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Citation for published version:

Digital Object Identifier (DOI):
10.1073/pnas.0800076105

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the National Academy of Sciences

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Catalytic organometallic anticancer complexes

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Organometallic complexes offer potential for the development of new materials with a wide range of applications (1, 2). Organometallic complexes can exhibit chemical reactivity not possessed by either the metal or organic ligands alone and this reactivity can be fine-tuned by subtle changes in the electronic and steric properties of the bound ligands, or by variation of the metal and its oxidation state. These features provide a versatile platform for drug design that is now being exploited in several areas. For example, the redox activity of the organometallic ferrocenylphenol substituents enhances the anticancer activity of tamoxifen derivatives by conferring an ability to generate reactive oxygen species in cells (3). Here we consider the anticancer activity of ruthenium(II) arene complexes. Such complexes are well known as catalysts in industry, for example, for hydrogenation reactions (4). Although organometallic complexes can catalyze hydrogenation of intracellular biomolecules (5), metal-centered catalysts might be readily poisoned and be ineffective in biological media.

Organometallic half-sandwich RuII arenes of the type [{η⁶-arene}Ru(YZ)(X)]+ where YZ is typically a chelating diamine (e.g., ethylenediamine, en) and X is a halide (e.g., Cl) exhibit anticancer activity in vitro and in vivo (6, 7). As with many metal-based anticancer drugs, the primary cellular target is thought to be DNA. The Ru–Cl bond is labile, but hydrolysis is suppressed extracellularly because of the high chloride concentration, ~104 mM (8). The intracellular chloride concentration is much lower, and hydrolysis can generate a reactive site on ruthenium (Ru-OH₂) for subsequent DNA binding. DNA binding leads to further “downstream” effects, ultimately resulting in cell death (9, 10). Changing the chelating ligand has dramatic effects on cytotoxicity, which is also influenced by the nature of the arene ligand (11). In this series of diaime complexes, changing the monodentate ligand X from chloride to iodide has little effect on activity; both are good leaving groups (12). This contrasts dramatically with the chlorido and iodido ruthenium(II) arene complexes reported here, which contain σ-donor/π acceptor 2-phenylazopyridines as N,N-chelating (YZ) ligands. Unlike their en analogues, these complexes exhibit different solution chemistry, and a different mechanism of cancer cell cytotoxicity, involving catalytic redox reactions.

Results

Complexes. We synthesized and characterized RuII complexes containing either p-cymene (complexes 1–3) or biphenyl (complexes 4–6) as the π-bonded arene, a bidentate phenylazopyridine ligand having either NMe₂, OH or H as para substituent on the phenyl ring, and I⁻ as the monodentate ligand (Fig. 1). The molecular structure of complex 4-MeOH was determined by x-ray crystallography [see supporting information (SI) Fig. S1]. Significant bond lengths and angles are reported in the SI Text (Table S1). The Ru–N bond lengths are comparable to analogues of analogous RuII arene phenylazopyridine chlorido complexes (13), as are the ruthenium-arene carbon distances (13). The Ru–I bond length (2.6984 (5) Å) is slightly shorter than in ruthenium(II) arene complexes, containing ethylenediamine as the chelating ligand (2.7116 (7) Å for [{η⁶-hexamethylbenzene}Ru(en)]I+ and 2.7166 (7) Å for [{η⁶-indan}Ru(en)]I+ (12)). These phenylazopyridine complexes possess “3-legged piano-stool” structures and are intensely colored as a result of RuII-to-ligand (4d⁵-π*) charge-transfer and intraligand π–π transitions (13).

Cytotoxicity. Complexes 1, 2, 4, and 5 containing p-cymene or biphenyl as arene and electron-donating groups (OH, NMe₂) on the phenylazopyridine ligand were highly cytotoxic to A2780 human ovarian and the A549 human lung cancer cell lines with IC₅₀ values of 2–6 μM, whereas complexes 3 (IC₅₀ > 100 μM) and 6 (IC₅₀ ≈ 40–50 μM), containing an unsubstituted phenylazopyridine ligand were much less cytotoxic (Table 1). Replacement of iodide by chloride led to a dramatic decrease in cytotoxicity (13), and the 2-phenylazopyridine ligands alone were relatively nontoxic, with the exception of Azpy-NMe₂ in the A549 cell line (Table 1).

