Organometallic Cages as Vehicles for Intracellular Release of Photosensitizers

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · DECEMBER 2011

Impact Factor: 12.11 · DOI: 10.1021/ja207784t · Source: PubMed

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Efficient Oxidation of Cysteine and Glutathione Catalyzed by a Dinuclear Areneruthenium Trithiolato Anticancer Complex†

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Supporting Information

ABSTRACT: The highly cytotoxic diruthenium complex $[(p\text{-}MeC_6H_4Pr^i)_2Ru_2(SC_6H_4\text{-}p\text{-}Me)_3]^+$ (**1**), water-soluble as the chloride salt, is shown to efficiently catalyze oxidation of the thiols cysteine and glutathione to give the corresponding disulfides, which may explain its high in vitro anticancer activity.

Organometallic complexes offer aspects for medicinal chemistry that are not available with organic drugs, in particular because of their coordination and redox properties.[1] For this reason, metal-based pharmaceuticals have found a steadily increasing interest ever since the discovery of the anticancer activity of *cis*-$Pt(NH_3)_2Cl_2$ (cisplatin).[2] The quest for alternative anticancer drugs, particularly stimulated by the serious side effects of platinum-based cancer therapies,[3] resulted in a variety of cytotoxic organometallics, of which areneruthenium complexes occupy a prominent position because of their unique combination of lipophilic and hydrophilic properties.[4]

The mode of action by which areneruthenium complexes exert their antitumoral or antimetastatic effects is not yet fully understood. By analogy with platinum complexes, it was originally expected that DNA binding was also the main reason for the anticancer activity of ruthenium complexes, but serum proteins have also been discussed as possible targets.[5] While the ability of ruthenium to bind to DNA has been demonstrated,[6] in particular for areneruthenium ethylenediamine complexes,[7] it was observed that DNA binding of ruthenium was weaker and different from that observed for platinum.[8] These findings suggest different modes of action depending on the type of complexes. Thus, the RAPTA-type areneruthenium complexes (Chart S1 in the Supporting Information, SI), originally designed to improve the aqueous solubility,[9] have been found to target thioredoxin reductase and cathepsin B, proteins that act as enzymes in the cells.[10]

Another mode of action has been found for areneruthenium iodoazopyridine complexes, which are surprisingly cytotoxic despite their inertness to ligand substitution: In a pioneering study, Sadler and co-workers demonstrated these complexes to act as catalysts for oxidation of the tripeptide glutathione, supposed to be at the origin of their anticancer activity.[11]

In this paper, we report the *p*-cymene *p*-toluenethiolato derivative $[(p\text{-}CH_3C_6H_4Pr^i)_2Ru_2(SC_6H_4\text{-}p\text{-}CH_3)_3]^+$ (**1**; Chart 1) of the dinuclear complex family $[(arene)_2Ru_2(SR)_3]^+$,[12] which we had found to be highly cytotoxic for human ovarian cancer cells (with IC_{50} values in the nanomolar range for both the cell line A2780 and cisplatin-resistant line A2780 CisR),[13] to act as an efficient catalyst for oxidation of the thiols cysteine (Cys) and glutathione (GSH).

Complex **1** has been synthesized as previously described[12] and characterized by standard spectroscopic methods. The stability of **1** was assessed by recording ^{1}H NMR spectra over 24 h at 37 °C. The spectra show (Figure S1 in the SI) that **1** is absolutely stable in D_2O, dimethyl sulfoxide (DMSO)-d_6, D_2O/DMSO-d_6 (95:5), with or without the addition of 100 mM NaCl (close to extracellular $[Cl^-]$).

The interactions between **1** and glucose, all amino acids, and nucleotides were monitored by NMR spectroscopy, and, surprisingly, only Cys and GSH induced changes in the ^{1}H and ^{13}C NMR spectra compared to the free components. Figure 1 shows the ^{1}H NMR spectra obtained upon titration of Cys into a solution of **1** in D_2O with 50 mM NaCl. In agreement with the ^{1}H NMR spectrum of free Cys, the addition of 1 equiv of Cys resulted in the appearance of new Cys resonances at δ 4.17 (Hα), 3.46 (Hβ'), and 3.27 (Hβ). These new ^{1}H signals increased over time, relative to the signals of free Cys, and, in agreement with the literature, were assigned as arising from cystine.[14]

The addition of 1 equiv of GSH resulted in the appearance of new Cys resonances at δ 4.78 (Hα), 3.33 (Hβ'), and 3.04 (Hβ) (Figure 2). As for Cys, these new signals increased over time, relative to the signals of free GSH, and, in agreement with the literature, were assigned as arising from oxidized glutathione (GSSG).[15]

