Biol Res 25: 21-25 (1992)

Bacterial chemistry. VI. Biological activities and cytotoxicity of 1, 3-dihydro-2H-indol-2-one derivatives

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The biosynthetic pigment from *Chromobacterium violaceum* BB-78, 1,3-dihydro-2H-indol-2-one and its derivatives exhibit biological activities such as antimicrobial action, low hemolytic effects on red blood cells and *in vitro* trypanocide activity. A relatively high cytotoxicity on V-79 hamster fibroblast cells of the biosynthetic pigment was found, although with the methylol derivative the toxicity was almost eliminated. The methylol derivative exhibited similar toxicity as Nifurtimox, a known, commercial trypanocide compound.

INTRODUCTION

Chromobacterium violaceum BB-78, found in soil and water in tropical environments, produces the pigment 3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1-3-dihydro-2H-indol-2-one (Caldas *et al.*, 1978; Durán and Faljoni-Alario, 1978) (Fig. 1). It has been found that pigment-I has phototherapeutic potential (Durán and Faljoni-Alario, 1980), antibiotic properties (Durán *et al.*, 1983) and, in a preliminary study *in vitro*, trypanocide action on *Trypanosoma cruzi* (Durán *et al.*, 1987, 1989; Riveros *et al.*, 1989).

The biosynthetic pathways of pigment synthesis are not completely known, although all the carbon and nitrogen atoms of pigment-I probably derive exclusively from L-tryptophan or its metabolites (Riveros *et al.*, 1987). The physiological role of pigment-I in the economy of the cells is also unknown (DeMoss, 1967). Nutritional requirements of the *Chromobacterium violaceum* from the Rio Negro, Brazil, have recently been carried out in order to increase the pigment-I production (Riveros *et al.*, 1988) and to facilitate the synthesis of new products (Riveros, 1989).

Now we report a systematic study of the action of pigment-I and their bromo-, acetyl-, and methylol-derivatives on *T. cruzi*, the actual evaluation of cytotoxicity and their action on a Brazilian strain of *T. cruzi* (Y-strain).

MATERIAL AND METHODS

A strain of *C. violaceum* from the Rio Negro, Manaus, Amazon, was obtained originally from Prof. L. R. Caldas and Prof. A. C. Leitão, from the Universidade Federal do Rio de Janeiro. Cells were cultured in thioglycollate medium, with 1.2% agar, pH 6.8, temperature 32° C, in Roux bottles. Abundant growth of pigmented colonies was obtained in 36 h of incubation. Pigment-I was extracted with acetone (5 g wet weight cells/1), concentrated to dryness under reduced pressure and extracted in a soxhlet apparatus with chloroform, ether and acetone (Riveros, 1989).

The 3-[5-(5-hydroxy-1H-indol-3-yl-)-2-oxo-3 (2H)-furanylidene]-1,3-dihydro-2H-indol-2-one (Pigment-II) was synthetized as described before (Durán *et al.*, 1983). The dibromide, tri-acetyl, tri-methylol and tetra-so-

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dium salt derivatives of pigment-I were synthetized as already described (Riveros, 1989) (see Fig. 1).

Antimicrobial properties of the pigment-I derivatives were tested against several microorganism in Mueller-Hinton plates, using inocula of 10^6 cells, and filter paper discs impregnated with the compound in isotonic NaCl solution. A minimal inhibitory concentration (MIC) for pigment-I was determined by serial dilution in tubes with an original concentration of pigment-I of 100 µg/ml.

Trypanosoma cruzi (epimastigotes, Tulahuen strain) (Badinez, 1945) was cultivated in liver infusion tryptose serum medium (LIT) (Camargo, 1964; Chiari and Camargo, 1983) at 28° C. Carefully homogenized saline solutions were immediately brought to a Coulter Electronic Counter (Mode A with a 100 u orifice threshold 5), for the determination of the total number of organism present. The compounds under study were applied to cultures with an initial count of 5 x 10^6 flagellates per ml of medium and the growth phase covered a period of 72 h. *Trypanosoma cruzi* (epimastigote, amastigote and trypomastigote Y strain) were obtained by the method described before (De Castro and De Meirelles, 1986; Filardi and Brener, 1982). Flagellates and percent mobility were measured directly by microscopic observations.

