Trypanocidal activity of tetradentated pyridine-based manganese complexes is not linked to inactivation of superoxide dismutase

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A B S T R A C T

Two tetradentated pyridine-based manganese complexes (Cpd2 and Cpd3) were previously reported to inhibit efficiently the growth of *Trypanosoma cruzi in vitro* and *in vivo*. Cpd3 was also shown to be a potent inhibitor of trypanosomal iron superoxide dismutase (Fe-SOD) and its trypanocidal activity linked to the inhibition of this enzyme. Here we investigated the anti-trypanosomal activity of the two compounds against bloodstream forms of *Trypanosoma brucei*. Both compounds displayed potent trypanocidal activity against *T. brucei* bloodstream forms with minimum inhibitory concentrations (MICs) and 50% growth inhibition (GI_{50}) values of 1 μM and 0.2-0.3 μM, respectively. Cpd2 and Cpd3 also showed cytotoxicity against HL-60 cells but based on GI_{50} values the human cells were 14 and 87 times less sensitive indicating moderate selectivity. In contrast to previous observation, Cpd3 did not inhibit Fe-SOD within trypanosomes and Cpd2 inhibited the enzyme only by 34%. As Fe-SOD together with ornithine decarboxylase play vital roles in the antioxidant defence in bloodstream forms of *T. brucei*, inhibition of both enzymes should be synergistically. Therefore, the interaction of Cpd2 and Cpd3 with the ornithine decarboxylase inhibitor eflornithine was determined. Both compounds were found in combination with eflornithine to produce only an additive effect. Thus, the observed lack of synergy between Cpd2/Cpd3 and eflornithine can be regarded as further indication that both compounds are not very strong inhibitors of trypanosomal Fe-SOD. Nevertheless, tetradentated pyridine-based manganese complexes are interesting compounds with promising anti-trypanosomal activity.

Keywords:
Trypanosoma brucei
Manganese complexes
Chemotherapy
Drug combination

1. Introduction

African trypanosomiasis is a neglected tropical disease affecting both humans (sleeping sickness) and their livestock animals (nagana disease). The infection is caused by flagellated protozoans of the genus *Trypanosoma*. The parasites live and multiply extracellularly in blood and tissue fluids (lymph and cerebrospinal fluid) of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina* sp.). Sleeping sickness and nagana disease occur in Africa between the latitudes of 14°N and 20°S, an area that corresponds to the range of tsetse flies (Molyneux et al., 1996). In this region millions of people and livestock animals are at risk of contracting the infection (Franco et al., 2014; Giordani et al., 2016). Trypanosomiasis has been and still is a major obstacle for economic and cultural development in affected rural areas in Africa (Steverding, 2008; WHO, 2017).

For treatment of sleeping sickness and nagana disease only a few drugs are available (Steverding, 2010; Giordani et al., 2016). All these drugs have been developed a long time ago and require parental administration, induce significant toxic side effects, have limited efficacy and are being increasingly subject to drug resistance (Matovu et al., 2001; Fairlamb, 2003; Delespaux and de Koning, 2007). For these reasons, new, more effective and safer drugs for the treatment of African trypanosomiasis are necessarily needed.

For the elimination of superoxide anion radicals ($O_2^{\bullet-}$), bloodstream forms of *Trypanosoma brucei* express four isoforms of iron-containing superoxide dismutases (Fe-SOD) (Wilkinson et al., 2006; Dufernez et al., 2006). Since trypanosomes lack catalase, the main function of the Fe-SOD seems to be the metabolism of $O_2^{\bullet-}$ released during the generation of the tyrosyl radical in the small subunit of ribonucleotide reductase (Fontecave et al., 1987) which, otherwise, would irreversibly inactivate this enzyme (Gaudu et al., 1996) essential for DNA synthesis. This role of the Fe-SOD in the parasite is supported by the observation that both the Fe-SOD and the small subunit of ribonucleotide reductase are down-regulated in cell-cycle-arrested short stumpy trypanosomes (Kabiri and Steverding, 2001). Moreover, only the down-regulation of the cytosolic Fe-SOD isoform (Dufernez et al., 2006) resulted in a significant reduction in the growth rate of trypanosomes (Wilkinson et al., 2006) supporting

the role of Fe-SOD in the protection of ribonucleotide reductase in bloodstream forms of *T. brucei*. Taken together, all these findings indicate that Fe-SOD is a validated drug target in trypanosomes.

