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Synthetic Chromanol Derivatives and Their Interaction with Complex III in Mitochondria from Bovine, Yeast, and Leishmania

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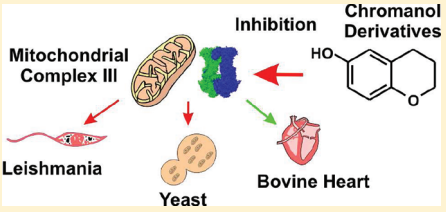
ABSTRACT: Synthetic chromanol derivatives (TMC4O, 6-hydroxy-2,2,7,8-tetramethyl-chroman-4-one; TMC2O, 6-hydroxy-4,4,7,8-tetramethyl-chroman-2-one; and Twin, 1,3,4,8,9,11-hexamethyl-6,12-methano-12*H*-dibenzo[*d*,*g*][1,3]dioxocin-2,10-diol) share structural elements with the potent inhibitor of the mitochondrial cytochrome (cyt) bc_1 complex stigmatellin. Studies with isolated bovine cyt bc_1 complex demonstrated that these compounds partially inhibit the mammalian enzyme. The aim of this work was to comparatively investigate these toxicological aspects of synthetic vitamin E derivatives in mitochondria of different species. The chromanols and atovaquone as reference compound were evaluated for their inhibition of the cyt bc_1 activity in mitochondrial fractions from bovine hearts, yeast, and *Leishmania*. In addition, compounds were evaluated *in vitro* for their inhibitory activity against whole-cell *Leishmania* and mouse peritoneal macrophages. In these organisms, the chromanols showed a species-selective inhibition of the cyt bc_1 activity different from that of atovaquone. While in atovaquone the side chain mediates species-selectivity, the marked differences for TMC2O and TMC4O in cyt bc_1 inhibition suggests that direct substitution of the chromanol headgroup will control selectivity in these compounds. Low micromolar concentrations of TMC2O ($IC_{50} = 9.5 \pm 0.5\ \mu M$) inhibited the growth of *Leishmania*, and an esterified TMC2CO derivative inhibited the cyt bc_1 activity with an IC_{50} of $4.9 \pm 0.9\ \mu M$. These findings suggest that certain chromanols also exhibit beyond their antioxidative properties antileishmanial activities and that TMC2O derivatives could be useful toward the development of highly active antiprotozoal compounds.



INTRODUCTION

Mammalian mitochondria attract increased attention in medical research because of their involvement in pathogenic events and because they are possible targets to cure diseases.[1,2] Functions of mammalian mitochondria, which were considered important to develop therapeutic approaches, include their ATP production and reactive oxygen species (ROS) production as well as their contribution to the intrinsic pathway of apoptosis. To prevent the resulting damage from mitochondrial ROS production, several natural and synthetic vitamin E-related antioxidants have been tested to protect mitochondria from lipid peroxidation.[3,4]

In spite of decades of intensive research about vitamin E, its function remains to be elucidated.[5] Among synthetic compounds related to vitamin E, highly efficient antioxidants such as the synthetic twin chromanol (Twin) and related compounds were identified.[6] An increasing number of reports demonstrated that antioxidants including vitamin E-related chromanols showed different effects at the cellular, tissue, and whole organism level, although their reaction rates with radicals were similar.[7,8] This raised questions about the bioactivity of such vitamin E derivatives, which is not related to the antioxidant activity.[8−12] Examples in this respect are the blocking of cardiac potassium channels by chromanol 293B[13] and the inhibition of succinate dehydrogenase by α-tocopherol succinate.[14,15] Neuzil et al. introduced the concept of tocopherols esterified to succinate as mitochondrially targeted anticancer agents (Mitocans).[16] These findings demonstrate that synthetic modification can turn vitamin E compounds into chemotherapeutic agents.

In the search for nonredox effects of chromanols (vitamin E-related compounds), it was noted that chromanols have a structural similarity to the potent inhibitor of mammalian mitochondria stigmatellin (Stig). That this similarity also implies the inhibiting properties in mammalian mitochondria was confirmed for the chromanol derivative TMC2O.[17] Although this compound has been far less active than Stig, it shares its binding with the mammalian mitochondrial complex III (cyt bc_1 complex). Besides application of chromanol derivatives to combat malignant cells,[14] which is based on selective toxicity, the same principle could also be useful to inhibit mitochondria of protozoal microorganisms. While strong inhibitors used in the research on mammalian mitochondria are too toxic for therapeutic inhibition of mitochondria from microorganisms, it needs to be clarified whether moderately active chromanol derivatives such

