Transfer hydrogenation catalysis in cells as a new approach to anticancer drug design

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Organometallic complexes are effective hydrogenation catalysts for organic reactions. For example, Noyori-type ruthenium complexes catalyse reduction of ketones by transfer of hydride from formate. Here we show that such catalytic reactions can be achieved in cancer cells, offering a new strategy for the design of safe metal-based anticancer drugs. The activity of ruthenium(II) sulfonamido ethylenamine complexes towards human ovarian cancer cells is enhanced by up to 50-fold in the presence of low non-toxic doses of formate. The extent of conversion of coenzyme NAD⁺ to NADH in cells is dependent on formate concentration. This novel reductive stress mechanism of cell death does not involve apoptosis or perturbation of mitochondrial membrane potentials. In contrast, iridium cyclopentadienyl catalysts cause cancer cell death by oxidative stress. Organometallic complexes therefore have an extraordinary ability to modulate the redox status of cancer cells.
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rganometallic complexes are well known as catalysts for organic chemical reactions, for example, olefin metathesis by Grubbs’ ruthenium carbene complexes and asymmetric hydrogenation of ketones by Noyori’s ruthenium arene complexes. The prospect of using organometallic complexes as catalytic drugs is attractive since this might allow low safe non-toxic doses of transition metals to be administered, and furthermore introduce novel mechanisms of action that can overcome resistance to current widely used platinum anticancer drugs. However, achieving catalytic activity in cells is challenging on account of the presence of many nucleophilic biomolecules, which might act as catalyst poisons. Here we show for the first time that Noyori-type ruthenium complexes can catalytically reduce coenzyme NAD in human ovarian cancer cells using non-toxic concentrations of formate as a hydride donor. Moreover, such catalysis greatly enhances the potency of the complexes and increases selectivity towards cancer cells versus normal cells. The mechanism of cancer cell death involves the generation of reductive stress, in contrast to organometallic iridium cyclometalated anticancer catalysts, which induce oxidative stress. It is apparent that organometallic complexes have a unique ability to modulate the redox status of cells.

First we considered the choice of organometallic transfer hydrogenation catalysts for cell studies. Several RuII, RhIII and IrIII complexes have previously been reported to catalyse the regioselective reduction of NAD+ to 1,4-NADH in water, using formate as a hydride source. Stekhan and Fish et al. elucidated the mechanism of reduction of NAD+ using Cp*RhIII bipyridine complexes (formate coordination, loss of CO2, hydride transfer to RhIII then to NAD+ in a weak-association complex). Su¨ss-Fink et al. reported the catalytic reduction of NAD+ using a series of RuII, RhIII and IrIII phanenthrolinyl catalysts. [(Cp*)Rh(phen)Cl]+ exhibits turnover frequencies (TOFs) of up to twice those of [(Cp*)Rh(bipy)Cl]+ in aqueous media. RhIII and RuII catalysts bearing dipirydyl amine ligands functionalized with maleimide are also active, but not as active as (Cp*)Rh(bipy)Cl]+ (ref. 18). In 2012, Hollmann et al. reported a tethered RhIII complex containing an analogue of the Noyori ligand TsDPPN immobilized in a poly(ethylene) polymer. The catalytic activity of the heterogeneous catalyst was lower than that of other soluble RhIII complexes.

Initially, we investigated the use of RuII sulfonamido ethylenamine complexes for catalytic hydrogenation of NAD+ in cancer cells but found them to be less effective than the RuII analogues, which were therefore explored in more detailed studies. We showed previously that the efficiency of [(η6-arene)Ru(R-SO2-En)Cl] catalysts depends on both the nature of the arene and on the sulfonamide substituent (R). For cell work, we compared complexes 1–4 with p-cymene (p-cym) as the arene and the sulfonamide substituent R = methyl (MsEn), 1), p-methylbenzene (TsEn, 2), p-trifluoromethylbenzene (TfEn, 3) and p-nitrobenzene (NbEn, 4), with analogues 5–7 containing o-terphenyl (o-terp) as the arene and R = methyl (MsEn, 5), p-methylbenzene (TsEn, 6), p-trifluoromethylbenzene (TfEn, 7), Fig. 1. The o-terp arene was chosen on account of its increased hydrophobicity (Supplementary Table 1) to enhance uptake into cells. The RuII-ethylenediamine complex 8 was studied for comparison since this compound has poor catalytic activity, but good anticancer activity in vitro and in vivo.

