Cytotoxicity and Potential Antiviral Evaluation of Violacein Produced by *Chromobacterium violaceum*

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Natural products are an inexhaustible source of compounds with promising pharmacological activities including antiviral action. Violacein, the major pigment produced by Chromobacterium violaceum*, has been shown to have antibiotic, antitumoral and anti-*Trypanosoma cruzi *activities. The goal of the present work was to evaluate the cytotoxicity of violacein and also its potential antiviral properties.The cytotoxicity of violacein was investigated by three methods: cell morphology evaluation by inverted light microscopy and cell viability tests using the Trypan blue dye exclusion method and the MTT assay. The cytotoxic concentration values which cause destruction in 50% of the monolayer cells (CC_{50}) were different depending on the sensitivity of the method. CC_{50} values were ≥ 2.07 ± 0.08 µM for FRhK-4 cells: ≥ 2.23 ± 0.11 µM for Vero cells; ≥ 2.54 ± 0.18 µM for MA104 cells; and ≥ 2.70 ± 0.20 µM for HEp-2 cells. Violacein showed no cytopathic inhibition of the following viruses: herpes simplex virus type 1 (HSV-1) strain 29-R/acyclovir resistant, hepatitis A virus (strains HM175 and HAF-203) and adenovirus type 5 nor did it show any antiviral activity in the MTT assay. However violacein did show a weak inhibition of viral replication: 1.42 ± 0.68%, 14.48 ± 5.06% and 21.47 ± 3.74% for HSV-1 (strain KOS); 5.96 ± 2.51%, 8.75 ± 3.08% and 17.75 ± 5.19% for HSV-1 (strain ATCC/VR-733); 5.13 ± 2.38 %, 8.18 ± 1.11% and 8.51 ± 1.94% for poliovirus type 2; 8.30 ± 4.24%; 13.33 ± 4.66% and 24.27 ± 2.18% for simian rotavirus SA11, at 0.312, 0.625 and 1.250 mM, respectively, when measured by the MTT assay.*

Key words: violacein - cytotoxicity - antiviral - MTT assay - herpes simplex virus type 1- poliovirus - rotavirus - hepatitis A virus - adenovirus

During the last few years efforts have been made to increase the number of substances with antiviral activity. Few substances are known which provide an effective treatment of viral infections in vivo (Balfour 1999). Also, the therapeutic potency of most of the antiviral agents encountered so far is counterbalanced by their severe side effects in humans (Glatthaar-Saalmüller et al. 2001) and the efficacy of these drugs is limited by increases in viral resistance (Pillay & Zambon 1998, De Logu et al. 2000). The search for antiviral substances with high efficacy, low toxicity, and minor side effects therefore must continue.

Natural products have been an abundant source of compounds which have proved useful in antiviral chemotherapy of infectious human diseases (Pujol et al. 1996, Bedoya et al. 2001) especially those originating from plant extracts and fermentation broths from soil bacteria, which provide compounds directly useful as drugs or as leads for the synthesis of new medicines (Nielsen 2002).

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Chromobacterium violaceum is a Gram(-) bacteria found in water samples and soils from tropical and subtropical regions of the world. Due to its biotechnological potential, *C. violaceum* had its genome sequenced by the Brazilian National Genome Project. The most notable characteristic of *C. violaceum* is the production of the chemically well characterized pigment named violacein (Bromberg & Duran 2001). Previous studies indicated antibiotic and antichagasic (Duran & Menck 2001), antitumoral (Melo et al. 2000), and antileishmanial (Leon et al. 2001) activities of violacein.

The aim of this study was to assess the cytototoxicity and the potential antiviral activity of violacein against the viruses: Herpes Simplex Virus type 1 (HSV-1) strains KOS, 29-R/acyclovir resistant and VR733/ATCC; Poliovirus type 2 (PV-2); Simian rotavirus SA11 strain, Hepatite A virus (HAV) strains HAF203 and HM175 and Adenovirus type 5 (AdV-5), a respiratory strain.

