

Trithiolato-Bridged Dinuclear Arene Ruthenium(II)- Glycoconjugates: Synthesis and Antiparasitic Activity

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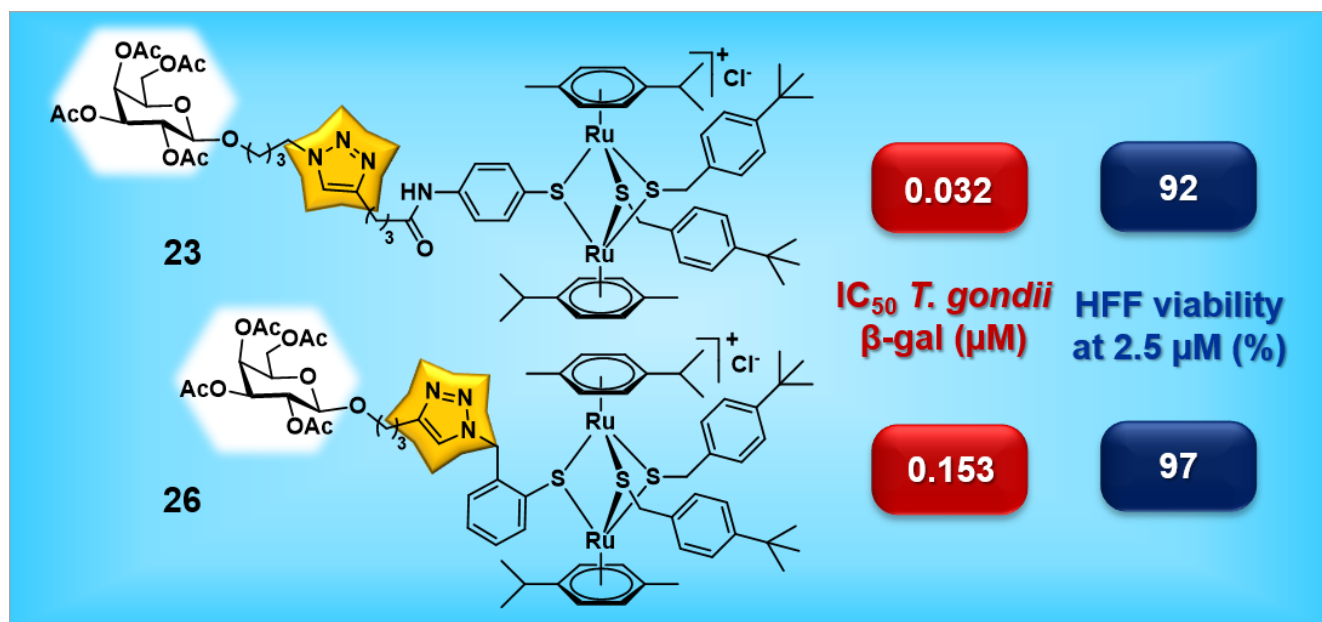
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Abstract

Eight novel carbohydrate-tethered trithiolato dinuclear ruthenium(II)-arene complexes were synthesized using CuAAC 'click' (Cu(I)-catalyzed azide-alkyne cycloaddition) reactions and, together with the diruthenium intermediates, were assessed for their *in vitro* activity against transgenic *Toxoplasma gondii* (*T. gondii*) tachyzoites constitutively expressing β -galactosidase (*T. gondii* β -gal), and for their cytotoxicity in non-infected host cells (human foreskin fibroblasts, HFFs). The results revealed that the biological activity of the hybrids was influenced by both the nature of the carbohydrate (glucose or galactose) attached to the ruthenium complex and the type/length of the linker between the two units. For seven selected diruthenium-carbohydrate conjugates, the values of the half-maximal inhibitory concentration (IC_{50}) on *T. gondii* β -gal and HFFs viability for a compound concentration of 2.5 μ M were measured. Remarkably, two galactose-diruthenium conjugates, **23** and **26**, performed significantly better than the corresponding unlabeled diruthenium complexes and the standard drug Pyrimethamine, with very low IC_{50} values (**23**: IC_{50} = 0.032 μ M, **26**: IC_{50} = 0.153 μ M, Pyrimethamine, IC_{50} = 0.326 μ M) and a very low toxicity on HFFs (viability 92% for **23** and 97% for **26**). Overall, our study shows that conjugation of carbohydrates to diruthenium compounds is a promising approach to develop new effective antiparasitic compounds with reduced toxicity.

Keywords: Trithiolato-bridged dinuclear ruthenium(II)-arene complexes, bioorganometallic, carbohydrates, CuAAC reactions, antiparasitic, *Toxoplasma gondii*, Human Foreskin Fibroblasts, toxicity.

Graphical Abstract



1. Introduction

The interest in the development of metal complexes for medicinal applications increased in the middle of the 20th century after the discovery of the anticancer properties of cisplatin[1,2]. Metal-based drugs are attractive due to their great versatility in terms of metal center, oxidation state, coordination number, in addition to the nature and geometric orientation of the ligands[3]. As the use of platinum-based drugs is limited due to shortcomings as the occurrence of chemoresistance and side effects associated to their high toxicity[4,5], this encouraged the research of compounds based on other metals as alternative to platinum anticancer therapeutics[1,6][7,8]. Parallel investigations aimed to enlarge the purpose of metal complexes with the identification of additional pharmacological properties, such as antibiotic[9,10] and antiparasitic[11-16].

Ruthenium complexes were found amid the most promising non-platinum chemotherapeutic alternatives[17,18]. Organometallic complexes based of the ruthenium(II)-arene scaffold constitute an important direction of study. Pioneered by RAPTA-C[19] ($\text{Ru(II)}[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)(\text{PTA})(\text{Cl})_2]$ (PTA = 1,3,5-triaza-7-phosphatricyclo-[3.3.1]decane)) and RM175[20] ($[\text{Ru(II)}(\eta^6\text{-C}_6\text{H}_5\text{-C}_6\text{H}_5)(\text{en})\text{Cl}]^+$ (en = ethylenediamine)) complexes, the Ru(II)-arene moiety has been used in a myriad of compounds to improve anticancer activity and selectivity[21-25], but also for other therapeutic applications[11,13,16,26].

A particular class of compounds containing this unit are the trithiolato-bridged dinuclear ruthenium(II)-arene complexes (**A-C** in Figure 1), which show not only high antiproliferative activity against cancer cells[27], but also promising antiparasitic properties[28,29]. The structure of these complexes is based on a trigonal bipyramidal Ru_2S_3 framework, with two ruthenium(II)-arene half-sandwich units. Two types of complexes can be distinguished, “mixed” (at least one of the bridge thiols is different, **A** in Figure 1) and “symmetric” (the three bridge thiols are identical, **B** and **C** in Figure 1)[27]. Former studies of this type of compounds on *Toxoplasma gondii*[28], *Neospora caninum*[29] and *Trypanosoma brucei*[30] identified high antiparasitic activity for some derivatives. For example, compounds **A-C** (Figure 1) inhibit *T. gondii* tachyzoites proliferation with IC_{50} values in nanomolar range (down to 1.2 nM for **A**) while not affecting the HFF (human foreskins fibroblasts) host cells viability.