**Results**

Regioselective catalytic reduction of NAD+ (2 mol equiv) to 1,4-NADH was observed in MeOH-d4/D2O (2:9 v/v) and D2O alone using complexes 1–4 and formate as the hydride source (25 mol equiv) by 1H nuclear magnetic resonance (NMR) at 310 K, pH 7.2 ± 0.1. The TOFs for the reactions (Table 1, determined as described in the Methods section) showed a general trend in which the more electron-withdrawing sulfonamides gave higher catalytic activity: NbEn > TfEn > TsEn > MsEn. Such a trend has been previously reported. The complexes studied in the present work show comparable TOFs (ca. 0.2–7 h⁻¹) for the reduction of NAD+ to those reported for [(η6-arene)Ru(N,N)Cl] complexes (ca. 0.006–10 h⁻¹), although much lower than those obtained using RhIII or IrIII complexes.

The catalytic reduction of NAD+ using complexes 5–7 under the same conditions was more rapid, being complete by the time the first 1H NMR spectrum was recorded. The reaction was therefore then carried out using only 6 mol equiv of NAD+, and the catalytic reactions were then complete within the first 10 min (5–7). Subsequently, formation of a brown precipitate was observed and, as a consequence, the TOF of these complexes could not be determined. However, it was apparent that the o-terp complexes were more active as catalysts than the p-cym complexes for which the highest activity was observed for 4, 20.5 min (for completion).

The catalytic mechanism was expected to involve initial binding of formate to Ru followed by transfer of hydride and (irreversible) release of CO2. Evidence for this was provided by complex 5 for which a Ru-H 1H NMR peak was detected at −5.5 p.p.m. (Supplementary Fig. 1) as well as mass spectrometry (MS) peaks assignable to both the formate and hydride adducts (Supplementary Table 2).

We then investigated the antiproliferative activity of complexes 1–7 in A2780 human ovarian cancer cells, and compared them with both complex 8 (RM175) and the clinically approved drug cisplatin. Complexes 1–4 were moderately active with half-maximal inhibitory concentration (IC50) values ranging from 11.9 to 14.7 μM (Fig. 2; Supplementary Table 3), some 6–12 × less potent than complex 8 (2.2 μM) and cisplatin (1.2 μM). The o-terp complexes 5–7 were slightly less active, with IC50 values in the range 12.4–21.2 μM. The potency order in both series is MsEn > TsEn > TfEn, the reverse of the order seen for catalytic activity above. However, no correlation would be expected since these antiproliferative experiments were not done under catalytic conditions.

The most notable difference in the properties of the ethylenediamine complex 8 and the sulfonamido ethylenamine complexes 1–7 is the ability of the sulfonamide substituent to stabilize an adjacent deprotonated Ru-bound N and in turn 16e− intermediates in the catalytic cycle. We thought that this change in electronic distribution would affect DNA binding, which is thought to play a major role in the activity of complex 8. Complex 8 binds strongly to guanine bases in DNA after initial aquation.

1H NMR studies showed that aquation of complexes 1–7 was complete in <5 min at 298 K, and therefore faster and more favourable than for 8. As might be expected from the increased charge density on Ru in the sulfonamide complexes, the pKa values of the aqua adducts of complexes 1–7 were higher (by about two units, range 9.5–9.9, Table 1; Supplementary Fig. 2) than for aquated 8 (pKa 7.7). The sulfonamide complexes would therefore exist mainly as aqua adducts over the pH range used in the present experiments (close to physiological, pH 7).

