Am. J. Trop. Med. Hyg., 101(6), 2019, pp. 1359–1368
doi:10.4269/ajtmh.19-0350

Vismione B Interferes with *Trypanosoma cruzi* Infection of Vero Cells and Human Stem Cell–Derived Cardiomyocytes

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Abstract. Traditional African medicine is a source of new molecules that might be useful in modern therapeutics. We tested ten limonoids, six quinones, one xanthone, one alkaloid, and one cycloartane, isolated from four Cameroonian medicinal plants, and one plant-associated endophytic fungus, against *Trypanosoma cruzi*, the etiological agent of Chagas disease (CD). Vero cells, or human-induced pluripotent stem cells (hiPSC)–derived cardiomyocytes (hiPSC-CM) were infected with *T. cruzi* trypomastigotes (discrete typing unit types I or II). Infection took place in the presence of drugs, or 24 hours before drug treatment. Forty-eight hours after infection, infection rates and parasite multiplication were evaluated by Giemsa stain. Cell metabolism was measured to determine functional integrity. In Vero cells, several individual molecules significantly affected *T. cruzi* infection and multiplication with no, or minor, effects on cell viability. Reduced infection rates and multiplication by the quinone vismione B was superior to the commonly used therapeutic benznidazole (BNZ). The vismione B concentration inhibiting 50% of *T. cruzi* infection (IC_{50}) was 1.3 μM. When drug was applied after infection, anti-*Trypanosoma* effects of vismione B [10 μM) were significantly stronger than effects of BNZ (23 μM). Furthermore, in hiPSC-CM cultures, infection and multiplication rates in the presence of vismione B (10 μM) were significantly lower than in BNZ (11.5 μM), without showing signs of cytotoxicity. Our data indicate that vismione B is more potent against *T. cruzi* infection and multiplication than BNZ, with stronger effects on established infection. Vismione B, therefore, might become a promising lead molecule for treatment development for CD.

INTRODUCTION

Chagas disease (CD) is a systemic, and often chronic, disease caused by the protozoan *Trypanosoma cruzi*. This parasite causes morbidity and mortality and is endemic to the Americas, of particular importance to Latin America, where at least six million people are estimated to be infected.[1–6] The incidence of CD in the United States and other nonendemic countries is rising, largely because of high emigration from endemic regions[7,8] and also because of blood transfusion, organ transplantation, and congenital transmission. It is estimated that > 300,000 individuals in the United States have CD, with up to 45,000 having cardiomyopathies.[2,3] Autochthonous cases have been reported in the southern tier of the United States, where a variety of potential animal reservoirs are described. Insect vectors, related to the triatomine insects responsible for transmission in Latin America, are found in 28 states.[9]

There are six *T. cruzi* (Tc) discrete typing units: TcI-VI.[10] Different *T. cruzi* strains seem to populate different organs, which might have implications for pathogenesis of chronic forms of the disease.[11]

Chagas disease presents with an acute phase, with only signs at the locus of the insect bite, followed by a lifelong chronic phase, with distinct clinical forms known as indeterminate (largely silent), later blooming with cardiac and/or digestive pathology.[6] The most common and severe manifestation of CD is the cardiac form, causing congestive heart failure, arrhythmias, and conduction abnormalities. This type of dilated cardiomyopathy is associated with thromboembolic events, often leading to stroke and sudden death. The current therapies (benznidazole [BNZ] or nitrofurans) are only recommended for treatment of the acute phase, and early in chronic infection, are toxic,[12–14] and have limited efficacy.[15]

For centuries, medicine relied on empirically discovered benefits of traditional medicinal plants without actual knowledge of the active compound or pharmacodynamics.

Of the plants that were used in this study, *Trichilia rubescens* Oliv. is a tree growing mainly in tropical areas of Africa and is used in Cameroonian folk medicine for the treatment of a variety of ailments, including jaundice, fever, gonorrhea, malaria, and to induce labor in pregnant women.[16] *Trichilia welwitschii* usage as a medicinal plant is not reported in the literature. However, decoction of the plant is used by local populations of Cameroon for the treatment of malaria (A. T. Tontsa, personal information).

Trichilia monadelpha genus have been used as timbers and herbal medicines by traditional healers in Cameroonian folk medicine for the treatment of various diseases such as abdominal pain, dermatitis, haemorrhoids, jaundice, gonorrhea, syphilis, and skin inflammation.[17] In Africa, *Psorospermum* species are used to treat wounds, spider or scorpion bites, skin diseases (such as scabies, dermatitis, and eczemas), and leprosy.[18]

As the discovery of medical effects was empirical, many benefits of plant ingredients remained unknown. In our modern world, traditional medicine turned out to be a valuable source of knowledge and unexplored pharmacologically active substances.[19] In previous studies, we showed that chemically defined substances, derived from Cameroonian medicinal plants have strong inhibitory effects on *Plasmodium falciparum*[20] or cancer.[21] Anthraquinones derived from those

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plants showed fungicidal or bactericidal properties.[22] Here, we studied effects of pure substances, derived from four Cameroonian medicinal plants (*T. rubescens*, *T. welwitschii*, *T. monadelpha*, and *Psorospermum densipunctatum*) and one *T. monadelpha*–associated fungus (*Colletotrichum gloeosporioides*) against *T. cruzi* infection in Vero cells or human-induced pluripotent cell-derived cardiomyocytes (hiPSC-CMs).

MATERIALS AND METHODS

Materials. Benznidazole, Giemsa solution, Bouin's fixative solution, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), and menadione were purchased from Sigma-Aldrich (St. Louis, MO). Fetal calf serum (FCS), RPMI 1640 medium, and B27 supplement (serum-free, contains insulin) were purchased from Gibco (New York, NY). Matrigel™ was purchased from BD Biosciences (San Jose, CA).

Plant material. Plants were collected at different sites of Cameroon and identified by Mr. Victor Nana (for *Trichilia* species) and Eric Ngansop (for *P. densipunctatum*), plant taxonomists of the Cameroon National Herbarium (Table 1).

Isolation and purification. References for isolation and characterization of molecules presently studied are provided in Table 2.

