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Characterization of the Activities of Dinuclear Thiolato-Bridged Arene Ruthenium Complexes against *Toxoplasma gondii*

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Abstract

The *in vitro* effects of 18 dinuclear-thiolato bridged arene ruthenium complexes, (1 mono-, 4 di- and 13-tri-thiolato compounds), originally designed as anti-cancer agents, were studied in the apicomplexan parasite *Toxoplasma gondii* grown in human foreskin fibroblast host cells (HFF). Some tri-thiolato compounds exhibited anti-parasitic efficacy at 250 nM and below. Among those, complex 1 and complex 2 inhibited *T. gondii* proliferation with IC_{50} values of 34 and 62 nM, respectively, and they did not affect HFF at dosages of 200 µM or above, resulting in selectivity indices of > 23'000. The IC_{50} values of complex 9 were 1.2 nM for *T. gondii* and above 5 µM for HFF. TEM detected ultrastructural alterations in the matrix of the parasite mitochondria at the early stages of treatment, followed by more pronounced destruction of tachyzoites. However, all three compounds applied at 250 nM for 15 days were not parasiticidal. By affinity chromatography using complex 9 coupled to epoxy-activated sepharose followed by mass spectrometry, *T. gondii* translation elongation factor-1 alpha and two ribosomal proteins, RPS18, and RPL27 were identified as potential binding proteins. In conclusion, organometallic ruthenium complexes exhibit promising activities against *Toxoplasma*, and potential mechanisms of action of these compounds as well as their prospective applications for the treatment of toxoplasmosis are discussed.

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43 **INTRODUCTION**

44 Organometallic compounds, especially platinum complexes, are widely applied as anti-cancer
45 chemotherapeutics (1). However, due to their drawbacks (i.e. severe side effects, insurgence
46 of tumor resistance, etc.), a variety of complexes of other transition metals such as copper,
47 gold or ruthenium have been investigated as potential alternative anti-cancer drug candidates
48 (2-10). Among the different metal complexes studied, arene ruthenium complexes showed
49 very promising anti-cancer properties with 50% inhibitory concentration (IC_{50}) values in the
50 low micromolar range, and certain selectivity for tumor cells over non-tumorigenic cells (11-
51 13). One such compound, namely RAPTA-C, is currently in pre-clinical evaluation (14).
52 Recently, some of us have shown that thiolato-bridged dinuclear arene ruthenium complexes,
53 in particular trithiolato dinuclear complexes of the type $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)_2\text{Ru}_2(\mu_2\text{-SR})_3]^+$ and
54 $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)_2\text{Ru}_2(\mu_2\text{-SR}^1)(\mu_2\text{-SR}^2)_2]^+$, were among the most cytotoxic ruthenium
55 complexes reported so far, with nanomolar IC_{50} values against both A2780 human ovarian
56 cancer cells and their cisplatin-resistant mutant variant A2780cisR (15-21). Interestingly, *in*
57 *vivo* studies on one of these compounds, namely $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)_2\text{Ru}_2(\mu_2\text{-SC}_6\text{H}_4\text{-}p\text{-Bu}^t)_3]^+$
58 (*diruthenium-1*) demonstrated a significant increase in survival of treated mice (22).

59 Arene ruthenium complexes also showed to be effective against bacteria (23), against
60 protozoan parasites including the two closely related apicomplexans *Neospora caninum* and
61 *Toxoplasma gondii*(24) and against helminths such as *Schistosoma mansoni* (25, 26) and
62 *Echinococcus multilocularis* (27). Interestingly, some ruthenium-clotrimazole (ctz) complexes
63 displayed high *in vitro* activity against *Leishmania major* and *Trypanosoma cruzi* and low
64 toxicity when assessed in normal mammalian cells (28). In addition to ruthenium, other
65 organometallic complexes have also been reported to display interesting anti-parasitic or/and
66 anti-infective activities (29-42). For instance, one manganese(I) tricarbonyl complex,
67 $[\text{Mn(CO)}_3(\text{bpy}^{\text{R,R}})(\text{ctz})]\text{PF}_6$,, showed submicromolar activity against *Staphylococcus aureus*

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68 and *S. epidermidis* with minimum inhibitory concentrations (MICs) of 0.625 μM. Moreover,
69 the related complex $[Mn(CO)_3(bpy^{R,R})(ktz)]PF_6$, (ktz = ketoconazole) was active against
70 *Trypanosoma brucei* with an IC_{50} value of 0.7 μM, while the IC_{50} value in mammalian cells
71 was more than 10 times higher (43).

72 Among the different above-mentioned pathogens, *T. gondii* is the most widespread parasite
73 worldwide, and infects approximately one third of the human population (44). In general, *T.*
74 *gondii* infestation remains without clinical symptoms in immune competent individuals, and
75 no treatment is required. However, *Toxoplasma* infection has been linked to neuropsychiatric
76 disease. Importantly, upon immunosuppression, or primary infection during pregnancy, *T.*
77 *gondii* can cause toxoplasmosis, a life-threatening disease affecting both humans but also
78 food and farm animals, which can lead to severe pathology including fetal malformation and
79 abortion. Current treatment options for toxoplasmosis include macrolide antibiotics and
80 sulfonamides (45), which inhibit protein biosynthesis and intermediary metabolism in the
81 apicoplast, a prokaryote-like organelle that is unique to apicomplexans (46). However, these
82 treatments are often characterized by adverse side effects, and do not eliminate the parasite,
83 thus do not act in a parasiticidal manner. It is therefore of high interest to investigate whether
84 dinuclear thiolato-bridged arene ruthenium complexes exhibit selective toxicity and
85 parasiticidal activity against *T. gondii*. Moreover, compounds with good efficacy against *T.*
86 *gondii* have good chances of being active against related apicomplexan parasites of high
87 medical and veterinary medical interest such as the coccidians *Cryptosporidium* and *Eimeria*,
88 and the closely related *Neospora caninum.*

89

90 **MATERIALS AND METHODS**

91 **Chemicals and synthesis of ruthenium complexes.** All reagents were commercially
92 available and were used as received. The complexes assessed in this study are shown in Fig.

