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Trypanosoma cruzi*: Antiproliferative effect of indole phytoalexins on intracellular amastigotes *in vitro

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Abstract

American trypanosomiasis (Chagas disease) continues to be a significant public health problem, and the therapeutic potential of current antichagasic agents (nifurtimox and benznidazole) is rather limited. Here we report on the antitrypanosomal effect of 1-methoxyspirobrassinol and other indole phytoalexins - secondary metabolites produced by Cruciferous plants. These compounds, that previously demonstrated antimicrobial and anticancer properties, displayed significant antiproliferative effects on intracellular amastigotes of *Trypanosoma cruzi* and may be prospective candidates for antichagasic drug design and development.

Keywords

Indole phytoalexins; *Trypanosoma cruzi*; Glutathione; Chagas Disease

1. Introduction

American trypanosomiasis (Chagas disease) caused by *Trypanosoma cruzi* infection is endemic to Mexico, Central and South America with overall prevalence estimated at 10–16 million cases. It continues to be a significant public health problem with about 15–40 million people at risk of infection and 25–30% at risk of developing chronic cardiac and/or gastrointestinal complications among the infected population (Urbina and Docampo 2003; World Health Organization 2005). Current chemotherapy for Chagas disease employs 5-nitrofurantoin nifurtimox and the 2-nitroimidazole benznidazole, which are of limited therapeutic potential and associated with side effects including anorexia, vomiting, peripheral polyneuropathy and allergic dermatopathy that could often lead to treatment discontinuation (Urbina and Docampo 2003). For this reason, continued search for new prospective antichagasic agents is warranted. In addition, new compounds are also desirable for sterilization of *T. cruzi*-infected blood to prevent transmission of Chagas disease via blood transfusions (Morales-Souza and Bordin 1996).

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Various natural and synthetic compounds have been tested in order to identify agents with growth inhibitory and/or toxic effects on *T. cruzi*. Among them, alkaloid piperine (Ribeiro *et al.* 2004), tetrahydrofuran lignans grandisin and veraguensin (Martins *et al.* 2003), flavonoids 5,4'-dihydroxy-7-methoxyflavanone and 5,4'-dihydroxy-3,6,7-trimethoxyflavone (Ribeiro *et al.* 1997), synthetic bisphosphonates (Szajnman *et al.* 2008) and aromatic diamidines (Silva *et al.* 2007) demonstrated activity *in vitro* against bloodstream trypomastigotes and/or intracellular amastigotes with IC₅₀ values usually at the low micromolar range. Rational antitrypanosomal drug discovery was facilitated by targeting biosynthesis of ergosterol, protein prenylation, cysteine protease cruzipain, phospholipid metabolism (Docampo 2001), trypanothione reductase, trypanothione synthetase, DNA topoisomerases, dihydrofolate reductase, glyceraldehyde 3-phosphate dehydrogenase, and hypoxanthine-guanine phosphoribosyltransferase (Docampo 2001; Urbina and Docampo 2003).

The oxidative defense system of *T. cruzi*, characterized by a limited ability to metabolize peroxides, and a pivotal role of trypanosomatid-specific thiol trypanothione (N¹,N⁸-bisglutathionylspermidine), opens therapeutic opportunities via reactive oxygen species (ROS)-mediated damage (Docampo 1990; Wilkinson *et al.* 2002). Specifically, DL-buthionine sulfoximine (BSO), a potent inhibitor of glutathione biosynthesis and, thus of trypanothione biosynthesis, reportedly depleted the trypanosomal glutathione levels, reduced trypanosome survival in mice infected by *T. brucei brucei* (Arrick *et al.* 1981), and increased survival rate and decreased parasitemia in mice infected by *T. cruzi* (Faúndez *et al.* 2008). In this paper we report on the antitrypanosomal effect of indole phytoalexins, plant defensive secondary metabolites with antimicrobial and anticancer properties, whose derivatives were recently found to have glutathione-depleting effects (Mezencev *et al.* 2008).