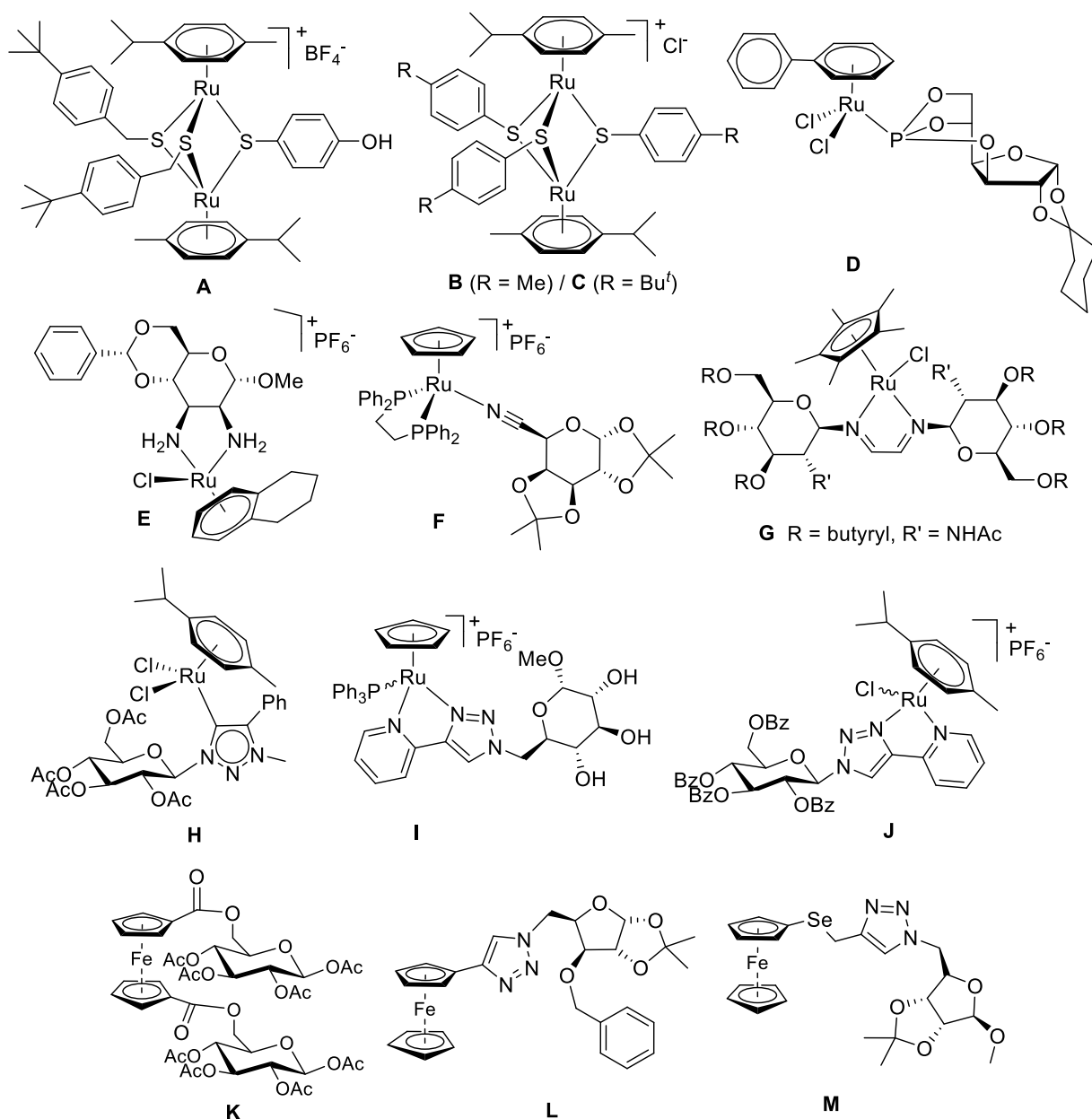


Figure 1. Structures of dinuclear thiolato-bridged arene ruthenium complexes active against *T. gondii* (A-C), of various ruthenium(II)-arene complexes presenting carbohydrate functionalized ligands and noteworthy anticancer activity (D-J), and of ferrocene-carbohydrate conjugates showing antimalarial, antibacterial and anticancer properties (K-M).

T. gondii is an obligate intracellular protozoan parasite of the phylum Apicomplexa that causes infections of medical and veterinary significance in humans and animals[31,32]. Infection is usually asymptomatic in immunocompetent individuals, but it may cause severe complications or even be fatal in immunocompromised patients[33]. Current common treatments for toxoplasmosis are not specific, require prolonged courses and have toxic side effects, and so, new therapeutic solutions are needed[33-36]. Unlike other pathogens, *Toxoplasma* has adapted to replicate in all nucleated cells of a wide range of vertebrates, regardless of their cellular metabolism, and thus displays an exceptional metabolic

robustness[37,38]. Accordingly, tackling the parasite auxotrophies and metabolic peculiarities can constitute an interesting therapeutic strategy[37].

Distinctively delivering metal complexes to a specific target or defined type of cells may be useful for diagnosis and therapy of pathological states. Anchoring bioactive molecules to organometallic complexes is a strategy aiming to improve the compounds' selectivity and biological activity[39,40]. Carbohydrates contribute to cell-cell recognition and adhesion, have a crucial role in cellular energy supply, and can bind to specific proteins (e.g., lectins, glucose transporters, and glycoenzymes). Consequently, their conjugation to metal complexes appears as a rational choice for drug design as this type of modification can promote biocompatibility and increase water solubility. Carbohydrate-metal hybrids show promise in medicinal chemistry[41-43] but also in catalysis[44,45]. Apart the metal, its oxidation state and coordination mode[46-48], various structural adjustments were considered as the type of the carbohydrate[49-54] and its substitution position[55], the presence and nature of the protecting groups[56-58].

Tumor cells demand of carbohydrates is augmented to sustain their fast-growing and high proliferation rate, which causes an increased glycolytic process known as the Warburg effect[59]. The cancer cells glucose metabolism can be exploited for targeted therapy[60], and consequently, glycoconjugates of various metal complexes were explicitly designed for selective uptake by cells overexpressing glucose transporters[61-63]. Apart cancer-specific treatment[39,41-43,64-67], alternative utilizations of this type of hybrids, as for example antiparasitic therapy, also received a lot of interest[68-70].

The medicinal applications of ruthenium complexes holding carbohydrate functionalized ligands was the focus of numerous studies[65,71-74]. Different carbohydrate units, various coordination modes, as well as presence/absence of protecting groups were experimented (Figure 1). For example, Ru(II)-arene complexes as **D** (Figure 1) with carbohydrate-derived phosphorus-containing ligands, have demonstrated promising antiproliferative activity and selectivity *in vitro*[75,76], with cytotoxicity dependent on the lipophilicity. Half-sandwich Ru(II) derivative **E**[77], bearing a mannose fragment as a diaminobidentate leg ligand, and complex **F**[78,79], with a galactose fragment *N*-coordinated *via* a nitrile group, exhibited promising antiproliferative activity on various cancer cells.

In the case of Ru(II)-arene tetrazene complexes as **G** (Figure 1)[80], bearing glucose moieties on their periphery, the presence of the acyl-protecting groups on the carbohydrate increased cancer cells cytotoxicity. Complex **H**[81] (Figure 1), containing a glucosyl functionalized 1,2,3-triazolyldiene *N*-heterocyclic carbene ligand, showed medium anticancer activity but good selectivity and water solubility.

Complexes like **I**[82] (Figure 1), with methyl α -D-mannopyranoside and methyl α -D-glucopyranoside to a pyridyl-2-triazole N–N structural motif were shown to exploit the glucose transporters for cellular uptake in cancer cells, associated to high cytotoxicity. Ruthenium(II) half-sandwich complexes like **J**[83] (Figure 1) with bidentate monosaccharide ligands, were cytostatic in various cancer cell lines, the carbohydrate moiety having a determining role, while large hydrophobic protective groups were needed for biological activity.

