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Abstract

Hypnophilin and panepoxydone, terpenoids isolated from *Lentinus strigosus*, have significant inhibitory activity on *Trypanosoma cruzi* trypanothione reductase (TR). Although they have similar TR inhibitory activity at 10 µg/mL (40.3 µM and 47.6 µM for hypnophilin and panepoxydone, respectively; ~100%), hypnophilin has a slightly greater inhibitory activity (~71%) on *T. cruzi* amastigote (AMA) growth *in vitro* as well as on *in vitro* phytohemagglutinin (PHA)-induced peripheral blood mononuclear (PBMC) proliferation (~70%) compared to panepoxydone (69% AMA inhibition and 91% PBMC inhibition). Hypnophilin and panepoxydone at 1.25 µg/mL had 67% inhibitory activity on *Leishmania* (*Leishmania*) *amazonensis* amastigote-like (AMA-like) growth *in vitro*. The panepoxydone activity was accompanied by a significant inhibitory effect on PHA-induced PBMC proliferation, suggesting a cytotoxic action. Moreover, incubation of human PBMC with panepoxydone reduced the percentage of $CD16^+$ and $CD14^+$ cells and down-regulated $CD19^+$, $CD4^+$ and $CD8^+$ cells, while hypnophilin did not alter any of the phenotypes analyzed. These data indicate that hypnophilin may be considered to be a prototype for the design of drugs for the chemotherapy of diseases caused by Trypanosomatidae.

Key words: Chagas disease; Leishmaniasis; Chemotherapy; Immunomodulation; Hypnophilin; *Lentinus strigosus*

Introduction

Programs to promote the development of pharmaceutical products for the treatment of so-called neglected diseases have been implemented around the world (1). Neglected diseases include diseases that disproportionately affect millions of poor populations in developing countries, such as tuberculosis, malaria, leishmaniasis, and trypanosomiasis. There is an urgent need for novel and improved drugs against these tropical diseases, specifically those caused by protozoa (2). Trypanosomatidae alone are responsible for an infected population of nearly 30 million, and more than 400 million are at risk depending on medical care and

on new perspectives of chemotherapy (3).

The drugs currently used for the treatment of Chagas disease are two nitroaromatic heterocyles, nifurtimox® [Nfx, Lampit Bayer, which is produced and used predominantly in Central America (4)] and benznidazole (Bnz, Rochagan®, Roche, Switzerland) that display little or no activity during chronic infection, despite their beneficial effects during acute disease (3,5,6). The drugs of choice for the treatment of leishmaniasis are sodium stibogluconate (Pentostam®, GlaxoSmithKline, UK), meglumine antimoniate (Glucantime®, Sanofi-Aventis, France), pentamidine (Astellas

Healthcare Inc., USA) and liposomal amphotericin B® (Bristol-Myers Squibb, USA) but they sometimes fail to act (3). Furthermore, these drugs have several side effects that reduce their clinical use (4,7).

Trypanothione reductase (TR) is an essential enzyme specific for the parasitic protozoa of the order Kinetoplastida. The enzyme catalyzes the NADPH-dependent reduction of glutathionylspermidine conjugates. It is a key enzyme of the parasite's thiol metabolism in which the ubiquitous glutathione/glutathione reductase system is replaced by trypanothione and TR. Reduction of trypanothione disulfide is crucial for the maintenance of a reducing intracellular milieu. These considerations render the parasite-specific TR an attractive target molecule for the development of structure-based drug against Chagas disease, African sleeping sickness, other diseases caused by trypanosomes, and leishmanias (8,9). Studies have also been devoted to the characterization of the impact of new drug prototypes on the human immune system (10).

We initially screened more than 3000 extracts of plants and fungi and observed that the extract of *Lentinus strigosus*, a Basidiomycete fungus belonging to the Polyporaceae family (11,12), contained the most promising drug activity because of its strong TR-inhibitory activity. This TR-bioguided assay led to a detailed investigation of two secondary metabolites produced by the fungus *L. strigosus* named panepoxydone and hypnophilin (12).

In the present study, we provide evidence for the differential cytotoxic effect of these drugs on the proliferation of human peripheral blood mononuclear cells (PBMC) stimulated by phytohemagglutinin (PHA) and on the immunophenotyping of normal PBMC. These compounds also had a significant antiparasitic effect against *Trypanosoma cruzi* amastigotes (AMA) and AMA-like forms of *Leishmania* (*Leishmania*) *amazonensis.* We also report for the first time the effect of hypnophilin and panepoxydone on leukocyte subpopulations, demonstrating a differential cytotoxic effect on lymphocytes. Our findings suggest that hypnophilin, a terpenoid from *L. strigosus*, is a potential prototype for Chagas disease and leishmaniasis chemotherapy.

