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Synthesis, spectroscopic characterization, photochemical and photophysical properties and biological activities of ruthenium complexes with mono- and bi-dentate histamine ligand
Synthesis, spectroscopic characterization, photochemical and photophysical properties and biological activities of ruthenium complexes with mono- and bi-dentate histidine ligand†

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The monodentate cis-[Ru(phen)2(hist)2]2+ 1R and the bidentate cis-[Ru(phen)2(hist)]2+ 2A complexes were prepared and characterized using spectroscopic (1H, (1H−1H)COSY and (1H−13C)HSQC NMR, UV-vis, luminescence) techniques. The complexes presented absorption and emission in the visible region, as well as a tri-exponential emission decay. The complexes are soluble in aqueous and non-aqueous solution with solubility in a buffer solution of pH 7.4 of 1.14 × 10−1 mol L−1 for (1R + 2A) and 6.43 × 10−4 mol L−1 for 2A and lipophilicity measured in an aqueous–octanol solution of −1.14 and −0.96, respectively. Photolysis in the visible region in CH3CN converted the starting complexes into cis-[Ru(phen)2(CH3CN)2]2+. Histamine photorelease was also observed in pure water and in the presence of BSA (1.0 × 10−6 mol L−1). The bidentate coordination of the histamine to the ruthenium center in relation to the monodentate coordination increased the photosubstitution quantum yield by a factor of 3. Pharmacological studies showed that the complexes present a moderate inhibition of AChE with an IC50 of 181 μmol L−1 (referred to risivagin, IC50 1.9 μmol L−1 and galantamine IC50 0.006 μmol L−1) with no appreciable cytotoxicity toward to the HeLa cells (50% cell viability at 925 μmol L−1). Cell uptake of the complexes into HeLa cells was detected by fluorescence confocal microscopy. Overall, the observation of a luminescent complex that penetrates the cell wall and has low cytotoxicity, but is reactive photochemically, releasing histamine when irradiated with visible light, are interesting features for application of these complexes as phototherapeutic agents.

Introduction (hist) has been broadly established as having important roles in mammalian physiology, for example, as a bioregulatory agent in vasodilation and neuronal signaling. Moreover, histamine has shown encouraging anti-tumor and anti-metastatic properties and implications for Alzheimer’s disease therapy. Unfortunately, the limiting factor is the histamine intolerance. In this context, ruthenium–polypyridine complexes can be seen as useful delivery agents of histamine and, in particular, are promising for the photochemical delivery of histamine to desired physiological targets. These complexes are also interesting for research tools because the coordination to the Ru center can occur through either N1 or N3 of the imidazole ring giving rise to the adjacent (1A) and remote (1R) isomers. Furthermore, due to the rotation of the CH2CH2NH2 side chain of the imidazole ring of the histamine, two possible conformations of the 1A and 1R isomers, the trans and gauche conformers, may coexist in the solution. The gauche conformer is expected to be more stable because of the proximity of the NH2 group to the acid proton of the NH imidazole and for this reason it is expected to be the major species. The 1A isomer, the combination of lower stability of the trans conformer associated to higher lability of the Ru–N3 bond facilitates the formation of the bidentate Ru–hist complex. Scheme 1 shows the possible modes of coordination of histamine to the metal center Ru(n).

Having this background, herein we describe the synthesis of a ruthenium–polypyridine complex containing histamine in an aqueous solution, and evidence is presented for a monodentate and bidentate coordination mode to the ruthenium center. In addition, we also report the synthesis, in an aqueous basic solution, of the analogous complex having only the bidentate coordinated histamine, as well as a comparison of the structures obtained using NMR (1H, (1H−1H)COSY and (1H−13C)HSQC) spectroscopy. The photophysical and photochemical properties of the complexes are also reported.

†Electronic supplementary information (ESI) available: NMR spectroscopic data. See DOI: 10.1039/c2dt12136k
To address the biological activity of these complexes at the physiological medium, we investigated their aqueous solubility, hydrophobicity as well as in vitro inhibitory effect on cell proliferation and cell uptake into the HeLa cells line. The kinetics of the interaction of the complexes with acetylcholinesterase was also evaluated.

