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Ruthenium(II) phosphine/diimine/picolinate complexes: Inorganic compounds as agents against tuberculosis

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A B S T R A C T

This paper describes the synthesis and characterization of four new ruthenium complexes containing 1,4 bis(diphenylphosphino)butane (dpbb), 2-pyridinecarboxylic acid anion (pic) and the diimines [(2,2'-bipyridine (bipy), 4,4'-dimethyl-2,2'-bipyridine (Me-bipy), 4,4'-dichloro-2,2'-bipyridine (Cl-bipy) and 1,10-phenanthroline (phen)] as ligands, with formulae [Ru(pic)(dpbb)(bipy)] PF 6 ([SCAR01]), [Ru(pic)(dpbb)(Me-bipy)] PF 6 ([SCAR02]), [Ru(pic)(dpbb)(Cl-bipy)] PF 6 ([SCAR03]) and [Ru(pic)(dpbb)(phen)] PF 6 ([SCAR04]). Additionally, the in vitro anti-Mycobacterium tuberculosis (MTB) activity, cytotoxicity and activity against in vitro infection of these complexes and two more complexes, cis-[Ru(pic)(dppe)2] PF 6 ([SCAR05]) and cis-[RuCl2(dppe)(bipy)] ([SCAR06]), and their free ligands are described and discussed. All compounds showed excellent MIC against MTB, low cytotoxicity and a selectivity index higher than 10. Also, all compounds showed significant intracellular inhibition and the compound SCAR05 showed a better activity than rifampin and SQ109. This is the first report of activity against in vitro infection of ruthenium compounds.

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1. Introduction

The tuberculosis (TB) mortality rates decreased globally in 2007, with 1.3 million HIV-negative TB patients dying in 2007 and 456,000 deaths among individuals infected with both TB and HIV. However, in 2007 there were an estimated 9.27 million incident cases of TB, the majority of which occurred in Asia and Africa. This is an increase from the previous year, when 9.24 million cases were recorded [1]. In addition, the highly drug-resistant organisms are virtually untreatable in immunocompetent patients, so when these organisms enter into contact with highly immunocompromised HIV-infected populations, the mortality rate within a few weeks of infection approaches 100% [2]. In the last 40 years, no new drugs have been developed specifically against mycobacteria [3] and, only in the last few years, some promising candidate drugs are being tested in the preclinical phase [4]. Thus, there is a great need to develop new therapeutic agents to treat tuberculosis, so as to reduce the total duration of treatment and to provide more effective treatment against multidrug resistance (MDR), extensive drug resistance (XDR) and latent tuberculosis infection [5]. There are currently two main approaches to developing a new anti-TB drug [6]. One of them is based on the synthesis of analogues of existing drugs, with the aim of shortening and improving TB treatment. The other involves a search for novel structures with which mycobacteria have never been challenged before [7]. Adopting this second approach, our research is focused on the synthesis and characterization of new compounds, specifically new metal complexes.

Medicinal chemistry is taking a growing interest in the development of metal complexes for use as drugs or diagnostic agents.
Owing to their wide spectrum of coordination numbers and geometries, as well as kinetic properties, metal complexes offer mechanisms of drug action that cannot be realized by organic agents [7,8]. Ruthenium compounds have already exhibited potential anti-tumour activity since the 1970s and 1980s, and, more recently, the Ru(II) complexes NAMI-A and KP 1019 (FFC14a) have entered clinical trials. Recent studies have indicated that the anti-tumour activity of Ru(II) complexes also depends on their reducibility to Ru(II) species. The in vitro activity of a homologous series of Ru(III) complexes increased with increasing ease of reduction [8].

In this laboratory, we have studied and reported on the anti-Mycobacterium tuberculosis (MTB) activity of several new complexes containing various metals [3,7,9–14]. Specifically, Ru(II) complexes have shown excellent activity against in vitro MTB, being up to 150 times more active than their free ligands 4,6-dimethyl-2-mercaptopyrimidine; 1,4 bis(diphenylphosphino)butane; 1,2-bis(diphenylphosphino)ethane, bis(diphenylphosphino) methane, triphenylphosphine, 2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine, 2-pyridinecarboxylic acid and, interestingly, in these complexes the activity was higher, when they contained three chelated ligands, than that of the precursor complexes containing two chelated and two chloride ligands [9,14].

This paper describes the synthesis and characterization of four new ruthenium(II) complexes containing 1,4 bis(diphenylphosphino)butane (dppb), the 2-pyridinecarboxylic acid anion (pic) and diimines (bipy, Me-bipy, Cl-bipy and phen), as ligands, with formulae [Ru(pic)(dppb)(bipy)]PF6 (SCAR01), [Ru(pic)(dppb)(Me-bipy)]PF6 (SCAR02), [Ru(pic)(dppb)(Cl-bipy)]PF6 (SCAR03) and [Ru(pic)(dppb)(phen)]PF6 (SCAR04). Additionally, the in vitro anti-M. tuberculosis (MTB) activity, cytotoxicity and activity against in vitro infection of these complexes and two more complexes, cis-[RuCl2(dppb)(bipy)]PF6 (SCAR05) and cis-[RuCl2(dppb)(bipy)] (SCAR06) [9,15], and their free ligands are presented and discussed.

2. Results and discussion

2.1. Synthesis of the complexes

The chemical reactivity of the 2-pyridinecarboxylic acid (Hpic) ligand with complexes such as cis-[RuCl2(dppb)(N-N)] [15] enabled the synthesis of complexes with the general formula [Ru(pic)(dppb)(N-N)]PF6 (N-N = bipy, Me-bipy, Cl-bipy or phen), containing three chelated ligands, under mild conditions, by simple chloride exchange (see Scheme 1).

