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Trithiolato-bridged dinuclear ruthenium(II)-arene conjugates tethered with lipophilic units: Synthesis and *Toxoplasma gondii* antiparasitic activity

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ABSTRACT

Trithiolato-bridged dinuclear ruthenium(II)-arene complexes are active against various parasites including *Toxoplasma gondii*. Lipids, isoprenoids and lipoate are metabolites scavenged by *T. gondii* from the host cell but also synthesized by the parasite, and these molecules can be appended to the diruthenium moiety in a conjugate approach aiming at compounds with improved antiparasitic activity and selectivity. The synthesis and *in vitro T. gondii* activity evaluation of 23 new trithiolato diruthenium complexes bearing various lipophilic units are reported. The influence of several structural elements as the nature of the lipophilic pendant and the type of the connecting bond between the two units on the conjugates' biological properties were examined. In a primary screening, the antiparasitic efficacy and cytotoxicity were assessed at 0.1 and 1 μM against transgenic *T. gondii* tachyzoites constitutively expressing β-galactosidase and on human foreskin fibroblasts (HFF) host cells. For 14 selected conjugates the half-maximal inhibitory concentration (IC_{50}) on *T. gondii* and their effect on HFF viability at 2.5 μM were determined. The decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a** efficiently inhibited parasite proliferation (IC_{50} values of 0.065, 0.127 and 0.123 μM, respectively) with no effect on HFF viability at 2.5 μM and deserve further attention.

1. Introduction

Toxoplasma gondii, an obligate intracellular parasite belonging to the phylum Apicomplexa, infects all warm-blooded animals including humans. If a total of 30% of the world's human population is estimated to be chronically infected with *Toxoplasma*, severe disease is rare. Nevertheless, *Toxoplasma* can be critical and cause life-threating disease as for example inducing important neurological deficits in immunosuppressed individuals [1–3], as well as, hydrocephalus, chorioretinitis, and blindness in congenitally infected newborns [4,5]. In farm animals, toxoplasmosis can lead to abortion, causing important economic burden [6,7]. The most frequently used treatments for *T. gondii* infection are combinations of antifolates, such as pyrimethamine and sulfadiazine. However, other options are necessary since therapy of *Toxoplasma* human infections faces many challenges including limited efficacy (medication only for the acute phase of the disease being inefficient in eliminating cysts in brain and muscles), poor tolerance, substantial side effects, and widespread drug resistance [8–10].

The auxotrophies and metabolic defects in *T. gondii* can be exploited as therapeutic approaches [11]. This parasite can replicate in every nucleated host cell by orchestrating metabolic interactions to derive crucial nutrients, in particular lipids [12,13]. Depending on its growth and survival needs, the parasite synthesizes lipids or scavenges them from the host environment [11,14-17]. The parasite diverts a variety of lipid precursors from the host cytoplasm and efficiently manufacture them into complex lipids [14], the apicoplast fatty acid synthesis being essential for organelle biogenesis and parasite survival [18]. The metabolites and final products that are both scavenged and synthesized by *Toxoplasma* comprise lipids,

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lipoate and isoprenoids (as farnesol and geranylgeraniol) [11]. *T. gondii* is an avid scavenger of lipids retrieved from the host cell [19], and deprivation from essential host lipids can induce parasite starvation [14]. The uncontrolled uptake of unsaturated fatty acids by the parasite and its vulnerability to lipid storage inhibition were suggested as potential strategies to restrict the parasite growth [11,14,19]. Additionally, lipid analogues with antiproliferative properties can be taken up by the parasites, which results in parasite membrane defects, and potential death [14].

Trithiolato-bridged dinuclear ruthenium(II)-arene compounds (as **A1**/**A2** and **B** in Fig. 1) are promising scaffolds as potential organometallic drugs. This type of dinuclear ruthenium(II) compounds are not only highly cytotoxic against human cancer cells [20] but also efficient against various protozoan parasites, such as *Toxoplasma gondii* [21], *Neospora caninum* [22] and *Trypanosoma brucei* [23]. The half-maximal proliferation inhibitory concentrations (IC_{50}) of complexes **A1** (R = Me), **A2** (R = Bu^t) and **B** against *in vitro* cultured *T. gondii* tachyzoites were 34, 62 and 1 nM, respectively, and these compounds did not impair the viability of human foreskin fibroblasts (HFF) used as host cells [21].

The requirement for compounds with larger therapeutic windows and reduced toxicity has stimulated the research into metal complexes and organometallics containing lipophilic units, which yielded compounds with interesting potential applications as anticancer drugs. The nature of the metal unit, of the attached lipophilic compound, and of the connection between the two parts highly influences the biological properties and some relevant examples are presented in Fig. 1. Various Pt(II) and Pt(IV) complexes presenting lipophilic units as ligands or attached to ligands were reported [24,25-29], and miriplatin (**C** in Fig. 1) was approved for the treatment of hepatocellular carcinoma in Japan in 2009 [30–32]. The length of the lipophilic chain was essential to the anticancer properties of Pt(IV) complexes **D** [25,33] and **E** [26,34] (Fig. 1). Ruthenium(II)- and osmium(II)-arene complexes bearing pyridine ligands modified with medium and long alkyl chains like **F** [35] and **G** [36] (Fig. 1), were generally poorly active against cancer cells. In contrast, complexes **H** and **I** [37], containing 1,2,3-triazolylidene-*N*-heterocyclic carbine ligands modified with *n*-hexyl and *n*-dodecyl chains, and **J** [38], with long-chain isonicotinic ester group, exhibited high anticancer activity. Trithiolato diruthenium complex **K** [39] (Fig. 1), with octane-1-thiol bridges, showed high antiproliferative activity on cancer cells, but reduced selectivity. If Pt(IV) complex **L** [40] (Fig. 1), with two α-lipoic acid units as axial ligands, exhibited increased cancer cell activity, half-sandwich Ir(III) complex **M** [41] (Fig. 1), functionalized with α-lipoate on a bidentate 2,2-bipyridine leg ligand, showed no cancer cells toxicity.

In this study, the intramolecular combination of the trithiolato diruthenium scaffold with different lipophilic derivatives essential for the survival and growth of *T. gondii* was challenged [11]. The conjugation of the trithiolato-bridged diruthenium organometallic unit with nutrients/metabolites for which the parasite has a high affinity is aimed at obtaining compounds with improved antiparasitic efficacy and selectivity. Trithiolato diruthenium complexes are stable and the bridge thiols can be easily derivatized by organometallic complex functionalization [42–46]. The pool of lipophilic compounds considered for the modification includes isoprenoids (geraniol, farnesol and geranylgeraniol), lipoic acid, as well as various carboxylic acids with linear, medium long, saturated and unsaturated chains. Another structural variation addressed was the type of connection (ester *vs* amide) between the diruthenium unit and the lipophilic appendices.

The new diruthenium lipophilic conjugates, along with the diruthenium intermediates, and the lipophilic compounds used for the modifications, were screened *in vitro* against *T. gondii* tachyzoites expressing β-galactosidase (*T. gondii* β-gal) grown in human foreskin fibroblasts (HFF) host cells, with additional assessment of HFF viability using the alamarBlue assay. The compounds exhibiting promising antiparasitic activity and selectivity were further subjected to dose-response (IC_{50}) determination on *T. gondii* β-gal and HFF cytotoxicity assessment at 2.5 μM.