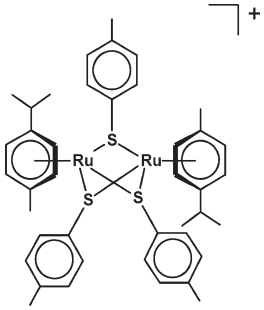
Interestingly, titrations with Cys and GSH revealed that only the ^{1}H and ^{13}C chemical shifts of the α-CH and β-CH_2 groups of Cys were affected, whereas the chemical shifts of the complex and the other amino acids remained unperturbed (Figures 2 and 3 and S2 in the SI). These results strongly suggest that Cys and GSH do not form stable adducts with **1**, which was further evidenced by DOSY spectra (Figure S3 in the SI). From these ^{1}H NMR titration experiments, it became apparent that **1** can act as a very efficient catalyst for oxidation of Cys to cystine and of GSH to GSSG:

$$RSH + RSH \xrightarrow{1} RS{-}SR + H_2 \quad (1)$$

In order to evaluate the catalytic performance of **1**, we followed the ^{1}H NMR spectra of mixtures of **1** with Cys and GSH with the ratio 1:100, respectively, over time. The catalytic

Received: September 5, 2011
Published: October 07, 2011

Chart 1. Complex 1[a]



[a]The counteranion is Cl^-.

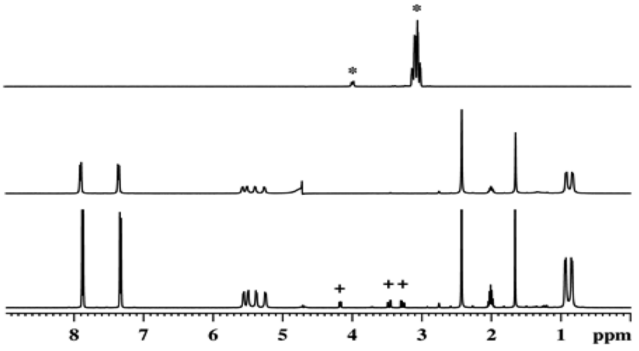


Figure 1. ^{1}H NMR spectra of Cys (top), **1** (middle), and a mixture of **1** and Cys (ratio 1:1, bottom) recorded at 37 °C in D_2O/DMSO-d_6 (95:5) after 2 h of incubation. The resonances of Cys are indicated by * and the resonances of cystine by +. The residual water signal is visible around 4.7 ppm.

conversion of GSH to GSSG may be directly related to the anticancer activity. Cancer cells are known to have a higher GSH pool than healthy cells, and in all living cells, more than 90% of the total GSH pool is in the reduced form (GSH) and less than 10% exists in the disulfide form (GSSG). An increased GSSG-to-GSH ratio is considered to be indicative of oxidative stress, which damages all components of the cell, including proteins, lipids, and DNA and which may lead to apoptosis.[16] For Cys, the ^{1}H NMR spectra showed that the reaction led to a steady oxidation of Cys to cystine after only 14 h, which led to a turnover frequency after 50% conversion (TOF_{50}) of 8.1 h^{-1} (Figure 3). We point out here that the catalytic reaction of complex **1** could also be performed using a ratio of 1:1000. The reaction led to a TOF_{50} of 80.9 h^{-1} (Figure S5 in the SI). Steady oxidation was evidenced by the complete disappearance of the original resonances of Cys at δ 4.03 (Hα), 3.17 (Hβ'), and 3.09 (Hβ) (Figure 2). The formation of cystine during the reaction was further confirmed by electrospray ionization mass spectrometry (ESI-MS) spectra (Figure S6 in the SI).

For GSH, the ^{1}H NMR spectra revealed steady oxidation of GSH to GSSG after 16 h (Figure 3), with TOF_{50} being 7.4 h^{-1} (Table 1). Steady oxidation was also evidenced by the complete disappearance of the original resonances of Cys in GSH at δ 4.61 (Hα), 3.02 (Hβ'), and 2.96 (Hβ) (Figure 2). Formation of GSSG during the reaction was further confirmed by ESI-MS spectra (Figure S7 in the SI). It is worth pointing out that, for an identical complex/GSH ratio (1:100), TOF_{50} obtained for

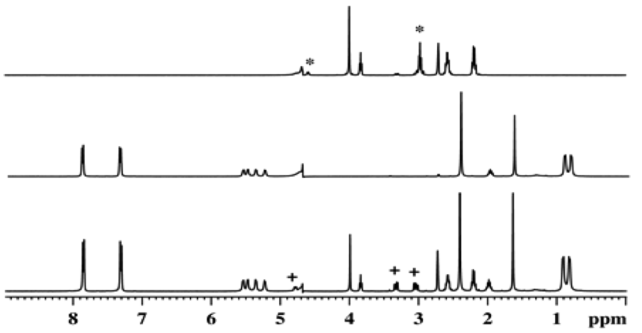


Figure 2. ^{1}H NMR spectra of GSH (top), **1** (middle), and a mixture of **1** and GSH (ratio 1:1, bottom) recorded at 37 °C in D_2O/DMSO-d_6 (95:5) after 2 h of incubation. The resonances of Cys (GSH) are indicated by * and the resonances of cystine (GSSG) by $^+$. The residual water signal is visible around 4.7 ppm.

