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The influence of tumour microenvironmental factors on the efficacy of cisplatin and novel platinum(IV) complexes

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Abstract

The chemotherapeutic drug cisplatin is an important treatment for many types of solid tumours, in particular non-small cell lung cancer (NSCLC). Platinum(IV) complexes offer several advantages to cisplatin due to their requirement for reduction to the active platinum(II) form to elicit cytotoxicity. This should minimise non-specific effects and facilitate higher amounts of the active complexes reaching the target DNA. Hypoxia and a quiescent cell population are features of the tumour microenvironment known to lead to resistance to many chemotherapeutic agents. It is unclear how these microenvironmental factors will impact on the efficacy of novel platinum(IV) complexes. Consequently, the cytotoxicities of several platinum drugs were determined in monolayer and tumour spheroid cultures derived from NSCLC lines. Platinum(IV) reduction potential correlated well with cytotoxicity. The complex containing a chloro axial ligand demonstrated the greatest potency and the drug with the hydroxy ligand was the least effective. Although drug cytotoxicity was not enhanced under hypoxic conditions, both cisplatin and the platinum(IV) complexes retained full potency. In addition, all of the platinum drugs retained the ability to evoke apoptosis in quiescent cells. In summary, unlike many anticancer drugs, the platinum(IV) complexes retain cytotoxic potency under resistance-inducing tumour microenvironmental conditions and warrant further investigation as more selective alternatives to current platinum-based therapy for the treatment of solid tumours.

Keywords: Platinum(IV); Cisplatin; Hypoxia; Tumour microenvironment; Quiescent cells; Solid tumour models

1. Introduction

The platinum(II) drug, cisplatin (*cis*-$[PtCl_2(NH_3)_2]$, Fig. 1) is one of the most important chemotherapeutic drugs in clinical use, demonstrating anticancer activity against a broad range of solid tumours including NSCLC [1]. The mechanism of action of cisplatin remains to be completely elucidated but it is widely accepted that cytotoxicity primarily results from the formation of DNA intrastrand adducts [2,3]. Recognition proteins detect the DNA damage and initiate DNA repair. If the damage is irreparable, cell death is triggered provided that the apoptotic pathway remains intact [4,5].

Toxicity and drug resistance present significant drawbacks to the use of cisplatin in cancer treatment. Toxicities include nephrotoxicity, myelosuppression, ototoxicity, anaphylaxis and peripheral neuropathies [6]. Resistance is considered to be multifactorial and includes reduced intracellular drug accumulation, increased inactivation by glutathione and metallothionein, increased DNA damage repair and alterations in apoptotic pathways [4,5]. The low bioavailability and cellular uptake of cisplatin, combined with the high reactivity of the compound also limit the efficacy of the drug. The highly reactive nature of cisplatin results from rapid hydrolysis of the parent compound to the activated, aquated form $[cis\text{-}[PtCl(H_2O)(NH_3)_2]]^+$ in

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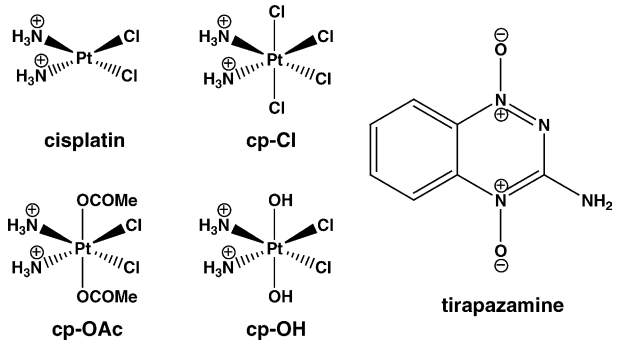

Fig. 1. Chemical structures of cisplatin, platinum(IV) compounds and tirapazamine. Cisplatin: *cis*-$[PtCl_2(NH_3)_2]$; cp-Cl: *cis*-$[PtCl_4(NH_3)_2]$; cp-OAc: *cis*, *trans*, *cis*-$[PtCl_2(OAc)_2(NH_3)_2]$; cp-OH: *cis*, *trans*, *cis*-$[PtCl_2(OH)_2(NH_3)_2]$; tirapazamine: 3-amino-1,2,4-benzotriazine 1,4-dioxide.

biological solutions. As a result, cisplatin reacts with a number of cellular components, such as proteins, RNA, membrane phospholipids and cytoskeletal microfilaments [4], which reduce the pool of drug available to complex with DNA.

Platinum(IV) compounds have great potential as anticancer agents as they have several advantages over platinum(II) drugs. It is generally accepted that platinum(IV) compounds are reduced to the active platinum(II) counterpart prior to reaction with DNA [7]. This should theoretically give an increased opportunity for the complex to arrive at the target DNA intact, reducing non-specific toxicity and increasing the desired activity [8]. The rate of reduction is highly dependent on the nature of the axial groups and to a lesser degree, the non-leaving equatorial moiety [9]. The reduction from platinum(IV) to platinum(II) occurs most readily for complexes with chloro axial groups ($E_p = -260$ mV) followed by those with acetato ligands ($E_p = -635$ mV) and least readily for those with hydroxy groups ($E_p = -880$ mV) (see Fig. 1) [9,10]. A number of biological reducing agents are believed to participate in the conversion of platinum(IV) complexes to the active platinum(II). These include glutathione, L-methionine, L-cysteine, ascorbate, metallothionein and albumin [8].