2. Results and discussion

2.1. Chemistry

The synthesis of the carboxy, hydroxy and amino diruthenium intermediates **1**–**3** (Fig. 2 and Schemes 1-3), of the ethyl ester **7a** ([(η^6-*p*-MeC_6H_4Pr^i)$_2$Ru$_2$(μ_2-SCH$_2$$C_6H_4$-*p*-Bu^t)$_2$($\mu_2$-SC$_6H_4$-*p*-CH$_2CO_2$Et)]Cl), of the acetic ester **10a** ([(η^6-*p*-MeC_6H_4Pr^i)$_2$Ru$_2$(μ_2-SCH$_2$$C_6H_4$-*p*-Bu^t)$_2$($\mu_2$-SC$_6H_4$-*p*-OA*c*)]Cl) and of the acetic amide **10b** ([(η^6-*p*-MeC_6H_4Pr^i)$_2$Ru$_2$(μ_2-SCH$_2$$C_6H_4$-*p*-Bu^t)$_2$($\mu_2$-SC$_6H_4$-*p*-NHA*c*)]Cl) (Fig. 2) were previously described [42,45,47].

A first series of ester conjugates, **4a**-**6a** and **8a**, were synthesized by reacting the diruthenium carboxy intermediate **1** with the terpenoids geraniol (**4**), farnesol (**5**) and geranylgeraniol (**6**), and with *n*-butanol (**8**) in the presence of EDCI (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) as coupling agent and DMAP (4-(dimethylamino)pyridine) as basic catalyst. Compounds **4a**-**6a** and **8a** were isolated in 36–61% yield (Scheme 1).

Similar reaction conditions were used for the obtainment of a second series of ester conjugates, **9a** and **11a**-**17a**, synthesized by the reaction of the trithiolato diruthenium hydroxy derivative **2** with α-lipoic (**9**), butyric (**11**), hexanoic (**12**), decanoic (**13**), myristic (**14**), stearic (**15**), oleic (**16**) and elaidic (**17**) acids, respectively (Scheme 2). Compounds **9a** and **11a**-**17a** were isolated in 58–88% yield. Reactions of the diruthenium hydroxy derivative **2** with linear poly-unsaturated acids were also attempted but the ester conjugates could not be isolated due to important degradation during the purification process.

The amide derivatives **9b** and **11b**-**20b** were synthesized by the reaction of the diruthenium amino derivative **3** with α-lipoic (**9**), butyric (**11**), hexanoic (**12**), decanoic (**13**), myristic (**14**), stearic (**15**), oleic (**16**), elaidic (**17**), linoleic (**18**), α-linolenic (**19**) and γ-linolenic (**20**) acids, in the presence of EDCI and HOBt (1-hydroxybenzotriazol) as coupling agents, in basic conditions (DIPEA, *N*,*N*-diisopropylethylamine) (Scheme 3). Compounds **9b** and **11b**-**20b** were isolated in 48–91% yield.

All compounds were fully characterized by ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectroscopy, high resolution electrospray ionization mass spectrometry (HR ESI-MS) and elemental analysis (see the Experimental Chemistry section in the *Supporting Information* for full details). Mass spectrometry data corroborated the spectroscopic information with the trithiolato diruthenium lipophilic ester (**4a-6a, 8a, 9a, 11a**-**17a**), and amide (**9b, 11b**-**20b**) conjugates showing molecular ion peaks corresponding to [M-Cl]$^+$ ions.

For the assessment of the biological activity, the compounds were prepared as stock solutions in dimethylsulfoxide (DMSO). Similar to former reports [42,43,45,47], the ^{1}H NMR spectra of the functionalized diruthenium lipophilic conjugates **8a, 13a**-**15a, 17a, 13b**-**15b**, and **17b**-**19b** in DMSO–d_6, recorded at 25 °C 5 min and more than 1 month after sample preparation showed no noteworthy modifications (see Figures S2 and S3 in the *Supporting Information*), demonstrating a very good stability of the compounds in this highly complexing solvent. Structurally comparable ester conjugates with coumarin and BODIPY fluorophores connected *via* ester bonds to the trithiolato diruthenium unit were recently studied [42,46]. Only very limited solvolysis of the ester bonds was noticed after 168 h for some compounds, and it was concluded that the fluorophore diruthenium conjugates exhibit high stability in the

