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A comparative study of hydrophilic phosphine hexanuclear rhenium cluster complexes' toxicity

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The octahedral rhenium cluster compound $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ has recently emerged as a very promising X-ray contrast agent for biomedical applications. However, the synthesis of this compound is rather challenging due to the difficulty in controlling the hydrolysis of the initial $\text{P}(\text{C}_2\text{H}_4\text{CN})_3$ ligand during the reaction process. Therefore, in this report we compare the *in vitro* and *in vivo* toxicity of $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ with those of related compounds featuring the fully hydrolysed form of the phosphine ligand, namely $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$ ($\text{Q} = \text{S}$ or Se). Our results demonstrate that the cytotoxicity and acute *in vivo* toxicity of the complex $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ solutions were considerably lower than those of compounds with the fully hydrolysed ligand $\text{P}(\text{C}_2\text{H}_4\text{COOH})_3$. Such behavior can be explained by the higher osmolality of $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$ versus $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$.

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Introduction

Recent studies indicate that hexanuclear rhenium cluster complexes with the general formula $[\{\text{Re}_6\text{Q}_8\}\text{L}_6]$ (where Q is S, Se, or Te and Ls are some organic or inorganic apical ligands) have high potential for therapeutic and diagnostic applications in different fields of biology and medicine due to their luminescence, photosensitising and antitumor properties as well as due to highly efficient absorption of X-rays.^{1–13} This set of outstanding physical properties led to intensive studies of the biological properties of rhenium octahedral clusters. As an example, we have recently developed the cluster complex $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ that demonstrated high radiodensity and high water solubility.⁹ Importantly, our *in vitro* studies have shown that this compound has low cytotoxicity as it does not internalise into cells.

In vivo studies have further confirmed low acute toxicity and the rapid elimination of the compound from the body through the urinary tract after intravenous injection.^{9,11} This compound has therefore excellent potential for application as an X-ray contrast agent.

This compound is synthesised under hydrothermal conditions from tris-(2-cyanoethyl)phosphine ($\text{P}(\text{C}_2\text{H}_4\text{CN})_3$) and $\text{Na}_4[\{\text{Re}_6\text{Se}_8\}(\text{OH})_6]$, upon which the organic ligand precursor undergoes hydrolysis. Unfortunately, under these operating conditions, it is difficult to control the hydrolysis of $\text{P}(\text{C}_2\text{H}_4\text{CN})_3$ and reproducibly obtain only two of three nitrile groups for each ligand being hydrolysed to form a carboxylate and one to form an amide. However, we have noticed that a similar hydrothermal reaction between $[\{\text{Re}_6\text{Q}_8\}(\text{OH})_6]^{4-}$ ($\text{Q} = \text{S}$ or Se) and $\text{P}(\text{C}_2\text{H}_4\text{COOH})_3$ carried out in aqueous solutions led to the formation of the corresponding rhenium cluster complexes having tris-(2-carboxyethyl)phosphines as ligands – $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$. Moreover, these compounds can also be obtained directly from $\text{P}(\text{C}_2\text{H}_4\text{COOH})\text{-HCl}$ and $\text{Na}_4[\{\text{Re}_6\text{Q}_8\}(\text{OH})_6]$ with high purity and good yield.¹⁴ Therefore, in this report we compare the biological properties of compounds $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$ ($\text{Q} = \text{S}$ (1) or Se (2)) *in vitro* and *in vivo* with those of closely related $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ (3) in order to evaluate what effect (if any) the level of hydrolysis and the cluster core composition (*i.e.* $\{\text{Re}_6\text{S}_8\}^{2+}$ vs. $\{\text{Re}_6\text{Se}_8\}^{2+}$) has on cellular internalisation and toxicity, and acute toxicity.