Recently, it was reported that the tetradentated pyridine-based manganese complexes Cpd2 and Cpd3 (Fig. 1) display *in vitro* and *in vivo* anti-trypanosomal properties against the related trypanosome species *T. cruzi* (Olmo et al., 2016). One of the compounds, Cpd3, was also shown to be a potent inhibitor of Fe-SOD (Olmo et al., 2016). In this study, we investigated the trypanocidal activity of Cpd2 and Cpd3 against bloodstream form of *T. brucei* and analysed whether their anti-trypanosomal actions were due to the inactivation of Fe-SOD in the parasites.

2. Materials and Methods

2.1. Reagents

Suramin sodium salt, resazurin sodium salt, phenylmethanesulfonyl fluoride (PMSF) and pyrogallol were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Eflornithine (DL-α-difluoromethylornithine, DFMO) was ordered from Enzo Life Sciences Ltd. (Exeter, Devon, UK),

2.2. Chemistry

Compounds Cpd2 ([$Mn(CF_3SO_3)_2$-^{dMM}PDP]; ^{dMM}PDP = (*S,S′*)-4-methoxy-2-[[2-[1-[(4-methoxy-3,5-dimethyl-2-pyridyl)methyl]pyrrolidin-2-yl]pyrrolidin-1-yl]methyl]-3,5-dimethyl-pyridine) and Cpd3 ([$Mn(CF_3SO_3)_2$-^{dMM}BPMCN]; ^{dMM}BPMCN = (*R,R′*)-*N,N′*-bis[(4-methoxy-3,5-dimethyl-2-pyridyl)methyl]-*N,N′*-dimethyl-cyclohexane-1,2-diamine) were synthesised according to previously published methods (Cussó et al., 2013; Olmo et al., 2016). The structure of the compounds are shown in Fig. 1. Because of the limited water solubility, stock solutions of Cpd2 and Cpd3 were prepared in DMSO.

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103 *2.3. Cell Culture*

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105 Bloodstream forms of the *T. brucei* clone 427-221a (Hirumi et al., 1980) and human
106 myeloid leukaemia HL-60 cells (Collins et al., 1977) were grown in Baltz medium (Baltz et
107 al., 1985) supplemented with 16.7% heat-inactivated bovine serum. Trypanosome and human
108 cells were maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

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110 *2.4. Toxicity assay*

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112 Toxicity assays were performed according to the method described by Merschjohann et al.
113 (2001) with slight modifications. In brief, cells were seeded in 96-well plates in a final volume
114 of 200 µl of Baltz medium containing various concentration of test compounds (tenfold
115 dilutions from 10^{-4} M to 10^{-10} M) and 1 % DMSO. Wells containing medium and 1% DMSO
116 served as controls. The initial cell densities were 1×10^4/ml for bloodstream-form
117 trypanosomes and 5×10^4/ml for human myeloid HL-60 cells. After 24 h incubation, 20 µl of
118 0.5 mM resazurin in PBS (sterile filtered) was added and the cells were incubated for a further
119 48 h. Subsequently, the absorbance was read on a microplate reader using a test wavelength of
120 570 nm and a reference wavelength of 630 nm. The 50 % growth inhibition (GI_{50}) value, i.e.,
121 the concentration of a compound necessary to reduce the growth rate of cells by 50 % compared
122 to the control, was determined by linear interpolation according to the method described by
123 Huber and Koella (1993). The minimum inhibitory concentration (MIC) value, i.e., the
124 concentration of a compound at which all cells were killed, was determined microscopically
125 by inspecting each well thoroughly for the presence of motile trypanosomes or unlysed HL-60
126 cells.

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128 *2.5. Fe-SOD activity assay*

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The effect of Cpd2 and Cpd3 on the activity of Fe-SOD within bloodstream forms of *T. brucei* were determined as follows. Live trypanosomes (2.5×10^7/ml) were incubated in Baltz medium with 100 µM Cpd2 or Cpd3 in the presence of 0.92% DMSO at 37 °C. Control cultures were treated with 0.92% DMSO alone. After 3 h incubation, cells were washed three times with PBS/1% glucose, lysed in lysis buffer (5 mM Tris, 0.1 mM Na_4-EDTA, pH 7.8, 400 µM PMSF) (5×10^7/100 µl) for 10 min on ice followed by high-speed centrifugation to clear the lysate. It should be noted that after the 3 h incubation no dead cells were observed in any of the samples (Cpd2-treated cells, Cpd3-treated cells and control cells).

