved by inclusion complex formation. Recent studies show that cyclodextrin complexation results in enhanced drug delivery to the membranes and, consequently, enhanced drug bioavailability [7]. Formulations using Glibenclamide/β-CD [8], Griseofulvin [9] and Clotrimazole [10] improved their biological activities due to their solubility

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Violacein (1)

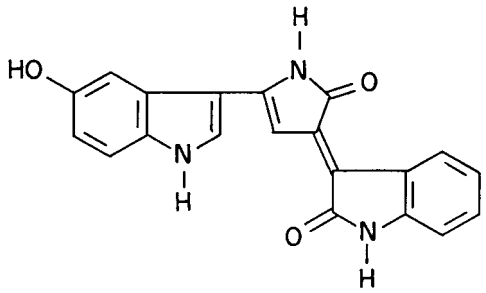

Figure 1. The violacein structure.

by complexation with β-CD. Very few studies relating to indole derivatives and inclusion complexes with β-CD have been published [11, 12].

The formation of inclusion compounds between violacein and β-CD was studied by diffusion measurements and circular dichroism. The results of the two experiments were in good concordance with the formation of 1 : 1 and 1 : 2 complexes. The size of molecules involved was monitored using diffusion coefficient and circular dichroism measurements and computational calculations indicated a preference for the inclusion of the most polar part of the molecule to form the 1 : 2 inclusion complex [13].

Violacein (3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene]-1,3-dihydro-2H-indol-2-one) (Figure 1) was isolated from the Amazon river, Manaus, Amazon State, in Brazil, characterized and purified as described recently [14].

Violacein exhibited trypanocidal [15, 16], antibiotic [17] and antitumoral activities [18–20]. Its toxicity was also studied [16]. After *in vitro* testing in a panel cell line by the National Cancer Institute Developmental Therapeutics Program, the response parameter IG_{50} (50% growth inhibition) for ovarian cancer IGROV1 (0.62×10^{-8} M), non-small cell lung cancer NCI-H460 (0.58×10^{-8} M) and colon cancer KM12 (0.27×10^{-9} M) were found [18, 20].

Due to several biological activities of violacein and the inclusion complex previously demonstrated with β-CD [13], the aim of the present paper is to study any improvement of its solubility, enhancement of its biological activities as an antioxidant and the diminution of its toxicity through violacein/β-CD inclusion complex formation. The aim is to apply this new fomulation as an antitumor drug and to explore the feasibility of the carrier in reducing the toxicity and enhancing the antitumor efficacy of violacein.

2. Materials

β-Cyclodextrin was purchased from SIGMA Chemical Co. and was used as received. Violacein was prepared and purified as previously published [14].

The violacein/β-CD inclusion complex was prepared as previously published [13] as follows: a stock solution of β-CD (10^{-3} M) was prepared with distilled water and dilutions were made from this stock solution to obtain the different desired concentrations. A stock solution of violacein (10^{-3} M) in 0.003% DMSO in pure ethanol was added to aliquots of the β-CD solution to give the desired final concentration.

3. Methods

3.1. CYTOTOXICITY ASSAYS

(a) *Cell culture*. Chinese hamster V-79 lung fibroblasts were grown as previously reported [15, 21, 23].

(b) *Cell growth*. Cells were seeded at 3×10^4 cells/mL in the wells of 24-well plates and incubated at 37 °C in a 5% CO_2 humidified atmosphere. Forty-eight hours later, semiconfluent cultures were exposed to the test at eight different concentrations ranging from 0 to 5 μM for violacein or the complex with β-CD. Control experiments with the same amount of β-CD in the 0 to 5 μM range were carried out. The cells were washed twice with PBS solution (the PBS solution contained 0.14 M NaCl; 3.36×10^{-3} M KCl; 8.10×10^{-3} M Na_2HPO_4; 1.84×10^{-3} M KH_2PO_4; 1.26×10^{-3} M $CaCl_2$ and 0.53×10^{-3} M $MgCl_2$; pH = 7.2). The cells were then fixed with trichloroacetic acid and submitted to alkaline hydrolysis (0.5 M NaOH). The absorbance of the lysate was recorded at 260 nm in order to quantify nucleic acid. The IC_{50} corresponds to the drug concentration which caused a 50% decrease of DNA content

(c) *Reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used to assess the mitochondrial dehydrogenase activity of viable cells* [22]. MTT assay: V-79 cells (3×10^4 cells/mL) were seeded in 24 well plates and incubated until semiconfluence. The culture medium was removed and replaced with a medium containing violacein (0–5 μM) or in the presence of β-CD. The cells were incubated for an additional 24 h and the culture medium was removed and replaced with a medium containing 0.5 mg/mL of MTT and incubated for 5 h. The culture medium was removed and 1 mL of ethanol was added to each well to solubilize the formazan formed. The plates were gently shaken for 10 minutes and the absorbance was measured at 570 nm [24].