Author contributions: S.J.D., A.H., and P.J.S. designed research; S.J.D., A.H., and S.E.M. performed research; S.E.M. and S.P. contributed reagents/analytic tools; S.J.D., A.H., S.E.M., S.P., and P.J.S. analyzed data; and S.J.D., A.H., S.P., and P.J.S. wrote the paper.

Conflict of interest statement: The University of Edinburgh has filed a patent application relating to these ruthenium complexes. This has been licensed to Oncosense Ltd.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0800076105/DCSupplemental.

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Solution Chemistry. We investigated whether the complexes could undergo activation by hydrolysis. We used conditions that mimic the concentration, pH, exposure time, and temperature used in biological cell tests, and thus monitored 100 μM solutions of the complexes in 10 mM phosphate buffer (containing 95% D2O/5% MeOD, pH 7.3) and at 310 K over 24 h by 1H NMR. For iodo complexes 1, 2, 4, and 5 containing phenylazopyridine ligands with OH or NMe2 substituents, no changes in the spectra occurred (e.g., Fig. S2 for complex 4) suggesting that no hydrolysis took place. This was confirmed by ESI-MS studies of the NMR sample where only the intact cation was detected [1, m/z found 588.75 (M−1), calcd 589.04; 2, m/z found 561.70 (M−1), calcd 561.99; 4, m/z found 608.71 (M−1), calcd 609.03; 5, m/z found 581.65 (M−1), calcd 581.98]. However, the NMR spectra of the unsubstituted phenylazopyridine complexes 3 and 6 changed over the 24-h period (Figs. S3 and S4, respectively). For 6, all peaks for the intact cation disappeared and the only assignable peaks in the spectrum were due to free biphenyl. For 3, the peaks for the intact cation decreased in intensity and peaks for free p-cymene were observed. The slow decomposition of 3 and 6 (50 μM, 10 mM phosphate buffer, 310 K, 24 h) was confirmed by UV-Vis spectroscopy (Fig. S5).

Importantly, with 104 μM NaCl present (close to extracellular [Cl−]), no changes in the UV-Vis spectrum of complex 4 were observed over 24 h at 310 K (see Fig. S6), suggesting that no substitution of iodide by chloride takes place.

Table 1. IC50 values (μM) for [η6-arene]Ru(azpy-R)PF6 complexes, their corresponding chloride analogues [η6-arene]Ru(azpy-R)ClPF6 and free phenylazopyridine ligands for A2780 ovarian and A549 lung cancer cell lines, and the first and second (where observed) electrochemical reduction potentials (DMF vs. Ag/AgCl)

<table>
<thead>
<tr>
<th>Complex*</th>
<th>A2780</th>
<th>A549</th>
<th>Ered, V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1-CI)</td>
<td>4 (&gt;100)</td>
<td>3 (&gt;100)</td>
<td>−0.40, −1.00</td>
</tr>
<tr>
<td>4 (4-CI)</td>
<td>3 (44)</td>
<td>2 (49)</td>
<td>−0.36</td>
</tr>
<tr>
<td>Azpy-NMe2</td>
<td>&gt;100</td>
<td>14</td>
<td>−1.28</td>
</tr>
<tr>
<td>2 (2-CI)</td>
<td>4 (58)</td>
<td>4 (&gt;100)</td>
<td>−0.33, −0.77</td>
</tr>
<tr>
<td>5 (5-CI)</td>
<td>5 (18)</td>
<td>6 (56)</td>
<td>−0.26, −0.72</td>
</tr>
<tr>
<td>Azpy-OH</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>nd†</td>
</tr>
<tr>
<td>3 (3-CI)</td>
<td>&gt;100 (&gt;100)</td>
<td>&gt;100 (&gt;100)</td>
<td>−0.22, −0.74</td>
</tr>
<tr>
<td>6 (6-CI)</td>
<td>39 (&gt;100)</td>
<td>51 (&gt;100)</td>
<td>−0.18, −0.67</td>
</tr>
<tr>
<td>Azpy</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>−1.31 (vs. SCE)‡</td>
</tr>
</tbody>
</table>

*Iodo complexes 1-6 (and their chlorido analogues 1-CI to 6-CI in parentheses).
†nd, not determined.
‡Ref. 14.