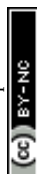
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Results

The critical step in the evaluation of the biological properties of compounds (1), (2) and (3) is the determination of their cytotoxicity. The effect of the cluster complexes on the metabolic activity of the cells was determined using the MTT colorimetric assay. We used three different cell lines as models to evaluate the toxic effect of these compounds: two cancer cell lines – human larynx carcinoma (Hep-2) and human epithelioid cervix carcinoma cell line (HeLa) – and a normal cell line – peritoneal macrophages (MPH). The Hep-2 and HeLa cells were chosen since these cell lines have already been used for the evaluation of the cytotoxicity of several hexanuclear metal clusters and cluster containing materials^{8–11,15–18} and therefore the results obtained allow for the comparison of cytotoxicity between different cluster based materials. Macrophages are cells of the mononuclear phagocytic system responsible for the clearance of pathogens and chemical compounds by phagocytosis and pinocytosis and they are also involved in inflammatory reactions.^{19,20} Finding a non-toxic *in vivo* bioimaging agent for macrophages could provide researchers with new tools to advance the evaluation of disease (*e.g.* cardiovascular disease, in particular ischemia, arthritis and other inflammation processes) and therapies.^{21,22}

The results obtained by the MTT test in the concentration range 0.0056–5.76 mM are presented in Fig. 1, while values of the half maximal inhibitory concentration (IC_{50}) are summarised in Table 1. The data show that compound (2) has the highest cytotoxic effect, while compound (3) has the lowest cytotoxic effect on all three model cell lines. The MTT assay also demonstrates that the peritoneal macrophages are noticeably more sensitive to (1) and (2) than HeLa and Hep-2 cells. Finally, all three studied cluster complexes showed no suppressive effect on the cell viability at concentrations from 0.0056 to 0.18 mM.

Since octahedral rhenium cluster complexes demonstrate luminescence in the red region, it is easy to monitor the cellular internalisation of these compounds using confocal microscopy. Indeed, it has already been shown before that compound (3) does not enter Hep-2 cells.¹¹ Here we have undertaken similar studies on Hep-2, HeLa and MPH cell lines to evaluate whether there is any difference in both their internalisation in healthy and cancerous cells and between cluster complexes with different cores and apical ligands. Fig. 2 shows the representative confocal microscopy images of the cells incubated with 0.18 mM solutions of (1)–(3) for 24 hours. These images show that for both cancerous cell lines, there was no uptake of any of the studied compounds (Fig. 2G–R), while there is clear evidence of intense red photoluminescence located in the cytoplasm of the macrophages (Fig. 2A–F).

Acute toxicity studies were undertaken on Balb/C mice, *i.e.* the same mice strain that was used for the acute toxicity studies of compound (3) before. The animals were randomly split into 9 groups with 10 mice in each group and treated intravenously using clusters (1) and (2) at doses 50, 150, 250 and 350 mg (Re) per kg or sham-injected with physiological

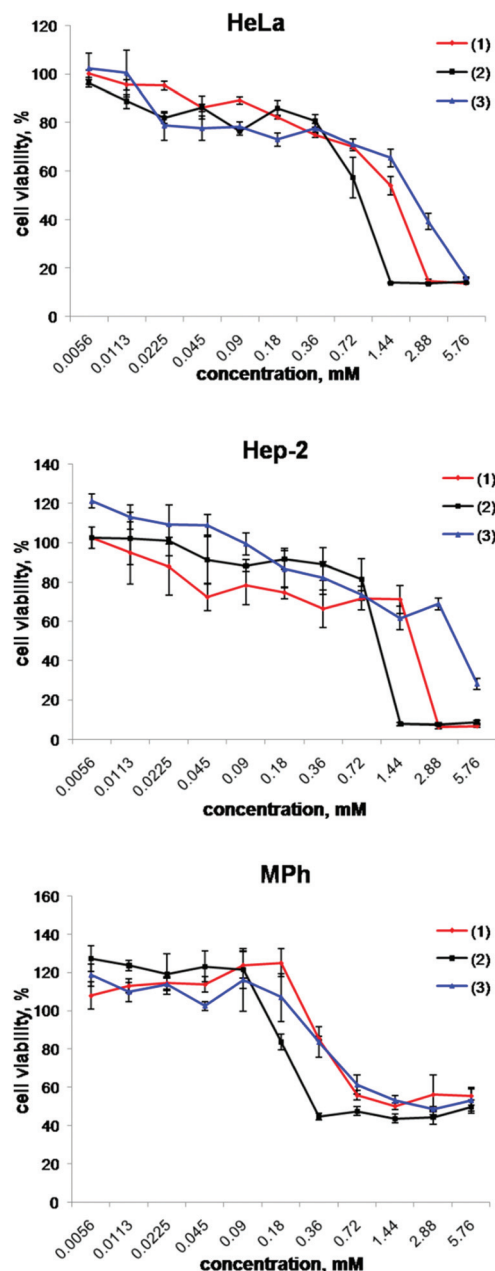


Fig. 1 Cell viability response of HeLa, Hep-2 and MPH cells using the MTT assay for (1), (2) and (3).