Material and Methods

Panepoxydone and hypnophilin

Panepoxydone and hypnophilin were isolated from an ethyl acetate extract of *L. strigosus* (Schwein) Fr. (voucher code UFMGCB975) as previously described (12). All spectral data (MS, IR, 1H-, and 13C-NMR) of the crystals obtained for this study were in agreement with that published for panepoxydone and hypnophilin (12) (Figure 1). The compounds were dissolved in dimethylsulfoxide (DMSO), diluted in RPMI and added to the culture at the desired final concentrations. A control experiment was performed using 0.05% DMSO, the maximum concentration of DMSO used in the present study.

Measurement of recombinant *T. cruzi* trypanothione reductase

The colorimetric assay was performed in 96-well plates (Costar 9017, Corning, USA) by the method of Hamilton et al. (13) using DTNB (17.5 nmol) as the colorimetric reagent. Each assay well (250 µL) contained enzyme (6 mU), trypanothione (0.25 nmol) and NADPH (50 nmol) using HEPES buffer (40 mM, pH 7.5) with 1 mM EDTA. The experiments were conducted in triplicate and the results are reported as percent enzyme inhibition. The control drug was clomipramine (6.5 µM, Sigma, USA), a known TR inhibitor (14). The assays were conducted after 30 min at 30°C of pre-incubation of the compounds with the enzyme before the addition of the NADPH co-factor and the substrate trypanothione. Measurements were made at 412 nm in the kinetic mode every 10 s for 5 min in triplicate in two independent experiments.

Measurement of *T. cruzi* amastigote β-galactosidase activity

The assay was performed by the method of Buckner et al. (15). *T. cruzi* (Tulahuen strain) expressing the *Escherichia coli* β-galactosidase gene was grown on a monolayer of mouse L929 fibroblasts. Cultures to be assayed for β-galactosidase activity were grown in RPMI 1640 medium, pH 7.2-7.4, without phenol red (Gibco BRL, USA) plus 10% fetal bovine serum, 2 mM glutamine and 40 µg/mL gentamicin. For the bioassay, 4000 L929 cells in 80 µL supplemented medium were added to each well of a 96-well microtiter plate. After incubation overnight, 40,000 trypomastigotes in 20 µL were added to the cells and incubated for 2 h. The medium containing extracellular parasites was replaced with 200 µL of fresh medium and the plate was incubated for an additional 48 h to establish the infection. The medium was then replaced with solutions of the compounds at different concentrations in fresh medium (200 µL), and the plate was incubated for 96 h. Then, 50 µL 500 µM chlorophenol red glycoside in 0.5% Nonidet P40 was added to each well, the plate was incubated for 24 h, and absorbance at 570 nm was measured. Controls with uninfected cells, untreated infected cells, and medium only were run in parallel. The results are reported as percent reduction of absorbance at 570 nm in the experimental wells compared to control wells with untreated infected cells (percent inhibition = $(1 - Abs_{exp} / Abs_{contr}) \times 100$). Triplicate analyses were run in the same plate, and the experiments were repeated at least once.

Percent inhibition of the viability of amastigote-like forms of *L.* (*L.*) *amazonensis* by hypnophilin and panepoxydone

Promastigotes of *L.* (*L.*) *amazonensis* (IFLA/BR/196/ PH-8 strain) were obtained from lesions of infected hamsters. The parasites were grown at 26°C in Schneider's medium, pH 7.2, and then stimulated to differentiate into

the amastigote forms by increasing the temperature (32°C) and lowering the pH of the Schneider's medium to 6.0. After 7 days under these conditions, 90% of the promastigotes had transformed into amastigote-like forms, which were then used in the bioassays. Amastigote density was adjusted to 1 x 10^8 parasites/mL, and 90 μL added to each well of 96-well plates. Ten microliters of the test and control solutions was added to attain the desired concentrations. The plates were incubated at 32°C for 72 h, and cell viability was then determined using the methyl thiazolyl tetrazolium (MTT) assay (16). The results are reported as percent inhibition in relation to the controls without the drug. Amphotericin B at 0.2 μg/mL (Fungisons Bristol-Myers Squibb B, Brazil) was used as a positive control. All assays were performed in triplicate.