Electrochemical Reductions. In general, the complexes exhibited two electrochemical reductions (in dimethylformamide), the first at −0.2 to −0.4 V and a second at −0.7 to −1 V (Table 1). Reductions were also observed near to the solvent cutoff (−2 V), but were not considered further.

In all cases the first reduction (confirmed as a one-electron reduction for complex 3, n = 0.98, Fig. S7) was essentially irreversible (no reverse scan peak at 0.1 Vs–1, Fig. S8). The reduction potential becomes more positive as the arene is changed from p-cymene to biphenyl and also as the chelating azo ligand is changed from azpy-NMe2 to azpy-OH to azpy (Table 1). Based on literature assignments for other ruthenium–phenylazopyridine complexes (14, 15), this reduction can be assigned to addition of an electron into the π* orbital centered on the azo group of the phenylazopyridine ligands to form the azo anion radical (−N=N− + e− → (−N=N−)−). The relative order therefore reflects the decreasing π-acceptor capability of the substituted azo ligands with the addition of electron-donating groups onto the phenyl ring (16). The irreversible second reduction step is assigned to conversion to the dianionic species (−N=N−)2−. Azo groups usually give rise to two separate electrochemical reductions in polar aprotic solvents (17). In aqueous media the two-electron reduction of azo groups is accompanied by proton transfer to give hydrazo groups (NH=NH) in a single step (17).

Reactions with Glutathione. In cells, the major reducing agent is the tripeptide glutathione (GSH), a thiol present at millimolar concentrations (18). We were thus interested to follow the reactions of the ruthenium complexes with GSH. HPLC and electrospray ionization (ESI)-MS studies showed that the initial step in the reaction of excess GSH with [(η6-biphenyl)Ru(azpy-NMe2)]PF6, complex 4, and the corresponding chlorido complex involves complete substitution of halide by G−, to give [(η6-biphenyl)Ru(azpy-NMe2)GS]− (m/z obs 788.0, calcd 789.2; Fig. S9). Half-lives for this substitution were determined as 1.3 min (kobs = 8.73 × 10−3 s−1) for the chlorido complex 4-CI, and 17.5 min for the iodido complex 4 (kobs = 6.58 × 10−4 s−1; 298 K by UV-Vis spectroscopy). Thus iodide substitution by G− occurs 14 times more slowly than chloride substitution. During 1H NMR studies of reactions of [(η6-biphenyl)Ru(azpy-NMe2)]PF6, 4, and [(η6-biphenyl)Ru(azpy-OH)]PF6, 5, with glutathione (carried out in deoxygenated solutions under N2 or Ar to avoid autoxidation), it became apparent that these complexes can act as catalysts for the oxidation of GSH to GSSG.

The NMR spectra showed that incubation of 10 mM GSH with 100 μM 4 or 5 (in 10 mM phosphate, pH 7.2, 85% H2O, 10% D2O, 5% acetone-d6, 310 K, followed over 24 h) led to a steady oxidation of 3.7 mM (turnover frequency 0.30 h−1, R2 = 0.98) and 4.6 mM, (turnover frequency = 0.37 h−1, R2 = 0.99), respectively, of GSH to GSSG (Fig. S10), as evidenced by the appearance of new peaks at δ 3.30 ppm corresponding to the β-CH2 of GSSG (Fig. S10 inset). The formation of GSSG was further confirmed by ESI-MS spectra of these solutions after 24 h incubation, which showed peaks consistent with the presence of glutathione (m/z obs 305.2, calcd for G− 305.1) and glutathione disulfide (m/z obs 611.1, calcd for GSSG 611.2).