3.2. ACUTE TOXICITY ASSAY

Escherichia coli (ATCC 25922 strain) was used in the acute toxicity assay and the method for determination of bacterial cytotoxicity by flow injection analysis [25] proved to be a reliable and rapid assessment of the toxicity of the compounds by analyzing the alteration of the amount of CO_2 produced and trapped in the culture medium.

3.3. ANTIOXIDANT CAPACITY ASSAY

Studies on erythrocyte lipid peroxidation were carried out by the thiobarbituric acid (TBA) method. This method measures the malonaldehyde content [26]. The red cells were twice washed in 50 times their volume of buffered saline. After the second washing the packed-cell volume (PCV) was adjusted to 2.5%. The cell suspension was pre-incubated at 37 °C for 1 h in a shaking water bath. The suspending medium was removed by centrifugation and the cells were resuspended in fresh buffered saline. The PCV was adjusted to 1 mM of the basis of hemoglobin estimation. Buffered saline solutions of the same pH were used throughout. The final cell suspension (1 mM Hb) was exposed to tert-butylperoxide (1 mM) for 30 min. The same experiments in different concentrations with violacein (0–600 μM) and in the presence of violacein/β-CD were carried out.

Lipid peroxidation was measured indirectly by measuring malonaldehyde (MDA) concentrations. To 4 mL of cell suspension 2 mL trichloroacetic acid (final conc. 12.5%) was added and the mixture was centrifuged. An aliquot (4 mL) of the supernatant was transferred to a boiling tube fitted with an air condenser and 1 mL of thiobarbituric acid solution (1%) was added. The mixture was placed in a boiling-water bath for 15 min then immediately cooled under tap water. The absorption at 532 nm was measured (ε mM = 156). The malonaldehyde concentrations were expressed in nmol MDA per g hemoglobin [26].

4. Results and Discussion

Chromobacterium violaceum is a gram-negative bacterium found in samples of water and soil from tropical and subtropical regions of the world. Violacein, a major pigment produced by this bacterium, exhibited several biological activities as mentioned before. Growth curves of *C. violaceum* at 10% and 50% of dissolved oxygen tension were obtained [27]. Violacein production per bacterium started and had a peak at the exponential growth phase, with a 3-fold higher production in the 50% than the 10% experiment. In the presence of either catalase or ascorbic acid the violacein production underwent a 2 h delay. These results indicate that violacein was an antioxidant produced by *C. violaceum* for autoprotection against reactive oxygen species that were responsible for cellular oxidative damage [27].

In order to understand this phenomenon, studies on erythrocyte lipid peroxidation by the TBA method were carried out. This study showed that violacein