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93 1. The symmetrical trithiolato complexes 1-7 were synthesized following a slightly modified
94 published protocol (17). The dinuclear complex $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)\text{Ru}_2(\mu\text{-Cl})\text{Cl}_2]$ was first
95 dissolved and heated in refluxing technical grade ethanol, and a solution of 6 equivalents of
96 the corresponding thiol SR in 5 mL technical grade ethanol EtOH was added dropwise (R =
97 4-$C_6H_4CH_3$: 1; 4-$C_6H_4Bu^t$: 2; 4-C_6H_4OH: 3; 3,4-$C_6H_3(OMe)_2$: 4; 4-mco, mco =
98 methylcoumarinyl: 5; 3-C_6H_4Cl: 6; 3-$C_6H_4NH_2$: 7). The resulting mixture was refluxed for 18
99 h. After cooling to room temperature, the solvent was removed under reduced pressure. The
100 oil obtained was purified by column chromatography on silica gel using a mixture of
101 dichloromethane and ethanol (5:1) as the eluent. The "mixed" trithiolato complexes 8-13 were
102 synthesized in two steps, as previously described (19, 47). First, the neutral dichlorido
103 dithiolato intermediates $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)_2\text{Ru}_2(\mu_2\text{-SCH}_2\text{-C}_6\text{H}_4\text{-R})_2\text{Cl}_2]$ are obtained from the
104 reaction of the *p*-cymene ruthenium dichloride dimer $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)\text{Ru}_2(\mu\text{-Cl})\text{Cl}_2]$ with 2
105 equivalents of the respective thiol SCH_2R (R = C_6H_5: 8; R = 4-$C_6H_4CH_3$: 9; R = 4-C_6H_4OMe:
106 10; R = 4-C_6H_4F: 11; R = 4-C_6H_4Cl: 12; R = 4-C_6H_4Br: 13 in ethanol at 0 °C, according to the
107 published method (49). These intermediates react in refluxing ethanol during 15 h with 6
108 equivalents of 4-Mercaptophenol 4-HS-C_6H_4-OH to give the corresponding mixed trithiolato
109 complexes $[(\eta^6\text{-}p\text{-MeC}_6\text{H}_4\text{Pr}^i)_2\text{Ru}_2(\mu_2\text{-S-C}_6\text{H}_4\text{-OH})(\mu_2\text{-SR})_2]^+$ 8-13. The dithiolato complexes
110 14-17 and the monothiolato complex 18 were synthesized according to published methods
111 (48, 49). The resulting complexes 1 – 18 (Fig. 1,) which were isolated as chloride or
112 tetrafluoroborate salts are air-stable, orange to red solids and were dried in vacuum. The
113 analytical data matched those previously reported in the literature (15, 17, 47, 48).

114 **Host cell cultivation and parasite cultures**. If not stated otherwise, all tissue culture media
115 were purchased from Gibco-BRL (Zurich, Switzerland), and biochemical reagents were from
116 Sigma (St. Louis, MO). Human foreskin fibroblasts (HFF) and Vero cells (green monkey
117 kidney epithelial cells) were maintained in RPMI-medium containing 10% fetal calf serum
118 (FCS) (Gibco-BRL, Zürich, Switzerland) and antibiotics as described earlier (24). *T. gondii*

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119 beta-gal (transgenic *T. gondii* RH expressing the beta-galactosidase gene from *E. coli* (50))
120 were maintained in Vero cells, and were isolated and separated from their host cells as
121 described (24).

122 ***In vitro* assessment of drug efficacy.** To study the effects of compounds against *T. gondii*
123 tachyzoites *in vitro*, 0.5 mM stock solutions of complexes were prepared in water, sterile
124 filtered, and stored at 4 °C.

125 For assessment of drug efficacy against *T. gondii* tachyzoites, parasites were isolated (24) and
126 assays were performed using HFF as host cells (24). In short, 5 x 10^3 HFF cells / well) were
127 grown to confluence in a 96 well plate in phenol-red free culture medium at 37 °C with 5%
128 CO_2. Cultures were infected with freshly isolated *T. gondii* beta-gal tachyzoites beta-gal
129 tachyzoites (1 x 10^3 / well) and drugs were added at the time point of infection. Initial
130 assessments of drug efficacy were done by exposing parasite cultures to 2500 nM, 250 nM,
131 25 nM or 2.5 nM of each compound for a period of three days, or water was added as a
132 control. For IC_{50} determinations, 6 selected complexes (1-5, and 9) were added at
133 concentrations ranging between 0 and 2000 nM. After three days at 37 °C/ 5% CO_2, plates
134 were centrifuged at 500 g, medium was removed, and cell cultures were lysed in PBS
135 containing 0.05% Triton-X-100. After addition of 10 µL of 5 mM chlorophenol red-β-D-
136 galactopyranoside (CPRG; Roche Diagnostics, Rotkreuz, Switzerland) dissolved in PBS, the
137 absorption shift was measured at 570 nm wavelength at various time points on a VersaMax
138 multiplate reader (Bucher Biotec, Basel, Switzerland). The activity, measured as the release of
139 chlorophenol red over time, was proportional to the number of live parasites down to 50 per
140 well as determined in pilot assays. IC_{50} values were calculated after the logit-log-
141 transformation of relative growth and subsequent regression analysis by the corresponding
142 software tool contained in the Excel software package (Microsoft, Seattle, WA).

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143 In one time course experiment, 9 (100 nM) was added to HFF monolayers either 10 min prior
144 to infection or 1 h, 5 h or 24 h post-infection with *T. gondii* tachyzoites. The proliferation of
145 tachyzoites was measured after 2 days of culture as described above.

146 For long term treatment assays, *T. gondii* infected HFF grown in T25 culture flasks were
147 exposed to 250 nM of 1, 2 or 9 for a period of 15 days, after which the cultures were washed
148 with medium and were further maintained in medium devoid of drugs. Regrowth of parasites
149 was monitored on a daily basis by light microscopy

150 Cytotoxicity assays on non-infected confluent HFF were performed also in 96 well plates by
151 exposing HFF to a concentration range of 2.5 nM, 25 nM, 250 nM and 2.5 µM of each
152 compound, and assessment of the viability by AlamarBlue assay as described (51).

153 **Transmission electron microscopy (TEM).** HFF (5 x 10^4 per inoculum) cultured in T25
154 tissue culture flasks for 24 h were infected with 10^5 *T. gondii* beta-gal tachyzoites, and 200
155 nM of 1, 2 or 9 were added at 24 h post-infection. After 6, 24 or 48 h, cells were harvested
156 using a cell scraper, and they were placed into the primary fixation solution (2.5 %
157 glutaraldehyde in 100 mM sodium cacodylate buffer pH 7.3) for 2 h. Specimens were then
158 washed 2 times in cacodylate buffer and were post-fixed in 2% OsO4 in cacodylate buffer for
159 2 h, followed by washing in water, pre-staining in saturated uranyle acetate solution, and step
160 wise dehydration in ethanol. They were then embedded in Epon 812-resin, and processed for
161 TEM as described (24). Specimens were viewed on a Phillips 400 transmission electron
162 microscope operating at 80 kV.

