COMMUNICATION

A hypoxia efficient imidazole-based Ru(II) arene anticancer agent resistant to deactivation by glutathione†

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A slow hydrolyzing imidazole-based RuIII-arene complex [(L)RuII(η6-p-cym)(Cl)](PF6) (1) with excellent stability in the extracellular chloride concentration shows better activity under hypoxia and strong resistance to glutathione (GSH) in vitro under hypoxic conditions. It arrests the cell cycle in sub G1 and G2/M phases and leads to apoptosis.

Ruthenium-based anticancer agents have been one of the most appreciated anticancer drugs after the platinum drugs for their remarkable activity against cancer, the availability of different oxidation states at normal physiological conditions and less risk of side effects. Several π-bonded arene bound ruthenium(II) complexes show high potency against various forms of cancer. RuIII complexes have also demonstrated potential as anti-cancer agents. NAMI-A,1,9–11 is in clinical trials due to its potential to stop the metastasis of cancer cells, especially for solid tumors, although it has relatively poor IC50 values in vitro. In contrast, KP1019 and NKP1339 are active in primary tumors.1,9–11 In vivo experiments in mice show that the [RuIII(η6-arene)Cl2(pta)] (pta is 1,3,5-triaza-7-phosphaadamantane) (RAPTA) complex is also a promising candidate to reduce the growth of lung metastases.7 Binding of Ru anticancer agents with albumin and transferrin in the blood stream is thought to help their delivery to cells.1,3,12–14 It is mostly believed that the Ru in oxidation state +II is the active form. The presence of a reducing agent like glutathione (GSH), or ascorbic acid in pancreas,1,15,16 causes the reduction of RuIII to RuII and increases the rate of aquation and binding with biomolecules.4,9,17 The redox processes however also help to generate reactive oxygen species (ROS), which can destroy GSH pools,1,18 thus destroying the cellular redox balance.19 Yet, the presence of a higher concentration of glutathione (viz. in resistant cells) can inhibit the RuIII complexes by binding to the metal center, thus rendering them inactive.20,21 Ru complexes have the potential to be a good alternative to cisplatin for treating cisplatin-resistant cancers, but they also have affinity towards the thiolate sulphur of cysteine and glutathione, which inhibits their anticancer activity,20,21,22,23 leading to failure of chemotherapeutics.24–26 In addition, the situation is more complicated due to hypoxia, viz. in carcinomas, sarcomas, and lymphomas, since many anticancer agents show less activity in hypoxia, viz. cisplatin.27

The activity of RuIII arene complexes has been tuned mostly by a change of arene,17,28,29 or change of the other ligands,30–32 including the halide ion.17,33 Several RuII arene complexes are active against cisplatin-resistant cell lines.34–36 Among the several ways to tune the activity of RuIII arene complexes, we planned to introduce steric bulk in the auxiliary ligand to slow down the hydrolysis. The ligand used for this purpose was a sterically hindered imidazole based Schiff base ligand (L = N-((1H-imidazol-2-yl)methylene)-2,6-diisopropylaniline). Our attempt provided us a p-cymene (p-cym)-bound ruthenium(II) complex of L (1) (Fig. 1). The compound is slow to hydrolyze and has excellent stability in saline solution. The compound shows promising anticancer activity as per our initial studies using three different carcinoma cell lines (MCF-7, A549, HeLa). We found that in hypoxic conditions the activity of 1 is enhanced and the complex is strongly resistant to deactivation by the cellular reductant L-glutathione.

Complex 1 crystallizes in the monoclinic space group, P21/n (see ESI, Table S1†). Each unit cell contains four complexes. In each molecule, one vertex of the tetrahedral structure is occupied by a chloride, two with ligand L and another one by the p-cymene with a η6 bonding, but all the distances between carbons of p-cymene and metal atoms are not the same (see ESI, Table S2†). This may be due to the steric hindrance of the isopropyl group of p-cymene with the closest isopropyl group.
PF₆ group is present in the lattice per molecule of the 30% probability level. Hydrogen atoms and counter anion have been omitted for clarity.

of the other ligand (L). The NMR spectra also support the above fact that the two methyl groups of the isopropyl in p-cymene are no longer equivalent (see ESI, Fig. S1, S2†). One PF₆ group is present in the lattice per molecule of the complex, since the RuII is in the +2 oxidation state.

