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# Polypyridylruthenium(II) complexes exert *in vitro* and *in vivo* nematocidal activity and show significant inhibition of parasite acetylcholinesterases

Madhu Sundaraneedi[a], Ramon M. Eichenberger[b], Rafid Al-Hallaf[b], Dai Yang[b], Javier Sotillo[b], Siji Rajan[a], Phurpa Wangchuk[b], Paul R. Giacomin[b], F. Richard Keene[b,c], Alex Loukas[b], J. Grant Collins[a,1], Mark S. Pearson[b,*,1]

[a] School of Physical, Environmental and Mathematical Sciences, UNSW (ADFA), Canberra, Australian Capital Territory, 2612, Australia
[b] Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, Queensland, 4878, Australia
[c] School of Physical Sciences, University of Adelaide, Adelaide, South Australia, 5005, Australia

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ABSTRACT

Over 4.5 billion people are at risk of infection with soil transmitted helminths and there are concerns about the development of resistance to the handful of frontline nematocides in endemic populations. We investigated the anti-nematode efficacy of a series of polypyridylruthenium(II) complexes and showed they were active against L3 and adult stages of *Trichuris muris*, the rodent homologue of the causative agent of human trichuriasis, *T. trichiura*. One of the compounds, $Rubb_{12}$-mono, which was among the most potent in its ability to kill L3 ($IC_{50}$ = 3.1 ± 0.4 μM) and adult ($IC_{50}$ = 5.2 ± 0.3 μM) stage worms was assessed for efficacy in a mouse model of trichuriasis by administering 3 consecutive daily oral doses of the drug 3 weeks post infection with the murine whipworm *Trichuris muris*. Mice treated with $Rubb_{12}$-mono showed an average 66% reduction ($P$ = 0.015) in faecal egg count over two independent trials. The drugs partially exerted their activity through inhibition of acetylcholinesterases, as worms treated *in vitro* and *in vivo* showed significant decreases in the activity of this class of enzymes. Our data show that ruthenium complexes are effective against *T. muris*, a model gastro-intestinal nematode and soil-transmitted helminth. Further, knowledge of the target of ruthenium drugs can facilitate modification of current compounds to identify analogues which are even more effective and selective against *Trichuris* and other helminths of human and veterinary importance.

## 1. Introduction

More than 4.5 billion people worldwide are at risk of infection by soil-transmitted helminths (STHs) and over one-third of this number are infected with these parasites (*Ascaris lumbricoides* – 0.8 billion infections), whipworm (*Trichuris trichiura* - 0.46 billion) and hookworms (*Necator americanus* and *Ancylostoma duodenale* - 0.44 billion) (Pullan et al., 2014). STHs are prevalent in more than 100 countries (Gan et al., 2009), particularly in developing tropical and subtropical regions (Sub-Saharan Africa, East Asia and South America) where the hygiene conditions and sanitation practises are poor.

STH infections rarely cause death; rather, they cause chronic and insidious effects on the host's health with clinical manifestations correlating with infection intensity. Heavy infection causes extensive morbidities such as intestinal disturbances, nutrition loss, physical and intellectual growth retardation and severe anaemia, particularly in pre- and school-aged children (Brooker et al., 1999; Stoltzfus et al., 2000). STH remains a serious problem in public health with a global burden of 4.98 million years lost to disability (YLDs) (Pullan et al., 2014), which approaches that caused by malaria.

Despite the public-health importance of these infections, these diseases have been neglected by the medical and international communities, predominantly because they are concentrated in very poor communities and the diseases can often be overshadowed by other public health or social issues. But, in recent years, the control of STH has become more important in public health management, and combat strategies have been proposed after the World Health Assembly resolution in 2001, with the administration of anthelmintics being the cornerstone of these programmes (Loukas and Bethony, 2008).

Five drugs (albendazole, mebendazole, pyrantel pamoate,

* Corresponding author.
*E-mail address:* mark.pearson@jcu.edu.au (M.S. Pearson).
[1] Both authors contributed equally.