Given this activation pathway for platinum(IV) complexes, it may be expected that their efficacy will display sensitivity to alterations in cellular status within a solid tumour. Indeed, many forms of drug resistance are directly attributed to the tumour microenvironment [11–13]. For example, hypoxia arises when an aberrant vasculature and poor blood flow leads to an insufficient supply of oxygen to the tumour tissue [14]. In order to survive the reduction in oxygen tension, cells respond to hypoxia in numerous ways, such as the production of angiogenic growth factors [15] and by switching from aerobic to anaerobic metabolism [16]. Much of the cellular hypoxic response is orchestrated by hypoxia-inducible transcription factor 1 (HIF-1), which binds to hypoxia response elements in target genes [17,14].

Hypoxia induces resistance to many classes of anticancer drugs through multiple mechanisms [18]. However, hypoxia could potentially have a negative or positive influence on the efficacy of platinum-based therapy as it decreases cellular glutathione levels [19,20] but increases the production of metallothionein [21] and the activity of DNA-repair enzymes [22]. The effect of hypoxia on the cytotoxicity of cisplatin is a controversial issue with many studies presenting conflicting data [23–25]. The influence of tumour hypoxia on the efficacy of platinum(IV) compounds has not yet been investigated.

Quiescent cells, another feature of solid tumours, pose a significant barrier to successful chemotherapeutic intervention [26]. Growth arrest appears to be a consequence of microenvironmental factors, e.g. hypoxia [27], mediated through expression of cyclin dependent kinase inhibitors $p27^{kip1}$ and $p18^{INK4C}$ [28,29]. The apoptotic effects of the majority of conventional anticancer drugs result from targeting the cellular proliferation machinery [6]. Therefore, quiescent cells have a negative influence on drug efficacy since the chemotherapeutic response is proportional to the proliferating cell fraction of the tumour [30,31]. The drug insensitivity of quiescent cells also leads to the additional problem of regrowth resistance where after treatment the quiescent cells resume proliferation and repopulate the tumour [26,32,12]. However, some reports suggest that cisplatin retains significant potency in quiescent cells [33,34]. The significance of proliferation status regarding the efficacy of platinum(IV) compounds remains to be evaluated.

The aim of the present study was to determine the influence of hypoxia and proliferation status on the efficacy of cisplatin and platinum(IV) complexes in NSCLC cell lines. NSCLC lines can differ considerably with regard to protein expression profiles [35,36] so we chose to use both NCI-H520 and NCI-H226 cells. To evaluate the contribution of multicellular factors on drug potency in hypoxia, both monolayer and tumour spheroid (TS) cultures were utilised. Drug efficacy in the tissue was evaluated by measuring the ability to generate a cytotoxic response. The sensitivity of quiescent monolayers to cisplatin and platinum(IV)-induced apoptosis was also assessed.

2. Materials and methods

2.1. Materials

Methylene Blue, Sulforhodamine B sodium salt (SRB) and fatty acid free BSA were purchased from Sigma, UK. Pimonidazole and the primary pimonidazole antibody (Hypoxyprobe-1 Mab1) were supplied in the Hypoxyprobe-1 kit from Chemicon International Inc. (Temecula, CA, USA). Brij 35 was obtained from Fischer Scientific and Haematoxylin and Aquamount were obtained from VWR International, UK. SeaPlaque® agarose was obtained from Cambrex, UK.

2.2. Synthesis of compounds

cis-$[PtCl_2(NH_3)_2]$ (cisplatin) [37], *cis*-$[PtCl_4(NH_3)_2]$ (cp-Cl), *cis*, *trans*, *cis*-$[PtCl_2(OAc)_2(NH_3)_2]$ (cp-OAc), *cis*, *trans*, *cis*-$[PtCl_2(OH)_2(NH_3)_2]$ (cp-OH) [9] and 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine) [38] were synthesised as reported previously (Fig. 1).

2.3. Cell lines, culture and TS production

NCI-H226 and NCI-H520 human NSCLC cells were obtained from Clare Hall Laboratories, UK. H226 cells were passaged in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and penicillin (100 IU/ml)/streptomycin (100 mg/ml), all were obtained from Life Technologies Ltd., UK. H520 cells were passaged in modified RPMI 1640 medium (1.5 g/l sodium bicarbonate, 10 mM HEPES, 4.5 g/l glucose, 1 mM sodium pyruvate) purchased from ATCC (LGC Promochem, UK), supplemented as above.

TS of H226 cells were grown using the liquid overlay technique in 96-well tissue culture plates. The 96-well plates were given a 100 μl base-coat of 0.75% (w/v) agarose that had been prepared in RPMI 1640 medium (without FBS). Freshly trypsinised H226 cells taken from exponentially growing cultures were overlaid on solid agarose base-coats at a density of 4×10^3 cells/100 μl of RPMI 1640 medium (supplemented as described for monolayers). The cells were kept stationary for 24 h (37°, 5% CO_2) after which the plates were transferred to a Titramax 100 (Heidolph Instruments) and shaken at 300 rpm for a further 24–48 h to allow TS to form.

TS of H520 cells were grown in spinner flasks (Techne, UK). A mother dish was prepared by coating the base of a T75 cm^2 cell culture flask with 20 ml base-coat of 0.75% (w/v) agarose prepared in medium. Exponentially growing H520 cells were added in 20 ml of medium at a density of 1.5×10^6 cells/ml and the flask was left stationary for 24 h. Any large cell aggregates were removed by sedimentation and the remaining small aggregates were transferred to a 500 ml spinner flask in 100 ml of medium. The medium was refreshed (50 ml) every other day and the TS were grown for 7–8 days, at which point they were transferred to the wells of agarose base-coated 96-well plates in 100 μl of medium.

2.4. Hypoxic incubations

Hypoxic incubations were performed in a 3.5 l anaerobic chamber containing an Anaero*Gen*™ sachet (Oxoid, UK). The chamber was placed in an incubator at 37 °C and O_2 levels within the jar were continuously monitored using a calibrated Mini O2DII oxygen sensor (Analox, UK).

