Antimicrobial activity of ruthenium-based intercalators

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Abstract

Multidrug-resistance of bacterial pathogens is a major problem and there is a clear need for the development of new types of antibiotics. Here we investigated the antimicrobial activity of Ruthenium(II) based DNA-intercalating complexes. These complexes were found to have no activity *in vitro* against the Gram-negative bacterium *Escherichia coli*, but the complexes were clearly active against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*. *In vivo* activity has also been demonstrated for one of the compounds using a simple infection model, the nematode *Caenorhabditis elegans*. Importantly, this also showed that the compound tested was not toxic to the nematodes.

Keywords: DNA intercalator, Ruthenium complexes, antimicrobial agents, methicillin-resistant *Staphylococcus aureus*, *Caenorhabditis elegans*

1. Introduction

Multidrug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or extended spectrum beta lactamase (ESBL) producing *Escherichia coli* are a significant problem in modern healthcare (Karageorgopoulos and Falagas, 2009). Resistance against antimicrobial agents develops quickly, even against synthetic compounds that bacteria have never encountered previously (Tsiodras et al., 2001). There is therefore a real need to continuously look for new types of antibacterial compounds.

Several avenues are being followed to find new compounds that inhibit growth or virulence of pathogenic bacteria. One type of compound that has not been studied in great detail are metal complexes. Some early studies showed activity of metal chelates against a range of Gram-negative and Gram-positive bacteria (Dwyer et al., 1952; Dwyer et al., 1969). In infection models using rodents these compounds showed promise in particular in the topical treatment of bacterial infections (Dwyer et al., 1969). Other studies have shown that platinum-containing complexes have antibacterial activity. These are, however, quite toxic to eukaryotic cells, and compounds such as cisplatin are now mainly used in the treatment of cancer (Boulikas and Vougiouka, 2003). We recently showed that a major groove binder, a dinuclear iron(II) supramolecular helicate, has activity against Gram-positive bacteria (Richards et al., 2009). In that case it was also demonstrated that the helicate was able to bind to the chromosomal DNA of bacteria, but it was not clear whether it was DNA-binding alone that that lead to its bactericidal activity.

Ruthenium(II) polypyridyl complexes do not change structure under physiological conditions and are stable in strong acids and bases. They are readily synthesised and in many cases can be resolved into stable enantiomeric forms. Ru(II) complexes of the form $[Ru(P_L)_2(I_L)]^{2+}$ (where P_L, peripheral ligand; I_L, intercalating ligand) have been shown to interact with DNA, although the mode and extent of the interactions vary, depending on the

type of ligands coordinated and the concentrations used for the binding studies. Spectroscopic experiments to elucidate the type, strength, location and geometry of binding of mononuclear Ru(II) complexes to DNA were the focus of research in the 1980s and 90s (see Barton, 1985; Eriksson et al., 1994; Hiort et al., 1993; Rehmann and Barton, 1990, and references therein). These studies have shown that phen (1,10-phenanthroline) as the intercalating ligand is amongst the weakest moieties capable of any extent of intercalation. When the intercalating ligand is dppz (dipyrido[3,2-a:2'3'-c]phenazine), the complex has much higher affinity for DNA due to the greatly increased overlap of the extended phenazine rings with the aromatic bases of nucleic acids. The ligands dpq (dipyrido[3,2-d:2',3'f]quinoxaline and dpqC (dipyrido[3,2-a:2'3'-c](6,7,8,9-tetrahydro) phenazine) have affinities between that of phen and dppz. Properties of these Ru(II) complexes are also determined by the peripheral ligands, which can influence, for instance, DNA binding and/or enantioselectivity. In this study we have analysed the antimicrobial activity of three Ru(II) complexes (Fig. 1): $[Ru(phen)_2(dpq)]^{2+}$, $[Ru(bpy)_2(dpqC)]^{2+}$ (bpy = 2,2'-bipyridine), and $[Ru(2,9\text{-}Me_2phen)_2(dppz)]^{2+}$ (2,9-Me_2phen = 2,9-dimethyl-1,10-phenanthroline). We have used classical antibiotic resistance tests as well as a nematode infection model and found that in particular $[Ru(2,9\text{-}Me_2phen)_2(dppz)]^{2+}$ has good antimicrobial activity, both *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Strains and growth conditions