MATERIALS AND METHODS

Compound - Violacein was isolated and purified from *C. violaceum* (CCT3496/JMC3496) as described by Duran et al. (1994). Violacein was dissolved in absolute ethanol (Merck) and 0.003% of dimethyl sulphoxide (DMSO, Merck) and stored at 4ºC protected from light until tested. The suitable dilutions for testing were made in cell culture medium as stated below and the stock solution was quantified by using a spctrophotometer (Pharmacia, Ultrospec 3000) at 577 nm.

Cell culture and viruses - The cell lines used were

Vero cells (Adolpho Lutz Institute, Brazil), HEp-2 cells (Biological Science Institute, University of São Paulo, Brazil), MA104 cells (Biological Science Institute, University of São Paulo, Brazil) and FRhK-4 cells (Macquarie University, NSW, Australia). All the cell lines were grown in 199 Medium (Sigma) supplemented with 10% fetal bovine serum (FBS - Gibco BRL), penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.025 µg/ml) (Gibco BRL). The cell cultures were maintained at 37ºC in a humidified 5% CO_2 atmosphere. The following viruses were used: Herpes Simplex Virus type 1 (HSV-1) strains KOS and 29-R/acyclovir resistant (Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Rennes, France); HSV-1 strain VR733 (American Type Culture Collection, Rockville, MA, US); Poliovirus type 2 (PV-2) - a vaccinal strain Sabin II (Adolpho Lutz Institute, Brazil); Simian rotavirus SA11 strain (RV-SA11); Hepatitis A virus (HAV) strains HAF203 and HM175 (Federal University of Rio de Janeiro); and adenovirus type 5 (AdV-5) (Biological Science Institute, University of São Paulo, Brazil). HSV-1 strains and PV-2 were propagated in Vero cells; RV-SA11 was propagated in MA104 cells in the presence of trypsin (Sigma, 5 µg/ml); AdV-5 was propagated in HEp-2 cells and HAV strains were propagated in FRhK-4 cells. Stock viruses were prepared as described previously (Barardi et al. 1998, Simões et al. 1999) and the supernatant fluids were harvested, titrated and stored at -80ºC until used. HSV-1 and AdV-5 titers were obtained by the limit-dilution method and expressed as 50% tissue culture infections dose per ml ($TCID_{50}$/ml) (Reed & Müench 1938); PV-2 titer was performed by the plaque method (Burlenson et al. 1992) and expressed as plaque forming units (pfu/ml); HAV and RV-SA11 titers were performed by immunofluorescence assay and expressed as focus forming units per ml (ffu/ml) (Barardi et al. 1998).

Cytotoxicity evaluation

Cell morphology evaluation by inverted light microscopy (Simões et al. 1999) - Vero or MA104 or HEp-2 or FRhK-4 cell cultures ($2x10^5$ cells/ml) were prepared in 96-well tissue culture plates (Corning, US). After a 24 h period of incubation at 37ºC in a humidified 5% CO_2 atmosphere cell monolayers were confluent, the medium was removed from each well and replenished with 200 µl of violacein dilutions per well (1:2) ranging from 5 to 0.078 µM prepared in 199 medium. For cell controls 200 µl of 199 medium without violacein was added. All cultures were incubated at 37ºC in a humidified 5% CO_2 atmosphere for 72 h. Cell morphology was observed daily for microscopically detectable morphological alterations, such as loss of confluency, cell rounding and shrinking, and cytoplasm granulation and vacuolization. Morphological changes were scored and CC_{50} values (= cytotoxic effects on 50% of cultured cells) were estimated from graphic plots (concentrations required to cause visible alterations in 50% of intact cells).

Cell viability test by Trypan blue dye exclusion method (Walum et al. 1990) - Vero or MA104 or HEp-2 or FRhK-4 cell cultures ($2x10^5$ cells/ml) were grown in 12-well tissue culture plates (Corning, US). After a 24 h period of incubation, the same procedure for violacein cytotoxicity assay described above was followed by using 1 ml of violacein dilutions per well. After 72 h the medium was removed, cells were trypsinized and an equal volume of 0.4% (w/v) Trypan blue dye aqueous solution was added to cell suspension. Viable cells were counted under the phase contrast microscope. CC_{50} values were estimated from graphic plots from data of % viable cells when compared to cell controls.

