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In Vitro Effects of Novel Ruthenium Complexes in *Neospora caninum* and *Toxoplasma gondii* Tachyzoites

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Upon the screening of 16 antiproliferative compounds against *Toxoplasma gondii* and *Neospora caninum*, two hydrolytically stable ruthenium complexes (compounds 16 and 18) exhibited 50% inhibitory concentrations of 18.7 and 41.1 nM (*T. gondii*) and 6.7 and 11.3 nM (*N. caninum*). To achieve parasiticidal activity with compound 16, long-term treatment (22 to 27 days at 80 to 160 nM) was required. Transmission electron microscopy demonstrated the rapid impact on and ultrastructural alterations in both parasites. These preliminary findings suggest that the potential of ruthenium-based compounds should thus be further exploited.

Toxoplasma gondii and *Neospora caninum* are cyst-forming apicomplexan parasites that infect a wide range of hosts. In an immunocompetent host, infection with either parasite does not cause disease (1–3). *N. caninum* has emerged as one of the most important infectious causes of bovine abortion (4–6). In contrast, *T. gondii* causes toxoplasmosis in humans and many animal species, either in chronically infected individuals during a decrease in immunoreactivity or if a seronegative mother acquires a primary infection during pregnancy, leading to abortion or serious fetal abnormalities (7–9). Toxoplasmosis treatment is based on only a few chemotherapeutics with considerable adverse effects (10, 11). In *Neospora*-seropositive cattle, pregnancy and the associated immunomodulation are already sufficient to cause recrudescence, fetal damage, and abortion (2–6). Chemotherapy has been considered a promising option if effective drugs can be identified (12, 13). Several compounds were investigated *in vitro* (14–16), but only a few were evaluated in small-animal models (14, 17–24).

We have evaluated compounds originally synthesized as anticancer drugs. Currently used metal complexes (25–31) exhibit considerable toxicity. This has stimulated the interest in other compounds with more acceptable toxicity, such as ruthenium complexes (32–36). Effects of ruthenium compounds on some bacteria and parasites have been studied (37–46). "Classical ruthenium complexes" contain heteroatom ligands (e.g., azole derivatives), and NAMI-A and KP-1019 have been evaluated in phase I clinical trials for cancer treatment (47–49). Organometallic complexes are defined by at least one metal-carbon bond. The η^6-arene ruthenium(II) phosphite complexes 5, 6, 12, and 15 to 18 were characterized earlier (50), while [Ru(η^6-*p*-cymene)(bipyridine)Cl][Cl] 11 was synthesized as shown previously (51). Based on our experiences in the design of selective inhibitors of CYP11B2 (53) and CYP11B1 (54), the pyridine-based compounds 4, 7 to 10, and 14 were from a small in-house library of CYP enzyme inhibitors. 2,2′-Bipyridine 3 was obtained from Joachim W. Heinicke, Ernst Moritz Arndt University of Greifswald, Greifswald, Germany. The cytotoxic lipophilic imidazolium salt 1,3-bis(2,4,6-trimethylphenyl)imidazolium chloride 3 was synthesized as described previously (54–56). The arylimidamide DB745 2 (23) was kindly provided by David Boykin, Georgia State University, Atlanta, GA. The chemical structures and molecular masses of the drugs are shown in Fig. 1.

Maintenance of human foreskin fibroblasts (HFF) and Vero (African green monkey) cells and viability assessments by alamarBlue cytotoxicity assays were performed as described previously (50). Transgenic β-galactosidase-expressing *T. gondii* (RH) and *N. caninum* (Nc-1) tachyzoites (kindly provided by David Sibley, Washington University, St. Louis, MO) were maintained by serial passage in Vero cells (23, 24). Investigation of the inhibitory potential of the compounds was done as previously described (23, 24, 57). In short, confluent HFF grown in flat-bottom 96-well plates were infected with *T. gondii* or *N. caninum* tachyzoites at 10^3 parasites per well. After 2 h, compounds 2 to 18 (1 μM for initial screening and 0.5 nM to 1 μM for 50% inhibitory concentration [IC_{50}] determinations) were added, and after 72 h of cultivation, parasite proliferation was assessed by the addition of chlorophenol red–β-D-galactopyranoside (Roche Diagnostics, Rotkreuz, Switzerland) in phosphate-buffered saline. A_{570} was measured in a VersaMax 96-well multiplate reader (Bucher Biotec, Basel, Switzerland) at various time points (23, 24).