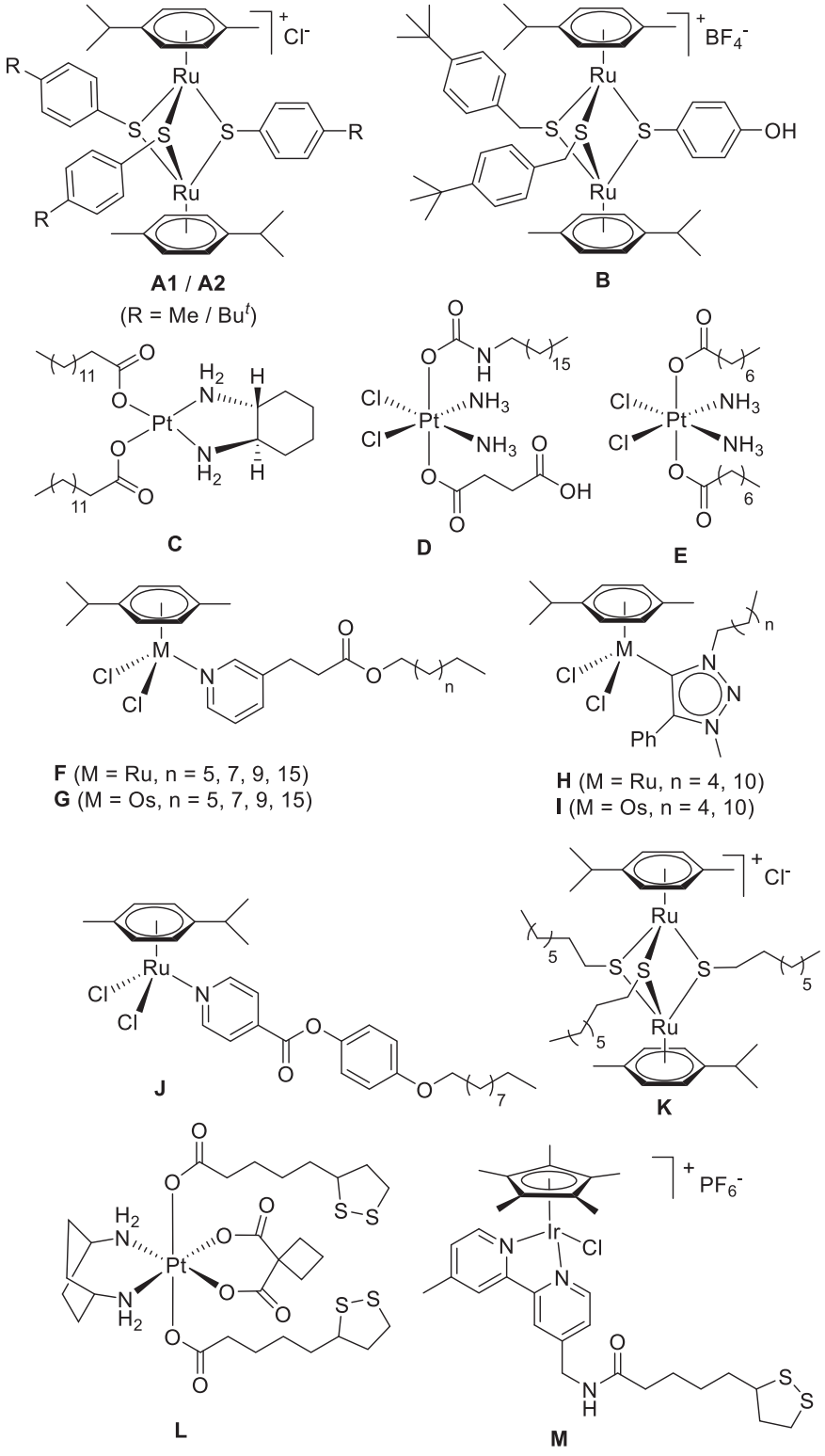


Fig. 1. Structure of trithiolato dinuclear ruthenium(II)-arene complexes **A1**/**A2** and **B** exhibiting anti-toxoplasma activity, of Pt(II) (**C**) and Pt(IV) complexes (**D** and **E**) with lipophilic ligands, of Ru(II)- and Os(II)-arene complexes with lipophilic pendants **F-I**, of the trithiolato dinuclear ruthenium(II)-arene complex **J** with lipophilic bridging thiols, and of **K** and **M** Pt(IV) and Ir(III) complexes containing lipoic units.

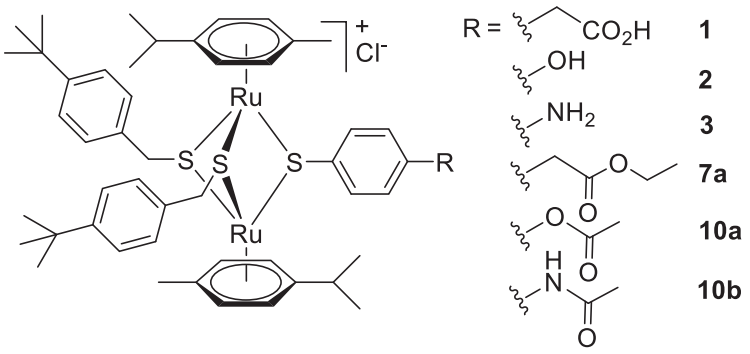


Fig. 2. Structure of the carboxy, hydroxy and amino diruthenium intermediates **1**–**3** and of the ester and amide compounds **7a, 10a** and **10b**, included in this study for comparison.

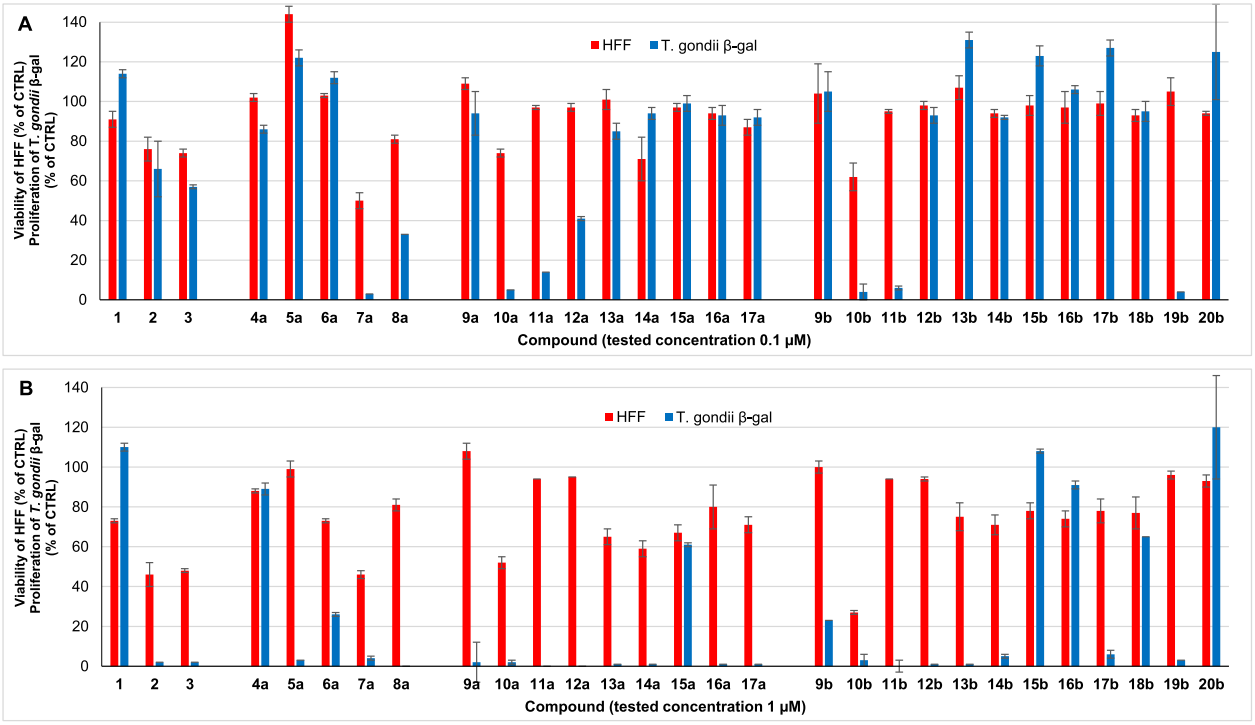


Fig. 3. Clustered column chart showing the *in vitro* activities at 0.1 µM (A) and 1 µM (B) of the 29 diruthenium compounds on HFF viability and *T. gondii* β-gal proliferation. Non-infected HFF monolayers treated only with 0.1% DMSO exhibited 100% viability and 100% proliferation was attributed to *T. gondii* β-gal tachyzoites treated with 0.1% DMSO only. For each assay, standard deviations were calculated from triplicates. Data for compounds **1–3, 7a, 10a** and **10b** were previously reported [42,45,47].

conditions used for the biological evaluations. Therefore, it was assumed that the ester conjugates are suitably stable for the first *in vitro* biological activity evaluation.

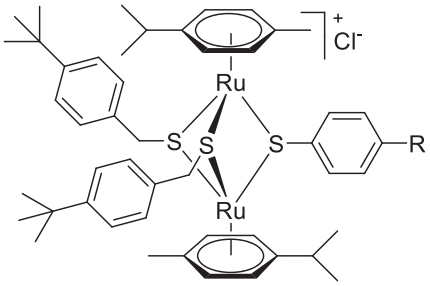
2.2. In vitro *activity against toxoplasma gondii and human foreskin fibroblasts*

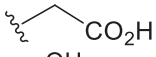
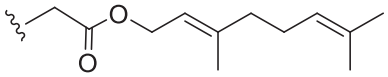
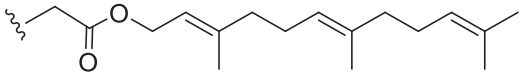
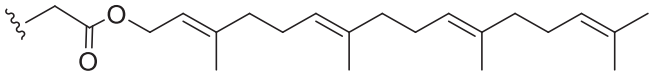
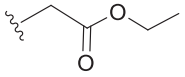
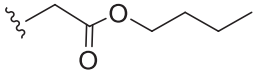
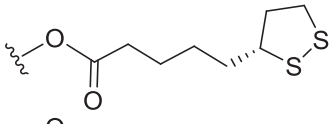
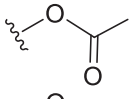
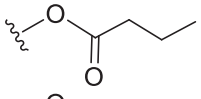
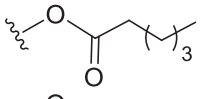
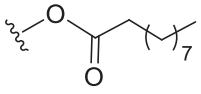
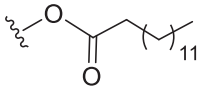
The impact upon *T. gondii* β-gal tachyzoites (transgenic *T. gondii* tachyzoites constitutively expressing β-galactosidase) grown in HFF (human foreskin fibroblasts) and non-infected HFF of 23 new conjugates (12 ester derivatives, **4a**-**6a, 8a, 9a, 11a**-**17a**, and 11 amide derivatives, **9b, 11b**-**20b**) and of the 15 lipophilic derivatives used for the synthesis of the hybrid molecules was investigated. The diruthenium intermediates **1**–**3** (Fig. 2 and Schemes 1-3), the ethyl ester **7a**, the acetic ester **10a** and the acetic amide **10b** (Fig. 2) were previously evaluated against *T. gondii* β-gal under similar conditions [42,45,47], and the corresponding values are given in Table 1 and Fig. 3 for comparison.