The high affinity of the malaria parasite for glucose was also exploited in targeting the parasite by glucose derivatives, such as ferrocenyl carbohydrate conjugates[69]. For example, compound **K**, disubstituted with acetyl-protected glucose moieties, showed moderate antimalarial activity *in vitro* in both *Plasmodium falciparum* chloroquine-resistant and non-resistant strains[69]. Conjugates **L** (Figure 1), derived from ferrocenyltriazole and carbohydrates, exhibited moderate to good cytotoxicity towards various cancer cell lines and antibacterial activity against both Gram-positive and Gram-negative pathogens[84]. Significant cytotoxicity against various cancer cell line was also showed by triazole bridged ferrocene-selenoribose conjugate **M**[85] (Figure 1) obtained *via* click chemistry.

This study continues the quest of trithiolato-bridged dinuclear ruthenium(II)-arene compounds presenting improved therapeutic value (in terms of antiparasitic efficacy/host cells toxicity balance) by exploiting the conjugate strategy and the parasite auxotrophies and specific metabolic needs. Furthermore, the investigation of carbohydrate metabolism in *T. gondii* has received a lot of interest[86-89] and seen the high energetic demand accompanying parasite growth and proliferation, carbohydrates can constitute an appealing choice among the metabolites able to promote the internalization of the organometallic unit in the parasite. Consequently, pending carbohydrate-containing units to the Ru(II)-arene moieties appears a judicious choice for drug design.

The synthesis of the trithiolato diruthenium complexes is generally straightforward and efficient[90-92], and this scaffold is robust to chemical modification and easily adaptable to the conjugate strategy as demonstrated by the various series of hybrids with peptides[93], drugs[94,95], fluorophores[92]·[96] or metabolites[97]. Ester and amide couplings[92,98], but also CuAAC (Cu(I)-catalyzed azide-alkyne cycloaddition) click reactions[95]·[97] proved to be useful tools for the functionalization of the diruthenium trithiolato unit at the level of the bridge thiols. CuAAC offer the advantage of mild reaction conditions, compatible with various ligands[46,48,81,99-101] but also with organometallics[84,102-106], and enables the construction of libraries of compounds⁹⁵·[107-109]. For example, this type of modification allowed the obtainment of RAPTA-type ruthenium(II)-arene anticancer compounds[81,99], but also the synthesis of various ligands used to generate carbohydrate-functionalized 1,2,3-triazolylidene ruthenium(II)-arene catalysts[44,45]. Likewise, various platinum, ruthenium and ferrocene glycoconjugates for bio-applications were obtained using click reactions[81-85,110]. Additionally, trithiolato diruthenium(II)-arene compounds suitably substituted with alkyne or

azide groups were already used in CuAAC reactions for obtaining conjugates with molecules of interest e.g., various nucleic bases or drugs[95][97].

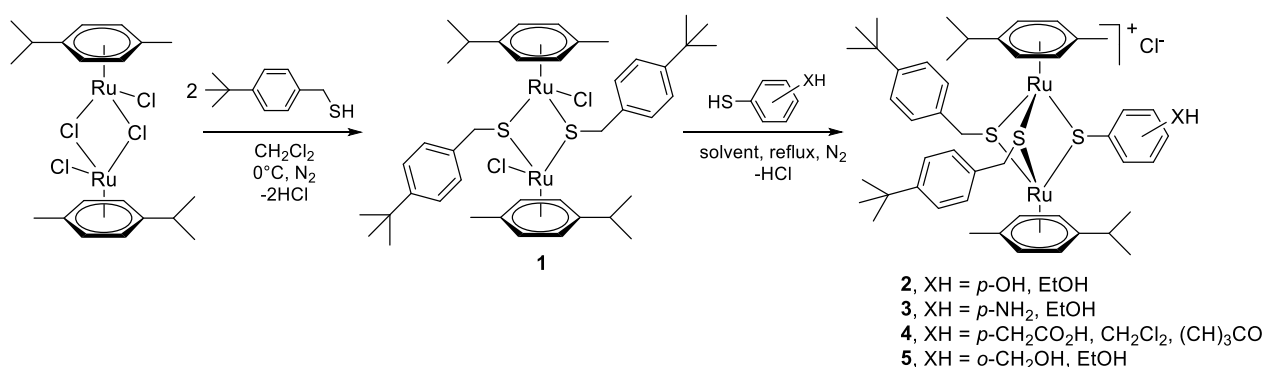
This research challenges the obtainment of carbohydrate-conjugates based on the trithiolato dinuclear scaffold as potential antiparasitic drugs. For this purpose, a carbohydrate moiety was pended on one of the bridge thiols using 'click' reactions. The nature of the carbohydrate (acetyl protected glucose or galactose) and the type and length of the linker between the two units, were addressed as sources of variability. The new diruthenium hybrids and intermediates were screened *in vitro* against *T. gondii* tachyzoites expressing β -galactosidase (*T. gondii* β -gal) grown in human foreskin fibroblasts (HFF) with complementary assessment of HFF viability using alamarBlue assay. The compounds showing promising antiparasitic activity and selectivity were further subjected to dose-response (IC_{50}) determination on *T. gondii* β -gal.

2. Results and discussion

2.1. Synthesis

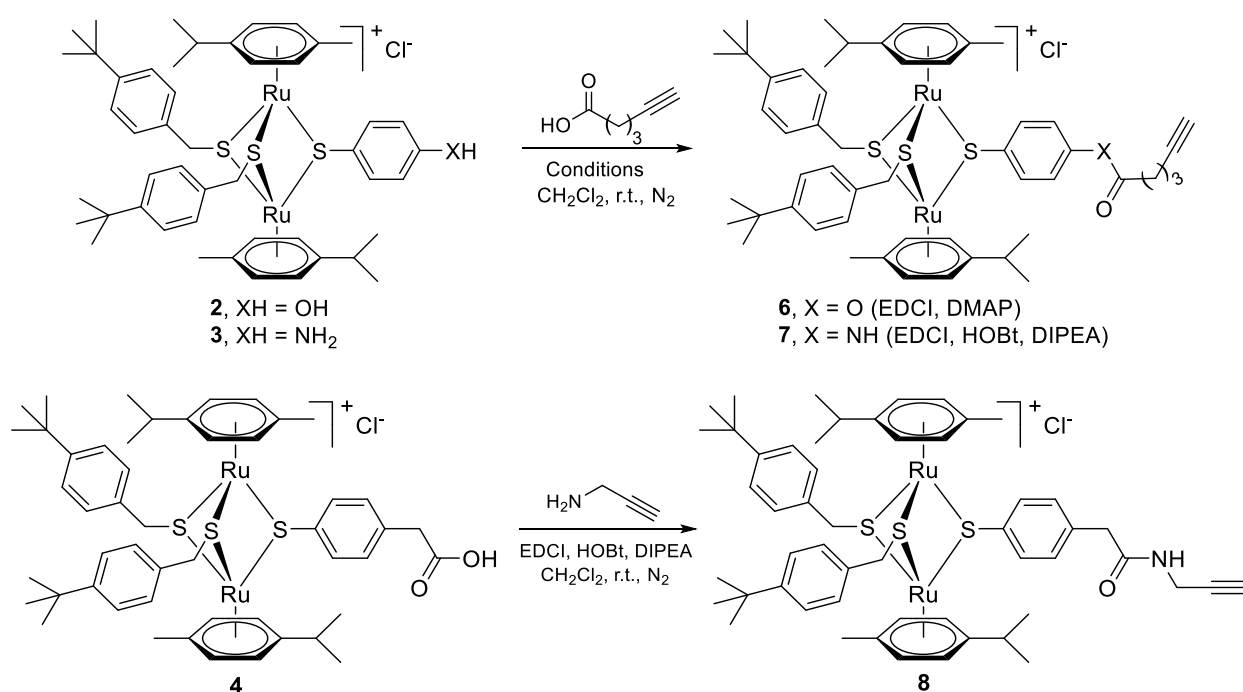
For CuAAC reactions, alkyne and azide partners are needed, and when appropriately substituted, both the diruthenium moiety and the carbohydrate can play either role. With this aim, various diruthenium and carbohydrate intermediates were synthesized.