Initial screening at 1 μM revealed that only ruthenium-based compounds 16 and 18 completely inhibited the proliferation of both *T. gondii* and *N. caninum* (Fig. 2A and B), exhibiting dose-dependent effects (Fig. 2C and D). IC_{50}s (Table 1) show that *N. caninum* was slightly more susceptible. Treatment with all of the other compounds resulted in increased parasite proliferation (Fig. 2A and B), most likely because of nonlethal metabolic stress. At a concentration of 1 μM, neither of the com-

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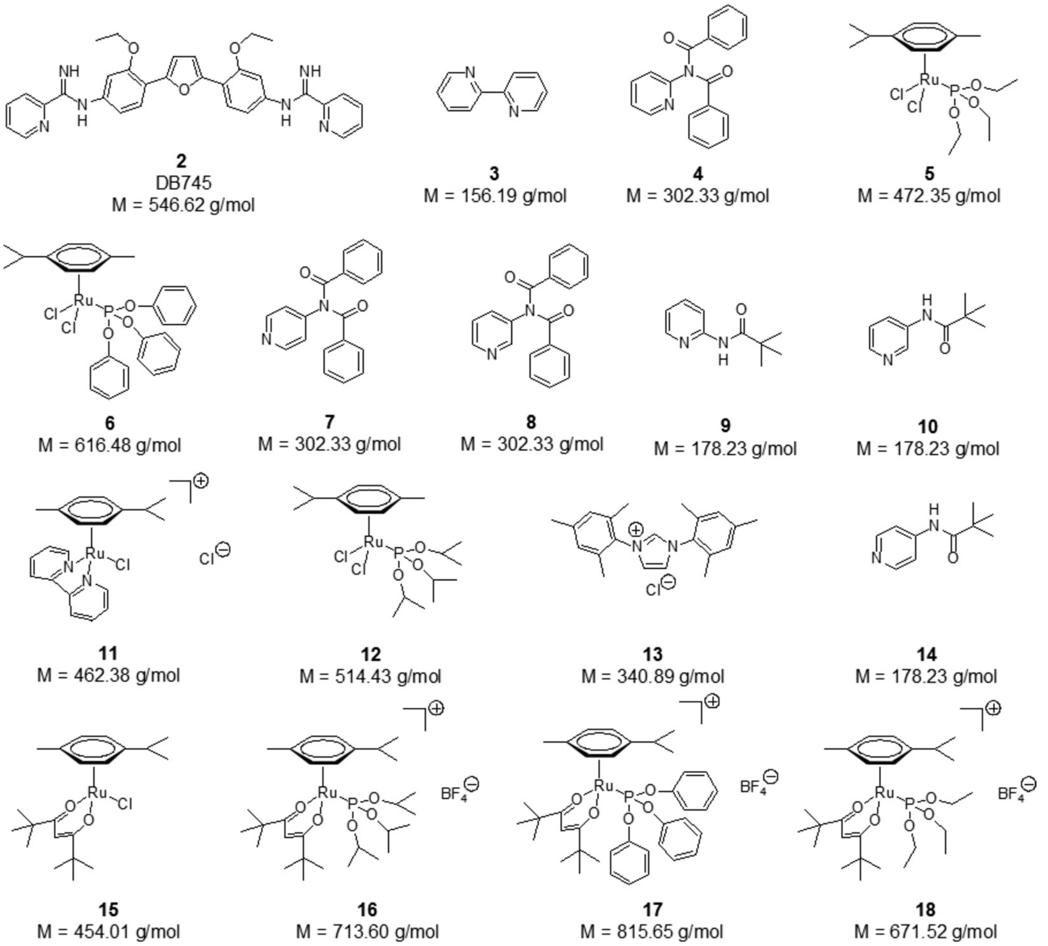

FIG 1 Structures and molecular masses (M) of the compounds investigated in this study. Note that compound 2 (DB745) was used as a positive control in the assessment of parasite toxicity (see Fig. 2) and was replaced with Triton X-100 in the assessment of HFF host cell toxicity (see Fig. 3).