2.2. Structural studies

The X-ray structure of SCAR01 was determined and an ORTEP view showing the atom numbering scheme is shown in Fig. 1. Selected bond lengths and angles are presented in Table 1. The crystal structure of the complex consists of separate discrete molecular units and solvated water molecules (w). Complexes have shown excellent activity against in vitro MTB, being up to 150 times more active than their free ligands 4,6-dimethyl-2-mercaptopyrimidine; 1,4 bis(diphenylphosphino)butane; 1,2-bis(diphenylphosphino)ethane, bis(diphenylphosphino) methane, triphenylphosphine, 2,2'-bipyridine, 4,4'-dimethyl-2,2'-bipyridine, 2-pyridinecarboxylic acid and, interestingly, in these complexes the activity was higher, when they contained three chelated ligands, than that of the precursor complexes containing two chelated and two chloride ligands [9,14].

This paper describes the synthesis and characterization of four new ruthenium(II) complexes containing 1,4 bis(diphenylphosphino)butane (dppb), the 2-pyridinecarboxylic acid anion (pic) and diimines (bipy, Me-bipy, Cl-bipy and phen), as ligands, with formulae [Ru(pic)(dppb)(bipy)]PF6 (SCAR01), [Ru(pic)(dppb)(Me-bipy)]PF6 (SCAR02), [Ru(pic)(dppb)(Cl-bipy)]PF6 (SCAR03) and [Ru(pic)(dppb)(phen)]PF6 (SCAR04). Additionally, the in vitro anti-M. tuberculosis (MTB) activity, cytotoxicity and activity against in vitro infection of these complexes and two more complexes, cis-[RuCl2(dppb)(bipy)]PF6 (SCAR05) and cis-[RuCl2(dppb)(bipy)] (SCAR06) [9,15], and their free ligands are presented and discussed.

2.3. Characterization of the compounds

The 31P{1H} NMR spectra of complexes SCAR01-4 in CH3Cl/CD2O in a capillary presented typical AB spin systems exhibiting chemical shifts close to 39.0 (d) and 37.3 (d), with ΔP,P = 33.5 Hz, indicating the magnetic nonequivalence of the two phosphorus atoms.
we suggest that in the new complexes the high-
ness of these new complexes in cyclic voltammetric experiments was
reversible one-electron RuII/RuIII redox process was observed for
for the Ru-P2
P1 distance of 2.347(3) Å (32.0 ppm, with2
responding to the
ence of the pic ligand coordinated to the Ru(II) center. Bands cor-
3200-3100 cm
visible region, one band and a shoulder were observed, respectively
of comparison with the free ligands (dppb, N
complexes showed four absorptions in the UV region (two bands
Table 2
Table 2. Precursor complexes of general formula cís-[RuCl2(dpbp)(N-N)] show a pair of doublets at 43.0 and
32.0 ppm, with Jp-p = 32.0 Hz, in their 31P{1H} spectra[15,22]. In
the precursors, the high-field doublet corresponds to the P trans N,
as previously described [15]. These assignments are based on an
empirical linear correlation established between the crystallo-
determined Ru–P distances in a series of Ru-dpbp complexes and the corresponding 31P chemical shifts observed in
solution, in which the chemical shifts become more high-field with
increasing Ru–P bond length [15,16,19]. In light of this information, we
suggest that in the new complexes the high-field doublet belongs to
the P trans to nitrogen from bipy (N-N), because the Ru-
P1 distance of 2.347(3) Å (trans N-N) is longer than that observed for
the Ru-P2 trans O of the pic ligand (2.324(2) Å (see Table 1).

The mass spectra (ESI-MS) of complexes SCAR01-4, delivered in
a CH2Cl2 solution, showed the parent molecular ions at m/z values
[M – PF6+] consistent with the assigned formulation (see experi-
mental data). No fragmentation was observed under the experi-
mental conditions described.

The IR spectra of the new complexes also confirmed the pres-
ence of the pic ligand coordinated to the Ru(II) center. Bands cor-
responding to v(CO) (COO) and ν(CO) vibrational modes appeared
close to 1660 and 1334 cm
, respectively, for all complexes. The band attributable to ν(OH), which appeared at
3200-3100 cm
in the free ligand spectrum, was absent in those of
complexes SCAR01-4, indicating that the pic ligand is coordinated in
the deprotonated form [23]. The electronic spectra of SCAR01-4
complexes showed four absorptions in the UV region (two bands
and two shoulders), assigned as intra-ligand transitions by means of
comparison with the free ligands (dpb, N–N and pic). In the
visible region, one band and a shoulder were observed, respectively
close to 420 and 490 nm. These absorptions can be attributed to
metal-to-ligand charge transfer transitions, probably involving
both diimine (N–N) and pic ligands. The electrochemical behavior
of these new complexes in cyclic voltammetric experiments was
similar to that observed for their precursors [15,29]. A quasi-
reversible one-electron RuII/RuIII redox process was observed for
each complex. As expected, the E1/2 values found for the new
complexes were considerably more anodic than those observed for
the respective precursors, by approximately 0.60 V (Table 2), indicat-
ing that the Ru center was more stable in the new derivatives
than in their precursors. This stabilization is assumed to be due to

The replacement of two monoanionic donor chlorides by a single
monocharged chelating pic unit, resulting in positively-charged
product complexes [24]. Among the four new complexes, the E1/2
values clearly correlate with the diimine pKa values, such that the
complex containing the most acidic ligand (Cl-bipy) showed the most
stable ruthenium center (highest E1/2).

