Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

A New Promising Application for Highly Cytotoxic Metal Compounds: η^6-Areneruthenium(II) Phosphite Complexes for the Treatment of Alveolar Echinococcosis

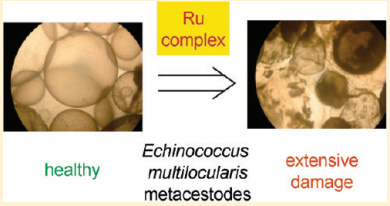
Tatiana Küster,[‡] Nadine Lense,[†] Fabienne Barna,[‡] Andrew Hemphill,*[,‡] Markus K. Kindermann,[†] Joachim W. Heinicke,[†] and Carsten A. Vock*[,†]

[†]Institute of Biochemistry—Inorganic Chemistry, Ernst-Moritz-Arndt University of Greifswald, Felix-Hausdorff-Strasse 4, D-17487 Greifswald, Germany

[‡]Institute of Parasitology, Vetsuisse Faculty, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland

Supporting Information

ABSTRACT: Two series of η^6-areneruthenium(II) phosphite complexes were prepared, characterized, and evaluated in vitro for their toxic potential against *Echinococcus multilocularis* metacestodes. Neutral complexes of general formula $[(\eta^6\text{-}p\text{-cymene})RuCl_2\{P(OR)_3\}]$ (R = Et, ^{i}Pr, Ph) with two easily exchangable chloride ligands showed only minor toxicity, whereas the substitution of these moieties against a β-diketonate (2,2,6,6-tetramethylheptanedionate) ligand led to hydrolytically stable complex salts of type $[(\eta^6\text{-}p\text{-cymene})Ru(\beta\text{-diketonate})\{P(OR)_3\}][BF_4]$ (R = Et, ^{i}Pr, Ph) with comparable in vitro toxicity (50% PGI release at c = 1.4 − 4.7 μM) to the reference drug nitazoxanide (50% PGI release at c = 1.2 μM). In addition, the latter complexes were highly toxic against rat hepatoma cells (IC_{50} = 0.40−2.0 μM) and less toxic against human foreskin fibroblasts (IC_{50} = 1.1−2.9 μM) and Vero cells (IC_{50} = 1.2−8.9 μM). The measured cytotoxicities against mammalian cells are, to the best of our knowledge, among the highest ever observed for ruthenium-based complexes. In conclusion, complex salts of type $[(\eta^6\text{-}p\text{-cymene})Ru(\beta\text{-diketonate})\{P(OR)_3\}][BF_4]$ might be interesting candidates for further development toward anthelmintic drugs and/or highly cytotoxic metal compounds.



INTRODUCTION

Alveolar echinococcosis (AE)[1] is a rare but life-threatening disease caused by the larval (metacestode) stage of the fox tapeworm *Echinococcus multilocularis* (*E. multilocularis*). The distribution of *E. multilocularis* is largely restricted to the Northern hemisphere, and highest prevalences occur in Central Asia, Russia, North-Western China, and parts of Europe and Japan. Although AE is present in higher developed countries, the number of patients is most likely underestimated. For instance, the incidence rate for Germany, 0.07/100.000 persons, is probably underscored by a factor of 3−5.[2] This has contributed to the fact that the development of new drugs against echinococcosis has not been a major focus of the pharmaceutical industry. The reported incidence in Switzerland had been (0.02−1.4)/100.000 population for many years[3,4] but has increased to 2.6/100.000 from 2001 to 2005.[5]

Infection in humans occurs by ingestion of food contaminated with parasite eggs containing an oncosphere, originating from the feces of natural definitive hosts such as fox, cat, or dog. After activation of the oncospheres in the stomach, parasites are able to penetrate the mucosa and enter blood and lymphatic vessels, finally reaching the liver as the main target organ. Here, metacestode development and proliferation occurs. AE exhibits cancer-like features, such as tumor-like growth of the vesicle-like metacestodes and occasionally metastatic spread into other organs.[6] Consequences are serious if the disease is not radically treated by surgery and/or chemotherapy. Surgery, intensively accompanied by chemotherapeutical treatment, is the therapy of choice. Benzimidazole carbamates like mebendazole **1** and albendazole **2** (Figure 1) are currently the most important drugs; however, they act parasitostatic rather than parasitocidal, and recurrence rates after treatment interruption are high. Thus, benzimidazole therapy often is life-long.[4,7] Moreover,

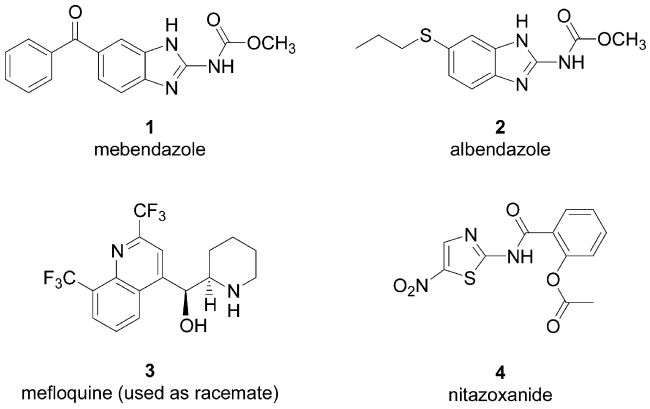


Figure 1. Structures of mebendazole **1**, albendazole **2**, mefloquine **3**, and nitazoxanide **4**.

Received: November 17, 2011
Published: April 10, 2012

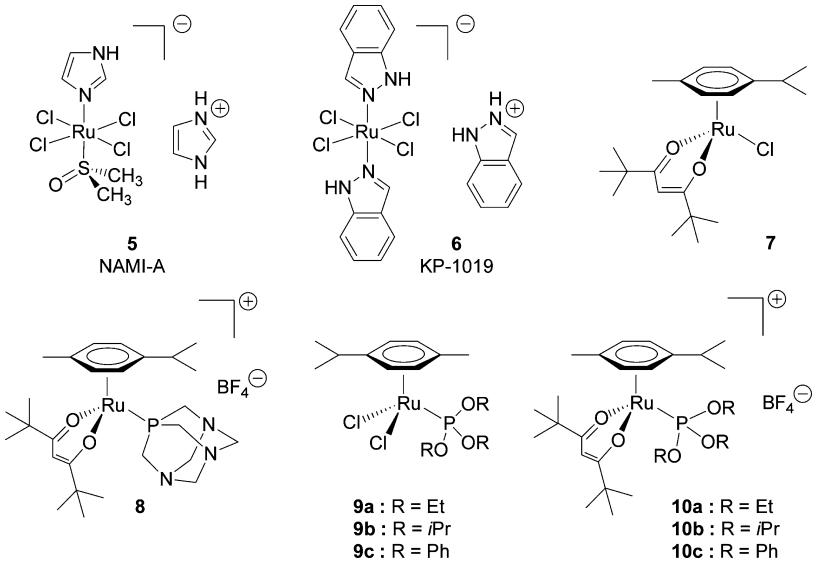
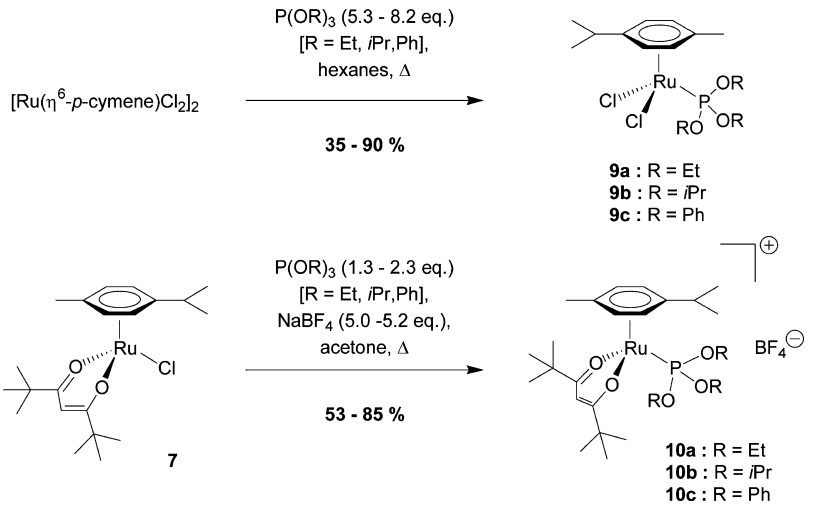


Figure 2. Structures of the antimetastatic ruthenium(III) complex NAMI-A **5**, the cytotoxic complex KP-1019 **6**, Sadler's cytotoxic complex **7**, the hydrolytically stable complex **8**, and the representatives of the substance classes **9** and **10** evaluated within this publication.

Scheme 1. Syntheses of Complexes 9 and 10



there is no alternative chemotherapeutic option for unresponsive patients or patients that do not tolerate the application of **1** or **2**. More recently, several other promising drug candidates for the treatment of AE have been evaluated,[8−10] among them the well-known antimalarial drug mefloquine **3**[9] and the broad-spectrum anti-infective agent nitazoxanide **4** (Figure 1).[10] However, in human patients, **4** (nitazoxanide) has not held its promises achieved in the mouse model.[8] **3** (mefloquine) appears to be a valuable alternative for **2** (albendazole) as assessed in mice,[9] but this needs to be verified in human patients, where side effects could pose a serious problem. Therefore, alternative treatment options are urgently required.