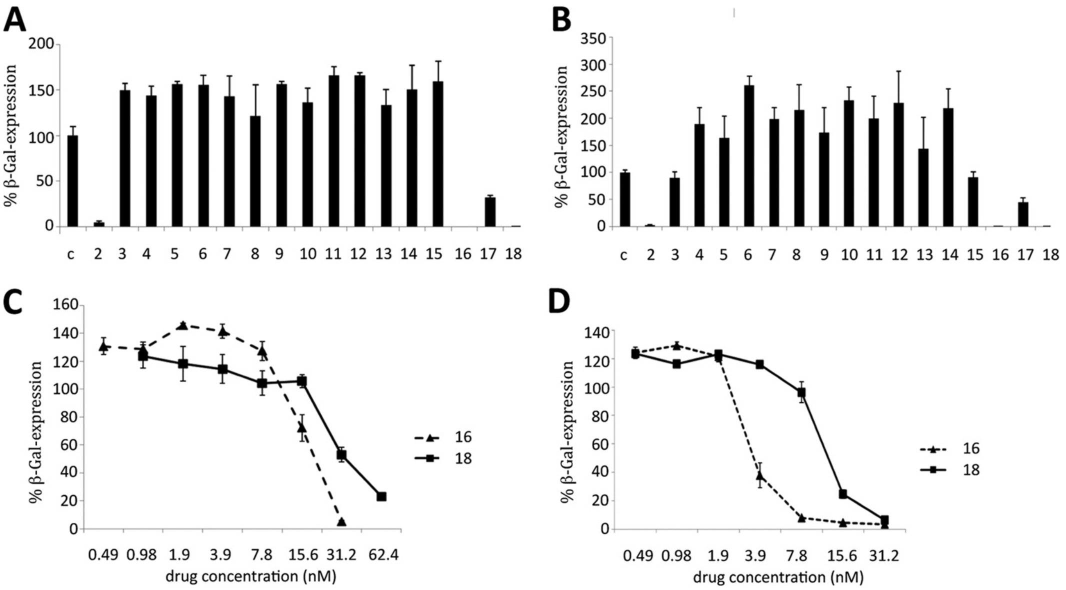

FIG 2 Proliferation-inhibitory effects upon *T. gondii* and *N. caninum* tachyzoites. Compounds 2 to 18 were added to transgenic *T. gondii* (A) and *N. caninum* (B) tachyzoites expressing β-galactosidase at 2 h postinfection of HFF monolayers at a concentration of 1 μM. Parasite proliferation was assessed by measurement of β-galactosidase activity after 72 h. Results are presented as percentages of β-galactosidase activity relative to that of a control containing the appropriate amount of dimethyl sulfoxide (c = 100%). (C, D) Compounds 16 and 18 were further assessed in dose-response experiments with *T. gondii* (C) and *N. caninum* (D), and measurements were done as described above.

TABLE 1 IC$_{50}$s of compounds 16 and 18 in noninfected HFF and in *N. caninum* and *T. gondii* tachyzoites expressing β-galactosidase grown in HFF monolayers[a]

	IC$_{50}$[b] for:		
Compound	*N. caninum*	*T. gondii*	HFF
16	6.7	18.7	2.4
18	11.3	41.1	6.95

[a] Parasite proliferation was assessed by measuring β-galactosidase activity (23, 24, 45), and HFF cell vitality was assessed by alamarBlue assay (38).

[b] IC$_{50}$s are given in nM for parasites and in μM for HFF.

pounds caused excessive cytotoxicity in noninfected HFF (Fig. 3A; Table 1). Exposure of extracellular *T. gondii* tachyzoites to 250 nM compound 16 for 1 to 2 h resulted in a pronounced (>90%) reduction of parasite numbers, while compound 18 had no effect (Fig. 4A). Both compounds had a severe impact on *N. caninum* infectivity (Fig. 4B). Pretreatment of host cells prior to infection was also investigated. Confluent HFF treated with compound 16 or 18 were washed, infected with tachyzoites, and cultured for 72 h. *T. gondii* proliferation was not affected (Fig. 5A), but *N. caninum* tachyzoites were severely impaired (Fig. 5B). This indicated that these compounds were taken up by the host cells as described earlier for other ruthenium-based drugs (58) and for dicationic pentamidine derivatives such as DB750 (59) and DB745 2 (23, 24).

