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Research Article

Antiparasitic and Immunomodulatory Activities of 1,1-bis(4-Hydroxyphenyl)-2-Phenyl-but-1-ene and Its Protected and Free 2-Ferrocenyl Derivatives

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Strategy, Management and Health Policy				
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ABSTRACT The ferrocenyl diphenol 1 [1,1-bis(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene] displays strong cytotoxic effects against a variety of cancer cells. In the present study we have evaluated the immunomodulatory and antiparasitic activities of compound 1 and its protected dipalmitate analogue 2. We have furthermore compared the antiparasitic results of 1 and 2 with the organic analogue, 3 [1,1-bis(4-hydroxyphenyl)-2-phenyl-but-1-ene], where the ferrocenyl group has been replaced by a phenyl ring. When assayed against normal (noncancerous) splenocytes, all compounds were considered nontoxic. Compound 1 inhibited NO production by macrophages, inhibited concanavalin A-induced lymphoproliferation, and was active against *Leishmania amazonensis* and *Trypanosoma cruzi*. Compound 2 had lower activity in all assays performed. Surprisingly, compounds 1 and 2 exhibited similar and significant activity against *Plasmodium falciparum*, with IC_{50} values of 3.50 and 1.33 µM, respectively. Compound 3 showed an inverse activity profile, being active against *T. cruzi* but far less active against *P. falciparum*. Drug Dev Res 71:69–75, 2010. © 2009 Wiley-Liss, Inc.

Key words: ferrocene; malaria; bioorganometallic chemistry; immunomodulatory; nitric oxide; Chagas disease; *Plasmodium falciparum*; *Trypanosoma cruzi*; *Leishmania amazonensis*

INTRODUCTION

The discovery of the antiproliferative effects of simple ferricenium salts on Ehrlich ascites tumors in 1984 [Köpf-Maier et al., 1984a,b] inspired an enormous body of work on the anti-cancer properties of ferrocene-containing compounds [Hillard et al., 2007a; Metzler-Nolte and Salmain, 2008, and references therein]. Ferrocenyl phenols in particular demonstrated strong antiproliferative activity [Hillard et al., 2007a,b;
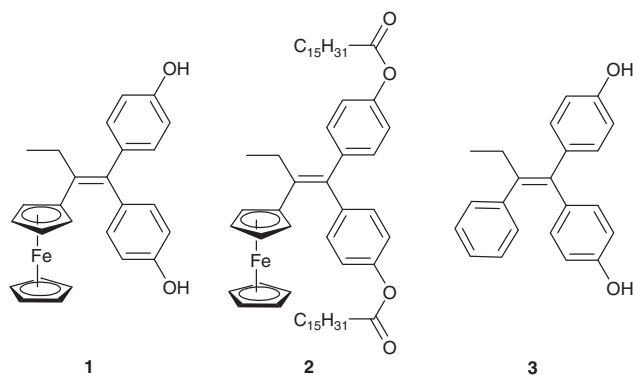
Fig. 1. Compounds studied in this report.

Pigeon et al., 2009; Plażuk et al., 2009; Vessières et al., 2005]. One of the most efficacious compounds, [1,1-bis(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene] **1** (Fig. 1), is cytotoxic against hormone-dependent MCF-7 and hormone-independent MDA-MB-231 breast cancer cells, with an IC$_{50}$ value for the latter of $\sim$0.5 μM [Vessières et al., 2005]. Compound **1** is also toxic against 9L gliosarcoma transfected in rat flank and brain, when encapsulated into lipid nanocapsule delivery devices [Allard et al., 2008, 2009]; it displays activity against malignant melanoma cells in vitro [Michard et al., 2008]. We have shown that the anticancer properties of such compounds may arise from the oxidation chemistry of the ferrocene moiety, ultimately leading to a quinoid species [Hillard et al., 2006, 2008]. We believe this to be a key active metabolite, particularly due to the observation that quinones play a major role as reactive oxygen species (ROS) enhancers, redox catalysts, and electrophiles [Asche, 2005; Bolton et al., 2000; De Abreu et al., 2002; Goulart et al., 2003, 2004; Hillard et al., 2008; Monks and Jones, 2002; Ferreira et al., 2009], and for this reason are commonly encountered in chemotherapeutic agents.