163 **Coupling of compound 9 to epoxy-activated sepharose, affinity chromatography and**
164 **identification of a drug-binding protein by liquid chromatography tandem mass**
165 **spectrometry (LC–MS/MS) analysis.** To prepare a complex-9-sepharose matrix, 20 mg of
166 complex 9 were added to 0.5 mg of epoxy-sepharose suspended in 2 mL of coupling buffer
167 ($NaCO_3$ 0.1 M, pH 9.5) followed by an incubation for two days at 37 °C on a shaker.

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168 Furthermore, a mock epoxy-sepharose column was prepared by treatment with coupling
169 buffer without complex 9 and blocking with ethanolamine. Prior to the runs, both columns
170 were combined in a tandem (mock column first, then complex-9-column) and washed with 25
171 mL of PBS equilibrated at 20 °C.

172 To identify potential binding proteins both from *T. gondii* and from the host cell, three T75-
173 flasks containing HFF monolayers were infected with 2 x 10^7 *T. gondii* tachyzoites and
174 incubated for 3-4 days. Then, cells were harvested by scraping and pelleted (1,000 g, 10 min,
175 4◦C). For protein extraction, frozen pellets were resuspended in 1 ml ice cold PBS containing
176 1% Triton-X-100 and 1 mM phenyl-methyl-sulfonyl-fluoride. Suspensions were vortexed
177 thoroughly, and centrifuged (15,200 x g), 10 min, 4◦C). Extraction of pellets was repeated
178 twice. Supernatants were combined (5–10 mg of total protein) and subjected to affinity
179 chromatography by loading onto the column tandem at a flow rate of 0.25 mL/min. The
180 columns were washed with PBS until a flat baseline was detected (ca. 20 mL PBS). The
181 columns were separated, and proteins binding to the columns were eluted with a pH shift
182 (glycine Cl^- 100 mM, pH 2.9). Fractions (3 mL) were taken before, during and after elution
183 and precipitated overnight with 80% acetone at –20 °C. The precipitates were solubilized in
184 30 µL of Laemmli buffer and were separated by 10% sodium dodecyl sulphate
185 polyacrylamide gelelectrophoresis (SDS-PAGE) using a Hoefer Minigel 250 Apparatus (GE
186 Healthcare, Little Chalfont, UK). Proteins were visualized by silver staining.

187 For mass spectrometry analysis, colloidal Coomassie staining was applied and selected
188 protein bands were cut out with a clean scalpel, placed into Eppendorf tubes containing
189 ethanol/distilled water (1:4) and were stored at 4 °C. In-gel digestion/liquid chromatography
190 tandem mass spectrometry (LC–MS/MS) analysis was performed by the Mass Spectrometry
191 and Proteomics Facility at the Department of Clinical Research of the University of Bern
192 (Bern, Switzerland). The sequences obtained were blasted against the UniProt database
193 (www.uniprot.org).

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195 **RESULTS**

196 ***In vitro* efficacy of Ru(II) complexes.** The tri-thiolato complexes 1-5 and the mixed complex
197 9 inhibited the proliferation of *T. gondii* with IC_{50} values of approximatively 500 nM or below
198 (Table 1). The tri-thiolato complex 7 and the mixed complexes 8 and 10-13 had no
199 measurable anti-parasitic activity or were toxic for host cells already at concentrations of 250
200 nM or 2500 nM. The same was true for the di-thiolato complexes 14-17 and the mono-
201 thiolato complex 18. The activity of the complexes against *T. gondii* parallels to a certain
202 extent the results previously found against several cancer cell lines: the IC_{50} values of 7 were
203 two orders of magnitude larger than that of the other complexes (20), and the mono- and
204 dithiolato complexes were found to be only moderately cytotoxic in vitro against cancer cell
205 lines (IC_{50} values between 0.2 and 2.5 μM) (48, 49).

206 Complexes 1, 2 and 9 appeared as the most active with IC_{50} values of 34, 62 and 1.2 nM,
207 respectively (see Table 1). Accordingly, host cell toxicity was investigated for these three
208 complexes. In the presence of 1, HFF vitality was decreased to 63% of the control value at a
209 concentration of 250μM, which was the highest concentration used in these assays. Thus, an
210 extrapolated, but purely theoretical, IC_{50} value of 800 μM was calculated for 1, since the
211 solubility limit in water-based solutions is around 500 μM. 2 did not affect vitality of HFF up
212 to a concentration of 250 μM. 9, exhibiting the by far lowest IC_{50} values, had an IC_{50} for HFF
213 of approximately 5 μM. Thus, all three complexes affected *T. gondii* tachyzoites at low
214 nanomolar concentrations, and these effects were parasite-specific, with a high selective
215 toxicity index: > 23,000 for 1, > 16,000 for 2, and > 5,000 for 9. Interestingly, a long-term
216 treatment with compound 9 at 250 nM 9 over a period of up to 15 days did not eliminate all
217 parasites, since regrowth of tachyzoites was observed 5-10 days after releasing drug pressure

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218 for all three compounds. This indicates that these compounds acted in a parasitostatic rather
219 than parasiticidal manner.

220 **Ultrastructural changes induced by Ru(II) complexes show that one of the primary**
221 **target organelles in *T. gondii* tachyzoites is the mitochondrion.** To obtain more detailed
222 information on the subcellular effects of these 3 thiolato-bridged dinuclear arene ruthenium
223 complexes, TEM was performed on drug-treated HFF infected with *T. gondii* (Fig. 2, 3). Non-
224 treated parasites, exemplified in Fig. 2 were located intracellularly and were undergoing
225 proliferation by endodyogeny within a parasitophorous vacuole (PV), surrounded by a distinct
226 PV membrane. These parasites exhibit the typical apicomplexan structural features, including
227 rhoptries, dense granules, micronemes, and a conoid at the anterior part. The parasite
228 mitochondrium, filled with a structured electron dense matrix, could be readily identified in
229 these non-treated parasites (Fig. 2C). In cultures exposed to 1, alterations within the
230 mitochondria of *T. gondii* were evident already after 6 h of treatment, showing a progressive
231 degeneration of the electron-dense intra-mitochondrial matrix (Fig. 3B-C). The interior
232 ultrastructural organization of these mitochondria was largely distorted and only membranous
233 residues were present in some cases. However, the outer membrane of the mitochondria was
234 still intact, and parasites maintained their overall shape. After 48 h of treatment with 1, *T.*
235 *gondii* tachyzoites had lost their characteristic shape, and parasites displayed a largely
236 distorted morphology, no internal organelles were recognizable anymore, and the PV and its
237 membrane were essentially lost. However, host cell mitochondria exhibited a normal
238 morphology with clearly discernable cristae (Fig. 3D). Similar results were obtained in *T.*
239 *gondii* infected cultures treated with 2 (data not shown). For treatments with 9, mitochondrial
240 changes were not noted in *T. gondii* tachzyoites already after 6 h of treatment (data not
241 shown), but similar alterations as observed during treatments with 1 became evident after 24-
242 48 h of 9 exposure (Fig. 3E, F). However, intact parasites could also be observed in cultures
243 treated with all three complexes. Overall, this suggested that these three ruthenium complexes

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244 induced largely similar ultrastructural changes by inducing distinct alterations in the
245 mitochondria, and could thus act with (a) similar or identical mechanism(s) of action.