Perhaps surprisingly, the model base 9-ethylguanine had a similar affinity for complexes 1–4 as complex 8 (as determined by 1H NMR, equilibrium constants 60–105 mM⁻¹ compared with 60 mM⁻¹ for 8, Supplementary Table 4), and binding (to N7, Supplementary Fig. 3) was rapid (pH 7.2, 310 K). The adducts were also characterized by electrospray ionization–MS (Supplementary Table 5). No binding to 9-methyladenine was
no binding of complex 2 (as a representative of the series) to calf thymus DNA was detected by inductively coupled plasma-mass spectrometry (ICP–MS) measurements of bound Ru even after a 27-h incubation at 310 K (mol ratio 1:3 complex:DNA base pairs, 2 mM cacodylate buffer pH 7.4, 2 mM NaCl). The absence of strong binding to calf thymus DNA was also apparent from circular and linear dichroism and melting temperature studies (Supplementary Table 6; Supplementary Figs 4 and 5). This weak binding is probably a consequence of unfavourable steric and electronic interactions between the DNA double helix and the sulfonamido side chain in comparison with the unsubstituted ethylenediamine analogue 8. Hence, we can conclude that DNA is not likely to be a target for the sulfonamide complexes.

Next, we investigated the antiproliferative activity of the complexes towards A2780 human ovarian cancer cells in
were co-administered with different concentrations of sodium formate. (Fig. 2). Cellular accumulation of Ru under similar conditions. (1/3 IC50) and three different concentrations of sodium formate (0.5, 1 and 2 mM). The antiproliferative activity of complexes 1–7 was enhanced by co-administration with formate (Supplementary Tables 7 and 8; Fig. 2), consistent with a direct contribution to the activity from catalytic transfer hydrogenation. The antiproliferative activity of complex 8 was also enhanced by the co-administration of formate, but to a much lower extent (Fig. 2). The largest decrease in cell survival, of ca. 50%, was observed for complexes {[p-cym]Ru(MeEn)Cl} (1) and {[p-terp]Ru(TsEn)Cl} (2), dropping from ca. 69% to 1% when the concentration of formate was increased from 0 to 2 mM (Fig. 2). Complexes containing o-terp also showed a significant enhancement of antiproliferative activity, with complex 7 giving the greatest decrease in cell survival (ca. 16-fold). In contrast, the presence of formate only decreased cell survival induced by complex 8 (RM175) from ca. 70% to 39%.

Next, we investigated whether the formate-induced increase in activity was accompanied by intracellular conversion of NAD⁺ to NADH might occur. First we showed that formate itself at concentrations from 0 to 2 mM is non-toxic to the cells (Fig. 3). Then A2780 cells were co-incubated with equivalent concentrations of complexes 1–8 (1/3 IC50) and three different concentrations of sodium formate (0.5, 1 and 2 mM). The antiproliferative activity of complexes 1–7 was enhanced by co-administration with formate (Supplementary Tables 7 and 8; Fig. 2), consistent with a direct contribution to the activity from catalytic transfer hydrogenation. The antiproliferative activity of complex 8 was also enhanced by the co-administration of formate, but to a much lower extent (Fig. 2). The largest decrease in cell survival, of ca. 50%, was observed for complexes {[p-cym]Ru(MeEn)Cl} (1) and {[p-terp]Ru(TsEn)Cl} (2), dropping from ca. 69% to 1% when the concentration of formate was increased from 0 to 2 mM (Fig. 2). Complexes containing o-terp also showed a significant enhancement of antiproliferative activity, with complex 7 giving the greatest decrease in cell survival (ca. 16-fold). In contrast, the presence of formate only decreased cell survival induced by complex 8 (RM175) from ca. 70% to 39%.

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Cells, and living organisms in general, maintain a tight balance for their redox system by controlling the levels of oxidants and antioxidants. In particular, cancer cells are under constant oxidative stress, and high levels of reactive oxygen species such as OH, O$_2^*$, and H$_2$O$_2$ are common as a result of disturbed mitochondrial function$^{32,33}$. As a consequence, neoplastic tissues are especially sensitive to further changes in the redox balance, for example, those caused by changes in the NAD$^+$/NADH ratio. In contrast, non-cancerous cells have normal-functioning mitochondria that allow them to adjust to changes in their redox balance. This difference provides a strategy for conferring selectivity on anticancer agents that attack cancer cell metabolism$^{34-36}$.

