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COMMUNICATION

Combining a Ru(II)-arene complex with a NO-releasing nitrate-ester ligand generates cytotoxic activity

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A Ru(II) arene complex with a NO-releasing 4-nitrooxymethyl-pyridine ligand shows increased cytotoxicity against the non-small cell lung cancer cell line A549 as compared to either the free ligand or the unfunctionalized complex. EPR spin-trapping studies show that NO release is selective, being limited in phosphate buffered saline or human serum, but promoted by glutathione.

Nitric oxide (NO) is a cellular signaling molecule, whose role in cancer has been widely studied.[1] Both tumoricidal and tumorigenic effects of NO have been reported and this biphasic behaviour has been linked to concentration, with higher levels typically leading to antineoplastic activity.[2] A variety of pathways have been proposed for the anticancer activity of NO including promotion of apoptosis, and inhibition of proliferation and angiogenesis.[2b,3] Specific mechanisms include generation of reactive nitrogen species, particularly peroxynitrite ($ONOO^-$), and modulation of DNA repair mechanisms in tumors.[1,2b]

NO-donor compounds are used clinically as vasodilators, with prominent examples including organic nitrates and the iron nitrosyl complex sodium nitroprusside.[3-4] A variety of inorganic NO donors have been reported. These include a number or ruthenium nitrosyl complexes with vasodilatory effects and, in some reports, anticancer activity.[4a,5] While medicinal metal-based NO-donor complexes typically contain directly coordinated nitrosyl groups, a more versatile approach employs coordination of ligands with NO-donating functional groups. This has been used successfully to enhance the cytotoxic activity of platinum complexes with NO-donor nitrate-ester or furoxan functionalized ligands.[6] Herein, we describe the application of this strategy to ruthenium anticancer complexes.

Ruthenium anticancer agents are currently considered the most promising metal-based alternatives to platinum chemotherapeutics.[7] Recently, development in this area has increasingly focused on organometallic Ru(II) arene complexes.[8] Notable examples include complexes of the type $[(\eta^6\text{-arene})Ru(L)Cl]^+$, where L is a bidentate oxygen and/or nitrogen donor ligand,[7c,9] and "RAPTA" compounds $[(\eta^6\text{-arene})RuCl_2(pta)]$, where pta = 1,3,5-triaza-7-phosphaadamantane.[10] Complexes of the first type show very promising *in vitro* cytotoxic activity, whereas RAPTA compounds have drawn attention for their antimetastatic activity. The pharmacological properties of these and related anticancer Ru(II) arene complexes are modulated by their ligands. In general, the arene groups provide a bulky hydrophobic surface, which can influence both interactions with biological targets and cellular accumulation.[8b,11] Exchangeable ligands, such as chloride, enable direct coordination to protein and nucleic acid targets, following initial hydrolysis.[7c,12] Non-exchangeable ancillary ligands have been used to install different types of pharmacophores, including enzyme inhibitors, biological species, and photoactivators.[13]

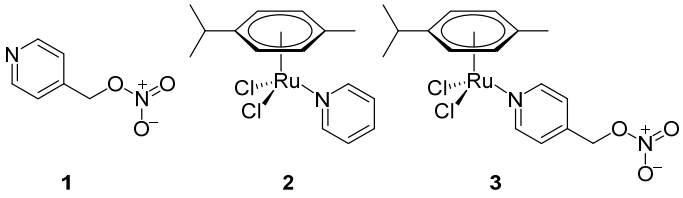

Fig. 1 4-nitrooxymethylpyridine ligand (**1**), Ru(II) cymene complex with pyridine auxiliary ligand (**2**), and nitrate-ester functionalized complex (**3**).

In this work we have used the ancillary ligand 4-nitrooxy-methylpyridine (**1**) (**Fig. 1**) to generate an analogue of RAPTA-type complexes (**3**) with the ability to selectively release nitric oxide. The result is synergistic activation of cytotoxicity since neither the unfunctionalized Ru(II) arene complex (**2**) nor the ligand (**1**) are active independently. This approach contrasts with typical combination chemotherapy, where compounds are coadministered, by linking pharmacophores so that the whole is greater than the sum of the parts.