Cell viability test by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Mosmmann 1983, Takeuchi et al. 1991, Sieuwerts et al. 1995 with minor modifications) - Vero or MA104 or HEp-2 or FRhK-4 cell cultures ($2x10^5$ cells/ml) were prepared in 96-well tissue culture plates (Corning, US). After a 24 h period of incubation, the same procedure for violacein cytotoxicity assay described above was followed by using 200 µl of violacein dilutions per well. After 4 days, at 37ºC in humidified 5% CO_2 atmosphere, medium was removed by suction from all wells and 50 µl of MTT (Sigma, 1mg/ml) solution prepared in 199 medium was added to each well and the plates were incubated for 4 h at 37ºC. The MTT solution was removed without disturbing the cells and 100 µl of DMSO was added to each well to dissolve formazan crystals. After gently shaking the plates for 5 min, whereby crystals were completely dissolved, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, Elx 800, US) at 540 nm. The percentage of cytotoxicity was calculated as [(A-B)/Ax100], where A and B are the absorbances of control and treated cells, respectively. The CC_{50} was defined as the concentration that reduced the absorbance of treated cells to 50% when compared to cell controls.

Antiviral assay

Cytopathogenicity inhibition - Vero or MA104 or HEp-2 or FRhK-4 cell cultures ($2x10^5$ cells/ml) were prepared in 96-well tissue culture plates (Corning, US). After a 24 h period of incubation, the same dilutions of violacein (100 µl per well) described above for cytotoxicity assays were added just before the inoculation of 100 µl of each one of the following viruses: HSV-1, strains KOS, ATCC/VR-733 and 29R; PV-2; HAV, strains HM175 and HAF203; RV-SA11 and AdV-5. RV-SA11 was previously treated with trypsin (5 µg/ml) at 37ºC for 20 min (Estes et al. 1981). Respective controls of violacein toxicity, normal cells and viruses were run simultaneously. Plates were incubated for a period of time corresponding to four cycles of replication of each virus: 72 h for HSV-1, 24 h for PV-2, 48 h for rotavirus, 132 h for AdV-5 and HAV and were examined daily by inverted light microscopy for the appearance of cytopathic effect (CPE) on Vero, MA104, HEp-2 and FRhK-4 cells, respectively. The percentages of viral inhibition caused by violacein were calculated in relation to each virus control. Acyclovir [9-(2-hydroxyetho-xymethyl) guanosine, Sigma, 10, 5 and 2.5 µg/ml] was used as positive control for HSV-1 inhibition.

MTT method - Vero or MA104 or HEp-2 or FRhK-4 cell cultures ($2x10^5$ cells/ml) were prepared in 96-well tissue culture plates (Corning, US), and the violacein dilutions, as described above were added just before the inoculation

of 100 µl of each one of the same viruses used before. Plates were incubated for 2 days for PV-2, 3 days for RV, 4 days for HSV-1 and 5 days for AdV-5 and HAV. The same method used to evaluate cell viability with MTT as described above was followed. The percentages of protection were calculated spectrophotometrically as [(A-B)/(C-B)x100], where A, B and C indicate the absorbances of violacein, virus and cell controls, respectively. Acyclovir [9-(2-hydroxyethoxymethyl) guanosine, Sigma, 10, 5 and 2.5 µg/ml] was used as positive control for HSV-1 inhibition.

Data analysis - The 50% cytotoxic concentrations (CC_{50}) of violacein were estimated from concentration-effect curves after linear regression analysis. The percentages of viral inhibition by violacein in relation to each tested virus represent mean ± standard error of the mean values of three different experiments. Student's t-test ($p < 0.05$) was carried out as appropriate.

RESULTS AND DISCUSSION

Assessment of cytotoxicity is clearly an important part of the evaluation of a potential antiviral agent since a useful compound should show neither acute nor long-term toxicity against the host. Such a compound should be completely selective for virus specific processes with no or few effects on cellular metabolism (Simões et al. 1999).