The specific role of formate was confirmed by comparison with acetate. The activity of neither complex 2 nor 8 towards human ovarian cancer cells was affected significantly by acetate under similar conditions (Fig. 2; Supplementary Table 11). Acetate, unlike formate, cannot act as a source of hydride for catalytic hydrogenation reactions.

We then investigated whether complex 2 in combination with formate could convert NAD$^+$ to NADH in A2780 human ovarian cancer cells. In the first experiment, A2780 cells were incubated for 24 h with complex 2 and three different concentrations of sodium formate (0.5, 1 and 2 mM). Neither the complex nor formate alone had a significant effect on the intracellular NAD$^+$/NADH ratio (Fig. 3). However, the NAD$^+$/NADH ratio decreased significantly after co-administration of 2 and increasing amounts of formate (from 4.5 to 1.1 with 2 mM formate, Fig. 3).

An apparent TOF (TOF$_{ap}$) of $0.19 \pm 0.01$ h$^{-1}$ was determined for the conversion of NAD$^+$ to NADH in A2780 cancer cells treated with 4.5 \mu M complex 2 and 2 \mu M sodium formate for various times (0, 2, 4, 12, 18 and 24 h). However, the interpretation of this value is not straightforward since many other processes in cells may influence the concentrations of NAD$^+$ and NADH. The NAD$^+$/NADH ratio decreased rapidly for the first 4 h, from 4.78 ($t=0$ h, untreated) to 0.83 ($t=4$ h), followed by a 4-h period in which the decrease was less pronounced, reaching a final ratio of 0.74 after 12 h (Fig. 3). After 12 h, a slight recovery was observed, with the ratio reaching 0.98 after 24 h. This might be due to gradual poisoning of the catalyst (reaction with other biomolecules) or degradation.

Flow cytometry analysis of A2780 cells exposed to complex 2 and 2 \mu M sodium formate showed that the levels of reactive oxygen species (ROS) in treated cells are comparable to those arising from treatment with N-acetyl-l-cysteine, a well-known reductant (Fig. 4a). The intracellular generation of ruthenium hydride species may lead not only to the reduction of NAD$^+$ (and NADP$^+$), but also to the reduction of other biomolecules such as ketones and imines$^{20,37,38}$.

Induction of apoptosis in A2780 cells exposed to complex 2 and sodium formate was investigated using flow cytometry. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) dual-staining allowed the detection of four different populations: viable cells, non-viable cells and early-stage and late-stage apoptosis. Early-stage apoptosis is characterized by changes in the symmetry of the phospholipid membrane, and late stage by further disruption of the integrity of the cell membrane so it becomes permeable to PI. In the case of cells exposed to complex 2 and 2 \mu M of sodium formate, no population of cells in either of these two stages of apoptosis was detected after 24 h (Fig. 4b), as was the case after treatment with sodium formate alone.

**Figure 3** Perturbation of the NAD$^+$/NADH ratio in A2780 cancer cells exposed to 1/3 × IC$_{50}$ of complex 2 and sodium formate. (a) Variation in the concentration of formate (0, 0.5, 1 and 2 mM) in the absence (left) and presence (right) of complex 2, 24 h exposure. (b) Graph showing the linear correlation between the concentration of sodium formate and the NAD$^+$/NADH ratio. (c) Effect of exposure time (0, 2, 4, 12, 18 and 24 h) on the NAD$^+$/NADH ratio. (d) Variation in the concentration of NAD$^+$ and NADH with time. All the experiments were performed as duplicates of triplicates in independent experiments and the error bars were calculated as the s.d. from the mean. An independent two-sample t-test with unequal variances, Welch’s test, was used to define the statistical difference between the values obtained for the NAD$^+$/NADH ratio experiment. *$P<0.05$, **$P<0.01$. 