LC-MS attempts to detect intermediates in the catalytic cycle were unsuccessful, probably because they are transient. Intermediates may be formed in the reaction during the course of the reaction, suggesting that a gas had been produced. A variety of methods were used in attempts to detect H2 during the course of the reaction (colorimetric assays, NMR, and gas chromatography). However, none of these led to H2 detection (see SI Text). The GC analysis showed that small amounts of O2 were present in the head space of reactions despite thorough initial purging with N2 or Ar. No H2O2 was detected in the reaction mixtures by using peroxide test sticks.
increase the levels of ROS inside A549 cancer cells. Under similar conditions, neither of the free ligands Azpy-NMe2 nor Azpy-OH caused any oxidation of GSH to GSSG. Hence, the redox activity of 2-phenylazopyridine ligands depends on being coordinated to ruthenium.

Detection of Reactive Oxygen Species (ROS) in A549 Cancer Cells. The molecular probe 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) is taken up by live cells, is hydrolyzed to 2′,7′-dichlorodihydrofluorescein (DCFH), and in the presence of ROS is oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (19). This probe measures general oxidative stress (20). ROS is oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (19). This probe measures general oxidative stress (20). Fig. 2 shows the increase in fluorescence detected over time after exposure to 25 μM Ru compounds (1, 2, 4, and 5), Ru-A ([((η⁶-bip)Ru(en)Cl]PF₆), Ru-B ([((η⁶-tha)Ru(en)Cl]PF₆), and H₂O₂. For each compound the fluorescence was averaged over 12 wells (n = 12).

Fig. 2. Relative increase in DCF fluorescence detected over time after exposure to 25 μM Ru compounds (1, 2, 4, and 5), Ru-A ([((η⁶-bip)Ru(en)Cl]PF₆), Ru-B ([((η⁶-tha)Ru(en)Cl]PF₆), and H₂O₂.

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Discussion

Cytotoxicity Without Hydrolysis. Many examples are known of metal anticancer complexes containing metal–halide bonds that are activated in cells by hydrolysis and subsequently bind to DNA as a target, for example, PtII diam(m)ine complexes, including the drug cisplatin (21), and diamino RuII arene complexes (22). In contrast, the organometallic iodido RuII arene azopyridine complexes studied in this article are inert toward hydrolysis and yet are still cytotoxic.

For example, complexes 1, 2, 4, and 5 are all resistant to hydrolysis over 24 h at 310 K and at micromolar concentrations and physiological pH. Complexes containing unsubstituted azpy (3 and 6) were not as cytotoxic toward the two cancer cell lines. This low activity can be correlated with their instability in phosphate-buffered solutions (Figs. S3–S5). High cytotoxicity is therefore associated with iodido azopyridine complexes that are highly stable in aqueous media. The corresponding chlorido azpy complexes are much less stable in water, with slow hydrolysis of both Ru–Cl and Ru–arene bonds occurring [t(1/2) 9–21 h (13)], and these complexes are much less cytotoxic (Table 1). Iodide is less electronegative than chloride, behaves more like a simple two-electron donor, and forms stronger bonds to RuII. The increased electron density at the ruthenium center is therefore available for backdonation onto the arene, which also strengthens the RuII–arene bonds. Hydrolysis rates of RuII arenes decrease when a π-acceptor-chelating ligand is incorporated into the complex, as we have discussed elsewhere (13).

The dramatic increase in cytotoxicity of iodido complexes compared with chlorido complexes observed here is not seen for RuII arene complexes containing the σ-donor ethylenediamine as the chelating ligand; compare, for example, [([η⁶-benzene]Ru(η⁶-en)Cl]PF₆, IC₅₀ = 17 μM, [([η⁶-benzene]Ru(η⁶-en)]PF₆, IC₅₀ = 20 μM (12). In the case of ethylenediamine complexes, the Ru–I bonds are converted to Ru–Cl bonds during the cytotoxicity assays because of the large excess of NaCl in the cell culture medium. In contrast, the iodido phenylazopyridine complexes do not appear to react with excess NaCl (see Fig. S5 for 4).