Results and discussion

1. Preparation, structure and electronic characteristics

The reaction of the complex cis-RuCl$_2$(phen)$_2$ with 2 equiv. of histamine at a H$_2$O : EtOH (1 : 1) ratio led to the formation of a monodentate and bidentate complex and a small amount of the conformers of these complexes. All attempts to separate the complexes and synthesize only the monodentate complex have failed so far, even when an excess of histamine was used. However, the major product of the synthesis is the 2A complex, possibly because a 6 membered ring is formed when the terminal NH$_2$ coordinates with a metallic center. The pure bidentate complex, 2A, was obtained by the reaction of cis-RuCl$_2$(phen)$_2$ with the histamine ligand in a 2 : 1 ratio in an aqueous solution, pH 10. The coordination modes of the histamine to the ruthenium center were confirmed by NMR spectroscopic measurements. The monodentate complex was found to have two histamine ligands coordinated cis- to one another by the N1 nitrogen atom of the imidazole ring giving rise to the remote isomer (1R), whereas in the bidentate complex the chelation occurred through the N3 nitrogen atom of the imidazole ring and the N of the amine terminal NH$_2$ of the CH$_2$CH$_2$NH$_2$ side chain producing the adjacent isomer, 2A. The assignments were made on the basis of $^1$H, ($^1$H–$^1$H) COSY and ($^1$H–$^{13}$C)HSQC experiments, Fig. 1, Scheme 1, Table 1 and Fig. S1, S2, S3, S4.†

(a) $^1$H NMR. The analysis of the $^1$H-NMR spectra of both mono and bidentate complexes revealed the differences between the signals from the imidazole ring and those of the CH$_2$CH$_2$NH$_2$ side chain of the imidazole ring of both compounds. The H1a, H1a’ and H2a, H2a’ (CH) ring protons of imidazole of the monodentate complex, 1R, showed signals at 7.40 and 6.57 ppm while in the bidentate complex, 2A these signals showed a slight upfield effect to 7.01 and 6.10 ppm as is shown
in Table 1 and Fig. 1. Most significant is the fact that in the monodentate complex, the amine terminal NH2 of the CH3CH2NH2 side chain of the imidazole ring is probably involved in intra-molecular interaction with the NH(imidazole ring), as observed for free histamine. This interaction could be affecting the signal of the proton from the imidazole NH group to the Ru center.

The corresponding shifts in the H3a and H4a (CH2) protons of the CH3CH2NH2 side chain of the imidazole ring from 2.48 and 2.70 ppm in the monodentate to 2.74 and 3.00 ppm in the bidentate coordination are indicative of the decrease in electron density at these sites due to delocalization of the negative charge to the Ru center.

For both complexes, the phenanthroline aromatic ring protons show signals between 9.49 and 7.39 ppm. A characteristic of the monodentate complex is that the two phen ligands lie in magnetically equivalent positions. Thus, due to the same chemical environment, the proton signals H3, H3′ and H4, H4′ are identified at the same position. In contrast, for the bidentate complex, the H3 and H4 protons near to the NH2 group appear at 9.49 ppm and 8.08 ppm, whereas the H3′ and H4′ protons near to the imidazole ring are identified at 8.71 ppm and 7.79 ppm, respectively. Owing to the combination of inductive and steric effects, the protons H3 and H4 experience an upfield effect with respect to those in the monodentate complex.

The spectroscopic techniques used to characterize the mixture (1R + 2A) synthesized in this work showed the presence of five species in the solution. We were not able to separate and isolate these species, however on the basis of integrating the 1H NMR signals we identified these species as 2A (bidentate, 61%), 1R (monodentate, 30%) and three conformers, which originated from monodentate histamine coordination (9%). The presence of the complexes 1R and 2A is clearly observed by the NOE-NMR experiments, as shown in and discussed in the next section. The conformers were observed only in the 1H NMR spectrum of the mixture (1R + 2A) as less intense signals in the region of the imidazole ring and in the phenanthroline ligand.