Table 1 The IC_{50} of (1), (2) and (3) on the Hep-2, HeLa cells and peritoneal macrophages determined by the MTT assay

Compound	IC_{50} values, mM		
	MPH	HeLa	Hep-2
(1)	1.27 ± 0.03	1.42 ± 0.07	1.80 ± 0.64
(2)	0.36 ± 0.06	0.82 ± 0.03	1.07 ± 0.08
(3)	1.72 ± 0.58	1.66 ± 0.14	5.60 ± 0.17



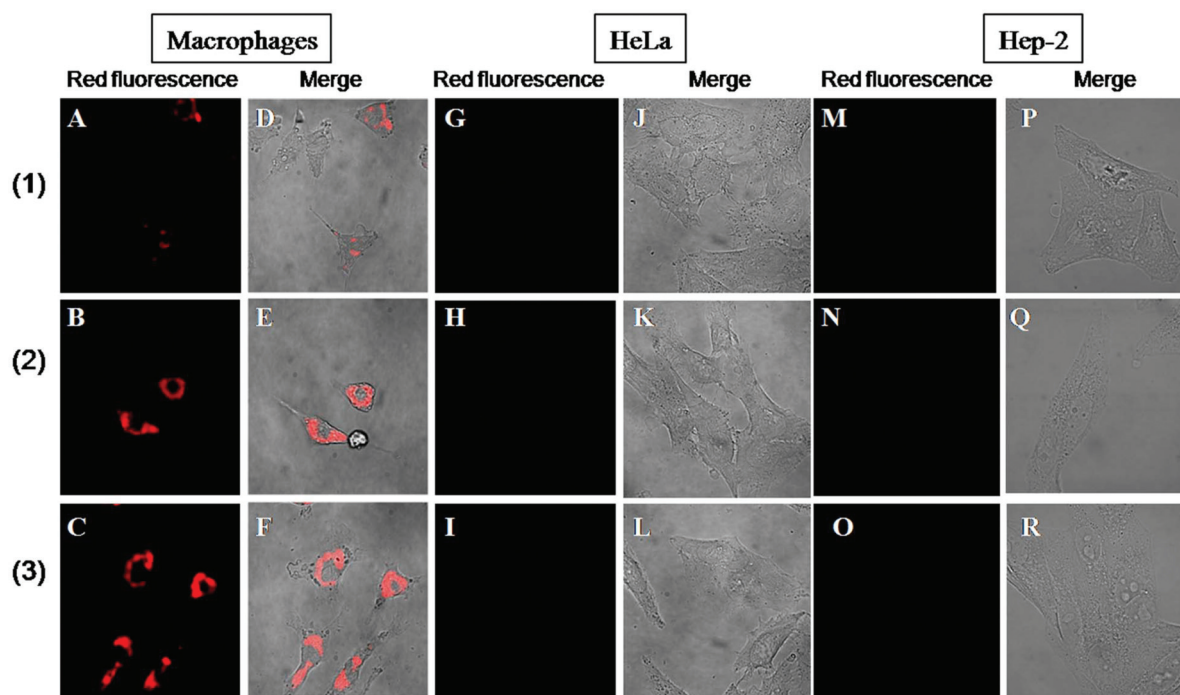


Fig. 2 Confocal fluorescence microscopy of peritoneal macrophages (A–F), HeLa (G–L) and Hep-2 (M–R) after incubation with (1), (2) and (3) for 24 hours. Red fluorescence and merged (phase contrast and fluorescence) images.

saline. The survival rate of the mice after the treatment (Fig. 3) shows no evidence of toxicity for doses up to 150 mg (Re) per kg of the complexes (1) or (2). Within the groups that were treated with 250 mg (Re) per kg of (1) and (2), 6 and 4 mice, respectively, died within the first day. 350 mg (Re) per kg of both clusters was the lethal dose for all ten mice in the group. Finally, we have not observed any changes in the behavior of any of the animals that survived the treatment in any of the groups.