The bacterial strains used in this study are *Escherichia coli* MC4100 (Peters et al., 2003), *Bacillus subtilis* 168 (Kunst et al., 1997), *Enterococcus faecalis* BS385 (van Merode et al., 2006), *Enterococcus faecium* E1162 (Heikens et al., 2007), and *Staphylococcus aureus* MRSA252, MRSA41 and MSSA160 (Feil et al., 2003). Strains were maintained on Luria-Bertani (LB) broth (Fisher) or Brain Heart Infusion (BHI) broth (Fisher).

2.2 Synthesis of Ru(II) complexes

$[Ru(phen)_2(dpq)]Cl_2$ (Greguric et al., 1997), $[Ru(bpy)_2(dpqC)](PF_6)_2$ (Collins et al., 1998), and $[Ru(2,9\text{-}Me_2phen)_2(dppz)]Cl_2$ (Greguric et al., 2000) were synthesised and characterised as previously described. In all cases racemic mixtures were used in this work.

2.3 Disc susceptibility tests

Mueller-Hinton (MH; Fisher) agar plates were seeded with approximately 10^5 bacteria. Discs (Whatmann 3MM chromatography paper, 6 mm) impregnated with 30 µg compound were applied to these plates. As negative controls discs with solvent only (dimethyl sulfoxide) were applied, all of which gave no zone of inhibition. Plates were incubated for 18 hours at 37 ^oC and the diameter of the clearing zone around the discs was measured.

2.4 Minimal inhibitory concentration and minimal bactericidal concentration

The MIC (minimal inhibitory concentration) values were determined with a macrobroth dilution method using MH broth as previously described (Andrews, 2001). MBC (minimal bactericidal concentration) values were determined by plating 10 µL of each of the broth

cultures from the MIC tests and incubating those plates at 37^{o}C for 24 hours; the MBC was defined as the lowest concentration at which no growth was observed.

2.5 Time-kill assays

Cells were grown to mid-exponential growth phase in MH broth and then diluted to give a final concentration of approximately 10^6 cells per mL. Compound was added and samples were taken at regular intervals. To remove the compound, cells were immediately collected by centrifugation (2 min at 14,000 *g*) and washed with fresh medium. The viable count was determined by plating serial dilutions, and the number of colony forming units (CFU) per mL was calculated.

2.6 *C. elegans* rescue and toxicity assays

For nematode infection and rescue assays, a strain that is hypersensitive to pathogens was used, *Caenorhabditis elegans glp-4(bn2) sek-1*(*km4*) (Moy et al., 2006). The strain, which was obtained from the Caenorhabditis Genetics Centre (CGC) at the University of Minnesota, also has a mutation leading to temperature-sensitive sterility so that it does not produce any progeny during the assay – this simplified the scoring of survival rates. For normal growth nematodes were maintained at 15 ^{o}C on nematode growth medium, using *E. coli* OP50 as a source of food (Brenner, 1974). For infection, *C. elegans* worms were age-synchronised by isolating eggs through treatment with hypochlorite/NaOH and hatching the eggs overnight in M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 0.25 g/L $MgSO_4.7H_2O$). Next, worms were deposited on lawns of *E. coli* OP50 grown on NGM plates and incubated at 25 ^{o}C. When worms reached the L4-stage they were removed from the plates with M9 buffer and washed 3 times. Approximately 30 worms were deposited in each well of a 24-well plate containing 1 mL LB with 50 mg/L ampicillin (to inhibit growth of *E. coli*) and 10^6 cells *S.*

aureus MRSA252. After 1 hour $[Ru(2,9\text{-}Me_2phen)_2(dppz)]^{2+}$ was added, and the plates were incubated at 25 ^{o}C without agitation. Worm survival was scored daily.