The investigation of ruthenium complexes as anticancer[11−17] and, to a minor extent, also as antibacterial[18,19] and antitrypanosomal[20,21]/antiplasmodial[22,23] agents in chemotherapy has become a very important field of research over the past 20 years. NAMI-A **5** (Figure 2) was the first ruthenium complex to reach phase I clinical trials as anticancer agent in 2004, and its antimetastatic potential was successfully proven.[24] More recently, KP-1019 **6** (Figure 2), a Ru(III) complex developed by Keppler et al. with structural similarity to **5** (NAMI-A), has shown its anticancer properties also against primary tumors.[25,26] The high cytotoxic potential of η^6-areneruthenium(II) β-diketonate complexes like **7** (Figure 2) has been extensively studied for the first time by Sadler and coworkers in 2006.[27] In previous research, we investigated whether the presence of an easily exchangeable ligand like chloride on the ruthenium center is a prerequisite for the cytotoxic activity of the compound. It turned out that a hydrolytically stable complex **8** (Figure 2), with the labile chloro ligand being substituted by the by far more stable PTA group (PTA = 1,3,5-triaza-7-phosphaadamantane), conserved the cytotoxic potential, and no significant hydrolysis in differently concentrated saline solutions at 37 °C took place even after several days.[28] The 2,2,6,6-tetramethylheptanedionate (^{t}Bu$_2$acac) ligand in **8** offered the best hydrolytic stability in a series of different diketonates tested. Therefore, to avoid the problems of multiple metal species being present in solution due to facilitated ligand exchange, we are focusing on hydrolytically stable complexes, with chelating ligands like ^{t}Bu$_2$acac being of substantial interest for structural design.

dx.doi.org/10.1021/jm300291a | J. Med. Chem. 2012, 55, 4178−4188

Table 1. Some Representative ^{1}H, ^{13}C, and ^{31}P NMR Data for Complexes 9a−c and 10a−c[a]

compd	^{1}H	^{13}C	^{31}P
9a	5.37 (d, J = 6.0 Hz, 2 H, 3-H, 5-H) 5.51 (d, J = 6.0 Hz, 2 H, 2-H, 6-H)	88.65 (d, $J_{C,P}$ = 6.1 Hz, C-2, C-6) 89.23 (d, $J_{C,P}$ = 6.5 Hz, C-3, C-5)	112.1
9b	5.32 (d, J = 6.0 Hz, 2 H, 3-H, 5-H) 5.46 (d, J = 6.0 Hz, 2 H, 2-H, 6-H)	88.50 (d, $J_{C,P}$ = 5.7 Hz, C-2, C-6) 88.62 (d, $J_{C,P}$ = 6.6 Hz, C-3, C-5)	106.8
9c	5.08 (d, J = 6.1 Hz, 2 H, 3-H, 5-H) 5.41 (d, J = 6.1 Hz, 2 H, 2-H, 6-H)	88.61 (d, $J_{C,P}$ = 6.2 Hz, C-2, C-6) 88.94 (d, $J_{C,P}$ = 7.3 Hz, C-3, C-5)	104.9
10a	5.77 (m_c, 4 H, 2-H, 3-H, 5-H, 6-H) 5.68 (s, 1 H, COC*H*CO)	89.30 (d, $J_{C,P}$ = 7.3 Hz, C-2, C-6) 92.19 (d, $J_{C,P}$ = 5.9 Hz, C-3, C-5) 92.61 (d, $J_{C,P}$ = 1.5 Hz, COCHCO)	114.5
10b	5.71 (br. d, J = 6.2 Hz, 2 H, 3-H, 5-H) 5.75 (br. d, J = 6.2 Hz, 2 H, 2-H, 6-H) 5.73 (s, 1 H, COC*H*CO)	88.84 (d, $J_{C,P}$ = 8.0 Hz, C-2, C-6) 92.30 (d, $J_{C,P}$ = 5.3 Hz, C-3, C-5) 93.37 (COCHCO)	107.7
10c	5.36 (br. d, J = 6.3 Hz, 2 H, 3-H, 5-H) 5.82 (br. d, J = 6.3 Hz, 2 H, 2-H, 6-H) 5.88 (s, 1 H, COC*H*CO)	89.97 (d, $J_{C,P}$ = 5.9 Hz, C-2, C-6) 92.00 (d, $J_{C,P}$ = 5.9 Hz, C-3, C-5) 93.40 (COCHCO)	107.7

[a]Assignments of the signals belonging to protons or carbons on the cymene system have been made according to literature data.[33]

Growth of metacestodes in human liver after infection with *E. multilocularis* is a process very similar to growth of primary tumors and metastases occurring with progressing cancer. Since ruthenium complexes have been successfully evaluated as antitumor agents,[11−17] it is reasonable to assume beneficiary effects against diseases of similar phenotype. However, to the best of our knowledge, ruthenium compounds have never been evaluated as chemotherapeutics against infections with *E. multilocularis* or similar parasitic diseases. Furthermore, although the high solubility of some ruthenium phosphite complexes in water is known,[29] complexes with simple phosphite ligands astonishingly have not been biologically evaluated yet. An exception is the work of Hartinger and Nazarov et al.,[30−32] who used phosphite moieties to attach sugar molecules to ruthenium centers in order to improve water solubility of the complexes. In this paper, we report on the in vitro efficacy of η^6-areneruthenium(II) phosphite complexes of general formula **9** and **10** against *E. multilocularis* metacestodes in combination with cytotoxicity studies on human foreskin fibroblasts, Vero cells (a monkey kidney epithelial cell line), and rat hepatoma cells.

RESULTS

Synthesis and Spectroscopic Characterization. Sadler's complex **7** was synthesized as previously reported.[27,28] Treatment of $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ with excess 2,2,6,6-tetramethylheptanedione and excess Na_2CO_3 in acetone, followed by evaporation of the solvent, extraction of the residue with CH_2Cl_2, and crystallization from CH_2Cl_2/hexane afforded **7** in good yield. Synthesis of complexes **9** was accomplished using a slightly modified literature-known protocol by Hodson and Simpson.[33] Heating of the ruthenium precursor $[RuCl_2(\eta^6\text{-}p\text{-cymene})]_2$ with an excess of the corresponding phosphite in hexane under reflux for several hours furnished the desired complexes **9a−c** as orange to red solids in 64−90% yield (Scheme 1). Spectral data of complexes **7** and **9a−c** were in accordance with literature data.

The new, previously unreported phosphite complexes of general formula $[Ru(\eta^6\text{-}p\text{-cymene})(^tBu_2acac)\{P(OR)_3\}][BF_4]$ **10** were prepared in analogy to our previously described PTA derivatives (e.g., compound **8**).[28] Suspensions of complex **7**, the corresponding phosphite ligand, and $NaBF_4$ in acetone were heated to reflux several times for a short period. This procedure turned out to be superior in comparison to continuous heating regarding the formation of decomposition products. Subsequent evaporation of the solvent, extraction of the residue with CH_2Cl_2, evaporation of the solvent, and crystallization of the residue from EtOAc/hexane afforded the desired products as yellow to orange-yellow solids in 53−85% yield (Scheme 1). A structurally similar complex, namely, $[Ru(\eta^6\text{-}p\text{-cymene})(acac)\{P(OMe)_3\}][BF_4]$, with acac being unsubstituted acetylacetonate ligand, has already been described in 1990 by Carmona et al.;[34] however, no biological data were presented, and our previous investigations[28] indicated low stability of ruthenium complexes with unsubstituted acetylacetonate ligand against hydrolysis.

Characteristic ^{1}H, ^{13}C, and ^{31}P NMR data for complexes **9a−c** and **10a−c** are summarized in Table 1. Assignments of the signals belonging to protons or carbons on the cymene system have been made according to literature data.[33] As can be seen, the presence or absence of the diketonate ligand has only minor influence on the chemical shift of the ^{31}P resonance signal. However, some interesting effects can be observed for ^{1}H and ^{13}C resonances belonging to the *p*-cymene system. For complexes **9**, two distinct doublets are observed for the aromatic cymene protons in ^{1}H NMR spectra, whereas almost no difference in chemical shift can be observed for the equivalent proton resonances in complexes **10** (with exception of **10c**). In contrast, the two ^{13}C resonances for C-3/C-5 and C-2/C-6 appear at almost identical chemical shifts ($\Delta\delta$ = 0.12−0.58 ppm) for complexes **9**, whereas the gap between the signals is increased for complexes **10** ($\Delta\delta$ = 2.03−3.46 ppm). With respect to the resonances of free *p*-cymene, which can be observed at δ = 7.13 ppm (2-H, 3-H, 5-H, 6-H)[28] and δ = 126.3 ppm (C-2, C-6)[35] and δ = 129.1 ppm (C-3, C-5),[35] a weaker coordination of the *p*-cymene moiety to the ruthenium center can be assumed for complexes **10** in comparison with complexes **9**. This interesting fact might be explained by increased steric demand delivered by the additional β-diketonate ligand and/or by stronger π-donation of ruthenium into the acetylacetonate system in comparison to simple chloride ligands, which should lead to a reduced π-donation into the *p*-cymene system and weakened binding of the aromatic moiety. This observation is in agreement with our own earlier results.[28] Regarding the backbone of the β-diketonate ligand, a low-field shift is observed for ^{1}H and ^{13}C signals belonging to the COC*H*CO group from **10a** to **10c**. The effect is more pronounced for the ^{1}H NMR resonances.

In Vitro Toxicity of Complexes 7, 9a−c, and 10a−c against *E. multilocularis* Metacestodes. At first, primary screening at a drug concentration of c = 20 μM was carried out. Toxicity of the compounds was assessed by measuring phosphoglucose isomerase (PGI) activity liberated by dying parasites into the culture medium.[36] **4** (nitazoxanide) (Figure 1), a drug that is effective against *E. multilocularis* metacestodes in vitro,[10] was used as reference. The results of the primary screening are depicted in Figure 3.

As can be seen from the data, incubation of metacestodes with complexes of type **9** exhibited no significant in vitro toxicity at c = 20 μM. At best, compound **9c** led to the release

dx.doi.org/10.1021/jm300291a | J. Med. Chem. 2012, 55, 4178−4188

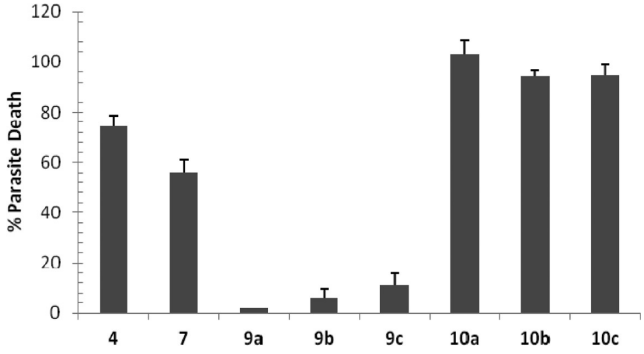


Figure 3. Results of primary in vitro drug screening against metacestodes of *E. multilocularis*. Drug treatments lasted 5 days. **4** (nitazoxanide) was used as reference. PGI activity in medium supernatants is given as percentage in relation to the activity achieved upon incubation of metacestodes in Triton X-100.[36]

of approximately 12% of PGI activity compared to completely destroyed parasites. In contrast, treatment with Sadler's complex **7** resulted in almost 56% PGI release. The best results were obtained for ruthenium complexes of type **10**, with 90−100% PGI release, indicating that all parasites were killed after 5 days. The highest toxicity was observed for compound **10a**.

In addition to toxicity assessments via PGI assay, the effects of the compounds on *E. multilocularis* metacestodes were visualized at day 5 of treatment. The micrographs for all tested compounds are shown in Figure 4. Metacestodes treated with compounds **4**, **10a**, **10b**, and **10c** exhibited dramatic morphological alterations and loss of turgor, indicating that their viability was severely impaired. For compound **7**, these alterations were also visible, but also seemingly unaffected metacestodes could be detected. In contrast, after application of compounds **9a**, **9b**, and **9c**, metacestodes remained with unaltered morphology.