2.4. Anti-M. tuberculosis activity assay

The in vitro anti-MTB activities of the free ligands and the
phosphine-diimine-picoline ruthenium complexes were tested against
MTB H37Rv ATCC 27294 and the MICs are reported in
Table 3. The MICs of complexes SCAR01-05 are comparable to or
better than those of some “second” and “first” line drugs used in
current therapy [6,25,26]. SQ 109, an antibacterial compound
derived from ethambutol, with MIC of 1.56 µM [27], and TMC 207,
a diarylquinoline with MIC of 0.81 µM [28], both of which are new
promising drug candidates, presently in the human clinical trial
phase, are examples of in vitro activity comparable to the ruthen-
iun complexes presented here. Even though the complex SCAR06
showed the worst inhibitory result among the tested compounds, it
had activity comparable to or better than the “second” line drugs [25].
Comparing the complexes SCAR01 and SCAR06, we observe that
SCAR01 is 5.5 times more active than SCAR06, probably due to
the absence of the Hpic ligand in the latter. The ligands dppe and
phen are the only ones that have good MICs (15.70 and 11.80 µM
respectively). However, these values are respectively 71 and 16
times higher than those of their complexes.

2.5. In vitro cytotoxicity

The cytotoxicity results (Table 3), expressed as the IC50 for
the J77A1 cell line, show that all of the tested ruthenium complexes
exhibited low cytotoxicity (IC50 ranging from 3.4 to 104 µM). The
free ligands are also not very cytotoxic (IC50 ranging from <50 to
2538 µM). The selectivity index (SI) of each compound was deter-
mined as the ratio of IC50 to MIC (Table 3). According to Orme et al.
[29], candidates for new drugs must have an index equal to or
higher than 10, together with MIC lower than 6.25 µg/mL (or the
molar equivalent) and a low cytotoxicity. SI is used to estimate the
therapeutic window of a drug and to identify drug candidates for
further studies. Thus, all the ruthenium complexes studied here,
with SI ranging from 15.20 to 40.10, are very promising new anti-
tuberculosis drug candidates, and therefore they were all tested in
the in vitro infection model [29].

2.6. In vitro infection model

The in vitro intracellular activities of the phosphine-diimine-
picoline ruthenium complexes were tested against MTB Erd-
mann (ATCC 35801) containing the Luciferase Plasmid and the
inhibitory values are reported in Table 4. The compounds were
tested in macrophages at those concentrations: at their MICs and 4
times higher and lower. All complexes presented significant in-
tracellular activity at various concentrations. The complexes SCAR02
and SCAR05 were the most active because they showed the best
activity at lower concentrations (0.13 µM = 70.50% inhibition and
0.06 µM = 47.60% inhibition, respectively). These complexes also
exhibited the best activity at concentrations equal to and higher
than the MIC. The comparison between the intracellular activity of
compound SCAR05 and rifampin (Table 4) showed a higher activity
in the new compound. Additionally, the intracellular activities of
compounds SCAR01-05 are better than those of 26 synthesized
compounds from a combinatorial library of 1,2-ethylendiamines,
and comparable to SQ 109 (4.72 µM = 99% intracellular inhibition),
which is the best compound in this library [27]. On the other hand, the compound SCAR06 showed low intracellular activity at 1.29 μM (19.40%), compared to the similar compound SCAR01, with 55.10% inhibition at a concentration of 0.95 μM (Table 4). This finding corroborates the previous observation about the importance of the presence of Hpic in the complex structure, to intensify the anti-tuberculosis activity.

2.7. In vitro structure–activity relationship (SAR)

Indeed, although Hpic is the ligand with the lowest activity among the tested ligands against MTB, its presence in the ruthenium complexes increased both extra and intracellular activities. For example, the substitution of the two Cl atoms by Hpic in the cis-[RuCl2(dppb)(bipy)] (SCAR06), resulting in [Ru(pic)(dppb)(bipy)]PF6 (SCAR01), increased the anti-TB activity five times. Therefore, it is likely that the structures of the complexes are responsible for the properties of the compounds, and important for their mechanism of action as tuberculostatics. The structural changes probably affect the reaction of the complexes with bacterial DNA, which is the likely target of these drugs. It is known that the interaction with DNA is the basis of the cytostatic effect of ruthenium compounds [30–32] and this interaction is modulated by the nature of the ligands present in the complexes. In the present case, the smaller partial electronic charge on the free oxygen atom of the pic ligand, in comparison with the charges on the chlorine atoms of the original compound [33], makes the former the worst hydrogen bond acceptor. This could be associated with a higher cytotoxicity [32] and probably the anti-MTB effect follows the same trend.

In order to preliminary address if interaction with DNA could be part of the mode of action of the complexes, experiments with CT DNA were carried out. Binding of SCAR01 to DNA was studied by combining atomic absorption determinations (for the metal) and electronic absorption measurements for DNA quantification. The complex showed a negligible covalent binding level to CT DNA (nmol Ru/mg DNA base pairs). Interaction with DNA was further explored through circular dichroism technique, viscosity measurements and atomic force microscopy (AFM). In Fig. 2, the circular dichroism spectra of SCAR01 at several ruthenium complex: DNA molar ratios are shown. After 24 h of incubation at 37°C, changes in molar ellipticity can be observed for the complex.

