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Comparative efficacy of novel platinum(IV) compounds with established chemotherapeutic drugs in solid tumour models

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Abstract

Platinum(II)-based anticancer drugs are associated with high reactivity and thus a poor biological stability. The platinum(IV)-complexes display potential advantages due to their greater stability and bioreductive activation, thereby allowing a greater proportion of the drug to arrive at the target intact. All compounds tested were able to produce cytotoxicity in monolayer cell cultures, however, the potencies of platinum(IV) drugs were lower than that observed for the platinum(II) compounds or established organic chemotherapeutic agents. There was no significant alteration in the potency of platinum(II) or (IV) compounds to produce cytotoxicity in multicellular tumour spheroids (MCTS) compared to monolayer cultures. All the organic and platinum-based cytotoxic agents produced, to varying degrees, either a retardation or reduction in MCTS growth. Proliferating cells were restricted to the outer two to three cellular layers in intermediate ($d = 350$ μm) and large ($d = 600$ μm) MCTS. Regardless of MCTS size, drug treatment produced a larger and more widely distributed proliferating cell population, consistent with the recruitment of quiescent cells to the proliferating pool following cytotoxic damage. Histology indicated that the predominant morphological change was that of apoptosis, although there was some drug-dependent effects such as the metaphase arrest produced by vinblastine and chromatin dispersal to the periphery of nuclei produced by doxorubicin. In summary, whilst the platinum(IV) derivatives were able to produce cytotoxicity via apoptosis, the introduction of a stable axial group significantly retarded the rate at which this occurred.

Keywords: Platinum drugs; Chemotherapy; Solid tumour models; Apoptosis

1. Introduction

Platinum complexes remain the mainstay of a number of chemotherapy strategies and have resulted in significant curative potential in testicular cancer [1]. Yet despite their potency and widespread clinical use [2], there are a number of pitfalls or limitations to platinum complex-based chemotherapy. These limitations are caused by resistance pathways, toxicological profiles and pharmacokinetic considerations. Resistance manifests in a number of forms including reduced cellular accumulation [3,4], inactivation from thiol containing reductants (e.g. glutathione and metallathionein) [5–7] and increased gene specific DNA repair pathways [8]. Toxicological profiles are similar to many anticancer agents, however, the nephrotoxicity of cisplatin remains perhaps the single greatest dose-limiting factor [9]. Low bioavailability, poor cellular uptake and high reactivity towards macromolecules in biological solutions limit platinum-based chemotherapy by affecting the pharmacokinetic parameters. The intracellular reactivity of cisplatin with membrane phospholipids, proteins, cytoskeletal microfilaments and RNA will also reduce the amount of compound available to react with the intended target DNA [10]. A large number of chemical and biological

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Abbreviations: MCTS, multicellular tumour spheroids; Pt(II), planar and four co-ordinate platinum complex; Pt(IV), octahedral and six co-ordinate platinum complex; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; 5-FU, 5-fluorouracil.

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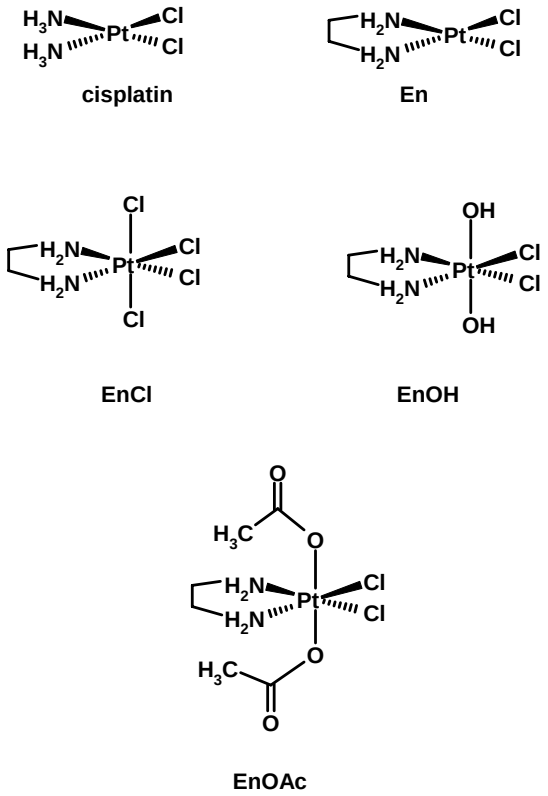


Fig. 1. Chemical structures of the platinum(II) and (IV) compounds. Structures of the Pt(II) and Pt(IV)-complexes used in the investigations (En and OAc correspond to ethane-1,2-diamine and acetato, respectively). En was used as the starting structure for the synthesis of Pt(IV) compounds with varying axial groups. The leaving groups in the active Pt(II)-complex are the chloride ligands. Cisplatin: *cis*-[$PtCl_2(NH_3)_2$]; En: [$PtCl_2$(en)]; EnCl: [$PtCl_4$(en)]; EnOH: *cis*,*trans*-[$PtCl_2(OH)_2$(en)]; EnOAc: *cis*,*trans*-[$PtCl_2(OAc)_2$(en)].

collaborations have thus far failed to generate structural analogues of cisplatin that combine this drugs efficacy, but with greater stability [11].