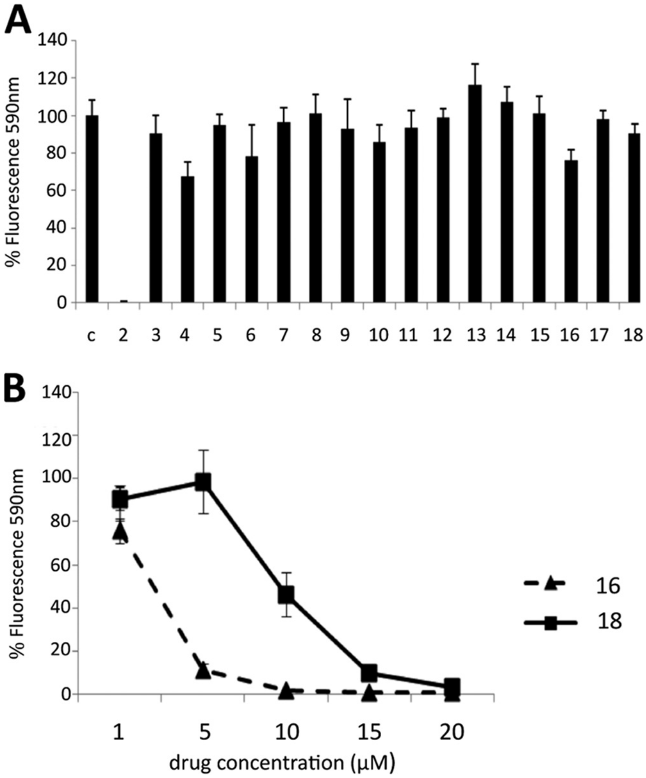

FIG 3 Cytotoxicity assessment of compounds 3 to 18 in HFF monolayers. (A) HFF were exposed to the drugs at a concentration of 1 μM for 72 h and viability measurements were done by alamarBlue assay. Results are presented as percentages of fluorescence measured relative to that of a control containing the appropriate amount of dimethyl sulfoxide (c = 100%). Note that compound 2 represents the positive cytotoxicity control (addition of 1% Triton X-100). In panel B, HFF were exposed to different concentrations (0 to 20 μM) of compound 16 or 18 for 72 h and measurements were done as described above.

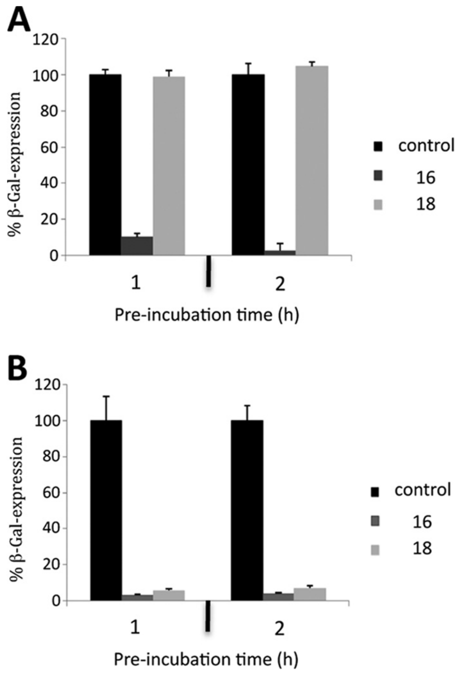

FIG 4 Effects of preincubation of extracellular tachyzoites. Extracellular *T. gondii* (A) and *N. caninum* (B) tachyzoites were exposed to compound 16 or 18 for 1 or 2 h and then added to HFF for 2 h. Subsequently, cultures were further maintained in the absence of the drugs for 72 h and proliferation was assessed by β-galactosidase activity measurement. Note that compound 16 affected both *T. gondii* and *N. caninum* extracellular tachyzoites, while compound 18 was active only against *N. caninum*. Results are presented as percentages of β-galactosidase activity relative to that of a control containing the appropriate amount of dimethyl sulfoxide (control = 100%).