The hydrolysis of the labile halide group in such complexes in general renders the complex active towards DNA binding. The 1H NMR study of complex 1 shows that the complex is ca. 32% hydrolyzed after 28 h (see ESI, Fig. S4†) in a 3:7 v/v DMSO-d₆-D₂O mixture, and initially up to 2.5 h, we could not see any peak for the hydrolyzed product. This slow hydrolysis may be associated with the steric hindrance rendered by the ligand due to the presence of isopropyl groups. The hydrolysis rate in 20 mM phosphate buffer solution at pH 7.4 containing 4 mM NaCl and 1% acetonitrile is 0.0115(5) h⁻¹ and hence the t₁/₂ is 60(3) h (Table 1, see ESI, Fig. S5†). In water containing 1% acetonitrile, the t₁/₂ of 1 is 4.5(1) h. 1 is stable up to 10 days in 110 mM saline solution as per the 1H NMR data (see ESI, Fig. S6†), which is encouraging for an active anticancer agent and relatively less commonly found. The properties of 1 and the available data in literature on this type of RuII complexes bearing the general formula, RuII(arene)(ligand)(halide), suggests that in general, there does not appear to be a strong correlation between the rate of hydrolysis and cytotoxicity (see ESI, Table S3†). However, when $t_{1/2}$ is less than an hour, the complexes are more cytotoxic (see ESI, Table S3†) with a few exceptions,

<table>
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<tr>
<th>Table 1 Rate of hydrolysis and half lives of 1 at pH 7.4 and 6.7 measured by UV-vis spectroscopy in 20 mM phosphate buffer solution in the presence of 40 mM or 4 mM salinea</th>
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<tbody>
<tr>
<td>pH 7.4 NaCl (mM)</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>Watera</td>
</tr>
<tr>
<td>Half-life ($t_{1/2}$) h</td>
</tr>
<tr>
<td>Dissociation rate (k) h⁻¹</td>
</tr>
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</table>

Data presented are the mean of three independent experiments. a Data presented are average of two experiments instead of three.

The correlation of $t_{1/2}$ values in water shows that our complex is also an exception to the generally observed trend. Recently a TiIV isopropoxide complex reported by Tshuva et al. shows that complexes stable towards hydrolysis in aqueous medium may be active as per the in vitro studies.40 From the above results, it appears that the hydrolysis of a complex in a biological environment may not be a simple phenomenon as predicted through hydrolysis studies in buffer.