2. Materials and methods

2.1 Compounds

Brassinin (1), 1-methoxybrassinin (2), cyclobrassinin (3), spirobrassinin (4), *cis*-1-methoxyspirobrassinol methyl ether (non-natural diastereoisomer) (5), *trans*-1-Boc-2-deoxy-2-(1-piperidyl)spirobrassinol (6) and camalexin (7) (Figure 1) were synthesized following previously reported procedures (Ayer *et al.* 1992; Kutschy *et al.* 1998; Kutschy *et al.* 2002; Mezencev *et al.* 2008; Pedras *et al.* 2000; Suchý *et al.* 2001; Takasugi *et al.* 1988). Stock solutions of these compounds were prepared in DMSO at concentration 40 mM.

2.2 Drug screening

The effect of compounds on the intracellular form of *T. cruzi* was tested on *T. cruzi*-infected γ -irradiated rat myoblasts L₆E₉ (Nadal-Ginard 1978) in accordance with a previously reported protocol (Szajnman 2008; Yan and Moreno 1998). Gamma-irradiated L₆E₉ myoblasts (1×10^7 cell/plate) in DMEM medium containing 20% fetal bovine serum were plated in 12-well tissue culture plates and incubated at 35°C in a 7% CO₂ atmosphere for 24 h. Cell monolayers were challenged with 4.17×10^6 trypomastigotes/well. One well without parasites and 2 wells with parasites and no drug added were left for controls. After 2 h of incubation, cultures were washed twice with Hanks solution and the culture medium was replaced, so that the remaining extracellular trypomastigotes were removed. At this time, 0.5–1.0 μ Ci [5,6-³H] uracil/well (specific activity, 40–50 Ci/mmol, Perkin Elmer) and the tested drugs (0.5; 1; 5; 20 and 50 μ M) were added, and cultures were incubated for an additional 72-h period. Incorporation of the [³H]uracil into trichloroacetic acid (TCA)-precipitable material was measured at this time. The supernatant from the monolayers was removed by aspiration, the cells were dissolved with 1.3 ml of 1% sodium dodecyl sulfate containing 100 μ g of cold uracil per ml, and 3.0 ml 5% ice-cold TCA were then added. The resulting precipitates were maintained at 4°C for at least 15 min and were collected on glass filters (Whatman GF/C) by using a sampling manifold

(Millipore, Bedford); the filters were washed twice with 5% ice-cold TCA and once with 95% ethanol, dried, placed in 4 ml scintillation cocktail (Eco-Lume), vortexed and counted. The percent of inhibition of trypanosomes (I) was calculated as follows: $I = 100 - 100 \times [(C_d - C_0)/(C_c - C_0)]$ where C_d , C_0 and C_c are counts per minute of drug-treated culture, uninfected control and untreated infected control samples, respectively. All experiments were performed for each compound in at least two biological replicates.

2.3 Drug cytotoxicity

Cytotoxicity of compounds **1–7** on cultured rat myoblasts L₆E₉ was evaluated by microscopic examination of cell cultures after 72-h incubation with tested compounds at the same concentrations as used for screening of antitrypanosomal activity. Compounds that at certain concentration induced remarkable cell detachment from originally confluent myoblast cultures were labeled as cytotoxic at that concentration.

2.4 Determination of IC₅₀

IC₅₀ values (concentrations of tested agents that inhibited growth of trypanosoma cultures to 50% of the untreated control) were determined from percent inhibition vs. concentration data using non-linear regression analysis with variable Hill slope by employing GraphPad Prism for Windows version 5.01 (GraphPad Software, Inc.).

2.5 Statistical analysis

Statistical significance of differences between mean IC₅₀ values was determined with unpaired two-tailed t-test and considered significant when $p < 0.05$.

2.6 Prediction of drug-likeness and toxicity

Prediction of the bioavailability of tested compounds as prospective oral drug candidates was performed using the Lipinski's Rule of Five (Lipinski et al. 2001). Parameters for this prediction: molecular weight (Mw), number of hydrogen bond donors (HBD), number of hydrogen bond acceptors (HBA), and log octanol/water partition coefficient (GlogP) were determined using the MetaDrug system (GeneGo, San Diego, CA) (Wishart 2007). In accordance with the Lipinski rule of five, compounds with $Mw \leq 500$, $HBD \leq 5$, $HBA \leq 10$ and $GlogP \leq 5$ were predicted to be orally bioavailable and marked as compliant. Cytotoxicity of tested compounds on human breast adenocarcinoma MCF-7 cell line and human oral maximum recommended therapeutic dose (MRTD) (Matthews et al. 2004) were predicted by quantitative structure activity relationship (QSAR) models using MetaDrug system.