Cytotoxicity through survival curves (ID_{50}) and DNA synthesis inhibition tests (D_{50}) on V-79 Chinese hamster cells were carried out by published methods (Hoffmann and Meneghini, 1978).

Hemolysis was measured by a previoously described method (Makita *et al.*, 1981).

Male albino mice (18-20 g) were inoculated intraperitoneally with 5 x 10^4 blood trypomastigotes of Y strain. Dose of 100 mg kg^{-1} of pigment-V was given 7 days by intraperitoneal route, beginning 24 h after inoculation (Filardi and Brener, 1982).

Pigment-V was tested in axenic cultures upon both proliferative forms of *T. cruzi* (epimastigotes, amastigotes, obtained from infected J-7746-8 cultures and trypomastigotes) following the described methodology (De Castro and De Meirelles, 1986).

RESULTS AND DISCUSSION

Antimicrobial activities of pigment-I derivatives (Fig. 1) are shown in Table I. No effects were found at levels of 100 µg/ml on *Enterobacter aerogenes* and *Escherichia coli*, and at the level of 50 µg/ml on *Providencia sp, Klebsiella pneumoniae* and *Salmonella typhi*. Antimicrobial activity was found for all pigment-I derivatives with the exception of pigment-IV, which was inactive against all the strains tested. Pigment-I, II, III, V and VI showed activity against Gram-positive and Gram-negative microorganisms, with a more pronounced effect being observed with Gram-positive bacteria (Table I). The low minimal inhibitory concentration for pigment-I acting on *Staphylococus aureus* and *Bacillus subtilis* is an interesting result.

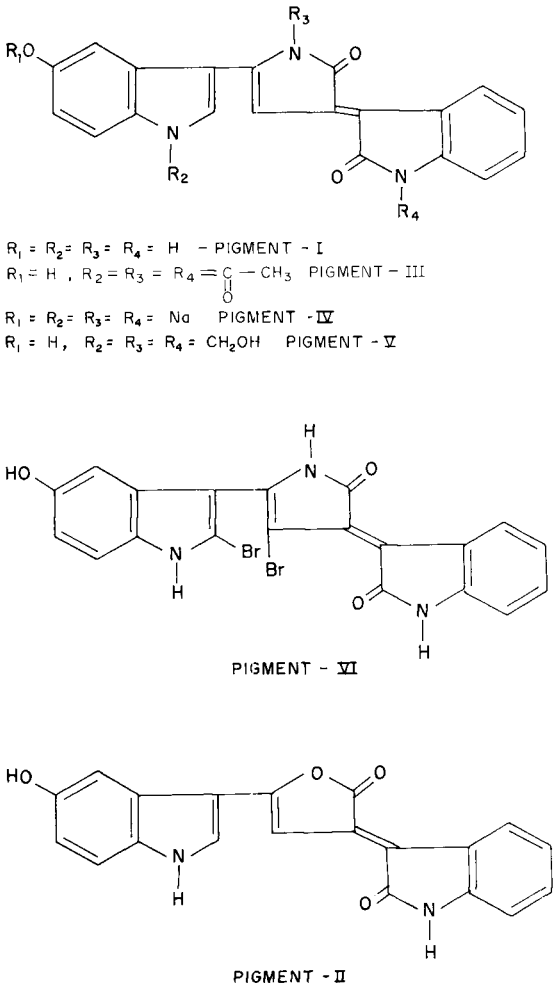

Fig. 1: Structures of Pigment-I and derivatives.