246 **Complex 9 affects extracellular parasites and interferes in adhesion, invasion or**
247 **intracellular establishment, but does not act efficiently against *T. gondii* proliferation**
248 **once parasites reside inside the host cell.** Since long-term treatment studies as well as TEM
249 suggested that these ruthenium complexes did not act parasiticidal, we wanted to determine
250 whether these compounds affected host cell invasion, intracellular proliferation, or both. For
251 this, HFF monolayers were infected with *T. gondii* tachyzoites, and 9 (100 µM) was added
252 either concomitantly with the infection, or after 1 h, 5 h or 24 h post infection (Fig. 4). 9
253 efficiently inhibited tachyzoite proliferation when added at the time point of infection and
254 also when applied at 5 h post-infection, but only partially when added 24 h post-infection.
255 Thus 9 acted mainly during first steps of the infection process (e.g. host cell invasion and
256 intracellular establishment), and only with limited efficacy once parasites resided inside the
257 host cell.

258 **Complex 9 interacts with ribosomal proteins from *T. gondii* and from the host cell.** By
259 affinity chromatography on complex-9-epoxy-sepharose, two major bands of approximately
260 50 kDa and 20 kDa were identified that were not present in the eluate of the mock column
261 (Fig. 5 A). Mass spectrometry analysis identified ribosomal proteins of host and parasite
262 origin as major components of the 20-kDa-band (Table 1). The composition of the 50 kDa
263 band was more heterogeneous. As quantified both via protein match score summation and via
264 protein score – the major component of the 50 kDa-band was *T. gondii* elongation factor 1-
265 alpha (TgEF1-alpha; Table 1) with a unique peptide coverage of nearly 50% of the sequence
266 (Fig. 5B). The second most abundant protein was its human homologue. Moreover, other
267 proteins of human origin were identified in this fraction (Table 1).

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269 **DISCUSSION**

270 We here report on a series of 18 dinuclear thiophenolato-bridged arene ruthenium complexes,
271 which exhibit highly promising *in vitro* activities against *T. gondii* tachyzoites. The
272 organometallic complexes studied in this work have been previously described (15, 17, 47,
273 48). Very importantly, recent studies by some of us have shown that these dinuclear arene
274 ruthenium complexes are inert to ligand substitutions and remain stable for long period in
275 water solutions or in organic solvents like DMSO (16, 21). These ruthenium complexes had
276 been originally generated for the treatment of cancer cells. Cancer cells and protozoan
277 parasites, including *Toxoplasma*, share several features: they both live and multiply in a host
278 organism and do not immediately kill their hosts, they have a potentially infinite proliferative
279 capacity, and escape in immune-compromised tissues. Cancer cells are largely resistant to
280 apoptosis, while *Toxoplasma* and *Neospora* are known to interfere in the programmed cell
281 death machinery of their host cell (52). Thus, we hypothesize that a potentially lucrative
282 starting point for the discovery of novel drug candidates against *T. gondii* and other
283 protozoans is to examine compounds that are being developed against cancer.

284 Among the 18 compounds studied, the trithiolato complexes 1, 2 and 9 were highly
285 efficacious against both parasites with IC_{50}s ranging between 1.2 and 62 nM. In addition,
286 these compounds exhibited a highly favorable selective toxicity index of up to 23'000. TEM
287 demonstrated that one of the first organelles that exhibited ultrastructural alterations upon
288 treatment with these compounds was the tachyzoite mitochondrion, which lost its interior
289 membranous matrix and cristae already after 6-24 h. More severe distortion, including a
290 complete breakdown of other organelles within the parasite cytoplasm and a general
291 disintegration of the tachzoites and the parasitophorous vacuole and its membrane, was
292 observed after 48 h.

293 In comparison to other drugs, the *in vitro* results on complexes 1, 2 and 9 are encouraging.
294 Pyrimethamine, sulfadiazine and atovaquone, compounds currently clinically used against

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295 toxoplasmosis, inhibited *T. gondii* beta-gal with IC_{50} values of 1 mM, 80 µM and 19-50 nM,
296 respectively (50). The calcium dependent protein kinase inhibitor BKI-1294, highly active
297 against *T. gondii* and *N. caninum* infections in mice, inhibited *T. gondii* and *N. caninum* beta-
298 gal proliferation under identical conditions with an IC_{50} of 137 and 40 nM, respectively (53).
299 Two previously identified organometallic ruthenium complexes exhibited IC_{50} values of 18
300 and 41 nM (24) however, with selective toxicity indeces below 100. As can be noticed from
301 the calculated Log P values (Table 1) and as previously observed against cancer cells (17), the
302 efficacy of inhibition is, to some extent, correlated to the lipophilicity of the complexes.
303 Unlike against A2780 and A2780cisR cancer cells, the most lipophilic complex, 2, is not the
304 most potent one against *T. gondii*, possibly suggesting that the different chemical nature of the
305 cell and *T. gondii* outer membranes could influence the uptake of dinuclear thiolato-bridged
306 arene ruthenium complexes.

307 While 1, 2 and 9 were highly efficacious against *T. gondii* and exhibited an excellent selective
308 toxicity, we obtained evidence that these compounds did not act in a parasiticidal manner.
309 Removal of the drugs after continuous treatments at 250 nM lasting up to 15 days did not
310 result in complete elimination of viable tachyzoites, and re-growth of parasites was observed
311 within 5-10 days after releasing the drug pressure. This was confirmed by TEM, where a
312 small number of largely intact tachyzoites were still found after 48 h of continuous *in vitro*
313 treatment. Similar results were previously reported for dicationic arylimidamides (54) and
314 ruthenium phosphite complexes in *T. gondii* (24), and for buparvaquone, the BKI-1294 as
315 well as for artemisinin derivatives in the closely related *N. caninum* (55-57). In some of these
316 reports, rapid adaptation of *T. gondii* and *N. caninum* tachyzoites to increased concentrations
317 of drugs within a few days was documented (54, 56). This outstanding adaptive ability
318 represents a major obstacle for the development of efficacious drugs against these parasites.
319 Nevertheless, the lack of parasiticidal activity *in vitro* still allows for excellent *in vivo*

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320 efficacy, as documented for the BKI-1294 in pregnant murine infection models for *N.*
321 *caninum* (55, 57).