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as TMC2O show a different activity in mitochondria from those of mammals and microorganisms. Therefore, this study was designed to explore species selective inhibition of complex III in mitochondria from mammals, yeast, and *Leishmania* by chromanols in comparison with that of reference compounds, such as Stig, pentamidine, and atovaquone (ATQ). A further goal was to decide whether these compounds can be useful as a template for the synthesis of more active compounds.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), cytochrome c^{3+}, decylubiquinone, dithiothreitol (DTT), hemin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin/streptomycin, 3-(*N*-morpholino)propanesulfonic acid sodium salt (MOPS), Triton X-100, Schneider's medium, heat-inactivated fetal bovine serum (HFBS), sorbitol, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), and pentamidine were purchased from Sigma. DMSO, glucose, EDTA, K_2HPO_4, KCN, KH_2PO_4, NaN_3, NaCl, TRIS, peptone, and sucrose were obtained from Merck, Germany. Stigmatellin (Stig) and triethanolamine were from Fluka. Yeast extract, agar, sodium acetate buffer, and zymolyase were purchased from Amresco, Applichem, BDH, and Seikagaku Corporation, respectively. Atovaquone (ATQ, isolated from Malarone) was a generous gift from GlaxoSmithKline. The chromanols (TMC2O, TMC4O, and Twin) were synthesized as previously reported.[18,19] Terephthalic acid 1-methyl ester 4-(4,4,7,8-tetramethyl-2-oxo-1-benzopyran-6-yl) ester (TMC2OE005) was synthesized according to the following procedure. A 25-mL round-bottom flask with a magnetic stirrer was charged with dihydro-6-hydroxy-4,4,7,8-tetramethyl-1(2*H*)-benzopyran-2-one (0.1 g, 0.45 mmol), monomethyl terephthalate (1.20 equiv, 0.0972 g, 0.54 mmol), and dry dichloromethane (8 mL). To the cloudy mixture were added dicyclohexylcarbodiimide (DCC) (3 equiv, 0.2785 g, 1.35 mols) and a catalytic amount of 4-dimethylaminopyridine (DMAP) (0.14 equiv, 0.0079 g, 0.064 mmol). The resulting cloudy solution was stirred at room temperature overnight and then filtered. The solvent was evaporated *in vacuo*, and the residual oil was subject to flash chromatography (SiO_2, *n*-hexane/EtOAc/CH_2Cl_2 gradient) to afford the pure product (0.1041 g = 60% yield) as a white solid. TLC: R_f = 0.30 (SiO_2; *n*-hexane/CH_2Cl_2/EtOAc, v/v/v = 7:2:2). ^{1}H NMR: 1.32 (s, 6H, CH_3), 2.11 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 2.61 (s, 2H, CH_2), 3.97 (s, 3H, CH_3), 6.94 (s, 1H, Ar−H), 8.15 (d, 2H, Ar−H), 8.25 (d, 2H, Ar−H).

Pathogens and Cell Cultures. The following microorganisms were used: *Saccharomyces cerevisiae* DBY 747, *Leishmania tarentolae* strain P10 (Jena Bioscience), and *Leishmania amazonensis* MHOM/77BR/LTB0016. Cytotoxicity was tested on peritoneal macrophages from normal BALB/c mice.

Isolation of Submitochondrial Particles from Bovine Heart. Bovine heart submitochondrial particles (BH-SMP) were obtained from bovine heart mitochondria by sonication and stored in liquid nitrogen until use.[20]

Isolation of Submitochondrial Particles from Yeast. The cultivation of the *S. cerevisiae* strain DBY 747 and the preparation of yeast mitochondria were performed according to a published procedure.[21] Cells were harvested by centrifugation (5 min at 1464*g* and 20 °C), the pellet was resuspended in buffer I (10 mM TRIS, 10 mM DTT, pH 9.4) for 15 min at 37 °C, and after another centrifugation (5 min at 1464*g* and 20 °C) resuspended in buffer II (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4). After a third centrifugation step (5 min at 1464*g* and 20 °C), the weight of the cell pellets was determined. To prepare spheroblasts, pellets were suspended in buffer II complemented with 2 mg of zymolyase per gram of yeast cells. After incubation for 45 min at 28 °C, spheroblasts were collected by centrifugation (5 min at 1464*g* and 20 °C), resuspended in buffer II, sedimented again (5 min at 1464*g* and 20 °C), and homogenized in a small volume of buffer III (250 mM sorbitol, 10 mM TRIS) using a Wheaton Dounce tissue grinder. Cells and cell debris were removed by two centrifugations (5 min at 1464*g* and 4 °C). Mitochondria were finally collected from the supernatant by centrifugation at 11952*g* for 10 min at 4 °C. To prepare yeast submitochondrial particles (Y-SMP), mitochondrial pellets were suspended in 5 mL of buffer I (without DTT) and diluted to 25 mL with 10 mM Tris (pH 7.5). The suspension was kept on ice for 20 min followed by a centrifugation at 39500*g* for 10 min at 4 °C. The pellet was resuspended in 20 mL of sucrose buffer (250 mM sucrose and 10 mM Tris, pH 7.4), and sonicated 18 times for 20 s (Branson sonifier at maximum intensity) with interruptions of 10 s for heat dissipation. Subsequently, the suspension was centrifuged at 5400*g* for 10 min at 4 °C to remove mitochondria. SMP were sedimented from the supernatant by centrifugation at 195000*g* for 60 min at 4 °C. The obtained SMP pellet was homogenized in 1.5 mL of buffer (250 mM sucrose, 0.2 mM EDTA, and 50 mM potassium phosphate, pH 7.2) and stored in liquid nitrogen.