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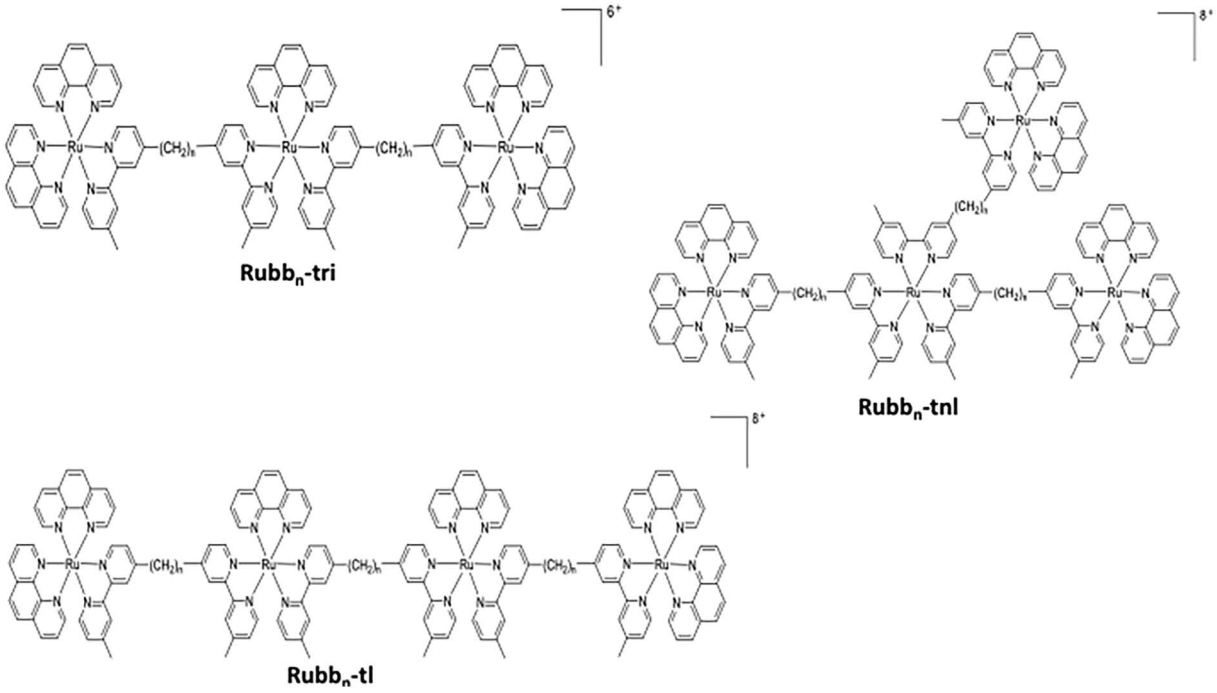


**Fig. 1.** The inert tri-nuclear (Rubb$_n$-tri), linear tetra-nuclear (Rubb$_n$-tl) and non-linear tetra-nuclear (Rubb$_n$tnl) ruthenium complexes.

levamisole, and ivermectin) are currently available for the treatment of STH, yet control programmes rely heavily on only the two benzimidazoles (albendazole and mebendazole) because of economical and operational feasibility (Tritten et al., 2011).

There is no direct evidence for emerging resistance to any of the current anthelmintics in human helminth populations, however, low cure rates have been reported for *T. trichiura* and hookworm infections (Keiser and Utzinger, 2010). Repeated and prolonged administration of these anthelmintics, necessitated by sub-optimal drug efficacies, increases the risk of resistance developing and has already been well documented among nematodes of veterinary importance, and is increasing in frequency as a consequence of extensive use of benzimidazoles over extended periods of time (Kaplan, 2004).

Among potential targets for nematode chemotherapy are acetylcholinesterases (AChEs). These enzymes catalyse the rapid breakdown of the neurotransmitter acetylcholine (ACh) in both central and peripheral nervous systems of eukaryotic organisms, and so control neuronal function (Massoulie et al., 1993). In addition to controlling cholinergic synapses, multiple isoforms of the enzyme are secreted from many nematodes in large amounts and have been implicated in mediating pathogenesis of nematode infection by modulating the host immune system through the disruption of host cholinergic signalling (Selkirk et al., 2005; Vaux et al., 2016) and providing acetate and choline precursors for helminth metabolism (Lee, 1996).

With respect to its termination of synaptic transmission, inhibition of AChE produces an excess accumulation of ACh and overstimulation of its receptors, causing uncoordinated neuromuscular function that often results in death due to respiratory paralysis (Thapa et al., 2017). As such, AChE inhibitors are widely used as pesticides (Kwong, 2002) and anthelmintics (Orhan, 2013). Indeed, metrifonate, an organophosphorus AChE inhibitor originally used as an insecticide, has also been used for the treatment of human and veterinary nematode infections (Thompson et al., 1996) but now has limited use as a therapeutic because of off-target toxicity (Kramer et al., 2014). Moreover, the frontline nematocides all target the neuromusculature of these parasites (Thompson et al., 1996) although they are not necessarily inhibitors of AChE.

In addition to organophosphates, mono-nuclear chemical complexes of the transition metal ruthenium have been shown to target and inhibit enzymes such as AChE (Vyas et al., 2014), and there are numerous recent studies documenting the efficacy of polypyridylruthenium(II) complexes against a variety of different microbial pathogens (Li et al., 2011; Pandrala et al., 2013; Gorle et al., 2014). Unlike their organophosphorus counterparts, ruthenium complexes are speculated to exert their inhibitory effects through a combination of electrostatic and hydrophobic interactions at the peripheral anionic (PAS) site of AChE, which is located at the gate of the enzyme's catalytic gorge (Bourne et al., 2005), and not through direct interaction with the active site. Ruthenium complexes are thought to be less toxic to human cells than small-molecule inhibitors because of this mode of inhibition and also because the overall neutral charge in the outer membrane leaflet of eukaryotic cells (Mason et al., 2007) creates a greatly reduced capacity for electrostatic interaction with the metal compounds (Gorle et al., 2016).