322 All three compounds had a profound impact on the ultrastructure of the parasite mitochondria,
323 which lost their characteristic electron dense matrix and cristae upon 6-24 h after initiation of
324 drug treatments. After 48 h, this impacted on the entire tachyzoites, leading in most cases to
325 severe alterations and death. Of note, mitochondria are also targeted by other drugs currently
326 used against apicomplexans, such as atovaquone, buparvaquone and decoquinate, which have
327 been shown to impair cytochrome b/c1 complex in *Toxoplasma, Plasmodium* and *Theileria*
328 parasites (58-61).

329 The mitochondrion represents an attractive drug target. The disruption of mitochondria has
330 been recently investigated as a potential novel chemotherapeutic mechanism for cancer
331 treatment, because it circumvents upstream apoptotic pathways that may be mutated or
332 lacking in cancer cells (62). Moreover, cancer cells have higher mitochondrial membrane
333 potentials, rendering them more susceptible to mitochondrial perturbations than non-
334 immortalized cells (63). On the basis of these factors, numerous mitochondria-targeting
335 agents have been developed in order to disrupt the mitochondrial membrane potential and to
336 further permeabilize the mitochondrial outer membrane. Some ruthenium(II) complexes can
337 induce mitochondria–mediated apoptosis in cancer cells (64-67). However, while in
338 mammalian cells the mitochondrion represents the main ATP-generating organelle that allows
339 complete oxidation of carbohydrates, lipids and amino acids via the tricarboxylic acid (TCA)
340 cycle and the electron transport chain, the situation in apicomplexans appears slightly
341 different. Apicomplexans have a single tubular mitochondrial network that also hosts part of
342 the heme biosynthesis, iron-sulfur cluster assembly, and lipoic acid salvage, and the
343 mitochondrion participates in the synthesis of many metabolic intermediates including
344 pyrimidines (68)

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345 How exactly the mitochondrion is targeted by our ruthenium complexes is not known.
346 Affinity chromatography using extracts from *T. gondii*-infected HFF lead to the identification
347 of TgEF1-alpha as well as its human homologue as major complex-3-binding partners. This is
348 not surprising since EF1-alpha is expressed in all eukaryotic cells and is highly conserved
349 (69). In eukaryotic cells, EF1-alpha promotes the GTP-dependent transfer of aminoacylated
350 tRNA to the ribosome A site, hence represents an essential component of protein synthesis. In
351 addition, other activities have been attributed to EF1-alpha in different eukaryotes, which are
352 associated with vital cellular functions such as cell growth, motility, protein metabolism,
353 signal transduction, DNA replication/repair protein networks and apoptosis (70-72). In
354 *Trypanosoma brucei* and *T. gondii,* EF1-alpha mediates the specificity of mitochondrial t-
355 RNA import (73, 74) and disruption of this process could lead to the observed mitochondrial
356 alterations.

357 In another apicomplexan parasite, *Cryptosporidium parvum*, CpEF1-alpha was shown to
358 localize to the apical region of *C. parvum* sporozoites, and antibodies directed against
359 CpTEF1-apha inhibited host cell invasion (75). The same was shown for *T. gondii* (76) Our
360 study also showed that complex 9 had a profound efficacy when applied at the early stages of
361 host cell infection, namely either during, or 1-5 h after, exposure of *T. gondii* tachyzoites to
362 host cells, but more limited efficacy was noted when added 24 h after infection. This would
363 be consistent with a mode of action that is relevant for invasion or early host cell
364 establishment. In addition, vaccination of mice with recombinant TgEF1-1alpha and a DNA
365 vaccine coding for TgEF1-alpha lead to significantly prolonged survival times in *T. gondii*
366 infected mice (76, 77) underlining the importance of TgEF1-alpha for the infection process.

367 As outlined in Table 1, other ribosomal proteins both of host and parasite origin, and various
368 other host proteins, were found to bind to 9 as well. This may explain the low, but still
369 detectable, host cell toxicity of 9.

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In conclusion, we have identified three promising dinuclear thiolato-bridged arene ruthenium complexes with promising and highly specific anti-parasitic activity, as assessed against *T. gondii*. These complexes induce severe mitochondrial alterations within 6-24 h of drug treatment, efficiently inhibit proliferation, but do not act in a parasiticical manner. One of these complexes, compound 9, interacts with TgTEF1-alpha and other parasite and host ribosomal proteins. Further studies will focus on the interactions of 9 and other promising ruthenium complexes with putative apicomplexan drug targets, and on the use of these drugs *in vivo*.

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Figure legends

FIG. 1. Structures of complexes 1-18 used in this study. Note that compounds 1, 2 and 9 were further characterized.

FIG. 2. Ultrastructure of *T. gondii* tachyzoites grown in HFF. A is a low magnification view of infected HFF, the boxed area is shown at a higher magnification in B. Tachyzoites proliferate within a parasitiophorous vacuole, surrounded by a parasitophorous vacuole membrane. Nuc = nucleus, dg = dense granules, mic = micronemes, rop = rhoptries, mito = mitochondrion. The boxed area in B shows the mitochondrial matrix and is enlarged in C. Bar in A = 1.8 µm, B = 0.3 µm, C = 0.1 µm

FIG. 3. Ultrastructure of *T. gondii* tachyzoites grown in HFF and treated with ruthernium compounds 1 and 9. Treatments were carried out using 200 nM of compounds 1 (A-D) or 9 (E, F). A is a low magnification view of parasites treated with compound 1for 6 h, the boxed areas are enlarged in B and C. D shows parasites exposed to compound 1 for 48 h. E and F show parasites exposed to compound 9 during 24 h. Note the distinct alterations in the mitochondria (mito) in B, C and E, and the still intact host cell mitochondria (h-mito) in D¨. The boxed area in F is enlarged in E. Bar in A, F = 1 µm, B, C, E = 0.4 µm, D = 0.8 µm,

FIG. 4. Compound 9 inhibits *T. gondii* tachyzoite proliferation only when applied early during infection. HFF monolayers grown in 96 well plates were treated with compound 9 (100 nM) either 10 min prior to infection or 1 h, 5 h or 24 h post-infection with *T. gondii* tachyzoites. The proliferation of tachyzoites was measured after 2 days of culture by beta-galactosidase assay as described in materials and methods