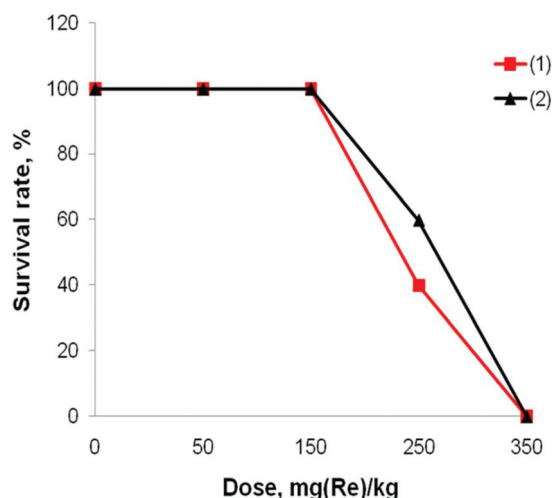


Fig. 3 Survival rate of mice injected intravenously with 0.1 mL of the cluster complexes (1) or (2) at different doses ($n = 10$).

The median lethal dose (LD_{50}) was determined to be 256 ± 25 mg (Re) per kg and 232 ± 24 mg (Re) per kg for the complexes (1) and (2), respectively. The LD_{50} for complex (3) was earlier found to be 4.67 ± 0.69 g kg^{-1} ,¹¹ which corresponds to 1.59 ± 0.23 g (Re) per kg. Thus, from all three compounds, complex (3) is the least toxic for both *in vitro* and *in vivo*.

One of the factors that can significantly affect the acute toxicity of such closely related compounds may be the osmolality of their aqueous solutions. Therefore, we measured the osmolality of the solutions of (1) and (2) in the same concentrations that were used for treatment of mice (10, 30, 50, 70 mg (Re) per mL) and compared the results with those of complex (3) (Table 2).

Table 2 The osmolality of aqueous solutions of the cluster complexes (1), (2) and (3) at different concentrations

Compound	C, mg (Re) per mL	C, mM	Osmolality, mOsm kg^{-1}
(1)	70	62.7	580 ± 1
	50	44.8	398 ± 2
	30	26.9	257 ± 4
	10	9.0	96 ± 1
(2)	70	62.7	613 ± 5
	50	44.8	425 ± 2
	30	26.9	255 ± 1
	10	9.0	99 ± 1
(3)	70	62.7	279 ± 2
	50	44.8	198 ± 1
	30	26.9	122 ± 1
	10	9.0	51 ± 1



The solutions of the cluster complex (3) at all measured concentrations showed osmolality lower than that of serum ($285 \pm 10 \text{ mOsm kg}^{-1}$). However, the solutions of the cluster complexes (1) and (2) possessed an acceptable osmolality only at the concentrations of 10 and 30 mg (Re) per mL. At concentrations of 50 and 70 mg (Re) per mL the osmolality of the solutions was higher than the osmolality of serum.

Discussion

Compounds $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$, where Q = S (1) and Se (2) are chemical derivatives of the compound $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ (3) that was earlier proposed as a potentially good X-ray contrast agent. Specifically, there are two major differences between compounds (1), (2) and (3): (i) the overall charge of the cluster anions of (1) and (2) is significantly higher than that of (3) due to the chemical composition and charge of the organic ligand, *i.e.* three anion $\text{P}(\text{C}_2\text{H}_4\text{COO})_3^{3-}$ rather than two anion $\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2^{2-}$ and (ii) the content of the cluster core in (1) is different from (2) and (3). To evaluate the effect of these differences in chemical composition on the biological properties of the cluster complexes, we have studied cellular toxicity and cellular internalisation using three different cell lines and acute toxicity in mice.