Many methods have been developed for determining the antiviral activity of compounds in cell culture. Cytotoxicity evaluation in vitro is usually made by using cell viability assays, such as the uptake of a dye by dead cells after breakdown of the cellular permeability barrier (ex. Trypan blue, eosin Y, etc.) or mitochondrial function (ex. MTT or XTT assay), but other parameters, such as changes in cell morphology under microscopic examination have also been used as indicators of compound toxicity. These endpoints have been established for many years and in many cell types (Vlietinck et al. 1997, Eisenbrand et al. 2002).

The reduction of MTT ([3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide]) in the cell assesses the functional intactness of mitochondria based on the enzymatic reduction of the tetrazolium salt by the mitochondrial dehydrogenase in viable cells (Denizot & Lang 1986).

MTT and neutral red are probably the most commonly used colorimetric indicators of cell viability and they have been used to evaluate cytotoxicity in a quantitative way in contrast with cell morphology evaluation by inverted light microscopy which is qualitative and more subjective (Smee et al. 2002).

The toxicity of violacein to Vero, MA104, HEp-2 and FRhK-4 cells was investigated by three different methods: determination of effects on cell morphology (direct microscopic observation and scoring), and the cell viability tests by using the Trypan blue dye exclusion method and the colorimetric MTT assay.

Preliminary studies had already demonstrated that violacein presents cytotoxicity to V79 fibroblasts (CC_{50} = 7 µM; MTT assay) (Melo et al. 2000).This cytotoxicity was also verified with the cell lines used in this study, and Table I shows the different CC_{50} values obtained with violacein, depending on the method used.

Violacein showed a concentration-response relationship since the cytotoxicity increased gradually with the increase of its concentration as shown by the tested methodologies (Figure).

The results of cytotoxicity evaluation were different depending on the cell line used, and CC_{50} values obtained by microscopic evaluation of cell morphology and by the Trypan blue exclusion method were significantly different from CC_{50} values obtained by the MTT assay ($p < 0.05$ – *t*-test) (Table I). It is important to note that if the cell viability tests were carried out under the same conditions, the results would probably have been closer. When microscopic evaluation of cell morphology and the Trypan blue method were used, the incubation time was 72 h. On the other hand, when MTT assay was made, the incubation time was higher (4 days). So, the differences found may well be a function of the time in which violacein was in contact with the cells.

According to Smee et al. (2002) the use of a dye is very important to assess toxicity rather than solely relying on visual scoring. Cells may appear almost normal when observed microscopically, and yet be toxified by the test compound. In contrast, sometimes cells show morphological alterations and yet dye uptake methods can indicate values equivalent to untreated cultures.

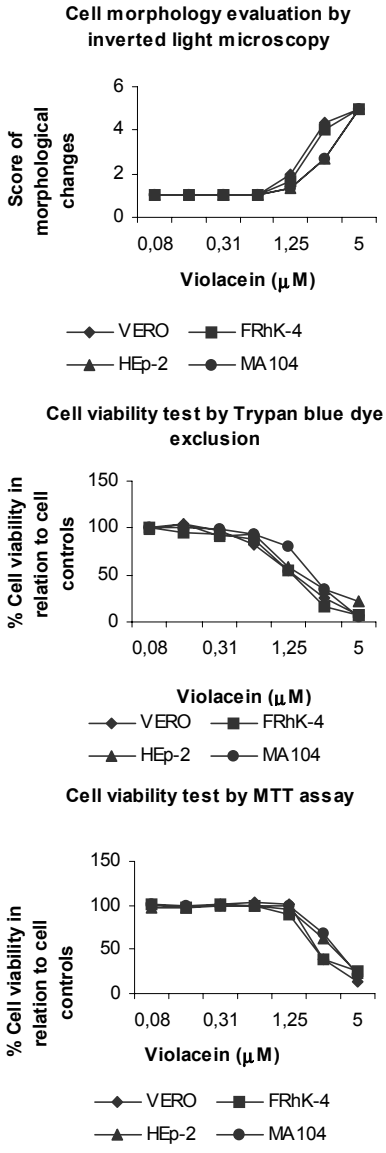
Sometimes MTT underestimates the toxicity of certain substances. As this dye undergoes enzymatic conversion in viable cells, it is possible that certain compounds might inhibit this process making them to appear less cytotoxic than they really are (Smee et al. 2002). Moreover, compounds that are naturally coloured, as violacein, may interfere with a test based upon colorimetry, thus it is important to use more than one cytotoxicity method in