The activity of Fe-SOD in cell extracts was determined indirectly by the inhibition of pyrogallol autoxidation according to the methods described by Marklund and Marklund (1974). In brief, 100 µl measuring buffer (100 mM Tris, 2 mM EDTA, pH 8.0), 30 µl water and 50 µl cell extract (2.5×10^7 cell equivalents) or 50 µl lysis buffer (negative control) were pipetted into wells of a 96-well plate. The background absorbance was read on a microplate reader at 450 nm. Then, 20 µl of a 2 mM pyrogallol solution in 1 mM HCl was added and the increase in absorbance at 450 nm was recorded every minute over a period of 20 min.

2.6. Determination of drug interactions and isobologram construction

The interactions between compounds Cpd2/Cpd3 and the anti-sleeping sickness drug eflornithine (DFMO) were determined by a modified isobolographic method (Fivelman et al., 2004). Based on GI_{50} values, the maximum concentration of individual drugs was set at $5 \times GI_{50}$ ensuring that the GI_{50} was at the midpoint of the serial dilution. The highest concentrations of solutions were prepared in proportions of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 of compounds Cpd2/Cpd3 and DFMO, which were then twofold serially diluted (6 dilution steps). The assay was set up in 96-well plates and wells without Cpd2/Cpd3 and DFMO served as controls. All wells contained 1% DMSO. The initial cell density was 1×10^4 trypanosomes/ml. The assay was evaluated and GI_{50} values calculated as described above.

The fractional inhibitory concentration index (FICI) at the GI_{50} value was calculated as $FICI = GI_{50(combination)}/GI_{50(alone)}$. The sum of FICIs (ΣFICI) was computed as $\Sigma FICI = FICI_{(drug}$

$_{A)}$ + $FICI_{(drug\ B)}$. The mean sum of FICIs (xΣFICI) was averaged over the ΣFICIs. Isobolograms were built by plotting the FICI of each drug ratio. The xΣFICIs were used to classify the interactions according to Odds (2003). An xΣFICI of ≤ 0.5, between 0.5 and 4, and of ≥4 indicates synergy, indifference and antagonism, respectively.

3. Results and discussion

3.1. In vitro *trypanocidal evaluation of Cpd2 and Cpd3*

The trypanocidal and cytotoxic activity of the two tetradentated pyridine-based manganese complex compounds Cpd2 and Cpd3 was evaluated with bloodstream forms of *T. brucei* and human myeloid leukaemia HL-60 cells. Both compounds showed a concentration-dependent inhibitory effect on the *in vitro* growth of *T. brucei* bloodstream forms with MIC values of 1 μM and GI_{50} values between 0.2-0.3 μM (Fig. 2 and Table 1). The general cytotoxicity of Cpd2 and Cpd3 towards human HL-60 cells was substantially lower with MIC values of 10 μM and 100 μM and GI_{50} values in the low micromolar and mid micromolar range, respectively (Fig. 2 and Table 1). As a result, the MIC and GI_{50} ratios of cytotoxic to trypanocidal activity (selectivity indices) of the compounds were 10 and 14 for Cpd2, and 100 and 87 for Cpd3, respectively (Table 1). The activity profile of Cpd2 and Cpd3 was in good agreement with their recently reported activities against the related protozoan parasites *T. cruzi* and against African green monkey kidney epithelial (Vero) cells (Olmo et al., 2016). However, compared with *T. cruzi* trypomastigote forms, *T. brucei* bloodstream forms were 6 and 14 times more sensitive to Cpd2 and Cpd3, respectively.

Compared to suramin, one of the drugs used for treatment of sleeping sickness, compounds Cpd2 and Cpd3 were about 10 times less trypanocidal (Fig. 2 and Table 1). Under same experimental conditions, the MIC and GI_{50} values of suramin for bloodstream forms of *T. brucei* were 0.1 μM and 0.035 μM, respectively. As suramin is nontoxic to mammalian cells (MIC and GI_{50} values of suramin for HL-60 were >100 μM; Table 1), the anti-sleeping sickness drug has much higher selectivity indices than compounds Cpd2 and Cpd3 (the MIC and GI_{50}

ratios of cytotoxic to trypanocidal activity of suramin were >1000 and >2853, respectively; Table 1).