Isolation and purification of compounds. Trichilia rubescens*.* Vilasinin-type limonoids are the prominent class of limonoids isolated and characterized from leaves, roots, and stem bark of *Trichilia rubescens.* Isolation was performed as described previously.[20,24,25]

Trichilia welwitschii*.* Dregeanin DM4, Rohituka 3, and Trichilia lactone D5, belonging to the prieurianin class of limonoids were isolated from seeds of *T. welwitschii*. Isolation was performed as described previously.[32]

Trichilia monadelpha *and associated endophytic fungi* C. gloeosporioides*.* Monadelphin A, a gedunin-class limonoid, was isolated from leaves of *T. monadelpha* as described previously.[30] Cytochalasin D was obtained from the endophytic fungus *C. gloeosporioides,* associated with *T. monadelpha* following an experimental procedure described previously.[29]

Psorospermum densipunctatum*.* Air-dried powered roots (2.32 kg), leaves (824 g), and stems (2,166 kg) of *P. densipunctatum* were separately extracted by maceration at room temperature for 48 hours, using methanol as the solvent. Each suspension was filtrated and resulting solutions were concentrated under reduced pressure. Crude residue of 105 g, 106 g, and 79 g from roots, leaves, and stems were received, respectively. Crude methanol extract of the roots from *P. densipunctatum* (105 g) was subjected to flash column chromatography on silica gel (Merck, Darmstadt, Germany, 230–400 mesh) and eluted with hexane/ethyl acetate (AcOEt) (3:1), hexane/AcOEt (1:1), hexane/AcOEt (1:3), and AcOEt, resulting in four fractions labeled F1 (19 g), F2 (3 g), F3 (3 g), and F4 (6 g). Fraction F1 (19 g) was also subjected to column chromatography on silica gel (Merck, 60–200 mesh) and eluted with hexane/AcOEt mixtures of increasing polarity. Three hundred twenty-four fractions of 150 mL each were collected and monitored by thin layer chromatography, using mixtures of hexane/AcOEt of increasing polarity as a mobile phase. Subfractions 31, 32–33, 45–52, and 56–60 were left to crystallize at room temperature to provide, after filtration, vismione B (RPD13), 11-hydroxy-5-methoxy-2, 2,9-trimethyl-2H-anthra [1,2-b]-pyran-7,12-dione (RPD3), 2-géranylémodine (RPD6), or 3-géranyloxyémodine (RPD7), respectively.

In a similar way, from the methanolic crude extract of leaves (106 g), four fractions labeled F1 (23 g), F2 (2 g), F3 (1 g), and F4 (5 g) were obtained from flash column chromatography on silica gel (Merck, 230–400 mesh) and eluted with hexane/AcOEt (3:1), hexane/AcOEt (1:1), hexane/AcOEt (1:3), and AcOEt, respectively. Fraction F1 (23 g) was subjected to column chromatography on silica gel (Merck, 60–200 mesh) and eluted with hexane/AcOEt mixture of increasing polarity. Subfraction 24 was left at room temperature to crystallize and gave vismiaquinone (FePD1). The residues obtained from subfractions 26–29 and 48–58 (0.46 g) were both eluted with a mixture of hexane/AcOEt (1:39) and yielded 3-methoxyemodine (FePD6).

Crude methanolic extract from the stem (79 g) was subjected to repeated column chromatography on silica gel (Merck, 60–200 mesh) and eluted with hexane/AcOEt mixture of increasing polarity to yield one compound identified as 2,8-dihydroxy-3-méthoxylxanthone (TPD_3), obtained from subfractions 67–82. All molecules studied are listed in Table 2, with citations for more procedural details.

The structures of all pure isolated compounds were determined based on their ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data in conjunction with their mass spectral data and confirmed by comparison of these data and physical constants with those previously published (see Table 2).

General experimental procedures. Optical rotations were recorded on a Perkin-Elmer Model 2000 polarimeter (Perkin-Elmer, Waltham, MA). Melting points were determined on a Buchii melting point apparatus and are uncorrected. Infra-red spectra were recorded on a Bruker Fourier transform/infrared spectrophotometer. One- and 2 -dimensional NMR spectra were recorded on a Bruker AV-300 and AV-500 spectrometer (Bruker, Billerica, MA) equipped with 5-mm 1H (300 MHz and 500 MHz) and 13C (75 MHz and 125 MHz) probes, operating at 300 and 75 MHz, and 500 and 125 MHz, respectively, either in deuterated chloroform, deuterated methanol, or deuterated pyridine with tetramethylsilane as an internal standard. High-

TABLE 1
Identification of raw material used for these studies (plants and endophytic fungus)

Plant	Herbarium (collected in)	Year of collection	Voucher/GenBank number
Trichilia rubescens	Mbankomo (Eloumden Mont)	March 2014	38705/HNC
Trichilia welwitschii	Mbankomo (Simbock)	July 2009	31288/HNC
Trichilia monadelpha	Mbankomo (Eloumden Mont)	June 2014	66909/HNC
Psorospermum densipunctatum	Alatening foothills (Bamenda)	August 2013	62831/HNC
T. monadelpha/Colletotrichum gloeosporioides	Mbankomo (Eloumden Mont)	August 2015	KY792086

HNC = Herbier National du Cameroon, Cameroon National Herbarium.