3. Results and Discussion

1-methoxybrassinin (**2**) demonstrated the highest potency against intracellular *T. cruzi* (Table 1) and its mean IC₅₀ value was significantly different than that of cyclobrassinin ($p=0.0035$), spirobrassinin ($p=0.0331$), *cis*-1-methoxyspirobrassinol methyl ether ($p=0.0003$), *trans*-1-Boc-2-(N-piperidyl)-2-deoxyspirobrassinol ($p=0.0008$), and camalexin ($p=0.0467$), while the difference between IC₅₀ values of 1-methoxybrassinin (**2**) and brassinin (**1**) was not statistically significant ($p=0.2909$). The results imply that the dithiocarbamate side chain contributes to the antitrypanosomal potency as its cyclization results in increased IC₅₀ values. Similarly, the methoxy group seems to increase the antitrypanosomal potency within the series of tested compounds, as demonstrated by the higher potency of compounds **2** and **5** compared to compounds **1** and **4**, although these differences were not found to be statistically significant. 1-methoxybrassinin (**2**) exhibited a higher potency than the currently used antichagasic agents nifurtimox, benznidazole, and the well-known antiparasitic agent 4-phenoxyphenoxyethyl thiocyanate (WC-9) (Cinque *et al.* 1998), whose IC₅₀ values under similar experimental

conditions are 2.8, 3.7, and 12 μM , respectively (Szajnman et al. 2008; Yan and Moreno 1998).

In our experiments, no tested compound, with the exception of compound **6**, was found to induce cytotoxicity in cultured myoblasts at concentrations up to 50 μM ; however, compound **6** displayed cytotoxicity in cultured myoblasts treated for 72 hrs at concentrations 20 μM and above. IC_{50} values predicted for cytotoxicity of tested compounds on MCF-7 cells demonstrate that the potency of their general cytotoxic effect is comparable to that of the traditional antichagasic agents nifurtimox and benznidazole (Table 2). While the MRTDs of tested compounds are generally lower than that corresponding to nifurtimox and benznidazole, the differences are small and all predicted MRTDs are within the same order of magnitude. The effective human doses of the tested compounds are not yet known; therefore, their therapeutic windows cannot be compared with those of nifurtimox and benznidazole. However, the therapeutic indices estimated from the ratio of IC_{50} (cytotoxicity for MCF-7) to IC_{50} (antichagasic) for the most potent tested compound **2** (6.9), nifurtimox (2.4) and benznidazole (4.2) suggest that compound **2** likely possesses a more favorable safety profile than both these traditional drugs.

Furthermore, 1-methoxybrassinin (**2**) as well as all other tested indole phytoalexins were found to comply with the Lipinski's Rule of Five, which indicates that these compounds are likely to be orally bioavailable (bioactive) (Table 2). Similarly, all tested compounds have their molecular weights within 100–350 and GlogP values within 1–3 which suggests optimal lead compound properties for further lead optimization. Moreover, brassinin and 5-bromo-brassinin were reported to be orally bioavailable, with 5-bromo-brassinin exhibiting sustained serum levels for up to 8 hrs and *in vivo* anticancer activity after oral administration to MMTV-*Neu* mice (Banerjee et al. 2008). For these reasons, 1-methoxybrassinin and its derivatives may be interesting compounds for antichagasic drug design and development.