Herein, we demonstrate the AChE-inhibitory action of two mononuclear and a series of di-, tri- and tetra-nuclear polypyridylruthenium (II) (ruthenium) complexes linked by the bis[4(4′-methyl-2,2′-bipyridyl)]-1,n-alkane ligand ("bb$_n$"; n = 7, 10, 12 and 16) against extracts of *T. muris* and *Ancylostoma caninum* (helminths used as models of human trichuriasis and hookworm infection, respectively) and both adult and L3 stage *T. muris* parasites *in vitro*. We also show the *in vivo* efficacy of one of these ruthenium complexes (Rubb$_{12}$-mono) in a mouse model of trichuriasis, providing evidence that drugs based on these compounds could be a valuable addition to the chemotherapeutic arsenal against both human and veterinary nematode infections.

## 2. Materials and methods

### 2.1. Nomenclature and preparation of ruthenium complexes

$[Ru(phen)_2(Me_2bpy)]^{2+}$ (phen = 1,10-phenanthroline; $Me_2$bpy = 4,4′-dimethyl-2,2′-bipridine) and the mononuclear (Rubb$_n$-mono), dinuclear (Rubb$_n$-di), trinuclear (Rubb$_n$-tri), tetranuclear linear (Rubb$_n$-tl) and tetranuclear non-linear (Rubb$_n$-tnl) polypyridylruthenium(II) complexes (Fig. 1) were synthesised using the appropriate bis[4′-(4-methyl-2,2′-bipyridyl)]-1,n-alkane ligand (bb$_n$) as

previously described (Gorle et al., 2014). All ruthenium complexes were chloride salts and were dissolved in $H_2O$ at stock concentrations of 1 mM.

### 2.2. Animals and parasites

Six-to eight-week old male B10.BR mice were purchased from the Animal Resource Centre, Canningvale, Western Australia and allowed to acclimatise for one week before infection. Mice were orally infected with 200 µl of PBS containing approximately 200 live embryonated *T. muris* eggs. All animals were kept in groups of 5 mice in cages with free access to water and food and with a 12-h light/dark cycle. Third stage larvae (L3) and adult worms were harvested from the caecum by sacrificing the mice after three weeks and five weeks, respectively. Both stages were washed with PBS containing 2 × antibiotic/antimycotic (AA) and used for either extract preparation (adult) or *in vitro* experiments (adult and L3).

### 2.3. Parasite extract preparation

Freshly harvested adult *T. muris* or *A. caninum* (thawed from -80 °C stocks) were homogenised (10 parasites/200 µl) in PBS containing 1% Triton X-100, 40 mM Tris-HCl, pH 7.4, at 4 °C using a TissueLyser II (Qiagen) and the supernatant collected by centrifugation at 15,000 *g* for 60 min at 4 °C. Protein concentration was determined using the Pierce BCA Protein Assay kit (Thermofisher), aliquoted and stored at -80 °C until use.

### 2.4. Enzyme activity in parasite extracts and inhibition assays

AChE activity in Triton X-100-soluble adult *T. muris* and *A. caninum* extracts was determined in a Polarstar Omega microplate reader (200 µl final volume in 96-well plates) using the Ellman method (Ellman et al., 1961). Extracts were serially diluted (40-10 µg) in AChE assay buffer (0.1 M sodium phosphate, pH 7.4), 2 mM acetyl-thiocholine (AcSCh) and 0.5 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were added and absorbance was measured (405 nm) every 10 min for 5 h at 37 °C. Specific activity was calculated using the initial velocity of the reaction. For AChE inhibition assays, extract equal to a specific activity of 0.55 nmol/min/well (*T. muris* - 30 µg, *A. caninum* – 20 µg) were diluted in AChE assay buffer to a final volume of 170 µl and pre-incubated with ruthenium complexes (10 nM–100 µM) for 20 min at RT. AcSCh and DTNB were added at 2 mM and 0.5 mM, respectively and absorbance was measured (405 nm) every 10 min for 5 h at 37 °C. Inhibition for each sample was calculated relative to the negative control (reactions without ruthenium complexes) and reactions were performed in duplicate.

### 2.5. In vitro activity of ruthenium complexes against adult and L3 stage T. muris

Ruthenium complexes were dissolved in sterile PBS for use in the assay. Adult or L3 stage worms were transferred in groups of 4 into each well of a 48-well plate containing 0.5 ml pre-warmed RPMI medium supplemented with 2 × AA and cultured with ruthenium complexes (50 µM). Control worms were treated with the PBS vehicle. All worms were incubated at 37 °C and 5% $CO_2$ for 72 h and monitored every 24 h for motility by microscopic examination (20 × ). The viability of the worms was evaluated according to previously reported protocols (Tritten et al., 2012; Keiser et al., 2013) using a motility scale from 0 to 3 (0 = dead, 1 = very low motility, 2 = low motility, 3 = normal motility). $IC_{50}$ values of the two most effective ruthenium complexes were then similarly tested at different concentrations ranging from 3.125 to 50 µM. Assays were conducted in duplicate and data is represented as the average of these two experiments ± SE. Albendazole was used at 200 µg/ml - the $IC_{50}$ previously determined against adult and L3 stage *T. muris* (Tritten et al., 2011) - as a positive control in all experiments.