The iodido complexes also react more slowly with GSH than the chlorido complexes. In cells, GSH exerts a major protective role as a detoxifier of electrophilic substances (E), such as metals, forming thioether conjugates GS-E (18). These conjugates are eliminated via an ATP-dependent GS-E pump (23). GS-conjugation is the major deactivation mechanism for platinum complexes such as cisplatin (24). The greater resistance of the Ru–I bond to conjugation may further enhance their potency compared with chlorido complexes.

Ligand-Based Redox Reactions. The coordination of phenylazopyridine ligands to arene-ruthenium centers shifts their reduction potentials to biologically accessible values and results in cytotoxicity. Phenylazopyridine ligands can accept two electrons in one electrochemically accessible, lowest unoccupied molecular orbital, which is primarily azo in character (25). Coordination of these ligands to the dipositive ruthenium center allows ligand-based reduction to occur at a much more positive potential; the first electrochemical reduction for the free ligand azpy-NMe2, for example, occurs at -1.28 V, compared with -0.40 V for complex 1 and -0.36 V for complex 4 (Table 1). Catalytic Activity Toward Glutathione. Ruthenium complexes 4 and 5 oxidize GSH to GSSG catalytically, unlike the free ligands azpy-NMe2 and azpy-OH. Here we have shown that under physiologically relevant conditions micromolar concentrations of organometallic azpy complexes can deplete millimolar amounts of GSH over 24 h by catalytic oxidation to GSSG.

Fig. 3. Percentage cell survival after 24 h exposure to ruthenium compounds at 1 μM (4), or 5 μM (1, 2, and 5), and cisplatin (CDDP) as a control (5 μM) and 96 h recovery for A549 lung cancer cells (lighter) and A549 cells pretreated with 5 mM N-acetylcysteine for 2 h before drug addition to increase intracellular thiol levels (darker). The error bars are standard deviations from an average of three wells.

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A possible catalytic cycle for the conversion of GSH into GSSG is shown in Fig. 4. The initial step involves the conjugation of GSH across the azo double bond and subsequent reduction of the azo group to an hydrazo group with GSH oxidation. Such a process is reported to occur for the azo ester C$_6$H$_5$N=NCOOCH$_3$ (26) and for the diamide (CH$_3$)$_2$NCON=CON(CH$_3$)$_2$ (27). However for these reported reactions, a stoichiometric amount of complex is required, that is, the compounds are not catalytic. Because no H$_2$ was detected during the course of the reaction, it seems likely that the second step in which the azo N=NCOO group is regenerated, and the catalytic cycled is completed, proceeds via concomitant hydrogenation of dissolved O$_2$ to generate H$_2$O$_2$. Given that no H$_2$O$_2$ was detected in the reaction mixture, it is reasonable to propose that iodide (displaced from Ru by the initial substitution reaction with GSH) causes its decomposition (I$^-$/H$_2$O$_2$/H$_2$O$_2^*$/I$^-$/H$_2$O$_2$/H$_2$O; I$^-$/H$_2$O$_2$/H$_2$O$_2^*$/I$^-$/H$_2$O). Such catalytic behavior of iodide toward H$_2$O$_2$ is well known (28) and explains the observed gas bubbles (O$_2$).

Examples in the literature of azo groups facilitating the oxidation of GSH include the Hodgkins and non-Hodgkins lymphoma drug procarbazine [CH$_2$NH–NHCH$_2$C$_6$H$_4$CONHCH$_2$(CH$_2$)$_2$], an hydrazo compound that is easily oxidized to the corresponding azo compound and re-reduced via GSH oxidation to GSSG (29). The reoxidation of this hydrazo group is facilitated by O$_2$ inside cells, which is hydrogenated to form H$_2$O$_2$.

Oxidation of GSH to GSSG was not induced by the free ligands. This is perhaps not surprising because electron-donating groups attached to azo groups (here OH or NMe$_2$) decrease their ability to oxidize GSH (17). Coordination to Ru$^{II}$ is necessary to make the azo nitrogen atoms positive enough for the redox reaction to occur.