TABLE 2
Pure substances used in this study

Our code	Original codes	Names	Class	Sources	MW	Literature
Lim1	TGR5	Trichirubine A	Limonoid	*Trichilia rubescens*	452	23
Lim2	RAMTRE2	Rubescin D	Limonoid	*T. rubescens*	422	20,21
Lim3	TR8	Rubescin B	Limonoid	*T. rubescens*	406	24
Lim5	PTR4	Rubescin F	Limonoid	*T. rubescens*	438	25
Lim6	TR4	TS3	Limonoid	*T. rubescens*	420	20,21,26
Lim7	TR12	Rubescin C	Limonoid	*T. rubescens*	464	24
Lim8	TM-SB-60-1-1	Cytochalasin D	Alkaloid	*Colletotrichum gloeosporioides*	507	27–29
Lim9	TMFE2	Monadelphin A	Limonoid	*Trichilia monadelpha*	572	30
Lim10	TWF10	Dregeanin DM4	Limonoid	*Trichilia welwitschii*	584	31,32
Lim11	TWF9	Rohituka-3	Limonoid	*T. welwitschii*	600	31,32
Lim12	TWF5	Trichilia lactone D5	Limonoid	*T. welwitschii*	704	31,32
Lim13	TWT1	28,29-bis,norcycloarten-3β, 4α, 6α-triol	Cycloartane	*T. welwitschii*	430	33
A	RPD_7	3-geranyloxyemodine	Quinone	*Psorospermum densipunctatum*	406	22
B	RPD_{13}	Vismione B	Quinone	*P. densipunctatum*	354	34
C	RPD_6	2-geranylemodine	Quinone	*P. densipunctatum*	406	35
D	RPD_3	2,9-trimethyl-2*H*-anthra [1,2-b]-pyran-7,12-dione	Quinone	*P. densipunctatum*	350	36
E	$FePD_1$	2-isoprenyl-3-methoxyemodine	Quinone	*P. densipunctatum*	352	22
F	$FePD_6$	3-methoxyemodine	Quinone	*P. densipunctatum*	284	22
G	TPD_3	1,7-dihydroxy-6-methoxylxanthone	Xanthone	*P. densipunctatum*	284	37

resolution mass spectrometry (Electrospray Ionization and Electronic Impact) was performed on a Varian mass spectrometer (Varian Inc., Palo Alto, CA). Silica gels (Merck, 230–400 and 70–230 mesh), Sephadex LH-20 (Merck), and reverse-phase RP-18 (Merck) were used as stationary phases for flash and column chromatography. Thin-layer chromatography analyses were performed on silica gel 60F254–precoated alumina sheets (0.2 mm layer thickness). Spots were visualized under a UV lamp (254 nm and 365 nm) or by heating after spraying with 10% H_2SO4 reagent. Mixtures of *n*-hexane, AcOEt, methylene chloride, and methanol were used as eluent solvents.

***Trypanosoma cruzi* strains.** In the present study, the *T. cruzi* strains Y (TcII, ATCC 50832) and Sylvio (TcI, ATCC 50800) were used.

Isolation of trypomastigotes. Culture-derived trypomastigotes (TCTs) of the TcI or TcII strains were obtained from monolayers of Vero cells (CCL-81; ATCC, Manassas, VA), which had been infected at a ratio of 5:1 (TCTs/Vero cells). Vero cells were incubated at 37°C in RPMI 1640, enriched with 5% inactivated FCS, supplemented with antibiotics (penicillin 500 µ/mL and streptomycin 0.5 mg/mL). Parasites were collected from culture supernatants by centrifugation at 1,000 × *g* for 10 minutes and the sediment was suspended in RPMI 1640 with 5% FCS. Parasites were counted using a Neubauer chamber, and the number was adjusted according to assay needs.

Differentiation of cardiomyocytes from hiPSCs. Reprogramming with Sendai virus was used to generate hiPSC lines from peripheral blood mononuclear cells (PBMCs) of healthy individuals, according to previously published protocols.[38,39] Human-induced pluripotent stem cells lines were differentiated into hiPSC-CMs using a 2-dimensional monolayer differentiation protocol and characterized as described previously with respect to gene expression profiles, protein expression profiles, and electrophysiologic profiles.[40,41] The cells were maintained in a 5% CO_2/95% air environment as described previously.[38–40]

Infection with T. cruzi*.* Monolayers of Vero cells or hiPSC-CM were prepared on Matrigel-coated 8-well chamber slides at a density of 2×10^5 cells/well and cultivated for 48 hours at 37°C in a 5% CO_2 atmosphere. When using hiPSC-CM, chamber slides were precoated with Matrigel, and RPMI 1640 containing 5% FCS was supplemented with B27. Infection was carried out at a target effector ratio of 1:5 (cell:parasite), with 24-hour interaction time in the presence of drugs, before replacing the medium with RPMI 1640 with 5% FCS ± B27.

In another study, the infection was pre-established by infecting cells for 24 hours before treatment. Twenty-four hours after infection, the cells were washed with phosphate-buffered saline, fixed with Bouin's solution, and Giemsa stained. The number of infected cells (defined as at least 1 amastigote/cell) and the number of amastigotes per infected cells (multiplication) were determined for each vision field by microscopy. About 25–40% of all cells in control wells (no drugs) were infected by TcII, whereas TcI infected 80% of cells when used at the same infection ratio. For comparison with drug-treated wells, control infections were regarded as 100%. Figures display a representative experiment for each molecule studied, although molecules were studied two or three times each.

Cell metabolism. Viability of uninfected cells was determined by XTT metabolic assay.[42] Cells (10^5) were seeded into each well of a 96-well plate and allowed to settle for 24 hours at 37°C, 5% CO_2, and 80% humidity. After medium change, drugs were added, and the cells were incubated for 24 hours at 37°C, 5% CO_2, and 80% humidity. XTT–menadione (200 µg/mL and 40 µM, respectively) in RPMI 1640 with 5% FCS (containing B27 for cardiomyocyte tests) was added to each well and incubated at 37°C. Absorption was determined at 490 nm using a plate reader (Opsys MR, DYNEX Technologies, Chantilly, VA).

Statistical analysis. Results were analyzed using Student's *t* test if two groups of equal size were compared or by Student's *t* test with Welsh modification if the two groups had unequal sample sizes.