In a primary screening, transgenic *T. gondii* tachyzoites constitutively expressing β-galactosidase (*T. gondii* β-gal) were cultured in HFF monolayers and exposed to concentrations of 1 and 0.1 µM of each compound. In parallel, the cytotoxicity of the compounds was evaluated at the same concentrations in non-infected HFF. As a measure of the parasite proliferation, β-galactosidase activity was determined, while the impact on non-infected HFF was assessed using the alamarBlue assay. Non-infected HFF monolayers treated only with 0.1% DMSO exhibited 100% viability, and 100% proliferation was attributed to *T. gondii* β-gal tachyzoites treated with 0.1% DMSO. The results for the diruthenium intermediates **1**–**3** and the corresponding ester **4a**-**17a** and amide **9b**-**20b** conjugates are summarized in Table 1 and Fig. 3. The data for the lipophilic compounds used for conjugation are shown in Table S1 and Figure S1 (*Supporting information*).

The lipophilic derivatives (Figure S1 and Table S1) were non-toxic to HFF and *T. gondii* at the concentrations used for the primary screening. For the conjugates, both the nature of the lipophilic derivatives and that of the connector between the two

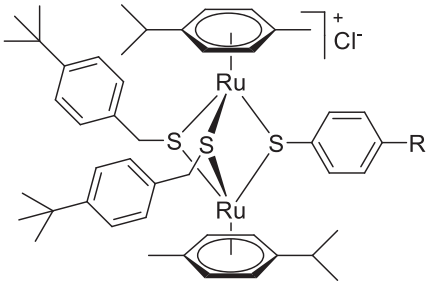
Table 1
Results of the primary cytotoxicity/efficacy screening of the diruthenium compounds in non-infected HFF cultures and *T. gondii* β-gal tachyzoites cultured in HFF. The compounds selected for the determination of the IC_{50} values against *T. gondii* β-gal are labelled with*. For each assay, standard deviations were calculated from triplicates.

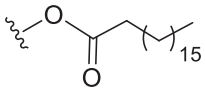
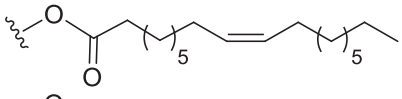
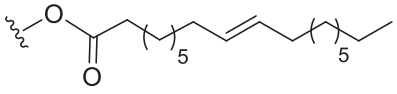
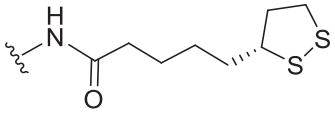
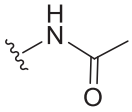
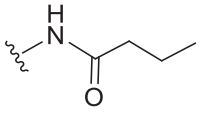
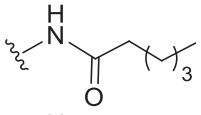
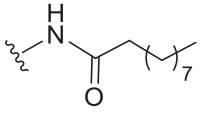
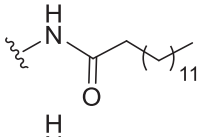
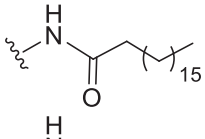
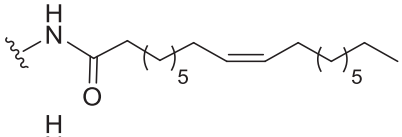
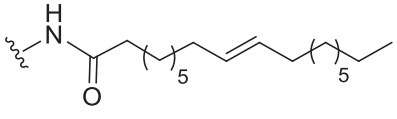


Compound	R	HFF viability (%)		*T. gondii* β-gal growth (%)	
		0.1 μM	**1** μM	**0.1** μM	**1** μM
Diruthenium intermediates					
1[a,*]		91 ± 4	73 ± 1	114 ± 2	110 ± 2
2[a,*]		76 ± 6	46 ± 6	66 ± 14	2 ± 0
3[a,*]		74 ± 2	48 ± 1	57 ± 1	2 ± 0
First series of ester conjugates					
4a		102 ± 2	88 ± 1	86 ± 2	89 ± 3
5a*		144 ± 4	99 ± 4	122 ± 4	3 ± 0
6a		103 ± 1	73 ± 1	112 ± 3	26 ± 1
7a[a]		50 ± 4	46 ± 2	3 ± 0	4 ± 1
8a*		81 ± 2	81 ± 3	33 ± 0	0 ± 0
Second series of ester conjugates					
9a		109 ± 3	108 ± 4	94 ± 11	2 ± 10
10a[a,*]		74 ± 2	52 ± 3	5 ± 0	2 ± 1
11a*		97 ± 1	94 ± 0	14 ± 0	0 ± 0
12a*		97 ± 2	95 ± 0	41 ± 1	0 ± 0
13a*		101 ± 5	65 ± 4	85 ± 4	1 ± 0
14a*		71 ± 11	59 ± 4	94 ± 3	1 ± 0

(*continued on next page*)

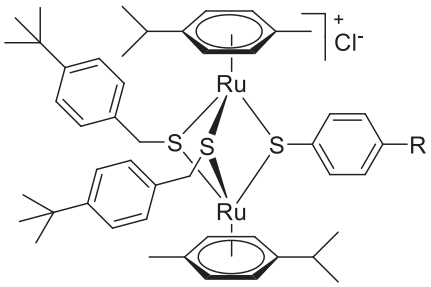
Table 1 (*continued*)

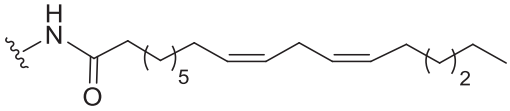
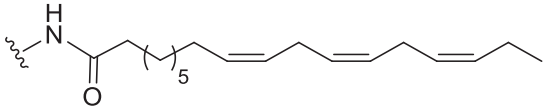
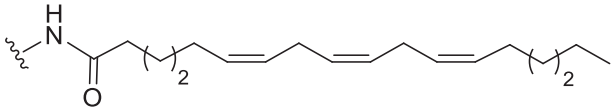


Compound	R	HFF viability (%)		*T. gondii* β-gal growth (%)	
15a		97 ± 2	67 ± 4	99 ± 4	61 ± 1
16a*		94 ± 3	80 ± 11	93 ± 5	1 ± 0
17a*		87 ± 4	71 ± 4	92 ± 4	1 ± 0
Amide conjugates					
9b		104 ± 15	100 ± 3	105 ± 10	23 ± 0
10b[a]		62 ± 7	27 ± 1	4 ± 4	3 ± 3
11b*		95 ± 1	94 ± 0	6 ± 1	0 ± 3
12b*		98 ± 2	94 ± 1	93 ± 4	1 ± 0
13b*		107 ± 6	75 ± 7	131 ± 4	1 ± 0
14b*		94 ± 2	71 ± 5	92 ± 1	5 ± 1
15b		98 ± 5	78 ± 4	123 ± 5	108 ± 1
16b		97 ± 8	74 ± 4	106 ± 2	91 ± 2
17b		99 ± 6	78 ± 6	127 ± 4	6 ± 2