We were able to prepare and isolate the 2A complex using a basic medium (pH = 10), because in this condition the NH2 is found in a deprotonated form and, consequently, the bidentate coordination was the preferred form. It can be observed that the bidentate coordination of the histamine ligand does not enable the CH3CH2NH2 group to rotate and as a consequence, the minority conformers obtained in the mixture synthesis are not observed by 1H NMR.

(b) 1D-nOesy experiments. Further insight into the bidentate complex formation came from the 1D-Noesy experiment, Fig. 2 and 3. Irradiation at 7.00 ppm resonance in the bidentate complex leads to an increase in the intensity of the signals at 2.74 and 3.00 ppm, related to the CH3CH2NH2 side chain of the imidazole ring of the histamine ligand, indicating that these protons are spatially close to each other, but very distant from the phenanthroline ligand because no effect is observed in the phenanthroline signals, Fig. 2a. In contrast, the irradiation of 6.10 ppm proton signals caused nOe in the phenanthroline signals at 7.79 and 8.71 ppm, which are related to H4′ and H3′ protons. nOe for the terminal NH2, in 2.26 ppm, from the CH3CH2–NH2 group can also observed, which is spatially closed to the irradiated signals, Fig. 2b.

The 1D-nOesy experiments were also performed for the monodentate complex by irradiating at 7.40 ppm and 6.57 ppm resonances, Fig. 3. The irradiation of 7.40 ppm only shows nOe in the phenanthroline signals (9.39 and 8.04 ppm), related to H3 and H4 protons, Fig. 3a. To note, the irradiation of 7.4 ppm signal is not very selective, because the H1a from the 1R complex and also the H9 proton from the 2A complex are affected. The result of this non-selective irradiation leads to the observation of signals in 7.83 and 8.38 ppm, related to protons H10 and H8 from the 2A complex and a nOe at 2.70 ppm related to protons H4a. By irradiating the 6.57 ppm signal, a nOe can be detected at 2.48 ppm, which is related to H3a protons from a CH3CH2NH2 side chain of the imidazole ring of the histamine, a nOe for H3 and H4 protons from phenanthroline (9.39 and 8.04 ppm, respectively) and a nOe for the H1a proton (7.41 ppm) from the imidazole ring of the other ligand, Fig. 3b.

The NMR experiments show that monodentate coordination of the histamine ligand occurs via Ru–N1(imidazole ring), i.e., it forms a stable complex with a remote isomer, 1R, whereas the

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**Table 1** 1H NMR (1D and 2D-COSY) chemical shift of 2A and 1R in CD3CN

<table>
<thead>
<tr>
<th>(1R)</th>
<th>H</th>
<th>δH (mult, Hz)</th>
<th>(2A)</th>
<th>H</th>
<th>δH (mult, Hz)</th>
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<tr>
<td>3,3′</td>
<td>9.39 (dd; 1.0/5.2)(2H)</td>
<td>3</td>
<td>9.49 (dd; 1.1/5.1)(1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,5′</td>
<td>8.65 (dd; 1.2/8.2)(4H)</td>
<td>3′, 5, 5′</td>
<td>8.71 (m)(3H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3′</td>
<td>8.04 (dd; 5.2/8.1)</td>
<td>4</td>
<td>8.08 (dd; 5.2/8.1) (1H)</td>
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<td></td>
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<tr>
<td>6,6′</td>
<td>6.09 (d; 8.8) (2H)</td>
<td>6,6′</td>
<td>8.00 (dd; 8.8) (1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,7′</td>
<td>8.18 (d; 8.8) (2H)</td>
<td>7,7′</td>
<td>8.16 (d; 8.8) (1H)</td>
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<tr>
<td>8,8′</td>
<td>8.37 (dd; 0.8/7.6)(3H)</td>
<td>8,8′</td>
<td>8.38 (dd; 0.8/8.2)(2H)</td>
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<td></td>
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<tr>
<td>9,9′</td>
<td>7.39 (dd; 5.2/8.1)(4H)</td>
<td>9,9′</td>
<td>7.41 (m; 5.3/8.2)(2H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,10′</td>
<td>7.79 (dd; 1.2/5.2)(1H)</td>
<td>10,10′</td>
<td>7.83 (dd; 1.2/5.2)(1H)</td>
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<td></td>
</tr>
<tr>
<td>1a</td>
<td>7.40 (s) (2H)</td>
<td>1a</td>
<td>7.00 (s; 1.2)(1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>6.57 (s) (2H)</td>
<td>2a</td>
<td>6.10 (d; 1.2) (1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td>2.48 (t; 6.6)</td>
<td>3a</td>
<td>3.00 (q; 4.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>2.70 (t; 6.6)</td>
<td>4a</td>
<td>2.74 (m)</td>
<td></td>
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</tr>
<tr>
<td>NH</td>
<td>10.35</td>
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</table>
bidentate histamine form leads to the adjacent isomer, 2A, Ru–N3(imidazole ring).