### Table 3

Anti-MTB activity (MIC), cytotoxicity (IC50), and selectivity index (SI) of the ruthenium complexes and their free ligands.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Compounds</th>
<th>MIC</th>
<th>IC50</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/mL</td>
<td>μM</td>
<td>μg/mL</td>
</tr>
<tr>
<td>SCAR01</td>
<td>[Ru(pic)(dppb)(bipy)]PF6</td>
<td>0.91</td>
<td>0.95</td>
<td>31.30</td>
</tr>
<tr>
<td>SCAR02</td>
<td>[Ru(pic)(dppb)(Me-bipy)]PF6</td>
<td>0.49</td>
<td>0.50</td>
<td>11.70</td>
</tr>
<tr>
<td>SCAR03</td>
<td>[Ru(pic)(dppb)(Cl-bipy)]PF6</td>
<td>0.78</td>
<td>0.76</td>
<td>31.30</td>
</tr>
<tr>
<td>SCAR04</td>
<td>[Ru(pic)(dppb)(phen)]PF6</td>
<td>0.63</td>
<td>0.74</td>
<td>19.50</td>
</tr>
<tr>
<td>SCAR05</td>
<td>cis-[Ru(pic)(dppb)(bipy)]PF6</td>
<td>0.20</td>
<td>0.22</td>
<td>3.90</td>
</tr>
<tr>
<td>SCAR06</td>
<td>cis-[RuCl2(dppb)(bipy)]</td>
<td>3.90</td>
<td>5.17</td>
<td>78.20</td>
</tr>
<tr>
<td>Ligand</td>
<td>Hpic</td>
<td>&gt; 50</td>
<td>&gt; 406</td>
<td>312.50</td>
</tr>
<tr>
<td>Ligand</td>
<td>dppb</td>
<td>&gt; 50</td>
<td>&gt; 117.20</td>
<td>625</td>
</tr>
<tr>
<td>Ligand</td>
<td>phen</td>
<td>6.25</td>
<td>15.70</td>
<td>156.30</td>
</tr>
<tr>
<td>Ligand</td>
<td>bipy</td>
<td>2.34</td>
<td>11.80</td>
<td>8.80</td>
</tr>
<tr>
<td>Ligand</td>
<td>Me-bipy</td>
<td>25</td>
<td>160.10</td>
<td>9.80</td>
</tr>
<tr>
<td>Ligand</td>
<td>Cl-bipy</td>
<td>25</td>
<td>111.06</td>
<td>9.80</td>
</tr>
</tbody>
</table>

* Data reported previously [9,14].

### Table 4

Intracellular activity against MTB Erdmann ATCC 35801 with pSMT1 plasmid, of ruthenium compounds and rifampin at various concentrations.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Compounds</th>
<th>Concentrations Range</th>
<th>Intracellular inhibitory activities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μg/mL</td>
<td>μM</td>
</tr>
<tr>
<td>SCAR01</td>
<td>[Ru(pic)(dppb)(bipy)]PF6</td>
<td>3.64</td>
<td>3.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.91</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.23</td>
<td>0.24</td>
</tr>
<tr>
<td>SCAR02</td>
<td>[Ru(pic)(dppb)(Me-bipy)]PF6</td>
<td>1.96</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>SCAR03</td>
<td>[Ru(pic)(dppb)(Cl-bipy)]PF6</td>
<td>3.12</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.78</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.20</td>
<td>0.19</td>
</tr>
<tr>
<td>SCAR04</td>
<td>[Ru(pic)(dppb)(phen)]PF6</td>
<td>2.52</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.63</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16</td>
<td>0.19</td>
</tr>
<tr>
<td>SCAR05</td>
<td>cis-[Ru(pic)(dppb)(bipy)]PF6</td>
<td>1.04</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>SCAR06</td>
<td>cis-[RuCl2(dppb)(bipy)]</td>
<td>15.6</td>
<td>20.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.90</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98</td>
<td>1.29</td>
</tr>
<tr>
<td>RMP</td>
<td>Rifampin</td>
<td>0.40</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>0.04</td>
</tr>
</tbody>
</table>
These changes in the wavelength and the ellipticity of free DNA indicate modifications in the secondary structure of DNA as a consequence of its interaction with the complexes. Although the substitution of ligands by N atoms of DNA bases could be responsible, the most probable hypothesis is that weak interactions occur between the ligands and DNA, through hydrogen bonds.

The comparison of the AFM images of pBR322 DNA incubated with SCAR01 (Fig. 3a) and of the free plasmid (Fig. 3b), show typical modifications in the form of the DNA, namely super coiling, kinks and compaction. These modifications indicate that SCAR01 interacts with DNA. Additionally, the variation of viscosity with time at 25 °C shows a decrease in the viscosity, which implies that there is no intercalation of the complex between base pairs of the DNA. All these results are in agreement with the structure of SCAR01 that seems not to allow neither covalent binding to DNA bases nor intercalation between bases.

It is known from the literature, that the KP1019 compound, a tumour-inhibiting drug with a new mode of action, which probably involves accumulation in transferrin receptor-(over) expressing tumour cells via transferrin receptor, subsequent reduction to RuIII species, reacts with DNA and induction of apoptosis via intrinsic mitochondrial pathway [34]. Bratsos et al. presented some ruthenium complexes containing 1-(2-Picolyl)-substituted 1,2,3-triazole ligands showing their anticancer properties probably involving hydrolysis of the chloride ligand and subsequent reaction with DNA [35]. Furthermore, another recent study showed the cellular uptake and subcellular distribution including adduct formation with genomic DNA and uptake into mitochondria of two ruthenium(III)-based drugs in clinical trials, KP1019 and NAMI-A, indicating that these ruthenium drugs have distinct differences with respect to cisplatin, especially cisplatin-resistant cells. In comparison to the sensitive cells, KP1019 exhibits higher cytotoxicity and a slightly changed metabolism of the drug, whereas NAMI-A treatment results in increased intracellular ruthenium levels and a higher number of ruthenium-DNA adducts [36]. These data show that in fact the DNA can be a target for ruthenium complexes but interaction with proteins cannot be discarded as possible targets for ruthenium and other metal ions complexes.