2.5. Cytotoxicity measurements in cell monolayers and TS

2.5.1. Cytotoxicity in cell monolayers

H226 and H520 cells were seeded at a density of 10^3 cells/well in 96-well tissue culture plates in 100 μl of the appropriate medium and left to attach for 24 h. Drugs were prepared in medium at twice the desired concentration to give a final concentration range of 1–300 μM and 100 μl aliquots were added to each well. The cells were exposed to hypoxia/normoxia (5% CO_2) for 16 h at 37 °C, the medium was removed and the cells were washed twice with fresh medium and incubated under normoxic conditions for a further 6-day recovery period.

To determine the cytotoxicity of the drugs, the amount of cellular protein was determined using the SRB assay. The medium was removed from the cells and the cells were fixed in 100 μl 10% trichloroacetic acid (TCA) for 30 min at 4 °C. The TCA was removed from the cells and the cells were washed five times with tap water. One hundred microlitres of SRB (0.075% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 15 min. SRB was removed and the plates were washed four times with 1% acetic acid. Plates were air-dried and the SRB-stained monolayers were solubilised in 200 μl of 10 mM Trizma base. Plates were shaken for 10–20 s. and the absorbance measured at 540 nm using a Spectra Max 250 microplate reader (Molecular Devices, UK).

2.5.2. Cytotoxicity in TS

Drugs were added to the TS ($d \sim 350$ μm) and the incubations under hypoxic/normoxic conditions were performed exactly as described for the monolayers above. After the 16 h incubation, individual TS were moved to a new 'uncoated' well in a 48-well plate and 500 μl of fresh medium was added. The TS were incubated for 6 days to allow cellular outgrowth from the tissue. Following the incubation period, the medium was aspirated and replaced with 200 μl 5 g/l methylene blue in methanol to fix and stain the cells. The wells were washed five times with tap water and the radial outgrowth was measured using a graduated microscope eyepiece graticule (Pyser-SGI, UK). The radius of the TS was subtracted from the measurements and the degree of outgrowth was expressed as a percentage of that obtained in the absence of drug treatment. It was not possible to use this assay for H520 TS since during the post-dosage incubation period no radial outgrowth occurred. Therefore, to obtain a measurement of the total number of cells present, the methylene blue stained TS were solubilised in 200 μl of 2% SDS overnight on a shaker at room temperature. A 100 μl aliquot was taken from each well and placed in the well of a 96-well plate and the absorbance was measured at 650 nm using a Spectra Max 250 microplate reader.

2.6. Assessment of drug-induced apoptosis in proliferating and quiescent cells

Apoptosis was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, UK). This is a photometric enzyme-immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after induced cell death. Cells were seeded in 96-well plates at a density of 10^4 cells/well in 100 μl of medium and left for 24 h to attach. For analysis of apoptosis in quiescent cells, after attachment the medium was aspirated, the cells were washed twice in medium containing 0.1% fatty acid free BSA instead of serum and the cells were incubated in 100 μl of this medium for 72 h. Prior to drug addition, the medium was aspirated from the wells. A volume of 200 μl of the platinum drugs was added in medium (without serum) at concentrations of 1, 10 and 100 μM. After 16 h incubation in the presence of drug, the apoptosis assay was performed in accordance with the manufacturers' instructions. The fold elevation in cytoplasmic nucleosomes, compared to untreated cells, was expressed as an enrichment factor. For the assessment of apoptosis in proliferating cells, serum-containing medium was used throughout.

2.7. Histological procedures

TS were harvested, washed in PBS and fixed overnight in neutral buffered formalin (pH 7.0). The TS were transferred to small plastic casting moulds for embedding, allowed to settle and the formalin removed. The moulds were filled with melted 2% (w/v) agarose in 4% (v/v) formaldehyde. The agarose was allowed to set on ice before being removed from the mould and placed in a tissue cassette, which was routinely processed (Histopathology Department, John Radcliffe Hospital, Oxford, UK). The processed, agarose-embedded, TS were embedded in paraffin wax and 5 μm sections were cut. For monolayers, cells were seeded on coverslips placed at the bottom of 6-well plates. Coverslips were removed using a scalpel blade and forceps and the monolayers were fixed in neutral buffered formalin.

2.8. Pimonidazole staining for tissue hypoxia

Prior to exposure to hypoxia, 100 μM of pimonidazole hydrochloride was added to the medium containing the cells or TS. For the TS, processing was carried out as described and sections were completely de-waxed and rehydrated with PBS. Sections were washed with PBS/0.2% Brij 35 and antigen retrieval achieved with 0.01% Pronase at 40 °C for 40 min. Sections were washed with PBS/0.2% Brij 35 at 4 °C between all subsequent steps. Endogenous proxidase activity was inhibited with Peroxidazed 1 (BioCarta, Europe) and the primary antibody (Hypoxyprobe-1 Mab1) was added for 40 min at 25 °C. Mach 2 goat-anti-mouse HRP conjugate (BioCarta, Europe) was added for 45 min and detection was achieved using DAB substrate chromogen (DakoCytomation, UK). Sections were counterstained with haematoxylin and mounted with aquamount.

2.9. Proliferation status of monolayers: BrdU incorporation

The cellular proliferation status of cells grown as monolayers was determined by measurement of BrdU incorporation into DNA using a 5-bromo-2′-deoxy-uridine labelling and detection kit II (Roche Diagnostics, UK). Cells were seeded on coverslips and left to adhere for 24 h. Quiescent cells were generated as described in Section 2.6. The cells were incubated with BrdU (10 μM) for 12 h, washed with PBS and fixed in neutral buffered formalin (pH 7.0). Antigen retrieval was achieved by placing slides in 1 M HCl at 60 °C for 10 min and then washing twice in PBS. Endogenous proxidase activity was inhibited with Peroxidazed 1. The monolayers were washed with PBS and incubated with the monoclonal anti-BrdU antibody Bu20a for 45 min. Mach 2 goat-anti-mouse HRP conjugate was added for 45 min and detection was achieved using DAB substrate chromogen. Sections were counterstained with haematoxylin and mounted with aquamount.