To gain more insight about the pathway of action, CT DNA binding titration was carried in 1:9 v/v DMF:50 mM Tris-HCl/NaCl (pH = 7.4). The binding constant ($K_b$) of 2.31(3) × 10³ M⁻¹ (see ESI, Fig. S7†) shows that the interaction is moderate. Although the interaction with CT DNA is not too high, literature data suggest that the cytotoxicity of a similar family of complexes is due to the formation of adducts with DNA bases especially N7 of guanine.41 This interaction was in spite of 50 mM NaCl being present in the buffer, which would render the hydrolysis of 1 very slow. Hence, we may say that either the complex is able to interact with DNA even without undergoing hydrolysis or the presence of DNA may assist the hydrolysis, leading to more complex–DNA interaction. The lipophilicity of 1 showed that it is more lipophilic, based on the partition coefficient (log $D$), as compared to the ligand L (2.0(1)). The log $D$ value for 1 is 3.2(1), which is predicted to be within the optimum range for a molecule to be a good drug.42 Hence, the slow hydrolysis and the log $D$ value are encouraging for good cytotoxicity. When we probed 1 for cytotoxicity against HeLa (human cervical carcinoma), MCF-7 (human breast adenocarcinoma) and A549 (human lung adenocarcinoma) cell lines, we found that 1 is significantly active in all the above (Table 2, ESI, Fig. S8†). Since L is not cytotoxic up to 300 μM, the toxicity is due to formation of the complex [RuII(η⁶-p-cym)(Cl)](PF₆). It is known that having a good in vitro cytotoxicity profile in normoxia may be a good indication, but cytotoxicity may worsen under hypoxic conditions due to hypoxia-induced resistance.27,43 Hence, we probed the
activity of 1 under hypoxic conditions in MCF-7 and A549 cells. We found that the IC₅₀ values were 9.1 ± 0.3 µM (p < 0.01) and 15.6 ± 1.4 µM (p < 0.05) against MCF-7 and A549 cells, respectively [Table 2, see ESI, Fig. S9†], showing that there is ca. 35% increase in activity in hypoxia (Table 2). Carcinomas may have a interstitial pH of ca. 6.7; 44 hence, the hydrolysis of 1 was also studied at pH 6.7 using UV-Vis spectroscopy, which showed that the rate of hydrolysis at pH 6.7 was significantly more than the rate at pH 7.4 (Table 1). The results suggest that a change in pH really affects the rate of hydrolysis and the increase in the rate of hydrolysis may be one of the reasons for the enhancement of activity under hypoxia. 45 In contrast, cisplatin shows a decrease in activity (ca. 15-30%) using the same hypoxic conditions (Table 2), which is well supported by the literature. 43 In order to understand if it is a rather general property of Ru⁴⁺ complexes to be equally or better active in hypoxia, based on the suggestion of a Reviewer, we synthesized and characterized the [Ru⁴⁺(en)(η⁶-p-cym)Cl]²⁺ (en = ethylenediamine) of Sadler et al. and then probed its activity against MCF-7 and A549 cells (Table 2). The results show that the complex may be considered to be almost equally active (in A549 cells) or better (in MCF-7 cells) under hypoxic conditions. The deactivation by glutathione appears to be cell dependent, since it is not deactivated in A549 cells under hypoxic conditions in the presence of glutathione but is deactivated in MCF-7 cells. The difference in activity is statistically significant under normoxic and hypoxic conditions. A recent study also suggests that having similar activity in normoxia and hypoxia itself is an appreciable quality for an anticancer agent, 27 since many anticancer agents show a decrease in activity 43 in hypoxia. Hence, 1 may be considered as a potent anticancer agent with more activity in hypoxia.

Complex 1 exhibits strong resistance to deactivation by l-glutathione, which is a major deactivating agent for most Pt and Ru anticancer agents or Pt-based clinical drugs. 21, 27, 46 In hypoxic conditions, 1 in the presence of 1 mM of reduced l-glutathione (ca. 60–100 molar equivalent with respect to the IC₅₀ in hypoxia) exhibits IC₅₀ values of 10.8 ± 1.2 µM (p < 0.03) against MCF-7 cells and 16.7 ± 0.5 µM (p < 0.01) against A549 cells [see ESI, Fig. S10†]. It shows that the deactivation by glutathione is up to ca. 19% for 1 using such a huge excess of glutathione, whereas under the same conditions with only 20 molar equivalents of l-glutathione cisplatin is deactivated by ca. 50% in the MCF-7 and A549 cell lines (Table 2). It should be noted here that even in the presence of a large excess of reduced l-glutathione in hypoxia, the IC₅₀ value is still better than that observed in normoxia for complex 1. The electronic and steric effects of the ligand in 1 and the kinetic nature of Ru⁴⁺ may be making the hydrolysis rate slow and the approach by glutathione difficult, leading to no binding with glutathione. The study on the [Ru⁴⁺(en)(η⁶-p-cym)Cl]²⁺ complex by us also suggests that Ru⁴⁺ may have the potential to be used in the development of hypoxia-active anticancer agents.

The NMR studies support that although the complex 1 slowly hydrolyzes, it does not bind to glutathione when reacted with 20 molar equivalent of reduced l-glutathione (see ESI, Fig. S14†). Instead, the formation of the glutathione dimer slowly takes place over 8–10 h, which may be due to the presence of a trace amount of oxygen, since we find the same dimer formation even in the absence of any complex in the solution (although the N₂ purging times were quite longer, ca. 30–45 min) [see ESI, Fig. S14†]. The results support that glutathione hardly affects the cytotoxicity of 1.