Redox-active and/or organometallic moieties are furthermore important actors in antiparasitic compounds. For example, ferrocenyl derivatives of penicillin and cephalosporin have shown an improved therapeutic spectrum against drug-resistant organisms [Edwards et al., 1975, 1976]. Ferroquine, the ferrocenyl analogue of the antimalarial chloroquine, is extremely toxic against several strains of chloroquine-resistant *Plasmodium falciparum*, and is now in phase II clinical trials [Biot et al., 1997, 2009; Dive and Biot, 2008]. Organometallic ruthenium derivatives of chloroquine also show activity against chloroquine-resistant strains of *P. falciparum* [Sánchez-Delgado et al., 1996; Rajapakse et al., 2009] and Chagas disease [Sánchez-Delgado et al., 1993], a debilitating disease caused by the flagellate protozoan *Trypanosoma cruzi*. Quinone

compounds likewise have found applications in Chagas disease [Goulart et al., 1997], likely due to the unique sensitivity of *T. cruzi* to the action of intracellular generators of H$_2$O$_2$. *T. cruzi* possesses a redox defense system based upon trypanothione and trypanothione reductase, which regenerates trypanothione from its oxidized (disulfide) form. It lacks catalase and glutatione peroxidase and is therefore more sensitive to H$_2$O$_2$-induced oxidative stress than its biological hosts. This mechanism is also operative in *Leishmania amazonensis* [Castro-Pinto et al., 2008; Lima et al., 2004], the parasite responsible for cutaneous and visceral leishmaniasis.

Immunopathologies such as autoimmune and allergic processes are diseases with high incidence and in which currently available drugs, although efficacious in most instances, produce undesirable side effects, resulting in complications to patients, especially after long-term use. The search for new bioactive molecules is of great interest, due to the need of new medicines for more effective treatment of pathologies with lower toxic effects. In the context of antiparasitic compounds, it is especially desirable to find a drug candidate that kills the parasite in the body while dampening the immune response of the body.

We now report the results of our investigation of the free ferrocenyl phenol **1** and its dipalmitate analogue **2** (Fig. 1), in terms of immunomodulatory activity (inhibition of lymphoproliferation and activated macrophage nitric oxide production), as well as their activity against *L. amazonensis*, *T. cruzi*, and *P. falciparum*. The role of the ferrocene moiety in cytotoxicity and antiprotozoal effects was evaluated by comparing the antiprotozoal results with those of the organic analogue of the free phenol, **3** (Fig. 1).

MATERIALS AND METHODS
Preparation of Compounds

Compounds **1** [Vessières et al., 2005], **2** [Allard et al., 2009], and **3** [Yu and Forman, 2003] were synthesized as previously reported. Samples were purified by semi-preparative HPLC or silica gel chromatography before use.

Animals

Male and female 4–6 weeks old BALB/c mice were used as spleen cell donors and for macrophage isolation. They were housed in standard environmental conditions and maintained at the animal facilities at Gonçalo Moniz Research Centre, FIOCRUZ (Salvador, Brazil). Mice were provided rodent diet and water ad libitum. All mice were handled and sacrificed according to the National Institutes of Health

(NIH) guidelines for laboratory animals, and protocols were approved by the Ethics Committee for Animal Use of FIOCRUZ.

Cytotoxicity Assay

Splenocytes from BALB/c mice (6×10^5 cells/well) were cultured in 96-well plate in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) and $50\,\mu g/ml$ of gentamycin (Novafarma, Anápolis, GO, Brazil). Each substance was evaluated in five concentrations starting at $100\,\mu g/ml$, in triplicate. Cells were incubated for 24 h at $37°C$ and 5% CO_2. After this period, cultures were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD) to determine [³H]-thymidine incorporation using a beta radiation counter (Plate Chameleon V Microplate Reader, Hidex, Turku, Finland). The viability of the cells was determined by the thymidine incorporation; cytotoxicity was calculated in relation to the [³H]-thymidine incorporation of untreated cultures.