RESULTS

Effects of ten limonoids, one alkaloid, or one cycloartane on infection of Vero cells with the *T. cruzi* Y strain (TcII). We infected Vero cells with trypomastigotes of the TcII strain either alone or in the presence of each of the 10 limonoids

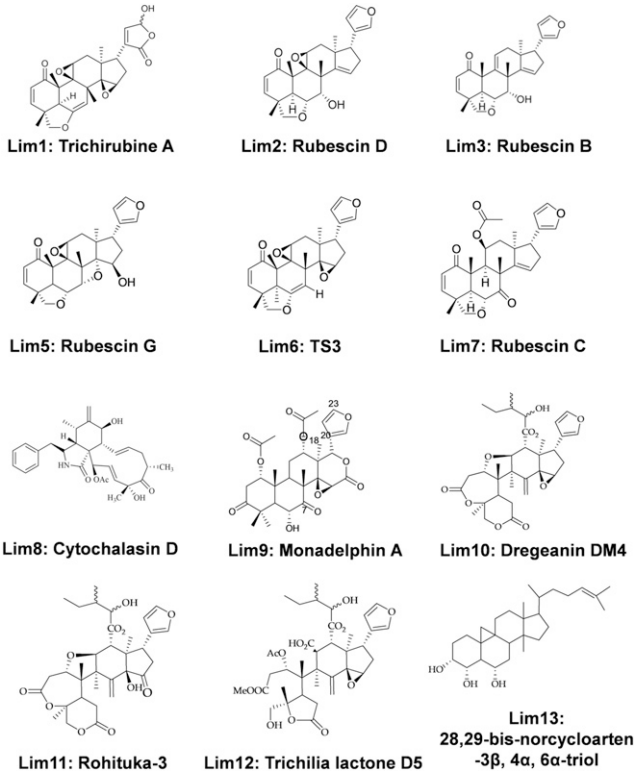

FIGURE 1. Chemical structures of 11 limonoids and cytochalasin D used in this study.

(Lim1, 2, 3, 5, 6, 7, 9, 10, 11, and 12), the alkaloid Cytochalasin D (Lim8), or the cycloartane 28,29-bis,norcycloarten-3β, 4α, 6α-triol (Lim13), at 10 μM (Table 2, Figure 1). For comparison, we infected Vero cells in the presence of BNZ at concentrations reflecting high (6 mg/L) or low (3 mg/L) therapeutic drug levels.[43] Six mg/liter or 3 mg/L are equivalent to 23 μM or 11.5 μM, respectively. Our results show that BNZ treatment resulted in a dose-dependent response against TcII infection (Figure 2A). Ten of the 12 test substances showed significant inhibition of TcII infection, whereas one limonoid (Lim9) did not inhibit and one limonoid (Lim6) caused a complete loss of Vero cells during infection (Figure 2A). Of the substances active against TcII infection, six also inhibited TcII multiplication (Lim3, 5, 7, 11, 12, and 13) (Figure 2B). Only Lim6 (TS3), and the alkaloid Lim8 (Cytochalasin D) showed pronounced interference with uninfected Vero cell metabolism (Figure 2C). Of all test substances, only Lim5 (Rubescin F) inhibited Vero cell infection more than BNZ (11.5 μM), but inhibition was lower than achieved by BNZ (23 μM) (Figure 2A). Regarding TcII multiplication, none of the test substances showed better results than BNZ (11.5 μM) (Figure 2B).

Effects of six quinones and one xanthone on infection of Vero cells with the *T. cruzi* Y strain (TcII). Vero cells were infected with trypomastigotes of the TcII strain either alone, or in the presence of each of the six quinones (Figure 3A–F), or one xanthone (Figure 3G) (Table 2, Figure 3), at 10 μM. For comparison, BNZ was used at concentrations reflecting high (23 μM) or low (11.5 μM) therapeutic drug levels. Four of the six quinones showed significant inhibition of TcII infection, whereas two quinones (Figure 3A and D) and the xanthone (Figure 3G) did not inhibit (Figure 4A). Of the substances active

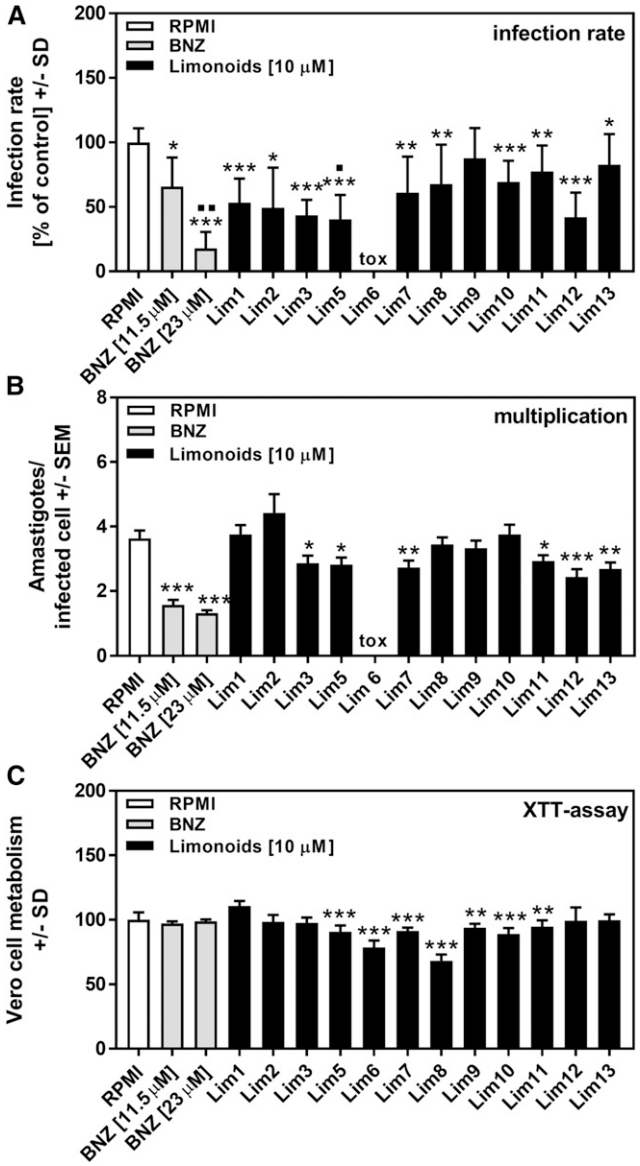

FIGURE 2. Effects of limonoids on infection of Vero cells with the *Trypanosoma cruzi* Y strain (TcII). Vero cells (2×10^5/well) were infected with TcII at a ratio of five trypanosoma/cell for 24 hours at 37°C, 5% CO_2, and 80% humidity. Infections took place in the presence of RPMI 1640 medium without drugs, benznidazole (BNZ) (11.5, or 23 μM), or Lim substances (Table 2) at (10 μM). Twenty-four hours after infection, the cells were washed and fresh medium without drugs or TcII was added. Infection rates (**A**) and multiplication (**B**) were determined 48 hours after infection. Host cell viability was determined by XTT assay (**C**). Statistics: Welch *t*-test. Comparison: asterisks (**A**, **B**, **C**), RPMI 1640 vs. all other bars; squares (**A**), BNZ (11.5 μM) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two, or three asterisks or squares = $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively. Tox: complete loss of Vero cells during infection.