The results of the MTT assay have shown that compound (3) is the least cytotoxic for both the cancerous cell lines HeLa and Hep-2 cells and the healthy cell line MPH, while confocal microscopy has revealed that HeLa and Hep-2 cells do not take up any of the studied cluster complexes, while intense red luminescence located in the cytoplasm was observed from macrophages treated with the clusters. Thus, confocal microscopy imaging confirms the uptake of the clusters by MPH. The lack of uptake of the metal cluster by cancer cells is easily explained by the high negative charge of the cluster anion that prohibits cellular internalisation due to repulsive force with the cellular membrane. On the other hand it was already shown that macrophages are efficient in the uptake of both positively and negatively charged particles.^{23,24} Macrophages are cells of the mononuclear phagocytic system, which are distributed extensively in the tissue and play an important anti-inflammatory role. Cellular inflammation is one of the major symptoms of many developing pathologies including atherosclerosis, type 2 diabetes, Alzheimer's disease, cancer and other illnesses.^{25–28} One of the main approaches currently used for the detection and imaging of inflammation is based on the CT visualisation of hard tissue. X-ray imaging based on the use of radiopaque agents accumulated selectively in macrophages was recently offered as an alternative method for the detection of cellular inflammation in soft tissues.^{29–33} The ability of (1), (2) and (3) to easily accumulate in macrophages makes these moieties potentially good agents for the visualisation of cellular inflammation.

According to our *in vivo* toxicity studies on Balb/C mice, complex (3) is considerably less toxic than (1) and (2). Despite

this, all these compounds demonstrate similar cellular internalisation patterns and the difference in their cellular toxicity is not significant. One of the possible explanations of the noticeably increased acute toxicity of (1) and (2) is the difference in the osmolality of the cluster solutions caused by a significant increase of the cluster anion charge, which consequently leads to a higher number of ions in a solution. Indeed, variations of plasma osmolality may interfere with the cell membrane polarisation or function and induce cell shape changes that may also modify the behavior of blood cells.^{34,35} Moreover, an intravenous injection of a hyperosmolar solution may produce significant changes in the acid–base equilibrium and the electrolyte concentrations,³⁶ as well as increase the viscosity of the blood, disrupt the hemodynamics and induce thrombosis. The measured osmolality of the solutions (1) and (2) in the concentrations used for the treatment of mice was found to be about two-fold higher than that of the equivalent solutions of (3) and, apparently such an increase in osmolality was enough to increase the acute intravenous toxicity (*i.e.* LD₅₀ values) of the cluster complexes (1) and (2) by the factor of ~6 in comparison with complex (3).

Experimental

Materials and methods

Neutral aquahydroxo complexes $[\{\text{Re}_6\text{Q}_8\}(\text{H}_2\text{O})_4(\text{OH})_2]$ (Q = S or Se) were prepared according to a literature procedure.³⁷ $\text{Na}_4[\{\text{Re}_6\text{Q}_8\}(\text{OH})_6]$ was obtained by the reaction of the corresponding complex $[\{\text{Re}_6\text{Q}_8\}(\text{H}_2\text{O})_4(\text{OH})_2]$ with an aqueous solution of NaOH followed by precipitation of $\text{Na}_4[\{\text{Re}_6\text{Q}_8\}(\text{OH})_6]$ with an excess of ethanol.⁹ $\text{Na}_2\text{H}_8[\{\text{Re}_6\text{Se}_8\}(\text{P}(\text{C}_2\text{H}_4\text{CONH}_2)(\text{C}_2\text{H}_4\text{COO})_2)_6]$ (3) was synthesised by the hydrothermal reaction between $\text{Na}_4[\{\text{Re}_6\text{Se}_8\}(\text{OH})_6]$ and $\text{P}(\text{C}_2\text{H}_4\text{CN})_3$ (130 °C, 48 h) and purified by the method described before.^{9,11} $\text{Na}_2\text{H}_{14}[\{\text{Re}_6\text{Q}_8\}(\text{P}(\text{C}_2\text{H}_4\text{COO})_3)_6]$ (Q = S (1) or Se (2)) were synthesised by the hydrothermal reaction between $\text{Na}_4[\{\text{Re}_6\text{Se}_8\}(\text{OH})_6]$ and $\text{P}(\text{C}_2\text{H}_4\text{COOH})_3\cdot\text{HCl}$ (130 °C, 48 h) and purified by the methods described before.¹⁴

Osmolality of complexes (1), (2) and (3)

The osmolalities of aqueous solutions (1)–(3) were measured with a freezing point depression osmometer (OCKP-1, KIVI Osmometry, Saint Petersburg, Russian Federation). Each point was measured three times and the average value was determined.