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690 **FIG. 5.** Identification of complex 9-binding proteins. A, SDS-PAGE and silver staining of
691 tandem (mock- and compound 9-sepharose) affinity chromatography of a protein extract
692 prepared from *T. gondii* infected HFF. Soluble extract and non-binding fraction (flow-
693 through) are shown on the left, followed by wash and eluate fractions of the mock columns
694 and the complex 9-sepharose column. The two arrows point to the two bands of 50 kda and
695 20 kDa, which were cut out and analyzed by LC-MS. B shows the amino acid sequence of the
696 50 kDa band identified as TgEF1-alpha, the peptide sequences identified by LC-MS are
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Tables

Table 1. The efficacies of dinuclear thiolate-bridged arene ruthenium complexes against *T. gondii* beta-galactosidase expressing tachyzoites, host cell (HFF) cytotoxicity, and respective physicochemical data. Chloride salts of the corresponding thiols of the complexes [1-6] were used for all experiments. For the determination of efficacies, confluent HFF monolayers grown in a 96-well plate were treated with the complexes at various concentrations, and were infected with *T. gondii* beta-gal tachyzoites (10^3 per well). After 3 days, beta-galactosidase activity or host cell viability were determined, IC_{50} values were calculated as described, and are presented with 95% confidence intervals. The LogP values correspond to the values that were calculated for the thiols RSH groups [ref17]. nd = not done

Complex	*T. gondii* beta-gal IC_{50} (nM)	HFF IC_{50} (µM)	LogP *(RSH)*
1	34 ± 4	800	2.98 ± 0.28
2	62 ± 10	>1000	4.21 ± 0.29
3	130 ± 20	nd	2.38 ± 0.32
4	120 ± 20	nd	2.83 ± 0.42
5	540 ± 60	nd	1.68 ± 0.29
9	1.2 ± 0.5	5‘129	nd

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717 Table 2. Results of mass spectrometry analysis of the two major bands shown in Fig. 5. AC, UniProt accession number; ID, UniProt identifier;

718 PMSS, protein match score summation.

Band	AC	ID	PMSS	Protein Score	unique peptides	Coverage (%)	Protein Mass (Da)	Description
20 kDa	P46783	RS10_HUMAN	79	147	5	32.7	18898	40S ribosomal protein S10
	P30050	RL12_HUMAN	72	161	5	43.0	17819	60S ribosomal protein L12
	S8FA78	S8FA78_TOXGO	46	86	3	25.5	17821	Ribosomal protein RPL12
	V4YUP9	V4YUP9_TOXGO	30	69	3	21.8	16331	Ribosomal protein RPL27
	P62269	RS18_HUMAN	29	67	3	19.1	17719	40S ribosomal protein S18
	S8EUB1	S8EUB1_TOXGO	26	61	3	21.8	17723	Ribosomal protein RPS18
	P61254	RL26_HUMAN	25	55	3	16.6	17258	60S ribosomal protein L26
	P62851	RS25_HUMAN	24	35	2	13.6	13742	40S ribosomal protein S25
	Q5SGD8	Q5SGD8_TOXGO	18	37	2	11.1	19983	Tgd057
50 kDa	S8GV85	S8GV85_TOXGO	323	518	17	47.5	49006	Elongation factor 1-alpha
	P68104	EF1A1_HUMAN	143	196	8	22.9	50141	Elongation factor 1-alpha 1
	O14773-2	TPP1_HUMAN	71	120	5	27.2	34464	Isoform 2 of Tripeptidyl-peptidase 1
	P63261	ACTG_HUMAN	36	77	4	14.7	41793	Actin, cytoplasmic 2
	P06576	ATPB_HUMAN	32	61	3	8.3	56560	ATP synthase subunit beta, mitochondrial
	P22234	PUR6_HUMAN	26	47	2	6.1	47079	Multifunctional protein ADE2
	P16989-2	YBOX3_HUMAN	22	42	2	8.3	31947	Isoform 2 of Y-box-binding protein 3
	O75821	EIF3G_HUMAN	18	32	2	5.0	35611	Eukaryotic translation initiation factor 3 subunit G
	Q9Y6N5	SQRD_HUMAN	17	31	2	6.2	49961	Sulfide:quinone oxidoreductase, mitochondrial

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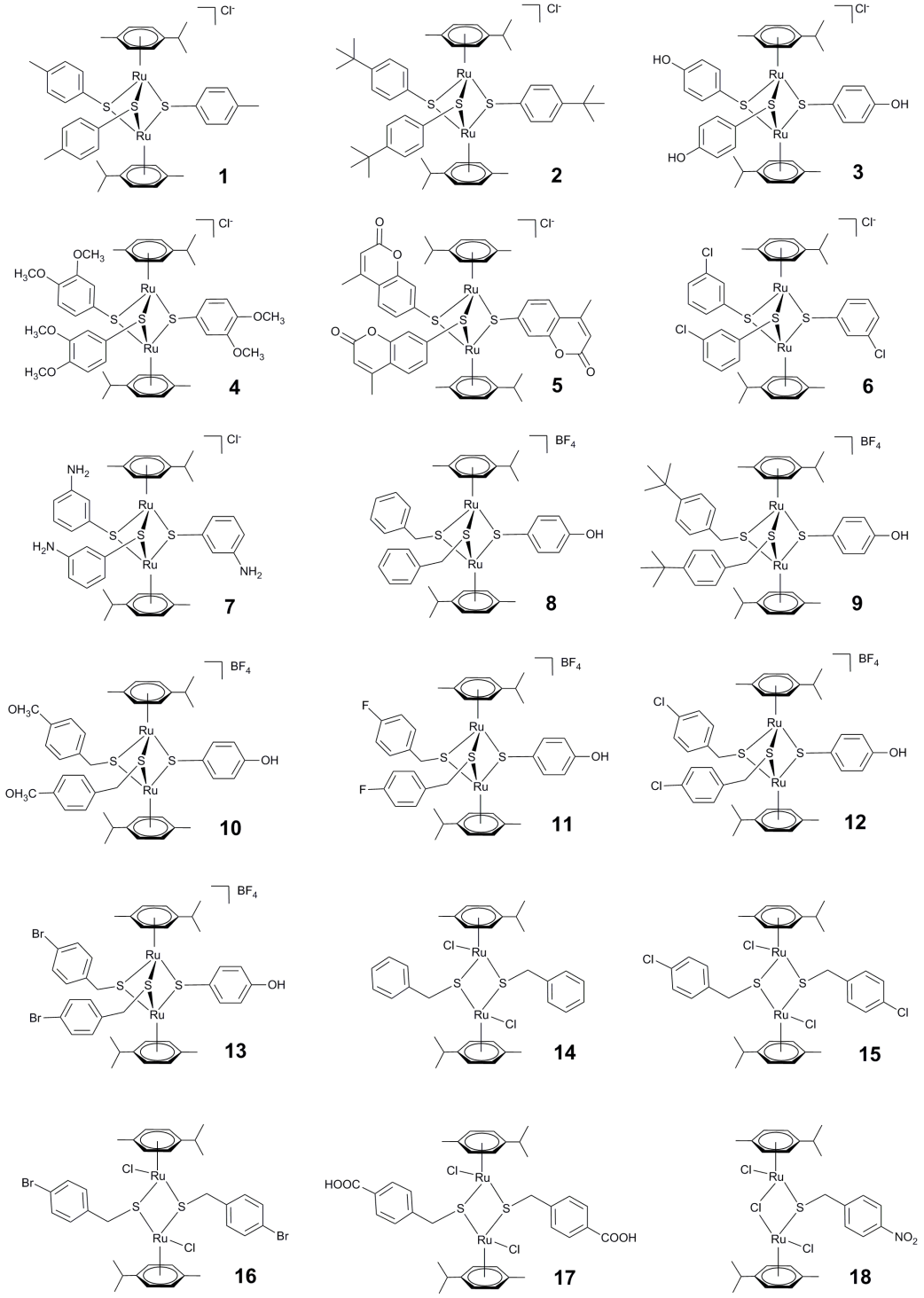