Percent inhibition of human PBMC proliferation by hypnophilin and panepoxydone

Hypnophilin and panepoxydone at 10 μg/mL were used to determine their effect on *in vitro* PHA-induced PBMC proliferation. PBMC were obtained from healthy adult volunteers and processed as previously described (17). The assay was performed by the method of Souza-Fagundes et al. (10). Briefly, the cultures were incubated with samples in the presence or absence of dexamethasone (positive control) and cell proliferation and viability were determined by a colorimetric assay using MTT (16). The results are reported as percent inhibition or stimulation compared to PHA-induced lymphocyte proliferation without added drugs.

Immunophenotyping of PBMC

PBMC (2 x 10^6 cells/mL) were cultured in polypropylene tubes (Falcon, 2063, Becton Dickinson, USA) in the presence of 1.25 μg/mL panepoxydone or hypnophilin for 18 h. Immunophenotyping was performed using 2 μL of the following antibody clones (Becton Dickinson): Leu-4 FITC (CD3), Leu-3a FITC (CD4), Leu-2a FITC (CD8), and Leu-M3 PE (CD14). The isotype controls used were phycoerythrin-conjugated IgG1 and FITC-conjugated IgG2. Data acquisition and analysis were performed with a single-laser flow cytometer (FACscan, Becton Dickinson) using the CellQuest software. Ten thousand events were counted for each determination. Laser scatter dot plots (size versus granularity) were used to identify and select the PBMC subpopulations. Two-dimensional fluorescence dot plots (FL1 versus FL2) were used to identify labeled cell subpopulations after staining with fluorochrome-conjugated monoclonal antibodies.

All experimental protocols were approved by the Ethics Committee of Fundação Oswaldo Cruz, Ministry of Health, Brazil.

Statistical analysis

Each experiment with PBMC and purified cells was run in triplicate and repeated at least five times on different days using PBMC from different individuals. Data are reported as means ± SEM. All data for each experiment were analyzed by the Student *t*-test. Statistical significance was considered when P was ≤0.05. The 50 and 90% effective concentrations (EC50 and EC90, respectively) of panepoxydone and hypnophilin against parasite growth were determined by a sigmoidal Hill function with the GraphPad Prism software (version 5; GraphPad Software Inc., USA) and linear regression analysis with the MS EXCEL® version 2007 (Microsoft Inc., USA).

Results

Inhibition of *in vitro* TR activity, *T. cruzi* amastigote growth, *L.* (*L.*) *amazonensis* growth and PHA-induced human PBMC proliferation by panepoxydone and hypnophilin

The chemical structures of panepoxydone and hypnophilin isolated from the organic extract of *L. strigosus* are shown in Figure 1. We evaluated their inhibitory effect *in vitro* in order to mimic the *in vivo* situation of parasite growth. Human PBMC proliferation assays were performed to investigate the impact of new drug prototypes on the human immune system (Figure 1). Both compounds at 10 μg/mL (47.6 and 40.3 μM for panepoxydone and hypnophilin, respectively) significantly inhibited TR activity, growth of *T. cruzi* or *L.* (*L.*) *amazonensis* amastigotes, as well as the PHA-induced proliferation of human PBMC.
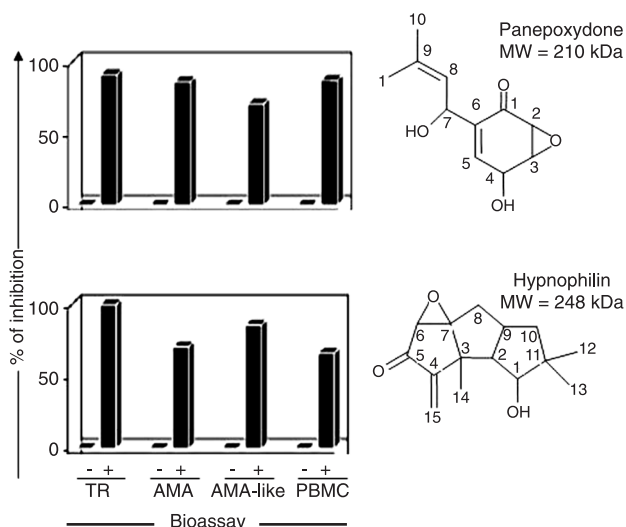

Figure 1. Effect of panepoxydone and hypnophilin on trypanothione reductase (TR) activity, on the viability of amastigotes of *Trypanosoma cruzi* (AMA) and of amastigote-like forms of *Leishmania* (*Leishmania*) *amazonensis* (AMA-like), and on phytohemagglutinin-induced human peripheral blood mononuclear cell (PBMC) proliferation. The concentration of each compound added to the cultures (+) was 10 μg/mL. Untreated cultures are shown as (-). Data are reported as means. The structures of the compounds are shown on the right.