Phytoalexins are secondary metabolites produced by certain plants in response to pathogen attack or various forms of stress. Unlike constitutively occurring antimicrobial phytoanticipins that represent inbuilt plant chemical barriers to infection, phytoalexins are not present in healthy plants and are synthesized from remote precursors only in tissues colonized by pathogens (Paxton 1981). Phytoalexins are generally lipophilic low molecular weight compounds with non-specific and not particularly potent antimicrobial (antifungal) activity (Smith 1996), and their mode of action against plant pathogens is not well understood, but it likely involves disruption of cell membranes among a variety of other biochemical and physiological processes (Rogers et al. 1996). Indole phytoalexins from cruciferous plants, such as brassinin (**1**), 1-methoxybrassinin (**2**) and cyclobrassinin (**3**) isolated from Chinese cabbage (Takasugi et al. 1986), spirobrassinin (**4**) from Japanese radish (Monde et al. 1995) and camalexin from false flax (**6**) (Browne et al. 1991) have been shown to exhibit antibacterial and antifungal activity against plant and some human pathogens including *Bipolaris leersiae*, *Magnaporthe grisea*, *Phoma lingam*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium nivale*, *Cladosporium cucumerinum*, *Escherichia coli*, *Bacillus subtilis*, *Listeria monocytogenes* and *Xanthomonas campestris* (Pedras et al. 2007). In addition, various indole phytoalexins, including brassinin, spirobrassinin, methoxybrassinin and camalexin demonstrate *in vitro* cytostatic/cytotoxic effects against cultured human solid tumor and leukemia cell lines (Mezencev et al. 2003; Moody et al. 1997; Pilátová et al. 2005). Compound **6**, which is a synthetic 2-amino analog of the natural phytoalexin 1-methoxyspirobrassinol, was found to exhibit anticancer effects across the NCI₆₀ panel of human cancer cell lines representing leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer, and to induce depletion of total cellular glutathione in breast cancer MCF-7 cells (Mezencev et al. 2008). The previously reported antitrypanosomal effect of the glutathione-depleting agent BSO (Arrick et al. 1981; Faúndez et al. 2008) gave the rationale for testing the

antiproliferative effects of compound 6, as well as some other indole phytoalexins against *Trypanosoma cruzi*. This is the first report of the antitrypanosomal effect of these compounds and their possible use for further drug design and development. Although their mode of action likely includes a glutathione-depleting effect, it still remains to be elucidated, which will be the subject of our further studies.

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Figure 1. Brassinin (1), 1-methoxybrassinin (2), cyclobrassinin (3), spiobrasinin (4), *cis*-1-methoxyspiobrasininol methyl ether (non-natural diastereoisomer) (5), *trans*-1-Boc-2-deoxy-2-(1-piperidyl)spiobrasininol (6) and camalexin (7)

Table 1

IC₅₀ values [μM] of tested compounds on the amastigote form of *T. cruzi* grown in L₆E₉ rat myoblasts. Drug treatment : 72 hrs; SEM = standard error of mean; n = number of biological replicates

Tested Compound	IC ₅₀ ± SEM [μM] (antichagasic)
Brassinin (1)	3.8 ± 1.8 (n=2)
1-methoxybrassinin (2)	1.2 ± 0.33 (n=3)
Cyclobrassinin (3)	11.3 ± 1.6 (n=3)
Spirobrassinin (4)	16.1 ± 5.3 (n=2)
<i>cis</i> -1-methoxyspirobrassinol methyl ether (5)	9.2 ± 0.6 (n=3)
<i>trans</i> -1-Boc-2-deoxy-2-(1-piperidyl)spirobrassinol (6)	9.2 ± 0.5 (n=2)
Camalexin (7)	11.3 ± 4.1 (n=2)

Table 2

Molecular weight (Mw), number of hydrogen bond donors (HBD), number of hydrogen bond acceptors (HBA), predicted log octanol-water partition coefficient (GlogP), compliance with Lipinski rule of five (RuleOf5), predicted maximum recommended therapeutic dose (MRTD) and predicted cytotoxicity for MCF-7 cells (IC₅₀).

Compound	Mw	HBD	HBA	GlogP	RuleOf5	MRTD[mg/kg.day]	IC ₅₀ [μM]
(1)	236.02	1	2.07	Compliant	1.62		10.2
(2)	266.11	2	1.83	Compliant	1.74		8.3
(3)	234.01	0	2.07	Compliant	1.91		9.8
(4)	250.01	2	0.95	Compliant	2.14		17.4
(5)	296.10	2	1.42	Compliant	2.14		11.7
(6)	419.20	2	1.89	Compliant	1.17		21.9
(7)	200.01	0	2.07	Compliant	1.78		8.7
Nifurtimox	287.10	5	0.76	Compliant	3.09		6.6
Benznidazole	260.10	4	0.94	Compliant	3.02		15.5