![Diagram](image1.png)

![Diagram](image2.png)

![Diagram](image3.png)

![Diagram](image4.png)
We also used flow cytometry to investigate whether exposure of cells to complex 2 and sodium formate induced variations in the mitochondrial membrane potential. The induction of apoptosis is closely related to mitochondrial changes. In the intrinsic pathway of apoptosis activation, changes in the mitochondrial membrane potential allow release of cytochrome c into the cytosol, which promotes the subsequent activation of the caspase cascade 39. No changes in the mitochondrial membrane potential were observed (Fig. 4c). This is consistent with a mechanism of action based on the perturbation of the redox state of cells, and the lack of apoptosis.

In the absence of apoptosis after 24 h of drug exposure, we investigated the induction of necrosis by flow cytometry. A2780 cells exposed to complex 2 in the presence and absence of 2 mM sodium formate tested negative for increased fluorescence of the 7-ADD stain in the FL2-red channel, indicating that, under these experimental conditions, there is no cellular necrosis either (Fig. 4d). Furthermore, the same technique was used to confirm cellular membrane integrity. In this case, cells exposed to complex 2 alone or in combination with 2 mM sodium formate showed low fluorescence in the FL2 channel reading for CytoPainter red, which reacts with exposed amines in compromised membranes (Fig. 4e). Other mechanisms that could be considered in future work include the induction of autophagy 40 and pathogenic mitochondrial oxidation 41, both of which have been linked to reductive stress, but it is also possible that the cell death observed here involves new pathways and cannot be simply mapped onto known mechanisms.

Discussion
It is challenging to design metal complexes that might exhibit catalytic activity in living cells 5–7. Catalysts for chemical transformations work efficiently under well-defined conditions, whereas cells contain a wide range of nucleophiles, as well as oxidants and reductants, which might readily poison the catalyst via substitution or redox reactions. Nevertheless, some success has been reported 42–45. For example, manganese macrocycles such as M40403 can act as superoxide dismutase mimics and decompose superoxide into O2 and H2O2 in cells 46,47. Iron porphyrin complexes can catalyse the reduction of azides to amines 48, and copper peptide complexes can catalyse the degradation of RNA in hepatitis models 42,49. Cobalt complexes, which cleave peptide bonds 50–52, can decompose amyloids present in diseases such as Alzheimer’s or Parkinson’s 53,54. In particular, organoametallic compounds have shown success in the last few years. For example, Ru complexes such as [(η6-arene)Ru(azpy)I]+ (azpy = N,N-dimethylphenyl- or hydroxyphenylazopyridine) catalytically oxidize glutathione (GSH) to glutathione disulfide (GSSG), increasing the levels of ROS in cells 55.

Meggars et al. 56,57 have also shown that organoruthenium complexes such as [(Cp*)Ru(COD)Cl] (COD = cyclooctadiene) or the photoactivatable complex [(Cp*)Ru(η6-pyrene)PF6] can catalyse the cleavage of allylcarbamates from protected imines. More recently, the catalytic cleavage of allylcarbamates in cells has been greatly improved by the use of complexes of the type [(Cp)Ru(QA-R)(η3-allyl)]PF6 (QA = 2-quinolinecarboxylate; R = π-donating groups). The latter complexes can activate protected fluorophores or even protected anticancer drugs in cells 58, an approach that could also provide reduction in toxicity for anticancer drugs. An interesting strategy for avoiding poisoning and increasing the stereoselectivity of organometallic catalysts is to incorporate them in the pocket of a host protein, as illustrated by Ward et al. using biotin-labelled Noyori-type IrIII complexes and streptavidin as the protein host 59.

In this work, we have shown for the first time that Noyori-type catalytic transfer hydrogenation can be achieved in living cells, using RuII arene complexes with a chelated sulfonylethylamine ligand and formate as the hydride donor to convert coenzyme NAD+ into NADH, and thereby modulate the NAD+ /NADH redox couple.

Formate at non-toxic concentrations not only initiates the catalysis, but also enhances the anticancer potency of the
by changes in the NAD

apoptosis, alters the MMP and generates oxidative stress via ROS production. In contrast, the IrIII complex accepts hydride from NADH, causes apoptosis, alters the MMP and generates oxidative stress via ROS production.