The parasitostatic and/or parasiticidal activities of compounds 16 and 18 were studied as described previously (23, 24). In short-term experiments (Table 2), confluent HFF were infected with *T. gondii* or *N. caninum*, and at 2 h postinfection, 100, 250, or 500 nM compound 16 or 18 was added for 72 h of incubation, after which time the drug-containing medium was replaced with normal medium. Microscopy showed that both compounds failed to eliminate all of the tachyzoites, but compound 16 was more effective than compound 18. The abilities of *T. gondii* and *N. caninum* cells to adapt to compounds 16 and 18 were explored by slowly increasing the drug concentrations (Table 3). Infected HFF were initially cultured in the presence of compound 18 (50 nM for *T. gondii*; 12 nM for *N. caninum*) and compound 16 (20 nM and 10 nM, respectively), and drug levels were increased by 10 to 30 nM, typically every 3 to 4 days. Microscopy again demonstrated the higher efficacy of compound 16 (Table 3).

Inspection of drug-treated infected HFF by transmission electron microscopy (TEM) revealed distinct ultrastructural alterations in both parasites (Fig. 6 and 7). Untreated *T. gondii* (Fig. 6A and B) and *N. caninum* (Fig. 7A and B) form parasitophorous vacuoles containing proliferating tachyzoites. In cultures treated with compound 16, the drug rapidly induced

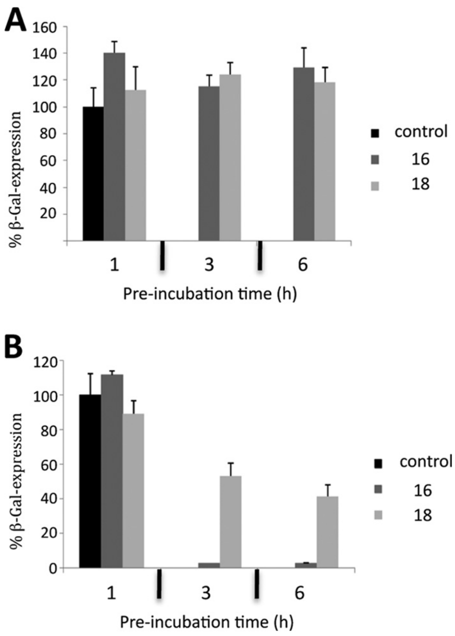

FIG 5 Effects of preincubation of HFF host cell monolayers prior to infection on proliferation of *T. gondii* and *N. caninum* tachyzoites. HFF monolayers were exposed to compounds 16 and 18 for 1, 3, or 6 h; washed; and infected with *T. gondii* (A) or *N. caninum* (B) tachyzoites. Proliferation of parasites was assessed after 72 h by measurement of β-galactosidase activity. *T. gondii* proliferation (A) was not affected, while proliferation of *N. caninum* tachyzoites was impaired severely by compound 16 pretreatment and partially also by compound 18 (B). Results are presented as percentages of β-galactosidase relative to a control containing the appropriate amount of dimethyl sulfoxide (control = 100%).

TABLE 2 Short-term treatment of *N. caninum* and *T. gondii* tachyzoites grown in HFF is not parasiticidal[a]

Parasite and compound	Concn (nM)	Posttreatment culture time (days)
N. caninum		
Control		3
16	100	8
	250	9
	500	10
18	100	6
	250	7
	500	8
T. gondii		
Control		2
16	100	2
	250	6
	500	7
18	100	2
	250	2
	500	3

[a] T25 tissue culture flasks containing confluent HFF monolayers were infected with 8×10^5 *N. caninum* or *T. gondii* tachyzoites. At 2 h postinfection, compound 16 or 18 was added and cultivation continued for 72 h in the presence of each compound at 100, 250, or 500 nM. The cultures were then washed with medium to remove the drugs and then incubated further in the absence of the compounds as indicated in Table 3. Posttreatment culture time indicates the numbers of days of culture in the absence of drugs until the reemergence of parasite proliferation was detected by light microscopy.