### 2.6. Enzyme inhibition studies on ruthenium complex-treated adult T. muris

Groups of five, freshly isolated adult *T. muris* were cultured in the presence of sub-lethal concentrations of either $Rubb_{12}$-mono and $Rubb_{12}$-tri (6.25 µM in PBS) or PBS alone in RPMI medium supplemented with 2 × AA. Three sets of five worms were used for each drug tested. Worms were collected after 24 h when they were still motile. Triton X-100-soluble extracts were made from each set of five worms (so, three extracts for each drug tested), quantified using the Pierce BCA Protein Assay kit (Thermofisher) and immediately assayed for AChE activity. Each assay was technically replicated three times. For each enzyme assay, activities of drug-treated parasites were expressed relative to worms cultured without ruthenium complexes (negative controls). Data is the average of assays run on triplicate extracts and three technical replicates of each assay ± SE.

### 2.7. Tolerability study

In order to determine the appropriate dose of $Rubb_{12}$-mono to be used in the *in vivo* study, the maximum tolerated dose was determined for the B10.BR mouse strain. $Rubb_{12}$-mono was administered to groups of three male mice for three consecutive days. The doses ranged from 5 to 20 mg/kg. Animals were closely monitored for adverse clinical signs throughout the study and mice showing adverse effects were euthanised using $CO_2$ asphyxiation. The highest dose that did not cause any adverse clinical signs for three consecutive daily doses was considered to be the maximum tolerated dose (MTD).

### 2.8. In vivo efficacy of $Rubb_{12}$-mono

The *in vivo* efficacy of $Rubb_{12}$-mono was tested in two independent trials. For each trial, groups of nine male B10.BR mice (6-8 weeks old) were each orally infected with 200 µL of PBS containing approximately 200 live embryonated *T. muris* eggs. At 28 days p.i., one group was given three consecutive daily oral doses (200 µl) of $Rubb_{12}$-mono (2 × 10 mg/kg in PBS and 1 × 5 mg/kg in PBS) and the other (negative control group) was given three consecutive daily oral doses (200 µl) of PBS. Mice were sacrificed at 35 days p.i. and worms were harvested from the caecum and counted manually using light microscopy (20 × magnification). The worm burden reduction (WBR) was calculated as: $[[(a - b)/a] \times 100]$, where $a$ = average worm count in the negative control group and $b$ = average worm count in a treated group. Faecal samples (approximately 0.1 g) from each individual mouse were collected 24 h before necropsy, homogenised in 2 ml of saturated NaCl overnight at 4 °C and the number of eggs counted in triplicate using a Whitlock McMaster counting chamber. This number was used to determine the number of eggs per gram (EPG) of faeces and reduction calculated as for WBR. Extracts were prepared from equal numbers of worms harvested from each individual mouse of each group (trial 1 control – n = 9 mice, trial 1 treatment – n = 6 mice, trial 2 control – n = 9 mice, trial 2 treatment – n = 7 mice) and assayed in triplicate for AChE activity. For each trial, differences in AChE activity was calculated by comparing the average of all extracts from the treatment group with the average ( ± SE) of all extracts from the control group.

### 2.9. Statistical analyses

Statistical analyses were performed using Graphpad Prism 7. Inhibition curves and $IC_{50}$ values were generated using sigmoidal dose-response (variable slope) with a non-linear fit model. One-way ANOVA with Dunn's multiple comparison was used to determine significance ($p$), which was set at 0.05. In the case where only two groups were compared (*in vivo* studies), student's *t*-test with Welch's correction was

**Table 1**
Inhibition and the potency ($IC_{50}$) of the series of ruthenium complexes against AChE activity in adult *T. muris* extracts. Ruthenium complexes that showed the highest potency are highlighted (bold).

| Compound | AChE inhibition (%)[a, b] | $IC_{50}$ (μM)[b] |
|---|---|---|
| $[Ru(phen)_2(Me_2bpy)]^{2+}$ | 0.7 ± 0.6 | 31.26 ± 1.17 |
| $Rubb_7$-tri | 81.6 ± 0.3 | 0.29 ± 0.00 |
| $Rubb_7$-tl | 76.9 ± 0.5 | 0.32 ± 0.02 |
| **$Rubb_7$-tnl** | **93.2 ± 0.5** | **0.12 ± 0.02** |
| **$Rubb_{10}$-tri** | **86.8 ± 0.7** | **0.06 ± 0.00** |
| $Rubb_{12}$-mono | 5.0 ± 0.3 | 2.30 ± 0.25 |
| $Rubb_{12}$-di | 68.7 ± 0.9 | 0.35 ± 0.08 |
| $Rubb_{12}$-tri | 65.4 ± 0.2 | 1.00 ± 0.05 |
| $Rubb_{12}$-tl | 81.2 ± 0.1 | 0.83 ± 0.06 |
| $Rubb_{12}$-tnl | 90.7 ± 0.8 | 0.24 ± 0.01 |
| $Rubb_{16}$-tri | 76.4 ± 0.7 | 0.47 ± 0.04 |
| $Rubb_{16}$-tl | 69.5 ± 0.7 | 0.47 ± 0.04 |
| $Rubb_{16}$-tnl | 91.9 ± 0.0 | 0.25 ± 0.00 |

[a] Inhibition of AChE activity assessed at 1 μM.
[b] Data represent the mean of duplicate experiments ± SE.