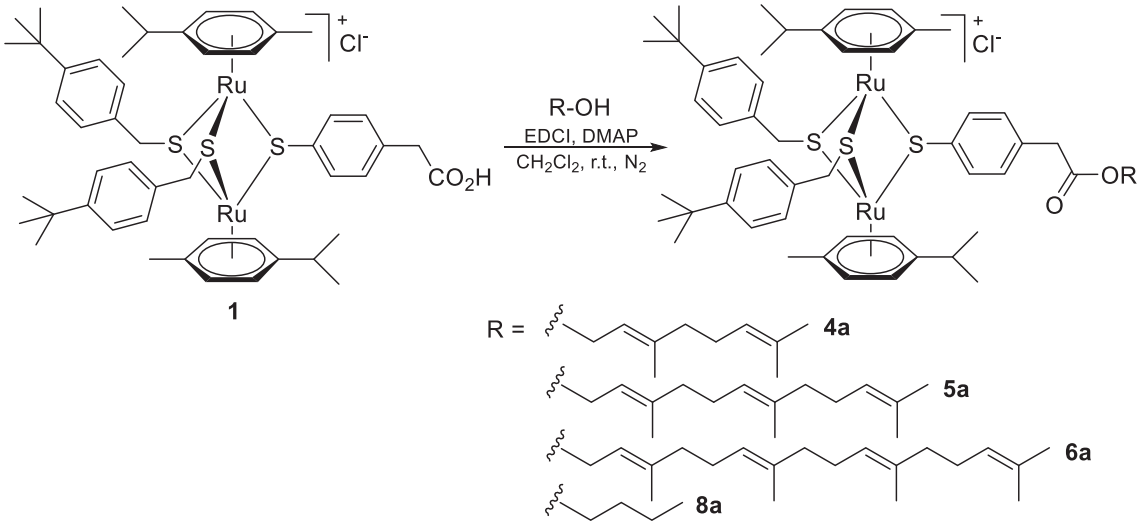
(*continued on next page*)

Table 1 (*continued*)



Compound	R	HFF viability (%)		*T. gondii* β-gal growth (%)	
18b		93 ± 3	77 ± 8	95 ± 5	65 ± 0
19b[a]		105 ± 7	96 ± 2	4 ± 0	3 ± 0
20b		94 ± 1	93 ± 3	125 ± 24	120 ± 26

[a] Data for compounds **1**–**3, 7a, 10a** and **10b** were previously reported [42,45,47].

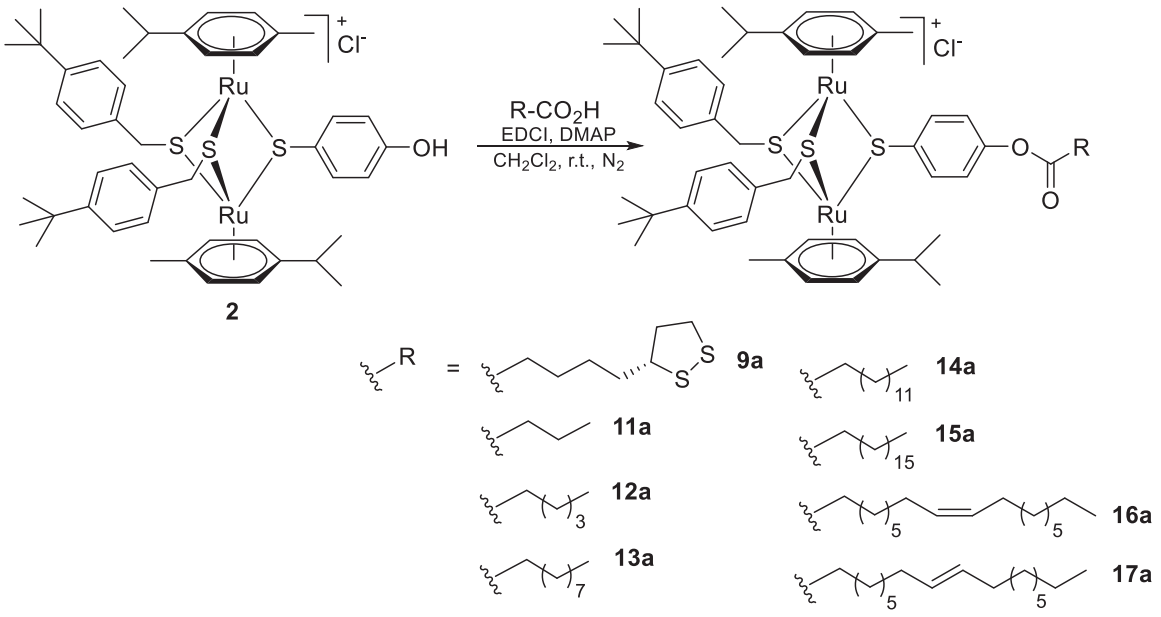


Scheme 1. Synthesis of the first series of ester derivatives **4a**-**6a** and **8a** starting from the diruthenium carboxy intermediate **1**.

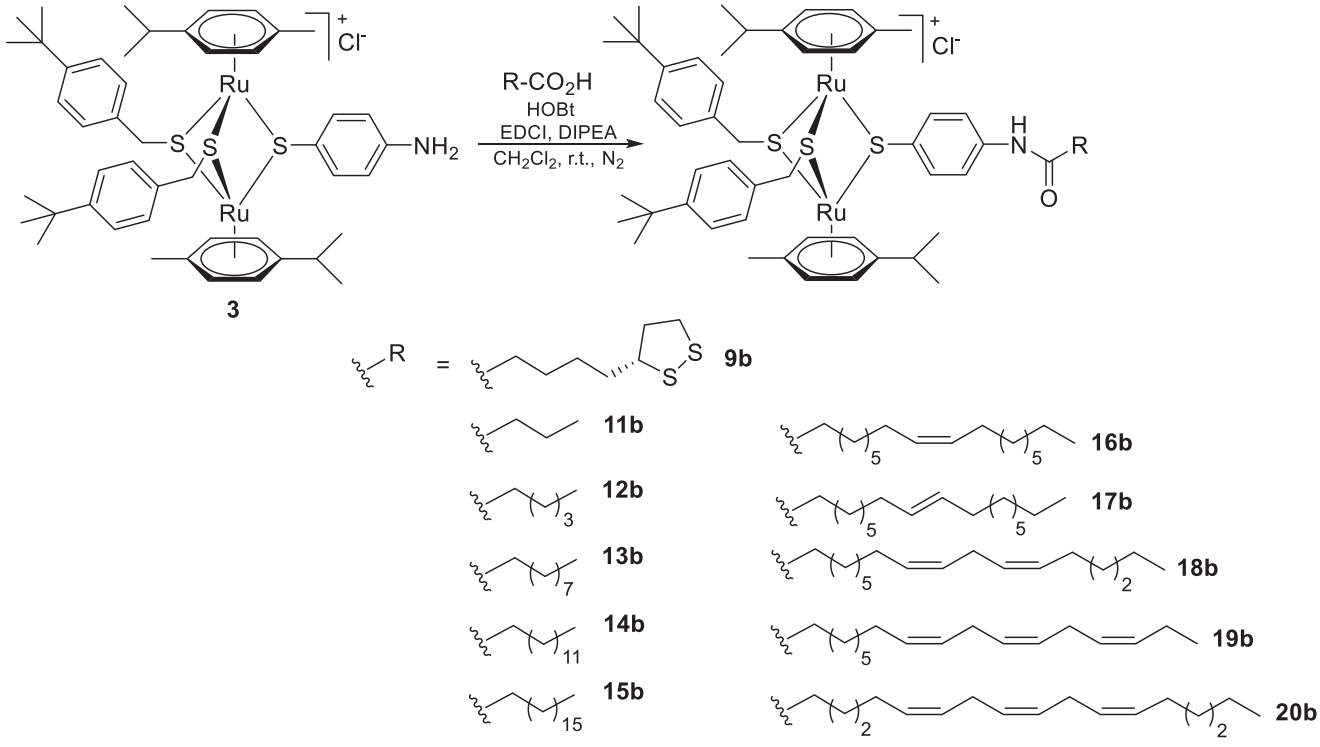
units strongly influenced the measured cytotoxicity and the antiparasitic activity.