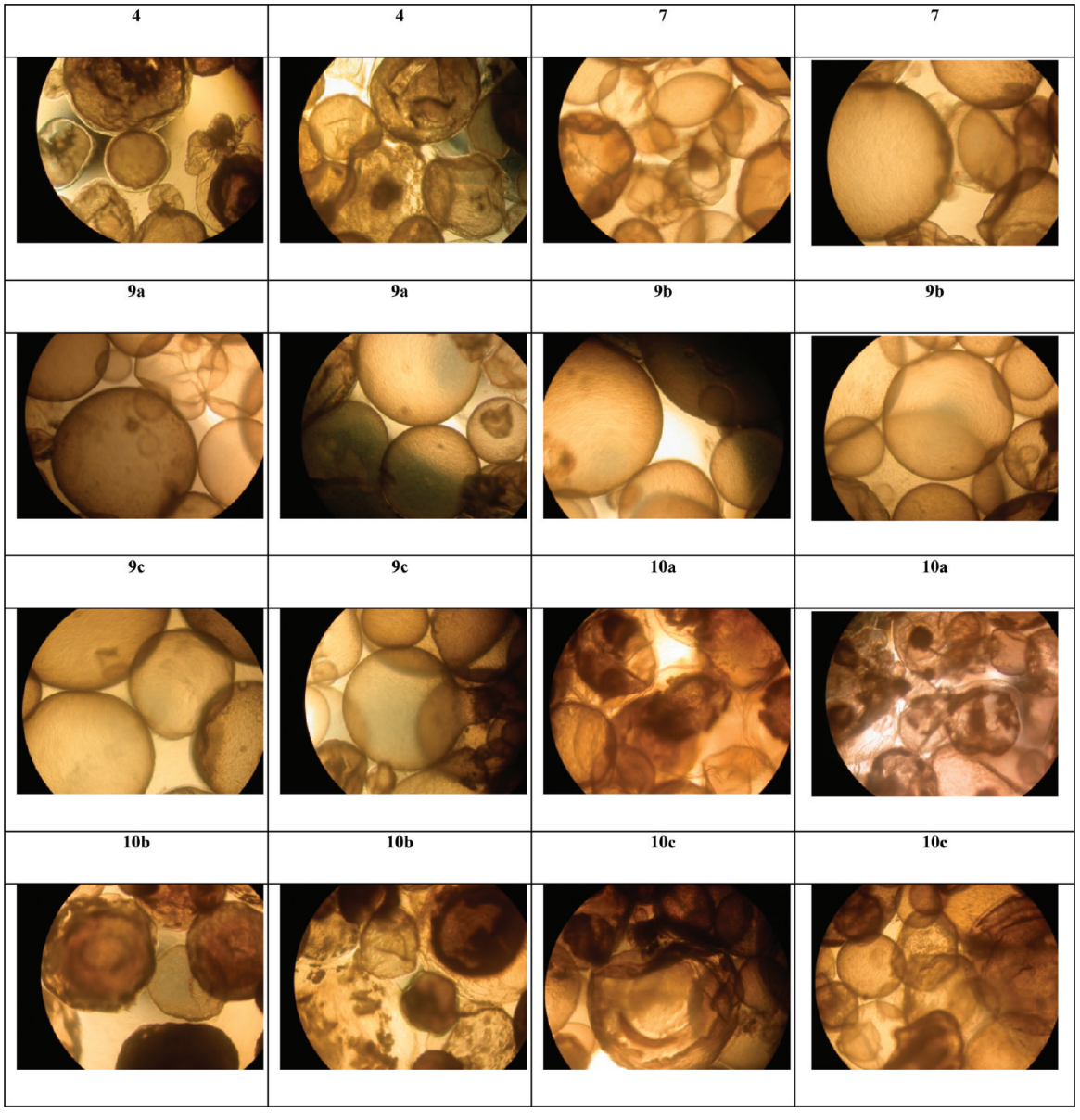


Figure 4. *E. multilocularis* metacestodes after incubation with reference **4** (nitazoxanide) and the ruthenium complexes **7**, **9a−c**, and **10a−c** for 5 days. Drug concentration was c = 20 μM. Note the extensive damage (vesicle collapse) that is observed with **4** but also with **10a**, **10b**, and **10c**.

dx.doi.org/10.1021/jm300291a | *J. Med. Chem.* 2012, 55, 4178−4188

As seen in Figure 5 (upper part), the effects of ruthenium complexes were dose-dependent. While complex **7** caused only

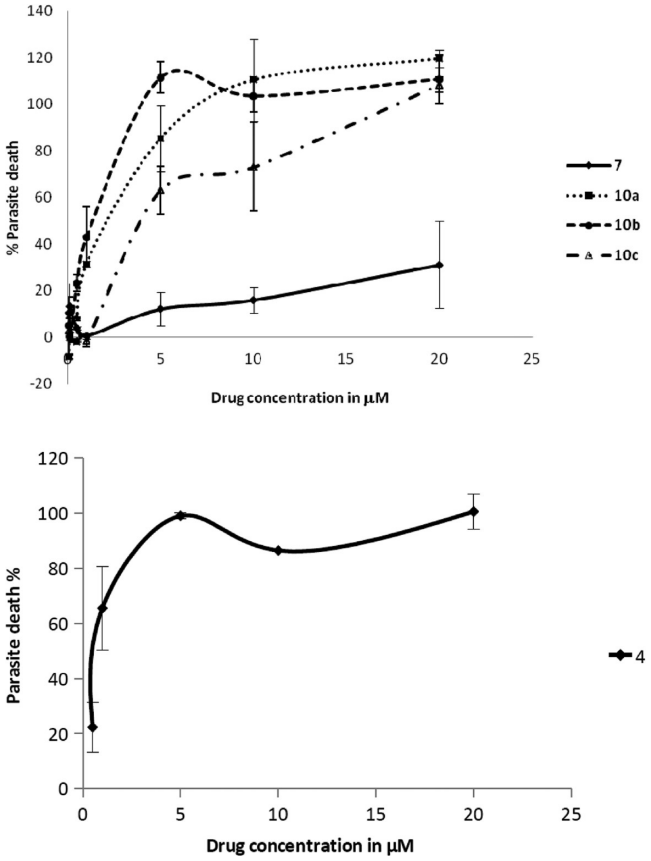


Figure 5. Dose-dependent effect of complexes **7** and **10a–c** (upper part) and **4** (nitazoxanide) (lower part) on parasite metacestodes, as measured by PGI assay. The % parasite death indicates the value of PGI activity measured in culture supernatants in relation to the values obtained in cultures treated with 1% Triton X-100.

limited PGI release (50% PGI release is achieved at c = 39.6 μM), complexes **10a–c** were very effective even below 5 μM. Complex **10a** was the most effective, with 50% PGI release achieved at c = 1.4 μM. For complexes **10b** and **10c**, similar toxicity was observed; concentrations for 50% PGI release of c = 4.2 μM (**10b**) and c = 4.7 μM (**10c**) indicate, however, only slight differences in the toxicities of all complexes **10**. The observed parasitocidal activities, and in particular for complex **10a**, are comparable to the highly effective drug **4** (nitazoxanide) (50% PGI release at c = 1.2 μM), which has been used as reference.

Toxicity of Complexes and 10a, 10b, and 10c against Mammalian Cell Lines. To evaluate the toxicity of the prepared ruthenium complexes against mammalian cell lines, a vitality assay (resazurin) was performed using cultivated human foreskin fibroblasts (concentrations ranging from 0.1 to 20 μM), Vero cells, and rat hepatoma cells (in both cases, concentrations ranging from 0.05 to 20 μM). **3** (Mefloquine) (Figure 1), a drug that is effective against *E. multilocularis* metacestodes in vitro and in vivo,[9] was used as reference with human foreskin fibroblasts. The obtained results are depicted in Figure 6; IC_{50} values were determined and are summarized in Table 2. As can be seen from the data, complexes **10** exhibit toxicities very similar to those of human foreskin fibroblasts (IC_{50} = 1.1–2.9 μM). The toxicity of mefloquine **3** appears to be about the same (IC_{50} = 2.1 μM). For Vero cells, which represent an immortalized, noncancerous epithelial cell line, a slightly more pronounced toxicity pattern is observed. When applied to this cell line, complex **10b** (IC_{50} = 1.2 μM) appears to be approximately 6–7 times more toxic than **10a** and **10c**. Interestingly, all complexes **10** show increased toxicity toward rat hepatoma cells in comparison to human foreskin fibroblasts and Vero cells, the most toxic being **10b** with an IC_{50} of 0.40 μM, followed by **10c** (IC_{50} = 1.3 μM) and **10a** (IC_{50} = 2.0 μM).

For all ruthenium complexes **10**, an increase of metabolic activity to more than 100% is observed when drug concentration is approaching cytotoxic values. A very pronounced effect is observed for **10b** with rat hepatoma cells, the metabolic activity being increased to almost 150%. However, this effect is due to increased metabolism of the cells in response to the applied toxic stress.

DISCUSSION

The ruthenium complexes being evaluated for their anti-metacestodal and cytotoxic properties can be divided into three subgroups. Sadler's cytotoxic neutral complex **7** with one chloride ligand prone to easy hydrolytic exchange has been the starting point and reference for all investigations. Neutral complexes **9** contain two chloride ligands and are therefore expected to more easily undergo more complex ligand exchange reactions. Charged complexes **10** are designed to be stable against ligand exchange under cell-free conditions; however, a metabolic activation or decomposition when submitted to cellular medium cannot, of course, be excluded.

When tested for in vitro activity against *E. multilocularis* metacestodes, compounds **9** showed only minor toxicity at c = 20 μM and were not further evaluated. Complex **7** was toxic (achieving 50% PGI release at c = 39.6 μM) for the parasites; however, the effect was not as pronounced as for the highly effective reference drug **4** (nitazoxanide) (50% PGI release at c = 1.2 μM). In contrast, complexes **10** exhibited marked metacestodicidal effects of almost the same magnitude (50% PGI release observed at c = 1.4–4.7 μM) as observed for **4**. Toxicity toward *E. multilocularis* metacestodes increases with stability of the complexes against hydrolysis (**9** < **7** < **10**). This is probably due to increasing "lipophilicity" (more exactly, formation of a more dense lipophilic sphere around the metal ion) and drug uptake, as ligand exchange by water increases hydrophilicity and prevents the cellular uptake by membrane penetration. Within the series of complexes **10**, the highest parasite toxicity is observed for complex **10a** (50% PGI release at 1.4 μM). Although the substituents on the phosphite ligand are larger for **10b** and **10c**, which should normally result in increased lipophilicity, the toxicity of both compounds is approximately 3-fold lower in comparison with that of complex **10a**. This indicates that simple phosphite ligands with small substituents might be the best choice to reach high toxicity levels in *E. multilocularis* metacestodes. However, with three complexes of type **10** being tested, the data set is too small for confirmation of this assumption. A broader study employing a larger variety of different phosphite ligands is necessary and will follow.