The structures of the complexes studied in this work are quite different to the compounds containing chloride in their coordination sphere, what cause different interactions with the DNA. In this case only the complex SCAR06 presents chloride coordinated to the ruthenium, but it was showed that only one trans to the phosphorus atom is dissociated from the metal [37,38].

In a previous study we reported that for the complexes [RuIIICl4(DMSO)(H-Hypoxanthine)] and [RuIIICl4(DMSO)[H-(N6-butyladenine)]] was possible to observe in the AFM images a strong aggregation in plasmidic DNA forms [39]. No nuclease activity was observed in any of these tested complexes and the modifications observed confirm that these complexes do not destroy the structure of the pBR322 plasmid DNA but they yield more coiled and/or more associated plasmids. As in that case, in our present work we can observe aggregation of the forms but in a lower degree; the complex does not destroy the structure of the pBR322 plasmid DNA, no nuclease activity is observed. The compacting of the forms, the supercoiled observed, are in good agreement with the modification observed in CD spectrum and viscosity.

**Fig. 2.** Circular Dichroism spectra of plasmid pBR322 DNA and plasmid pBR322 DNA incubated with the [Ru(pic)(dpbb)(bipy)]PF6 (SCAR01) complex for 24 h, at 37 °C at molar ratios 0.1, 0.3 and 0.5.

**Fig. 3.** (a) AFM image of the plasmid pBR322 DNA incubated with the [Ru(pic)(dpbb)(bipy)]PF6 (SCAR01) complex for 24 h, at 37 °C. (b) AFM image of the free pBR322 DNA.
measurements, which indicate interaction of the compound with DNA, probably by electrostatic forces.

3. Conclusions

The synthesis and characterization of four new ruthenium complexes has been described in this work. Additionally, biological studies of these four ruthenium complexes and two other ones together with those of the free ligands were presented. The results reported here confirm the biological activity of these ruthenium complexes and raise the possibility of these compounds becoming new drugs against TB.

4. Experimental section

4.1. Materials for synthesis

Solvents were purified by standard methods. All chemicals used were of reagent grade or comparable purity. RuCl₃·3H₂O, the ligands 1,4-bis(diphenylphosphino)butane (dpbb), 2,2’-bipyridine (bipy), 4,4’-dimethyl-2,2’-bipyridine (Me-bipy), 4,4’-dichloro-2,2’-bipyridine (Cl-bipy), 1-10-phenanthroline (phen) and 2- pyridylcarboxylic acid (Hpic) were used as received from Aldrich. The complexes cis-[RuCl₂(dpbb)(N-N)], N-N = bipy, Me- bipy, Cl-bipy or phen were prepared according to published procedures [15,22].

4.2. Instrumentation

The infrared spectra were collected from CsI pellets in a Bomem-Michelson 102 FTIR spectrometer, in the 4000–200 cm⁻¹ region. UV–visible (UV–Vis) spectra were recorded in a HP8452A (diode array) spectrophotometer. All NMR experiments were performed at 293 K in a Bruker spectrometer, 9.4 T, observing ¹H at 400.13, ¹³C at 100.61 and ³¹P[¹H] at 161.98 MHz. The NMR spectra were recorded in CDCl₃, with TMS (¹H and ¹³C) and 85% H₃PO₄ (³¹P{¹H}) as internal and external references, respectively. The splitting of proton, carbon, and phosphorus resonances, respectively, in the reported ¹H, ¹³C and ³¹P[¹H] NMR spectra, are defined as d = doublet. Mass spectra were obtained in a QuattroLC Mass Spectrometer (Micromass, triple-quadrupole, ESI/APCI, UK) equipped with a DAD source (Phenix 40 pump, CE Instruments, Italy). The mobile phase used to deliver the sample into the ion source by direct infusion was water/acetonitrile (40:60, v/v) (JT Baker, Mexico), used to deliver the sample into the ion source by direct infusion was water/acetonitrile (40:60, v/v) (JT Baker, Mexico), flowing at 100 µL/min. All analyses were performed with an Electrospray Ionization (ESI) probe in the positive ion mode. In each analysis, 10 µL of a standard solution were injected at a concentration of 1 µg/mL. Cyclic voltammetry experiments were carried out at room temperature in CH₃Cl containing 0.10 M Bu₄NClO₄ (TBAP) (Fluka Purum), with a Bioanalytical Systems Inc. BAS-100B/W electrochemical analyzer. The working and auxiliary electrodes were stationary Pt foils; a Luggin capillary probe was used and the reference electrode was Ag/AgCl. Under these conditions, ferrocene is oxidized at 0.43 V (Fc/Fc⁰). C, H and N contents were determined in the Microanalytical Laboratory of Universidade Federal de São Carlos, São Carlos (SP), with an EA 1108 Fisons Instruments CHNS microanalyser.