**Detection of Reactive Oxygen Species.** The dichlorofluorescein fluorescence experiments showed that the iodido Ru$^{II}$ arene $N,N$-dimethyl- and hydroxy-azopyridine complexes 1, 2, 4, and 5 cause a buildup of reactive oxygen species in A549 lung cancer cells (Fig. 2). Reactive Oxygen Species (ROS) present a higher reactivity than molecular oxygen (30), and encompass not only free radicals such as the superoxide (O$_2^-$), hydroperoxyl radical (HO$_2^*$) and the hydroxyl radical (HO$^*$), but also hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (1O$_2$). ROS are continuously generated in small quantities during normal cellular processes and are involved in several biological functions, such as cell growth and signaling (30). Excessive ROS generation inside cells is harmful, causing oxidation of biomolecules such as lipids, cell membranes, proteins, and DNA, and giving rise to oxidative stress (30). Importantly, no DCF fluorescence was detected for ruthenium arene complexes containing ethylenediamine (en) as chelating ligand, illustrating a fundamental difference in the mechanism of action of these two classes (azpy/en) of cytotoxic ruthenium arene complexes.

To assess the impact of ROS inside A549 lung cancer cells, intracellular thiol levels were increased by adding the antioxidant N-acetyl-l-cysteine, which is believed to act by raising intracellular concentrations of cysteine and hence GSH, and also by direct reaction of its thiol group with ROS (31). Treatment of cells with N-acetyl-l-cysteine increased the survival of cells treated with Ru$^{II}$ arene phenylazopyridine compounds (Fig. 3) providing evidence that ROS are implicated in cell death.
**Oxidative Stress Causes Cell Death.** Oxidative stress is caused by a serious imbalance between the levels of ROS in a cell and its antioxidant defenses in favor of the former (20). On the basis that GSH is the primary cellular antioxidant, it seems likely that the depletion of antioxidants is the primary mechanism by which ROS can be toxic. One such depletion, and by which oxidative stress occurs in the systems studied here. Because cancer cells (mainly multi-drug-resistant cells) are more sensitive to the additional exposure to ROS than normal cells (32), the proposal has been made that ROS can be used therapeutically to treat cancer (27, 31).

Several established anticancer drugs are known to generate ROS or other radicals, including adriamycin (33), arsenic trioxide (34), and cisplatin (35).

**Conclusions**

Organometallic complexes present versatile platforms for drug design. Appropriate choice of the metal, its oxidation state, and the ligands can allow fine-tuning of the steric and electronic properties of the complex and hence its biological activity and mechanism of action. In the present case we have assembled RuII complexes containing a π-bonded aren ligand together with a strongly chelated σ-donor/π-acceptor azopyridine ligand and iodide as a strong monodentate ligand to produce complexes that are relatively inert toward activation by hydrolysis and yet potently cytotoxic toward cancer cells.

These azopyridine complexes undergo activation by reduction. Whereas azopyridine ligands alone are difficult to reduce, the reduction potentials are biologically accessible when the azopyridine is coordinated to RuII. The complexes can induce redox reactions inside cancer cells leading to an increase in the accumulation of reactive oxygen species. Intriguingly iodido RuII arene azopyridine complexes can act as catalysts for the oxidation of the major intracellular reducing agent glutathione to glutathione disulfide, in a cycle that appears to involve ligand-centered reduction of the azo bond by glutathione forming an azo-hydrate intermediate, followed by formation of glutathione disulfide and regeneration of the azo bond. Catalytic drugs with ligand-centered mechanisms might have the advantage of not being so readily poisoned as metal-centered catalysts and therefore have greater potential for biological activity.

**Materials and Methods**

**Materials.** 4-(2-Pyridylazo)-N,N-dimethylaniline (azpy-NMe2), KI, NH4PF6, dichlorodihydrofluorescein diacetate, glutathione, and N-acetylcysteine were purchased from Sigma-Aldrich and ascorbic acid from Alfa Aesar. Methanol was anhydrous (Sigma-Aldrich) or dried over MgSO4, and anhydrous DMF (Aldrich) was used for electrochemical experiments. The starting materials ([η4-arene]RuCl2)2 with [η5-arene]RuCl3 were purchased from Sigma-Aldrich and acetic acid from Alfa Aesar. Methanol was anhydrous (Sigma-Aldrich) or dried over MgSO4, and anhydrous DMF (Aldrich) was used for electrochemical experiments. The starting materials ([η4-arene]RuCl2)2 with [η5-arene]RuCl3 were purchased from Sigma-Aldrich and acetic acid from Alfa Aesar.