TABLE I

Cytotoxicity of violacein on different cell lines expressed as CC_{50} values obtained from different methods

Method	Cell line	CC_{50} (µM) [a]
Cell morphology evaluation by inverted light microscopy	Vero	2.29 ± 0.23
	MA104	2.69 ± 0.12 [b]
	FRhK-4	2.42 ± 0.22
	HEp-2	2.78 ± 0.13 [b]
Cell viability test by Trypan blue dye exclusion method	Vero	2.23 ± 0.06 [b]
	MA104	2.54 ± 0.10 [b]
	FRhK-4	2.07 ± 0.05 [b]
	HEp-2	2.70 ± 0.12 [b]
Cell viability test by the MTT assay	Vero	2.96 ± 0.12
	MA104	3.55 ± 0.22
	FRhK-4	3.14 ± 0.32
	HEp-2	3.42 ± 0.05

a: 50% cytotoxic concentration; *b*: CC_{50} values were significantly different from CC_{50} values obtained by MTT assay ($p < 0.05$ – *t*-test). Data represent the mean ± standard error of the mean values of three separate experiments.



Cytotoxicity of violacein towards different cell lines investigated by different methods.

order to determine if results obtained using the dye agree with those obtained by visual scoring.

Cell viability test by the Trypan blue dye exclusion method is more suitable to cells grown in suspension than to monolayers because dead cells can detach from monolayers and are therefore lost from the assay (Wilson 2000). This fact could justify the low CC_{50} values (Table I) obtained by Trypan blue exclusion method when compared to the other cytotoxicity evaluation methods.

Various cell culture-based assays are available and can be successfully applied for the antiviral determination of synthetic or natural compounds (Vlietinck & Vanden Berghe 1998) which include visual quantitation of antiviral activity based upon the inhibition of the virus induced cytopathic effect (CPE) (Simões et al. 1999, Semple et al. 2001, Li et al. 2002,) or by less subjective measures, such as the colorimetric MTT assay (Takeuchi et al. 1991, Kaneko et al. 2001, Betancur-Galvis et al. 2002) and fluorometric assays (Smee et al. 2002).

For viruses that cause microscopically discernable CPE in cells, visual scoring of CPE inhibition is performed more frequently because it is rapid, and allows a number of compounds to be evaluated together using 96-well microplates (Smee et al. 2002).

MTT was first applied to quantify cellular proliferation (Mosmann 1983, Denizot & Lang 1986) and is now widely used for screening antitumoral (Carmichael et al. 1987, Todryk et al. 2001, Betancur-Galvis et al. 2002, Lotfi et al. 2002) and antiviral (Bedard et al. 1999, Kaneko et al. 2001, Takahashi et al. 2001, Glatthaar-Saalmüller et al. 2001) activities of a large number of natural products. This assay has several advantages: it is easy to perform, the evaluations are objective, it can be automated using a personal computer, and the cytotoxicity evaluation can be made in parallel with antiviral activity evaluation (Takeuchi et al.1991).

The potential antiviral action of violacein (0.078 to 2.5 μM) against HSV-1 (strains KOS, ATCC/VR733 and 29-R/acyclovir resistant), PV-2, RV-SA11, HAV (strains HM175 and HAF-203) and AdV-5 was studied by the cytopathogenicity inhibition test and the MTT assay.

Violacein showed no cytopathic effect inhibition of HSV-1 (strain 29-R/acyclovir resistant), HAV (strains HM175 and HAF-203) and AdV-5 neither demonstrated antiviral activity through the MTT assay. However violacein showed a weak inhibition of HSV-1 (strains KOS and ATCC/VR733), PV-2 and RV-SA11 replication by MTT assay (Table II) which is more sensitive and accurate than cytopathogenicity inhibition evaluation. The obtained percentages of viral inhibition were < than 50%, therefore it was not possible to estimate the EC_{50} values (50% effective concentration) necessary to calculate selectivity indices ($SI = CC_{50}/EC_{50}$).