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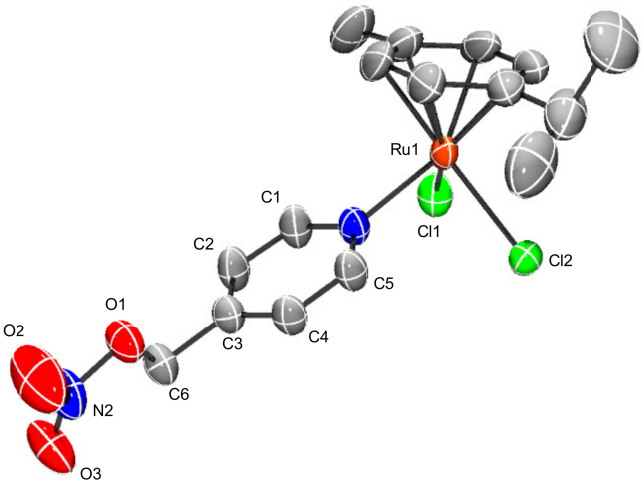

Fig. 2 Crystal structure of complex **3**. For clarity, a co-crystallizing chloroform solvent molecule has been omitted. Structure is drawn at the 50% probability level.

The new NO-donor compound derived from the Ru(II) arene complex $[(\eta^6\text{-}p\text{-cymene})Ru(pyridine)Cl_2]$[14] (**2**) was synthesized using the nitrate-ester functionalized pyridine ligand (**1**) to give complex **3**, $[(\eta^6\text{-}p\text{-cymene})Ru(4\text{-nitrooxymethylpyridine})Cl_2]$. Ligand **1** was synthesized using the literature procedure for the regioisomer 3-nitrooxymethylpyridine,[15] and was prepared as the HNO_3 salt. Complex **3** was prepared using the same procedure as the unfunctionalized compound **2**, demonstrating the stability and synthetic utility of ligand **1**. Both ligand **1** and complex **3** were characterized by elemental analysis, IR, and ^1H, ^{13}C NMR spectra (see supporting information).[16] The structure of complex **3** was confirmed by X-ray crystallography (**Fig. 2**).

The cytotoxicity of **1**, **2**, and **3** was investigated against A549 adenocarcinomic human alveolar basal epithelial cells, a non-small cell lung cancer cell line, using a fluorescent assay of cell viability (**Fig. 3**). Both ligand **1** and complex **2** failed to achieve 50% inhibition at even the highest tested concentrations of 1000 µM. However, the nitrate-ester functionalized complex **3** showed significantly higher cytotoxic activity, with an IC_{50} value of 180 µM. The cytotoxicity of Ru(II) arene complexes depends strongly on the nature of their ancillary ligands and the cell lines against which they are tested. Literature studies of Ru(II) arene complexes with the A549 cell line report IC_{50} values as low as sub-micromolar,[17] but often >100 µM.[18] As such, the activity of the NO-donor complex reported is not exceptional in its own right. However, the observation that the activity of **3** is so much greater than either the unfunctionalized compound **2** or the nitrate ester-ligand **1** alone, demonstrates that each component has a role to play in generating cytotoxicity. We suggest that the Ru arene promotes transmembrane transport, modulated by the relatively hydrophobic cymene ligand, which then enables intracellular release of NO by the nitrate ester ligand. Thus these components work synergistically to generate the activity of **3**.

Aqueous exchange of chloride ligands has been identified as an activating step for many Ru(II) arene complexes, which can enable binding to biomolecules including DNA.[8] UV-Vis spectra of **2** and **3** were measured in phosphate buffer (pH 7.4) and phosphate buffered saline (PBS, pH 7.4) with $[Cl^-]$ = 20 or 139 mM, corresponding to chloride concentrations in the cytoplasm and in standard PBS respectively. The spectra from each complex were very similar and did not vary with incubation over 90 min at 37 °C, in any of the solutions (see supporting information). This demonstrates that the nitrate ester group does not affect chloride ligand exchange.

Release of NO by **3** was studied using electron paramagnetic resonance (EPR) and spin trapping. Ferro-di(*N*-methyl-D-glucamine-dithiocarbamate) ($Fe(MGD)_2$) was used to trap NO, giving the stable complex $Fe(MGD)_2$-NO, which has a characteristic three-line spectrum due to hyperfine coupling from the ^{14}N (I = 1, 99.632 %) nucleus of NO (**Fig. 4a**). The time dependence of NO release was determined by double integration of EPR signals at selected incubation time points. This enabled quantitative comparison of NO-release in PBS, human serum, and in the presence of glutathione (GSH).