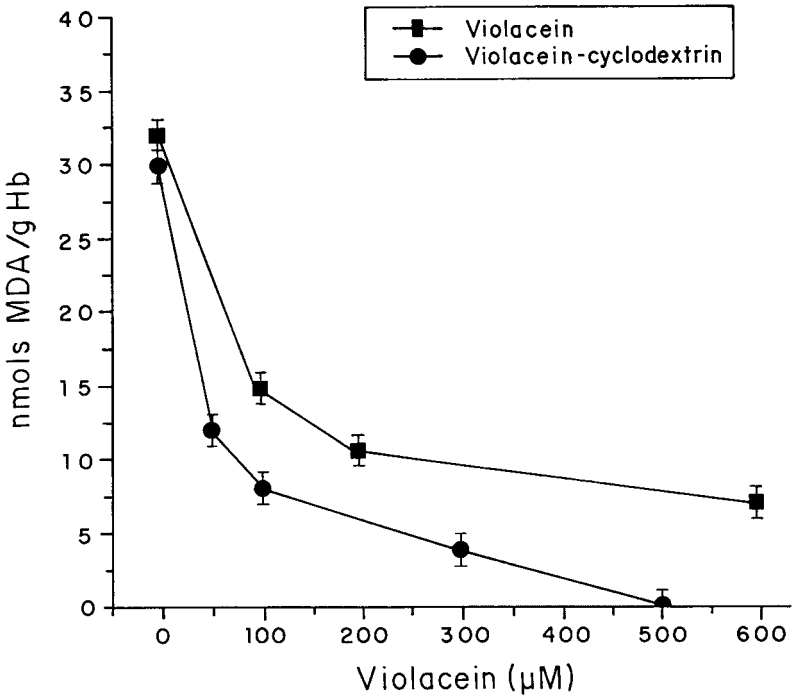

Figure 2. The effect of different concentrations of violacein (—■—) and violacein/β-cyclodextrin (1 : 2) molar ratio (—●—) on tert-butylperoxide (1 mM)-induced autooxidation. Pre-incubation time 30 min, pH 7.4, at 37 °C.

and violacein/β-CD (1 : 2) at 100 μM caused 50% and 80% inhibition of lipid peroxidation, respectively (Figure 2). At 500 μM the violacein/β-CD completely inhibited lipid peroxidation, but with free violacein at this concentration, only 65% inhibition was reached. This shows that β-CD enhanced the antioxidant activity of violacein, which is probably related to the intracellular protection by violacein in *C. violaceum* against oxidative stress [27].

According to previous studies, the IC_{50} values for the V-79 fibroblast culture cells was 1–5 μM [28]. Recently it was studied whether violacein toxicity causes apoptosis or necrosis. The results showed that violacein triggers apoptosis instead of necrosis in V-79 culture cells. The violacein induced morphological changes of the V-79 nuclei reflected chromatin condensation and a smaller DNA content [29]. Then it was important to verify the mechanisms of cytotoxicity of violacein because a therapeutic versus toxicological effect of a compound are important parameters in order to assess the possible pharmacological use as an antitumoral in trypanocide drug.

In order to better understand the toxicity of violacein, the multi-endpoints cytotoxicity and acute toxicity were applied. The reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used to assess the mitochondrial dehydrogenase activity of viable cells [21, 24], and the DNA or protein content is

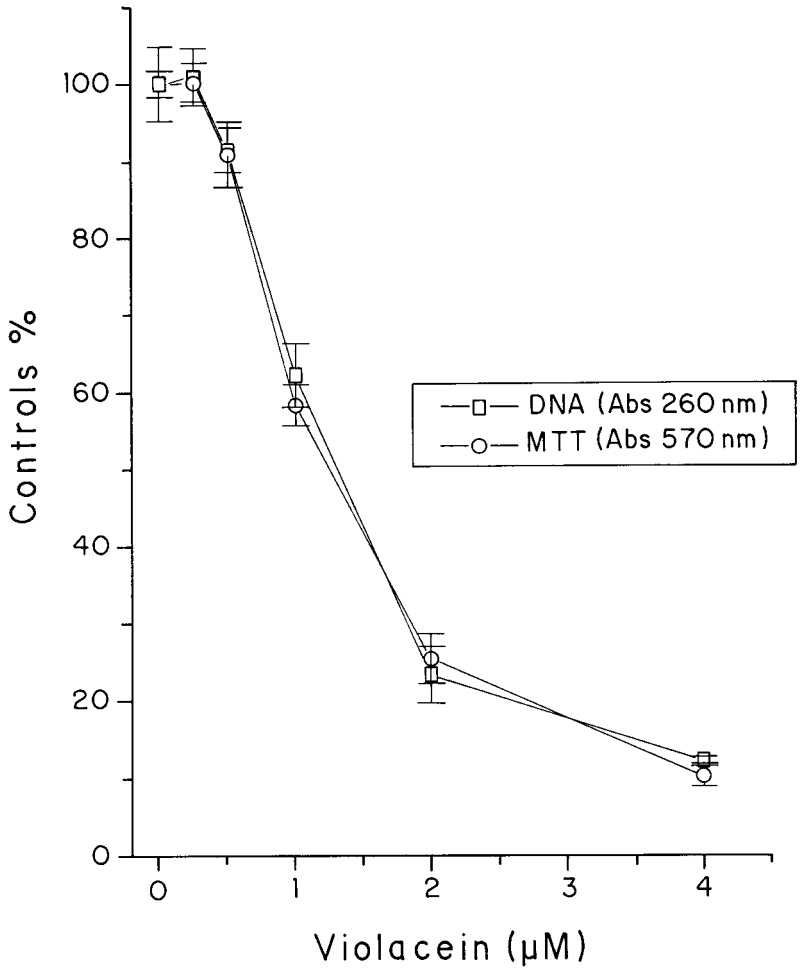

Figure 3. Multi-endpoint cytotoxidity assay: Treatment V79 cells for 24-h with violacein: MTT (—○—) and DNA (—□—) assays.

used to evaluate the content of cellular macromolecules, which are indicative of total cell number.