Isolation of Mitochondrial Fractions from *Leishmania*. *L. tarentolae* was grown according to established procedures.[22] A *L. tarentolae* culture (2700 mL) was centrifuged for 10 min at 478*g* and 4 °C, and the supernatant was discarded. The cell pellet was resuspended in buffer (10 mM TRIS-HCl, 0.3 M sucrose, 0.2 mM EDTA, and 0.2% BSA, pH 7.4) and washed in two extra centrifugation steps for 10 min at 478*g* and 4 °C. The washed cell pellet was incubated in lysis buffer (5 mM TRIS-HCl, pH 7.4) for 10 min while homogenizing in a dounce homogenizer. Cell debris was removed by centrifugation for 10 min at 1005*g* and 4 °C. The supernatant was centrifuged for 20 min at 13176*g* and 4 °C to sediment the mitochondrial fraction (MIT). The mitochondria were resuspended in 1 mL of buffer (250 mM sucrose, 50 mM KH_2PO_4, and 0.2 mM EDTA, pH 7.2) and stored in liquid nitrogen until use.

Inhibition of Decylubiquinol: Cytochrome *c* Oxidoreductase Activity. To measure the decylubiquinol($dUQH_2$):cyt c^{3+} oxidoreductase activity, the reduction of 100 μM cyt c^{3+} at 550 nm using 540 nm as the reference was monitored in buffer (250 mM sucrose, 50 mM KH_2PO_4, and 0.2 mM EDTA, pH 7.2) in the presence of 1 mM KCN, 1 mM NaN_3, and the artificial substrate 75 μM $dUQH_2$, which was prepared from decylubiquinone by reduction.[17] The residual $dUQH_2$:cyt c^{3+} oxidoreductase activity of SMP and MIT in the presence of various inhibitor concentrations was measured as follows: the inhibitors ATQ, TMC2O, TMC2OE005, TMC4O, Twin, and Stig were dissolved in DMSO. At first, SMP or MIT was added to 1 mL of buffer 50 s after starting the time scan, followed by the respective inhibitor 50 s later. The reaction was started after 150 s with $dUQH_2$ and was monitored for 100 s. The inhibition by TMC2OE005 was monitored in buffer (100 mM NaCl, 20 mM MOPS, and 0.05% Triton X-100, pH 7.2). The activity of noninhibited $dUQH_2$:cyt c^{3+} oxidoreductase activity was measured in the presence of the vehicle for the respective inhibitor. All inhibitor concentrations were tested in triplicate. The reduction rates for cyt c^{3+} were calculated from the time trace of the absorption difference at 550 nm − 540 nm ($\varepsilon_{550-540\ nm}$ = 19 mmol^{-1} L cm^{-1}). Reduction rates in the presence of the vehicle were set to 100%, and the remaining activity in the presence of inhibitors was expressed in %.

Mitochondrial Membrane Potential in *Leishmania*. The mitochondrial membrane potential ($\Delta\Psi m$) was monitored by using JC-1 dye as the probe.[23] JC-1 is a cationic mitochondrial vital dye that is lipophilic and becomes concentrated in the mitochondria in proportion to $\Delta\Psi m$ and ATP-generating capacity.[24] Briefly, promastigotes of *L. amazonensis* were collected after treatment (72 h) with TMC2O at 18 μM, valinomycin at 5 μM, Stig at 15 μM, and 1 μL of DMSO, and incubated for 10 min with 10 μM JC-1 at 37 °C, washed, and resuspended in medium. The fluorescence was observed under a Leitz Wetzler (Germany) fluorescence microscope at 400×.

For a quantitative JC-1 assay, *L. tarentolae* cell suspensions were incubated for 4 h with DMSO (control), TMC2O (50−200 μM), and

Stig (15 μM) at 25 °C in PBS. Then, cells were incubated with JC-1 (3 μM) for 30 min at 30 °C, centrifuged at 470*g* for 10 min at 25 °C, and resuspended in 1.2 mL of PBS. The fluorescence of JC-1 was assessed with a Hitachi F-4500 fluorescence spectrometer using 488 nm (slit 5 nm) as the excitation wavelength and recording the emission (slit 10 nm) at 530 nm ($F_{530\ nm}$) and 590 nm ($F_{590\ nm}$). From the emissions, the ratio $F_{590\ nm}/F_{590\ nm}$ was calculated.

Antipromastigote Assay for *L. amazonensis*. Exponentially growing cells (10^5 promastigotes/mL) were distributed in 96-well plates. Compounds dissolved in DMSO were added at a final concentration between 0.1 and 100 μg/mL and incubated at 26 °C. After 72 h, the parasites were incubated for 3 h with *p*-nitrophenol phosphate (20 mg/mL) dissolved in 1 M sodium acetate buffer at pH 5.5 with 1% Triton X-100 (BDH, Poole, England) at 37 °C. The absorbance was determined at 405 nm.

Cytotoxicity Assay for Peritoneal Macrophages. To investigate the toxicity in mammalian cells, the compounds were evaluated in mouse peritoneal macrophages, which are the host cells for the amastigote form of *Leishmania*. Resident macrophages were collected from peritoneal cavities of normal BALB/c mice in ice-cold RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with antibiotics and seeded at 30000 cells/well in U-shaped bottom plates of 96 wells. The cells were incubated for 2 h at 37 °C in 5% CO_2. Nonadherent cells were removed by washing with phosphate-buffered saline (PBS), and then compound dilutions in 1 μL of DMSO were added to 200 μL of medium with 10% heat-inactivated fetal bovine serum (HFBS) and antibiotics. Concentrations of the test products ranged from 1.5 to 100 μg/mL for 72 h. Macrophages treated with 1 μL of DMSO served as controls. The cytotoxicity was determined colorimetrically after addition of MTT (Sigma, St. Louis, USA). MTT solutions were prepared at 5 mg/mL in PBS and filter-sterilized at the moment of use. To each well, 15 μL of the MTT solution was added and after 3 h of incubation, the formazan crystals were dissolved by adding 100 μL of DMSO. The optical density was determined at a test wavelength of 560 nm and a reference wavelength of 630 nm.