In vitro growth inhibition assays. The antiproliferative activity of complexes 1–8 was determined in A2780 ovarian cancer cells and MRC5 fibroblasts. Briefly, 96-well plates were used to seed 5,000 cells per well. The plates were left to pre-incubate with drug-free medium at 310 K for 48 h before adding different concentrations of the compounds to be tested. A drug exposure period of 24 h was allowed. After this, supernatants were removed by suction and each well was washed with PBS. A further 72 h were allowed for the cells to recover in drug-free medium at 310 K. The sulforhodamine B (SRB) assay was used to determine cell viability. IC50 values, as the concentration that causes 50% cell death, were determined as duplicates of triplicates in two independent sets of experiments and their s.d. were calculated.

Co-administration of Ru complexes with formate or acetate. Cell viability assays were carried out with complexes 1–8 in A2780 ovarian cancer cells. These experiments were performed as described above, using a fixed concentration of formate and variable concentrations of the Ru complex. The SRB assay was used to determine cell viability. IC50 values were determined as duplicates of triplicates in two independent sets of experiments and their s.d. were calculated.

Ruthenium accumulation in cancer cells. Briefly, 1.5 × 10^6 cells per well were seeded on a six-well plate. After 24 h of pre-incubation, the complexes were added to give final concentrations equal to IC50/3 and a further 24 h of drug exposure was allowed. After this time, cells were washed, treated with trypsin-EDTA, counted and cell pellets were collected. Each pellet was digested overnight in concentrated nitric acid (73%) at 353 K; the resulting solutions were diluted using double-distilled water to give a final concentration of 5% HNO3 and the amount of Ru taken up by the cells was determined by ICP–MS. These experiments did not include any cell recovery time in drug-free media; they were all carried out as duplicates of triplicates and the s.d. were calculated. This experiment was also carried out using complex 2 and co-administering 2 mM sodium formate.

Ruthenium distribution in cancer cells. Cell pellets were obtained as described above, and were fractionated using the Fraction PREP kit from BioVision according to the supplier’s instructions. Each sample was digested overnight in concentrated nitric acid (73%) and the amount of Ru taken up by the cells was determined by ICP–MS. These experiments were all carried out in triplicate and the s.d. were calculated.

NAD+ \( \rightarrow \) NADH determination. Experiments to determine the NAD+ \( \rightarrow \) NADH ratio in A2780 ovarian cancer cells exposed to complex 2 were carried out using the NAD+ \( \rightarrow \) NADH assay kit from Abcam (ab65348) according to the manufacturer’s instructions. Briefly, A2780 cells were seeded in six-well plates at a density of 10^4 cells per well. After 24 h of pre-incubation, the complexes were added to each well independently, but within 5 min of each other. The antiproliferative activity of complexes 1–8 was determined in A2780 ovarian cancer cells and MRC5 fibroblasts. Briefly, 96-well plates were used to seed 5,000 cells per well. The plates were left to pre-incubate with drug-free medium at 310 K for 48 h before adding different concentrations of the compounds to be tested. A drug exposure period of 24 h was allowed. After this, supernatants were removed by suction and each well was washed with PBS. A further 72 h were allowed for the cells to recover in drug-free medium at 310 K. The sulforhodamine B (SRB) assay was used to determine cell viability. IC50 values, as the concentration that causes 50% cell death, were determined as duplicates of triplicates in two independent sets of experiments and their s.d. were calculated.