From the first series of esters, only the ethyl conjugate **7a** was toxic to the host cells at both tested concentrations, while hybrids **4a**-**6a** and **8a** showed reduced or no toxicity to HFF. The farnesyl conjugate **5a** exhibited the best parasite efficacy/cytotoxicity balance. When applied at 1 μM, farnesyl and *n*–butyl esters **5a** and **8a** were more active against the parasite compared to the geranyl and geranylgeranyl esters **4a** and **6a**, inhibiting *T. gondii* proliferation to less than 5%. Of note, the carboxy diruthenium intermediate **1** reduced HFF viability to 73% when applied at 1 μM without impact on the parasite growth. Comparatively, farnesyl ester **5a** had no effect on HFF viability at 1 μM, while efficiently inhibiting parasite growth to 3% at the same concentration.

The most interesting compounds from the second series of ester conjugates were the α-lipoic, butyric and hexanoic esters **9a, 11a** and **12a**, which showed no toxicity to HFF at 1 μM, while completely inhibiting the *in vitro* proliferation of *T. gondii* tachyzoites at 1 μM. When applied at 1 μM, oleic and elaidic ester hybrids **16a** and **17a** exhibited moderate toxicity to the host cells (viability of 80% and 71%), while completely impairing parasite growth. Decanoic and myristic esters **13a** and **14a** revealed a similar antiparasitic profile as **16a** and **17a**, while being more toxic to HFF (viability reduced to 65% and 59%). Interestingly, dissimilar to its analogues with shorter aliphatic chains (decanoic and myristic esters **13a** and **14a**), and to the conjugates with unsaturated chains of the same length (oleic and elaidic hybrids **16a** and **17a**), the stearic ester conjugate **15a** exerted reduced antiproliferative efficacy on *T. gondii* at 1 μM. Both the length and the nature of the appended



Scheme 2. Synthesis of the second series of ester derivatives **9a** and **11a-17a** starting from the diruthenium hydroxy intermediate **2**.



Scheme 3. Synthesis of the series of amide derivatives **9b** and **11b-20b** starting from the diruthenium amino intermediate **3**.

chain (saturated *vs* unsaturated) influenced the host cells toxicity and the antiparasitic activity. Compared to the hydroxy diruthenium intermediate **2**, the presence of the ester appendices in compounds **9a**-**17a** mostly decreased the HFF toxicity.

The most performant compounds in the amide conjugates series were the butyric and α-linolenic derivatives **11b** and **19b**, which completely inhibited the *T. gondii* β-gal proliferation at 0.1 μM, without affecting the host cells viability when applied at 1 μM. α-Lipoic, hexanoic and γ-linolenic amide conjugates **9b, 12b** and **20b** did not impair HFF viability, but only **12b** exhibited antiparasitic effect at 1 μM, while **9b** poorly reduced parasite growth and **20b** showed no effect at the same concentration. Decanoic (**13b**), myristic (**14b**), stearic (**15b**), oleic (**16b**), elaidic (**17b**) and linoleic (**18b**) amide conjugates exerted similar moderate toxicity to the host cells when applied at 1 μM (HFF viability between 71 and 78%). However, for these derivatives the nature of the appended unit strongly influenced the antiparasitic efficacy, with **13b, 14b** and **17b** reducing *T. gondii* proliferation to less than 6% when applied at 1 μM, while **15b, 16b** and **18b** exhibiting low efficacy against parasite proliferation. For the pendant moieties, the chain

length (**11b** *vs* **12b**-**14b** *vs* **15b**) but also the presence, the *cis-trans* geometry (**15b** *vs* **16b** and **17b**), the number (**17b, 18b** and **19b**) and the position of the double bonds (**19b** and **20b**) influence both the HFF cytotoxicity and, to a higher extent, the antiparasitic effect. Compared to the amino diruthenium intermediate **3**, apart from **10b**, all the other amide conjugates (**9b** and **11b**-**20b**) are less toxic to HFF.

For some appended units, a certain parallelism can be observed between the ester and the amide series. Thus, for similar effect on the host cells, α-lipoic ester **9a** inhibited parasite proliferation more efficiently than the corresponding amide **9b**. Acetyl ester and amide conjugates **10a** and **10b** were both more toxic to HFF compared to the other analogues of the series. Conjugates bearing short alkyl chain (butyric (**11a, 11b**) and hexanoic (**12a** and **12b**) esters and amides) do not impact the host cells viability at 1 μM, while efficiently inhibiting parasite proliferation when applied at the same concentration. Medium long alkyl chain derivatives (decanoic (**13a, 13b**) and myristic (**14a, 14b**) esters and amides) were efficient against *T. gondii* at 1 μM, while being moderately toxic to HFF. Compounds bearing the longest saturated chain, stearic ester, and amide **15a** and **15b**, exerted poor effect against *T. gondii* β-gal proliferation even at 1 μM with moderate toxicity on HFF. For the same chain length, the presence of a *trans* double bond in elaidic acid derivatives **17a** and **17b**, led to an increased antiparasitic efficacy compared to stearic ester and amide **15a** and **15b**. For the oleic derivatives **16a** and **16b** the *cis* double bond induced contrasting effects, only the oleic ester conjugate **16a** exerting an improved parasite antiproliferative efficacy compared to stearic ester **15a**.

The compounds inhibiting by at least 90% the *T. gondii* proliferation and reducing with less than 50% host cells growth were selected for a second screening consisting in IC_{50} determination on *T. gondii* β-gal and HFF cytotoxicity assessment at 2.5 μM, the measured data being summarized in Table 2.

The dose-response experiments allowed the identification of three promising compounds: the decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a**, . These compounds combine low IC_{50} values (0.065, 0.127 and 0.123 μM, respectively), and no effect on HFF viability when applied at 2.5 μM. Remarkably, these three compounds exhibit a better antiparasitic activity/toxicity balance compared to the corresponding diruthenium hydroxy derivative **2** from which they were obtained and are more efficient against *T. gondii* compared to the standard drug pyrimethamine.

From the first series of ester dyads, the farnesyl conjugate **5a** showed poor antiparasitic efficacy, but had no impact on the host cells, while the butyl hybrid **8a** was efficient against *T. gondii* but was also highly toxic to HFF when applied at 2.5 μM. These results indicate that, the nature of the pendant unit strongly influences the biological activity.