Antiamastigote Assay for *L. amazonensis*. The peritoneal macrophages were harvested and plated at 10^6/mL in 24-Well Lab-Tek (Costar, USA) and incubated at 37 °C and 5% CO_2 for 2 h. Nonadherent cells were removed, and stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio for 4 h. The cells were washed to remove free parasites, prediluted compounds were added, and the plates were further incubated for 48 h. The cells were fixed with methanol and stained with 20% Giemsa for microscopic reading. The total parasite burden (= average number of amastigotes per cell) in treated wells was compared with that in control wells.

Selectivity Index Calculation. The selectivity index (SI) ratio (IC_{50} for macrophage/IC_{50} for amastigotes) was used to compare the toxicity of the extracts for murine macrophage and the activity against intracellular *Leishmania* amastigotes

Statistical Analysis. In cellular experiments, the 50% of growth inhibition value (IC_{50}) was determined from the linear concentration−response curves, and the results were expressed as the mean ± standard deviation of at least two independent experiments. Statistical significances were evaluated by Student's *t* test. The concentrations at 50% inhibition (absolute IC_{50} values) for cyt bc_1 activities were obtained from the plots of remaining activity vs inhibitor concentration using a nonlinear regression of the activity data (Origin 6.1 (MicroCal), custom function) according to a four parameter logistic model (4PL, Hill-Slope model) following the recommendations of the National Institute of Health.[17]

RESULTS

The chroman derivatives used in this study are shown in Figure 1. They were selected because of their partial inhibitory activity in isolated mammalian cyt bc_1 complex and the fact that certain chromanols exhibited structural similarities to the potent inhibitor of mammalian mitochondria Stig. Furthermore, the reference compound ATQ was used, which was reported to have species selective inhibitory activities at the cyt bc_1 complex.[25]

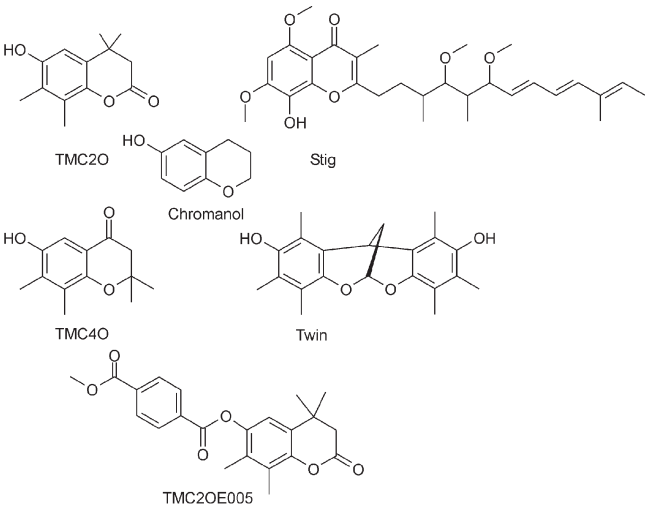

Figure 1. Chromanol compounds studied in this work: TMC2O (6-hydroxy-4,4,7,8-tetramethyl-chroman-2-one), TMC4O (6-hydroxy-2,2,-7,8-tetramethyl-chroman-4-one), Twin (Twin-chromanol, 1,3,4,8,9,11-hexamethyl-6,12-methano-12*H*-dibenzo[*d*,*g*][1,3]dioxocin-2,10-diol), Stig (stigmatellin, 2-((7*E*,9*E*,11*E*)-4,6-dimethoxy-3,5,11-trimethyl-trideca-7,9,11-trienyl)-8-hydroxy-5,7-dimethoxy-3-methyl-chromen-4-one), and TMC2OE005 (terephthalic acid 1-methyl ester 4-(4,4,7,8-tetramethyl-2-oxo-1-benzopyran-6-yl) ester).

Table 1. Antileishmanial and Cytotoxicity Activity of Chromanol Derivatives Studied in *L. amazonensis* Promastigotes/Amastigotes in Comparison with Peritoneal Macrophages from BALB/c Mice

	IC_{50}[a] ± SD (μM)			
compounds	promastigotes	amastigotes	macrophages	selectivity index[b]
ATQ	12.2 ± 0.8	4.0 ± 0.8	21.5 ± 0.7	5
TMC2O	14.0 ± 2.7	9.5 ± 0.5	87.6 ± 5.2	9
TMC4O	58.1 ± 1.8	9.9 ± 3.4	161.6 ± 8.9	16
Twin	10.5 ± 0.3	2.9 ± 0.9	66.3 ± 1.0	23
pentamidine	1.17 ± 0.02	3.8 ± 0.3	34.3 ± 4.9	9

[a] IC_{50}: concentration of compound that caused 50% of inhibition. [b] Selectivity index: IC_{50} for macrophages/IC_{50} for amastigotes.