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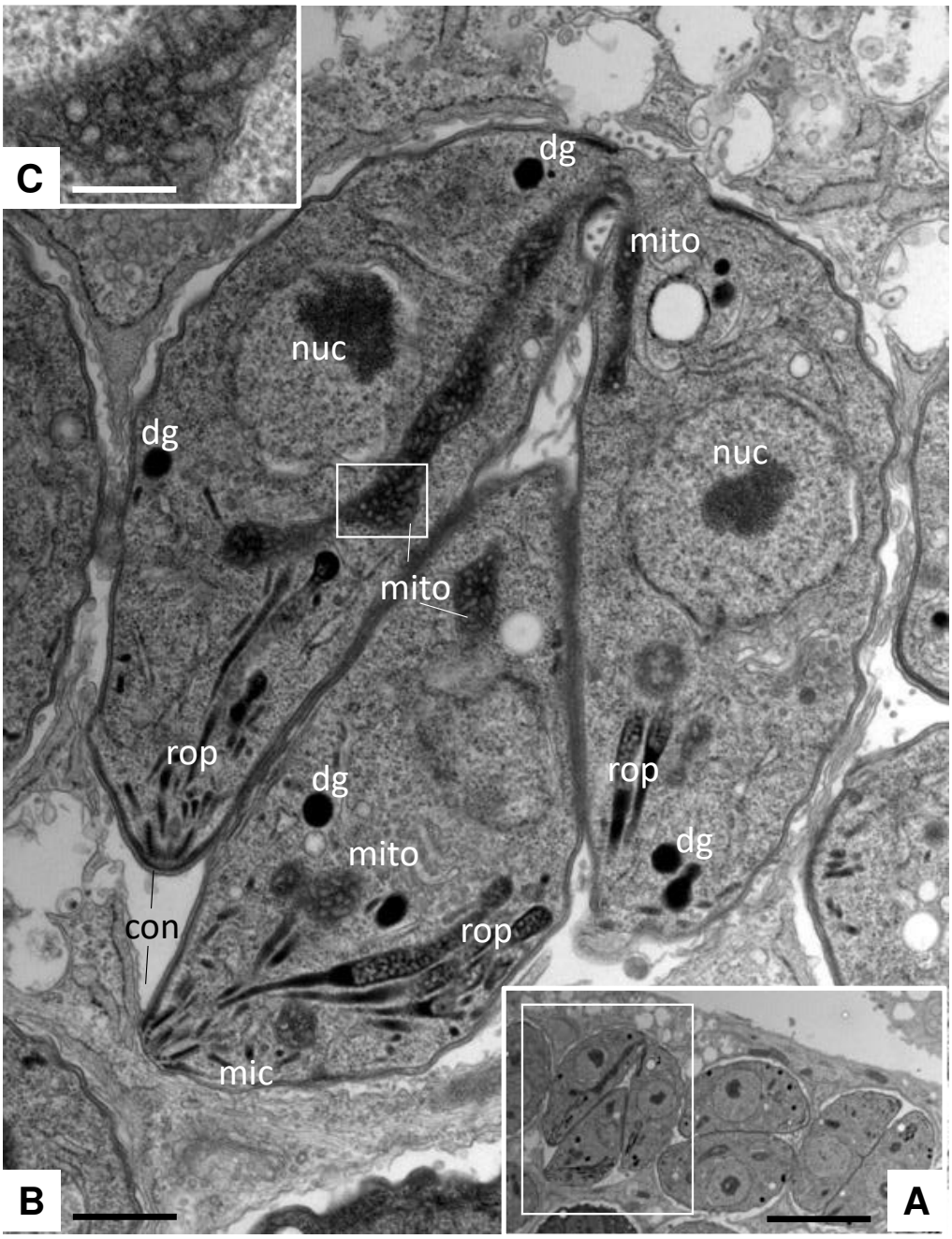