2.10. Data analysis

Data analysis was performed using GraphPad Prism^{TM} software. Drug toxicity in monolayers and TS was quantified using non-linear regression of the general dose–response equation:

$$F = F_{\min} + \frac{F_{\max} - F_{\min}}{1 + 10^{(\log_{10} \mathrm{IC}_{50} - D)}}$$

where F is the viable cell number, $F_{\max}$ is the maximum viable cell number, $F_{\min}$ is the minimum viable cell number, IC_{50} is the drug concentration causing a 50% decrease in cell viability or radial outgrowth and D is the logarithm of the drug concentration.

The viable cell number at each drug concentration was expressed as a percentage of the SRB or methylene blue absorbance (at 540 or 650 nm, respectively) obtained in control monolayer or TS wells in the absence of drug. The multicellular resistance factor (MCRF) refers to the ratio of IC_{50} values obtained in TS versus monolayers for each cell line:

$$\mathrm{MCRF} = \frac{\mathrm{IC}_{50}\,(\mathrm{TS})}{\mathrm{IC}_{50}\,(\mathrm{monolayer})}$$

Statistical comparisons between the mean values of two parameters were done using the Student's t-test. For multiple comparisons, a one-way ANOVA was used with a

Tukey's post hoc test. In both cases, a P-value <0.05 was considered statistically significant.

3. Results

3.1. Drug cytotoxicity in hypoxic monolayers of H520 and H226 cells

The compounds used in this study are shown in Fig. 1. The efficacy of the platinum(II) 'parent' drug cisplatin was compared to a range of platinum(IV) compounds. The hypoxia-selective topoisomerase II poison tirapazamine (TPZ) [39] was utilised as a positive control. Under hypoxic conditions the drug is reduced to a cytotoxic radical but when oxygen is present, the TPZ radical is back-oxidised to the non-toxic parent compound.

For exposure of cells to hypoxia, the Oxoid Anaero*Gen*™ system was employed. The efficiency and reproducibility of this system was evaluated by monitoring the O_2 levels within the jar over a 24-h period. The O_2 levels in the jar dropped rapidly from an initial value of 20.6% to <1% within 25 min and to 0.1% at 45 min (Fig. 2). The O_2 concentration remained stable at 0.1% for the next 3 h (Fig. 2) and for the remainder of the 24 h timecourse (data not shown), at which point the chamber was re-opened. Identical results were obtained for three independent incubations (Fig. 2), demonstrating that this system provided a reproducible hypoxic environment for the subsequent cell incubation experiments. The temperature within the jar did not rise above 37 °C during the incubation despite the reaction between the ascorbic acid component of the Anaero*Gen*™ sachet and O_2 being exothermic (data not shown).

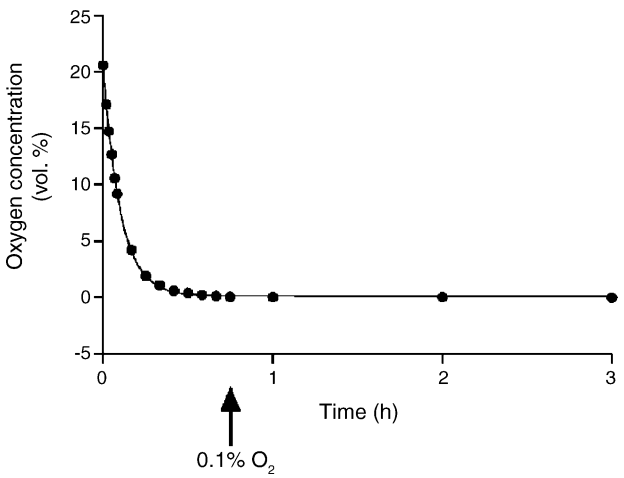

Fig. 2. O_2 levels during hypoxic incubations. The efficiency of the Oxoid Anaero*Gen*™ system to generate a hypoxic environment was evaluated by monitoring the O_2 levels within the chamber over a 24-h period, at 37 °C, using a calibrated Mini O2DII oxygen sensor. The timepoint at which the O_2 level equilibrated to 0.1% is indicated with an arrow. This 0.1% O_2 level was maintained for the full 24 h incubation. Only the first 3 h are shown for clarity. Almost identical results were obtained for three independent incubations (mean shown ±S.E.M., standard errors are within the boundaries of the symbols).

A 16 h incubation period with the test compounds followed by a 6-day recovery period was sufficient to induce considerable cytotoxicity in monolayers of both cell lines (Tables 1 and 2). A general trend observed was that the H226 monolayers (Table 1) were less sensitive to the cytotoxic effects of the drugs than the H520 monolayers (Table 2), as shown by the consistently higher IC_{50} values in the former. This difference was significant for some of the treatments, e.g. cisplatin, cp-OH (normoxia) but not for others (Table 2). Under normoxic conditions, cisplatin and cp-Cl, the most reactive of the platinum(IV) compounds, were equipotent with IC_{50} values of ~1 μM for both cell lines (Tables 1 and 2). The IC_{50} values for the other platinum(IV) drugs, cp-OAc (H226: 6.2 ± 1.1 and H520: 4.4 ± 1.1 μM) and cp-OH (H226: 14 ± 2 and H520: 7.5 ± 0.9 μM) displayed a negative correlation with propensity for reduction. The differences in the potencies between each of platinum(IV) compounds were statistically significant ($P < 0.05$) (Tables 1 and 2).