In a first step, the bioactivity of these compounds was studied in cellular systems related to leishmaniasis caused by protozoal microorganisms. *Leishmania* parasites exist as extracellular promastigotes in the vector and as intracellular amastigotes in mammalian macrophages. The influence of chromanol derivatives on the viability of promastigotes, amastigotes (*L. amazonensis*), and peritoneal macrophages from BALB/c mice was studied (Table 1). If only their antioxidant properties are important, no strong deleterious but rather protective effects would be expected. With respect to macrophages the reference compounds, ATQ and pentamidine were most toxic with IC_{50} values below 35 μM. In contrast, chromanols had IC_{50} values in macrophages above 60 μM indicating a lower toxicity. With the exception of

Table 2. Inhibition of the dUQH$_2$:cyt c^{3+} Oxidoreductase Activity by Chromanol Derivatives in Submitochondrial Particles from Bovine Heart (BH-SMP), *S. cerevisiae* (Y-SMP), and *L. tarentolae* (Lt-MIT)

	IC$_{50}$[a] ± SD (μ)]			selectivity index[b]	
com-pounds	BH-SMP[c]	Y-SMP[d]	Lt-MIT[e]	BH-SMP/Y-SMP	BH-SMP/Lt-MIT
Stig	0.00395 ± 0.0028	0.00198 ± 0.0010	0.47 ± 0.03	2	<1
ATQ	0.261 ± 0.082	0.030 ± 0.013	6.8 ± 1.1	9	<1
TMC2O	206 ± 18	1892 ± 79	74 ± 7	<1	3
TMC4O	190 ± 68	1286 ± 427	4251 ± 1470	<1	<1
Twin	418 ± 244	520 ± 31	322 ± 33	<1	1.3

[a] IC$_{50}$: concentration of compound that caused 50% of inhibition. [b] Selectivity index: IC$_{50}$ for mammalian mitochondria/IC$_{50}$ for mitochondria of microorganisms. [c] BH-SMP: submitochondrial particles (14 μg/mL i.T.) from bovine hearts. [d] Y-SMP: submitochondrial particles (39 μg/mL i.T.) from *S. cerevisiae*. [e] Lt-MIT: mitochondrial fraction (59 μg/mL i.T.) from *L. tarentolae*.

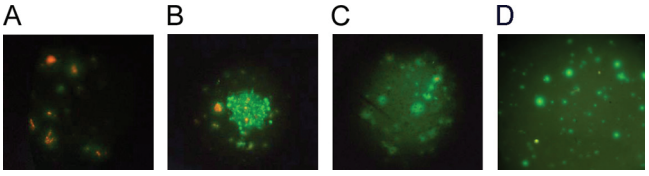

Figure 2. Effect of TMC2O on mitochondrial membrane potential in *L. amazonensis*. Promastigotes were incubated with vehicle or inhibitors for 72 h and after incubation with JC-1 analyzed by fluorescence microscopy. The green color corresponds to JC-1 monomers indicating low membrane potential, and the orange color indicates JC-1 aggregates, which are formed in mitochondria with high membrane potential. (A) Control cells treated with vehicle only, (B) cells incubated with TMC2O, (C) cells incubated with valinomycin, and (D) cells incubated with Stig.

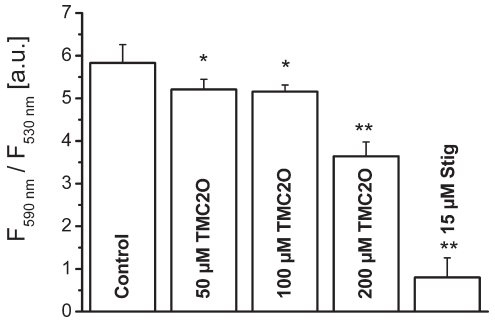

Figure 3. Quantitative effects of TMC2O on the mitochondrial membrane potential in *L. tarentolae*. Cells were incubated in the presence of different concentrations of TMC2O and Stig for 4 h, and after incubation with JC-1, the membrane potential was assessed by the measurement of the fluorescence emission ratio $F_{590\ nm}/F_{530\ nm}$. The control contained the corresponding amounts of DMSO, which was used as the vehicle for TMC2O and Stig. Data represent the mean values of $n = 4$ incubations ± SD. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

pentamidine, all compounds were slightly more active against amastigotes than promastigotes, while, IC$_{50}$ values for both stages of *L. amazonensis* were within the same magnitude. The IC$_{50}$ of chromanols and reference compounds for amastigotes were in the range of 2.9−9.9 μM. On the basis of a rather moderate toxicity of chromanols in macrophages, chromanols (TMC2O 9, TMC4O 16, and Twin 23) exhibited equal or higher selectivity indices than reference compounds (ATQ 5, pentamidine 9).