**Diffraction.** Data were collected at 150 K. Absorption corrections were applied to the data in SHELXL-97. The H atoms that are part of methyl and hydroxyl groups were located in a difference map, and the groups treated as rotating rigid bodies; other H atoms were placed in calculated positions.

**Electrospray Mass Spectrometry.** ESI-MS was obtained on a Micromas Platform II mass spectrometer and solutions were infused directly. The capillary voltage was 3.5 V and the cone voltage was typically varied between 10 and 45 V. The source temperature depended on the solvent used. Both positive and negative ion modes were used (as appropriate).

**UV-Vis Spectroscopy.** A Perkin-Elmer Lambda-16 UV/Vis spectrophotometer was used with 1-cm path length quartz cuvettes (0.5 ml) and a PTP1 Peltier temperature controller. Spectra were recorded at 310 K and were processed by using UV/Winlab software for Windows 95.

**pH Measurements.** The pH values of aqueous solutions were measured at 298 K by using a Corning pH meter 240 equipped with a Thermo micro combination KCl or KNO3 electrode calibrated with pH 4, pH 7, and pH 10 buffer solutions (Sigma-Aldrich). The pH meter readings for D2O solutions were recorded without correction for the effect of deuterium on the glass electrode, and are termed pHH.

**Cyclic Voltametry and Coulometry.** Electrochemical studies were performed with General Purpose Electrochemical System (GPES) Version 4.5 software connected to an Autolab system containing a PSTAT20 potentiostat. All of the electrochemical techniques used a three-electrode configuration. The reference electrode was Ag/AgCl in a solution of 0.1 M TBA[BF4] in DMF against which E1/2 for the ferrocinium/ferrocene couple was measured to be 0.55 V. The working and counter electrodes were a platinum microdisk (0.5-mm diameter) and a large surface area platinum wire, respectively. Coulometric experiments were performed in a conventional H-type cell by using large surface-area Pt working and counter electrodes. All solutions were purged with dry N2 before electrochemical study.

**High Performance Liquid Chromatography (HPLC).** A Hewlett-Packard series 1100 quaternary pump and a Rheodyne sample injector with a 1.0-ml loop, an HP 1100 series UV/Vis detector, and HP 1100 series Chemstation with an HP enhanced integrator were used. Analytical separations were carried out on a PLRP-S reversed-phase column (250 mm × 7.5 mm, 100 Å, 8-μm, Polymer Labs) with detection at 286 nm. The mobile phases were A, water (for HPLC application, Fisher Chemicals) containing 0.1% TFAH; and B, acetonitrile (for HPLC application, Fisher Chemicals) containing 0.1% TFAH. The flow rate was 3.5 ml/min. The gradient (solvent B) was 10–50% over 30 min, 50–90% to 40 min.

**Gas Chromatography (GC).** A 5-Å molecular sieve column on an Agilent 7890A GC system equipped with a thermal conductivity detector was used in attempts to detect H2 with N2 as a carrier gas (for details and other methods see SI Text).

**NMR Spectroscopy.** NMR spectra were recorded on either a Bruker Avance 600 MHz equipped with either a TCI z- or a TCI z-gradient cryoprobe, a Bruker Avance 800 MHz equipped with a TCI z-gradient cryoprobe, or a Bruker DPX 360 MHz spectrometer equipped with a TCI z-gradient cryoprobe, or a Bruker DPX 360 MHz spectrometer equipped with a TCI z-gradient cryoprobe, or a Bruker Avance 800 MHz equipped with a TCI z-gradient cryoprobe, or a Bruker DPX 360 MHz spectrometer equipped with a TCI z-gradient cryoprobe. The concentration and time previously reported to be nontoxic for this cell line (40), and the full procedure is reported in the ESI Text. The IC50 values were determined on A549 cells with increased thiol levels, cells were preincubated with 5 mM N-acetylcysteine for 2 h, a concentration and time previously reported to be nontoxic for this cell line (40), and confirmed by our controls. The N-acetylcysteine was removed, cells were washed twice in PBS, fresh cell medium was added, and IC50 values were then determined.