Figure 5 | The contrasting mechanisms of action of two classes of organometallic anticancer catalysts. Illustrated is the induction of reductive stress in cancer cells by the RuII arene complexes reported here, and oxidative stress by IrIII cyclopentadienyl complexes. Both processes are mediated by changes in the NAD+ \( \rightarrow \) NADH redox couple. RuII/formate donates hydride to NAD+ \( \rightarrow \), lowers the level of reactive oxygen species (ROS), but does not trigger changes in mitochondrial membrane potential (MMP) nor apoptosis. In contrast, the IrIII complex accepts hydride from NADH, causes apoptosis, alters the MMP and generates oxidative stress via ROS production.
1.0 \times 10^6 cells per well. After 24 h of pre-incubation, cells were treated with a fixed concentration of 2 equal to its I_{50} in co-administration with three different concentrations of sodium formate (0.5, 1, and 2 mM). These concentrations of sodium formate were also used on their own as a second set of negative controls. Drug exposure was allowed for 24 h, after which all supernatants were removed by suction and wells were washed with PBS. Cells were treated with trypsin, detached and counted before being pelleted by centrifugation. Cell pellets were extracted using a total 1/4 NAD+/NADH extraction buffer containing the green fluorescent reagent and filtered using a 10-kDa-molecular weight cutoff filter. Samples were split into two, the first half was used to determine total NAD+ (NAD+ + NADH), and the second half to determine NADH after heating the samples at 333 K for 30 min. The absorbance at 540 nm was normalized against the protein content in each sample determined using the Bradford assay.

**Time dependence of NAD+/NADH ratio.**

Experiments to determine the time dependence of the NAD+/NADH ratio in A2780 ovarian cancer cells exposed to complexes were carried out using the NAD+ /NADH assay kit as described above with the following modifications. The drug exposure time was variable and included time points at 0, 2, 4, 12, 18, and 24 h; the concentration of complex 2 was fixed at I_{50} levels, and the concentration of formate was 2 mM.

**ROS determination.**

Flow cytometry analysis of total induction of ROS in A2780 cells caused by exposure to complex 2 and sodium formate was carried out using the TUNEL Assay Kit (Enzo Life Sciences) according to the manufacturer's instructions. Briefly, 1.0 \times 10^6 A2780 cells per well were seeded in a six-well plate. Cells were pre-incubated in drug-free medium at 310 K for 24 h in a 5% CO2 humidified atmosphere, after which they were exposed to either sodium formate (2 mM), complex 2 (concentration equal to I_{50}) or a combination of both. After 24 h of drug exposure, supernatants were removed by suction and cells were washed and harvested. Staining was achieved by resuspending the cells in buffer containing the green fluorescent reagent. Cells were analysed in a Becton Dickinson FACScan Flow Cytometer using Ex/Em: 490/525 nm for the oxidative stress detection. Data were processed using Flowjo software. The experiment included cells treated with pyocyanin for 30 min as positive control.

**Mitochondrial membrane assay.**

Analysis of the changes of mitochondrial potential in A2780 cells after exposure to complex 2 and sodium formate was carried out using the JC-10 Mitochondrial Membrane Potential Assay Kit (Abcam) according to the manufacturer's instructions. Briefly, 1.0 \times 10^6 A2780 cells per well were seeded in six-well plates (1.0 \times 10^6 cells per well), pre-incubated for 24 h in drug-free media at 310 K, after which they were exposed to either sodium formate (2 mM), complex 2 (concentration equal to I_{50}) or a combination of both in triplicate experiments and the cells were left to incubate for a further 24 h under similar conditions. Supernatants were removed by suction and each well was washed with PBS before detaching the cells using trypsin-EDTA. Staining of the samples was done in flow cytometry tubes protected from light, incubating for 30 min at room temperature. Samples were immediately analysed on a Beckton Dickinson FACScan Flow Cytometer. Samples were split into two, the first half was used to determine ROS in A2780 cells caused by exposure to complex 2 and sodium formate were carried out using the CytoPainter assay (Abcam) according to the manufacturer's instructions. Briefly, A2780 cells were seeded in six-well plates (1.0 \times 10^6 cells per well), pre-incubated for 24 h in drug-free media at 310 K, after which they were exposed to either sodium formate (2 mM), complex 2 (concentration equal to I_{50}) or a combination of both. Cells were harvested using trypsin and stained in the dark using CytoPainter Red (Ex/Emission: 583/603 nm), which reacts with cell surface amines in compromised membranes. After staining, cell pellets were analysed in a Becton Dickinson FACScan Flow Cytometer.

**Mitochondrial membrane integrity.**

Flow cytometry analysis of cellular membrane integrity of A2780 cells caused by exposure to complex 2 and sodium formate were carried out using the CytoPainter assay (Abcam) according to the manufacturer's instructions.

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