3.2. Inhibitory evaluation of Cpd2 and Cpd3 on T. brucei *Fe-SOD*

The effect of compounds Cpd2 and Cpd3 on the activity of *T. brucei* Fe-SOD was determined using the pyrogallol autoxidation assay (Marklund and Marklund, 1974). This assay has previously been shown to determine readily Fe-SOD activity in cell extracts of bloodstream forms of *T. brucei* (Steverding and Scory, 2004). However, when trying to measures the effect of the compounds on Fe-SOD activity directly in trypanosome cell extracts, we observed that the presence of the tetradentated pyridine-based manganese complexes increased the autoxidation rate of pyrogallol in a concentration dependent manner (Fig. S1). Therefore, we decided to test the effect of Cpd2 and Cpd3 on the activity of Fe-SOD within bloodstream-form trypanosomes because this enabled us to remove the compounds by repeated washing and centrifugation of the cells. Each compound was co-incubated with the parasites for 3 h at 100 µM, a concentration that was 100 times the MIC value of 1 µM, at which all trypanosomes were killed in the growth inhibition assay (see above). After washing and lysis of the parasites, cell extracts were assayed for their ability to prevent the autoxidation of pyrogallol. Extracts of parasites that had been incubated with Cpd3 blocked completely the autoxidation of pyrogallol (Fig. 3). However, extracts of trypanosomes that had been treated with Cpd2 partially prevented the autoxidation of pyrogallol (Fig. 3). These results indicate that Cpd3 did not affect the activity of Fe-SOD in trypanosomes while Cpd2 inhibited the enzyme in the parasites to some extent. Based on the uninhibited autoxidation of pyrogallol in the absence of cell extract, calculations revealed that Cpd2 inhibited the Fe-SOD within trypanosomes by approximately 34%.

These findings are in disagreement to recent observations that Cpd3 caused 100% inhibition of *T. cruzi* Fe-SOD at 25 µM and that Cpd2 showed no significant inhibition of the enzyme up to 100 µM when assayed directly in cell-free extracts (Olmo et al., 2016). This discrepancy can only be explained by the different assays employed to determine the activity

of Fe-SOD. Olmo et al. (2016) used the cytochrome c assay (Beyer and Fridovich, 1987) to measure the activity of Fe-SOD in crude cell extracts. In this assay, Fe-SOD-mediated inhibition of ferricytochrome c reduction in the presence of an $O_2^{\bullet-}$ generating system (xanthine/xanthine oxidase) is determined. However, this assay method has several pitfalls that could affect the outcome of the test (Beyer and Fridovich, 1987). First, crude cell extracts may contain quinones and other compounds that can mediate electron transfer from xanthine oxidase to cytochrome c (McCord and Fridovich, 1970). In this context it should be mentioned that Olmo et al, (2016) were using cell extracts prepared form *T. cruzi* epimastigotes, the life cycle stage of the parasite expressing a functional respiratory chain. Second, since the activity of Fe-SOD is recorded by the inhibition of the reduction of cytochrome c, any compound that directly inhibits xanthine oxidase would be mistaken for an active Fe-SOD. Third, any compound that interacts with cytochrome c, the indicating scavenger for $O_2^{\bullet-}$, would affect the outcome of the assay. Fourth, any compound that reacts with $O_2^{\bullet-}$ producing molecules that can reduce cytochrome c would replace $O_2^{\bullet-}$ which cannot be scavenged by the Fe-SOD. Such compounds would compete with the Fe-SOD for $O_2^{\bullet-}$ and as a result the enzyme would appear less effective creating the impression that Fe-SOD is inhibited by the compounds. As Cpd2 and Cpd3 had originally been designed to be capable of generating highly oxidising molecules (Garcia-Bosch et al., 2012; Cussó et al., 2013), it is quite possible that the two compounds can react with any components of the xanthine oxidase-cytochrome c assay. Evidence for this is provided by observations that both Cpd2 and Cpd3 can oxidise cytochrome c (Fig. S2). Therefore, the observed inhibition of *T. cruzi* Fe-SOD by Cpd3 reported by Olmo et al. (2016) is most likely due to interference of the compound with the xanthine oxidase-cytochrome c assay. In contrast, by incubating live trypanosomes instead of cell extracts with Cpd2 and Cpd3, we were able to remove the compounds before cell lysates were subjected to assaying the activity of Fe-SOD.