The DNA content is a useful method to quantify the cellular material. The cellular growth of V79 fibroblast exposed to different concentrations of violacein were measured. Figure 3 shows the dose-response in the DNA content and the IC_{50} value was 1.30 μM in the absence of β-CD. The dose-response of reduction of MTT of violacein by the V79 cells is represented in Figure 3. This figure shows that the IC_{50} values for violacein was 1.23 μM, identical with the DNA values mentioned above.

The cytotoxicity of 1.2 μM violacein was not modified when the molar ratio of violacein/β-CD was 1 : 1, but was significantly decreased in a molar ratio of 1 : 2 (Figure 4).

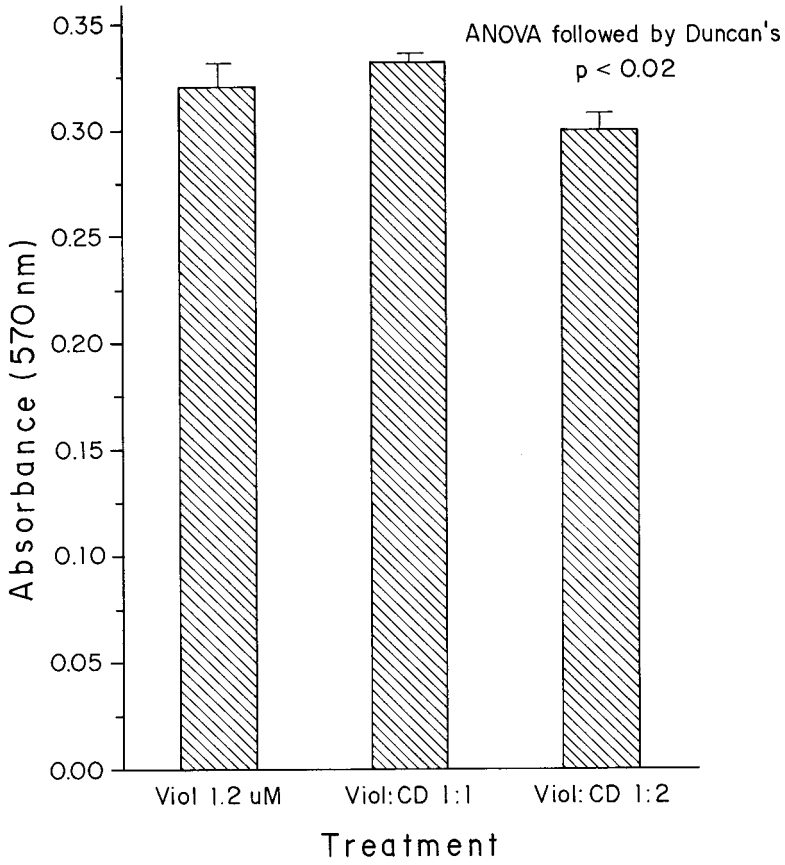

Figure 4. Cytotoxicity of 1.2 μM violacein in a free form, molar ratio of 1 : 1 and 1 : 2 with β-cyclodextrin (from left to right).

The acute toxicity of the violacein against *E. coli* was very low (around 10%) at 400 μM either in the absence or presence of a 1 : 1 molar ratio of violacein/β-CD, similar to that observed in the V-79 culture cell in the cytotoxicity experiments. It seems to us that violacein exhibited a very specific effect in different bacterial strains as previously observed in *Mycobacterium tuberculosis* H37Ra which exhibited minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 180 μM and 373 μM, respectively [30]. The same selectivity was observed when compared with fibroblast in this paper.

Thus the study of the violacein/β-CD complex resulted in a reduced cytotoxicity and better solubility of the violacein. The exact pharmacokinetics of the complex in terms of systemic absorption may have to be further confirmed by bioavailability studies and studies to determine whether the complex enhances the antitumor activity is currently in progress.

Acknowledgements

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