against TcII infection, only one inhibited TcII multiplication (B, vismione B) (Figure 4B). Vismione B showed moderate interference with Vero cell metabolism (Figure 4C), whereas no microscopically visible signs of toxicity were observed for vismione B when examining Giemsa-stained cells (data not shown). Interestingly, vismione B activity against TcII infection, as well as against TcII multiplication, was significantly

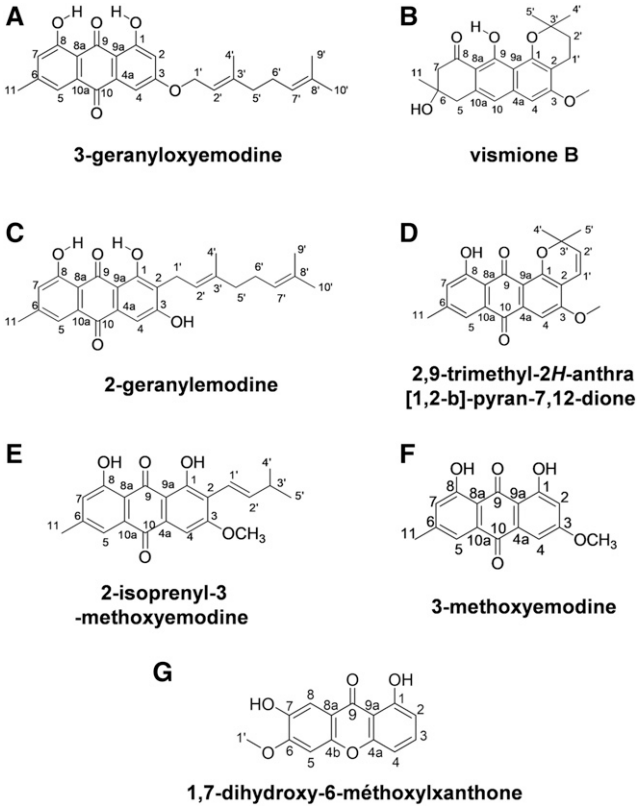

FIGURE 3. Chemical structures of six quinones (**A**–**F**) and one xanthone (**G**) used in this study.

more pronounced than the activity of BNZ, even at a concentration of 23 μM.

Vismione B dose-dependently interferes with TcII infection and multiplication. Because vismione B showed the most impressive effects on TcII infection and multiplication among all substances tested, we decided to study it in more detail. Vismione B showed dose-dependent activity against TcII infection (Figure 5A), as well as TcII multiplication (Figure 5B). The IC_{50} for vismione B on TcII-infected Vero cells was found to be about 1.3 μM (Figure 5A).

Vismione B interferes with pre-established TcII infection. When Vero cells were infected with TcII 24 hours before drug treatment and incubated for an additional 24 hours, we found no effects of BNZ (11.5 μM) on the presence of viable TcII amastigotes in cells, and only small effects of BNZ (23 μM) (Figure 6A). By contrast, vismione B (10 μM)–treated cells rarely contained viable amastigotes, as determined by Giemsa staining and microscopy (Figure 6A). If viable amastigotes were present, there were few and no significant differences between BNZ (11.5 or 23 μM) or vismione B (10 μM)–treated cells (Figure 6B), indicating effects on TcII multiplication by both substances, as also seen in Figure 4B.

Vismione B interferes with TcII and TcI infection of hiPSC-CMs. Human-induced pluripotent cell-derived cardiomyocytes were treated with BNZ or vismione B during infection, similar to the experimental setup shown in Figures 4 and 5. Vismione B 10 μM interfered with TcI infection significantly better than BNZ 11.5 μM and equal to BNZ 23 μM (Figure 7A and B). Regarding TcII infection, vismione B (10 uM) performed better than BNZ (11.5 μM or 23 μM) (Figure 7B).