Initial studies with MCF-7 cells show that 1 arrested cells at the sub G1 phase as well as in the G2/M phase (Table 3, see ESI, Fig. S15†), unlike cisplatin, which arrests MCF-7 cells only in the G2/M phase. 47 The accumulation of a significant popu-

![Table 2](image)

<table>
<thead>
<tr>
<th>Complex</th>
<th>Normoxia IC₅₀ (µM) ± S.D.</th>
<th>Hypoxia IC₅₀ (µM) ± S.D.</th>
<th>Hypoxia + Glutathione IC₅₀ (µM) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.8 ± 1.2</td>
<td>9.1 ± 0.3</td>
<td>10.8 ± 1.2</td>
</tr>
<tr>
<td>L</td>
<td>23.2 ± 0.4</td>
<td>15.6 ± 1.4</td>
<td>16.7 ± 0.5</td>
</tr>
<tr>
<td>C1</td>
<td>43.9 ± 2.6</td>
<td>31.7 ± 1.7</td>
<td>49.3 ± 1.8</td>
</tr>
<tr>
<td>CDDP</td>
<td>15 ± 1</td>
<td>19 ± 2</td>
<td>29 ± 2c</td>
</tr>
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The IC₅₀ values were calculated by non linear curve fitting in dose response inhibition—variable slope model using graph pad prism. S.D. = standard deviation. The statistical significance (p) of the data is <0.05 or better. ²Hypoxia (1.5% O₂). ³Not determined. ⁴With 1 mM of reduced l-glutathione. ⁵20 molar equivalent of reduced l-glutathione used with respect to IC₅₀ dosage of the respective cell line in hypoxia.
lotion of cells in the sub G1 phase indicates that 1 may follow the apoptotic pathway. The cleavage of chromatin DNA into internucleosomal fragments is one of the important biochemical characteristics of apoptotic cells. The ladder assay of 1 against MCF-7 cells shows that the DNA is cleaved to form nucleosome-sized fragments of approximately 180–200 base pairs (see ESI, Fig. S16†), confirming that 1 induces apoptosis in the MCF-7 cells. The optical microscopy images of MCF-7 cells treated with 1 for 24 h show chromatin condensation and nucleus swelling as shown with bright arrows in DAPI images and with dark arrows in merged images (see ESI, Fig. S17†). The data are supportive of apoptotic killing of cancer cells by complex 1.

Conclusions

To summarize, [(L)RuII(η6-p-cym)(Cl)(PF6)] (1) of the sterically hindered Schiff base L is highly stable in 110 mM NaCl solution, emphasizing its stability in the extracellular space. 1 is slow to hydrolyze at the normal physiological pH of 7.4 in 4 mM NaCl with a t1/2 of (60 h). The complex shows an encouraging in vitro cytotoxicity profile and may be a potent anticancer agent because it is more active under hypoxic conditions and resists deactivation by glutathione in both of the probed carcinoma cell lines, MCF-7 and A549. The enhancement of activity under hypoxia may be related to the increased rate of hydrolysis at pH 6.7. The presence of L renders 1 resistant to hydrolysis and deactivation by reduced glutathione. The resistance to deactivation is further supported by the in vitro activity of 1 in the presence of an excess (60–100 equivalent of IC50) of glutathione for both MCF-7 and A549 cells. The hypoxia activity of [RuII(en)(η6-p-cym)Cl](PF6) of Sadler et al. also shows a promising trend as per our studies on MCF-7 and A549 cells. Therefore, this work shows that RuII may be exploited to design hypoxia-active anticancer agents, and steric hindrance may be exploited to improve the cytotoxicity profile of RuII arene complexes in high concentrations of glutathione. In fact, the resistance to glutathione, steric hindrance and slow hydrolysis may play a synergistic role. These results are highly encouraging and warrant more work with L and its analogues to generate RuII complexes by changing the halide and the arene to understand the effect of steric hindrance on the rate of hydrolysis and its dependence on pH, better activity in hypoxia and resistance to deactivation by 1-glutathione.

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Notes and references

6 P. J. Dyson, Chimia, 2007, 61, 698–703.