**Table 2**
Inhibition and the potency ($IC_{50}$) of the series of ruthenium complexes against AChE activity in adult *A. caninum* extracts. Ruthenium complexes that showed the highest potency are highlighted (bold).

| Compound | AChE inhibition (%)[a, b] | $IC_{50}$ (μM)[b] |
|---|---|---|
| $[Ru(phen)_2(Me_2bpy)]^{2+}$ | 56.8 ± 0.0 | 0.6 ± 0.0 |
| $Rubb_7$-tri | 71.4 ± 1.0 | ND |
| **$Rubb_7$-tl** | **76.6 ± 0.1** | **0.2 ± 0.0** |
| $Rubb_7$-tnl | 47.1 ± 0.5 | 0.8 ± 0.0 |
| $Rubb_{10}$-tri | 58.5 ± 0.1 | 1.0 ± 0.0 |
| $Rubb_{12}$-mono | 18.4 ± 3.4 | 18.1 ± 0.2 |
| $Rubb_{12}$-di | 57.1 ± 1.0 | 0.8 ± 0.0 |
| $Rubb_{12}$-tri | 55.4 ± 0.4 | 0.9 ± 0.0 |
| **$Rubb_{12}$-tl** | **59.7 ± 0.2** | **0.6 ± 0.0** |
| $Rubb_{12}$-tnl | 39.4 ± 0.7 | 1.4 ± 0.1 |
| $Rubb_{16}$-tri | 43.6 ± 0.2 | ND |
| $Rubb_{16}$-tl | 37.0 ± 1.0 | 1.0 ± 0.1 |
| $Rubb_{16}$-tnl | 27.6 ± 2.1 | 3.5 ± 0.3 |

[a] Inhibition of AChE activity assessed at 1 μM.
[b] Data represent the mean of duplicate experiments ± SE.

used.

### 2.10. Animal ethics approval

The James Cook University (JCU) animal ethics committee approved all experimental work involving animals (ethics approval number A2271). Mice were raised in cages in the JCU quarantine facility for the duration of the experiments in compliance with the 2007 Australian Code of Practice for the Care and use of Animals for Scientific Purposes and the 2001 Queensland Animal Care and Protection Act. All reasonable efforts were made to minimise animal suffering.

## 3. Results

### 3.1. Inhibition of AChE activity in adult T. muris extracts

A series of ruthenium complexes were screened at 1 μM for AChE inhibitory activity in *T. muris* adult parasite extract. Except for $[Ru(phen)_2(Me_2bpy)]^{2+}$ and $Rubb_{12}$-mono, all complexes inhibited 65-93% AChE activity (Table 1). The $IC_{50}$ for each ruthenium complex against AChE activity was also determined. Except for $[Ru(phen)_2(Me_2bpy)]^{2+}$, all complexes exhibited $IC_{50}$ values between sub- to low-micromolar concentrations with $Rubb_{10}$-tri and $Rubb_7$-tnl being the most potent, having $IC_{50}$ values of 61 and 120 nM, respectively (Table 1).

### 3.2. Inhibition of AChE activity in adult A. caninum extracts

Since the ruthenium complexes inhibited AChE activity in *T. muris* extract, we explored the inhibitory activities of the compounds in extracts of *A. caninum* (Table 2). The series displayed a more varied range of activity than was seen for *T. muris*, with AChE inhibition ranging from 18 to 76%. $Rubb_7$-tl and $Rubb_{12}$-tl showed the greatest inhibitory activity against *A. caninum* extracts, with $IC_{50}$ values of 0.2 ± 0.01 μM and 0.6 ± 0.03 μM, respectively. AChE inhibition increased with increasing nuclearity of the ruthenium centres only to the level of the dinuclear complex, with the inhibition by $Rubb_{12}$-mono < $Rubb_{12}$-di ≈ $Rubb_{12}$-tri ≈ $Rubb_{12}$-tl. The tetra-linear complexes showed greater activity compared to their non-linear counterparts and the inhibitory activity of the tri-nuclear complexes decreased with increasing chain length of the linking ligand ($bb_n$) (Table 2) (Fig. 2).

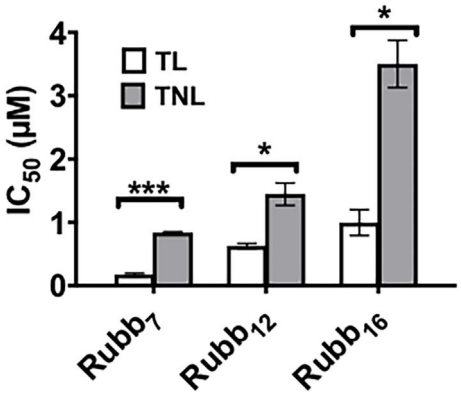


**Fig. 2. Activity of tetra-nuclear (linear - TL and non-linear - TNL) ruthenium complexes against AChE in *A. caninum* extracts.** $IC_{50}$ values are the mean of duplicate experiments ± SE. Significance determined by student's *t*-test. *P ≤ 0.05, ***P ≤ 0.001.