For the second series of ester conjugates, neither the antiparasitic efficacy nor the HFF cytotoxicity correlated with the length of the anchored chain. For instance, derivatives **10a** and **12a** (from acetic and hexanoic acids) strongly impaired HFF viability when applied at 2.5 μM (viability reduced to 16 and 0%, respectively), while derivatives **11a** and **14a** (from butyric and myristic acids) had moderate effect on the host cells at the same concentration (viability of 72 and 78%, respectively). The decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a** were identified as the most effective compounds, not impairing the HFF viability and strongly inhibiting parasite proliferation. The decanoic hybrid **13a** had the lowest IC_{50} value (0.065 μM, 5 times lower than the IC_{50} of the standard drug pyrimethamine). The double bond geometry (*cis vs trans*) in oleic and elaidic esters **16a** and **17a** had negligible effect on the antiparasitic activity, the compounds exhibiting similar IC_{50} values of 0.127 and 0.123 μM, respectively, and no impact on the host cells. Overall, compared to the parent hydroxy diruthenium intermediate **2**, anchoring lipophilic chains connected through ester bonds in conjugates **11a, 13a, 14a, 16a** and **17a** led to lower host cell toxicity.

In the amide conjugates series, the analogues with the shortest chains, the butyric and hexanoic hybrids **11b** and **12b**, strongly impaired HFF viability when applied at 2.5 μM (viability 0%). In contrast, the medium-long chain decanoic **13b** and myristic **14b** amide conjugates were nontoxic to the host cells at 2.5 μM (viability 117 and 94%, respectively) but also exhibited low efficacy in inhibiting *T. gondii* β-gal proliferation (IC_{50} values of 0.515 and 0.495 μM, more than 1.5 times higher compared to that of the standard drug pyrimethamine). Relative to the amino diruthenium intermediate **3**, a reduction of the HFF cytotoxicity was observed only for the amide hybrids with medium-long alkyl chains **13b** and **14b**, while an opposite effect was observed for the derivatives with short chains **11b** and **12b**. The results obtained for the ester and amide conjugates demonstrate that the nature of the bond connecting the lipophilic unit to the diruthenium moiety strongly influence the biological activity.

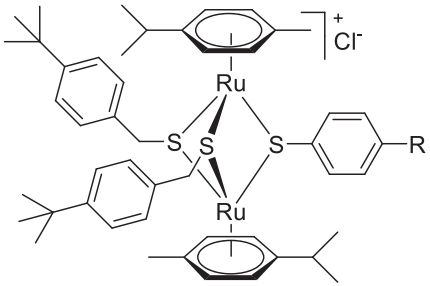
The infection and persistence of the *T. gondii* in host cells significantly rely on lipid metabolic activities [14,18], the parasite acquiring the necessary lipids through intricate and complex networks of synthetic and salvage pathways [48,49]. Related to lipid metabolism, previous studies suggested various lipophilic compounds as potential approach for toxoplasma therapy and prevention [19,50-53]. Anchoring lipophilic appendices on organometallic units can lead to improved biological properties (in terms of activity and selectivity) [25,26], the nature of the metal unit, that of the lipophilic pendants, as well as the connection mode having a determinant role [35–37]. Our results further confirm these observations even if no clear SAR (structure activity relationship) was identified for this library of compounds . No linear dependence between the chain length and the host cells toxicity was observed and an example is that of the acetic, butyric and hexanoic esters **10a, 11a** and **12a** with HFF viability at 2.5 μM of 16, 72 and 0%. Also, for the same type of attached lipophilic compounds esters **13a** and **14a** exhibit lower *T. gondii* IC_{50} values compared to the respective amides **13b** and **14b**. In comparison to the hydroxy diruthenium compound **2**, the decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a** showed lower host cell toxicity, for improved (**13a**) or similar (**16a** and **17a**) antiparasitic activity (Table 2).

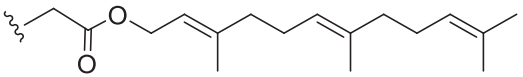
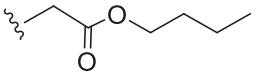
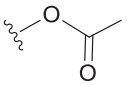
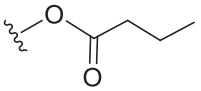
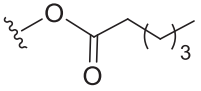
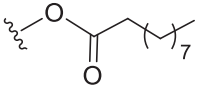
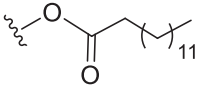
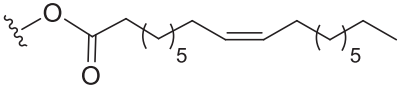
The exact mechanism of action of trithiolato diruthenium compounds is not yet known. These organometallic complexes are stable to hydrolysis and in the presence of biomolecules such as amino acids and DNA [20]. The oxidation of cysteine (Cys) and glutathione (GSH) (to form cystine and GSSG, respectively) was demonstrated in the presence of some compounds, but no correlation between the *in vitro* cytotoxicity and the catalytic activity on the glutathione oxidation was observed [54,55]. TEM (transmission electron microscopy) studies of different protozoan parasites (*Toxoplasma gondii, Neospora caninum, Trypanosoma brucei*) treated with trithiolato dinuclear ruthenium(II)-arene complexes revealed alterations in the mitochondrial ultrastructure pointing out this organelle as potential target [21–23]. Further tests are necessary to ascertain if the lipophilic conjugates **13a, 16a** and **17a** share the same targets as the non-modified diruthenium complexes as for example the hydroxy precursor **2**.

3. Conclusions

This report was focused on the synthesis and *in vitro* antiparasitic activity study of 49 compounds, among which 23 new conjugates based on trithiolato-bridged ruthenium(II)-arene scaffold tethered with various lipophilic derivatives. The influence of the nature of the appended lipophilic moiety and that of the chemical bond between the two units on the biological activity was assessed. A first screening, consisting in measuring the efficacy

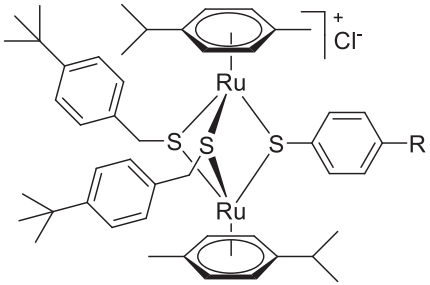
Table 2
IC_{50} values (μM) against *T. gondii* β-gal tachyzoite proliferation and effects on HFF viability at 2.5 μM, for selected compounds and pyrimethamine (as standard). *T. gondii* proliferation and HFF viability were quantified by β-galactosidase and alamarBlue assays, respectively.