Nitric Oxide Assay

Peritoneal exudate cells were obtained by washing the peritoneal cavity of BALB/c mice with cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) 5 days after injection of 3% thioglycolate in saline (1.5 ml per mouse). Peritoneal cells were washed twice with HBSS and resuspended in RPMI medium (GIBCO-BRL) supplemented with 10% FCS (Cultilab), L-glutamine (2 mM), RPMI 1640 vitamins solution (1%) (Sigma), sodium pyruvate (1 mM), HEPES (10 mM), 2-mercaptoethanol ($50\,\mu M$), and gentamycin ($50\,\mu g/ml$) (Sigma). Cells were plated (2×10^5 cells/well) in 96-well plates. After 1 h incubation at $37°C$, nonadherent cells were removed by washing with HBSS. Cultures were then stimulated with $500\,ng/ml$ lipopolysaccharide (LPS from *Escherichia coli* serotype 0111:B4, Sigma) in combination with $5\,ng/ml$ interferon-γ (IFN-γ; PharMingen, San Diego, CA) and treated with various concentrations starting at $100\,\mu g/ml$ of the pure substances, in a final volume of $200\,\mu l$. Cell-free supernatants were collected at 24 h of culture for determination of nitrite concentration, using the Griess method, as previously described [Ding et al., 1988].

Lymphoproliferation Assay

BALB/c mice splenocytes suspensions were prepared in complete RPMI medium and cultured in 96-well plates at 6×10^5 cells/well in $200\,\mu l$, in triplicate, in the presence of $2\,\mu g/ml$ concanavalin A (Con A; Sigma), alone or in various concentrations starting at $100\,\mu g/ml$ of analyzed compounds, as described in Figure 2. After 48 h, plates were pulsed with $1\,\mu Ci$ of [methyl-³H] thymidine for 12 h, and proliferation was assessed by measurement of ³H-thymidine uptake. The percentage of inhibition of lymphocyte proliferation by the compounds was determined in relation to the concanavalin A stimulated control [Costa et al., 2008].

Anti-*Leishmania amazonensis* Assay

L. amazonensis (MHOM/BR88/BA-125 Leila strain) promastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS, 5% sterile human urine, and $50\,\mu g/ml$ gentamycin, pH 7.2, at $26°C$ until logarithmic phase. Parasites were cultured in 96-well plates at 5×10^6 cells/well in $200\,\mu l$, in triplicate wells, alone or in the presence of the samples analyzed in five different concentrations (100, 33, 11, 3, and $1\,\mu g/ml$). After 5 days, a direct counting of viable parasites was carried out in a Neubauer chamber, using a phase-contrast microscope [Costa et al., 2008]. Amphoterycin B was used as the reference drug.

Anti-*Trypanosoma cruzi* Assay

T. cruzi (Y strain) epimastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS and $50\,\mu g/ml$ gentamicin, pH 7.2, at $26°C$, until logarithmic phase. Parasites were cultured in 96-well plates at 10^7 cells/well in $200\,\mu l$, in triplicate, alone or in the presence of the samples analyzed in five concentrations (100, 33.3, 11.1, 3.7, and $1.2\,\mu g/ml$). After 11 days, direct counting of viable parasites was carried out using a Neubauer chamber and phase-contrast microscopy [Leite et al., 2006]. Amphoterycin B was used as a reference drug.

Anti-*Plasmodium falciparum* In Vitro Assay

Compounds were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive [Junior et al., 1999]. All parasites were maintained in continuous culture of human erythrocytes (blood group A⁺) using RPMI medium supplemented with 10% human plasma [Trager and Jensen, 2005]. Parasites grown at 1–2% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at five concentrations, diluted with 4% DMSO in RPMI culture medium without hypoxanthine. Mefloquine was used in each experiment as a control for the *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the [³H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with [³H]-hypoxanthine uptake in drug-treated versus

untreated wells. All the assays were performed in triplicate, as described previously [Andrade-Neto et al., 2004; Zalis et al., 1998]. Mefloquine was used as a reference.

IC$_{50}$ Calculations

The inhibitory concentration for 50% (IC$_{50}$) of parasite growth was calculated based on a nonlinear regression (curve fit).

RESULTS

Cytotoxicity

The cytotoxicity of compounds **1**–**3** was tested against BALB/c mice splenocytes at five concentrations, starting at 100 μg/ml, as described above. IC$_{50}$ values (24-h incubation) for compounds **1** and **2** could not be determined due to the low cytotoxicity at the concentrations tested. In the case of compound **3**, an IC$_{50}$ value of 36 μg/ml (114 μM) was determined. The variable dose results are shown in Figure 2.

Immunomodulatory Activity Assays

The activity of compounds **1** and **2** on cultures of activated macrophages was evaluated. At 100 μg/ml, compound **1** almost completely inhibited nitric oxide production by macrophages activated by LPS and IFN-γ (Fig. 2), while compound **2** did not have a significant inhibitory activity on NO production, with an inhibition of only 18% at 100 μg/ml.