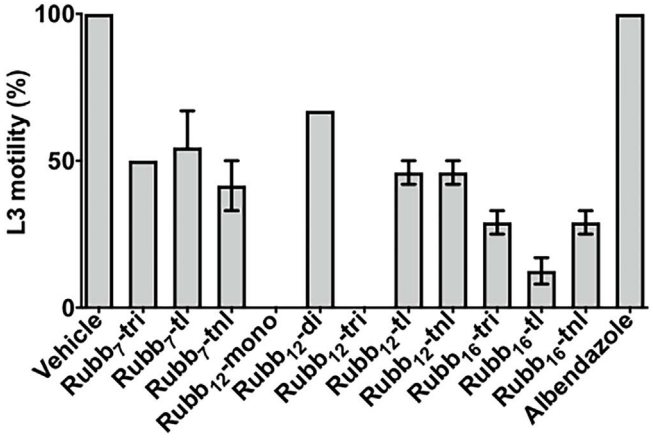


**Fig. 3. Activity of ruthenium complexes against *T. muris* third stage larvae (L3).** Survival of cultured parasites in RPMI medium after treatment with Ru complexes (50 μM) for 24 h. Albendazole was used at 200 μg/ml. Experiments were performed in duplicate and data represents the average of these two experiments ± SE.

### 3.3. In vitro effect of ruthenium complexes against L3 and adult T. muris

Four L3 *T. muris* larvae were cultured in the presence of each ruthenium complex (50 μM) and the parasite motility was monitored at 24 and 48 h (Fig. 3). Treatment with $Rubb_{12}$-mono and $Rubb_{12}$-tri complexes resulted in 100% worm death in 24 h, whereas most of the

**Table 3**
Potency of $Rubb_{12}$-mono and $Rubb_{12}$-tri against L3 and adult stages of *T. muris*.

| Compound | $IC_{50}$ (μM)[a,b] | |
|---|---|---|
| | L3 | adult |
| $Rubb_{12}$-mono | 3.1 ± 0.4 | 5.2 ± 0.3 |
| $Rubb_{12}$-tri | 2.3 ± 0.3 | 5.5 ± 0.7 |

[a] Data represent the mean of duplicate experiments ± SE.
[b] Values determined after 24 h (L3) and 48 h (adult) incubation with compounds.

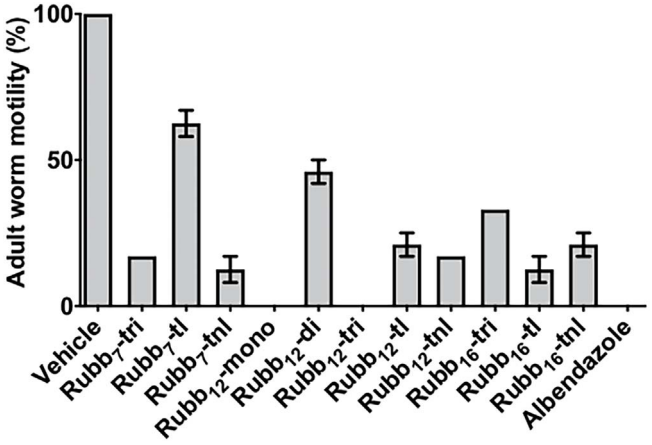


**Fig. 4. Activity of ruthenium complexes against *T. muris* adult parasites**. Survival of cultured worms in RPMI medium after treatment with Ru complexes (50 μM) for 24 h. Albendazole was used at 200 μg/ml. Experiments were performed in duplicate and data represents the average of these two experiments ± SE.

other complexes killed 100% of the worms after 48 h (data not shown). Subsequently, the most active complexes, $Rubb_{12}$-mono and $Rubb_{12}$-tri, were tested at different concentrations against L3 stage worms and displayed $IC_{50}$ values of 3.1 ± 0.4 and 2.3 ± 0.3 μM, respectively (Table 3). In identical experiments with adult parasites, $Rubb_{12}$-mono and $Rubb_{12}$-tri were the most effective, killing 100% of adult worms in 24 h (Fig. 4). $IC_{50}$ values were subsequently determined as 3.1 ± 0.4 and 2.3 ± 0.3 μM, respectively (Table 3).

### 3.4. Mechanism of action of ruthenium complexes against T. muris

Given the AChE inhibitory effect exhibited by ruthenium complexes against adult parasite extracts and their efficacy in anti-*Trichuris* assays *in vitro*, their mechanism of action was analysed in live adult worms. Extracts made from parasites cultured for 24 h in the presence of sub-lethal concentrations of $Rubb_{12}$-mono and $Rubb_{12}$ –tri (the two most effective compounds against both L3 and adult *T. muris*) were assayed for AChE activity, and significant reductions were seen in the treated worms ($Rubb_{12}$-mono - 42%, $P < 0.0001$; $Rubb_{12}$-tri - 44%, $P < 0.0001$) compared to controls (Fig. 5).

### 3.5. Tolerability of $Rubb_{12}$-mono

The tolerability of $Rubb_{12}$-mono in mice was evaluated using consecutive daily oral doses of 5, 10 and 20 mg/kg. According to standard practice, the MTD was considered to be the highest dose where no signs of physical stress were observed. $Rubb_{12}$-mono was not tolerated at 20 mg/kg (1 out of 3 mice died within 15 min) but did not show any toxicity at 10 mg/kg, even after three consecutive oral doses. The MTD of $Rubb_{12}$-mono was considered to be 10 mg/kg and was used as the dose in the *in vivo* efficacy study.