The dithiolato derivative **1**[111] (obtained from the ruthenium dimer $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)\text{RuCl}]_2\text{Cl}_2$) and 4-*tert*-butylbenzenemethanethiol) was further reacted with a second thiol (4-mercaptophenol, 4-aminobenzenethiol, 2-(4-mercaptophenyl)acetic acid, and 2-mercaptobenzyl alcohol, respectively) to provide the trithiolato-bridged dinuclear compounds **2-5**, as previously reported (Scheme 1)[90-92].



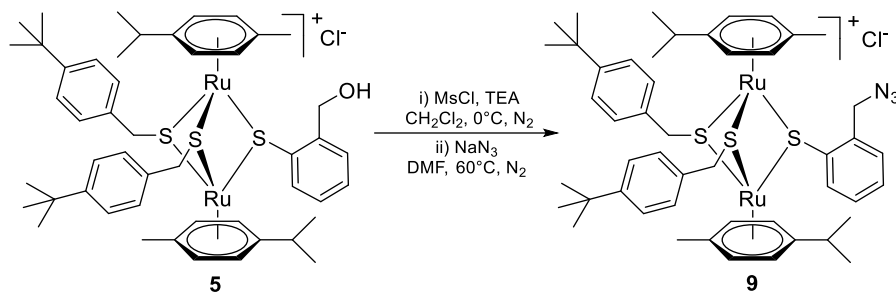
Scheme 1. Synthesis of the diruthenium intermediates bearing OH (**2**), NH_2 (**3**) $\text{CH}_2\text{CO}_2\text{H}$ (**4**), and $\text{CH}_2\text{-OH}$ (**5**) groups on one of the bridge thiols.

Intermediates **2-4** can be further modified using ester and amide coupling reactions as formerly described[92,95,98]. The ester alkyne analogue **6** was obtained in moderate yield (47%) from the reaction of **2** with 5-hexynoic acid using EDCI (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride) as coupling agent, in basic conditions (DMAP, 4-(dimethylamino)-pyridine) (Scheme 2, top). 5-Hexynoic acid was also reacted with the amino diruthenium derivative **3** using EDCI and HOBT (1-hydroxybenzotriazole) as coupling agents, in basic conditions (DIPEA *N,N*-diisopropylethylamine), to afford amido alkyne compound **7** as reported[97] (Scheme 2, top). Similar reaction conditions were used for the synthesis of amide **8** from carboxylic acid diruthenium derivative **4** and propargylic amine following a formerly described protocol (Scheme 2, bottom)[95,97].



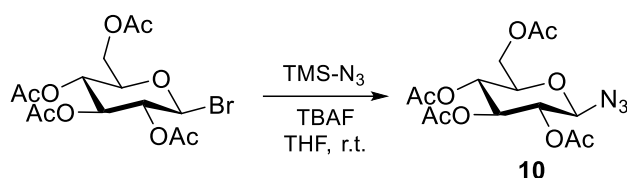
Scheme 2. Synthesis of the alkyne functionalized ester and amide diruthenium compounds **6**, **7** and **8**.

The azide trithiolato diruthenium derivative **9** (Scheme 3), was obtained following a two steps pathway starting from alcohol **5** using a reported protocol[97]. First, the hydroxy group was activated by mesylation (MsCl, methanesulfonyl chloride) in basic condition (TEA, triethylamine), followed by the nucleophilic substitution with azide (NaN₃).



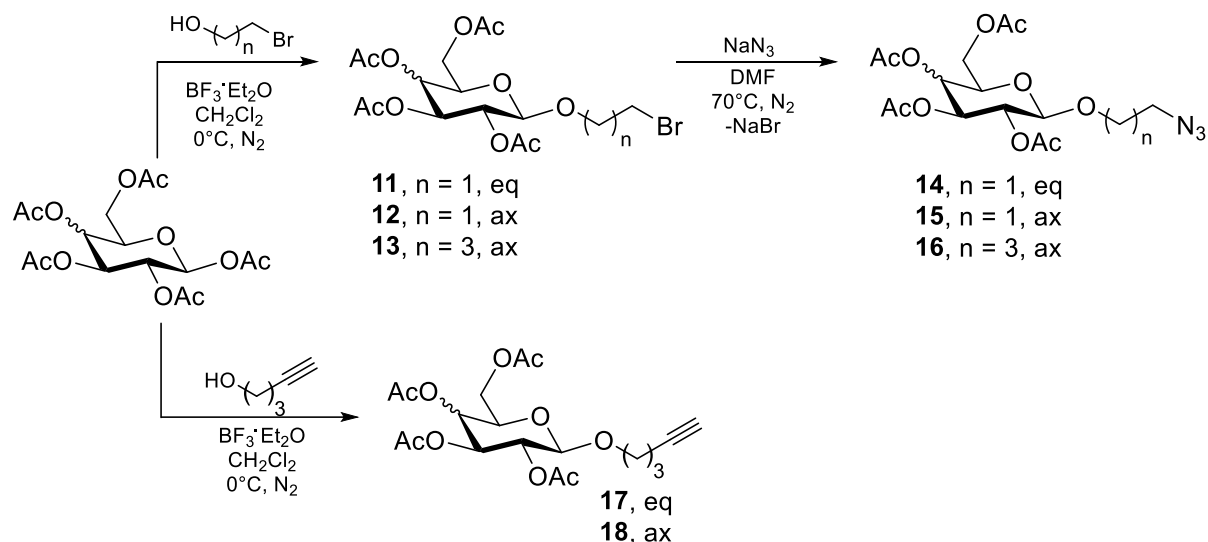
Scheme 3. Synthesis of the azide functionalized diruthenium complex **9**.

Appropriate carbohydrate derivatives bearing azide and alkyne groups were also synthesized (Schemes 4 and 5). Azido glucose compound **10** (2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl azide), was synthesized from commercially available 2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl bromide following a literature protocol (Scheme 4)[112]. The reaction was realized with TMS- N_3 (trimethylsilylazide) in THF in the presence of TBAF (tetrabutylammonium fluoride) in catalytic amounts, and **10** was isolated in moderate yield (51%).



Scheme 4. Synthesis of the azido glucose derivative **10**.

The azide compounds **14-16** were obtained following a two-step procedure previously described[55,58,113] (Scheme 5, top). First, β -D-glucose pentaacetate and β -D-galactose pentaacetate were glycosylated with 2-bromoethanol and 4-bromo-1-butanol. The reactions were realized in the presence of $BF_3 \cdot Et_2O$ (boron trifluoride diethyl etherate) as Lewis acid catalyst[55] and allowed the obtainment of the ether glycosides **11-13** in low to moderate yields (32, 41, and 47%, respectively). In the second step the bromine atom on the pending chain of **11-13** was substituted with azide (NaN_3)[58,113], derivatives **14-16** being isolated in 47, 74 % and quant. yields, respectively.