The structures and isomeric forms of the two complexes were further supported by DFT optimized geometries of 1R, 1A and 2A (see below).

Complex 1R was identified but could not be separated from 2A. For this reason its spectroscopic, photochemical and photophysical properties were evaluated by comparing the results obtained in the mixture (1R + 2A) with those of 2A obtained separately.

(c) Absorption and emission properties. The thermal stability of these complexes in the dark was proved by the absence of changes in the electronic absorption spectra in all solvents tested over a period of 24 hours. The UV-vis spectra of the complexes, obtained in acetonitrile, show intense bands at higher energy associated mainly with \( \pi-\pi^* \) electronic transitions (<350 nm) and a broad absorbance at 400–600 nm, characteristic of MLCT processes. Excitation in the visible region band, in acetonitrile and at room temperature, produces a relatively intense emission at 640 nm. The excitation spectrum (\( \lambda_{em} = 640 \) nm) resembles the absorption spectrum and the intensity of emission is solvent dependent: it is higher in CH2Cl2 and lower in CH3CN. The emission quantum yields in CH3CN for (1R + 2A) and 2A complexes are similar but lower\(^{11} \) than [Ru(phen)3]2+ having 0.010 values. The fit of the emission decay profiles measured in the CH3CN solution to a monoexponential curve resulted in high values of reduced-\( \chi^2 \) values (1.864 for (1R + 2A) and 1.670 for 2A). Furthermore, the plot of residuals showed a non-random distribution of the differences between the fitted curve and the experimental data (Fig. 4A). A best fit was obtained with a bi-exponential curve, with reduced-\( \chi^2 \) values close to 1.0 and a random distribution of residuals (Table 2 and Fig. 4B). The decays were described by a long lifetime, 248.5 ns for (1R + 2A) and 137.3 ns for 2A, and a short lifetime component, 35.5 ns for (1R + 2A) and 20.5 ns for 2A and the contribution from the long lifetime accounts for 98.5% of the total emission. The normalized pre-exponential factor of the long lifetime was equal to 0.90 in both samples, meaning that there is a relative ratio of 0.9:0.1 between the initial concentrations of the long lifetime and the short lifetime emitting species. The emission lifetimes of both complexes are shorter than that for [Ru(phen)3]2+ (500 ns)\(^{11} \) and we observe that while the relative populations of the long and the short lifetime species are the same in both complexes, the lifetime values increase by a factor of 1.9 in (1R + 2A) compared to 2A.
2. Continuous photolysis

The complexes are photoreactive and the changes in their electronic absorption and emission spectra in 420 nm light irradiation are consistent with histamine release and the formation of a solvated complex, Fig. 5 and 6. The maximum wavelength of the photoproduct reproduces the maxima absorption of a solvated complex, Fig. 5 and 6. The maximum absorption matches the values reported in the literature. Furthermore, the signals at 9.77 ppm in the $^1$H NMR obtained after 40 min irradiation of the complexes correspond to the cis-[Ru(phen)$_2$(CH$_3$CN)$_2$]$^{2+}$ ion complex (Fig. 5C and 6C).