3. Results

3.1 *In vitro* antibacterial activity

Three Ru(II) complexes were tested for their antimicrobial activity: $[Ru(phen)_2(dpq)]^{2+}$, $[Ru(bpy)_2(dpqC)]^{2+}$, and $[Ru(2,9\text{-}Me_2phen)_2(dppz)]^{2+}$. Initial tests on these compounds were performed using disc diffusion tests. All three Ru-complexes were active on the Gram-positive *B. subtilis*, with $[Ru(2,9\text{-}Me_2phen)_2(dppz)]^{2+}$ showing the largest zone of inhibition, followed by $[Ru(bpy)_2(dpqC)]^{2+}$ and $[Ru(phen)_2(dpq)]^{2+}$. In contrast, none of the compounds were active against the Gram-negative bacterium *E. coli*, suggesting that the compounds are only active on Gram-positive bacteria.

B. subtilis is non-pathogenic, and it was therefore of interest to look at the sensitivity of Gram-positive pathogens towards the ruthenium intercalators. To test this we focused on clinical isolates of enterococci (*Enterococcus faecalis* BS385 and *Enterococcus faecium* E1162), and *S. aureus*. Of the enterococcal strains, *E. faecium* E1162 is resistant to several antibiotics, including ampicillin, kanamycin, and tetracycline, while the *S. aureus* isolates MRSA41 and MRSA252 are resistant to ampicillin, erythromycin, kanamycin, and ciprofloxacin for instance (Holden et al., 2004; data not shown). Disc diffusion tests again showed a similar trend as observed for *B. subtilis*, with $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ the most active compound and $[Ru(phen)_2(dpq)]^{2+}$ the least active. Against enterococci only $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ was active.

To investigate this further we determined the MIC and MBC values. With *B. subtilis* and *S. aureus* strains this again showed the same order of activity of the compounds, with $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ the most active and $[Ru(phen)_2(dpq)]^{2+}$ the least (Table 1). The

lowest MIC value was observed with $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ on *S. aureus* MRSA252, with a value of 2 mg/L. MBC values were in all cases 2- or 4-fold the value of the MIC values, indicating that the compounds are mostly bactericidal.

Time-kill curves were performed in order to determine the rate of killing. For this purpose we only tested $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ on *S. aureus* MRSA252, at a concentration of 0.5 × MIC (1 mg/L), 4 × MIC (= MBC, 8 mg/L) and 16 × MIC (= 4 × MBC, 32 mg/L). As shown in Fig. 2 the rate of killing is clearly concentration-dependent. Without or with a final concentration of 1 mg/L $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ cells continue to grow. At a concentration of 8 mg/L the bacteria die, with after 120 min an approximately 10-fold reduction in living bacteria. At a concentration of 32 mg/L the bacteria die more rapidly, with more than 99.9% of cells killed after 120 min (*i.e.* >3-log10 CFU/mL killing).

3.2 Antibacterial activity in *C. elegans*

C. elegans is a soil nematode that feeds on bacteria. It has been shown to be a useful infection model for several human pathogens (for a recent review, see O'Callaghan and Vergunst, 2010). It has been shown that virulent strains of *S. aureus* kill *C. elegans* through colonization of the gut, but if exposure to the bacteria is limited to less than 8 hours then the lifespan of the nematodes is normal (Sifri et al., 2003). We therefore tested the ability of the most active complex in the *in vitro* screens, $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$, to rescue *C. elegans* from *S. aureus* infection. In the assay the nematodes were exposed for 1 hour to *S. aureus* cells after which different concentrations of $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ were added. As shown in Fig. 3, without compound all nematodes died within 6 days. Addition of 1 mg/L compound, which is below the MIC value, somewhat improved the rate of survival after 3 to 4 days, but at longer times of incubation the survival rate was not improved. By way of contrast, when either 8 or 32 mg/L $[Ru(2,9\text{-}Me_2\,phen)_2(dppz)]^{2+}$ was added nearly 80% of worms were still

alive after 6 days. That percentage was similar to the survival of worms not exposed to *S. aureus* and metal complex (data not shown), showing that $[Ru(2,9\text{-}Me_2\ phen)_2(dppz)]^{2+}$ is not toxic to *C. elegans*. Notably, we also tested toxicity of the compounds towards *C. elegans* in the presence of their normal food (*E. coli* OP50). Even at the highest concentration of 128 µg/ml the nematodes appeared not to be affected for at least 72 hours, and survival rates with or without compounds were identical (data not shown).