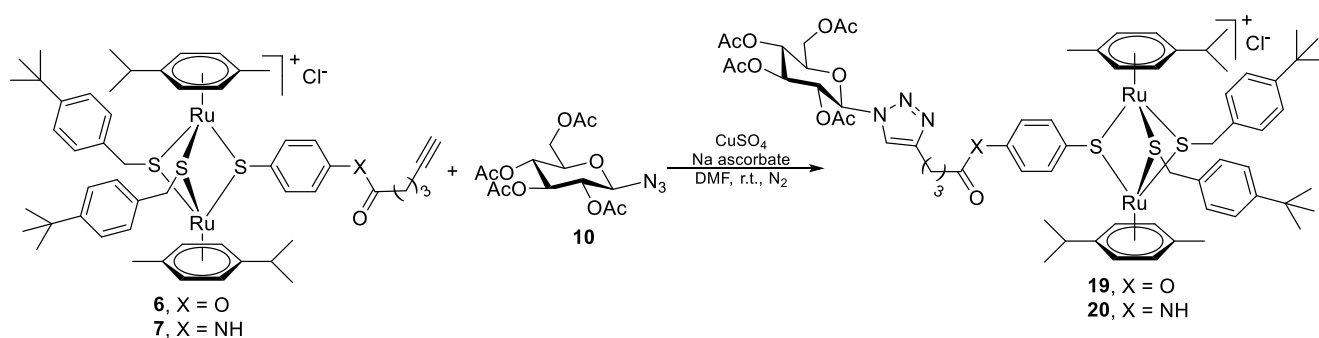


Scheme 5. Synthesis of the glucose and galactose azide **14-16**, and alkyne **17** and **18** derivatives (eq = equatorial, ax = axial).

The alkyne functionalized carbohydrates **17** and **18** were synthesized (Scheme 5, bottom) from β -D-glucose pentaacetate and, respectively, from β -D-galactose pentaacetate and 4-pentyn-1-ol in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ [114] and were isolated in medium yields (64 and 46%).

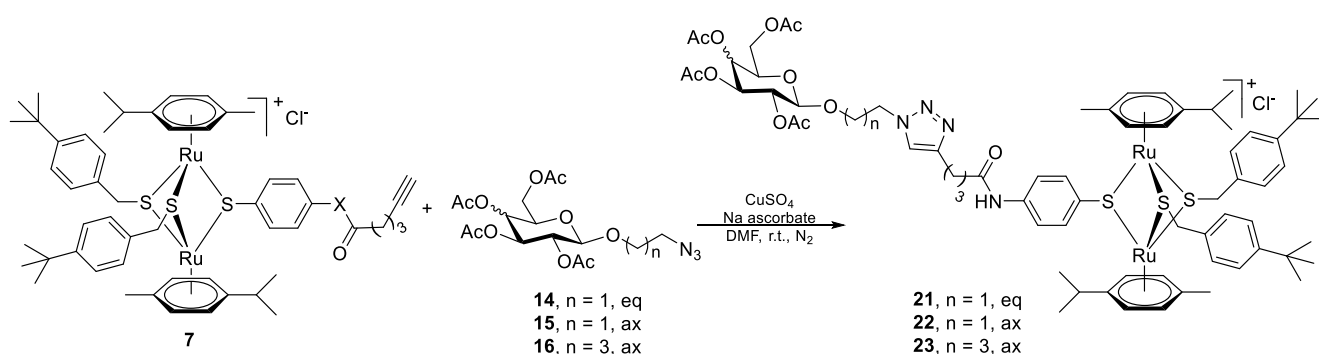
The carbohydrate units were attached to the trithiolato diruthenium scaffold using click 1,3-dipolar cycloadditions employing adapted protocols[115-117], in the presence of CuSO_4 as catalyst and sodium ascorbate as a reducing agent, in DMF under inert conditions. Complexes **6-9** bearing either alkyne or azide pendant group, were reacted with the appropriately functionalized carbohydrate derivatives **10** and **14-18** (Schemes 6-9) leading to eight new trithiolato dinuclear conjugates **19-26**.

Thus, alkyne functionalized diruthenium compounds **6** and **7** were reacted with glucose derivative **10** presenting an azide group directly anchored to the glucopyranosyl ring (Scheme 6). Amide conjugate **20** was isolated in good yield (72%), while difficulties were encountered in the purification of ester analogue **19** which was recovered in poorer yield (28%).

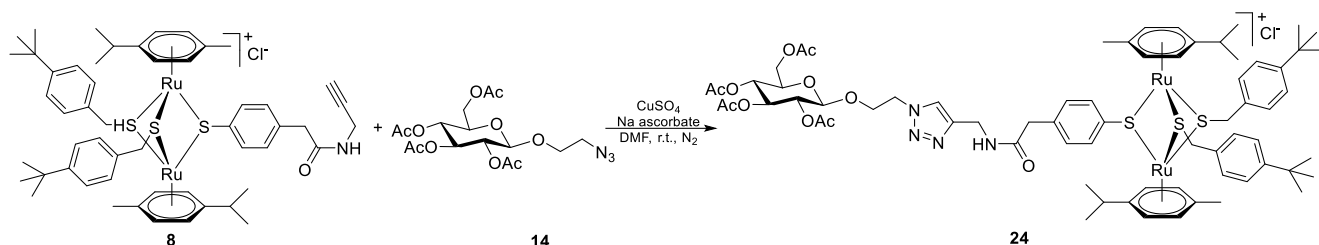


Scheme 6. Synthesis of the diruthenium glucose conjugates **19** and **20** from the alkyne ester and amide derivatives **6** and **7**.

Other glycoconjugates were synthesized using alkyne intermediate **7** (Scheme 7) and two types of modifications were envisioned: i) the nature of the carbohydrate (glucose in **21** vs galactose in **22**), and ii) the presence of spacers of different length between the azide group and the glucopyranosyl ring (galactose derivatives **22** and **23**). Conjugates **21-23** were isolated in good yields of 66, 65 and 74%, respectively. Neither the steric hindrance nor the nature of the carbon atom on which the azide group was anchored (**10** vs **14**) play a key role on the yield (**20** vs **21-23**). Similarly, the reaction of the diruthenium propargyl amide derivative **8** with galactose azide **14** allowed conjugate **24** in 75% yield (Scheme 8).

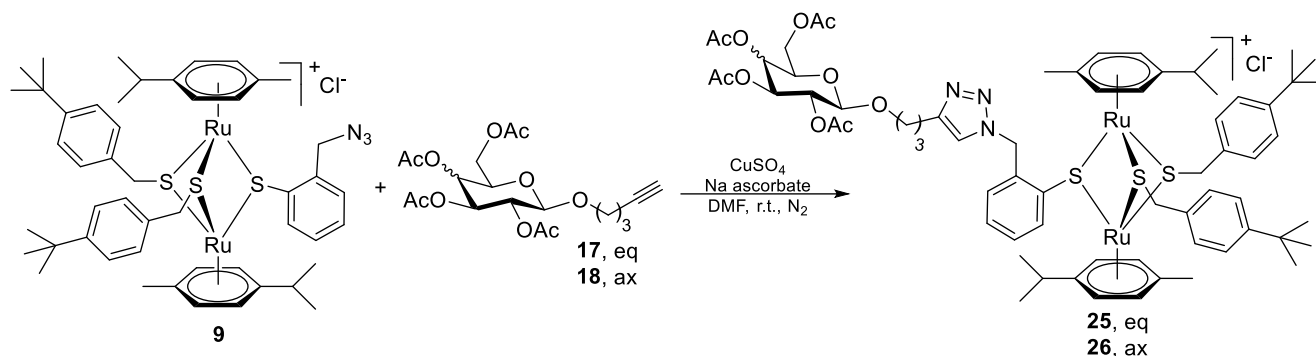


Scheme 7. Synthesis of the glucose **21**, and galactose **22** and **23** diruthenium conjugates.