Since it was shown in previous studies that TMC2O acts in mammalian mitochondria by binding to the isolated complex III, the inhibition of this enzyme in mitochondrial fractions from mammals (BH-SMP) and *L. tarentolae* (Lt-MIT) was studied. Furthermore, SMP from *S. cerevisiae* (Y-SMP) were included since they were used as model system for plasmodial parasites. Decrease of the dUQH$_2$: cyt c^{3+} oxidoreductase by chromanol derivatives is a measure of their inhibitory activity on MIT. In the presence of the vehicle, the activity of the cyt bc_1 complex in the subcellular fraction is set at 100%; the activities after adding test compound are expressed as the percentage of residual activity, from which an IC$_{50}$ value is calculated.

In a first set of experiments, TMC2O was compared to Stig and ATQ in BH-SMP and Y-SMP (Table 2). Stig exhibited no selectivity for the microorganism, but the tests confirmed the inhibitory preference of ATQ for the yeast cyt bc_1 complex in comparison with that of mammalian cyt bc_1 complex. In general, chromanol compounds possess a higher IC$_{50}$ value than ATQ. The IC$_{50}$ values of TMC2O and TMC4O were about 10 times higher for Y-SMP than that for BH-SMP. In contrast, Twin inhibited Y-SMP and BH-SMP at a similar strength, however, at high concentrations.

In a next step, mitochondrial fractions from *L. tarentolae* were used for inhibition experiments (Table 2). Stig was less effective against Lt-MIT than against BH-SMP. TMC2O inhibited the activity of Lt-MIT with a selectivity index of about 3. Other chromanols were less effective against Lt-MIT.

To study the effect of TMC2O on mitochondrial functions inside *L. amazonensis* promastigotes, the $\Delta\Psi m$ in these cells was assessed by the dye JC-1 in combination with fluorescence microscopy (Figure 2). Cells treated with the vehicle only (Figure 2A) show orange spots, which correspond to mitochondria with high membrane potential, surrounded by the green cytosol (low potential). If cells were treated with TMC2O (Figure 2B), with the ionophore valinomycin (Figure 2C, positive control) or with the cyt bc_1 inhibitor Stig (Figure 2D, positive control), the intracellular orange spots disappeared in most cells. This suggests that TMC2O, valinomycin, and Stig caused a breakdown of mitochondrial $\Delta\Psi m$ in these cells. In order to quantitatively study the influence of TMC2O on the mitochondrial membrane potential, *L. tarentolae* cells were incubated for 4 h with different concentrations of inhibitors and subsequently stained with JC-1. The fluorescence emission ratio $F_{590\ nm}/F_{530\ nm}$ reflects the quantitative ratio of JC-1 dimers to JC-1 monomers (Figure 3). The sensitivity of this parameter to the cyt bc_1 inhibitor Stig confirms that this is a direct measure of the mitochondrial membrane potential. TMC2O decreases the $F_{590\ nm}/F_{530\ nm}$ ratio in a concentration dependent manner.

Since TMC2O based on the IC_{50} values in Lt-MIT has a rather moderate activity, the question was whether substitution of this molecule could lead to more active TMC2O derivatives. As a proof of concept, a series of TMC2O derivatives were obtained by esterification of the 6-OH group with several bulky residues and were studied in mitochondrial fractions. Among them, the terephthalic acid 1-methyl ester 4-(4,4,7,8-tetramethyl-2-oxo-1-benzopyran-6-yl) ester (TMC2OE005) exhibited an IC_{50} for the cyt bc_1 activity in *L. tarentolae* mitochondria of 4.9 ± 0.9 μM, while inhibition in Y-SMP (113 ± 7 μM) and BH-SMP (66 ± 5 μM) was less strong (Figure 4). The IC_{50} of TMC2OE005 in Lt-MIT is more than 10 times lower than the IC_{50} of TMC2O (74 ± 7 μM). It was thus evident that additional side chains can significantly increase the efficiency of TMC2O. Therefore, this derivative is quite a promising candidate for further synthetic optimization, which is the topic of future work.

DISCUSSION

Mitochondria are present in all eukaryotes and are involved in bioenergetic, metabolic, biosynthetic, and signaling processes. In general, mitochondria of protozoal parasites exhibit a typical structure similar to those of mammalian mitochondria composed of four electron transport complexes (I−IV) in the inner mitochondrial membrane.[26,27] Basic functions of the mitochondrial complexes III and IV in protozoal microorganisms, such as *Plasmodium*, were similar to mammalian complexes,[28,29] although protozoal mitochondrial activities may strongly depend on their life cycle.[30] The mitochondrial cyt bc_1 complex was discovered as a putative antiplasmodial target in the 1980s, and the drug ATQ was approved for the treatment of *Pneumocystis carinii* pneumonia and *Toxoplasma gondii* infections in 1994.[31,32] Until today, this naphthoquinone drug is the only approved compound for this target in humans. Recently, it has been used in combination with proguanil for the prophylaxis and treatment of malaria (Malarone, Glaxo).[33] In addition, limited activity of ATQ against *Leishmania* has been demonstrated.[34−36]

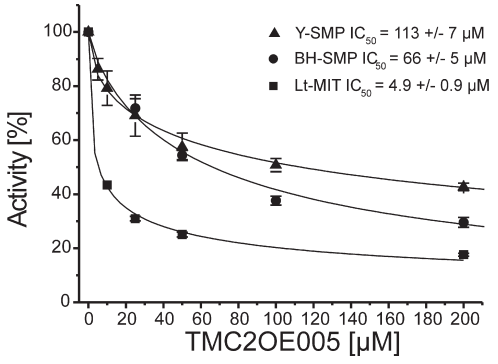

Figure 4. Residual $dUQH_2$:cyt c^{3+} oxidoreductase activity in Lt-MIT, BH-SMP, and Y-SMP in the presence of an TMC2O ester (TMC2OE005) showing a decreased IC_{50} compared to that of TMC2O. The concentrations of Lt-MIT, Y-SMP, and BH-SMP were 59, 149, and 34 μg/mL i.T., respectively. Data represent the mean ± standard deviation of three independent experiments.