TABLE I

Antimicrobial activity of pigment-I derivatives

	Pigments					
	I	II	III	IV	V	VI
Microorganism	MIC	At 50 µg/ml				
Enterobacter aerogenes ATCC 10006	100	–	+++	–	+++	++
Escherichia coli ATCC 25922	100	+	++	–	+++	++
Providencia sp	50	+++	+++	–	+++	+++
Klebsiella pneumoniae ATCC 3383	50	+	++	–	+++	++
Salmonella thyphi	50	++	+++	–	+++	+++
Staphylococus aureus	6	+	+++	–	+++	+++
Bacillus subtilis	6	++	+++	–	+++	+++

Petri plates of 80 mm
+ = 10 mm; ++ > 10 mm; +++ > 15 mm; –, no effect.
MIC: minimal inhibitory concentration expressed in µg/ml.

In a very preliminary experiment by Caldas *et al.* (1978), exposure of *T. cruzi* to impure pigment-I at a concentration of 75 µg/ml killed 100% of an exponential phase culture in a few minutes.

In a culture of *T. cruzi* (Tulahuen strain) with an initial count of 5 x 10^6 flagellates per ml of LIT medium, the growth phase covered a period of 72 h, merging then into a stationary phase where the population density attained 20 x 10^6 individuals per ml. When the culture attained 5 x 10^6, the flagellates were treated with the pigment-I derivatives in the 10 µg/ml to 40 µg/ml range (Fig. 2). Small effects on the growth curve of *T. cruzi* in a LIT medium at 10 µg/ml with pigment-I to pigment-VI were observed (not shown). Pigment-IV was inactive in all concentrations. However, at 20 µg/ml a significant effect on *T. cruzi* growth with pigment-I and VI, and total inhibition of growth with the pigment-V at this level of concentration were observed (ID_{50} 27 µM). At the level of 40 µg/ml, pigment-III, V and VI exhibited a total inhibition of *T. cruzi* growth.

By the method of Makita *et al.* (1981), hemolysis of erythrocytes was found to be 7% at 0.07 mM (42 µg/ml) and 20% at 0.5 mM (171.5 µg/ml). More drastic effects were observed with commercial phototherapeutic compounds such as hematoporphyrins, gentian violet and methylene blue. A study of cytotoxicity of pigment-I derivatives acting on V-79 hamster fibroblast cells, measured by means of a survival curve or a DNA synthesis inhibition test, showed a relative high toxicity of pigments I and VI (Table II). However, pigments III and V exhibited low

TABLE II

Cytotoxicity of pigment-I derivatives and Nifurtimox on V-79 Hamster fibroblast cells

Compounds	ID_{50} (µM)	D_{50} (µM)
Pigment-I	12.5 ± 0.7	113.1 ± 11.8
Pigment-III	20.0 ± 0.5 (a)	Not determined
Pigment-V	20.0 ± 0.7 (a)	450.0 ± 35 (c)
Pigment-VI	13.0 ± 0.9 (a)	48.0 ± 45 (c)
Nifurtimox	Non toxic (b)	450.0 ± 45 (c)

a) Concentration in which 50% inhibition is reached with no further effect at higher concentration (same values observed). b) At 30 µM. c) Experiment carried out in a range of 0-100 µM and extrapolated to D_{50} (since no toxicity of 50% was reached in the concentration range studied). For details see Durán *et al.* (1989).