Likewise, compound **1** exhibited strong inhibitory activity in the proliferation of Con A-activated lymphocytes (Fig. 2), with an IC$_{50}$ value of 9 μM, while compound **2** showed weak activity. The inhibitory activity of compound **1** on lymphocytes was not due to a cytotoxic effect, since the cytotoxicity levels were below the levels of immunomodulatory activities (Fig. 2).

Antiprotozoal Activity

The antiprotozoal activity of compounds **1** and **2** was investigated using two trypanosomatid parasites: *T. cruzi* and *L. amazonensis*, and chloroquine-resistant *P. falciparum*. Compound **1**, but not **2**, was active against *T. cruzi* and *L. amazonensis*. In contrast, both compounds were highly active against *P. falciparum*, with lower IC$_{50}$ values compared with those for *T. cruzi*

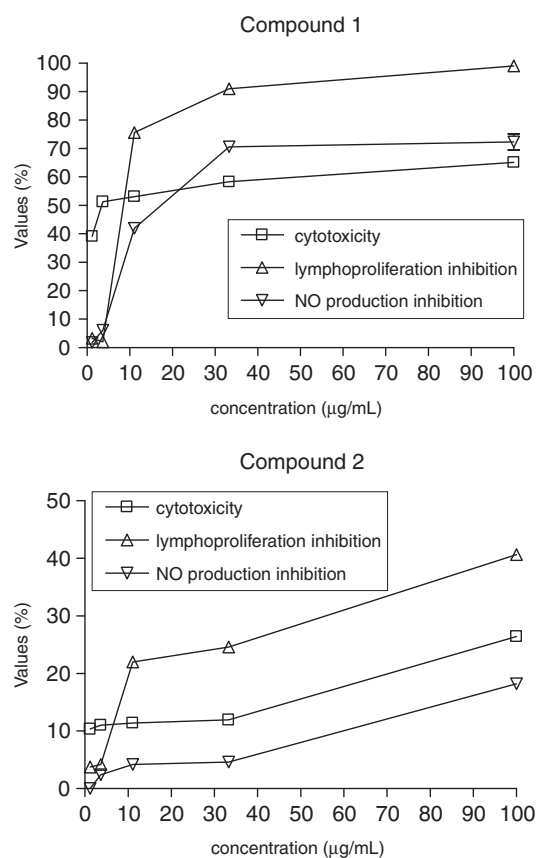and *L. amazonensis* (Table 1). Interestingly, compound **3** displayed an opposite profile, being more active

Fig. 2. Cytotoxicity and immunomodulatory activity of compounds **1** and **2**. Mouse splenocytes were cultured during 48 h in the presence or absence of different concentrations of the compounds tested and concanavalin A. Inhibition was calculated based on values of untreated, Con A-stimulated cultures, as described in Materials and Methods. NO production inhibition was determined in cultures of peritoneal murine macrophages stimulated with LPS+IFN-γ and treated with compound **1**, at different concentrations, measuring nitrite concentrations in culture supernatants by the Griess method, as described in Materials and Methods. For cytotoxicity assays, splenocytes of BALB/c mice were cultured during 24 h in the presence or absence of compounds and [³H]-thymidine. The percentage of viability was calculated comparing values of [³H]-thymidine incorporation of cultures treated with compounds to those of untreated cultures. Values represent the means±SD of values obtained from three individual experiments.

TABLE 1. Antiparasitic Activity of Compounds 1–3

Compound	IC$_{50}$ *L. amazonensis* μg/ml (μM)	IC$_{50}$ *T. cruzi* μg/ml (μM)	IC$_{50}$ *P. falciparum* μg/ml (μM)
1	30.3 (71.4)	26.38 (62.2)	1.5 (3.50)
2	Not active	Not active	1.2 (1.33)
3	15.31 (48.4)	2.62 (8.3)	28.66 (90.6)
Amphotericin B	0.061 (0.068)	0.138 (0.149)	—
Mefloquine	—	—	0.018 (0.043)

against *T. cruzi* and *L. amazonensis*, but was less active against *P. falciparum*.