The release of NO from nitrate esters *in vivo* typically involves either enzymatic or nonenzymatic activation. Thiols, such as GSH, have been identified as activators for the non-enzymatic release of NO.[4a] The likely mechanism of GSH activation involves an initial $2e^-$ reduction of the nitrate via oxidation of two equivalents of GSH to give glutathione disulphide (GSSG), which leads to release of a nitrite ion (NO_2^-).[19] Subsequent reduction of NO_2^- then yields NO. GSH is found in elevated levels in many tumor types, and is distributed heterogeneously within cells leading to higher levels in certain organelles, which can confer resistance to chemotherapeutic treatments.[20] Cellular concentrations of GSH in A549 cells have been reported to be around 5 mM[21] which is a significant excess with respect the concentrations of **3** used in the cytotoxicity assays. To reflect this situation, complex **3** was incubated with a five-fold excess of GSH at 37 °C in PBS. The concentration of **3** used (5 mM) also enabled NO release experiments with sufficient signal-to-noise to be conducted in

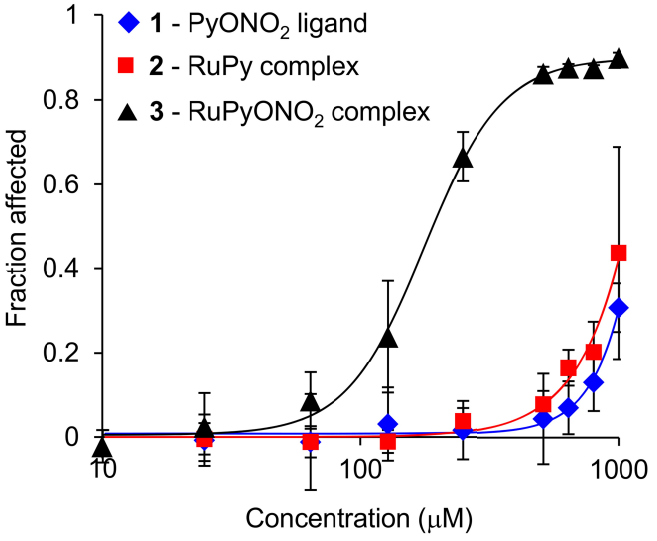

Fig. 3 Dose-response curves for the cytotoxic activity of ligand **1**, and complexes **2** and **3** against the non-small cell lung cancer cell line A549.