The quantum yields, at 420 nm irradiation, of the histamine photoreaction (eq 1R) and in CH$_3$CN. Excitation at 472 nm, and emission at 640 nm. Also shown, the instrument response function (green line) and curves (red line) from the fit to monoexponential decay (A) and bi-exponential decay (B). The plots of the residuals are presented for both fits.

Table 2. The time-resolved fluorescence parameters (lifetime $\tau_1$ and normalized pre exponential factor $b_1$) obtained from the fit of intensity decay data to biexponential curves, evaluated by $\chi^2_{\text{red}}$ values. The percentile values in the table give the contribution to the total fluorescence emission.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
<th>$b_1$</th>
<th>$b_2$</th>
<th>$\chi^2_{\text{red}}$</th>
<th>%1</th>
<th>%2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>137.3 ± 0.1</td>
<td>20.5 ± 0.9</td>
<td>0.904</td>
<td>0.996</td>
<td>1.071</td>
<td>98.4</td>
<td>1.6</td>
</tr>
<tr>
<td>1R + 2A</td>
<td>258.1 ± 0.4</td>
<td>35.5 ± 1.5</td>
<td>0.902</td>
<td>0.998</td>
<td>1.142</td>
<td>98.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The UV-vis and luminescent characteristics of the complexes are maintained in pure water and in an aqueous buffer solution (Tris/HCl, pH 7.4) in the absence and presence of bovine serum albumin (see below). This is relevant in terms of developing drug delivery systems considering that serum albumin has been investigated as the binding and transport protein of metallodrugs for their targets in physiologic medium.

2. Biological activity

Solubility and lipophilicity. The experimental solubility ($S$) in aqueous buffer tris–HCl solution (pH 7.4 and pH 3.6) and partition coefficient ($log P$) in octanol–water solution are listed in Table 2. The complexes show negative values of $log P$, indicating that they are hydrophilic, whereas the uptake experiments, shown below, indicate their ability to pass through the cell membrane. As expected, there is a direct correlation between the $S$ and $log P$ values.

In vitro acetylcholinesterase (AChE) inhibition. The role played by histamine on the cognitive and cholinergic system, leads us to examine the effect of complexes on the AChE enzyme activity. The experimental plots of absorbance of substrate by complex (1R + 2A) at different concentrations shown in Fig. 8A showed typical substrate-saturation curves. The inhibition mode of AChE was studied using the Lineweaver–Burk plot, Fig. 8B. From the
plots, the kinetic parameters: maximum rate constant, \(1/\text{rate} \text{ versus } 1/[\text{substrate}]\) and the apparent Michaelis–Menten constant \((k_m)\) were calculated. The values of \(K_m\) (0.48, 0.25, 0.21 and 0.18 \(\mu\text{mol L}^{-1}\)) for concentrations of 0, 15, 25 and 50 \(\mu\text{mol L}^{-1}\), respectively are indicative of a reversible and mixed noncompetitive type of inhibition.

These results demonstrated that the complexes do not compete with the substrate for the active site of the enzyme, but influence the binding of the substrate with the enzyme inhibiting it. Probably, the active site inhibitor of complexes and substrate are close, leading to a change in conformation of the enzyme that affects the enzyme–substrate interaction.\(^{24}\) The intersection of the linear plot of \(1/\text{rate} \text{ versus } \text{concentration}\) with the \(x\)-axis was able to estimate the inhibitor constant,\(^{25}\) \(K_i\) as 38.5 \(\mu\text{mol L}^{-1}\).

The \(IC_{50}\) (50% AChE inhibitory effect) of \((1R+2A)\) was determined to be 21 \(\mu\text{mol L}^{-1}\).