The comparative cytotoxic efficacy of the platinum drugs was also assessed after an equivalent period of drug exposure in an atmosphere of 0.1% O_2. After the 16 h incubation, positive immunohistochemical staining for cellular pimonidazole adducts confirmed that the H226 monolayers were hypoxic during the incubation period (Fig. 3B). The same result was obtained for H520 monolayers (data not shown). No adducts were detected in cells exposed to pimonidazole under normoxic conditions (Fig. 3A). Hypoxic exposure in the absence of drug did not alter the viability of cells relative to normoxic control cells (data not shown). Exposure of the monolayers to the platinum drugs under hypoxic conditions had no influence on the efficacy of these compounds in either of the cell lines (Tables 1 and 2) as both the extent of cytotoxicity (>85%) and the drug potency were the same in normoxia and hypoxia. In contrast, the efficacy of the positive control drug tirapazamine was significantly increased under hypoxia with a ~10-fold decrease in IC_{50} in both H226 monolayers (43 ± 10 μM versus 5.1 ± 1.0 μM) and H520 monolayers (33 ± 3 μM versus 3.8 ± 1.0 μM) (Tables 1 and 2). A concurrent increase in the extent of cell kill was also observed under hypoxia (H520: 83 ± 1% versus 94 ± 2% and H226: 66 ± 2% versus 98 ± 1% $P < 0.05$).

3.2. Drug cytotoxicity in hypoxic H520 and H226 TS

The 3D cellular organisation of TS provides a diffusional barrier to chemotherapeutic drugs that is absent in monolayers [12]. In addition, the cells within the TS are subjected to microenvironmental stresses and display regional variations in growth and protein expression characteristics [40]. These factors contribute to the decrease in drug efficacy observed for many anticancer drugs in TS, a phenomenon called multicellular resistance (MCR)

Table 1
Efficacy of compounds in normoxic and hypoxic H226 monolayers and TS

Drug	H226							
	Monolayer		TS (methylene blue)		TS (outgrowth)		MCRF	
	Normoxia IC_{50} (μM)	Hypoxia IC_{50} (μM)	Normoxia IC_{50} (μM)	Hypoxia IC_{50} (μM)	Normoxia IC_{50} (μM)	Hypoxia IC_{50} (μM)	Normoxia	Hypoxia
Cisplatin	$1.3 \pm 0.1^{c,d}$	1.5 ± 0.3^{d}	7.4 ± 1.5	5.1 ± 1.1^{d}	$14 \pm 2^{c,d}$	$10 \pm 1^{c,d}$	6.2	3.2
Cp-Cl	$1.3 \pm 0.3^{c,d}$	1.6 ± 0.1^{d}	6.5 ± 1.2	7.8 ± 2.4^{d}	$14 \pm 2^{c,d}$	$14 \pm 1^{c,d}$	5.0	4.7
Cp-OAc	$6.2 \pm 1.1^{a,b,d}$	8.4 ± 3.6	27 ± 5	26 ± 11	$78 \pm 20^{a,b,d}$	$76 \pm 27^{a,b,d}$	4.4	3.1
Cp-OH	$14 \pm 2^{a,b,c}$	$17 \pm 4^{a,b}$	95 ± 45	$102 \pm 27^{a,b}$	$151 \pm 4^{a,b,c}$	$167 \pm 9^{a,b,c}$	7.3	6.0
TPZ	43 ± 10	5.1 ± 1.0	No IC_{50} at max. conc	20 ± 6	No IC_{50} at max. conc	40 ± 3	NA	3.9

H226 cell monolayers or TS were incubated for 16 h under normoxic/hypoxic conditions in the presence of drug. After the incubation period, the monolayers were washed twice and 200 μl of fresh medium was added. TS were moved to uncoated wells of 48-well plates and 500 μl of fresh medium was added. Monolayers or TS were then incubated for a further 6-day recovery period. Drug cytotoxicity was determined in monolayers by staining the remaining cells with SRB and in TS by staining with methylene blue and measuring TS-associated dye or radial outgrowth. IC_{50} and MCRF values were determined from the dose–response curves as described in the materials and methods. Six replicates were performed for each drug concentration with the values obtained from three or more independent observations being shown as mean ± S.E.M. Statistically significant differences in IC_{50} values ($P < 0.05$) between platinum drugs within each treatment group are indicated.

[a] Different to cisplatin.
[b] Different to cp-Cl.
[c] Different to cp-OAc.
[d] Different to cp-OH.

[40,12]. After 16 h exposure of the H226 or H520 TS to the test compounds, the TS were allowed to recover for 6 days. The drug-induced cytotoxicity in TS comprising of H226 cells was determined using the standard outgrowth assay [41]. As mentioned in Section 2.5, an adapted methylene blue assay was required to determine the cytotoxicity in H520 TS. This assay was used in conjunction with the outgrowth assay for H226 TS for comparative purposes.

In the solid tissue environment of the TS, the drug cytoxicities measured under normoxic conditions using the methylene blue assay (Tables 1 and 2) displayed a similar trend to that observed in the monolayers, i.e. decreasing potency with decreasing drug reduction potential (Tables 1 and 2). Both cisplatin (1.1 ± 0.7 μM) and cp-Cl (1.5 ± 0.2 μM), but not cp-OAc, were significantly more potent in the H520 TS compared to cp-OH (21 ± 5 μM) (Table 2). A broadly similar set of results were obtained in the H226 TS (Table 1). As observed in the monolayers, the potency of the platinum(IV) complexes was marginally lower in the H226 TS. There was a significant difference in the IC_{50} values obtained for the two cell lines treated with cisplatin (H226: 7.4 ± 1.5 and H520: 1.1 ± 0.7 μM) or cp-Cl (H226: 6.5 ± 1.2 and H520: 1.5 ± 0.2 μM).