Dose-response relationships

The data in Figure 2A show that panepoxydone and hypnophilin both present a typical dose-response inhibition of TR, *T. cruzi* AMA, amastigotes of *L. (L.) amazonensis* (AMA-like), and PHA-induced human PBMC lymphoproliferation.

As shown in Table 1, the EC values for hypnophilin demonstrated a higher inhibitory effect on *T. cruzi* AMA
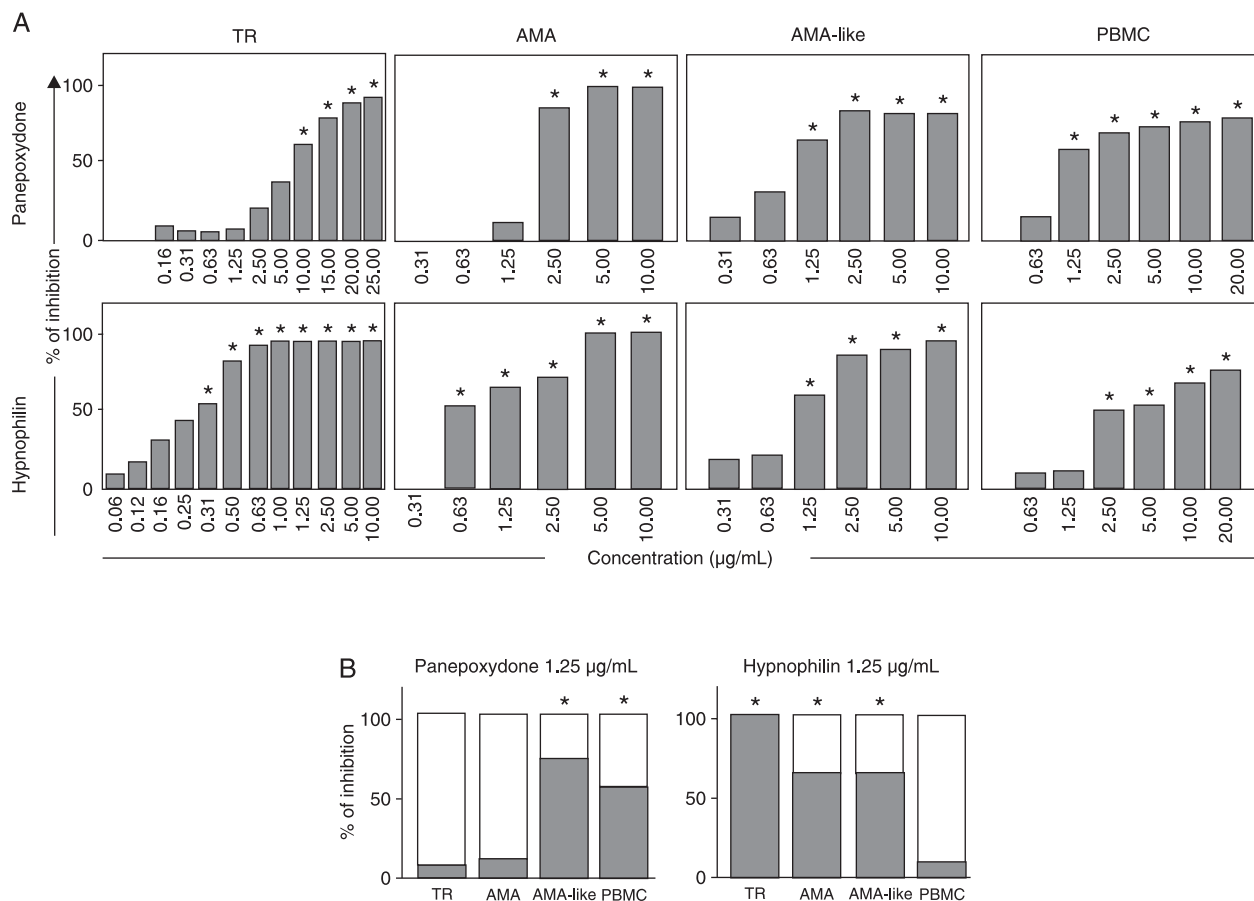