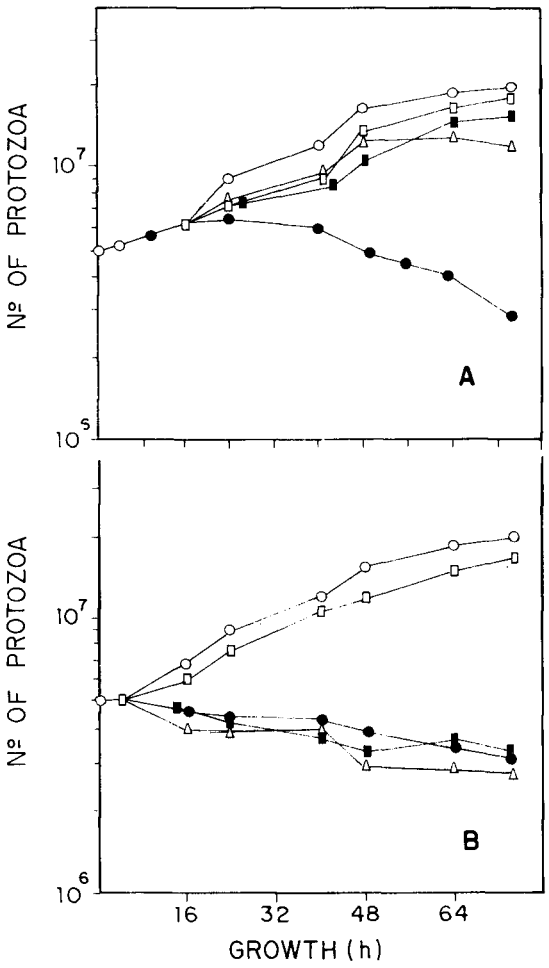

Fig. 2: Curve of growth of *T. cruzi* (epimastigote, Tulahuen strain) in LIT medium in the presence of pigment-I derivatives as described in the Experimental Section: (-o-) Growth Control; (-□-) Pigment-I; (-Δ-) Pigment VI; (-●-) Pigment-V and (-■-) Pigment III. A) 20 μg/ml; B) 40 μg/ml.

toxicity when compared with Nifurtimox under the same experimental conditions.

The substitution of a hydrogen atom on nitrogen by a methylol group (pigment-V) decreases the cellular toxicity of pigment-I. In addition, the latter compound is soluble in water which facilitates the *in vivo* experiments.

Obviously the toxicity observed in cell cultures (Table II) is not related to the trypanosoma growth inhibition or to the antibacterial activity. This is concluded from the small effect on inhibition at 40 μg/ml of pigment-I on *T. cruzi*, which showed a higher toxicity in culture cells.

Table III shows the percentage of mobility of *T. cruzi* under the action of the pigment-I derivatives. At the level of 20 μg/ml a very strong effect of pigment-V was observed.

Pigment-V and pigment-III definitely appear to be the best derivatives of pigment-I, not only because of their solubilities as in the case of pigment-V, but also because of their antimicrobial activities (MIC 4-6 μg/ml) for *Staphylococus aureus*, as well as its trypanocide *in vitro* activity (10-20 μg/ml). Also both compounds have lower toxicities on hamster fibroblast cells than pigment-I.

Pigment-V tested in axenic cultures presented an inhibitory effect upon both proliferative forms of *T. cruzi* (Y-strain). The pigment-V was effective upon the epimastigote form in a range of 25 to 500 μM with an ID_{50} of 75 μM after 24 h (approximately 3-fold more resistant than Tulahuen strain), while in

TABLE III

Efect of pigment-I derivatives on *T. cruzi* mobility

		Pigments (mobility %)				
Dose (μg/ml)	Control	I	III	IV	V	VI
0	95	-	-	-	-	-
10	-	86	86	95	86	86
20	-	86	70	80	0	80
40	-	80	30	76	0	50

At 48 h of incubation of epimastigote form of *T. cruzi* (Tulahuen strain).

the case of amastigotes, the range was 100 to 750 μM, with an ID_{50} of 500 μM, after 48 h, the latter being more resistant to pigment-V. Analysing the effect upon blood trypomastigote, we observed total lysis of the parasite only at 750 μM, after 24 h at 29° C.

In an initial approach to analyse the effect of pigment-V on *T. cruzi* host cell interaction, we observed damage to peritoneal macrophages above 25 μM. When male albino mice (18-20 g) were inoculated intraperitoneally with 5 x 10^4 blood trypomastigotes from Y-strain of *T. cruzi* and then injected for 7 days 100 mg/kg of pigment-V, only a 4% reduction of parasitemia (from a value of 15,303 parasites/mm^3 to a value of 14,721 parasites/mm^3) was found. Unfortunately, these results reveal very little activity of pigment-V against *T. cruzi* Y-strain from Brazil.

More detailed studies on the modes of action of pigments I, V and VI, and of new modifications of pigment-I against *T. cruzi* Y-strain in Brazil, are currently in progress.