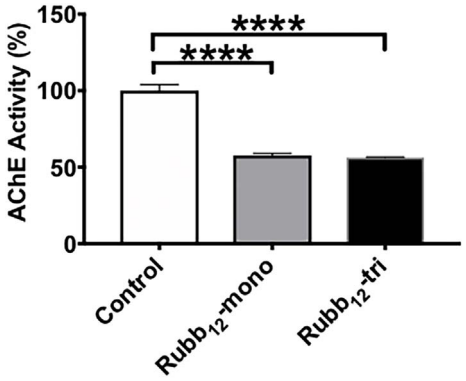


**Fig. 5. Action of $Rubb_{12}$-mono and $Rubb_{12}$-tri on adult *T. muris* AChE activity**. Worms were cultured in RPMI for 24 h in the presence of a sub-lethal dose (6.25 μM) of $Rubb_{12}$-mono or $Rubb_{12}$-tri or PBS (three groups of five worms were used for each treatment). Triton X-100-soluble extracts were made from each group of worms (so, three extracts per treatment) and 30 μg of each extract was used for enzyme activity assays. Data is the average of assays run on triplicate extracts and three technical replicates of each assay ± SE. Differences were measured by ANOVA. ****$P \leq 0.0001$.

### 3.6. In vivo efficacy of $Rubb_{12}$-mono against T. muris

Based on the significant *in vitro* activity against *T. muris* adults and L3 stage worms, $Rubb_{12}$-mono was further assessed for its *in vivo* efficacy using a mouse model of *T. muris* infection. Three weeks after infection, $Rubb_{12}$-mono was orally administrated once daily for 2 day at 10 mg/kg with a 5 mg/kg dose administered on the third day. Worm and faecal egg counts were assessed at 5 weeks p.i. (Fig. 6). For trial 1, $Rubb_{12}$-mono-treated mice achieved significant reductions in worm (35%, $P = 0.0396$) and egg (82%, $P = 0.0034$) counts, compared to PBS treated controls. Further, extracts made from worms recovered from treated mice showed significantly less AChE activity ($P = 0.0044$) than extracts from worms recovered from control animals. There was a non-significant decrease (11%, $P = 0.279$) in the worm burden of $Rubb_{12}$-mono-treated mice in trial 2 compared to controls and no difference in the AChE activity of extracts made from worms recovered from treated and control mice. However, the faecal egg count seen in treated mice was significantly lower (50%, $P = 0.0155$) than control animals from this trial. Over the two independent trials, the average reduction in faecal egg count was 66% ($P = 0.015$), compared to controls.

## 4. Discussion

Over 1.5 billion people are infected with soil-transmitted helminths (STHs), no anti-STH vaccines are available and there is evidence that resistance to the handful of drugs used to control this scourge is developing. In this study, we have investigated the activity of a series of ruthenium complexes against two model species of STHs - *T. muris* and *A. caninum* - have shown that these compounds are capable of inhibiting AChE activity in extracts made from the parasites and, at least for *T. muris*, the nematocidal action of ruthenium complexes could be partially due to AChE inhibition, an enzyme vital to the neuromuscular function of these helminths.

With regard to the AChE-inhibitory properties of ruthenium complexes against parasite extracts, no specific relationship between compound structure and activity was observed with *T. muris* extracts, although all the ruthenium complexes inhibited AChE activity at submicromolar to micromolar concentrations with specific tri- and tetranuclear complexes displaying the most potent activity. Conversely, a structure/activity relationship was observed for tetra-nuclear ruthenium complex inhibition of *A. caninum* extracts, with potency increasing with decreasing chain length between the ruthenium centres. The mononuclear ruthenium complexes are thought to interact with the