Figure 2. Dose-response effect of panepoxydone and hypnophilin on trypanothione reductase (TR), amastigotes of *Trypanosoma cruzi* (AMA), amastigote-like forms of *Leishmania* (*Leishmania*) *amazonensis* (AMA-like) and phytohemagglutinin-induced human lymphoproliferation (PBMC) assays (A). The concentration of 1.25 µg/mL was considered to guarantee higher AMA-like inhibitory capacity with a well-preserved PBMC response in the presence of the substances (B). The statistically significant differences (P < 0.05) were obtained by the paired Student *t*-test and are indicated by asterisks.

Table 1. *In vitro* activity (µg/mL or µM) of panepoxydone and hypnophilin against *Trypanosoma cruzi* amastigotes (AMA) and *Leishmania* (*Leishmania*) *amazonensis* amastigotes (AMA-like).

In vitro assays	Hypnophilin (µg/mL)		Panepoxydone (µg/mL)	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
AMA	0.66 (2.65 µM)	5.93 (23.89 µM)	1.79 (8.52 µM)	16.09 (76.58 µM)
AMA-like	2.16 (8.70 µM)	19.42 (78.26 µM)	0.84 (3.99 µM)	7.54 (35.89 µM)

Data are reported as means and molar concentration is given within parentheses. EC_{50} and EC_{90} = 50 and 90% effective concentrations.

growth *in vitro* (EC$_{50}$: 0.66 µg/mL or 2.65 µM and EC$_{90}$: 5.93 µg/mL or 23.9 µM) than panepoxydone (EC$_{50}$: 1.79 µg/mL or 8.52 µM and EC$_{90}$: 16.09 µg/mL or 76.58 µM). In contrast, panephoxydone demonstrated a higher inhibitory effect on AMA-like growth than hypnophilin (EC$_{50}$: 0.84 µg/mL or 3.99 µM and EC$_{90}$: 7.54 µg/mL or 35.89 µM) than hypnophilin (EC$_{50}$: 2.16 µg/mL or 8.70 µM and EC$_{90}$: 19.42 µg/mL or 78.26 µM).

These natural products showed a similar significant inhibitory effect at 1.25 µg/mL (Figure 2B). On the basis of the data in Figure 2A, we suggest that 1.25 µg/mL (6 µM panepoxydone and 5 µM hypnophilin) is the concentration guaranteeing higher TR, AMA and AMA-like inhibitory capacity as well as the least inhibition of PBMC proliferation. Interestingly, 5 µM hypnophilin is able to block 100% TR activity and about 70% of AMA and AMA-like growth, with minor inhibition of PHA-stimulated PBMC proliferation. In contrast, 6 µM panepoxydone had a lower (<10%) inhibitory effect against TR activity and AMA growth but presented 80% inhibition of AMA-like growth, but this was also associated with a significant inhibitory effect (50%) on PHA-induced PBMC proliferation, suggesting that 6 µM panepoxydone has cytotoxic activity (Figure 2B).

Hypnophilin displayed a non-cytotoxic immunomodulatory mechanism of action on *in vitro* human PBMC cultures

Figure 3A (PHA-induced proliferation) shows that both 6 µM panepoxydone and 5 µM hypnophilin inhibited PBMC proliferation, but this effect was more pronounced for panepoxydone. The statistically significant inhibition of proliferation (Figure 3B) was dose-dependent and occurred at a lower concentration of 0.31 µg/mL (1.3 µM) for panepoxydone compared to hypnophilin (2.5 µg/mL or 10.1 µM).

Incubation of human PBMC with panepoxydone significantly reduced the percentage of CD16$^+$ and CD14$^+$ cells and provoked down-regulation of CD19$^+$, CD4$^+$ and CD8$^+$ cells

We determined the effect of these compounds on lymphocyte subsets or monocytes by immunophenotyping and flow cytometry (Figure 3C). Panepoxydone, 1.25 µg/mL (6 µM), significantly reduced the percentage of CD16$^+$ (1.0 ± 0.0%) and CD14$^+$ cells (2.0 ± 1.0%) compared to untreated cells (11.0 ± 4.0 and 11.0 ± 5.0%, respectively) while hypnophilin did not alter any of the phenotypes analyzed. Moreover, 1.25 µg/mL (6 µM) panepoxydone altered the profile of lymphocyte subpopulations by down-regulating CD19$^+$, CD4$^+$ and CD8$^+$ molecules on the lymphocyte surface. Cytometric data illustrating these findings are presented in Figure 4. Taken together, these results suggest a potential for toxicity by panepoxydone and support the conclusion that hypnophilin had no cytotoxic effect on human PBMC.