Complexes were tested for their DNA interaction ability using native calf thymus DNA (CT DNA) (Type I) by a modification of a previously reported procedure [40,41]. CT DNA (50 mg) was dissolved in water (30 mL) (overnight). Solutions of the complexes in DMSO (spectroscopy grade) (1 mL, 10⁻³ M) were incubated at 37 °C with solution of CT DNA (1 mL) during 96 h. DNA/complexes mixtures were exhaustively washed to eliminate the un-reacted complex. Quantification of bound metal was done by atomic absorption spectroscopy on a Perkin Elmer 5000 spectrometer. Standards were prepared by diluting a metal standard solution for atomic absorption spectroscopy. Final DNA concentration per nucleotide was determined by UV absorption spectroscopy using molar absorption coefficient of 6000 M⁻¹ cm⁻¹ at 260 nm. The reported values are mean of three determinations. For the circular dichroism measurements, the SCAR01 complex was dissolved in an aqueous solution (prepared with Milli-Q water) of 4% DMSO (2 mg compound/5 mL). The stock solutions were freshly prepared before use. The samples were prepared by the addition of aliquots of these stock solutions to the appropriate volume of CT DNA in a TE buffer solution (50 mM NaCl, 10 mM tris-(hydroxymethyl)aminomethane (Tris–HCl), 0.1 mM H₄edta, pH 7.4) (5 mL). The amount of complex added to the DNA solution was expressed as the input molar ratio of Ru to nucleotide, calculated by formula (1)

\[
r_{i} = m_{\text{molec}} Am/C. Mr.V
\]

where \( m \) = mass of the compound (g); \( M_{\text{molec}} = \) mean nuclear mass per nucleotide (330 g/mol); \( Am = \) number of the metal atoms; \( C = \) concentration of DNA (g/mL); \( Mr = \) molar mass of the compound (g/mol); \( V = \) total volume of the sample (5 mL). A solution of free native DNA in TE was used as a blank. The CD spectra of DNA, in the presence and absence of complex (DNA concentration 20 mg/mL, molar ratios \( r_{i} = 0.10, 0.30, 0.50 \)), were recorded at room temperature, after 24 h incubation at 37 °C, on a JASCO J-720 spectropolarimeter with a 450 W xenon lamp; a computer was used for spectral subtraction and noise reduction. Spectra for each sample were scanned twice, from 220 to 330 nm. The plotted CD spectra are the average of three independent scans. The data are expressed as average residue molecular ellipticity (θ) in degrees cm² dmol⁻¹. Viscosity experiments were carried out in an AND-SV-1 viscometer in a water bath with a water jacket, to maintain the temperature at 25 °C. A range of 200–370 µL of 5 mM solutions of the compound were added to 2 mL of 100 mM CT DNA

Table 5

Crystallographic data and refinement details for [Ru(dpbb)(bipy)]PF₆·2H₂O (SCAR01).

<table>
<thead>
<tr>
<th>Empirical formula</th>
<th>C₄₄H₄₀N₃O₂P₂RuPF₆·2H₂O</th>
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<tbody>
<tr>
<td>Formula weight</td>
<td>986.80</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>298(2)</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P-1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
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</tr>
<tr>
<td>a (Å)</td>
<td>10.4187(5)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>21.9209(10)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>99.985(3)</td>
</tr>
<tr>
<td>α (°)</td>
<td>100.073(4)</td>
</tr>
<tr>
<td>β (°)</td>
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</tr>
<tr>
<td>γ (°)</td>
<td>2172.12(19)</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
</tr>
<tr>
<td>Density (calculated) (g cm⁻³)</td>
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</tr>
<tr>
<td>Absorption coefficient (mm⁻¹)</td>
<td>0.543</td>
</tr>
<tr>
<td>F(000)</td>
<td>1008</td>
</tr>
<tr>
<td>Crystal size (mm³)</td>
<td>0.27 × 0.25 × 0.20</td>
</tr>
<tr>
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</tr>
<tr>
<td>Limiting indices</td>
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</tr>
<tr>
<td></td>
<td>–12 ≤ k ≤ 12</td>
</tr>
<tr>
<td></td>
<td>–26 ≤ l ≤ 26</td>
</tr>
<tr>
<td>Reflections collected</td>
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</tr>
<tr>
<td>Independent reflections (Rin)</td>
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<tr>
<td>Data/restraints/parameters</td>
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</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>1.004</td>
</tr>
<tr>
<td>Final R indices (I &gt; 2σ(I))</td>
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</tr>
<tr>
<td></td>
<td>R₁ = 0.1208, wR₂ = 0.2359</td>
</tr>
<tr>
<td>Peak and hole/e Å⁻³</td>
<td>1.298 and –1.807</td>
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</tbody>
</table>
solution. The flow time was measured by a digital stop watch. For the Atomic Force Microscopy (TMAFM) experiment, plasmid pBR322 DNA was heated at 60 °C for 10 min to obtain the Open Circular form (OC form). Stock solution was 1 mg/mL in a buffer solution of HEPES (4 mM Hepes, pH 7.4/2 mM MgCl₂). Each sample contained 1 μL of pBR322 DNA at a concentration of 0.25 μg/μL, for a final volume of 40 μL. The amount of compound added is also expressed as r. AFM samples were prepared by placing a 3 μL drop of test solution onto freshly cleaved Muscovite green mica disks as the support. The drop was allowed to stand undisturbed for 3 min to favour the adsorbate—substrate interaction. Each DNA-laden disk was rinsed with Milli-Q water and blown dry with clean compressed argon gas directed normal to the disk surface. Samples were stored over silica gel prior to AFM imaging. All AFM observations were made with a Nanoscope III Multimode AFM (Digital Instruments, Santa Barbara, CA). Nano-crystal Si cantilevers of length 125 nm, with an average spring constant of 50 N/m, terminated with conical-shaped Si probe tips of apical radius 10 nm and cone angle 35° were utilized. High-resolution topographic AFM images were recorded in intermittent contact mode, at 1–3 Hz, in air at room temperature (relative humidity <40%), on various specimen areas of 2 × 2 μm.