alterations. Obviously metabolically impaired tachyzoites with numerous empty or lipid-containing inclusions with electron-dense granular or amorphous material were visible after 12 h (Fig. 6C). The nuclear membrane had a fuzzy appearance, and chromatin appeared to be preferentially located at the nuclear periphery. At 36 h, most *T. gondii* tachyzoites exhibited a completely disorganized cytoplasm, organelles were hardly discernible, and many parasites were embedded in a granular matrix (Fig. 6D). Similar alterations were evident in *N. caninum* tachyzoites treated with compound 16 (Fig. 7C to F), with effects being most pronounced at 36 h of treatment (Fig. 7E and F). While these observations indicated a critical metabolic impairment of parasites, the alterations observed do not really point toward a defined mode of action.

How compound 16 exerts its parasiticidal action remains unknown. Earlier studies indicated that ruthenium compounds interact with DNA (25, 27). However, more recent investigations showed that ruthenium compounds bind more strongly to proteins (60, 61) and potential targets in cancer cells were postulated, including cathepsin B, P-glycoprotein, and glutathione *S*-transferase P1 (62). Exploitation of the wealth of available knowledge about ruthenium compounds could represent a starting point for the development of drugs with antiparasitic properties (63).

Of the eight ruthenium complexes investigated, the set of phosphite complexes can be divided into hydrolytically labile (compounds 5, 6, and 12) and hydrolytically stable complexes (compounds 16 to 18). Only compounds 16 to 18 exerted antiparasitic effects. We assume that the hydrocarbon substituents around the ruthenium center form a lipophilic sphere that facilitates the uptake of the compounds, though they should

TABLE 3 *T. gondii* and *N. caninum* tachyzoites can adapt to increasing concentrations of compound 18 but not 16[a]

	Drug concn (nM)			
	T. gondii		*N. caninum*	
Drug treatment duration (days)	Compound 18	Compound 16	Compound 18	Compound 16
0	50	20	12	8
3	70	40	20	16
6	90	40	40	20
9	110	60	60	30
12	130	80	80	40
16	150	100	80	40
22	170	130	80	Medium
27	190	160	100	Medium
31	210	Medium	120	Medium
35	250	Medium	150	Medium
39	270	Medium	170	Medium
42	300	Medium	200	Medium
45	330	Medium	240	Medium

[a] T25 tissue culture flasks containing confluent HFF monolayers were infected and cultured initially in the presence of compound 16 or 18 at the concentrations indicated on day 0. Proliferation of parasites was monitored daily by light microscopy. At the time points indicated, the medium was replaced with new medium containing a slightly elevated concentration of the respective compound, the same concentration, or no drug at all. Every 6 to 10 days, cultures were trypsinized and seeded onto fresh HFF monolayers. The experiment was terminated on day 45.