In order to examine whether a selective toxic behavior toward metacestodes in comparison with normal mammalian cells can be achieved (which should be of importance when applying the drugs in vivo), complexes **10** were also evaluated

dx.doi.org/10.1021/jm300291a | J. Med. Chem. 2012, 55, 4178–4188

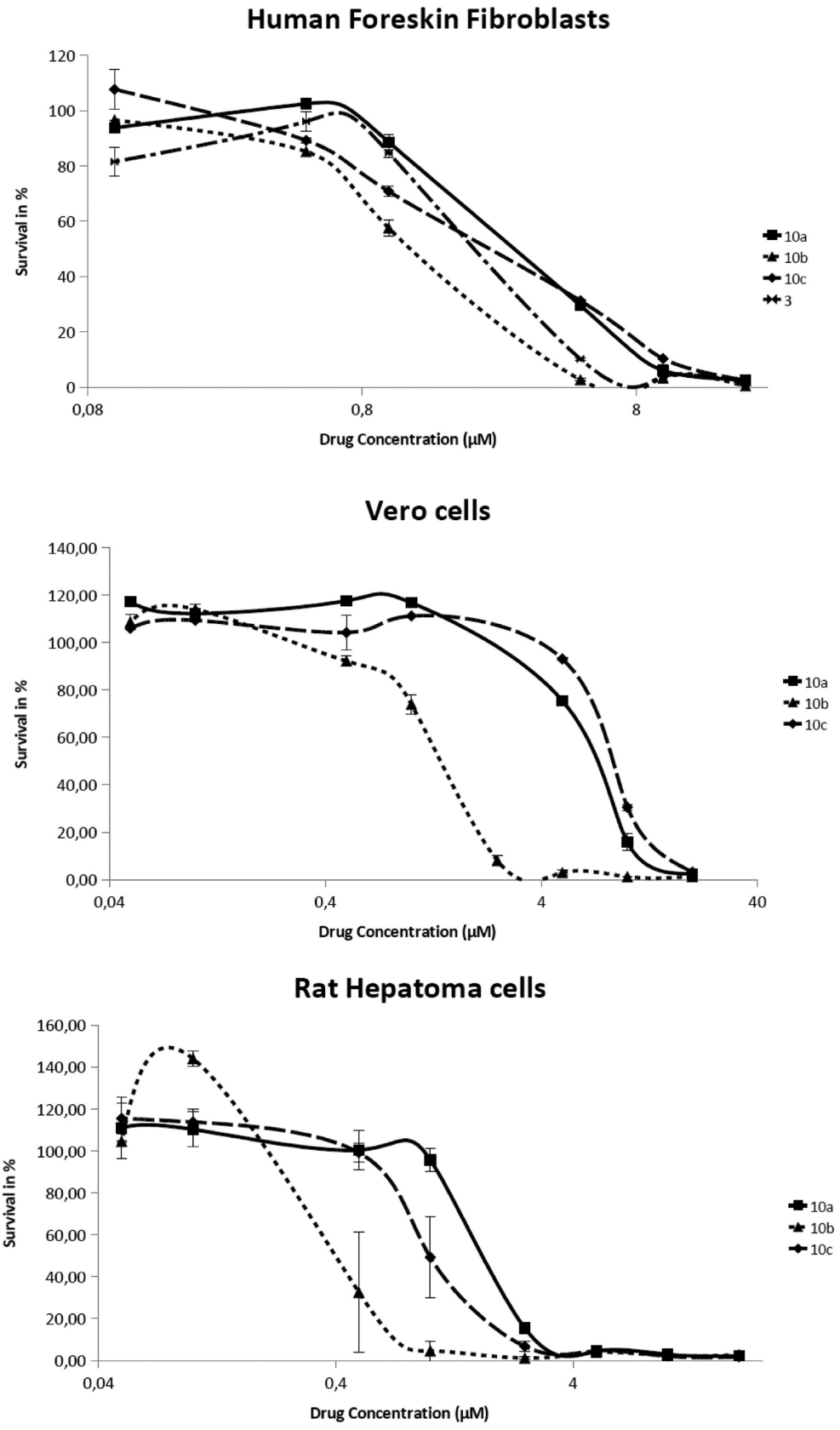


Figure 6. Vitality of human foreskin fibroblasts (upper diagram), Vero cells (middle diagram), and rat hepatoma cell cultures (lower diagram) at 72 h of treatment with mefloquine **3** and the ruthenium complexes **10a–c** at the indicated concentrations. The survival in % indicates the value of Alamar Blue fluorescence measured in cultures in relation to the values obtained in cultures treated with DMSO alone.

dx.doi.org/10.1021/jm300291a | *J. Med. Chem.* 2012, 55, 4178–4188

Table 2. IC_{50} (in μM) for Ruthenium Complexes 10a−c and 3 (Mefloquine) in Mammalian Cells, Determined via Alamar Blue Assay

	noncarcinogenic cell		
drug	human foreskin fibroblast	Vero cell (monkey)	cancer cell line rat hepatoma cell
10a	2.9	7.3	2.0
10b	1.1	1.2	0.40
10c	2.3	8.9	1.3
3	2.1		

against noncarcinogenic human and monkey cells, i.e., human foreskin fibroblasts and Vero cells, respectively. Ideally, compounds should show a high toxicity against *E. multilocularis* metacestodes and a very low toxicity against noncarcinogenic cell lines. Our observation was that complexes **10** display very similar in vitro toxicity to both metacestodes and fibroblasts, with complex **10b** being the most toxic derivative in fibroblasts and Vero cells. Interestingly, the level of toxicity is almost similar for complex **10b** in these cell lines, whereas complexes **10a** and **10c** are less toxic when applied to Vero cells. However, the toxicity of compounds against cell cultures cannot be automatically translated into the in vivo situation. This has been shown earlier for mefloquine, which exhibited profound cytotoxicity in vitro but not when applied to mice.[9]

We also evaluated the in vitro effects of complexes **10** against a cancer cell line, namely, rat hepatoma cells, and observed astonishingly high toxicities for all compounds, the most impressive one for **10b** with an IC_{50} of 0.40 μM. To the best of our knowledge, these findings belong to the highest toxicities ever observed for ruthenium-based complexes designed for use as cytostatics. For example, similar hydrolytically stable ruthenium complexes with sugar-derived phosphite ligands reported by Hanif, Hartinger, and Nazarov et al. had IC_{50} values far above 100 μM with human CH1, SW480, and A549 cancer cell lines.[30] Evaluation of our complexes on human cancer cell lines is pending; however, the promising data obtained with human fibroblasts raise our hopes for a positive outcome of these investigations, i.e., the observation of high to very high cytotoxicities.

The mechanism of the toxic action on both *E. multilocularis* metacestodes and mammalian cells has not yet been determined. A lot of investigations have been carried out by numerous research groups in order to specify the cellular target for ruthenium complexes with cytotoxic behavior; however, none of the proposed mechanisms (e.g., DNA binding, interaction with mitochondrial pathways) have been unambiguously identified and validated in vivo, and assumptions on them are sometimes highly speculative, as recently reviewed in detail by Bergamo and Sava.[11] Complexes **10** are designed to be stable against hydrolysis under cell-free conditions, which is supported by our own earlier long-time NMR studies on compound **8** in aqueous saline solutions.[28] DNA binding, a frequently proposed mechanism of action for metal-based cytotoxic compounds, requires liberation of at least one coordination site at the metal center, which is unlikely under cell-free conditions but of course possible if metabolic activation is considered. Complexes **9** are prone to easy chloride−water ligand exchange reactions, as shown elsewhere for similar ruthenium(II) PTA complexes;[37] however, compounds **9** were almost ineffective in our tests. Reduced cellular uptake of the η^6-areneruthenium(II) complexes with one or two water ligands due to high hydrophilicity might be a reasonable explanation. By addition of more lipophilic groups, like the ^{t}Bu$_2$acac chelate and subsequently the additional phosphite ligand, the properties of the compounds are changed in a twofold way: the possibility to form aqua complexes is reduced (for complex **7**) or excluded (as for complexes **10**), and the high charge of the Ru^{2+} cation is shielded by an almost centrosymmetric lipophilic sphere, which should even allow a direct transfer of the cation through cellular membranes by passive diffusion without a particular transporter system, which is normally required for ion uptake. From these observations, it is reasonable to assume an intracellular target to be responsible for the toxic effects. In addition, it might be assumed that the lipophilic sphere around the Ru^{2+} cation has the best shielding effect for branched isopropyl groups, which might explain the highest toxicity in mammalian cells for complex **10b**, although **10c** should be more lipophilic by normal definition.

As already mentioned above, similar hydrolytically stable complexes with sugar-derived phosphite ligands and oxalate as chelating moiety have been prepared and shown to have low binding to several biomolecules, which rationalized the observed low cytotoxicities.[30] However, our complexes **10** showed markedly different behavior. From our NMR studies, we assume a weaker binding of the cymene moiety for complex salts **10** in comparison with neutral complexes **9**. As a consequence, the loss of the η^6-arene system might be the most probable step of complex activation, liberating at the same time three possible coordination sites for biomolecules, e.g., DNA or proteins. The possibility of DNA binding with loss of the η^6-arene system as a mechanism of action for ruthenium(II) arene complexes has been shown by Dorcier and Dyson et al. in 2005.[38] In comparison to the much less toxic oxalato complexes,[30] a strong π-donation of the ruthenium into the conjugated cyclic ^{t}Bu$_2$acac system as well as to the phosphite ligand (stronger π-acceptor than phosphine ligands, e.g., PTA) is possible, which reduces the stabilization of the coordinated η^6-arene moiety and makes a loss of the group much more likely. The influence of strong π-accepting coligands has also been described by Dougan and Sadler et al. in 2006 for phenylazopyridine and phenylazopyrazolechloridoruthenium-(II) arene complexes.[39] Within this publication, the loss of the arene moiety has been studied by NMR spectroscopy to occur slowly in aqueous solution. However, one easily exchangeable chloride ligand was still available; this position has been blocked for our complexes **10** by the additional phosphite ligand. In addition to the π-accepting properties, the steric demand of the phosphite ligands might lead to additional repulsing forces, facilitating the loss of the arene moiety.

Although hydrolysis experiments in cell-free medium did not indicate any changes,[30] hydrolysis of the phosphite ligand in cells by suitable enzymes cannot be excluded.

In summary, considering the possibility of intracellular liberation of coordination sites at the ruthenium center, all generally assumed mechanisms of action, which involve binding of the ruthenium center to biomolecules like DNA, seem reasonable for our compounds. The combination of hydrolytic stability, lipophilic sphere-forming ligands, and careful optimization of both π-acceptor and steric properties of the ligands in order to generate an optimized coordinative stability for the η^6-arene system might be a useful approach to further rational design of new highly toxic η^6-areneruthenium(II) complexes. Complexes **10** may be regarded as optimized prodrugs, which

dx.doi.org/10.1021/jm300291a | J. Med. Chem. 2012, 55, 4178−4188

will deliver and presumably liberate reactive ruthenium species inside the cell with higher efficiency.