As observed in the cell monolayers, tissue hypoxia had no significant influence on the potencies of the platinum

Table 2
Efficacy of compounds in normoxic and hypoxic H520 monolayers and TS

Drug	H520					
	Monolayer		TS (methylene blue)		MCRF	
	Normoxia IC_{50} (μM)	Hypoxia IC_{50} (μM)	Normoxia IC_{50} (μM)	Hypoxia IC_{50} (μM)	Normoxia	Hypoxia
Cisplatin	$0.9 \pm 0.1^{c,d,*}$	$1.3 \pm 0.2^{c,d}$	$1.1 \pm 0.7^{d,*}$	$0.9 \pm 0.5^{d,*}$	1.2	0.7
Cp-Cl	$0.6 \pm 0.1^{c,d}$	$0.8 \pm 0.1^{c,d,*}$	$1.5 \pm 0.2^{d,*}$	2.7 ± 1.2^{d}	2.5	3.4
Cp-OAc	$4.4 \pm 1.1^{a,b,d}$	$4.9 \pm 0.9^{a,b,d}$	13 ± 5	13 ± 4	2.9	2.7
Cp-OH	$7.5 \pm 0.9^{a,b,c,*}$	$10 \pm 1^{a,b,c}$	$21 \pm 5^{a,b}$	$29 \pm 11^{a,b}$	2.8	2.9
TPZ	33 ± 3	3.8 ± 1.0	No IC_{50} at max. conc	10 ± 1	3.6	2.6

H520 cell monolayers or TS were incubated for 16 h under normoxic/hypoxic conditions in the presence of drug. After the incubation period, the monolayers were washed twice and 200 μl of fresh medium was added. TS were moved to uncoated wells of 48-well plates and 500 μl of fresh medium was added. Monolayers or TS were then incubated for a further 6-day recovery period. Drug cytotoxicity was determined in monolayers by staining the remaining cells with SRB and in TS by staining with methylene blue and measuring TS-associated dye. IC_{50} and MCRF values were determined from the dose–response curves as described in the materials and methods. Six replicates were performed for each drug concentration with the values obtained from three or more independent observations being shown as mean ± S.E.M. Statistically significant differences in IC_{50} values ($P < 0.05$) between platinum drugs within each treatment group are indicated.

[a] Different to cisplatin.
[b] Different to cp-Cl.
[c] Different to cp-OAc.
[d] Different to cp-OH.
[*] Statistically significant differences in IC_{50} values ($P < 0.05$) between the equivalent treatment in H226 monolayers or TS are indicated.