In contrast, chromanols were so far widely studied as antioxidants to protect mitochondrial functions. The best known natural chroman compounds in this respect are the tocopherols (chromanols, vitamin E). In spite of decades of intensive research, their function was not completely elucidated.[5,9] However, there are also other natural compounds based on the chroman structure. Semisynthetic chroman-4-one derivatives with alkyl or halogen substituents were shown to have fungicidal activity in plants.[37] Chromanone acids, e.g., natural chromanone compounds with a complicated substitution pattern isolated from the bark of *Calophyllum brasiliense*, have been reported to show antibacterial activity against *Bacillus cereus* and *Staphylococcus epidermidis* while being less cytotoxic to mammalian cells.[38] Furthermore, from plants of the genus *Piper* chromene (dehydrogenated chroman) derivatives with alkyl and methoxy substituents based on active compounds were extracted, which exhibit an antitrypanosomal activity.[39] The most active compound from this study shows an IC_{50} value of 2.82 μM against the epimastigote form of *Trypanosoma cruzi*, which is similar to the efficiency of TMC2O, TMC4O, and Twin (Table 1) against *Leishmania amastigotes*. However, in the study about the antitrypanosomal activity no specific target for chromenes was identified. Our experimental approach studying whole cell (Table 1 and Figures 2 and 3) and mitochondrial effects (Table 2 and Figure 4) suggests that in *Leishmania* mitochondrial complex III could be one of the targets of chroman compounds in these organisms.

In addition, previous studies with TMC2O and the isolated mammalian cyt bc_1 complex demonstrated that the Q_{out} pocket of the cyt bc_1 complex could be the location of inhibition. The amino acid sequences of the Q_{out} pocket for the species, which were studied in this work, are shown in Figure 5. The antiplasmodial effect of ATQ is based on different amino acid sequences at the substrate binding sites of the cyt bc_1 complex.[40] While the PEWY-loop (pos. 270−273; numbering corresponds to the mammalian sequences and the top scale in Figure 5) is widely preserved in mammals, yeast and *Plasmodia* (not shown), the Y273 is replaced by F273 in *Leishmania*. The amino acid sequence and probably also the three-dimensional structure of the *Leishmania* cyt *b* subunits show significant differences from their mammalian and yeast homologues (Figure 5).[40−42] Kessl et al. described that ATQ-sensitive species (*Plasmodium*, *Saccharomyces*, and *Pneumocystis*) have a phenylalanine (F) in position 277, while mammals have an alanine (A) at this position.[43] In contrast, *Leishmania* possess a leucine (L) at this position, which

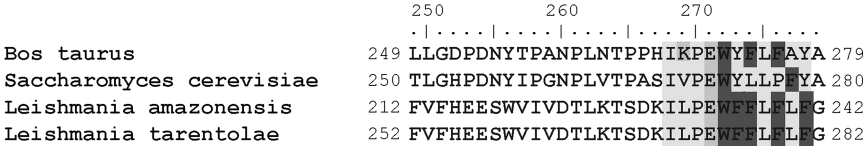

Figure 5. Part of the amino acid sequences for the mitochondrial cyt b protein around the Q_{out} binding pocket for ubiquinol at the cyt bc_1 complex of different species. The sequence alignment of the proteins from different species, *Bos taurus* [gi|56411145|], *Saccharomyces cerevisiae* [gi|30179770|], *L. amazonensis* [gi|146164406|], and *L. tarentolae* [gi|117865|], was performed using MegAlign from the DNA star package. Similar gray shades of the amino acids around the PEWY-loop indicate similar polarity.

is a nonpolar aliphatic amino acid similar to the alanine (A) in mammals.

Potent inhibitors for the mammalian cyt bc_1 complex, such as Stig, myxothiazol, and antimycin A, are not therapeutically useful because of their strong inhibition of the mammalian cyt bc_1 complex and their lack of selectivity for the protozoal cyt bc_1 complex (Table 2). Studies on the inhibitory properties of Stig derivatives in mammalian mitochondria demonstrated that the substituted alkyl side chain of Stig significantly modulates the IC_{50} values for the cyt bc_1 activity,[44] while the presence of an 8-hydroxy and 4-oxo group was essential for low IC_{50} values.[45]

Variations of the naphthoquinone inhibitors related to ATQ revealed that an alkyl chain boosts the inhibition of the cyt bc_1 complex and that certain substituents in the side chain vary the species selectivity between the yeast and bovine cyt bc_1 complex.[25] The inhibition observed for ATQ in our experiments is within the range of mammalian mitochondria as reported by Biagini et al.[46] For the yeast strain DBY 747, our value for ATQ is somewhat higher than the value reported by these authors (0.0035 μM).