Scheme 8. Synthesis of the diruthenium glucose conjugate **24**.

The trithiolato dinuclear intermediate **9**, with an azide in benzylic position on one of the bridge thiols, was reacted with the glucose and galactose alkyne derivatives **17** and **18** (Scheme 9), affording the carbohydrate conjugates **25** and **26**, isolated in moderate yields of 51 and 63%, respectively.



Scheme 9. Synthesis of the glucose and galactose diruthenium conjugates **25** and **26**.

All compounds were fully characterized by ^1H , ^{13}C nuclear magnetic resonance (NMR) spectroscopy, high resolution electrospray ionization mass spectrometry (HR ESI-MS) and elemental analysis (see the Experimental Section Chemistry in *Supporting Information* for full details). Mass spectrometry corroborated the spectroscopic data with the trithiolato diruthenium glucose and galactose conjugates **19-26** showing molecular ion peaks corresponding to $[\text{M}-\text{Cl}]^+$ ions.

For the assessment of the biological activity, the compounds were prepared as stock solutions in dimethylsulfoxide (DMSO). Similar to former reports[91,92,98], the ^1H NMR spectra of the functionalized diruthenium complexes **6**, **7**, **20**, **22**, **25** and **26** in $\text{DMSO}-d_6$, recorded at 25°C 5 min and more than 1 month after sample preparation showed no significant modifications (see Figure S1 in the *Supporting Information*), demonstrating a very good stability of the compounds in this highly complexing solvent.

2.2. *In vitro* antiparasitic activity

The biological activity of the carbohydrate azides and alkyne derivatives **14-16** and, respectively, **17** and **18** was not measured as these compounds were not isolated pure. Glucose and galactose conjugates **19-26**, glucose azide derivative **10** and diruthenium alkyne intermediate **6** were assessed for their *in vitro* biological activity in inhibiting proliferation of *T. gondii* β -gal, a transgenic strain that constitutively expresses β -galactosidase and for toxicity to HFFs (human foreskin fibroblast) used as host cells. The compounds were applied to infected or non-infected HFFs cultures for 72 h and at concentrations of 0.1 and 0.1 μM , the results being summarized in Table 1 and Figure 2. The trithiolato diruthenium complexes **2-5** and **9**, and alkyne intermediates **7** and **8** were evaluated previously against *T. gondii* β -gal under similar conditions[91,92,95,97], and the corresponding values are shown in Table 1 and Figure 2 for comparison. The viability of treated HFFs was measured by the alamarBlue metabolic assay, and the proliferation of *T. gondii* β -gal was quantified by the β -galactosidase colorimetric test. In

both cases, results are expressed as percentage (%) compared to control parasitic and host cells treated with 0.1% DMSO for which proliferation and viability were set to 100% (Table 1).

Table 1. Results of the primary efficacy/cytotoxicity screening of the azide derivative **10** and of trithiolato diruthenium compounds **2-9** and **19-26** in non-infected HFF cultures and *T. gondii* β -gal tachyzoites cultured in HFFs. 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. The compounds selected for determination of IC₅₀ values against *T. gondii* β -gal are tagged with *. For each assay, standard deviations were calculated from triplicates.

Compound	HFF viability (%)		<i>T. gondii</i> β -gal growth (%)	
	0.1 μ M	1 μ M	0.1 μ M	1 μ M
Diruthenium intermediates				
2^{a,*}	76 \pm 6	46 \pm 6	66 \pm 14	2 \pm 0
3^{a,*}	74 \pm 2	48 \pm 1	57 \pm 1	2 \pm 0
4^{a,*}	91 \pm 4	73 \pm 1	114 \pm 2	110 \pm 2
5^{a,*}	80 \pm 1	69 \pm 6	2 \pm 0	1 \pm 0
Alkyne and azide functionalized diruthenium compounds				
6*	101 \pm 1	100 \pm 1	4 \pm 0	0 \pm 0
7^{a,*}	101 \pm 0	96 \pm 0	21 \pm 2	0 \pm 0
8^{a,*}	71 \pm 2	46 \pm 6	52 \pm 13	3 \pm 1
9^a	96 \pm 1	64 \pm 1	9 \pm 1	1 \pm 1
azido glucose derivative				
10	98 \pm 1	99 \pm 0	101 \pm 1	99 \pm 0
Glucose and galactose diruthenium conjugates				
19*	102 \pm 1	97 \pm 1	9 \pm 1	0 \pm 0
20*	98 \pm 1	99 \pm 0	100 \pm 1	1 \pm 2
21*	100 \pm 1	101 \pm 0	87 \pm 1	1 \pm 0
22*	100 \pm 0	101 \pm 0	73 \pm 1	2 \pm 0
23*	99 \pm 1	101 \pm 1	85 \pm 1	1 \pm 0

24*	99 ± 1	98 ± 0	66 ± 2	3 ± 0
25	98 ± 1	97 ± 1	75 ± 2	27 ± 0
26*	98 ± 1	99 ± 1	89 ± 1	0 ± 0

*Compounds reported previously in ref[91,92,95,97].