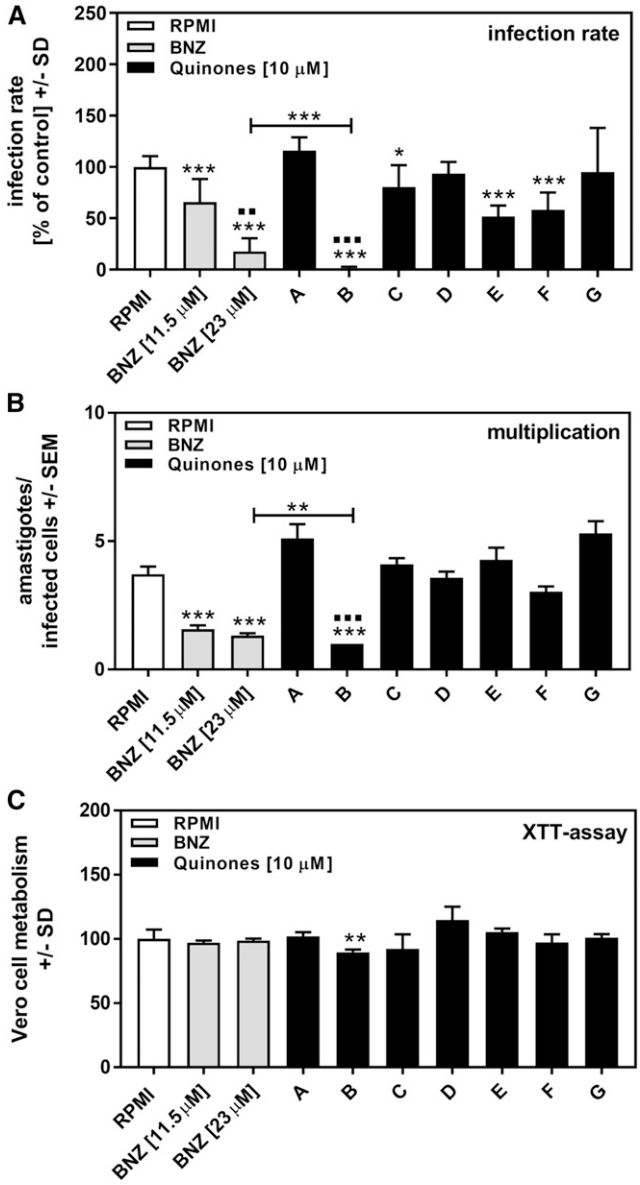

FIGURE 4. Effects of quinones A–F or the xanthone G on infection of Vero cells with the *Trypanosoma cruzi* Y strain (TcII). Vero cells (2×10^5/well) were infected with TcII at a ratio of five trypanosoma/cell for 24 hours at 37°C, 5% CO_2, and 80% humidity. Infections took place in the presence of RPMI 1640 medium without drugs, benznidazole (BNZ) (11.5 or 23 μM), quinones A-F, or the xanthone G (Table 2) at 10 μM. Twenty-four hours after infection, the cells were washed and fresh medium without drugs or TcII was added. Infection rates (**A**) and multiplication (**B**) were determined 48 hours after infection. Cell viability was determined by XTT assay (**C**). Statistics: Welch *t*-test. Comparison: asterisks (**A**, **B**, **C**), RPMI 1640 vs. all other bars; squares (**A**, **B**), BNZ (11.5 μM) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two, or three asterisks or squares = $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

Vismione B 10 uM showed equal effects to BNZ 11.5 μM on TcI multiplication but was less effective than BNZ 23 μM. Effects of vismione B (10 uM) on TcII multiplication were stronger than effects of BNZ (11.5 μM or 23 μM) (Figure 7D). We have noted that infection rates in controls were about 30% for TcII and about 80% for TcI, despite using the same Tc/cell ratio of five trypanosomas/cell for infection (data not shown). Higher

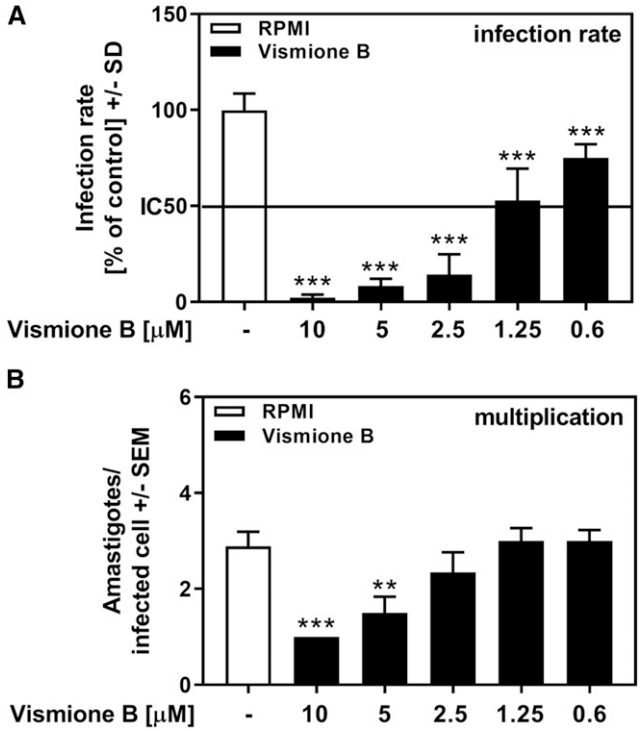

FIGURE 5. Vismione B dose-dependently interferes with TcII infection and multiplication. Vero cells (2×10^5/well) were infected with TcII at a ratio of five trypanosomas/cell for 24 hours at 37°C, 5% CO_2, and 80% humidity. Infections took place in the presence of RPMI 1640 medium without drugs or vismione B (0.6–10 μM). Twenty-four hours after infection, the cells were washed and fresh medium without drugs or TcII was added. Infection rates (**A**) and multiplication (**B**) were determined 48 hours after infection. Statistics: *t*-test. Comparison: RPMI vs. all other bars, two or three asterisks = $P \leq 0.01$ or $P \leq 0.001$, respectively.

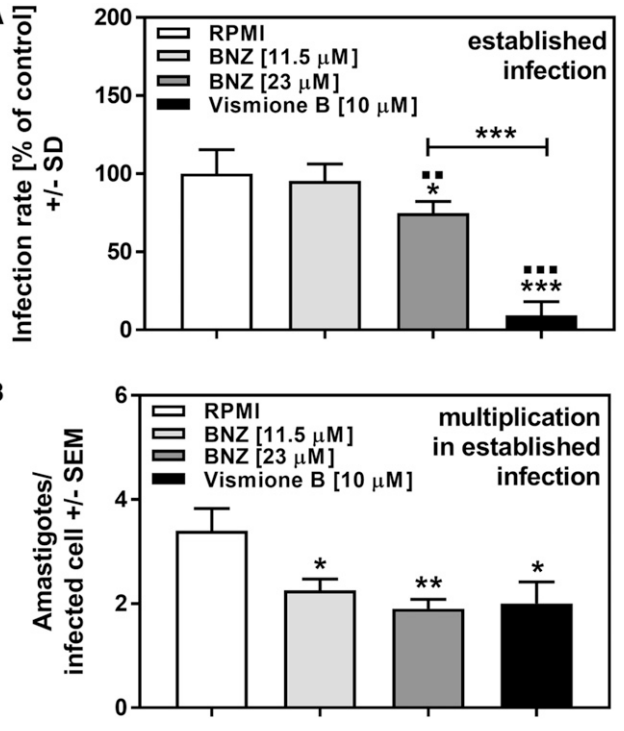

FIGURE 6. Vismione B interferes with established TcII infection. Vero cells (2×10^5/well) were infected with TcII at a ratio of five trypanosoma/cell for 24 hours at 37°C, 5% CO_2, and 80% humidity. Infected cells were washed and incubated in RPMI without drugs or treated with vismione B 10 μM or benznidazole (BNZ) (11.5 or 23 μM). Controls were incubated in the presence of RPMI, not containing TcII or drugs. Infection rates (A) and multiplication (**B**) were determined 24 hours after drug treatment. Statistics: Welch *t*-test. Comparison: asterisks (**A**, **B**), RPMI vs. all other bars; squares (**A**), BNZ (11.5 μM) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two, or three asterisks or squares = $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

infection rates for TcI were also reflected in the greater amounts of amastigotes in controls (about 13.1 for TcI, and 3.1 for TcII) (Figures 7B versus D). Cardiomyocyte metabolism was not affected by any of the substances or concentrations (Figure 7E).