Data shown in Table 2 demonstrate a strong inhibition of the cyt bc_1 complex by Stig in both BH-SMP and Y-SMP, underlining its property as a multispecies inhibitor, while it is much less effective in Lt-MIT. In contrast, TMC2O being effective in Lt-MIT is poorly inhibiting Y-SMP cyt bc_1 activity. Compared with BH-SMP, Stig required in Lt-MIT about 100 times higher concentrations for half the inhibition of cyt bc_1 activity (Table 2). This corresponds to the finding that trypanosomatids also are resistant to the Q_{out} reaction-center inhibitors myxothiazol and Stig.[47,48]

From these experimental data, one would not expect a contribution of the chroman headgroup to the species selectivity, although our data suggest a different species selectivity for the chromanol compounds TMC4O and TMC2O (Table 2). Among chromanols, TMC4O was ineffective to selectively inhibit Lt-MIT, while TMC2O and Twin inhibited Lt-MIT about 2−3 times stronger than the cyt bc_1 in BH-SMP. These data suggest that the inhibition pattern of chromanol compounds in *Leishmania* is in part different from their action in mammals and yeast.

Observed IC_{50} values for chromanols in cellular assays using *L. amazonensis* promastigotes and amastigotes in comparison with those of peritoneal mouse macrophages were in the low micromolar range (Table 1). The selectivity order was Twin > TMC4O > TMC2O, and their selectivity was higher than that of ATQ/pentamidine. These favorable properties of chromanols were essentially based on a lower toxicity in macrophages. ATQ has been reported to have an IC_{50} value of 41 μM against promastigotes of *L. infantum*,[49] which is slightly higher than our values for *L. amazonensis* (Table 1). This agrees with the limited efficacy of naphthoquinones (ATQ and lapachols) in leishmaniasis described in other studies.[50−52] Previous studies in the mammalian cyt bc_1 complex suggest that TMC2O could target this protein also in whole parasites. This interpretation is corroborated by the measurement of the $\Delta\Psi m$ after the incubation of *L. amazonensis* promastigotes with TMC2O compared to that of control cells (Figure 2). While in control cells, the $\Delta\Psi m$ is visible in the JC-1 staining (orange JC-1 dimers, Figure 2A); in TMC2O treated cells, most cells lack mitochondria with high membrane potential (no JC-1 dimers, Figure 2B). This was also confirmed for the quantitative study of the membrane potential in *L. tarentolae*. In these organisms, TMC2O (at shorter incubation times) displayed a concentration dependent decline of the membrane potential (Figure 3). Likewise, mitochondrial membrane potential was compromised in the presence of valinomycin and Stig (Figures 2C,D and 3). Since the $\Delta\Psi m$ is dependent on mitochondrial electron transfer, these data demonstrate that the antimicrobial activity of TMC2O in these parasites could be based on the inhibition of the cyt bc_1 electron transfer complex. For the other studied derivatives TMC4O and Twin, it appears to be more likely that their cytotoxicity (Table 1) is mediated by other targets than the cyt bc_1 complex.

As a proof of concept, TMC2O esters were synthesized, and their inhibition of the cyt bc_1 activity was tested in Lt-MIT. The low IC_{50} value for TMC2OE005 (Figure 4) demonstrates that modification of chromanol substituents can significantly contribute to inhibition strength.

In conclusion, tested chromanols possess an antileishmanial activity in the low micromolar range. At the cyt bc_1 drug target in *Leishmania*, a species selectivity quantitatively different from the established drug ATQ was observed. In contrast to findings for ATQ that the side chain of ATQ derivatives mediates species selectivity, the differences for TMC4O and TMC2O for cyt bc_1 inhibition in different species suggest that direct substitution of the chromane headgroup can also be important for species selectivity. Among the studied chromanols, TMC2O gave the most promising results in *Leishmania*. Experiments with esterified TMC2O derivatives in *L. tarentolae* mitochondria demonstrate that this chromanol moiety is a suitable starting point for further synthetic optimization.

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ABBREVIATIONS

ATP, adenosine triphosphate; ATQ, atovaquone; BH, bovine heart; BSA, bovine serum albumin; cyt, cytochrome; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; $dUQH_2$, decylubiquinol; $\Delta\Psi m$, mitochondrial membrane potential; EDTA, ethylenediaminetetraacetic acid; HFBS, heat-inactivated fetal bovine serum; IC_{50}, concentration at which inhibition of the activity was 50%; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; La, *Leishmania amazonensis*; Lt, *Leishmania tarentolae*; MIT, mitochondrial fraction; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OD, optical density; SMP, submitochondrial particles; Stig, stigmatellin; TMC2O, 6-hydroxy-4,4,7,8-tetramethyl-chroman-2-one; TMC2OE005, terephthalic acid 1-methyl ester 4-(4,4,7,8-tetramethyl-2-oxo-1-benzopyran-6-yl) ester; TMC4O, 6-hydroxy-2,2,7,8-tetramethyl-chroman-4-one; Tris, tris(hydroxymethyl)aminomethane; Twin,

twin-chromanol, 1,3,4,8,9,11-hexamethyl-6,12-methano-12*H*-dibenzo[*d*,*g*][1,3]dioxocin-2,10-diol); Y, yeast.

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