4. Discussion

S. aureus MRSA252 belongs to a clinically significant clone that is important for a large proportion of MRSA outbreaks in many countries, including the UK and the USA (Holden et al., 2004). It is resistant against several antibiotics, yet we show here that this strain and two other *S. aureus* isolates are sensitive to the Ru(II) complexes assayed in this work, with the most effective compound being $[Ru(2,9\text{-}Me_2\ phen)_2(dppz)]^{2+}$. These complexes were not active against *E. coli*; it is conceivable that they are, for instance, not able to cross the outer membrane of *E. coli*, but further experiments need to be performed in order to test this.

The time to kill *S. aureus* with $[Ru(2,9\text{-}Me_2\ phen)_2(dppz)]^{2+}$ was relatively fast as compared to some antibiotics that are used for the treatment of *S. aureus* infections. For instance, vancomycin has been shown to require 24 hours to kill 99.9% of *S. aureus* cells at a concentration of 4 × MBC (Murillo et al., 2006). The time we observed for $[Ru(2,9\text{-}Me_2\ phen)_2(dppz)]^{2+}$ to act is more comparable with *e.g.* quinolones such as levofloxacin or ciprofloxacin, which at similar concentrations require around 4 hours to reach 99.9% kill (Murillo et al., 2006; Gosbell, 2006).

Considering only the intercalating ligands of the three ruthenium complexes tested, the antibacterial activity was in the order dppz>dpqC>dpq. This is also the order of their DNA-binding affinity, with dppz having the highest affinity and dpq the lowest (Delaney et

al., 2002). This is consistent with the antibacterial activity of the Ru complexes being governed by their DNA-binding characteristics. Note, however, that the peripheral ligands can also affect affinity. For instance, the published value of the binding constant for $[Ru(phen)_2(dpq)]^{2+}$ (O'Donoghue et al., 2005) is higher than that of $[Ru(2,9\text{-}Me_2\ phen)_2(dppz)]^{2+}$ (Liu et al., 2001). A straightforward correlation between affinity and antimicrobial activity is therefore difficult to make. The reason for this may be that uptake in bacteria or binding to other targets such as proteins or ribosomes may also play a role.

In addition to the *in vitro* studies, we have shown that $[Ru(Me_2phen)_2(dppz)]^{2+}$ is active *in vivo* as it prevented death of *C. elegans* nematodes infected with *S. aureus*. Importantly, the compound was also not toxic to *C. elegans*, which is a good indicator that they are also not toxic in higher eukaryotes (Leung et al., 2008). It needs to be pointed out, however, that we have not tested the compounds against mammalian cell cultures and it is clear that further tests are necessary to confirm that the Ru(II) complexes are not cytotoxic. It is nevertheless worth noting that, although *C. elegans* is a very simple model system, it does share several features with higher animals and has neurons, muscles, intestines, epidermis, and an innate immune response system (Artal-Sanz et al., 2006). *C. elegans* has been shown to be very valuable in antimicrobial drug discovery, as exemplified in studies on *E. faecalis* and *Candida albicans* (Moy et al., 2006; Okoli et al., 2009). Whether the Ru(II) complexes tested here will indeed be a starting point for the development of compounds appropriate for clinical use will require further investigation by analysing their toxicity, uptake, and mode of action.

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Figure legends

Figure 1. Structures of **[**$Ru(phen)_2dpq]^{2+}$, $[Ru(bpy)_2dpqC]^{2+}$, and $[Ru(2,9\text{-}Me_2phen)_2dppz]^{2+}$

Figure 2. Time-kill assays of *S. aureus* MRSA252 in the presence or absence of $[Ru(Me_2Phen)_2dppz]^{2+}$. Circles, 32 mg/L; diamonds, 8 mg/L; triangles, 1 mg/L; squares, no compound added.