When compared to many commercially proven drugs in various countries to treat Alzheimer’s disease we can affirm that this complex is an inhibitor that is nine times stronger than the commercial drug rivastigmine\(^{26}\) (IC\(_{50}\) = 181.39 \(\mu\text{mol L}^{-1}\)), but it is much weaker than the drugs huperzine A (IC\(_{50}\) = 0.082 \(\mu\text{mol L}^{-1}\)), donepezil (IC\(_{50}\) = 0.001 \(\mu\text{mol L}^{-1}\)) and galantamine (IC\(_{50}\) = 0.006 \(\mu\text{mol L}^{-1}\)).

The absence of the inhibitory activity of either free phenanthroline, free histamine or precursor complex \(\text{cis-[Ru(phen)2Cl2]}\) suggests that the coordination of histamine to the fragment.

**Fig. 5** Continuous photolysis of \((1R+2A)\) in CH\(_3\)CN solution at 420 nm light irradiation: (A) change in the absorption spectrum; (B) change in the emission spectrum; (C) change in the \(^1\text{H-NMR spectra in the region of the imidazole ring proton, insert: in the region of phenanthroline. Irradiation times, } t_{irr} = 0–20 \text{ min.}\)

**Fig. 6** Continuous photolysis of \(2A\) in CH\(_3\)CN solution at 420 nm light irradiation: (A) change in the absorption spectrum; (B) change in the emission spectrum; (C) change in the \(^1\text{H-NMR spectra in the region of the imidazole ring proton, insert: in the region of phenanthroline.}\)

**Fig. 7** Continuous photolysis in aqueous buffer solution (Tris–HCl, pH 7.4) at 420 nm light irradiation for \((1R+2A)\). (A) Change in the absorption spectrum in water; (B) change in the emission spectrum in buffer pH 7.4 and (C) change in the emission spectrum in buffer pH 7.4 + BSA.
[Ru(phen)$_2$]$_2^+$/contributes to the inhibition of enzymes in a significant way.

In vitro HeLa cells proliferation inhibition. The complexes were assayed for cell proliferation inhibitory activity with the human cervical adenocarcinoma cell line HeLa-ATCC CCL-2, at concentrations of 1, 10, 100, 500, 1000 μmol L$^{-1}$, Table 3. The activity profiles of the two complexes show higher differences between them but they failed to prevent the cellular proliferation of the HeLa cell lines up to the concentration DL$_{50} > 650$ μmol L$^{-1}$, while the sensitivity of the cell line to doperzine is well known (DL$_{50} = 191$ μmol L$^{-1}$).27 According to the description above, free histamine has been shown to be involved in the proliferation of normal and malignant cells, in particular, it promotes HeLa cell growth.28–30

Cell uptake studies. The confocal microscopy fluorescent imaging of fixed HeLa cells with the complexes show that the complexes are located in the cellular interior, having a stronger emission (monitored at 660 nm) in the cytoplasm penetrating in the nuclear region (Fig. 9). These results indicate that the large size of the complex, its hydrophobicity (high $S$, small log $P$) and its positive charge (+2) does not prevent the complex cell uptake.

Conclusion

The observance of visible absorption, luminescence and high photoreactivity towards histamine dissociation combined with low cytotoxicity and cell uptake into HeLa line cells shows the potential utility of these complexes as drugs and/or research tools and promises a more effective delivery of histamine to specific physiological targets by controlling the irradiation area and intensities. Our results also offer the opportunity to map the reactivity of histamine in the physiologic medium using luminescence measurements.