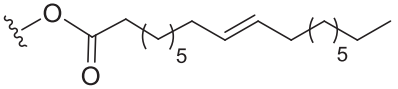
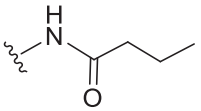
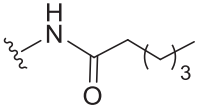
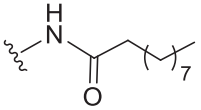
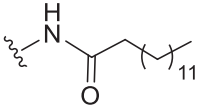
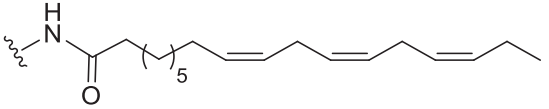


Compound	R	IC_{50} (μM)	[LS; LI][b]	SE[c]	HFF viability at 2.5 μM (%)[d]	SD[e]
Pyrimethamine		0.326	[0.396; 0.288]	0.051	99	6
Diruthenium intermediates						
2[a]		0.117	[0.139; 0.098]	0.051	56	6
3[a]		0.153	[0.185; 0.127]	0.049	51	5
First series of ester conjugates						
5a		0.695	[0.731; 0.661]	0.194	104	1
8a		0.086	[0.097; 0.076]	0.030	1	0
Second series of ester conjugates						
10a[a]		0.065	[0.101; 0.042]	0.092	16	5
11a		0.036	[0.050; 0.026]	0.062	72	0
12a		0.150	[0.199; 0.113]	0.062	0	0
13a		0.065	[0.072; 0.058]	0.029	118	1
14a		0.148	[0.170; 0.129]	0.037	78	0
16a		0.127	[0.153; 0.106]	0.058	123	2

(*continued on next page*)

Table 2 (*continued*)



Compound	R	IC_{50} (μM)	[LS; LI][b]	SE[c]	HFF viability at 2.5 μM (%)[d]	SD[e]
17a		0.123	[0.145; 0.104]	0.048	116	1
Amide conjugates						
11b		0.073	[0.098; 0.005]	0.060	0	0
12b		0.118	[0.126; 0.110]	0.019	0	0
13b		0.616	[0.618; 0.613]	0.001	117	21
14b		0.495	[0.501; 0.489]	0.012	90	4
19b		0.185	[0.240; 0.142]	0.262	90	2

[a] Data for pyrimethamine, **2, 3** and **10a** were formerly reported [42,45,47]. [b]Values at 95% confidence interval (CI); LS is the upper limit of CI and LI is the lower limit of CI. [c]The standard error of the regression (SE), represents the average distance that the observed values fall from the regression line. [d]Control HFF cells treated only with 0.25% DMSO exhibited 100% viability. [e]The standard deviation of the mean (six replicate experiments).

against *T. gondii* β-gal tachyzoites cultured in HFF and the determination of the cytotoxicity against host cells at 0.1 and 1 μM, engendered the selection of 14 conjugates. For these hybrids, the IC_{50} values against *T. gondii* and the evaluation of HFF viability after exposure to 2.5 μM led to the identification of the decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a** as the most promising members of this new library.

The results of this study show that both the nature of the lipophilic pending unit and the type of bond linking the two moieties impact the biological activity, even if no clear structure-activity relationship was identified. Tethering lipophilic compounds to the trithiolato diruthenium(II)-arene scaffold afforded conjugates exhibiting different antiparasitic efficacy/cytotoxicity profiles compared to the parent organometallic complexes **2**–**4** and, in some cases, an important reduction of the host cells cytotoxicity being observed. A fine structural tuning is necessary to obtain compounds with optimal properties, and the decanoic, oleic and elaidic ester conjugates **13a, 16a** and **17a** efficiently inhibit parasite proliferation while not impairing the HFF viability and show improved activity and cytotoxicity parameters compared to their respective diruthenium hydroxy precursor **2**.

4. Experimental

4.1. Chemistry

The chemistry experimental part, with full description of experimental procedures and characterization data for all new compounds, is presented in the *Supporting information*.

4.2. In vitro *activity assessment against* T. gondii *tachyzoites and HFF*

All tissue culture media were purchased from Gibco-BRL, and biochemical agents from Sigma-Aldrich. Human foreskin fibroblasts (HFF) were purchased from ATCC, maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Waltham, MA, USA) and antibiotics as previously reported [56]. Transgenic *T. gondii* β-gal (expressing the β-galactosidase gene from *Escherichia coli*) were kindly provided by Prof. David Sibley (Washington University, St. Louis, MO, USA) and were maintained, isolated, and prepared for new infections as described [56,57]. All the compounds were prepared as 1 mM stock solutions from powder, in 100% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). For *in vitro* activity and cytotoxicity assays, HFF were seeded at 5×10^3 cells /well and allowed to grow to reach confluence in DMEM complemented medium at 37 °C and 5% CO_2. Transgenic *T. gondii* β-gal tachyzoites were isolated and prepared for infection as reported [56]. *T. gondii* tachyzoites were released from host cells, and HFF monolayers were infected with freshly isolated parasites (1×10^3/well), and compounds were added concomitantly with infection. In the primary screening, *T. gondii* β-gal infected HFF monolayer cultures were treated with 0.1 and 1 µM of each compound, or with corresponding concentrations of DMSO (0.01 or 0.1% respectively) as controls, and incubated for 72 h at 37°C/5% CO_2 as previously described [45]. For the second screening, IC_{50} measurements for *T. gondii* β-gal were performed. The selected compounds were added concomitantly with infection in 8 serial concentrations 0.007, 0.01, 0.03, 0.06, 0.12, 0.25, 0.5, and 1 μM. After a period of 72 h of culture at 37 °C/5% CO_2, culture medium was aspirated, and cells were permeabilized by adding 90 μL PBS (phosphate buffered saline) with 0.05% Triton X-100. After addition of 10 μL 5 mM chlorophenolred-β-D-galactopyranoside (CPRG; Roche Diagnostics, Rotkreuz, Switzerland) in PBS, the absorption shift was measured at 570 nm wavelength at various timepoints using an EnSpire® multimode plate reader (PerkinElmer, Inc, Waltham, MA, USA). For the primary screening at 0.1 and 1 μM, activity was measured as the release of chlorophenol red over time and was calculated as percentage from the respective DMSO control, which represented 100% of *T. gondii* β-gal growth. In this assay, produced chlorophenol red is directly proportional to the number of tachyzoites which are expressing the β-galactosidase, and thus correlating with a drug activity at the given concentration. IC_{50} values were then calculated after the logit-log-transformation of relative growth and subsequent regression analysis. All calculations were performed using the corresponding software tool contained in the Excel software package (Microsoft, Redmond, WA, USA). Cytotoxicity assays using uninfected confluent HFF host cells were performed by the alamarBlue assay as formerly reported [58]. Confluent HFF monolayers cultured in 96 well-plates were exposed to 0.1, 1 and 2.5 μM of each selected compound. Non-treated HFF as well as DMSO controls (0.01%, 0.1% and 0.25%) were included. After 72 h of incubation at 37 °C/5% CO_2, the medium was removed, and plates were washed once with PBS. Then resazurin dissolved in PBS was added to a final concentration of 10 µg/mL. Plates were then measured at excitation wavelength 530 nm and emission wavelength 590 nM using an EnSpire® multimode plate reader (PerkinElmer, Inc). Fluorescence was measured at different timepoints. Relative fluorescence units were calculated from timepoints with linear increase.

Supplementary Materials

The Supporting Information is available free of charge on the …… website at https://doi.org/ ….. Primary cytotoxicity/efficacy screening of the lipophilic compounds in non-infected HFF cultures and T. gondii β-gal tachyzoites cultured in HFF. The chemistry experimental part with full description of experimental procedures. 1H NMR spectra of 8a, 13a-15a, 17a, 13b-15b, and 17b-19b recorded in DMSO–d6 at 25 °C as function of time. 1H NMR spectrum of 11a recorded in CDCl3 at 25 °C and HR ESI-MS spectrum of 11a.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Funding

The authors declare no competing financial interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2023.122624.

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