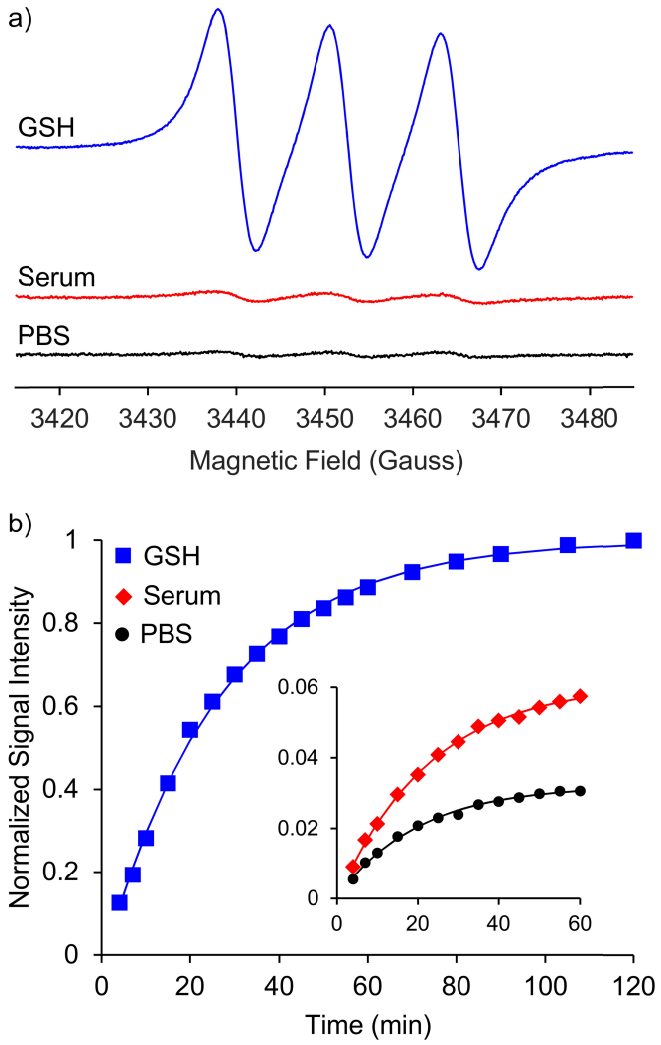

Fig. 4 EPR measurements of NO generated by complex **3** (5 mM), spin trapped with Fe(MGD)2 (10 mM), following incubation at 37 °C with: GSH (25 mM) in PBS, in human serum, and in PBS. a) EPR spectra after 10 min of incubation. b) time-course of signal intensity and fitting to 1st-order kinetics. Intensities normalized to signal amplitude from GSH sample after 2 hours of incubation.

PBS and human serum (see below). EPR signals from spin-trapped NO were readily observed with incubation in the presence of GSH (**Fig. 4a**), with the maximum intensity reached after 2 hours of incubation (**Fig. 4b**). The time dependence of the signal intensity was well fitted with first-order kinetics (**Fig. 4b**, **Table 1**).

To confirm the importance of GSH to NO release from compound **3**, spin trapping experiments were also performed in PBS only. Under these conditions only low levels of NO were detected (**Fig. 4**) with the concentration of NO detected after 1 hour of incubation only 3.1% of the maximum observed with GSH. Similar levels of NO-release have been reported from organic nitrate esters with incubation in PBS at 37 °C.[22] This demonstrates that **3** is relatively stable towards spontaneous release of NO in the absence of excess thiolates.

NO release was also measured in human serum to evaluate the effect of blood components. With incubation at 37 °C NO release was low, with only 5.7% of the maximum level with GSH observed after 1 hour. This is consistent with the low total thiol concentration in serum of ~0.4-0.6 mM, most of which is due to human serum albumin; low molecular weight thiols contribute only 12-20 μM, with the concentration of reduced GSH only around 3 μM.[23] Overall, the NO release studies demonstrate that these types of compounds could be administered intravenously without significant loss of NO in the blood stream, and could then be activated to generate NO once transported into more thiol-rich cellular environments. Similar thiol-dependent release of NO has been reported in recent studies of nitrate-ester and furoxan functionalized platinum complexes in the presence of L-cysteine.[6] This behaviour is also a feature of non-enzymatic NO generation from many organic NO donors.[4a]

Table 1. 1st order rate constants for NO-release by complex **3**, and relative amounts of NO released after 1 hour of incubation at 37 °C.

Condition	1st order k_{NO}	Release after 1 hr*
PBS	$8.4 \pm 1.2 \times 10^{-4}$ s^{-1}	3.1%
Human Serum	$7.3 \pm 0.7 \times 10^{-4}$ s^{-1}	5.7%
Excess GSH	$6.3 \pm 0.3 \times 10^{-4}$ s^{-1}	88.6%

* Calculated as the % of the maximum NO-release observed (excess GSH, 120 min = 100%).

In conclusion, the Ru(II)-arene complex **3**, containing a nitrate-ester pharmacophore, shows promising cytotoxicity against the A549 cell line and a potential pathway for selective intracellular activation by thiols. The observation that neither the unfunctionalized analogue complex **2** nor the nitrate ester ligand **1** show activity on their own demonstrates that their combination has a positive cooperative effect against cancer cells. This is consistent with NO donation by ligand **1** generating cytotoxicity, and the promotion of transport into cells by the Ru(II) arene moiety. The NO-releasing properties of **3**, determined by EPR spin-trapping studies, are enhanced in the presence of GSH, but are minimal in the absence of significant thiol concentration. This may improve the selectivity and cytotoxicity of the ruthenium complex in thiol-rich cells, such as cisplatin resistant cell lines, while minimizing NO loss in blood. Thus, the nitrate-ester ligand **1** provides a facile pathway to activating the cytotoxicity and selectivity of Ru anticancer complexes. Further development of this concept will involve studies of selectivity by probing activity in normal cells, and use of other structurally related pyridine-based nitrate esters.

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