3.3. Interaction of Cpd2 and Cpd3 with DFMO

As both Fe-SOD and ornithine decarboxylase have important roles in protecting bloodstream forms of *T. brucei* from oxidative damage but at different levels (Fe-SOD through the elimination of $O_2^{\bullet-}$ and ornithine decarboxylase through the biosynthesis of polyamines), it is reasonable to assume that combination of inhibitors against both enzymes should show synergy. In addition, combination therapy is nowadays the standard for the treatment of many diseases including parasitic protozoan infections. For example, the first-line recommended treatments of malaria and sleeping sickness are, according the World Health Organization, therapies based on the co-administration of two drugs (WHO 2016, 2017). The interaction of Cpd2 and Cpd3 with the ornithine decarboxylase inhibitor DFMO (eflornithine), a drug used in the treatment of the second stage (neurological phase) of sleeping sickness, was evaluated with the fixed isobologram method (Fivelman et al., 2004). The experimental design allowed the determination of FICI values for each combination. The Cpd2/DFMO and the Cpd3/DFMO combinations had ΣFICI values ranging between 1.16-1.44 and 1.37-1.72, respectively (Fig. 4). The xΣFICI of both combinations was similar with calculated values of 1.32±0.12 (Cpd2/DFMO) and 1.56±0.16 (Cpd3/DFMO) (Fig. 4) which were not statistically significantly different ($p = 0.0503$, student's t-test). As these xΣFICI values were within the range of 0.5 to 4, the interactions between Cdp2 and DFMO, and between Cpd3 and DFMO, were indifferent (additive) (Odds, 2003). Thus, the lack of synergistic interaction between the tetradentated pyridine-based manganese complexes and DFMO supports our finding that Cpd2 and Cpd3 are not strong and specific inhibitors of trypanosomal Fe-SOD.

Although Cpd2/DFMO and Cpd3/DFMO combinations did not result in the desirable synergistic interaction, an additive (indifferent) effect is also of value as it would permit decreased dosage while maintaining efficacy (Chou, 2006). It should be pointed out in this context that the recommended nifurtimox/DFMO combination therapy as first-line treatment of the second stage of sleeping sickness failed to show synergistic trypanocidal activity *in vitro* (Vincent et al., 2012).

3.4. Conclusions

We have shown that the two tetradentated pyridine-based manganese complex compounds Cpd2 and Cpd3 display promising trypanocidal activity against bloodstream forms of *T. brucei*. Importantly, one of the compounds, Cpd3, matches the activity criteria for drug candidates for African trypanosomiasis (GI_{50} <1 μM; selectivity >100) (Nwaka and Hudson, 2006). However, it should be mentioned that a cancer cell line was used in this study for determining the selectivity and that, compared with non-cancer cells, cytotoxicity of both compounds are therefore likely to be overestimated. For example, non-cancerous Vero cells are 3-20 times less sensitive to Cpd2 and Cpd3 (GI_{50}s = 78 and 88 μM, respectively; Olmo et al., 2016) than HL-60 cells employed in this investigation. Worth mentioning also is the previous finding that both compounds were able to cure a *T. cruzi* infection in mice with curative rates of 33-50% (Olmo et al., 2016).

In contrast to previous suggestions, we could not confirm that Cpd3 is an inhibitor of trypanosomal Fe-SOD but found that Cpd2 partially inhibits the enzyme. However, a partial inhibition of Fe-SOD by Cpd2 would certainly not be sufficient to cause killing of the trypanosomes. Thus, the mechanism of trypanocidal action of Cpd2 and Cpd3 remains to be established. As both compounds are powerful oxidising molecules, it is conceivable that they most likely interfere with the redox metabolism of bloodstream forms of *T. brucei*.

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Figure legends

Fig. 1. Chemical structure of the tetradentated pyridine-based manganese complexes Cpd2 and Cpd3.

Fig. 2. Effect of Cpd2, Cpd3 and suramin on the growth of bloodstream forms of *T. brucei* and human myeloid leukaemia HL-60 cells. Trypanosomes (closed symbols and solid lines) and HL-60 cells (open symbols and dashed lines) were incubated with varying concentrations of Cpd2 (circles), Cpd3 (triangles) or suramin (squares). After 72 h of culture, cell viability and proliferation was determined with the colorimetric dye resazurin. For clarity, only mean values of three independent experiments are shown. The standard deviations ranged between 0.0 to 14.7 percentage points.

Fig. 3. Effect of Cpd2 and Cpd3 on Fe-SOD activity within bloodstream forms of *T. brucei*. Trypanosomes were incubated with 100 µM Cpd2 (open squares), 100 µM Cpd3 (open triangles) or DMSO alone (positive control, open circles) for 3 h at 37 °C, then harvested, washed and lysed. The activity of Fe-SOD in cleared cell lysates was determined indirectly by the inhibition of pyrogallol autoxidation. To a solution containing 100 µl measuring buffer, 30 µl water and 50 µl cell lysate (2.5×10^7 cell equivalents), 20 µl of a 2 mM pyrogallol solution in 1 mM HCl was added and the increase in absorbance at 450 nm was followed photometrically. A negative control (closed circles) indicates the autoxidation of pyrogallol in the absence of cell lysate. A representative result from two independent experiments is shown.