4.3. X-ray crystallography

Orange [Ru(pic)(dppb)(bipy)]PF₆ (SCAR01) crystals were grown by slow evaporation of a dichloromethane/n-hexane solution. The crystal was mounted on an Enraf-Nonius Kappa-CCD diffractometer with graphite-monochromated Mo-Kα (λ = 0.71073 Å) radiation. The final unit—cell parameters were based on all reflections. Data collections were made with the COLLECT program [42]; integration and scaling of the reflections were performed with the HKL DENZO-SCALEPACK system of programs [43]. A semi-empirical (from equivalents) absorption correction was applied [43]. The structure was solved by direct methods with SHELXS-97 [44]. The model was refined by full-matrix least squares on F² by means of SHELXL-97 [45]. All hydrogen atoms were stereochemically positioned and refined with a riding model. The ORTEP view of SCAR01 is shown in Fig. 1 and was prepared with ORTEP-3 for Windows [46]. Hydrogen atoms on the aromatic rings were refined isotropically, each with a thermal parameter 20% greater than the equivalent isotropic displacement parameter of the atom to which it is bonded. The data collections and experimental details are summarized in Table 5. The determined bond lengths and angles are not sufficient for meaningful discussion; however, useful information may still be gleaned from the diffraction study with respect the coordination sphere of the metal.

4.4. Synthesis

The [Ru(pic)(dppb)(N-N)]PF₆ complexes (N-N = bipy, Me-bipy, Cl-bipy or phen) were prepared by allowing 50 mg (ca. 0.07 mmol) of the corresponding precursor cis-[RuCl₂(dppb)(N-N)] dissolved in 25 mL of methanol to react with excess 2-pyridinecarboxylic acid ligand (0.20 mmol; ≈ 24.6 mg) and 0.10 mmol (16.2 mg) of NH₄PF₆ under Ar atmosphere for 24 h. The final red solutions were concentrated to ca. 3 mL and water was added to precipitate an orange solid, which was filtered off, washed well with water (3 × 5 mL) and diethyl ether (3 × 5 mL) and dried in vacuo.

4.5. [Ru(pic)(dppb)(bipy)]PF₆ (SCAR01) yield

47 mg (75%). Anal. Calcld for C₄₄H₄₀F₆N₃O₂P₃Ru: exptl (calc) C, 55.12 (55.58); H, 4.74 (4.24); N, 4.46 (4.42). 3¹P{¹H} NMR: δ(ppm) 39.5 (d); 37.8 (d), 3¹P= 33.5 Hz. UV–Vis (CH₂Cl₂, 10⁻⁵ M): λ/nm ε/M⁻¹ cm⁻¹: 259sh (2.20 × 10⁴), 290 (2.58 × 10⁴), 301sh (2.22 × 10⁵), 350sh (6.40 × 10⁴), 422 (3.90 × 10³), 490sh (1.60 × 10³). ESI (m/z): 806 [C₄₄H₄₀N₃O₂P₃Ru]+.

4.6. [Ru(pic)(dppb)(Me-bipy)]PF₆ (SCAR02) yield

54 mg (70%). Anal. Calcld for C₄₆H₄₀F₆N₃O₂P₃Ru: exptl (calc) C, 56.25 (56.44); H, 4.50 (4.53); N, 4.23 (4.29). 3¹P{¹H} NMR: δ(ppm) 39.1 (d); 37.2 (d), 3¹P= 33.5 Hz. UV–Vis (CH₂Cl₂, 10⁻⁵ M): λ/nm ε/M⁻¹ cm⁻¹: 260sh (1.87 × 10⁴), 288 (2.20 × 10⁴), 300sh (1.88 × 10⁴), 352sh (5.90 × 10³), 416 (3.90 × 10³), 480sh (1.30 × 10³). ESI (m/z): 834 [C₄₆H₄₂N₃O₃P₃Ru]+.

4.7. [Ru(pic)(dppb)(Cl-bipy)]PF₆ (SCAR03) yield

61 mg (80%). Anal. Calcld for C₄₄H₃₈F₆N₃O₂P₃RuCl₂: exptl (calc) C, 51.73 (51.83); H, 4.17 (3.76); N, 4.21 (4.12). 3¹P{¹H} NMR: δ(ppm) 38.5 (d); 36.8 (d), 3¹P= 33.5 Hz. UV–Vis (CH₂Cl₂, 10⁻⁵ M): λ/nm ε/M⁻¹ cm⁻¹: 259sh (2.33 × 10⁴), 294 (2.36 × 10⁴), 337sh (9.60 × 10⁴), 453 (4.50 × 10⁵), 515sh (1.70 × 10⁵). ESI (m/z): 874 [C₄₄H₃₈N₃O₂P₃RuCl₂]+.

4.8. [Ru(pic)(dppb)(phen)]PF₆ (SCAR04) yield

56 mg (74%). Anal. Calcld for C₄₆H₄₀F₆N₃O₂P₃Ru: exptl (calc) C, 57.00 (56.68); H, 4.57 (4.14); N, 4.56 (4.31). 3¹P{¹H} NMR: δ(ppm) 40.2 (d); 38.0 (d), 3¹P= 33.6 Hz. UV–Vis (CH₂Cl₂, 10⁻⁵ M): λ/nm ε/M⁻¹ cm⁻¹: 234 (6.99 × 10⁴), 290 (2.91 × 10⁴), 300 (2.88 × 10⁴), 408 (4.06 × 10⁴). ESI (m/z): 830 [C₄₆H₄₀N₃O₂P₃Ru]+.