DISCUSSION

For decades, chemotherapy against CD was limited to BNZ and nifurtimox. Both drugs are predominantly used in treatment of acute and early chronic phase CD.[15,44] Long-term treatment with these drugs in the prevalent chronic phase of infection is limited because of the development of severe side effects. In the past years, a lot of effort has been put into investigating trypanosoma-specific drug targets, such as cruzipain,[45] or trypanothione, hoping to avoid severe side effects in long-term treatment. Inhibitors against both molecules are under investigation.[46] The use of amphotericin B, as well as combinations of azoles, for example, itraconazole and the antiarrhythmic amiodarone are under investigation.[47,48]

There are several reports of potent anti-trypanosomal substances derived from African medicinal plants. Examples are actinodaphnine and cassythine, two bioactive alkaloids from *Cassytha filiformis* (Lauraceae), which showed activity against *T. brucei* with an IC_{50} value of 2.2 μg/mL.[49] The sesquiterpenoids, muzigadiolide, muzigadial, 6α,9α-dihydroxy-4(13),7-coloratadiene-11,12-dial, mukaadial, and ugandensidial, derived from the East African medicinal plant *Warburgia ugandensis* (Canellaceae), showed activities against *T. brucei rhodesiense* with IC_{50} values ranging from 0.64 to 6.4 μM.[50] Artemetin, a flavonoid isolated from *Vitex simplicifolia* (Verbenaceae) leaves, exhibited promising trypanocidal activity with an IC_{50} value of 4.7 μg/mL.[51] Saropeptide acetate, an amide, isolated from *Zapoteca portoricensis* exhibited antitrypanosomal activity against *T. brucei rhodesiense* and *T. cruzi* with IC_{50} values of 3.63 and 41.65 μM, respectively.[52]

Here, we compared anti-*T. cruzi* effects of 19 compounds isolated from Cameroonian medicinal plants (*T. rubescens*, *T. welwitschii*, *T. monadelpha*, and *P. densipunctatum*) as well as one *T. monadelpha*–associated endophytic fungus (*C. gloeosporioides*) to effects of BNZ, the gold standard for therapy. The compounds investigated here belong to different classes of secondary metabolites (limonoids, quinones, xanthones, alkaloids, and cycloartanes).

Benznidazole, as well as many of our compounds, showed significant effects against *T. cruzi* infection of Vero cells. These findings are similar to previous studies showing effects of limonoids on trypanosomes.[53,54] We found that effects of most of the compounds tested here were less than the effects of BNZ. The only compound with significantly greater effects than BNZ on *T. cruzi* infection as well as *T. cruzi* multiplication in Vero cells and hiPSCs was vismione B. Vismione B is a