Fig. 4. Isobolograms showing the *in vitro* interactions between DFMO and Cpd2 (A) and between DFMO and Cpd3 (B) against bloodstream forms of *T. brucei*. Assays were performed by a fixed-ratio method based on GI_{50} values, with the combinations being tested at constant ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. Mean values ± standard deviations are shown from three independent experiments. The dashed line is the theoretical line that produced a sum of the

FICIs of 1 at all ratios tested and represents an additive effect of the two compounds. The xΣFICI values shown are the mean sums of the FICIs for the interactions tested.

Table 1

GI_{50} and MIC values and ratios of Cp2, Cp3 and suramin for *T. brucei* and HL-60 cells.

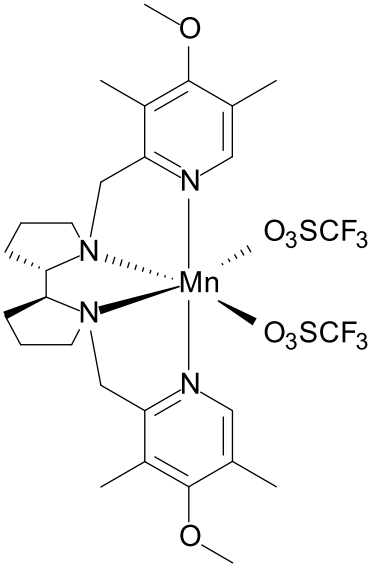
Compound	*T. brucei*		HL-60		Selectivity	
	MIC (µM)	GI_{50} (µM)[a]	MIC (µM)	GI_{50} (µM)[a]	MIC ratio[b]	GI_{50} ratio[c]
Cpd2	1	0.24±0.06	10	3.4±0.2	10	14
Cpd3	1	0.31±0.01	100	27.1±4.7	100	87
Suramin	0.1	0.035±0.001	>100	>100	>1000	>2857

[a] Mean values ± SD of three experiments.

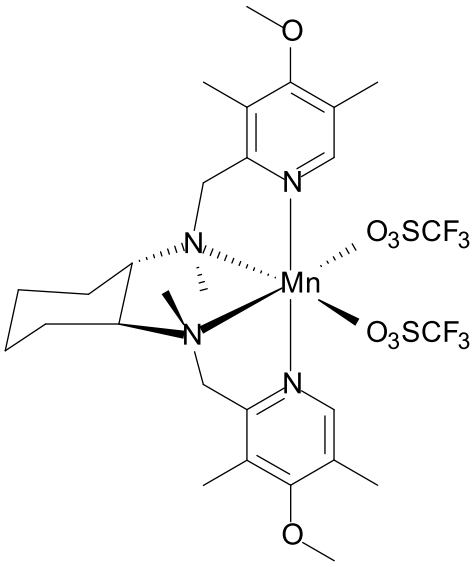
[b] Defined as $MIC_{(HL\text{-}60)}/MIC_{(T.\ brucei)}$.

[c] Defined as $GI_{50(HL\text{-}60)}/GI_{50(T.\ brucei)}$.

Fig. 1

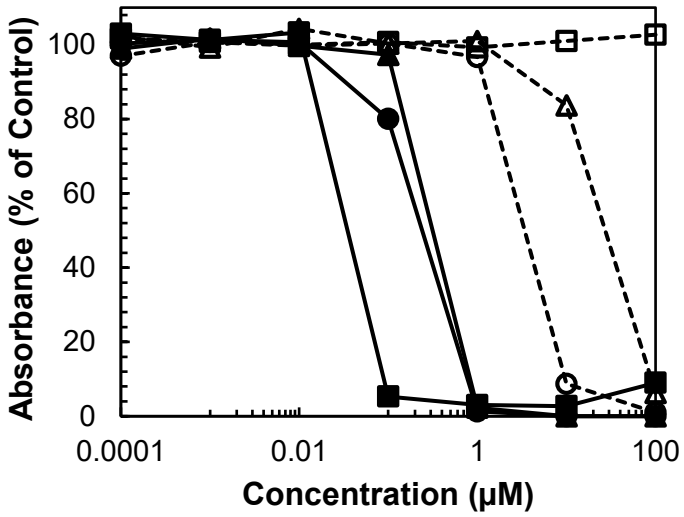


Cpd2



Cpd3

422 **Fig. 2**



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426 **Fig. 3**
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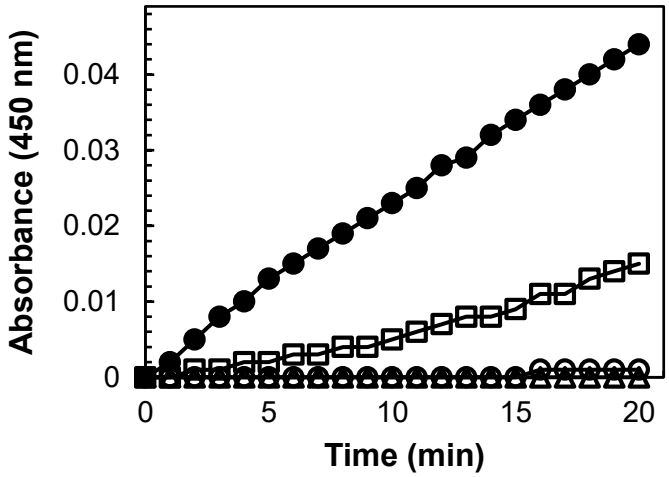