4.9. Anti-M. tuberculosis activity assay

The anti-MTB activity of the compounds was determined by the Resazurin Microtiter Assay (REMA) [47]. Stock solutions of the test compounds were prepared in dimethyl sulfoxide (DMSO) and diluted in Middlebrook 7H9 broth (Difco), supplemented with oleic acid, albumin, dextrose and catalase (OADC enrichment - BBL/Becton Dickinson, Sparks, MD, USA), to obtain final drug concentration ranges from 0.15 to 250 μg/mL. The serial dilutions were prepared in a Precision XS Microplate Sample Processor (Biotek®). The isoniazid was dissolved in distilled water, as recommended by the manufacturer (Difco laboratories, Detroit, MI, USA), and used as a standard drug. MTB H₃₇Rv ATCC 27294 was grown for 7–10 days in Middlebrook 7H9 broth supplemented with OADC, plus 0.05% Tween 80 to avoid clumps. Cultures were centrifuged for 15 min at 3150× g, washed twice, and resuspended in phosphate-buffered saline and aliquots were frozen at –80 °C. After 2 days, an aliquot was thawed to determine the viability and the Colony-Forming Unit (CFU) after freezing. MTB H₃₇Rv ATCC 27294 was thawed and added to the test compounds, yielding a final testing volume of 200 μL with 2 × 10⁶ CFU/mL. Microplates with serial dilutions of each compound were incubated for 7 days at 37 °C, after resazurin was added to test viability. Wells that turned from blue to pink, with the development of fluorescence, indicated growth of bacterial cells, while maintenance of the blue colour indicated bacterial inhibition [25,47]. The fluorescence was read (530 nm excitation filter and 590 nm emission filter) in a SPECTRAFluor Plus (Tecan®) microfluorimeter. The MIC was defined as the lowest concentration resulting in 90% inhibition of growth of MTB [25]. As a standard test, the MIC of isoniazid was determined on each microplate. The acceptable range of isoniazid MIC is from 0.015 to 0.06 μg/mL [25,47]. Each test was set up in triplicate.
4.10. Cytotoxicity assay

In vitro cytotoxicity assays (IC$_{50}$) were performed on the J774A.1 (ATCC TIB-67) mouse cell line, as recommended by Pavan et al. [3] The cells were routinely maintained in complete medium (RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus 100 U/mL penicillin and 100 μg/mL streptomycin), at 37 °C, in a humidified 5% CO$_2$ atmosphere. After reaching confluence, the cells were detached and counted. For the cytotoxicity assay, 1 x 10$^5$ cells/mL were seeded in 200 μL of complete medium in 96-well plates (NUNC). The plates were incubated at 37 °C under a 5% CO$_2$ atmosphere for 24 h, to allow cell adhesion prior to drug testing. The compounds were dissolved in DMSO (5%) and subjected to two-fold serial dilution from 1250 to 3.9 μg/mL. Cells were exposed to the compounds at various concentrations for 24 h. Resazurin solution was then added to the cell cultures and incubated for 6 h. Cell respiration, as an indicator of cell viability, was detected by reduction of resazurin to resorufin, whose pink colour and fluorescence indicates cell viability. A persistent blue colour of resazurin is a sign of cell death [25,47]. The fluorescence measurements (530 nm excitation filter and 590 nm emission filter) were performed in a SPECTRA Fluor Plus (Tecan) micro-fluorimeter. The IC$_{50}$ value was defined as the highest drug concentration at which 50% of the cells are viable relative to the control [3]. Each test was set up in triplicate.

4.11. Selectivity index

The selectivity index (SI) was calculated by dividing IC$_{50}$ for the mouse cells by the MIC for the pathogen; if the SI is ≥10, the compound is then investigated further [29].

4.12. In vitro infection model

The intracellular anti-MTB activity of the compounds was determined by in vitro infection model, as suggested by Snewin et al. [48] MTB Erdmann (ATCC 35801) containing the Luciferase toxicity assay, at 37 °C was routinely maintained in RPMI-1640 complete medium (see cytotoxicity assay). The cells were detached and counted. For the intracellular anti-MTB activity, 5 x 10$^5$ cells/mL were seeded in a 24-well plate (NUNC) (1 mL per well) at 37 °C and 5% CO$_2$, for 24 h. The plates were incubated at 37 °C under a 5% CO$_2$ atmosphere for 24 h to allow cell adhesion prior to drug testing. Bacterial suspensions were washed three times in phosphate-buffered saline (PBS) by centrifuging at 2200 × g for 10 min and resuspended in RPMI-1640 complete medium. Cells were infected by incubation with 1–5 bacteria per cell for 1 h at 37 °C and then washed three times with Hanks balanced salt solution (GIBCO BRL). The total number of cell-associated mycobacteria was initially determined, as follows. J774A.1 cells were lysed by the addition of 1 mL of sterile distilled water containing 0.1% Triton X-100 per well. The bacteria that were internalized in macrophages showed luminescence, confirming the infection. The complexes were diluted in RPMI-1640 complete medium at the following concentrations: MIC value, 4 times higher and 4 times lower than MIC (1 mL per well at each concentration). Our previous studies showed that incubation for 3 days is an optimal period. Thus, the plates were incubated for 3 days at 37 °C and 5% CO$_2$ and then washed and lysed. The RLU was determined and the percent inhibition by each compound at each concentration was calculated, based on the positive control test. As a standard test, the intracellular infection by rifampin was determined (Table 4). Each test was set up in triplicate.

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Appendix. Supplementary Material

Crystallographic data for the structural analysis for the complex [Ru(pic)(dpb)(bipy)]PF$_6$ 2H$_2$O (SCAR 01) discussed here have been deposited at the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK and is available on request quoting the deposition number CCDC 817301. These supplementary data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html.

References


15. Based on ZINDO1 calculation performed by one of us. Not published results.