Discussion

We have been investigating the biological activities of hypnophilin and panepoxydone, natural products isolated from *L. strigosus* (12), focusing on identifying new drugs for the treatment of leishmaniasis and Chagas disease. We demonstrate here that hypnophilin is an effective *in vitro* anti-*Trypanosoma* and anti-*Leishmania* compound, with minor toxicity against human PBMC. After appropriate study, this compound could be used as a lead compound for drug design studies against leishmaniasis and Chagas disease.

The EC$_{50}$ and EC$_{90}$ values for hypnophilin acting on AMA were 2.71 and 3.2 times significantly lower, respectively, compared to panepoxydone. In contrast, the EC$_{50}$ and EC$_{90}$ values for AMA-like were 2.57 times significantly lower for panepoxydone compared to hypnophilin.

Cytotoxicity assays using normal human PBMC demonstrated that hypnophilin had lower toxicity than panepoxydone. Under our experimental conditions, the results demonstrated that panepoxydone has a typical cytotoxic effect *in vitro*, suppressing the immune response, leading to a reduction of the percent of lymphocyte and monocyte subpopulations in PBMC cultures, whereas hypnophilin had a non-cytotoxic immunomodulatory mechanism of action.

There is a general consensus that the host immune response plays a pivotal role in controlling adverse events during the massive antigen release triggered by trypanosomicidal agents, by protective responsive that may control tissue damage, diminishing and/or modifying the inflammatory nature of the immune response elicited by the *T. cruzi* antigens released by the action of the drug (18). Hypnophilin at a 5 µM concentration has strong anti-*T. cruzi* activity associated with minor non-cytotoxic immunomodulation of human leukocytes and should be effective during the chronic phase of Chagas disease. It is important to note that the currently available treatment for Chagas disease with benznidazole or nifurtimox is effective mainly during the acute phase of infection, with little effect during the chronic phase (19,20). Moreover, the drugs that are available are more effective against the blood