According to May et al. (1991) violacein (with 10% of deoxyviolacein) showed activity against herpes and polioviruses. Duran (1998) also registered a patent for a formulation of cyclodextrin/violacein for treating bacterial, viral, trypanocidal infections and for antitumoral activity. Duran and Menck (2001), citing the patent registered by May et al. (1991), stated that 0.25 μg/ml of violacein inhibited HSV replication by 62%, and 0.063 μg/ml of violacein inhibited poliovirus replication by 56% in HeLa cells. This information from patent documents does not inform the methodologies used nor the virus strains and types or experimental conditions under which the results were obtained. No comparison with present data was therefore possible.

The results obtained in the present study differ from the above cited information in that higher concentrations (0.312 to 1.25 μM) of violacein have shown only a weak inhibitory action on HSV-1 (strains KOS and ATCC/VR733) and PV-2 replication (Table II).

The violacein cytotoxicity data themselves have intrinsic value in defining toxic effects (e.g. as an indicator of acute toxic effects in vivo) and are also important for designing more in depth in vitro studies (Eisenbrand et al. 2002). These data will be useful for a better understanding

TABLE II

Antiviral activity of violacein expressed as the percentual inhibition of viral replication when compared to controls by using the MTT assay

Viruses	Virus titre (MOI)	Violacein (μM)	% inhibition	ACV [a] (μg/ml)	% inhibition
HSV-1					
Strain 29-R	$5 \times 10^{5.64}$ $TCID_{50}$/ml (0.5)	2.5 - 0.078	NA	10-2.5	NA
Strain KOS	$5 \times 10^{6.25}$ $TCID_{50}$/ml (0.5)	1.250 0.625 0.312	21.47 ± 3.74 14.48 ± 5.06 1.42 ± 0.68	10 5 2.5	99.93±3.06 99.28± 0.72 96.50±0.82
Strain VR-733	$5 \times 10^{6.25}$ $TCID_{50}$/ml (0.1)	1.250 0.625 0.312	17.75 ± 5.19 8.75 ± 3.08 5.96 ± 2.51	10 5 2.5	101.60±2.77 101.81± 3.39 99.81± 2.19
RV-SA11	3.4×10^{7} ffu/ml (0.2)	1.250 0.625 0.312	24.27 ±2.18 13.33 ±4.66 8.30 ± 4.24	- - -	- - -
PV-2	5.6×10^{6} pfu/ml (0.3)	1.250 0.625 0.312	8.51 ± 1.94 8.18 ± 1.11 5.13 ± 2.38	- - -	- - -
AdV-5	5×10^{5} $TCID_{50}$/ml (0.03)	2.5 - 0.078	NA	-	-
HAV					
Strain HM175	2.5×10^{3} ffu/ml (0.02)	2.5 - 0.078	NA	-	-
Strain HAF203	2.5×10^{4} ffu/ml (0.02)	2.5 - 0.078	NA	-	-

ACV: acyclovir; *a*: positive controls for HSV-1 infection; NA: no activity; MOI: multiplicity of infection (virus titre/cell density). Data represent the mean ± standard error of the mean values of three separate experiments.

of other biological activities attributed to violacein, such as antibacterial, antitumoral and anti- *Trypanosoma cruzi* activities (Melo et al. 2000, Duran & Menck 2001).

One strategy used to improve biological activity is the incorporation of active substances into liposomes which offer a substancial improvement in the therapeutic indices of the molecules entrapped in them (Gulati et al. 1998). Some antiviral drugs and their liposomal formulations have been subjected to in vitro and in vivo studies and have been shown to result in an increase of drug absorption, less toxicity than conventional drug formulations and a more prolonged effect (Law et al. 2000, Wutzler et al. 2002). In this way, violacein-containing liposomes have been prepared and preliminary results show that its antiviral activity against HSV-1 is increased (data not shown). Complementary studies are in course in our laboratory.

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