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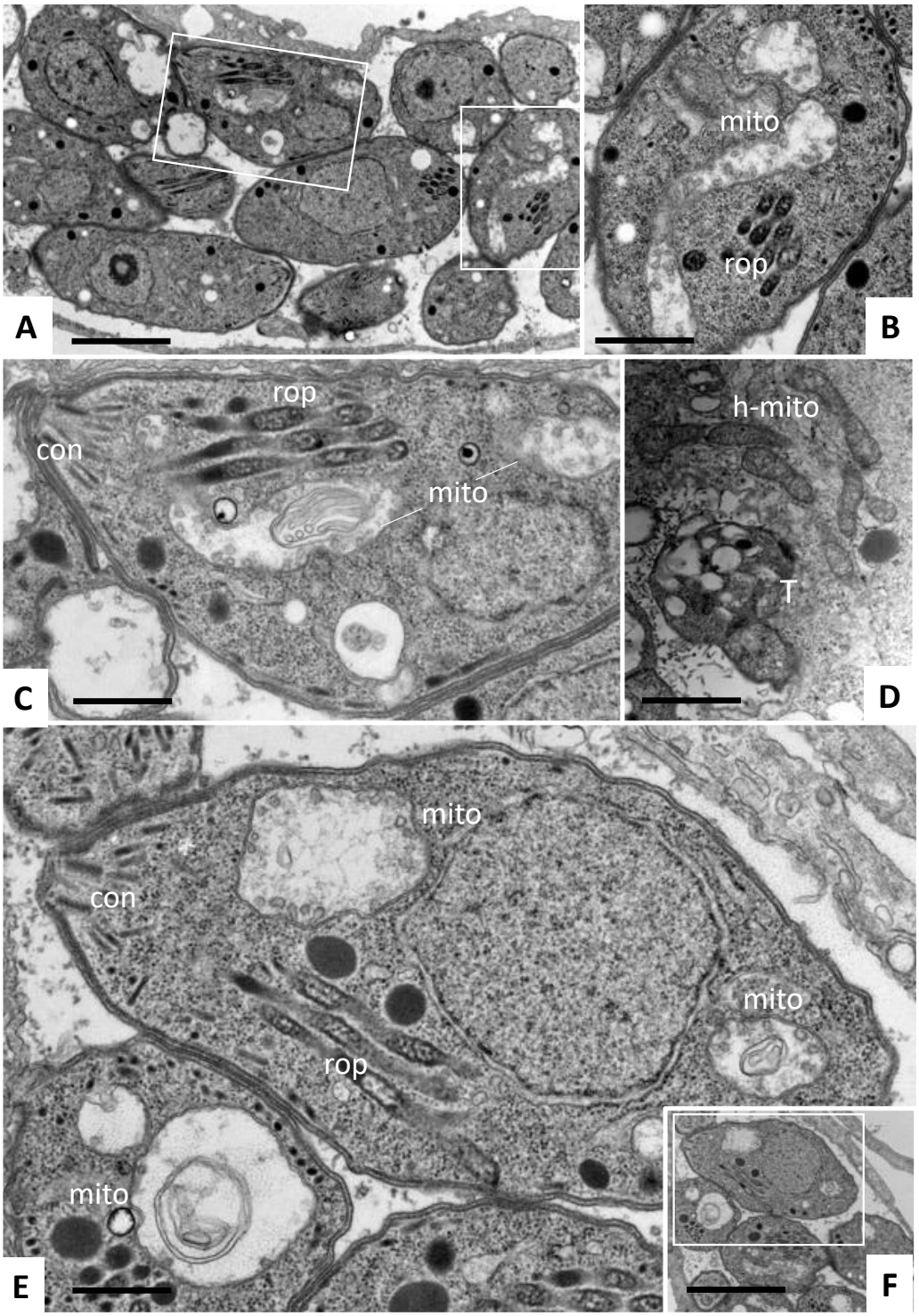

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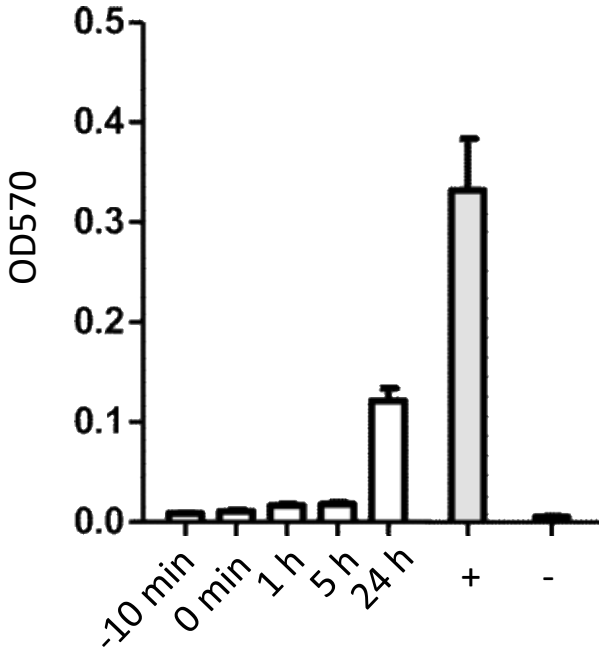

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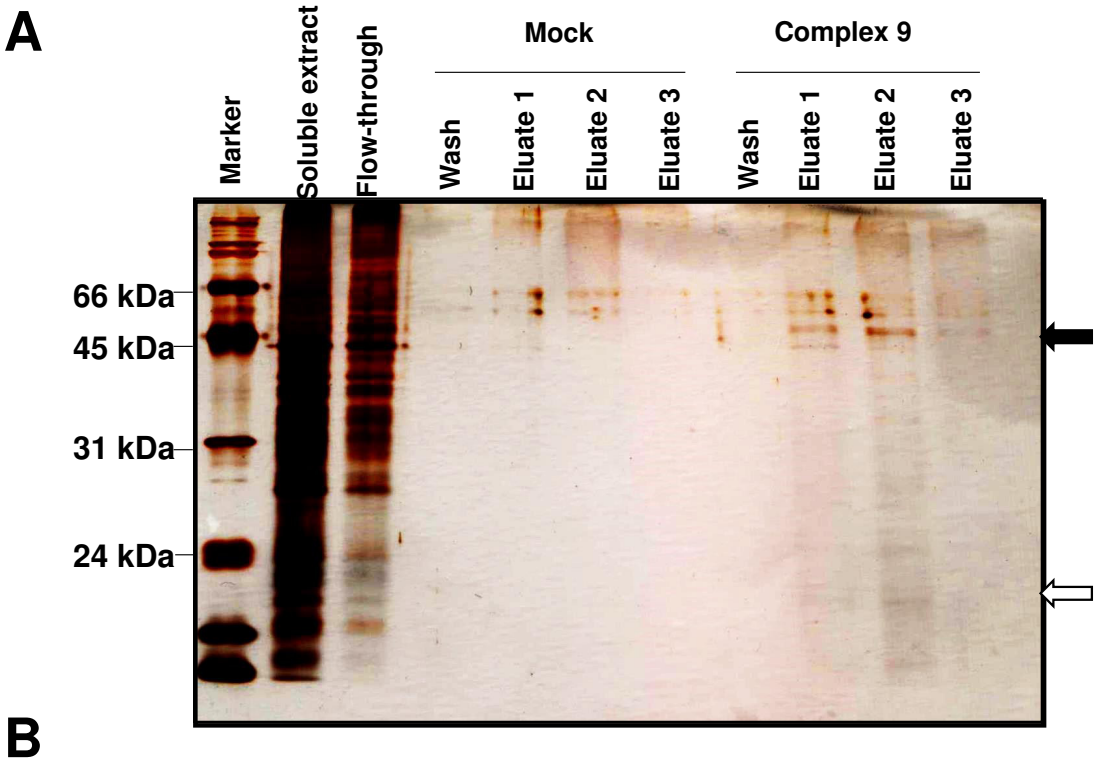

B

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VGVIKSVEKKEPGAGSKVTKSAVKAAKK

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