Experimental

General

All reactions were performed under a nitrogen atmosphere. RuCl$_3$–xH$_2$O, 1,10’-phenanthroline (phen), histamine (hist) and
lithium chloride were obtained from Aldrich; tetrabutylammonium perchlorate (TBAP) was obtained from Strem; bovine serum albumin (BSA) was obtained from Sigma. BSA was used without further purification (99% by electrophoresis). The buffer used was Trizma hydrochloride (Tris/HCl 50 mM), from Sigma-Aldrich. For the inhibition experiments were used the reagents: acetylcholinesterase (AChE) 500 UN, acetylthiocholine iodide and 5,5′-dithiobis(2-nitrobenzoic acid)(3,3′-6) (DTNB) from Fluka. HPLC grade acetonitrile and dichloromethane were distilled prior to use. cis-[Ru(phen)2(Cl)2] was prepared according to the literature method described for the 2,2-bipyridine complexes.31

Synthesis of cis-[Ru(phen)2(hist)](PF6)2·2H2O, complex (1R + 2A).

cis-[Ru(phen)2Cl]2 (0.20 g; 0.25 mmol) was dissolved in a 1 : 1 EtOH–H2O mixture (10 mL), and an amount of histamine (0.056 g; 0.50 mmol) was added. The solution was stirred under nitrogen atmosphere for 8 h under reflux. A stoichiometric amount of NH4PF6 was added to precipitate the complex and the resultant solution cooled at 0 °C overnight. The reddish-brown precipitate of cis-[Ru(phen)2(hist)]PF6 was filtered, washed with water and dried under vacuum (70% yield).

Synthesis of cis-[Ru(phen)2(hist)](PF6)2·2H2O, complex 2A

Complex 2A was obtained by the reaction of cis-[RuCl2(phen)2] with 1 equiv. of histamine after adjusting the pH to 10 with the addition of triethylamine (52.3 μL; 0.37 mmol). The compound, after isolation as the bis-hexafluorophosphate salt, was filtered, washed with water and dried under vacuum (60% yield). Anal. calcd for RuC25H24N2PF6·2H2O: C, 38.76; H, 3.25; N, 10.91. Found: C, 39.08; H, 3.32; N, 11.02%.

Spectroscopic techniques

Optical spectra were recorded on an Agilent 8453 UV-vis spectrophotometer. CHN elemental analyses were performed on an EA 1110 CHNS-O Carlo Erba Instrument at the Micro-analytical Laboratory at Universidade Federal de São Carlos (SP). NMR experiments were carried out in a CD3CN solution using a Bruker DRX-400 spectrometer. All chemical shifts (δ) are given in ppm units with reference to the hydrogen signal of the methyl group of tetramethylsilane (TMS) as internal standard and the coupling constants (J) are in Hz.

Monochromatic irradiations at 420 nm were generated either using a 200 W xenon lamp in an Oriel model 69911 Universal Arc Lamp source selected with an appropriate interference filter (Oriel) or a RMR-600 model Rayonet Photochemical reactor using RMR-4200 lamps. The experiments were carried out at room temperature in 1.00 cm path length 4 side quartz cells capped with a rubber septum. The magnetically stirred solutions (∼10−4−10−2 mol L−1 initial complex concentration) were deoxygenated with pure nitrogen. The progress of the photoreaction was monitored by spectroscopic (UV-vis, luminescence and 1H NMR) techniques.

Emission spectra were recorded on a Shimadzu RF-5301PC fluorescence spectrophotometer. Luminescence quantum yields were relatively measured using a standard Φ = 0.062 for [Ru(bpy)3]2+ in CH3CN.32

Time-correlated single-photon counting (TCSP) method was used to obtain fluorescence emission decay curves.33 The excitation source was a Tsunami 3950 Spectra Physics titanium–sapphire laser, pumped by a solid state Milenl X Spectra Physics laser. The repetition rate of the 5 ps pulses was set to 800 kHz using the pulse picker Spectra Physics 3980. The laser was tuned to give output at 945 nm and a second harmonic generator LBO crystal (GWN-23PL Spectra Physics) gave the 472 nm excitation pulses that were directed to an Edinburgh FL900 spectrometer, where the L-format configuration allowed the detection of the emission at a right angle from the excitation. The emission wavelength was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 2.20 ns, and measurements were made using time resolution of 0.245 ns per channel. A software provided by Edinburgh Instruments was used to analyse the decay curves, and the adequacy of the multi-exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced chi-square.