DISCUSSION

Interest in the use of organometallic compounds in medicine has grown exponentially, ever since the modern definition of bioorganometallic chemistry by Jaouen in 1985 [Jaouen, 2006]. Although the preponderance of studies focuses on anti-cancer compounds, there have been several studies on the application of such compounds as antiprotozoals, particularly ferrocene containing compounds [Baramee et al., 2006; Biot et al., 2000; Guillon et al., 2008; Itoh et al., 2000]. Notably, the organometallic compound ferroquine displays IC_{50} values in the nanomolar range against chloroquine-resistant *P. falciparum* strains; it is currently the most advanced anti-malaria project at Sanofi-Aventis [Biot et al., 2009]. In the present work we describe, for the first time, the antiparasitic and immunomodulatory activity of the ferrocenyl phenol **1**, previously shown to have strong antiproliferative effects against a variety of cancer cells lines. To test whether the pharmacological properties could be enhanced by increasing the lipophilicity of the molecule, the analogue **2**, where the phenol groups have been converted to long-chain esters, was also tested. Finally, to evaluate role of the ferrocene group, the organic analogue of **1** (**3**) was also studied.

All the compounds displayed low cytotoxicity against spleen cells, and an LC_{50} value could be calculated only for the organic compound **3** which was more toxic. Values for **1** and **2** could not be determined because the results obtained did not permit the calculation of the lethal doses, indicating that LC_{50} values for the ferrocenyl compounds are higher than $100 \, \mu g/ml$. A low cytotoxicity for **1** on normal brain cells has previously been observed in vitro [Allard et al., 2008], with an IC_{50} value of $50 \, \mu M$ found for newborn rat astrocyte primary cultures. This value is approximately 100 times higher than the value determined for MDA-MB-231 cancer cells or 9L-glioma cells. Indeed, in the current study, we observe that the purely organic compound **3** is more toxic to normal cells than those containing ferrocene.

Compound **1** displayed an elevated immunomodulatory activity, being able to inhibit the activation of lymphocytes and macrophages. These cells are important effectors of immune responses; therefore, the immunomodulatory potential of this compound in models of immune-mediated diseases, as well as its mechanism of action, should be investigated. Although some cytotoxic activity was seen (data not shown), the inhibitory activity of compound **1** in mammalian cells was not due to a toxic

effect, since the levels of cytotoxicity were below the values of inhibition of immune responses.

Both ferrocenyl compounds were active against chloroquine-resistant *P. falciparum*, with IC_{50} values within the range of $1.3–3.5 \, \mu M$. In addition, **1** was active against epimastigote forms of *T. cruzi* and promastigote forms of *L. amazonensis*. Compound **2**, however did not show activity against these protozoa. This mirrors the trend observed for compounds **1** and **2** against 9L glioma cells. While compound **1** and its acetate analogue showed strong and similar antiproliferative activity against such cells (IC_{50} around $0.5 \, \mu M$), the palmitate ester **2** was much less active ($IC_{50} = 20 \, \mu M$) [Allard et al., 2009]. The authors attributed the poor activity of **2** to the lack of hydrolysis of the palmitate ester in situ. This interpretation suggests that the free phenol function may be necessary for the activity of **1** against *T. cruzi* and *L. amazonensis*. Consistent with this interpretation, the organic compound **3** was found to be more active than the ferrocenyl **1**, suggesting that ferrocene does not impart any particular benefit against these protozoa. To our knowledge, such triphenyl ethylene compounds have never been studied in the context of Chagas disease (American trypanosomiasis). Because of its limited toxicity against human cells [Lubczyk et al., 2002] and its high toxicity against *T. cruzi* (Table 1) compound **3** could be an attractive lead compound in this context.

It is clear, however, that the ferrocenyl group is a key to the antiproliferative effects of compounds **1** and **2** against *P. falciparum* when compared with **3**, which shows only weak effects [Molyneaux et al., 2005]. Although the mechanism of ferroquine is not fully understood, it has been shown that the ferrocene moiety can generate reactive oxygen species (ROS) under oxidizing conditions, such as in the food vacuole of the *P. falciparum* [Biot et al., 2009]. The ability of compounds such a **1** to produce ROS in a variety of cell types is currently under study. The enhanced lipophilicity of **1** ($P_{o/w} = 5.0$) and **2**, compared with **3** ($P_{o/w} = 4.4$), could also play a role. Recent studies suggest that the activity of ferroquine results, at least in part, from its superior lipophilicity compared with chloroquine, and possibly from its localization in the lipidic sites of heme crystallization [Biot et al., 2009]. Because the lipophilicity of such compounds can be easily and systematically modified by the choice of protecting groups on the phenol moiety, a structure–activity relationship study is feasible.

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