Figure 3. Survival of *C. elegans* in the presence or absence of $[Ru(Me_2Phen)_2dppz]^{2+}$. Age-synchronised nematodes were incubated in the presence of 10^6 cells of *S. aureus* MRSA252 for one hour, after which compound was added. The number of alive and dead worms was scored daily. The error bars indicate the standard deviation from the average percentage of worms still alive. Circles, 32 mg/L; diamonds, 8 mg/L; triangles, 1 mg/L; squares, no compound added.

Table 1. MIC and MBC values[a]

Compound	***B. subtilis***		**MSSA160**		**MRSA41**		**MRSA252**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
$[Ru(phen)_2dpq]^{2+}$	>128	>128	64	128	ND[b]	ND	ND	ND
$[Ru(bpy)_2dpqC]^{2+}$	64	128	32	128	32	128	16	64
$[Ru(2,9\text{-}Me_2\ phen)_2dppz]^{2+}$	4	16	8	32	4	8	2	8

[a]MIC and MBC values indicated in mg/L.

[b]ND, not determined as the strains indicated were resistant to the compound in the disc tests.

Figures

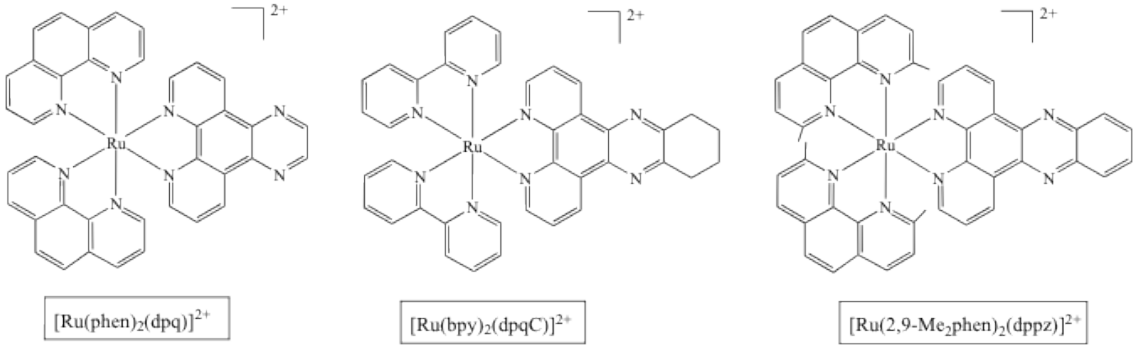

Figure 1

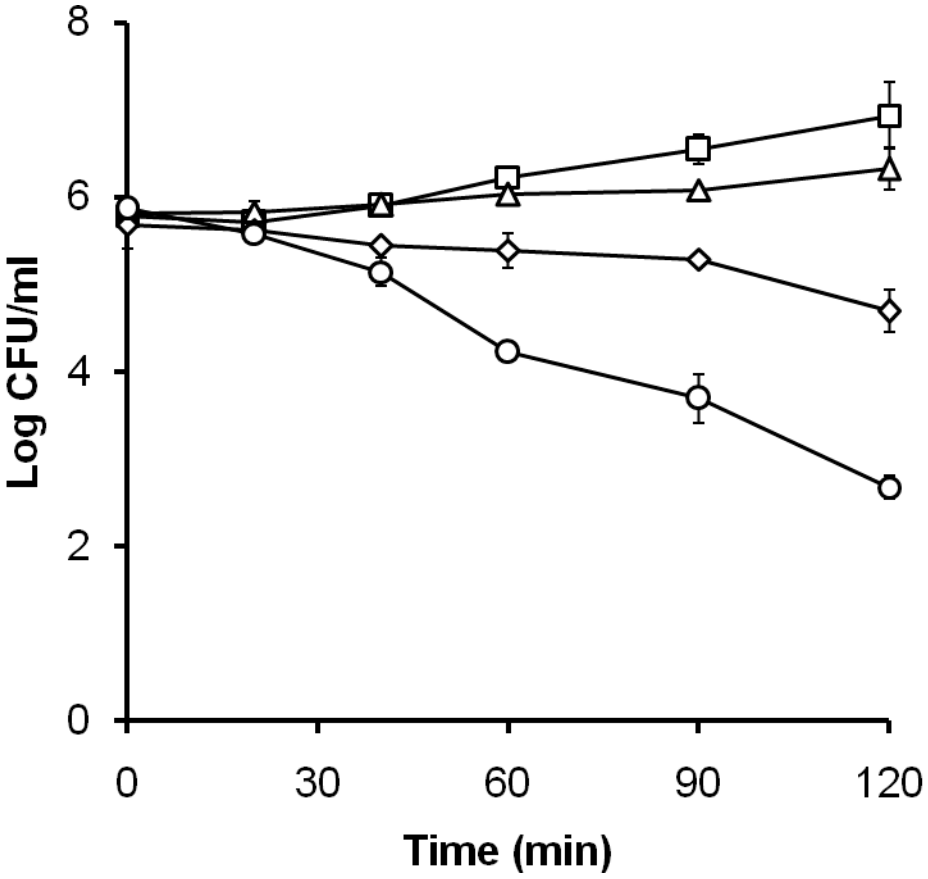

Figure 2

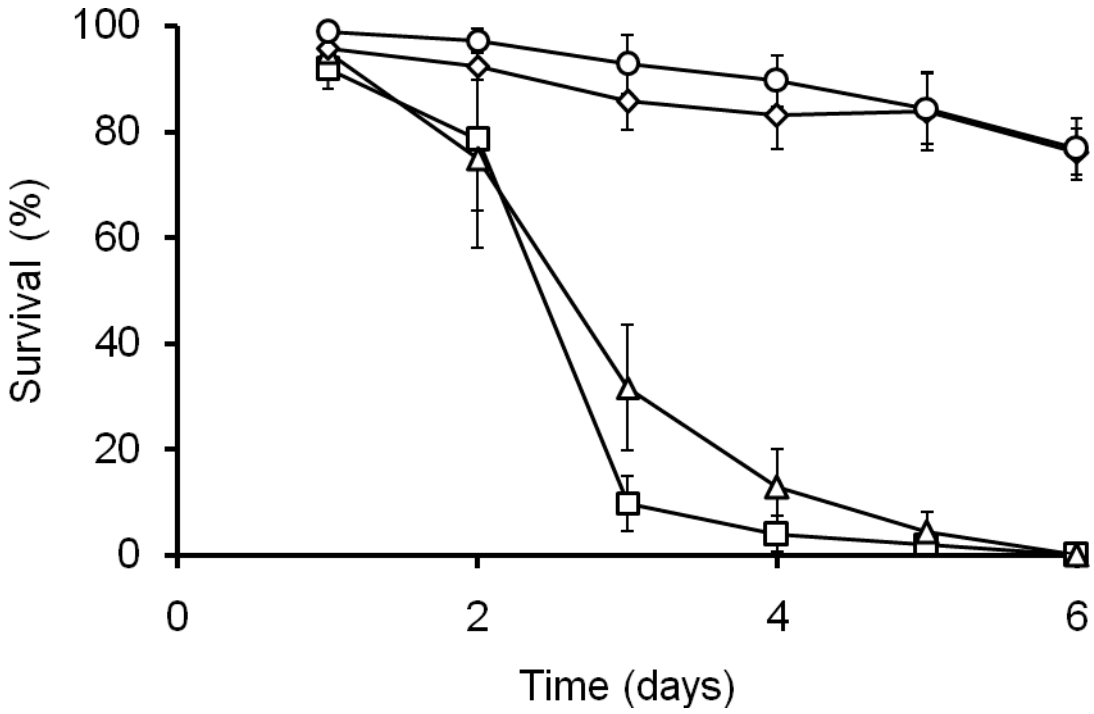

Figure 3