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431 **Fig. 4**
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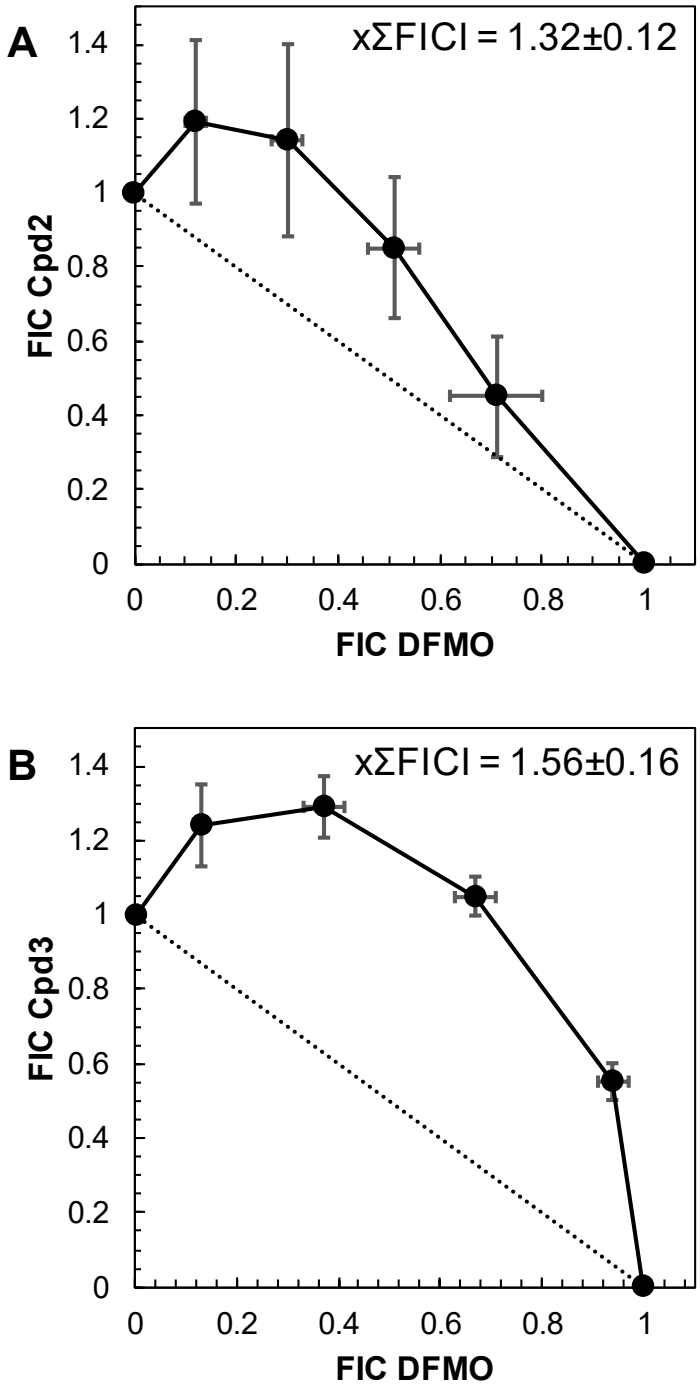

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Supplementary data:

Trypanocidal activity of tetradentated pyridine-based manganese complexes is not linked to inactivation of superoxide dismutase