**Detection of Reactive Oxygen Species (ROS) in A549 Cancer Cells.** The detection of ROS was based on earlier experimental protocols by Wang and Joseph (19) with some modifications. The full procedure is reported in the SI Text.

**Reactivity of Complexes 1–6 in Buffered Solutions.** Solutions of ruthenium complexes in MeOD were diluted in 10 mM phosphate buffer/D2O to give a final concentration of 100 μM Ru (95% D2O, 5% MeOD) and NMR spectra were recorded at 310 K after 15 min and after 24 h. The pH values of the samples were 7.35 (1), 7.38 (2), 7.38 (3), 7.40 (4), 7.31 (5), and 7.32 (6), and the samples were kept in the water bath at 310 K between NMR acquisitions. After 24 h, ESI/MS was obtained on samples of 1, 2, 4, and 5.

**Substitution of Iodide by Chloride.** Complex 4 was dissolved in water, sonicated for 15 min to ensure dissolution, filtered, and then diluted to ~30 μM. NaCl was added from a concentrated stock solution to give 100 mM NaCl and the
corresponding UV-Vis absorbance spectrum was recorded at hourly intervals over 24 h at 310 K.

UV-Vis Spectroscopy and HPLC of Reactions of Complexes 4 and 6 with GSH. Buffers used were filtered through Chelex-100 to remove iron and digested by using N2/Ar before dissolving GSH. Solutions of GSH were made fresh for each experiment and the pH adjusted to 7 with dilute NaOH/HCl, and the solutions degassed again. Aliquots of complex 4 or 6-C14 in MeOH were added to the solution (final concentrations: 50 µM Ru, 5 mM GSH, 10 mM phosphate buffer; 9.5 % H2O, 5 mM MeOH); the pH values were 7.06 (4) and 7.03 (4-C14) for UV-Vis experiments and 7.88 (4) and 7.89 (4-C14) for HPLC separations.

The rate of substitution of Ru-CIF by GSH was determined by UV-Vis spectroscopy, by fitting the change in absorbance at 597 nm with time (298 K) to the appropriate equation for pseudo-first-order kinetics by using Origin version 7.5 (OriginLab Corporation), to give the observed rate constant (kobs) and the corresponding half-life (t1/2). The extent of substitution of Ru-CIF by GSH was followed by HPLC. The new fraction appearing over time was collected and analyzed by ESI-MS.

Detection of GSH Oxidation to GSSG. Buffers were filtered through Chelex-100 to remove iron and digested by using N2/Ar before dissolving GSH. After dissolution the pH of the GSH solution was adjusted to 7 by addition of dilute NaOH/HCl and solutions were degassed again. Solutions of GSH were made fresh for each experiment and aliquots of complexes 4 or 5 dissolved in 11. Habitrem A, et al. (1983) Chemistry of ruthenium. 12. Reactions of ruthenium (II) organometallic arene complexes in human ovarian cancer. Br J Cancer 102:18269–18274.


ACKNOWLEDGMENTS. We thank Emily Jones and Daniel Cole (Oncosense Ltd.) for assistance with the cytotoxicity tests; Lucy Mooncroft, Paul Murray, Neil Robertson, and Lesley Yellowlees (University of Edinburgh) for assistance with the electrochemistry; Colin Murrell and Gerald Chapman (University of Warwick) for assistance with GC; Martin Wills and David Morris (University of Warwick) for discussions on H2 detection and reaction mechanisms; and also members of COST Action D39 for stimulating discussions. This work was supported by a Biotechnology and Biological Sciences Research Council CASE Award (to S.J.D.) and Oncosense Ltd.