flagellates than the intracellular amastigote forms (21). In addition, the drugs currently available to treat the different forms of leishmaniasis were introduced many decades ago and have significant drawbacks, especially in terms of efficacy, length of treatment, route of administration, toxicity, and cost (22). Therefore, we believe that our findings open new perspectives in the field of investigation of the action of natural products of Basidiomycota obtained from Brazilian ecosystems against Chagas disease and Leishmaniasis. Previous studies have identified panepoxydone as an agent interfering with the immune response inhibiting the activation of NF-kappaB (23,24). These findings have led to the proposal that panepoxydone may be useful as an anti-inflammatory agent. In the current study, we investigated, for the first time,
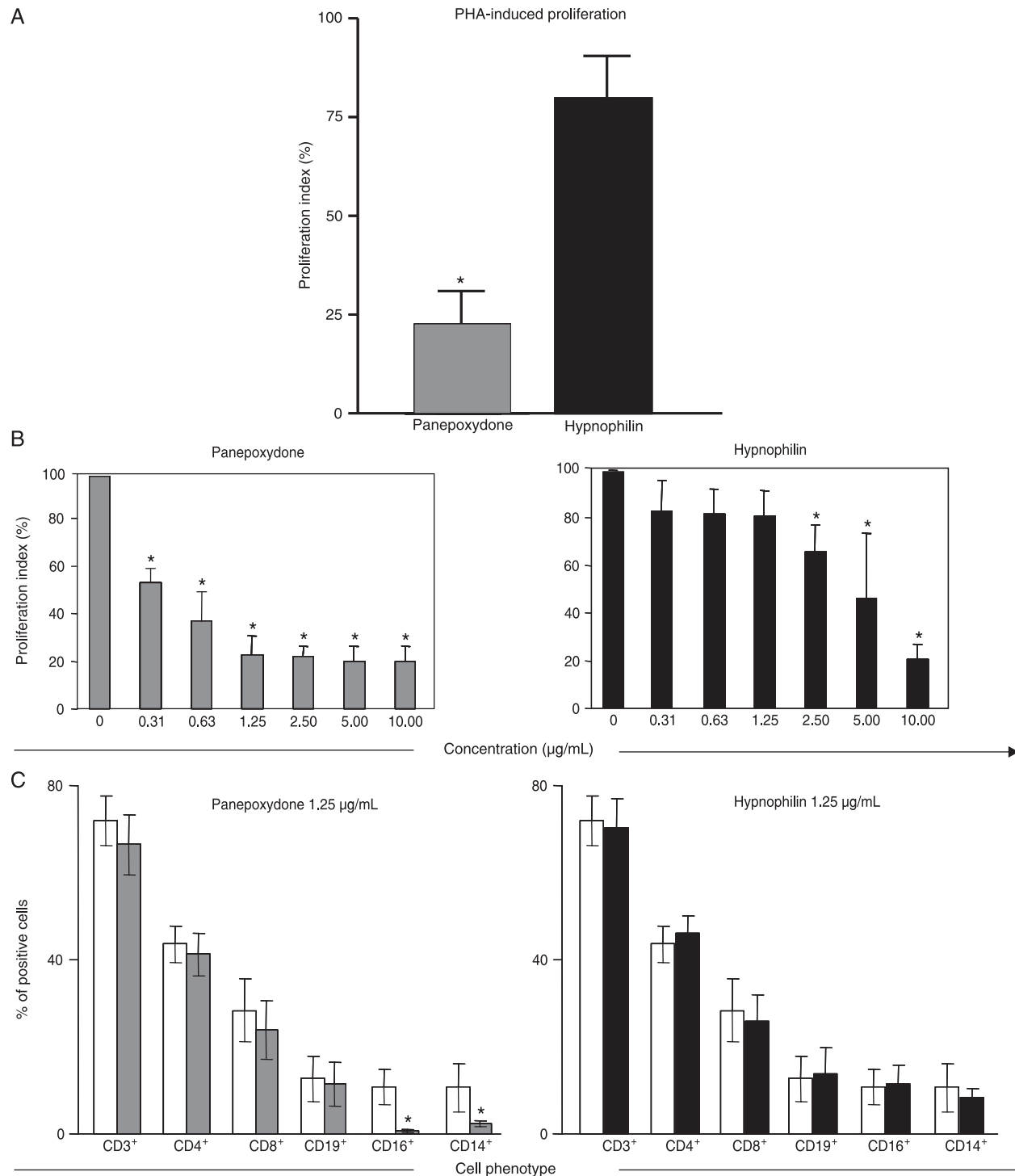
Figure 3. Effect of panepoxydone and hypnophilin on human phytohemagglutinin-induced proliferation and on peripheral blood mononuclear cell (PBMC) subpopulations. *A*, Human PBMC were incubated with 10 µg/mL panepoxydone (light gray columns) and 10 µg/mL hypnophilin (dark gray columns) for 72 h and cell proliferation was determined. *B*, Dose-response of proliferation to each compound. *C*, The effect of the substances (filled columns) compared to control (open columns) on lymphocyte subsets (CD3+, CD4+, CD8+, CD19+, CD16+ cells) and on monocytes (CD14+ cells) was determined after 18 h of incubation followed by flow cytometry analysis. Data are representative of four independent experiments performed in triplicate. *P < 0.05 for comparisons between subsets as well as between control cultures (100% positive; Student *t*-test).

the effects of panepoxydone on the proliferation of human PBMC stimulated with PHA and on human lymphocyte and monocyte immunophenotyping. Panepoxydone induced a reduction of the percentage of CD16$^+$ and CD14$^+$ cells compared to untreated cells. Quantitative flow cytometry experiments in the presence of hypnophilin at 1.25 µg/mL (5 µM) showed a reduced proliferation of PBMC stimulated with PHA and no apparent interference with the percentage