CONCLUSION

We have shown that hydrolytically stable ruthenium complexes are an interesting option for the treatment of alveolar echinococcosis. A high in vitro toxicity on *E. multilocularis* metacestodes was observed, the most toxic compound being complex **10a**. In vivo studies with experimentally infected mice are pending. Complexes **10**, in particular complex **10b**, also exhibited very high cytotoxic activity on rat hepatoma cells and might also be of interest for further development toward cytostatic compounds.

EXPERIMENTAL SECTION

All reactions were performed under an atmosphere of air without exclusion of oxygen and/or humidity. $[RuCl_2(\eta^6\text{-}p\text{-cymene})]_2$ was synthesized according to a literature protocol.[40] All other reagents and solvents were obtained from commercial sources and used without further purification. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 25 °C on a Bruker Avance II 300 MHz multinuclear FT-NMR spectrometer or on a Bruker Avance III 400 MHz Nanobay multinuclear FT-NMR spectrometer in $CDCl_3$. ^{1}H, ^{13}C, and ^{31}P chemical shifts are δ values and given in ppm. Coupling constants refer to H−H couplings (unless otherwise stated) and are given in Hz. ^{1}H and ^{13}C NMR spectra were referenced to internal solvents as follows: $\delta(CHCl_3, {}^1H) = 7.26$[41] and $\delta(CDCl_3, {}^{13}C) = 77.00$. For ^{31}P NMR spectra, H_3PO_4 (85%) was used as external standard. Mass spectra were recorded on a single-focusing mass spectrometer AMD40 (Intectra). Combustion (elemental) analyses were determined with a vario MICRO cube analyzer (*elementar*) using standard conditions. For all synthesized complexes, purity was confirmed by combustion (elemental) analysis to be ≥95%.

[Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac)Cl] (7). $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (300 mg, 0.490 mmol, 1.00 equiv) and Na_2CO_3 (260 mg, 2.45 mmol, 5.01 equiv) were suspended in acetone (15 mL). 2,2,6,6-Tetramethylheptanedione (0.510 mL, 457 mg, 2.49 mmol, 5.09 equiv) was added, and the mixture was stirred at room temperature for 3.5 h. Solvent was removed in vacuo. The solid residue was extracted with CH_2Cl_2 (4 × 20 mL). The extracts were filtered. Hexane (100 mL) was added, and CH_2Cl_2 was removed in vacuo until the beginning of crystallization. After storage at −25 °C overnight, the precipitate was filtered off with suction, washed several times with hexane, and dried in an air stream. The desired compound was obtained as reddish-brown solid (303 mg, 0.667 mmol, 68%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 18 H, 2 × C(CH_3)$_3$ {^{t}Bu$_2$acac}), 1.35 (d, J = 6.8 Hz, 6 H, 1-CH(CH_3)$_2$), 2.23 (s, 3 H, 4-CH_3), 2.89 (sept, J = 6.8 Hz, 1 H, 1-CH(CH$_3$)$_2$), 5.12 (d, J = 6.4 Hz, 2 H, 2-H, 6-H), 5.39 (s, 1 H, COCHCO {^{t}Bu$_2$acac}), 5.40 (d, J = 6.4 Hz, 2 H, 3-H, 5-H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$): δ = 17.73 (4-CH_3), 22.36 (1-CH(CH_3)$_2$), 28.46 (2 × C(CH_3)$_3$ {^{t}Bu$_2$acac}), 30.72 (1-CH(CH_3)$_2$), 40.71 (2 × C(CH$_3$)$_3$ {^{t}Bu$_2$acac}), 78.95 (C-2, C-6), 83.10 (C-3, C-5), 88.96 (COCHCO {^{t}Bu$_2$acac}), 96.80 (C-4), 99.03 (C-1), 196.0 (2 × CO {^{t}Bu$_2$acac}). EI-MS (70 eV): m/z (%) = 455.0 (24) $[M]^+$, 419.8 (30) $[M - Cl]^+$, 361.7 (87) $[M - C_4H_9Cl]^+$, 271.4 (98) $[M - C_{11}H_{19}O_2]^+$, 119.2 (55) $[C_9H_{11}]^+$, 57.2 (100) $[C_4H_9]^+$. Anal. Calcd for $C_{21}H_{33}ClO_2Ru$: C, 55.56%; H, 7.33%. Found: C, 55.53%; H, 7.06%. $C_{21}H_{33}ClO_2Ru$ (454.01).

[Ru(η^6-*p*-cymene)Cl$_2${P(OEt)$_3$}] (9a). To a suspension of $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (200 mg, 0.327 mmol) in hexane (40 mL), $P(OEt)_3$ (0.300 mL, 291 mg, 1.75 mmol, 5.35 equiv) was added at room temperature. The resulting mixture was heated to reflux for 4 h with stirring. After the mixture was cooled to room temperature, the precipitate was filtered off with suction, washed with hexane (3 × 15 mL), and dried in an air stream. The desired compound was obtained as orange amorphous solid (277 mg, 0.586 mmol, 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.24 (d, J = 6.9 Hz, 6 H, 1-CH(CH_3)$_2$), 1.28 (t, J = 7.1 Hz, 9 H, 3 × OCH$_2$CH_3 {P(OEt)$_3$}), 2.15 (s, 3 H, 4-CH_3), 2.92 (sept, J = 6.9 Hz, 1 H, 1-CH(CH$_3$)$_2$), 4.16 (quint, $J_{H,H} = J_{H,P}$ = 7.1 Hz, 6 H, 3 × OCH_2CH$_3$ {P(OEt)$_3$}), 5.37 (d, J = 6.0 Hz, 2 H, 3-H, 5-H), 5.51 (d, J = 6.0 Hz, 2 H, 2-H, 6-H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$): δ = 16.19 (d, $J_{C,P}$ = 5.8 Hz, 3 × OCH$_2$CH$_3$ {P(OEt)$_3$}), 18.26 (4-CH_3), 21.98 (1-CH(CH_3)$_2$), 30.34 (1-CH(CH$_3$)$_2$), 63.02 (d, $J_{C,P}$ = 6.2 Hz, 3 × OCH$_2$CH$_3$ {P(OEt)$_3$}), 88.65 (d, $J_{C,P}$ = 6.1 Hz, C-2, C-6), 89.23 (d, $J_{C,P}$ = 6.5 Hz, C-3, C-5), 100.5 (C-4), 109.1 (d, $J_{C,P}$ = 2.1 Hz, C-1). ^{31}P {^{1}H} NMR (121 MHz, $CDCl_3$): δ = 112.1. Anal. Calcd for $C_{16}H_{29}Cl_2O_3PRu$: C, 40.68%; H, 6.19%. Found: C, 40.52%; H, 6.16%. $C_{16}H_{29}Cl_2O_3PRu$ (472.35).

[Ru(η^6-*p*-cymene)Cl$_2${P(O^{i}Pr)$_3$}] (9b). To a suspension of $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (200 mg, 0.327 mmol) in hexane (40 mL), P(O^{i}Pr)$_3$ (0.400 mL, 363 mg, 1.74 mmol, 5.33 equiv) was added at room temperature. The resulting mixture was heated to reflux for 3.5 h with stirring. After the mixture was cooled to room temperature, the precipitate was filtered off with suction, washed with hexane (3 × 15 mL), and dried in an air stream. The desired compound was obtained as a red powder (118 mg, 0.229 mmol, 35%). A second crop of the desired product (orange to orange-red crystals) could be obtained by slow evaporation of the mother liquor (145 mg, 0.282 mmol, 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (d, J = 6.9 Hz, 6 H, 1-CH(CH_3)$_2$), 1.28 (d, J = 6.1 Hz, 18 H, 3 × OCH(CH_3)$_2$), 2.13 (s, 3 H, 4-CH_3), 2.91 (sept, J = 6.9 Hz, 1 H, 1-CH(CH$_3$)$_2$), 4.88 (dsept, $J_{H,H}$ = 6.1 Hz, $J_{H,P}$ = 8.1 Hz, 3 H, 3 × OCH(CH$_3$)$_2$), 5.32 (d, J = 6.0 Hz, 2 H, 3-H, 5-H), 5.46 (d, J = 6.0 Hz, 2 H, 2-H, 6-H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$): δ = 18.16 (4-CH_3), 22.09 (1-CH(CH_3)$_2$), 24.04 (d, $J_{C,P}$ = 3.8 Hz, 3 × CH(CH_3)$_2$ {P(O^{i}Pr)$_3$}), 30.38 (1-CH(CH$_3$)$_2$), 71.25 (d, $J_{C,P}$ = 7.7 Hz, 3 × CH(CH$_3$)$_2$ {P(O^{i}Pr)$_3$}), 88.50 (d, $J_{C,P}$ = 5.7 Hz, C-2, C-6), 88.62 (d, $J_{C,P}$ = 6.6 Hz, C-3, C-5), 101.0 (d, $J_{C,P}$ = 2.3 Hz, C-4), 108.4 (d, $J_{C,P}$ = 2.4 Hz, C-1). ^{31}P {^{1}H} NMR (121 MHz, $CDCl_3$): δ = 106.8. Anal. Calcd for $C_{19}H_{35}Cl_2O_3PRu$: C, 44.36%; H, 6.86%. Found: C, 44.23%; H, 6.76%. $C_{19}H_{35}Cl_2O_3PRu$ (514.43).