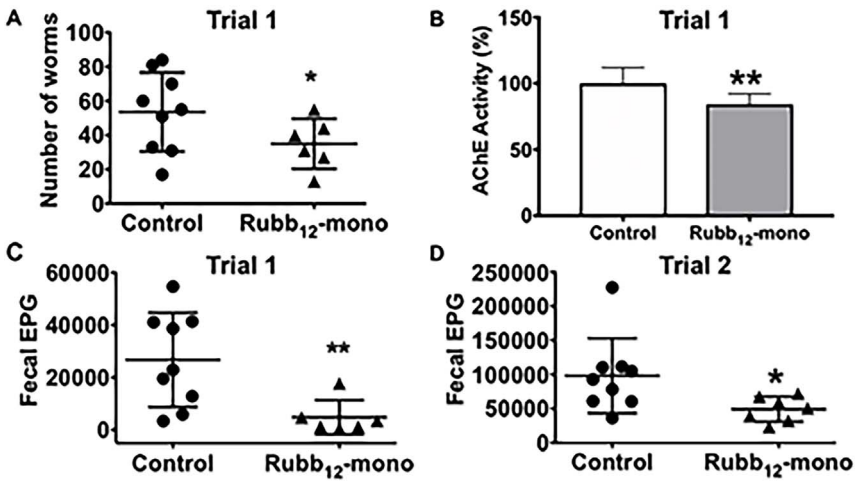


**Fig. 6. Treatment of *T. muris*-infected mice with $Rubb_{12}$-mono.** Decrease in trial 1 worm burden (A) was determined by comparing parasite burdens from treated and control mice. (B) AChE activity in Triton X-100 extracts made from worms recovered from control and treated mice. Extracts were made from equal amounts of worms collected from all surviving mice (control group – n = 9, treatment group – n = 6) and 30 μg of each extract was used to determine enzyme activity (performed in duplicate). Data is the mean of enzyme activity calculated from each individual extract for each group ± SE. Decrease in faecal egg count for (C) trial 1 and (D) trial 2 was determined by comparing faecal egg counts of control and treated mice. Symbols represent the average of triplicate counts from faeces obtained from individual mice. Differences were measured by unpaired *t*-test with Welch's correction. *P ≤ 0.05, **P ≤ 0.01. In trial 2, the decrease in adult worm burden and reduction in enzyme activity from parasites recovered was not significant.

PAS of AChE located at the rim of the active-centre gorge through a combination of electrostatic and hydrophobic interactions (Meggers, 2009). The tri- and tetra-nuclear complexes showed greater activity compared to mononuclear complexes, presumably due to the presence of the flexibly-linked multiple metal centres which may provide more interactions (electrostatic and hydrophobic) with the PAS, or each individual centre may contribute nonspecific additional points of contact. That the activity of the ruthenium complexes varied between *T. muris* and *A. caninum* extracts is likely due to differences in enzyme orthologues and the existence of multiple isoforms of AChE which are present in different helminth species (Arnon et al., 1999; Selkirk et al., 2005).

Encouraged by the activity of ruthenium complexes in *T. muris* adult parasite extracts, their effect against the L3 and adult stages *in vitro* were examined. With minor differences in sensitivity, $Rubb_{12}$-mono and $Rubb_{12}$-tri showed excellent activity against both stages of the parasite with both complexes exhibiting dose-dependent lethal effects. However, the results of motility experiments did not correlate with inhibition of enzyme activity in worm extracts, especially with $Rubb_{12}$-mono, which was one of the most effective compounds against both L3 and adult parasites, but not with regards to inhibiting AChE activity in extracts. This is possibly due to the differential accessibility of inhibitor to enzyme in an extract preparation compared to a living parasite. Another explanation is that the mechanism of action of ruthenium complexes is multi-faceted and not just confined to AChE inhibition. AChE activity significantly decreased in extracts made from cultured worms after treatment with sub-lethal concentrations of $Rubb_{12}$-mono and $Rubb_{12}$-tri ruthenium complexes, suggesting that ruthenium complexes are active against *T. muris* through AChE inhibition, but the possibility of the compounds exerting their nematocidal activity through interaction with other targets cannot be discounted given their documented ability to interact with molecules other than AChE, such as protein and lipid kinases (Meggers, 2009). There are numerous reports in the literature documenting the development of drug resistance in parasites due to mutation of the compound's target molecule (for example, benzamidazole resistance in nematodes due to single nucleotide polymorphisms in β-tubulin (Von Samson-Himmelstjerna et al., 2007) and mutation of a schistosome sulfotransferase resulting in resistance to oxamniquine (Valentim et al., 2013), and so the use of a drug that is directed against multiple molecular targets may decrease the chance of resistance evolving.

$Rubb_{12}$-mono was one of the most effective compounds with regards to *in vitro* anti-*Trichuris* activity and was therefore tested for efficacy in a mouse model of *Trichuris* infection. Surprisingly, $Rubb_{12}$-mono was found to be more toxic by oral administration than what has been reported for simple polypyridylruthenium complexes (eg: $[Ru(phen)3]^{2+}$) (Brandt et al., 1954). This is probabably due to a higher rate of absorption of $Rubb_{12}$-mono from the intestine into the blood. The complex showed promising *in vivo* activity at tolerated doses in mice, with treatment resulting in reductions in worm (only significant in tria1 1) and egg counts (significant in both trials), compared to controls. $Rubb_{12}$-mono-treated mice in trial 2 had a significantly ($P = 0.0103$) higher worm load than those in trial 1 and this may explain the reduced efficacy of $Rubb_{12}$-mono in the repeat trial. Regardless of the variation in parasite load, reduction in egg count was not only significant in both trials but also more pronounced than worm decrease, implying that egg count reduction was not just concomitant with worm burden reduction and that $Rubb_{12}$-mono treatment affected parasite maturation and fecundity. Further, parasites recovered from $Rubb_{12}$-mono-treated mice showed significantly decreased AChE activity than those harvested from control mice (consistent with *in vitro* results), suggesting that nematocidal activity was a result of drug-induced inhibition of enzyme activity. Since reductions in both worm and egg counts are required for efficacy of anthelmintics, the *in vivo* results suggest the potential of $Rubb_{12}$-mono as a drug lead for the development of novel nematocides to reduce infection pathology and interrupt disease transmission. Further, due to the uniquely shared neurobiology of helminths compared with higher eukaryotes (Thompson et al., 1996), we believe ruthenium complexes could exert broad spectrum anthelmintic activity while being relatively non-toxic and, due to the modular nature of these compounds, these drugs could be tailored to target specific regions of molecules, further enhancing their activity and selectivity.

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