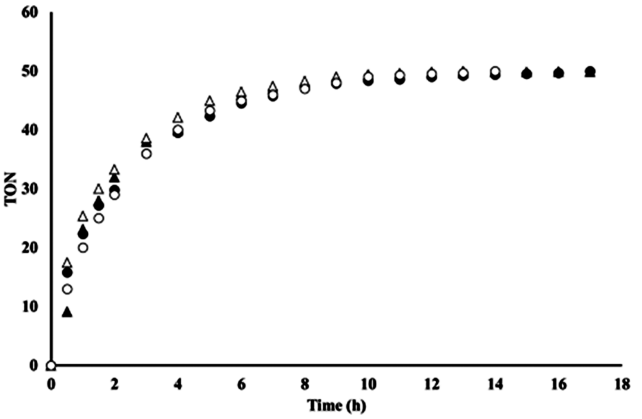


Figure 3. Turnover number (TON) for mixtures of **1**/Cys (▲) and **1**/GSH (●) (ratio 1:100) in D_2O at 37 °C with 50 mM NaCl under argon and **1**/Cys (△) and **1**/GSH (○) under the same reaction conditions as those above but under O_2.

Table 1. TOFs after 50% Conversion (TOF_{50}) for Oxidation of Cys to Cystine and of GSH to GSSG with 1 as a Catalyst (the Values Are Given per Ruthenium Atom)

	TOF_{50}/h^{-1} at 37 °C			
	p*D* = 4, 50 mM NaCl	p*D* = 7, 50 mM NaCl	p*D* = 10, 50 mM NaCl	p*D* = 7, 4 mM NaCl
Cys	8.1	8.1	7.2	6.5
GSH	7	7.4	6.9	6

complex **1** is about 1 order of magnitude higher than TOF obtained for iodo-containing ruthenium(II) arene organometallic derivatives (0.37 h^{-1}).[11] In addition, incubation of 10 mM GSH with these complexes led to steady oxidation of only 4.6 mM GSH to GSSG, whereas oxidation is complete for complex **1**. Moreover, complex **1** is stable during the reactions and can be recovered unchanged as the chloride salt, as shown by ^{1}H NMR spectra of the reaction of **1** with Cys and GSH recorded at t = 0 and 24 h (Figures S8 and S9 in the SI). To test the stability of the catalyst, 100 equiv of Cys was oxidized, and the reaction was reiterated five times using the same catalyst (Figure S10 in

dx.doi.org/10.1021/ic201941j |*Inorg. Chem.* 2011, 50, 10552–10554

the SI). The stability of **1** is remarkable, and TOF_{50} drops by only 15% after the fifth run.

The results for oxidation of Cys to cystine and of GSH to GSSG as a function of the pH and $[Cl^-]$ are shown in Table 1. It can be seen that TOF_{50} slightly decreases under basic conditions, whereas acidic conditions have no influence. The higher TOF_{50} values observed for **1** under acidic conditions open new avenues for further modifications with the requirement that future complexes exhibit efficient oxidation only under acidic conditions. Examples of complexes that undergo hydrolysis/activation only in cancer cells have recently been reported.[17] Likewise, the difference between $[Cl^-]$ in blood plasma and in the cytoplasm has been recently exploited for the design of ruthenium complexes that should only be activated or hydrolyzed once inside the cancer cells.[7b] However, Table 1 shows that TOF_{50} drops by about 20% in concert with decreasing $[Cl^-]$ from 50 to 4 mM. Further modifications of **1** will be required, aiming at higher TOF_{50} values for increasing chloride concentrations.

In the present study, we have shown that the dinuclear areneruthenium trithiolato complex **1** is inert toward biological model compounds and yet highly cytotoxic toward A2780 cancer cell lines. Supramolecular enzyme inhibition, although unlikely, cannot be completely ruled out. In line with areneruthenium iodoazopyridine complexes,[11] obviously, **1** has a different mechanism of cancer cell cytotoxicity, involving highly efficient catalytic oxidation of the major intracellular reducing agent GSH to GSSG. This complex might have the advantage of not being poisoned as metal catalysts and therefore might have greater potential for biological activity. Unlike the large majority of ruthenium complexes considered so far, complex **1** was found to be about 2 times more cytotoxic against the A2780 CisR cell line.[13] Interestingly, the intracellular GSH content was shown to be much higher in A2780 CisR cells.[18] Therefore, the highly efficient catalytic oxidation of GSH to GSSG might explain the better cytotoxicity of **1** against the cisplatin-resistant line A2780 CisR. Further studies to compare the cytotoxicity and catalytic activity with the nature of the bridging thiophenolato ligands and for fine-tuning of the influence of the pH and $[Cl^-]$ on TOF_{50} are under investigation.

■ ASSOCIATED CONTENT

Supporting Information. Analytical details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

■ ACKNOWLEDGMENT

J.F. thanks the Swiss National Science Foundation (Grant 200021-131887) for financial support.

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