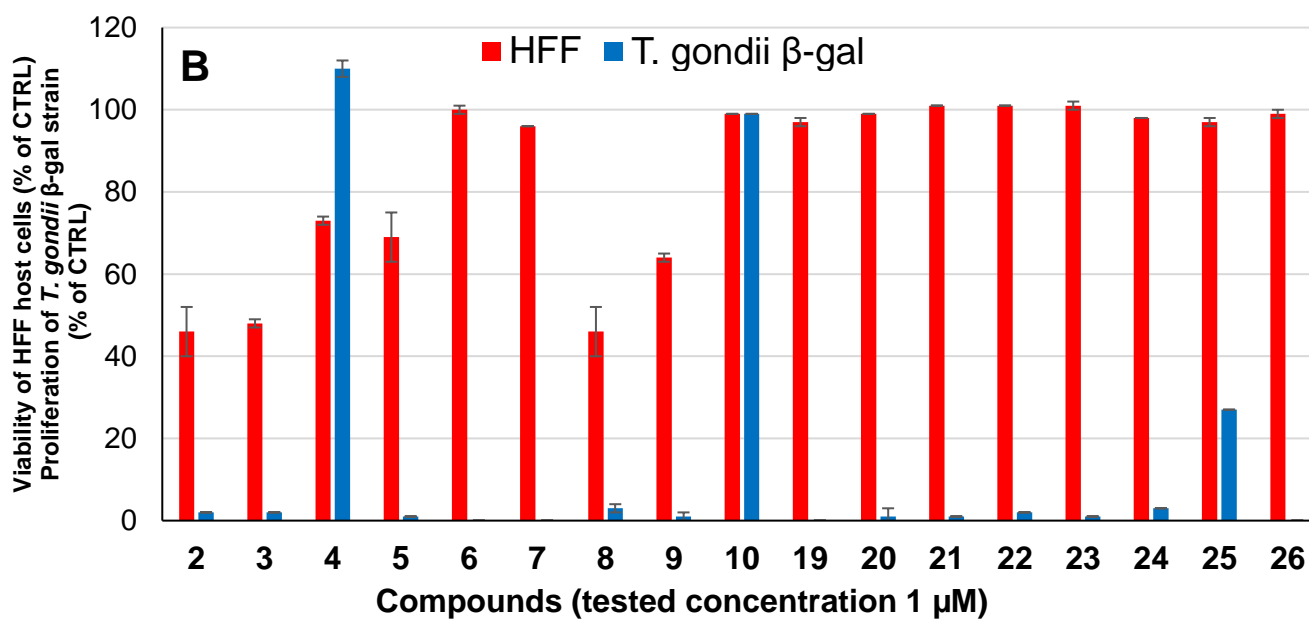
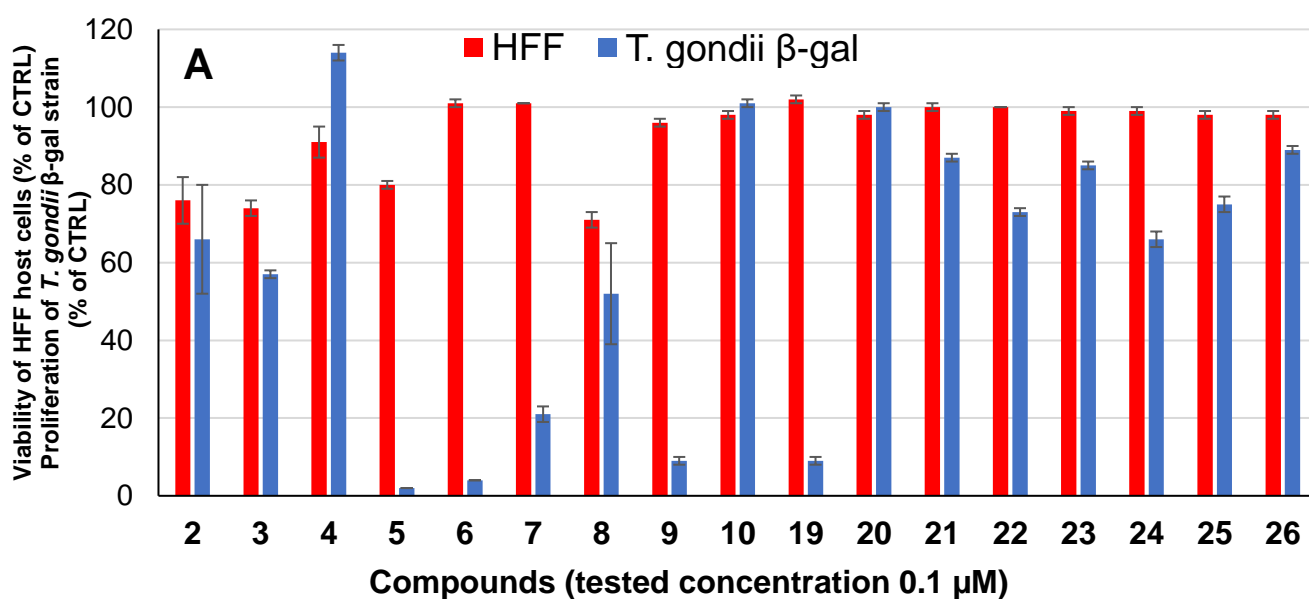


Figure 2. Clustered column chart showing the *in vitro* activities at 1 (A) and 0.1 (B) μM of the azide derivative **10** and of trithiolato diruthenium compounds **2-9** and **19-26** on HFF viability and *T. gondii* β -gal proliferation. For each assay, standard deviations were calculated from triplicates and are displayed on the graph. Data for compounds **2-5** and **7-9** were previously reported[91,92,95,97].

The biological activities of the hydroxy, amino and carboxy diruthenium compounds **2-4** were discussed elsewhere[92]. While **4** has limited effect on both HFF viability and parasite proliferation at the tested concentrations, **2** and **3** almost completely abolished parasite proliferation at 1 μM but are also toxic to the host cells at this concentration. Methylene hydroxy compound **5**[91] has a strong effect on the parasite proliferation even at 0.1 μM , while being less toxic to the host cells compared to phenol derivative **2**. Like their respective diruthenium hydroxy and amine intermediates **2** and **3**, both alkyne ester and amide derivatives **6** and **7** greatly impact *T. gondii* proliferation but affect significantly less the HFF viability. Related to carboxy analogue **4**, alkyne amide **8** shows a higher impact on parasite proliferation but also increased toxicity to the host cells. Diruthenium azide analogue **9** efficiently inhibits parasite proliferation even when applied at 0.1 μM but reduces HFF viability up to 64% when applied at 1 μM , while glucose azide derivative **10** exhibits neither antiparasitic activity nor toxicity at both tested concentrations.

In the first screening, none of the eight carbohydrate conjugates **19-26** impairs the host cells viability even at 1 μM . Apart from glucose conjugate **25**, all conjugates almost abolished the parasite proliferation when applied at 1 μM , but apart glucose conjugate **19**, all hybrid molecules had only a limited effect on *T. gondii* β -gal at 0.1 μM .

The relationship between the type of carbohydrate and/or the nature of the linker and the antiparasitic activity for the reported dyads follows no obvious trends, and prediction of efficacy related to the various chemical modifications is not straightforward. Some differences in anti-toxoplasma efficacy are observed when the conjugates are applied at 0.1 μM . For instance, glucose ester derivative **19** is significantly more active on *T. gondii* compared to the amide analogue **20**. Galactose functionalized compound **22** is more efficient in inhibiting the parasite proliferation compared to the corresponding glucose derivative **21**, while for the same carbohydrate an increase of the linker length has a negative effect on the antiparasitic activity (galactose conjugates **22** and **23**).

The compounds that simultaneously satisfied the following two criteria were selected for the evaluation of their IC_{50} values in *T. gondii* β -gal and the assessment of host cells toxicity after exposure to 2.5 μM : i) when the compound was applied at 1 μM , *T. gondii* β -gal growth was inhibited by 90% or more compared to control treated with 0.1% DMSO only, and ii) HFF host cell viability was not impaired by more than 50% for a compound applied at 1 μM . Based on the primary screening, seven glucose and galactose dyads **19-24** and **26** were selected. Pyrimethamine, currently used for the treatment of

toxoplasmosis, and which inhibited the proliferation of *T. gondii* β -gal tachyzoites with an IC₅₀ value of 0.326 μ M and did not affect HFF viability at 2.5 μ M (Table 2), was used as reference compound. The selection also included the diruthenium intermediate compounds **2-5** having free OH, NH₂ or CO₂H groups, along with two diruthenium alkyne ester and amide compounds **6** and **7**, and diruthenium azide **9**. The results are summarized in Table 2.

Table 2. Half-maximal inhibitory concentration (IC₅₀) values (μ M) on *T. gondii* β -gal for 15 selected compounds and pyrimethamine (used as standard), and their effect at 2.5 μ M on HFF viability.

Compound	IC ₅₀ <i>T. gondii</i> β -gal (μ M)	[LS; LI] ^b	SE ^c	HFF viability at 2.5 μ M (%) ^d	SD
Pyrimethamine^a	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
4^a	0.181	[1.482; 0.274]	0.954	99	2
5^a	0.038	[0.023; 0.060]	0.110	4	2
Alkyne and azide functionalized diruthenium compounds					
6	0.023	[0.030; 0.018]	0.503	7	1
7^a	0.038	[0.050; 0.029]	0.063	34	1
9^a	0.048	[0.058; 0.040]	0.139	11	1
Glucose and galactose diruthenium conjugates					
19	0.018	[0.031; 0.011]	0.387	29	3
20	0.011	[0.151; 0.080]	0.416	77	1
21	0.087	[0.135; 0.056]	0.274	0	0
22	0.298	[0.364; 0.244]	0.066	73	1
23	0.032	[0.044; 0.023]	0.278	92	2
24	0.328	[0.437; 0.247]	0.339	66	2
26	0.153	[0.178; 0.132]	0.476	97	2

^aData for pyrimethamine, and compounds **2-5**, **7** and **9** were previously reported[91,92,95,97]. **2-4** and **8** do not fulfil the first screening selection criteria, but the IC₅₀ values and viability of HFF at 2.5 μ M were determined for comparison purpose. ^bValues at 95% confidence interval (CI); LS is the upper limit of CI and LI is the lower limit of CI. ^cThe standard error of the regression (SE), stands for the average

distance that the observed values fall from the regression line. ^dControl HFF cells treated only with 0.25% DMSO exhibited 100% viability. ^eThe standard deviation of the mean (three replicate experiments).