[Ru(η^6-*p*-cymene)Cl$_2${P(OPh)$_3$}] (9c). To a suspension of $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (200 mg, 0.327 mmol) in hexane (20 mL), $P(OPh)_3$ (0.700 mL, 829 mg, 2.67 mmol, 8.17 equiv) was added at room temperature. The resulting mixture was heated to reflux for 4 h with stirring. After the mixture was cooled to room temperature, the precipitate was filtered off with suction, washed with hexane (3 × 15 mL), and dried in an air stream. The desired compound was obtained as an orange-red amorphous solid (260 mg, 0.422 mmol, 64%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.19 (d, J = 6.9 Hz, 6 H, 1-CH(CH_3)$_2$), 1.82 (s, 3 H, 4-CH_3), 2.72 (sept, J = 6.9 Hz, 1 H, 1-CH(CH$_3$)$_2$), 5.08 (d, J = 6.1 Hz, 2 H, 3-H, 5-H), 5.41 (d, J = 6.1 Hz, 2 H, 2-H, 6-H), 7.10−7.23 (m, 3 H, 3 × 4′-H {P(OPh)$_3$}), 7.27−7.33 (m, 12 H, 3 × 2′-H, 3 × 3′-H, 3 × 5′-H, 3 × 6′-H {P(OPh)$_3$}). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$): δ = 17.99 (4-CH_3), 22.14 (1-CH(CH_3)$_2$), 30.57 (1-CH(CH$_3$)$_2$), 88.61 (d, $J_{C,P}$ = 6.2 Hz, C-2, C-6), 88.94 (d, $J_{C,P}$ = 7.3 Hz, C-3, C-5), 103.1 (C-4), 109.4 (C-1), 121.8 (d, $J_{C,P}$ = 3.2 Hz, 3 × C-2′, 3 × C-6′ {P(OPh)$_3$}), 125.1 (3 × C-4′{P(OPh)$_3$}), 129.4 (3 × C-3′, 3 × C-5′{P(OPh)$_3$}), 151.5 (d, $J_{C,P}$ = 11.0 Hz, 3 × C-1′{P(OPh)$_3$}). ^{31}P {^{1}H} NMR (121 MHz, $CDCl_3$): δ = 104.9. Anal. Calcd for $C_{28}H_{29}Cl_2O_3PRu$: C, 54.55%; H, 4.74%. Found: C, 54.58%; H, 4.83%. $C_{28}H_{29}Cl_2O_3PRu$ (616.48).

[Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac){P(OEt)$_3$}][BF$_4$] (10a). $[Ru(\eta^6\text{-}p\text{-cymene})(^tBu_2acac)Cl]$ **7** (150 mg, 330 μmol, 1.00 equiv) and $NaBF_4$ (180 mg, 1.64 mmol, 4.97 equiv) were suspended in acetone (15 mL). $P(OEt)_3$ (120 μL, 701 μmol, 2.12 equiv) was added via syringe, and the resulting mixture was heated to reflux using a heating gun and then stirred at room temperature. After 15 and 30 min, the reaction mixture was heated again to reflux for a short time. Afterward, stirring was continued at room temperature for an additional 13 h. Solvent was removed in vacuo. The residue was extracted with CH_2Cl_2 (4 × 15 mL). The extracts were filtered and concentrated in vacuo. Addition of hexane and subsequent evaporation of the residual CH_2Cl_2 led to formation of an oil, which could be crystallized from EtOAc/hexane. The precipitate was filtered off with suction, washed several times with hexane, and dried in an air stream. The desired product was obtained as a dark yellow crystalline solid (189 mg, 281 μmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 18 H, 2 × C(CH_3)$_3$ {^{t}Bu$_2$acac}), 1.24 (d, J = 7.0 Hz, 6 H, 1-CH(CH_3)$_2$), 1.31 (t, J = 7.0 Hz, 9 H, 3 × OCH$_2$CH_3 {P(OEt)$_3$}), 2.07 (s, 3 H, 4-CH_3), 2.62 (sept,

dx.doi.org/10.1021/jm300291a | J. Med. Chem. 2012, 55, 4178−4188

J = 7.0 Hz, 1 H, 1-C*H*(CH_3)$_2$), 4.05 (quint, $J_{H,H}$ = $J_{H,P}$ = 7.0 Hz, 6 H, 3 × OC*H*$_2$CH$_3$ {P(OEt)$_3$}), 5.68 (s, 1 H, COC*H*CO {^{t}Bu$_2$acac}), 5.77 (m$_c$, 4 H, 2-H, 3-H, 5-H, 6-H). ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$): δ = 16.15 (d, $J_{C,P}$ = 6.6 Hz, 3 × OCH$_2$C*H*$_3$ {P(OEt)$_3$}), 17.25 (4-*C*H$_3$), 22.01 (1-CH(*C*H$_3$)$_2$), 28.41 (2 × C(*C*H$_3$)$_3$ {^{t}Bu$_2$acac}), 30.38 (1-*C*H(CH$_3$)$_2$), 41.48 (2 × *C*(CH$_3$)$_3$ {^{t}Bu$_2$acac}), 62.92 (d, $J_{C,P}$ = 7.3 Hz, 3 × OC*H*$_2$CH$_3$ {P(OEt)$_3$}), 89.30 (d, $J_{C,P}$ = 7.3 Hz, C-2, C-6), 92.19 (d, $J_{C,P}$ = 5.9 Hz, C-3, C-5), 92.61 (d, $J_{C,P}$ = 1.5 Hz, COC*H*CO {^{t}Bu$_2$acac}), 102.1 (C-4), 106.2 (d, $J_{C,P}$ = 1.5 Hz, C-1), 198.2 (2 × *C*O {^{t}Bu$_2$acac}). ^{31}P {^{1}H} NMR (162 MHz, $CDCl_3$): δ = 114.5. EI-MS (70 eV): m/z (%) = 635.1 (10) [M − 2 × F]$^+$, 469.0 (5) [M − 2 × F − P(OEt)$_3$]$^+$, 419.8 (13) [M − P(OEt)$_3$ − BF$_4$]$^+$, 119.2 (100) [C$_9$H$_{11}$]$^+$, 57.2 (50) [C$_4$H$_9$]$^+$. Anal. Calcd for $C_{27}H_{48}BF_4O_5PRu$: C, 48,29%; H, 7.20%. Found: C, 48.17%; H, 6.91%. $C_{27}H_{48}BF_4O_5PRu$ (671.52).

[Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac)(P(O^iPr)$_3$)][BF$_4$] (10b). [Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac)Cl] 7 (75.0 mg, 165 μmol, 1.00 equiv) and $NaBF_4$ (91.3 mg, 832 μmol, 5.04 equiv) were suspended in acetone (10 mL). P(O^iPr)$_3$ (50.0 μL, 218 μmol, 1.32 equiv) was added via syringe, and the resulting mixture was heated to reflux using a heating gun and then stirred at room temperature. After 15 and 30 min, the reaction mixture was heated again to reflux for a short time. Afterward, stirring was continued at room temperature for an additional 1.5 h. Solvent was removed in vacuo. The residue was extracted with CH_2Cl_2 (3 × 15 mL). The extracts were filtered and concentrated in vacuo. Addition of hexane and subsequent evaporation of the residual CH_2Cl_2 led to formation of an oil, which could be crystallized from EtOAc/hexane. The precipitate was filtered off with suction, washed several times with hexane, and dried in an air stream. The desired product was obtained as orange-yellow crystalline solid (89.0 mg, 125 μmol, 76%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 18 H, 2 × C(C*H*$_3$)$_3$ {^{t}Bu$_2$acac}), 1.24 (d, J = 6.8 Hz, 6 H, 1-CH(C*H*$_3$)$_2$), 1.31 (d, J = 6.1 Hz, 18 H, 3 × CH(C*H*$_3$)$_2$ {P(O^iPr)$_3$}), 2.02 (s, 3 H, 4-C*H*$_3$), 2.62 (sept, J = 6.8 Hz, 1 H, 1-C*H*(CH$_3$)$_2$), 4.62 (dsept, $J_{H,H}$ = 6.1 Hz, $J_{H,P}$ = 8.2 Hz, 3 H, 3 × C*H*(CH$_3$)$_2$ {P(O^iPr)$_3$}), 5.71 (br. d, J = 6.2 Hz, 2 H, 3-H, 5-H), 5.73 (s, 1 H, COC*H*CO {^{t}Bu$_2$acac}), 5.75 (br. d, J = 6.2 Hz, 2 H, 2-H, 6-H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$): δ = 17.27 (4-*C*H$_3$), 22.11 (1-CH(*C*H$_3$)$_2$), 24.30 (d, $J_{C,P}$ = 4.0 Hz, 3 × CH(*C*H$_3$)$_2$ {P(O^iPr)$_3$}), 28.53 (2 × C(*C*H$_3$)$_3$ {^{t}Bu$_2$acac}), 30.32 (1-*C*H(CH$_3$)$_2$), 41.38 (2 × *C*(CH$_3$)$_3$ {^{t}Bu$_2$acac}), 71.89 (d, $J_{C,P}$ = 9.3 Hz, 3 × *C*H(CH$_3$)$_2$ {P(O^iPr)$_3$}), 88.84 (d, J_{CP} = 8.0 Hz, C-2, C-6), 92.30 (d, $J_{C,P}$ = 5.3 Hz, C-3, C-5), 93.37 (COC*H*CO {^{t}Bu$_2$acac}), 102.7 (C-4), 104.1 (C-1), 198.8 (2 × *C*O {^{t}Bu$_2$acac}). ^{31}P {^{1}H} NMR (121 MHz, $CDCl_3$): δ = 107.7. EI-MS (70 eV): m/z (%) = 676.3 (4) [M − 2 × F]$^+$, 419.7 (12) [M − P(O^iPr)$_3$ − BF$_4$]$^+$, 119.2 (100) [C$_9$H$_{11}$]$^+$. Anal. Calcd for $C_{30}H_{54}BF_4O_5PRu$: C, 50.49%; H, 7.63%. Found: C, 50.62%; H, 7.37%. $C_{30}H_{54}BF_4O_5PRu$ (713.60).

[Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac){P(OPh)$_3$}][BF$_4$] (10c). [Ru(η^6-*p*-cymene)(^{t}Bu$_2$acac)Cl] 7 (75.0 mg, 165 μmol, 1.00 equiv) and $NaBF_4$ (94.0 mg, 856 μmol, 5.19 equiv) were suspended in acetone (10 mL). P(OPh)$_3$ (100 μL, 382 μmol, 2.31 equiv) was added via syringe, and the resulting mixture was heated to reflux using a heating gun and then stirred at room temperature. After 15 min, the reaction mixture was heated again to reflux for a short time. Afterward, stirring was continued at room temperature for an additional 2.5 h. Solvent was removed in vacuo. The residue was extracted with CH_2Cl_2 (3 × 15 mL). The extracts were filtered and concentrated in vacuo. Addition of EtOAc and hexane led to formation of a crystalline solid. The precipitate was filtered off with suction, washed several times with hexane, and dried in an air stream. The desired product was obtained as a yellow crystalline solid (70.7 mg, 86.7 μmol, 53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.14 (d, J = 7.0 Hz, 6 H, 1-CH(C*H*$_3$)$_2$), 1.20 (s, 18 H, 2 × C(C*H*$_3$)$_3$ {^{t}Bu$_2$acac}), 1.58 (s, 3 H, 4-C*H*$_3$), 2.20 (sept, J = 7.0 Hz, 1 H, 1-C*H*(CH$_3$)$_2$), 5.36 (br. d, J = 6.3 Hz, 2 H, 3-H, 5-H), 5.82 (br. d, J = 6.3 Hz, 2 H, 2-H, 6-H), 5.88 (s, 1 H, COC*H*CO {^{t}Bu$_2$acac}), 7.08−7.14 (m, 6 H, 3 × 2′-H, 3 × 6′-H {P(OPh)$_3$}), 7.17−7.24 (m, 3 H, 3 × 4′-H {P(OPh)$_3$}), 7.30−7.38 (m, 6 H, 3 × 3′-H, 3 × 5′-H {P(OPh)$_3$}). ^{13}C {^{1}H} NMR (101 MHz, $CDCl_3$): δ = 17.09 (4-*C*H$_3$), 21.95 (1-CH(*C*H$_3$)$_2$), 28.60 (2 × *C*(CH$_3$)$_3$ {^{t}Bu$_2$acac}), 30.53 (1-*C*H(CH$_3$)$_2$), 41.71 (2 × *C*(CH$_3$)$_3$ {^{t}Bu$_2$acac}), 89.97 (d, $J_{C,P}$ = 5.9 Hz, C-2, C-6), 92.00 (d, $J_{C,P}$ = 5.9 Hz, C-3, C-5), 93.40 (COC*H*CO {^{t}Bu$_2$acac}), 103.3 (C-4), 108.9 (d, $J_{C,P}$ = 2.2 Hz, C-1), 120.4 (d, $J_{C,P}$ = 5.1 Hz, 3 × C-2′, 3 × C-6′ {P(OPh)$_3$}), 125.8 (3 × C-4′ {P(OPh)$_3$}), 130.2 (3 × C-3′, 3 × C-5′ {P(OPh)$_3$}), 150.7 (d, $J_{C,P}$ = 11.0 Hz, 3 × C-1′ {P(OPh)$_3$}), 198.6 (2 × *C*O {^{t}Bu$_2$acac}). ^{31}P {^{1}H} NMR (121 MHz, $CDCl_3$): δ = 107.7. EI-MS (70 eV): m/z (%) = 419.9 (17) [M − P(OPh)$_3$ − BF$_4$]$^+$, 410.7 (10) [M − $C_{10}H_{14}$ − C_6H_5O − C_6H_4O]$^+$, 119.3 (100) [C$_9$H$_{11}$]$^+$. Anal. Calcd for $C_{39}H_{48}BF_4O_5PRu{\cdot}0.5H_2O$: C, 56.80%; H, 5.99%. Found: C, 56.89%; H, 6.12%. $C_{39}H_{48}BF_4O_5PRu$ (815.64).

In Vitro Culture of *E. multilocularis* Metacestodes. If not stated otherwise, all culture media were purchased from Gibco-BRL (Zürich, Switzerland) and biochemical reagents were from Sigma (St. Louis, MO, U.S.). *E. multilocularis* metacestodes dissected from experimentally infected Balb/c mice were crushed through a metal tea strainer. The metacestodes were incubated in antibiotic solution (20 μg/mL levofloxacin (Aventis, Meyrin, Switzerland), 20 μg/mL ciprofloxacin (Bayer, Zürich, Switzerland), in PBS) overnight. The next day, an amount of 5 × 10^6 rat hepatoma cells/mL (kindly provided by Klaus Brehm, Institute for Hygiene and Microbiology, University of Würzburg, Germany) was added to 1 mL of metacestodes, and medium (DMEM, 10% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate) was added to 50 mL. The metacestode/hepatoma cell cocultures were incubated in culture flasks at 37 °C, 5% CO_2, with medium and hepatoma cell changes once a week. Splitting of cultures was carried out when the total metacestode volume exceeded 20 mL. Metacestodes were used for experimental procedures when they reached diameters of 2−4 mm.

The rat hepatoma cells were maintained in the same medium. The cells were grown to total confluence, trypsinized, and diluted 1:20 in fresh culture medium once a week.

In Vitro Drug Treatment of *E. multilocularis* Metacestodes. *E. multilocularis* metacestodes were collected after 1−2 months of culture and were washed three times in PBS (Fluka Chemie, Buchs, Switzerland) in order to remove medium, debris, and broken vesicles. Treatments were carried out in medium without phenol red (DMEM, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, 2 mM L-glutamine), which was added to the same volume of vesicles and distributed to 24 well plates (Greiner Bio-One, Frickenhausen, Germany; 2 mL/well, ∼25−35 vesicles). The drugs were prepared as stocks of c = 10 mM in DMSO. Predilutions to 200 times the final concentration were prepared in medium and added to the metacestodes at c = 20 μM or lower for the dose-dependent assays. In parallel, specimens were viewed by light microscopy to assess potential drug-induced morphological damage.

Assessment of Parasite Toxicity by PGI Assay. Damage of vesicles incubated with selected drugs under axenic conditions was measured indirectly after 5 days of treatment by detecting the release of PGI, as previously described by Stadelmann et al.[36] The assay was performed in 96-well microtiter plates (Greiner Bio-One). Per well, 95 μL of assay buffer (100 mM Tris-HCl (pH 7.6), 0.5 mM NAD^+ (Fluka), 2 mM EDTA (Merck), and 1 U glucose 6-phosphate dehydrogenase) was mixed with 20 μL of each supernatant aliquot (see above). Measurements were performed in triplicate. As an assay inhibition control, the corresponding concentration of each compound was added to a reaction mix including metacestode fluid. The reaction was started by addition of fructose 6-phosphate (Fluka) to 1 mM. NAD^+ reduction to NADH was measured by reading the absorbance at 340 nm (A_{340}) each minute from 0 to 30 min on a 96-well plate reader (2300 EnSpire multilabel reader, Perkin-Elmer, Turku, Finland). Enzyme blanks (no substrate) and substrate blanks (no enzyme) were also included. Absorbance values of the enzyme blanks were subtracted from the enzyme reaction values afterward. The PGI activity of the untreated group was subtracted from the activity of the treated groups, as it represents the activity baseline. PGI activity was calculated from the corresponding linear regression parameters ($\Delta A_{340}/\Delta t$) and presented as percentage relative to the values obtained by treatment of vesicles with 1% Triton X-100. Linear regression analysis was performed using Excel (2007).

Assessment of Toxicity in Mammalian Cell Lines. Human foreskin fibroblasts were seeded in a 96-well microtiter plate (Greiner Bio-One) at a cell density of 10 000 cells/well. The cells were incubated at 37 °C, 5% CO_2 in DMEM, 10% FCS, 50 U/mL penicillin G, and 50 μg/mL streptomycin for 48 h when a confluence of around 100% was reached in a monolayer. Vero cells were cultivated similarly to the fibroblasts, but the initial density of cells per well was 5000, and the measurements were performed in DMEM without phenol red and supplemented with 1% L-glutamine. FCS and antibiotics were added to the medium as described above. Rat hepatoma cells were similarly seeded in a 96-well microtiter plate at a cell density of 10 000 cells/well. The complexes **10a**, **10b**, **10c**, and **3** (mefloquine) (in the case of human foreskin fibroblasts) dissolved in DMSO were added to final concentrations of c = 0.1, 0.5, 1, 5, 10, and 20 μM. As a negative control, the cells were treated with medium and the same amount of DMSO (final concentration of 2%) present in the treated groups. As a positive control, the cells were treated with 1% of Triton X-100. After incubation at 37 °C, 5% CO_2 for 3 days the cell vitality was assessed by an Alamar Blue assay. In short, a 200× solution (2 g/L) was made with resazurin and given to each well to a final concentration of 1×. Fluorescence at 595 nm was measured in a multilabel plate reader (2300 EnSpire multilabel reader, Perkin- Elmer, Turku, Finland) at 0 and 3 h after the addition of resazurin to the cells. The values obtained at 0 h were subtracted from the ones obtained at 3 h. The percentage of survival was calculated by standardizing the values to the untreated group (100%). For each incubation condition duplicates were measured. Linear regression analysis was performed using Excel (2007).

■ ASSOCIATED CONTENT

Supporting Information

Structures of compounds **7**, **9a−c**, and **10a−c**, including numbering patterns for spectral assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*For A.H. (for biological assays and quantification of biological effects): phone, (0041) 31-631-2384; fax, (0041) 31-631-2477; e-mail, andrew.hemphill@vetsuisse.unibe.ch. For C.A.V. (for concept of the work, synthesis, and spectroscopic characterization): phone, (0049) 3834-86-4343; fax, (0049) 3834-86-4377; e-mail, vockc@uni-greifswald.de.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Nadja Kriegel for measuring the dose−response curve of nitazoxanide **4** against *E. multilocularis* metacestodes (Figure 5, lower part). We are also indebted to Gabriele Thede and Mike Steinich for performing the NMR measurements and combustion (elemental) analyses. We are grateful to the Institute of Pharmacy, Ernst-Moritz-Arndt University of Greifswald, Germany, for the possibility of using the 400 MHz NMR spectrometer. This study was financially supported by the Swiss National Science Foundation grant 31-111780/1, the Bangerter Rhyner Foundation, Swiss Life Foundation, and Fondation Sana.

■ ABBREVIATIONS USED

AE, alveolar echinococcosis; *E.*, *Echinococcus*; PTA, 1,3,5-triaza-7-phosphaadamantane; $^{t}Bu_2$acac, 2,2,6,6-tetramethylheptanedionate; acac, acetylacetonate; PGI, phosphoglucose isomerase; EtOAc, ethyl acetate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