Dietmar Steverding, Karolina Kolosevska, Manuel Sánchez-Moreno

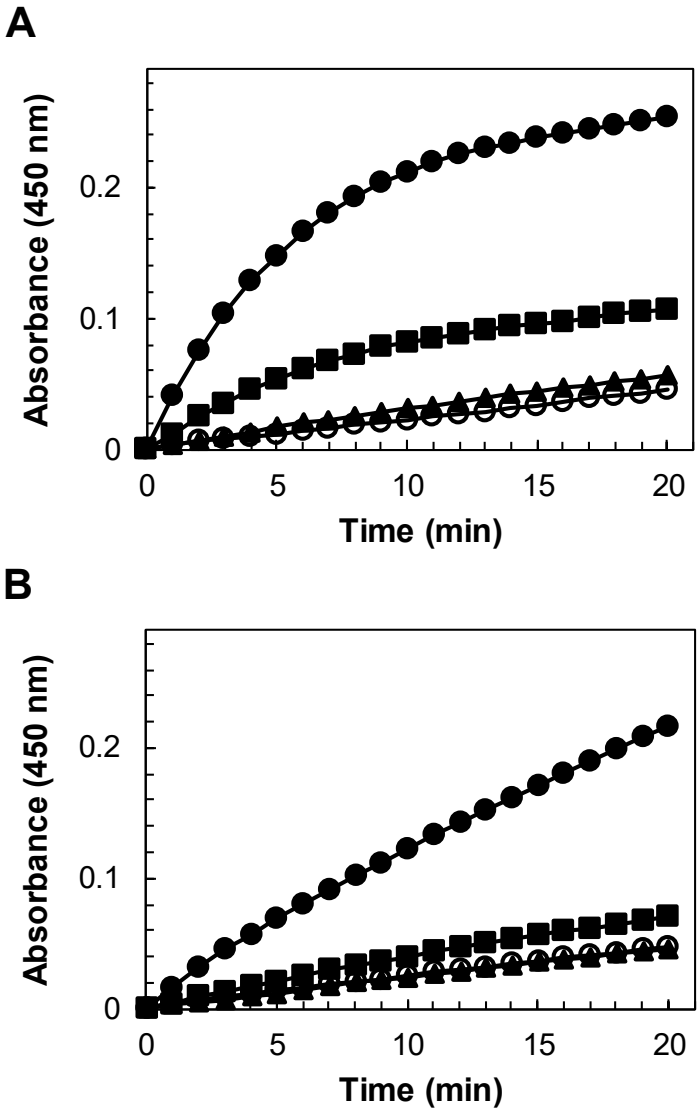

Fig. S1. Effect of Cpd2 and Cpd3 on the autoxidation of pyrogallol. Solutions containing different concentrations of the compounds in 100 µl measuring buffer (100 mM Tris, 2 mM EDTA, pH 8.0), 30 µl water, 50 µl lysis buffer (5 mM Tris, 0.1 mM EDTA, pH 7.8) and 1% DMSO were pipetted into wells of a 96-well plate. Solutions without compounds served as

controls. The background absorbance was read on a microplate reader at 450 nm. Then, 20 µl of a 2 mM pyrogallol solution in 1 mM HCl was added and the increase in absorbance at 450 nm was followed for 20 min. (A) Cpd2 and (B) Cpd3. Closed circles, 100 µM compound; closed squares, 10 µM compound; closed triangles, 1 µM compound; open circles, no compound (control). Note that an increase in absorbance indicates autoxidation of pyrogallol. A representative results from two independent experiments is shown.

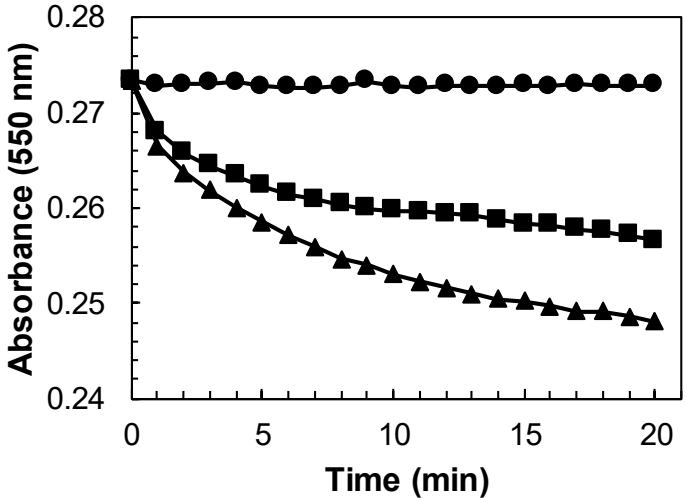

Fig. S2. Oxidation of cytochrome c by Cpd2 and Cpd3. Ferrocytochrome c (10 µM) was incubated with 100 µM Cpd2 (squares), Cpd3 (triangles) or no compound (solvent (DMSO) control; circles) in 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8. Changes in absorbance at 550 nm were recorded for 20 min with a Beckman Coulter DU® 800 spectrophotometer. Note that a decrease in absorbance indicates oxidation of ferrocytochrome c. A representative result from two independent experiments is shown.