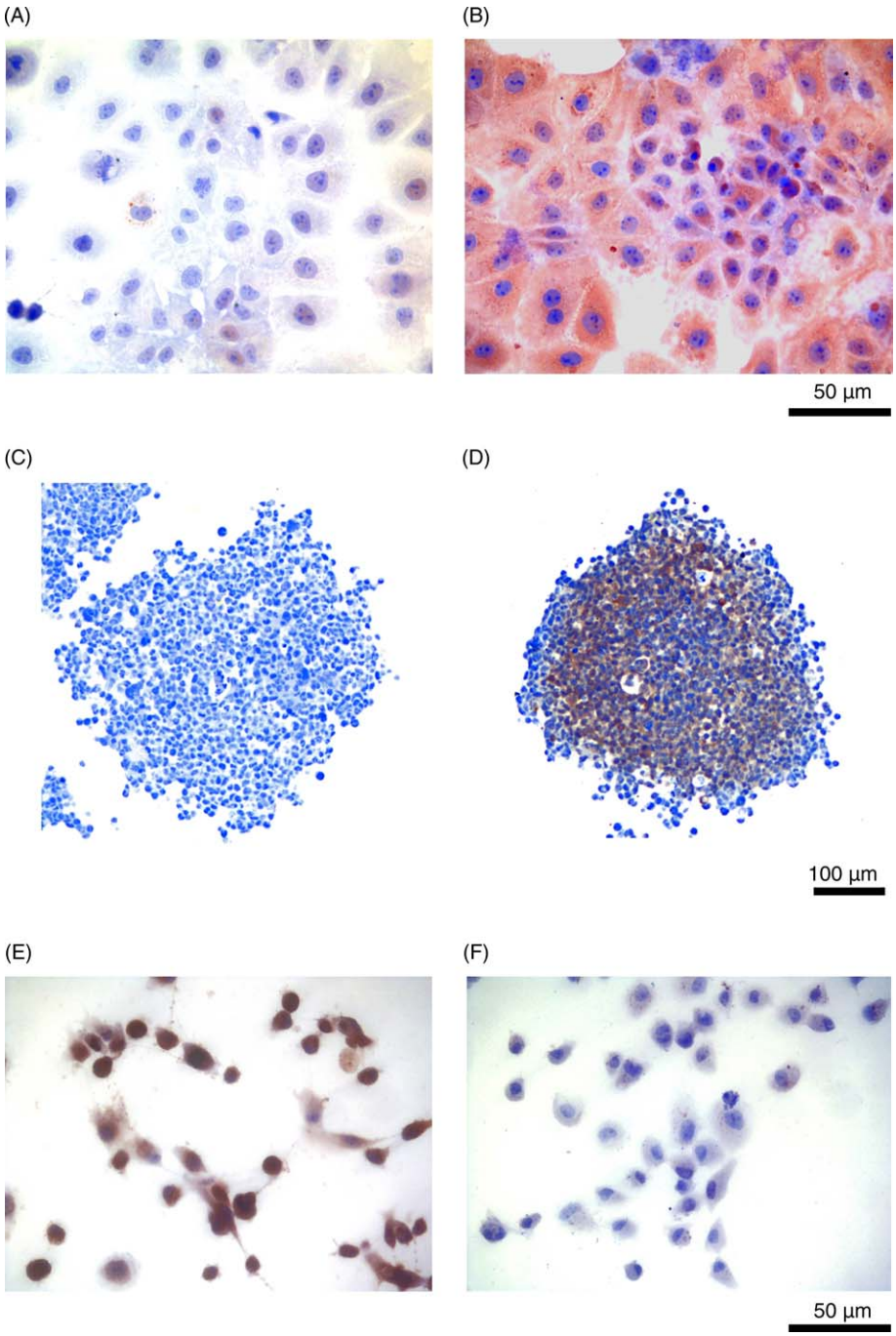

Fig. 3. Immunohistochemical detection of hypoxia and quiescence. Pimonidazole hydrochloride (100 μM) was added to the medium containing the cells or TS prior to a 16 h normoxic or hypoxic incubation. Pimonidazole adducts were detected with the primary antibody Hypoxyprobe-1 Mab1. Sections were counterstained with haematoxylin and mounted with aquamount. (A) H226 monolayer (normoxia); (B) H226 monolayer (hypoxia); (C) H520 TS (normoxia); (D) H520 TS (hypoxia). For assessment of cellular proliferation status, dividing or quiescent cells (generated as described in the text) were incubated with BrdU (10 μM) for 12 h. Detection of BrdU incorporated into DNA was achieved with the monoclonal anti-BrdU antibody Bu20a. (E) Proliferating H226 cells; (F) quiescent H226 cells.

drugs in the TS derived from either cell line. In addition, the extents of cytotoxicity were invariant at ∼100% for each compound. Tirapazamine, on the other hand, produced only partial cell kill under normoxic conditions in both TS types. In contrast, under hypoxic conditions, tirapazamine produced complete cell kill and measurable IC_{50} values were obtained in both H520 TS (10 ± 1 μM) and H226 (20 ± 6 μM). Positive immunohistochemical staining for cellular pimonidazole adducts confirmed that the H520 TS had been hypoxic during the incubation period (Fig. 3D). The same result was obtained for H226 TS (data not shown). No adducts were detected in TS exposed to pimonidazole under normoxic conditions (Fig. 3C).

The outgrowth assay was used as an alternative to the methylene blue assay for assessing cytotoxicity in H226 TS. The outgrowth assay assessed the proliferative capacity of the surviving cell population and the methylene blue assay quantified the total number of cells at the end of the experiment. Therefore, results obtained using the methylene blue assay were influenced by both the initial cell loss and the proliferative capacity of the surviving cells. When the outgrowth assay was used (Table 1), it demonstrated the same relative trend in drug cytotoxicity as obtained using the methylene blue assay (Table 1). However, this method provided greater discrimination of IC_{50} values that were not revealed with the methylene blue assay. In particular, the potencies of cp-OAc and cp-OH were significantly lower than observed for cp-Cl or cisplatin (Table 1). More importantly, hypoxia did not significantly alter the extent or potency of the platinum drugs to elicit cytotoxicity.

The MCRF for a drug reveals how the 3D organisation of the TS impacts on drug efficacy. In general, the drugs displayed higher MCRF values for the H226 cells (Table 1) than for the H520 cells (Table 2). Cisplatin was equally effective in H520 monolayers and TS (MCRF $\cong$ 1) (Table 2) but the H226 TS did exhibit some multicellular resistance (MCR) to cisplatin (MCRF = 6.2 (normoxia) and 3.2 (hypoxia)) (Table 1). The decreased drug sensitivity of the H226 TS and the increased MCR may result from the more compact TS (higher cell density) formed by this cell line in comparison to the H520 cells (experimental observation). Cisplatin is one of the few drugs that does not exhibit significant MCR [12,41], which may be explained by the rapid penetration of the drug through multicellular tissue layers.[1] A small degree of MCR (MCRF varying from 2.5 to 7.3) was observed for the platinum(IV) drugs for both TS types and in both conditions (Tables 1 and 2). Tirapazamine displayed MCR to a small degree in TS of both cell lines.

3.3. Drug efficacy in quiescent NSCLC monolayers

To assess the influence of cellular proliferation status on the efficacy of the platinum drugs, the level of apoptosis evoked in proliferating and quiescent H226 cells was determined by detection of histone-associated-DNA-fragments (mono- and oligonucleosomes). The absence of cell division in quiescent cells prevents the use of standard 'proliferation-based' cytotoxicity assays when examining the effects of drugs on quiescent cell populations. In the proliferating cells, a 16 h incubation with cisplatin, cp-Cl or cp-OAc evoked apoptosis in the H226 cells (Fig. 4A). The cp-OH complex, having the lowest reduction potential, was poor at inducing apoptosis. In all cases, the enrichment in nucleosomal fragments was most dramatic at the highest concentration tested (100 μM), for example a 50-fold elevation was seen for cp-Cl (Fig. 4A). Lower concentrations of drugs (1 and 10 μM) resulted in a much lower level of apoptosis for all of the compounds (Fig. 4A).