Cell culture

Mouse peritoneal macrophages (MPH) were obtained from Balb/C mice weighing 20–22 g. Animals were euthanized by cervical dislocation after a one week acclimation period (temperature 23 ± 2 °C and humidity 60%, free access to water and standard food). Mice were injected intraperitoneally with 3 mL of phosphate buffer saline (PBS), then the peritoneal lavage fluid was washed and the peritoneal macrophages were col-



lected and seeded in appropriate plates ready for *in vitro* experiments.

Human larynx carcinoma cell line (Hep-2) and human epithelioid cervix carcinoma cell line (HeLa) were purchased from the State Research Center of Virology and Biotechnology VECTOR (Russian Federation).

All cells were cultured in Eagle's Minimum Essential Medium (MEM, pH = 7.4) supplemented with a 10% fetal bovine serum (FBS) under a humidified atmosphere (5% CO₂ and 95% air) at 37 °C.

MTT-assay

MPh, Hep-2 and HeLa cells were seeded into 96-well plates at the concentration of $5-7 \times 10^3$ cells per well and incubated for 24 h under a 5% CO₂ atmosphere at 37 °C. The cells were then treated with solutions of (1), (2) and (3) taken in a broad range of concentrations (from 0.0056 to 5.76 mM) and incubated for 72 h under the same conditions. MTT reagent was added to each well to achieve a final concentration of 250 µg mL⁻¹, and the plates were incubated for 4 h. The formazan formed was then dissolved in DMSO (100 µL). The optical density was measured with a plate reader Multiskan FC (Thermo Scientific, USA) at a wavelength of 570 nm. The experiment was repeated three times on separate days.

Quantitative data obtained were analysed using Statistica 10.0 (StatSoft). Analysis between groups for the MTT assay was assessed by the Mann–Whitney U test. The *p* values <0.05 were considered statistically significant.

Confocal fluorescence imaging

The cells were seeded on microscope slides at the concentration of 1×10^5 cells per slide and incubated at 37 °C under a 5% CO₂ atmosphere for 24 h. The culture medium was then replaced for the culture medium containing 0.18 mmol of (1), (2) and (3) and the cells were incubated for 24 h. The cells were then washed with PBS and fixed in 4% paraformaldehyde. The cells were visualised using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Jena, Germany) equipped with a laser diode (405 nm) for fluorescence and with a 100× oil immersion objective.

Animals and housing conditions

All animal procedures were carried out in accordance with the protocols approved by the bio-ethics committee of Siberian Branch of the Russian Academy of Sciences, recommendations for proper use and care of laboratory animals (European Communities Council Directive 86/609/CEE) and principles of the Declaration of Helsinki. Mice were housed in stainless steel cages containing sterile paddy husk as bedding in ventilated animal rooms with free access to water and a commercial laboratory complete food. Animals were acclimatised for 1 week prior to the experimental use.

Median lethal dose assessment

Ninety 8-week-old Balb/C mice, each having a body weight of 20 ± 2 g, were randomised into 9 groups of 10 animals per

group (5 males and 5 females in each group) and received intravenous injection in the tail vein of 0.1 mL of an aqueous solution of the cluster complex (1) or (2) at doses 50, 150, 250 or 350 mg (Re) per kg, or sham-injected with the same volume of physiological saline. The solutions for intravenous infusion were used at the physiological pH = 7.4 that was achieved by the addition of NaOH. The animals were regularly observed for clinical signs and mortality for two weeks. After this period the mice were sacrificed.

Conclusions

In conclusion, *in vitro* studies have shown that the cytotoxicity and cellular internalisation behavior of complexes (1) and (2) are comparable to those of the complex (3). However, due to the increased osmolality of the complexes (1) and (2), their intravenous acute toxicity *in vivo* is significantly higher. Thus our study demonstrates that even a minor chemical change of the ligand environment of the cluster that alters the overall charge of the cluster anion can have a drastic effect with regard to the toxicity of the compound. Our study has also demonstrated that in terms of toxicity and internalisation, there is no significant difference between {Re₆S₈}²⁺ and {Re₆Se₈}²⁺. Moving forward, one way to produce highly effective X-ray contrast rhenium octahedral species of similar chemical structures, but reduced acute toxicity would be the esterification of all or some of the carboxylate groups of the organic ligands to reduce the overall charge of the complexes.

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