Acetylcholinesterase activity

The Michaelis–Menten kinetics were assayed according to the spectrophotometric method developed by Ellman et al.23 with minor modifications. A stock solution of 50 μmol L−1 was prepared by dissolving the appropriate amount of the complexes (1R + 2A) and 2A in methanol. The diluents in a concentration of 0–25 μmol L−1 were used for inhibition studies. A mixture containing 100 μL of the complex, 2.9 mL of a solution containing tris–HCl buffer, 50 mmol L−1 (pH 8.0), DTNB, 333 μmol L−1, NaCl, 0.1 mol L−1 and MgCl2, 0.02 mol L−1, and 15 μL of enzyme solution (0.025 units of AChE, prepared in 15 μmol L−1 solution of BSA in tris–HCl buffer, 50 mmol L−1, pH 8.0) was incubated for 15 min at room temperature. After this period, the reaction was initiated with the addition of 10 μL of acetylthiocholine iodide (25–150 μmol L−1). The hydrolysis of the substrate could be observed by the formation of a yellow compound (5-thio-nitrobenzoate). Absorbance was measured at 412 nm. Measurement were made after 5 min of the hydrolysis reaction. The inhibition type was justified by the linear Lineweaver–Burk equation. The constant parameters Km and Vmax were evaluated by the linear regression of the reaction (1/rate) versus the substrate concentration. The value of KI was then obtained from the intersection of the linear plot of (1/rate) versus complex concentration with the x-axis.

Solubility

Solubility of complexes (1R + 2A) and 2A in buffers tris–HCl, 50 mmol L−1, pH 3.6 and 7.4 were determined at 37.0 ± 0.5 °C. These experiments were carried out by adding an appropriate amount of complex until saturation in 2 mL of buffer solution. Suspensions were shaken for 24 h at 50 rpm until equilibrium was attained. Samples were centrifuged for 8 min in a centrifuge at 220 rpm. The concentration of the complex in the filtrate was determined by UV-vis spectrophotometer.
Lipophilicity

Lipophilicity was determined by partitioning octanol and buffer tris–HCl, 50 mmol L\(^{-1}\). The complex (100 ppm) was added to a solution containing 1 mL of octanol and 1 mL of buffer. Solutions were then placed in the shaker for 24 h at 50 rpm. Samples were centrifuged for 8 min at 220 rpm. The solutions were separated from the two phases and the concentration of the complex was determined in the organic and aqueous phases by ICP-OES (Perkin Elmer Optima 3000DV). The experiments were done in triplicate. Equation \( \log P = \log(C_o/C_w) \) was used to calculate \( P \): where \( C_o \) and \( C_w \) are the molar concentrations of complex in octanol and aqueous phases, respectively.

Cell culture

HeLa cells were maintained in Dulbecco’s Minimum Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, at 37 °C in a humid incubator with 5% CO\(_2\). In order to detach cells from the bottle, trypsin–EDTA solution was used.

Cytotoxicity

HeLa cells were seeded in 24 well plates at the initial density of 5 × 10\(^4\) cells per well. 18 h after plating, the cells were exposed to the complexes (1, 10, 100, 500 and 1000 μmol L\(^{-1}\)) in DMEM without phenol red and 1% FCS) for 3 h. The medium was then removed, cells were washed with PBS, and fresh medium was added. The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was performed 48 h after. Cells were incubated with 0.25 mg mL\(^{-1}\) MTT for 2 h. Formazan crystals were solubilized by adding 500 μL of DMSO. Absorbance was measured at 550 nm and the values of cell survival are presented as percent of control.

Confocal microscopy

HeLa cells were seeded in individual plates containing a glass coverslip (1 × 10\(^4\) cells per well). 18 h after, cells were exposed to the complexes (3.49 × 10\(^{-4}\) mol L\(^{-1}\)) in DMEM without phenol red and 1% FCS) for 4 h. The cells were then washed with PBS and the fluorescence images were obtained in a confocal microscope (Zeiss LSM510), using a set of filters with excitation at 488 nm and emission from 600–700 nm.

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References