ACKNOWLEDGEMENTS

Research support from FAPESP, CNPq, FINEP, PADCT, FONDICYT, OAS and UNESCO.

REFERENCES

BADINEZ O (1945) Contribución a la anatomía patológica en la enfermedad de Chagas experimental. *Biología* (Chile) *3:* 3-20

CALDAS L R, LEITÃO A A C, SANTOS S M, TYRREL R M (1978) Preliminary experiments on the photobiological properties of violacein. Internat Symp on Current Topics in Radiation and Photobiology, Academia Brasileira de Ciências, Rio de Janeiro, November, pp 121-132

CAMARGO E P (1964) Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic trypanosomes in liquid medium. *Rev Inst Med Tropical* (São Paulo) *6:* 93-100

CHIARI E, CAMARGO E P (1983) Culturing and cloning of *Trypanosoma cruzi*. Genes and antigenes of parasites. In: C M Morel, ed UNDP/WORK BANK/WHO Symposium. Rio de Janeiro, pp 23-26

DE CASTRO S L, DE MEIRELLE M DE D L (1986) Effect of drug on *Trypanosoma cruzi* and on the interaction with heart muscle cell *in vitro*. *Mem Inst Oswaldo Cruz* (Rio de Janeiro) *82:* 209-218

DeMOSS R D (1976) Violacein. *Antibiotics 2:* 77-81

DURAN N, CAMPOS V, RIVEROS R, JOYAS A, ERAZO E, HAUN M (1987) A new drug with antitrypanosomal activity from 1,3-dihydro-2H-indol-2-one. *Mem Inst Oswaldo Cruz* (Rio de Janeiro) *81:* 155

DURAN N, CAMPOS V, RIVEROS R, JOYAS A, PEREIRA M F, HAUN, M (1989) Bacterial chemistry III. Preliminary studies on trypanosomal activities of *Chromobacterium violaceum* products. *An Acad Brasil Ciên 61:* 31-36.

DURAN N, ERAZO S, CAMPOS V (1983) Bacterial chemistry II Antimicrobial properties from pigment of *Chromobacterium violaceum*. *An Acad Brasil Ciên. 55:* 231-234

DURAN N, FALJONI-ALARIO A (1978) Bacterial chemistry. Photochemical study on the *Chromobacterium violaceum* pigment. *Ciênc Cult* (Brazil) *30:* 609

DURAN N, FALJONI-ALARIO A (1980) Bacterial Chemistry I Studies of a potential phototherapeutic substance from *Chromobacterium violaceum*. *An Acad Brasil Ciên 52:* 297-302

FILARDI L S, BRENER Z (1982) A nitroimidazole-thiazole derivative with curative action in experimental *Trypanosoma cruzi* infection. *An Trop Med Parasitol 76:* 1582-1586

HOFFMANN M E, MENEGHINI R (1978) Action of hydrogen peroxide of human fibroblast in culture. *Photochem Photobiol 30:* 151-155

MAKITA Y, VERCECI A, HAUN M, DURAN N (1981) Synergistic effect of chlorpromazine and alkanals on the human red blood cells. *An Acad Brasil Ciên 53:* 837-840

RIVEROS R (1989) Quimica de bacterias, biossintese e atividade biológica de metabolitos por *Chromobacterium violaceum*. Doctoral Dissertation, Universidade Estadual de Campinas, S P, Brazil

RIVEROS R, HAUN M, CAMPOS V, DURAN N (1988) Bacterial chemistry-IV. Complete characterization of violacein, an antibiotic and trypanocide pigment from *Chromobacterium violaceum* (BB-78 strain). *Arq Biol Tecnol 31:* 375-487

RIVEROS R, HAUN M, DURAN N (1987) Studies on the biosynthesis of a new trypanocide from *Chromobacterium violaceum* (BB-78 strain). Arq Biol Tecnol 31: 83

RIVEROS R, HAUN M, DURAN N (1989) Effect of growth conditions on production of violacein by *Chromobacterium violaceum* (BB-78 strain). Braz *J Med Biol Res 22:* 569-577