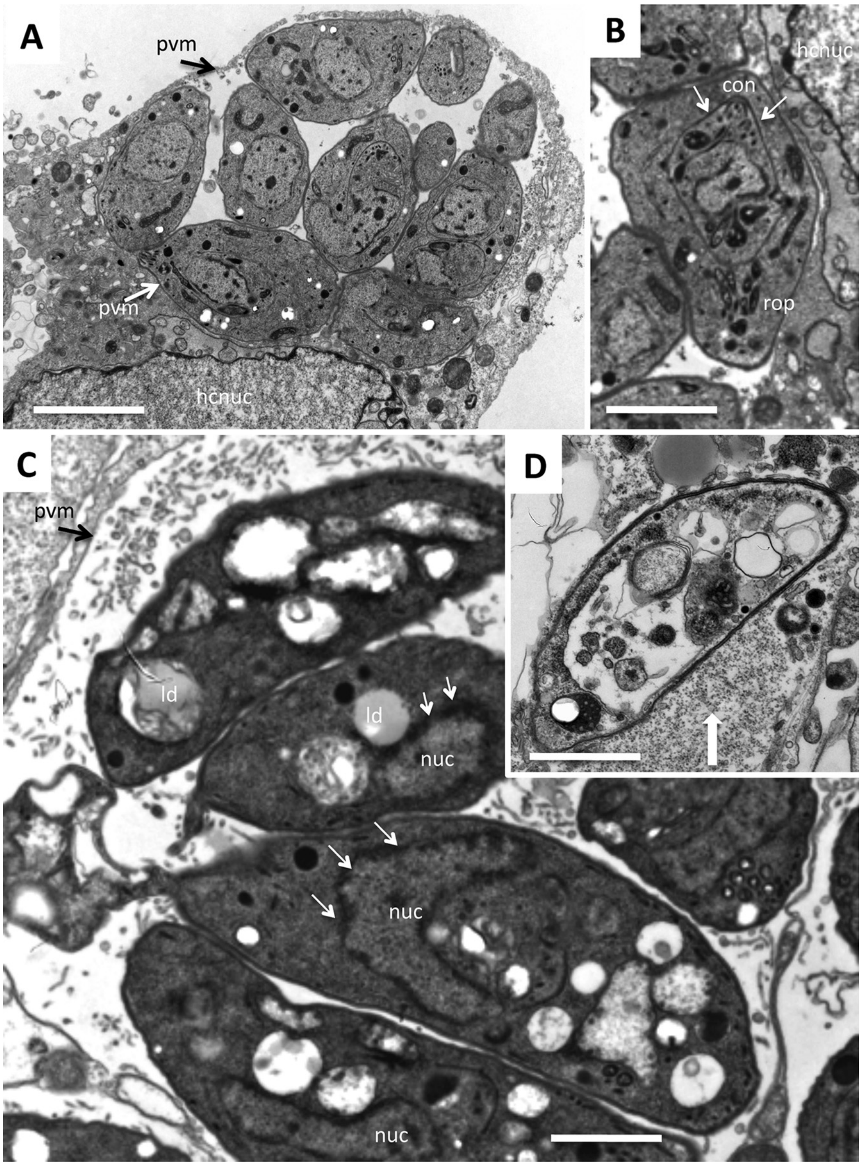

FIG 6 Effects of compound 16 on *T. gondii* ultrastructure. HFF monolayers grown to confluence in T25 culture flasks were infected with *T. gondii* tachyzoites, and after 48 h, they were treated with 100 nM compound 16 for 12 and 36 h, respectively; untreated infected cultures served as a control. Specimens were then processed for TEM as previously described (46, 47) and were viewed on a Philips 400T transmission electron microscope operating at 80 kV. (A, B) Numerous tachyzoites enclosed in a parasitophorous vacuole near the vicinity of the host cell nucleus (hcnuc), surrounded by a parasitophorous vacuole membrane (pvm). (B) Actively dividing tachyzoite with conoid (con) and rhoptry (rop) organelles. At 12 h after treatment started, clear alterations were observed (C). The tachyzoite cytoplasm is largely vacuolized, with vacuoles containing lipid droplets (ld) or membranous and electron-dense material. The white arrows point toward electron-dense chromatin deposits along the nuclear periphery of tachyzoites (nuc = nucleus). At 36 h of drug treatment (D), tachyzoites appear completely altered, exhibiting a disorganized cytoplasmic morphology, and intracellular parasites are often embedded in an electron-dense granular matrix (large white arrow in panel D). Bars: A, 1.4 μm; B, 0.8 μm; C, 0.5 μm; D, 0.8 μm.

not be able to penetrate membranes because of their ionic nature. For compound 16, an optimum arrangement and the formation of a ball-shaped sphere around the ruthenium center can be considered to be of importance. The smaller the surface of the molecule, the lower the chance of interaction with other molecules, e.g., in cell membranes. An additional aspect could be the impossibility of ligand exchange on the ruthenium center when almost perfect coverage is provided. The combination of bulky isopropyl groups on the phosphite ligand, *t*Bu residues on the 1,3-diketonate moiety, and a sterically demanding η^6-arene unit should effectively prevent nucleophilic attack on the metal center, leading to very good stability even in the presence of strong nucleophiles. Presumably, an attack on the phenyl moieties in compound 17 is easier (for example, by protonation following an S_EAr mechanism), which leads to decomposition of the triarylphosphite ligand and subsequent loss of the stability of the whole complex. In conclusion, the combination of reduced surface area and best shielding of the ruthenium