of human lymphocyte subsets and monocytes (Figure 4). Since the host immune response plays a pivotal role in the adverse events triggered by antigen release during treatment with trypanocidal drugs, the ability of hypnophilin to kill the intracellular forms of T. cruzi or L. (L.) amazonensis, while having no significant cytotoxic effect on human PBMC proliferation, suggests that this terpenoid may be a promising prototype for the development of new chemotherapeutic
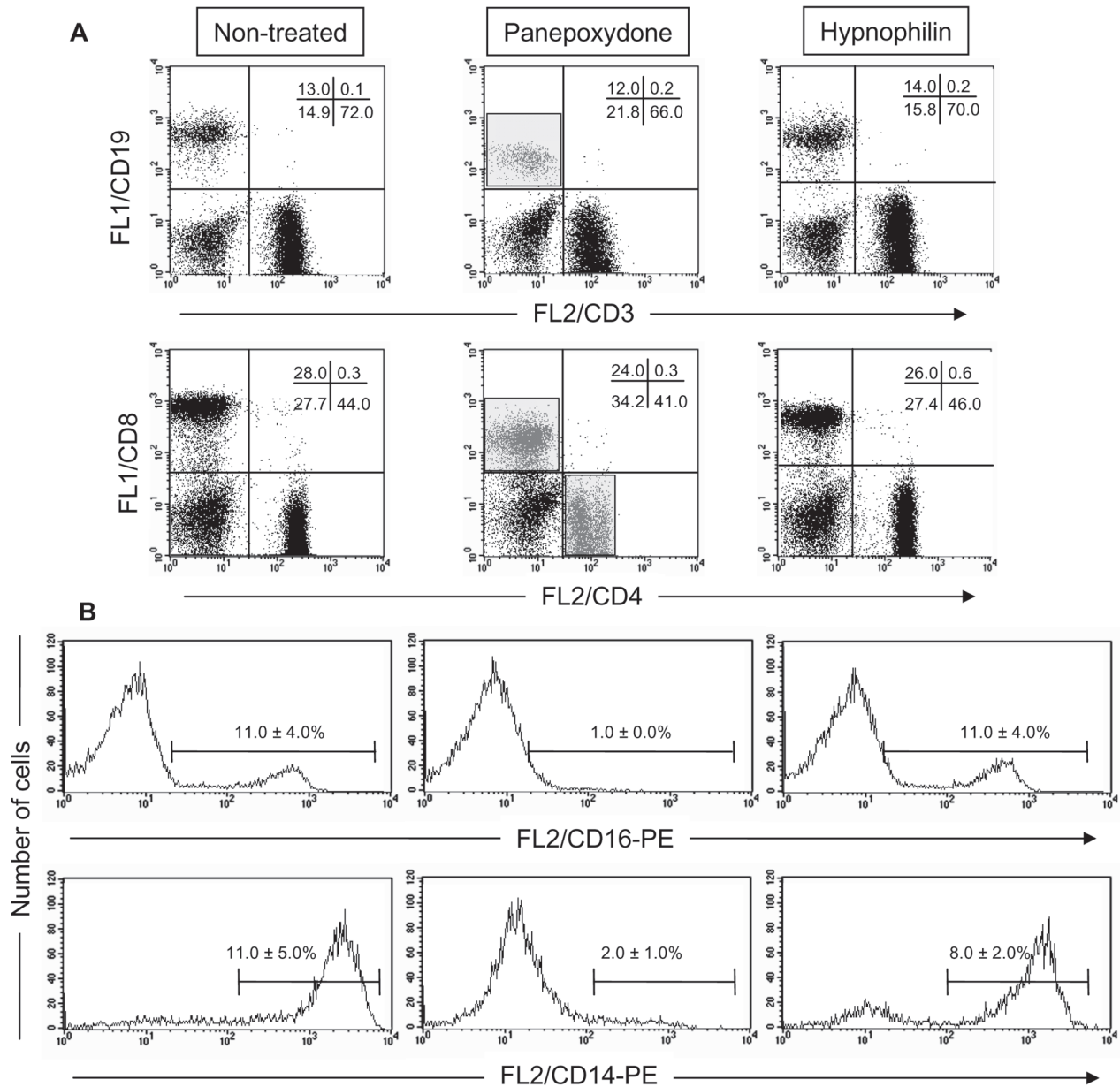

Figure 4. Representative data illustrating the effect of panepoxydone and hypnophilin on peripheral blood mononuclear cell subpopulations. Lymphocyte subsets (CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$ cells) are shown in dot plots (A) while CD16$^+$ cells and CD14$^+$ cells/monocytes are shown in histograms (B). The effect of the compounds on both lymphocyte subsets and monocytes was determined after 18 h of incubation followed by analysis by flow cytometry.

agents for Chagas disease and leishmaniasis. Additional studies are underway to characterize the molecular basis of hypnophilin-induced immunomodulatory activity.

Acknowledgments

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