■ REFERENCES

(1) Moro, P.; Schantz, P. M. Echinococcosis: a review. *Int. J. Infect. Dis.* **2009**, *13*, 125−133.
(2) Jorgensen, P.; an der Heiden, M.; Kern, P.; Schöneberg, I.; Krause, G.; Alpers, K. Underreporting of human alveolar echinococcosis in Germany. *Emerging Infect. Dis.* **2008**, *14*, 935−937.
(3) Eckert, J.; Deplazes, P. Alveolar echinococcosis in humans: the current situation in Central Europe and the need for countermeasures. *Parasitol. Today* **1999**, *15*, 315−319.
(4) Eckert, J.; Conraths, F. J.; Tackmann, K. Echinococcosis: an emerging or re-emerging zoonosis. *Int. J. Parasitol.* **2000**, *30*, 1283−1294.
(5) Schweiger, A.; Ammann, R. W.; Candidnas, D.; Clavien, P. A.; Eckert, J.; Gottstein, B.; Halkic, N.; Muellhaupt, B.; Prinz, B. M.; Reichen, J.; Tarr, P. E.; Torgerson, P. R.; Deplazes, P. Human alveolar echinococcosis after fox population increase, Switzerland. *Emerging Infect. Dis.* **2007**, *13*, 878−882.
(6) Mehlhorn, H.; Eckert, J.; Thompson, R. C. A. Proliferation and metastases formation of larval *Echinococcus multilocularis*. II. Ultrastructural investigations. *Z. Parasitenkd* **1983**, *69*, 749−763.
(7) Reuter, S.; Buck, A.; Manfras, B.; Kratzer, W.; Seitz, H. M.; Darge, K.; Reske, S. N.; Kern, P. Structured treatment interruption in patients with alveolar echinococcosis. *Hepatology* **2004**, *39*, 509−515.
(8) Hemphill, A.; Stadelmann, B.; Scholl, S.; Müller, J.; Spiliotis, M.; Müller, N.; Gottstein, B.; Siles-Lucas, M. *Echinococcus* metacestodes as laboratory models for the screening of drugs against cestodes and trematodes. *Parasitology* **2010**, *137*, 569−587.
(9) Küster, T.; Stadelmann, B.; Hermann, C.; Scholl, S.; Keiser, J.; Hemphill, A. In vitro and in vivo efficacies of mefloquine-based treatment against alveolar echinococcosis. *Antimicrob. Agents Chemother.* **2011**, *55*, 713−721.
(10) Reuter, S.; Manfras, B.; Merkle, M.; Härter, G.; Kern, P. In vitro activities of itraconazole, methiazole, and nitazoxanide versus *Echinococcus multilocularis* larvae. *Antimicrob. Agents Chemother.* **2006**, *50*, 2966−2970.
(11) Bergamo, A.; Sava, G. Ruthenium anticancer compounds: myths and realities of the emerging metal-based drugs. *Dalton Trans.* **2011**, *40*, 7817−7823.
(12) Ang, W. H.; Casini, A.; Sava, G.; Dyson, P. J. Organometallic ruthenium-based antitumor compounds with novel modes of action. *J. Organomet. Chem.* **2011**, *696*, 989−998.
(13) Suess-Fink, G. Arene ruthenium complexes as anticancer agents. *Dalton Trans.* **2010**, *39*, 1673−1688.
(14) Levina, A.; Mitra, A.; Lay, P. A. Recent developments in ruthenium anticancer drugs. *Metallomics* **2009**, *1*, 458−470.
(15) Hartinger, C. G.; Dyson, P. J. Bioorganometallic chemistry—from teaching paradigms to medicinal applications. *Chem. Soc. Rev.* **2009**, *38*, 391−401.
(16) Peacock, A. F. A.; Sadler, P. J. Medicinal organometallic chemistry: designing metal arene complexes as anticancer agents. *Chem. Asian J.* **2008**, *3*, 1890−1899.
(17) Jakupec, M. A.; Galanski, M.; Arion, V. B.; Hartinger, C. G.; Keppler, B. K. Antitumour metal compounds: more than theme and variations. *Dalton Trans.* **2008**, *37*, 183−194.
(18) Beckford, F.; Dourth, D.; Shaloski, M.; Didion, J.; Thessing, J.; Woods, J.; Crowell, V.; Gerasimchuk, N.; Gonzalez-Sarrias, A.; Seeram, N. P. Half-sandwich ruthenium arene complexes with thiosemicarbazones: synthesis and biological evaluation of [η^6-*p*-cymene)Ru(piperonal thiosemicarbazones)Cl]Cl complexes. *J. Inorg. Biochem.* **2011**, *105*, 1019−1029.
(19) Sathya, N.; Raja, G.; Priya, N. P.; Jayabalakrishnan, C. Ruthenium(II) complexes incorporating tridentate Schiff base ligands: synthesis, spectroscopic, redox, catalytic and biological properties. *Appl. Organomet. Chem.* **2010**, *24*, 366−373.
(20) Silva, J. J. N.; Guedes, P. M. M.; Zottis, A.; Balliano, T. L.; Silva, F. O. N.; Lopes, L. G. F.; Ellena, J.; Oliva, G.; Andricopulo, A. D.; Franco, D. W.; Silva, J. S. Novel ruthenium complexes as potential drugs for Chagas's disease: enzyme inhibition and in vitro/in vivo trypanocidal activity. *Br. J. Pharmacol.* **2010**, *160*, 260−269.

(21) Silva, J. J.; Pavanelli, W. R.; Pereira, J. C.; Silva, J. S.; Franco, D. W. Experimental chemotherapy against *Trypanosoma cruzi* infection using ruthenium nitric oxide donors. *Antimicrob. Agents Chemother.* **2009**, *53*, 4414−4421.
(22) Gabbiani, C.; Messori, L.; Cinellu, M. A.; Casini, A.; Mura, P.; Sannella, A. R.; Severini, C.; Majori, G.; Bilia, A. R.; Vincieri, F. F. Outstanding plasmocidal properties with a small panel of metallic compounds: hints for the development of new metal-based antimalarials. *J. Inorg. Biochem.* **2009**, *103*, 310−312.
(23) Martinez, A.; Rajapakse, C. S. K.; Naoulou, B.; Kopkalli, Y.; Davenport, L.; Sanchez-Delgado, R. A. The mechanism of antimalarial action of the ruthenium(II)-chloroquine complex $[RuCl_2(CQ)]_2$. *J. Biol. Inorg. Chem.* **2008**, *13*, 703−712.
(24) Rademaker-Lakhai, J. M.; Van den Bongard, D.; Pluim, D.; Beijnen, J. H.; Schellens, J. H. A phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. *Clin. Cancer Res.* **2004**, *10*, 3717−3727.
(25) Hartinger, C. G.; Zorbas-Seifried, S.; Jakupec, M. A.; Kynast, B.; Zorbas, H.; Keppler, B. K. From bench to bedside; preclinical and early clinical development of the anticancer agent indazolium *trans*-[tetrachlorobis(1*H*-indazole)ruthenate(III)] (KP1019 or FFC14A). *J. Inorg. Biochem.* **2006**, *100*, 891−904.
(26) Hartinger, C. G.; Jakupec, M. A.; Zorbas-Seifried, S.; Groessl, M.; Egger, A.; Berger, W.; Zorbas, H.; Dyson, P. J.; Keppler, B. K. *Chem. Biodiversity* **2008**, *5*, 2140−2155.
(27) Habtemariam, A.; Melchart, M.; Fernández, R.; Parsons, S.; Oswald, I. D. H.; Parkin, A.; Fabbiani, F. P. A; Davidson, J. E.; Dawson, A.; Aird, R. E.; Jodrell, D. I.; Sadler, P. J. Structure−activity relationships for cytotoxic ruthenium(II) arene complexes containing N,N-, N,O-, and O,O-chelating ligands. *J. Med. Chem.* **2006**, *49*, 6858−6868.
(28) Vock, C. A.; Renfrew, A. K.; Scopelliti, R.; Juillerat-Jeanneret, L.; Dyson, P. J. Influence of the diketonato ligand on the cytotoxicities of $[Ru(\eta^6\text{-}p\text{-cymene})(R_2acac)(PTA)]^+$ complexes (PTA = 1,3,5-triaza-7-phosphaadamantane). *Eur. J. Inorg. Chem.* **2008**, 1661−1671.
(29) Lastra-Barreira, B.; Diez, J.; Crochet, P. Highly water-soluble arene-ruthenium(II) complexes: application to catalytic isomerization of allylic alcohols in aqueous medium. *Green Chem.* **2009**, *11*, 1681−1686.
(30) Hanif, M.; Meier, S. M.; Kandioller, W.; Bytzek, A.; Hejl, M.; Hartinger, C. G.; Nazarov, A. A.; Arion, V. B.; Jakupec, M. A.; Dyson, P. J.; Keppler, B. K. From hydrolytically labile to hydrolytically stable Ru^{II}−arene anticancer complexes with carbohydrate-derived co-ligands. *J. Inorg. Biochem.* **2011**, *105*, 224−231.
(31) Hanif, M.; Nazarov, A. A.; Hartinger, C. G.; Kandioller, W.; Jakupec, M. A.; Arion, V. B.; Dyson, P. J.; Keppler, B. K. Osmium(II)− versus ruthenium(II)−arene carbo-hydrate-based anticancer compounds: similarities and differences. *Dalton Trans.* **2010**, *39*, 7345−7352.
(32) Berger, I.; Hanif, M.; Nazarov, A. A.; Hartinger, C. G.; John, R. O.; Kuznetsov, M. L.; Groessl, M.; Schmitt, F.; Zava, O.; Biba, F.; Arion, V. B.; Galanski, M.; Jakupec, M. A.; Juillerat-Jeanneret, L.; Dyson, P. J.; Keppler, B. K. In vitro anticancer activity and biologically relevant metabolization of organometallic ruthenium complexes with carbohydrate-based ligands. *Chem.—Eur. J.* **2008**, *14*, 9046−9057.
(33) Hodson, E.; Simpson, S. J. Synthesis and characterisation of $[(\eta^6\text{-cymene})Ru(L)X_2]$ compounds: single crystal X-ray structure of $[(\eta^6\text{-cymene})Ru(P\{OPh\}_3)Cl_2]$ at 203 K. *Polyhedron* **2004**, *23*, 2695−2707.
(34) Carmona, D.; Ferrer, J.; Oro, L. A.; Apreda, M. C.; Foces-Foces, C.; Cano, F. H.; Elguero, J.; Jimeno, M. L. Synthesis, X-ray structure, and nuclear magnetic resonance (^1H and ^{13}C) studies of ruthenium-(II) complexes containing pyrazolyl ligands. *J. Chem. Soc., Dalton Trans.* **1990**, 1463−1476.
(35) Bohlmann, F.; Zeisberg, R.; Klein, E. ^{13}C-NMR-spektren von monoterpenen. *Org. Magn. Reson.* **1975**, *7*, 426−432.
(36) Stadelmann, B.; Scholl, S.; Müller, J.; Hemphill, A. Application of an in vitro drug screening assay based on the release of phosphoglucose isomerase to determine the structure−activity relationship of thiazolides against *Echinococcus multilocularis* metacestodes. *J. Antimicrob. Chemother.* **2010**, *65*, 512−519.
(37) Scolaro, C.; Hartinger, C. G.; Allardyce, C. S.; Keppler, B. K.; Dyson, P. J. Hydrolysis study of the bifunctional antitumour compound RAPTA-C, $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2(pta)]$. *J. Inorg. Biochem.* **2008**, *102*, 1743−1748.
(38) Dorcier, A.; Dyson, P. J.; Gossens, C.; Rothlisberger, U.; Scopelliti, R.; Tavernelli, I. Binding of organometallic ruthenium(II) and osmium(II) complexes to an oligonucleotide: a combined mass spectrometric and theoretical study. *Organometallics* **2005**, *24*, 2114−2123.
(39) Dougan, S. J.; Melchart, M.; Habtemariam, A.; Parsons, S.; Sadler, P. J. Phenylazo-pyridine and phenylazo-pyrazole chlorido ruthenium(II) arene complexes: arene loss, aquation and cancer cell cytotoxicity. *Inorg. Chem.* **2006**, *45*, 10882−10894.
(40) Bennett, M. A.; Huang, T.-N.; Matheson, T. W.; Smith, A. K. (η^6-Hexamethylbenzene)-ruthenium complexes. *Inorg. Synth.* **1982**, *21*, 74−78.
(41) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **1997**, *62*, 7512−7515.