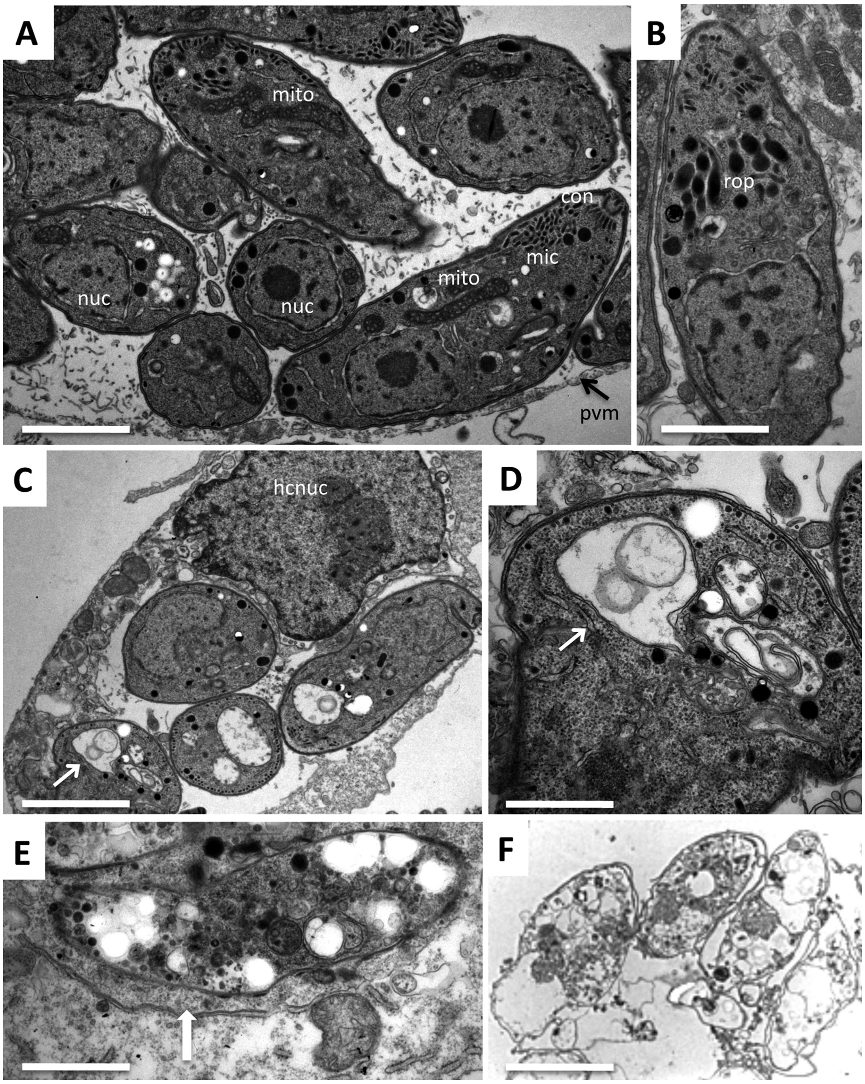

FIG 7 Effects of compound 16 on *N. caninum* ultrastructure. HFF monolayers were infected with *T. gondii* tachyzoites, and after 48 h, they were treated with 100 nM compound 16. (A, B) Nontreated *N. caninum* tachyzoites within a parasitophorous vacuole with intact nucleus (nuc), mitochondrion (mito), and anteriorly located conoid (con), micronemes (mic) and rhoptries (rop). After 12 h of treatment (C, D), the first alterations appear, such as the formation of cytoplasmic vacuoles (white arrows), and at 36 h, most tachyzoites appear completely altered (E), often embedded in granular material within the cytoplasm of host cells, but extracellular tachyzoite residues are also found (F). Bars: A, 0.8 μm; B, 0.6 μm; C, 1 μm; D, 0.3 μm; E, 0.8 μm; F, 1 μm.

center against nucleophilic attacks might explain the superior antiparasitic activity of 16.

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