The IC₅₀ values and the cytotoxicity of the diruthenium compounds **2-5**, **7** and **9** were measured previously.[91,92,95,97] IC₅₀ values ranged from 0.038 μ M (**5** and **7**) to 0.181 μ M (**4**), while the viability of HFFs was extremely variable, ranging from 4% (**5**) to 99% (**4**). The new alkyne ester **6** had the lowest IC₅₀ value (0.023 μ M) among all intermediates, but also strongly affected HFF viability when applied at 2.5 μ M.

Glucose conjugates **19** and **21** had very low IC₅₀ (0.018 and 0.087 μ M, respectively), but were toxic to host cells at 2.5 μ M (HFF viability of 29% for **19** and completely zero for **21**). Glucose hybrid **20** had the lowest IC₅₀ (0.011 μ M), and when applied at 2.5 μ M reduced host cells viability to encouraging 77%.

Galactose dyads **22** and **24** had only modest antiparasitic activity (IC₅₀ values of 0.294 and 0.328 μ M, respectively, comparable with those obtained for pyrimethamine) while being moderately toxic to HFF at 2.5 μ M (73 and 66%, significantly more cytotoxic compared to the standard pyrimethamine). Galactose conjugates **23** and **26** were the most promising of the series exhibiting not only high efficacy in inhibiting *T. gondii* β -gal proliferation (IC₅₀ values of 0.032 and 0.153 μ M, 10-fold and 2-fold lower compared to pyrimethamine, IC₅₀ = 0.326 μ M), but also low cytotoxicity on the host cells when applied at 2.5 μ M (HFF viability 92 and 97%, respectively).

It should be noted that galactose conjugate **23**, but also the glucose and galactose conjugates **20** and **21** affected the viability of HFFs less than the diruthenium intermediate alkyne **7** from which they were obtained by click reactions. A similar result was also obtained for the galactose conjugate **26** compared to the diruthenium intermediate azide **9**.

3. Material and Methods

3.1. Chemistry

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

3.2. Biological activity evaluation

3.2.1. Parasite Culture

All tissue culture media were purchased from Gibco-BRL, and biochemical agents from Sigma-Aldrich. Human foreskin fibroblasts (HFF) were obtained from the American Type Culture Collection (ATCC) and maintained in complete culture medium consisting in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Waltham, MA, USA) and antibiotics as previously described[118]. Transgenic *T. gondii* β -gal tachyzoites (expressing the β -galactosidase gene from *Escherichia coli*) from RH strain were kindly provided by Prof. David Sibley (Washington University, St. Louis, MO, USA) and were maintained by passages in HFFs cultures as previously described[118,119].

3.2.2. Preparation of the stock solutions

All compounds were prepared as 1 mM stock solutions from powder in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). For *in vitro* activity and cytotoxicity assays, HFFs were seeded at 5×10^3 /well in 96 well plates and allowed to grow to confluence in complete culture medium at 37°C and 5% CO₂. Transgenic *T. gondii* β -gal tachyzoites were freshly isolated from infected cultures as described[118], and 96-well plates containing monolayer HFFs were infected with 1×10^3 tachyzoites/well.

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

In a primary screening, each compound was evaluated at two concentrations 0.1 and 1 μ M and added to the media prior to the infection as previously described[98]. Control non-infected non treated HFFs cultures or *T. gondii* β -gal infected but not-treated cultures were cultivated in complete medium containing 0.01 or 0.1 % DMSO. 96-well plates were incubated for 72 hours at 37°C/5% CO₂ as previously described[98].

3.2.4. IC₅₀ Determination

For determination of IC₅₀ on *T. gondii* β -gal, eight serial concentrations ranging from 7 nM to 1 μ M were tested for each selected compound as previously described[92,95,96].

The β -galactosidase assay was performed exactly as previously reported[118]. Briefly, infected HFFs cultures in 96-well plates were lysed with PBS containing 0.05% Triton X-100. Then the substrate chlorophenolred- β -D-galactopyranoside (CPRG; Roche Diagnostics, Rotkreuz, Switzerland) was added in a final concentration of 0.5 mM. Absorption was measured at 570 nm wavelength using an EnSpire® multimode plate reader (PerkinElmer, Inc., Waltham, MA, USA).

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 previously reported[120]. Confluent HFF monolayers in 96-well plates were exposed to 0.1, 1 and 2.5 μM of each compound and incubated for 72 h at 37 °C/5% CO_2 . After this incubation step, the medium was removed, plates were washed once with PBS and 200 μL of resazurin (1:200 dilution in PBS) were added to each well. Plates were measured at excitation wavelength 530 nm and emission wavelength 590 nm using an EnSpire[®] multimode plate reader (PerkinElmer, Inc.). Fluorescence was measured at two different time points: T_0 as starting timepoint and T_{5h} as at 5 hours later. Relative fluorescence units were calculated from time points with linear increases.

4. Conclusions

This study was focused on the synthesis and *in vitro* anti-toxoplasma activity evaluation of eight new trithiolato-bridged arene-ruthenium(II) carbohydrate conjugates. Acetyl protected glucose and galactose moieties were pended on the diruthenium unit on one of the bridging thiols using CuAAC 'click' reactions and connectors of diverse types and lengths to obtain the carbohydrate-dyads. In the first screening, none of the conjugates affected the validity of host cells at 1 μM , suggesting reduced toxicity, and seven of the eight carbohydrate-diruthenium hybrids applied at 1 μM inhibited *T. gondii* β -gal growth by more than 90%. The second screening (IC_{50} values and toxicity to HFFs after exposure to 2.5 μM) led to the identification of two very promising acetyl protected galactose functionalized compounds **23** and **26**. Both conjugates not only highly exceeded (up to 10-fold) the anti-toxoplasma efficacy of the standard drug pyrimethamine for similar level of toxicity to HFF, but also had a significantly better balance antiparasitic activity/cytotoxicity compared to the corresponding non-carbohydrate tagged diruthenium complexes.

The type and length of the linker between the diruthenium core and the galactose unit also played a significant role in the biological activity, and finer structural adjustments will be needed to further improve the properties (more toxicity to *T. gondii*, less toxicity to healthy cells) of these carbohydrate conjugates.

In addition, the type of the carbohydrate and the presence of protecting groups is known to strongly influence the biological activity of other organometallic complex-carbohydrate conjugates[56,80,83]. Thus, the use of other carbohydrates than glucose and galactose, and the use of deprotected carbohydrates or carbohydrates carrying other protective groups can also be considered.

In conclusion, our study showed that conjugation of carbohydrates to diruthenium complexes is a valid strategy for obtaining novel ruthenium compounds with high antiparasitic efficacy and reduced cytotoxicity to host cells.

Supplementary Materials: The following supporting Information can be downloaded at . Synthetical procedures; Synthesis of the trithiolato-bridged dinuclear ruthenium(II)-arene intermediates **2-9**; Synthesis of the azide and alkyne functionalized carbohydrate intermediates **10-18**; Synthesis of the carbohydrate functionalized trithiolato-bridged dinuclear ruthenium(II)-arene complexes **19-26**. Figure S1. ¹H NMR Spectra of **6**, **7**, **20**, **22**, **25** and **26** recorded in DMSO-d₆ at 25°C as function of time.

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