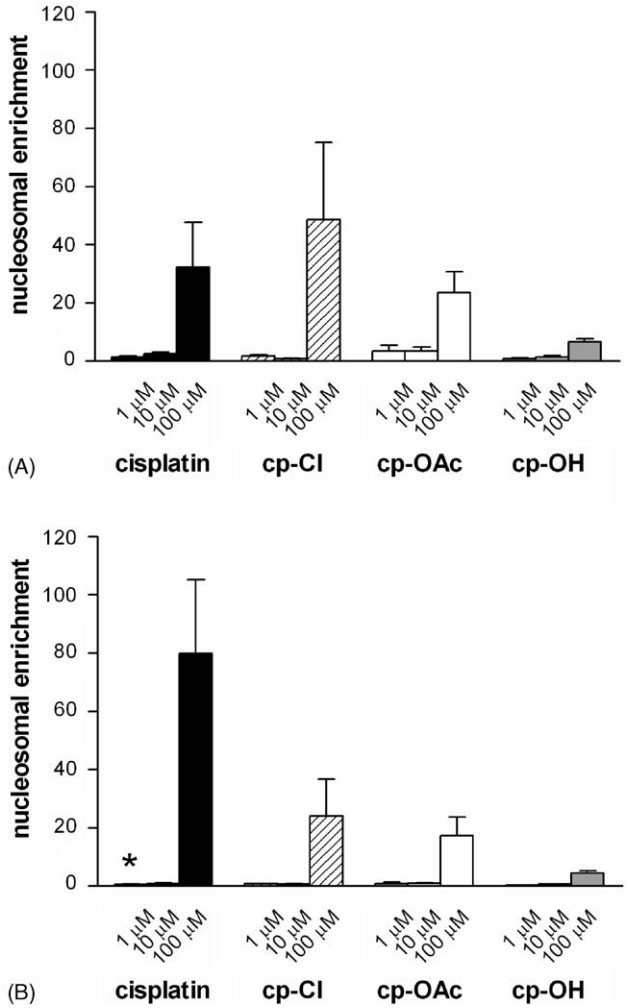

Fig. 4. Drug induced-apoptosis in proliferating and quiescent H226 cells, Cells were seeded in 96-well plates and after attachment, were switched from a proliferating to a quiescent state (as described in the methods). Monolayers were exposed to the appropriate drug for 16 h before cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) were quantified using a photometric enzyme-immunoassay performed in accordance with the manufacturers instructions. (A) Proliferating cells; (B) quiescent cells. * indicates significant difference between (A) and (B) ($P < 0.05$).

In quiescent cells, cisplatin was most effective at inducing apoptosis. There was a positive correlation in producing apoptosis with the drug reduction potential, both cp-Cl and cp-OAc evoked apoptosis less efficiently than cisplatin with the cp-OH being the least effective. As observed in the proliferating cells, the platinum drugs evoked apoptosis more effectively at 100 μM than at 1 or 10 μM as was clear from the cytoplasmic nucleosomal enrichment at this concentration (Fig. 4B). A significant increase in cisplatin-induced apoptosis was observed in proliferating cells over quiescent cells at 1 μM but not at higher drug concentrations (Fig. 4A and B). Importantly, cisplatin and the three platinum(IV) drugs retained the ability to induce apoptosis in quiescent NSCLC cells.

[1] S. Modok (manuscript in preparation).

4. Discussion

In NSCLC tumours, hypoxia is a prominent feature [42] and expression of HIF-1 regulated proteins, such as LDH [43], BNIP3 [44] or carbonic anhydrase IX [45] are associated with a poor prognosis. Many classes of chemotherapeutic drugs produce a decreased response in hypoxic cells (e.g. melphalan, bleomycin, etoposide and doxorubicin) [18], therefore, drugs capable of circumventing hypoxia-induced drug resistance are of considerable interest. Reports describing the influence of hypoxia on the cytotoxicity of the platinum(II) drug cisplatin have been contradictory [23–25]. In the present study, the potency of cisplatin was found to be unchanged by the oxygenation status of the exposed cells, which is in agreement with the findings of Kovacs et al. [24].

The potency of a series of novel platinum(IV) compounds under hypoxic conditions was evaluated for the first time. The requirement for the platinum(IV) complexes to be reduced in order to elicit the cytotoxic effects [7] forms the basis of the reduced non-specific cytotoxicity and improved selectivity of these drugs [8]. This also provides a rationale for testing them under the reducing conditions of a hypoxic tumour microenvironment. However, there was no change in platinum(IV) potency. Intracellular glutathione levels decrease under hypoxia [19,20] but metallothionein is up-regulated [21]. Both of these peptides can independently reduce platinum(IV) complexes to platinum(II) (though metallothionein to a lesser extent) [8] and can also inactivate platinum(II) drugs [5]. It appears that any changes in the expression of these peptides had no direct effect on platinum drug efficacy or resulted in no overall change in activation/inactivation of the complexes.

In addition to being selective for hypoxic cell killing, tirapazamine potentiates the effects of cisplatin under conditions of reduced oxygen [24], so perhaps tirapazamine in combination with a platinum(IV) drug may improve tumour selectivity further by reducing the non-specific toxicities that currently hamper platinum regimes [6].

In addition to regions of hypoxia, NSCLC tumours frequently contain $p27^{kip1}$-positive quiescent cells, particularly in the centre of tumour nodules, often adjacent to regions of necrosis [46]. The inherent insensitivity of quiescent cells to many anticancer drugs [47,33] and the ability of these cells to re-enter the cell cycle and repopulate the tumour after chemotherapy [26,32,12] is also a major treatment barrier.

The present study has shown that cisplatin retains the capacity to induce apoptosis in quiescent cells and the observation that platinum(IV) drugs share this activity may impact on the success of platinum(IV) treatment, which has the potential to provide greater selectivity than cisplatin in NSCLC by limiting non-specific toxicities. The basis for the retention of activity of platinum drugs in the tumour microenvironment is unclear. Many of the factors that contribute to tumour resistance for many drug classes, such as a dependence on oxygen for activity, pH gradients, poor diffusion and reduced cellular proliferation [18] appear to have less impact on platinum-based chemotherapies.

The present study has shown that neither cisplatin nor platinum(IV) compounds demonstrate decreased efficacy under tumour microenvironmental conditions. The fact that the drugs retained full potency in this environment, known to have a negative influence on many classes of anticancer drugs [11,18], suggests that further development of platinum(IV) complexes for the treatment of solid tumours is warranted.

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