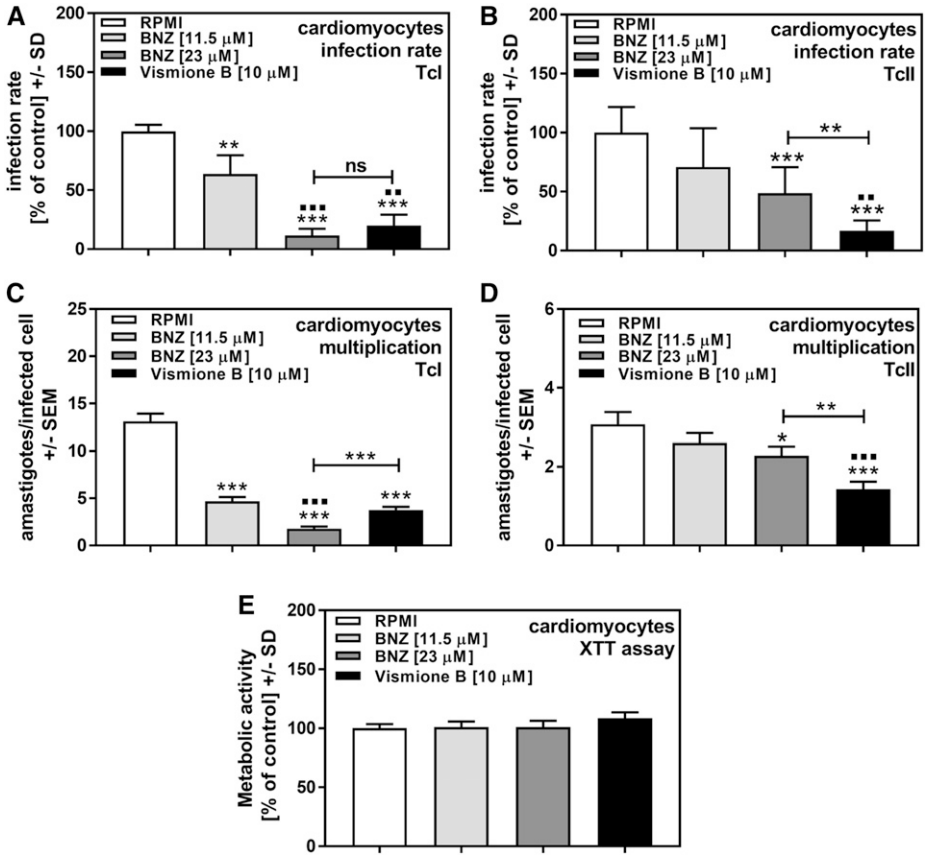

FIGURE 7. Vismione B interferes with TcII and TcI infection of human-induced pluripotent cell-derived cardiomyocytes. Human-induced pluripotent stem cell-derived cardiomyocytes (2×10^5/well) were infected with TcI or TcII at a ratio of five trypanosomas/cell for 24 hours at 37°C, 5% CO_2, and 80% humidity. Infections took place in the presence of benznidazole (BNZ) (11.5 or 23 μM) or vismione B (10 μM). Twenty-four hours after infection, the cells were washed and fresh medium without drugs, TcI, or TcII was added. Infection rates (**A**, **B**) and multiplication (**C**, **D**) were determined 48 hours after infection. Cell viability was determined by XTT assay (**E**). Statistics: Welch *t*-test. Comparison: asterisks, RPMI vs. all other bars; squares, BNZ (11.5 μM) vs. all other bars. Other comparisons as indicated by the ends of the brackets. One, two, or three asterisks or squares = $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

quinone that has previously been described to affect the malaria-causing parasite *P. falciparum*,[55] and to be toxic for human breast,[34] central nervous system, [34] and lung cancer cell lines.[34,55] We observed limited toxicity of vismione B on the continuous Vero cell line, confirming adverse effects of vismione B on rapidly replicating cells. It has to be noted that vismione B did not affect the metabolism of cardiomyocytes in our experiments, nor cardiomyocyte integrity as determined by Giemsa staining and microscopy.

Perhaps, the most striking finding of this study is the difference in activity against pre-established infection between BNZ and vismione B. Vismione B (10 μM) interfered with *T. cruzi* infection and multiplication to a significantly greater degree than BNZ (23 μM), a concentration that corresponds to levels in humans given the highest BNZ dose in therapy. This finding is especially interesting because BNZ has limited efficacy against chronic-stage Chagas myopathology.[12]

The IC_{50} of vismione B against *T. cruzi* infection of Vero cells was determined to be approximately 1.25 μM, whereas BNZ (11.5 μM) did not reach an IC_{50}. These data give rise to the estimate that vismione B might be about 10-fold more active against acute *T. cruzi* infection than BNZ. Regarding pre-established *T. cruzi* infection, vismione B (10 uM) was significantly more active against *T. cruzi* infection than BNZ 23 μM, with vismione B being about 15- to 20-fold more active than BNZ.

Trypanosoma cruzi is able to infect and replicate in many cell types in vitro, including PBMCs, human epithelial cells type 2, human umbilical vein endothelial cells, human cervical cancer cells (HeLa), and African green monkey kidney cells (Vero).[56] Vero cells are the most frequently used cell line to study *T. cruzi*.[56,57] Neonatal rat cardiomyocytes have been used for physiological studies,[58] but effects of trypanosoma on human cardiomyocytes might be different.[59] There is one study investigating effects of *T. cruzi* infection of human heart muscle cells.[60] Just recently, hiPSCs have been suggested as an in vitro system to investigate drug effects.[61] In that publication, da Silva Lara et al. used *T. cruzi* multiplication as a readout, showing long-term effects of BNZ on pre-established infection.

To our knowledge, nothing is known regarding a possible mechanism for the anti-trypanosomal activity of vismione B. *Trypanosoma cruzi* induces oxidative stress in its host cells, and although excessive oxidative stress is toxic for *T. cruzi*, it can be decreased to moderate levels by the *T. cruzi* aspartic proteinase TcAP1.[62] Moderate oxidative stress on the other hand is beneficial for Tc multiplication.[63] Some quinones have

been shown to interfere with cellular oxidative stress levels,[64,65] which would represent a disadvantage for proliferating *T. cruzi*. One might speculate that vismione B can affect oxidative stress levels as well.

We found higher infection rates of TcI compared with TcII, as well as a higher number of amastigotes present in controls of TcI-infected cells. Both strains are found in South America, as well as in the United States, with TcI being the predominant strain.[10,66] TcI infection rates of hiPSC-CM in the presence of vismione B (10 μM) were significantly lower than in control cells, and comparable with BNZ (23 μM), whereas effects on Tc multiplication were comparable with the effects of BNZ (11.5 μM), but not as good as BNZ (23 μM). So, although vismione B controlled infection and multiplication of the less infectious *T. cruzi* strain TcII in vitro significantly better than BNZ, the advantage over BNZ seems to be smaller when looking at TcI infection.

Preliminary data indicate that vismione B does not interfere with preformed *Aspergillus fumigatus* biofilm metabolism but inhibits yeast and bacterial growth (data not shown). Other anthranoid compounds, for example, vismione D have been shown to exert activities against *T. brucei rhodesiense*, *T. cruzi*, *Leishmania donovani*, and *P. falciparum*.[67] These findings and the fact that vismione B also inhibited *P. falciparum*[55] indicate that vismione derivatives have a wide range of activity against disease-causing agents. There also is evidence that vismiones have cytotoxic effects on tumor cells,[68] which might explain our observation that vismione B slightly affected the viability of fast dividing Vero cells, but not of cardiomyocytes (Figures 4C versus 7E). A logical next step would be to investigate effects of vismione B against *T. brucei* sp. and to investigate effects so far seen in vitro by using in vivo systems. Although to date there are no reports on tests of vismione B in vivo, other quinones have been tested in mice. Aloin, aloe-emodin, and rhein up to 2,000 mg/kg showed no adverse effects on mice, whereas at 200–400 mg/kg significantly reducing parasitemia and anemia during *T. congolense* infection.[69]

In conclusion, our data indicate that vismione B might have greater activity against *T. cruzi* infection than BNZ. Further tests are needed to determine biological effects in vivo.

Received May 6, 2019. Accepted for publication August 23, 2019.

Published online September 30, 2019.

Acknowledgments: We thank Roy Madigan, DVM, for providing the *T. cruzi* strain ATCC 50800 and Marife Martinez for technical assistance.

Financial support: This work was supported by the funding from National Institutes of Health (NIH) R01 HL141371, R01 HL141851 (JCW), and NIH K01 HL135455 (NS).

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