Of the thousands of complexes synthesised and tested in the pursuit of a successor to cisplatin, the majority have been Pt(II)-complexes (i.e. square planar and four co-ordinate). However, the Pt(IV)-complexes (octahedral, six co-ordinate) offer many potential advantages [12]. As shown in Fig. 1 the Pt(IV)-complexes contain functional groups above and below the plane of the ''equatorial'' leaving groups (e.g. Cl in cisplatin) and will be referred to as the ''axial'' groups. Several investigations have suggested that the anticancer activity of Pt(IV)-complexes requires reduction to the lower oxidation state Pt(II) state (for review see [12]). In fact, the presence of reducing agents such as glutathione, ascorbate and protein sulfhydryls increases the reactivity of Pt(IV)-complexes with DNA [13–15]. The rate at which Pt(IV)-complexes are reduced is critically dependent on the nature of the axial groups and to a lesser extent the non-leaving equatorial moiety. Complexes with chloro axial groups are the most rapidly reduced to the Pt(II)-state, whilst hydroxy groups are the least readily activated group [16]. Thus, by chemical variation of axial groups a number of Pt(IV)-complexes with lowered reactivity may be produced. The stability of such compounds should enable greater delivery to the site of action and prevent unwanted extra-tumour toxicity. In addition, the lipophilicity of the Pt(IV)-complexes may also be altered by axial substitutions to facilitate drug penetration within tumours and uptake into tumour cells. Despite the obvious ability to modify and rationally design Pt(IV)-based compounds, very few have reached the clinical trial stage. The notable exceptions are Iproplatin, Tetraplatin and JM216, which were discontinued due to lower activity compared to cisplatin, neurotoxicity and variable uptake, respectively [12]. All three generated significant promise during *in vitro* studies; for example analogues of JM216 produce cytotoxicity in monolayers with significantly higher potency than cisplatin [3]. Why have these and many other Pt(II) and Pt(IV)-complexes failed the transition from promising activities *in vitro* to anticancer efficacy *in vivo*?

A major limitation is the leap from relatively simplistic monolayer cytotoxicity systems used for preclinical screening to the complex environment of solid tumours. Unlike monolayer cell cultures, solid tumours are characterised by heterogenous cell populations (e.g. proliferating and quiescent), complex three-dimensional (3-D) architecture, high interstitial pressure and a hostile environment with gradients of nutrients, metabolites and cellular redox status [17]. These factors in combination often conspire to reduce chemotherapeutic efficacy by altering intratumour distribution, cellular uptake and drug stability. Prediction of novel drug behaviour in such an environment by extrapolation of pharmacokinetic properties observed in monolayer cultures is difficult and the usefulness of this system has been questioned [18]. Rational design of novel Pt(IV) compounds with ''desirable'' pharmacological properties requires that such factors be taken into account during preclinical evaluation. Many cancer cell lines can be readily grown as 3-D structures, known as MCTS to facilitate this type of evaluation [18,19]. The MCTS model system faithfully reproduces much of the cellular and environmental heterogeneity observed in avascular microregions of solid tumours [20,21], which may be systematically examined for their effects on drug efficacy.

The aim of the present manuscript is to characterise the relative ability of a series of novel Pt(IV)-complexes with varying axial groups to produce cytotoxicity in the MCTS model of solid tumours. In addition, the location and mechanism underlying their activity will also be investigated in this intact model system. The efficacy of these compounds will be compared to their ''parent'' Pt(II)-analogue and the established anticancer drug, cisplatin. Furthermore, the pharmacological spectrum of action will be related to the actions of a variety of organic-based anticancer drugs that produce cytotoxicity through antimetabolite activity (5-FU), DNA alkylation (chlorambucil),

topoisomerase II inhibition (doxorubicin) and mictrotubule disruption (vinblastine).

2. Materials and methods

2.1. Materials

RPMI-1640 medium, penicillin/streptomycin and fetal calf serum were obtained from Life Technologies. Methylthiazoletetrazolium (MTT), methylene blue trihydrate, vinblastine sulphate, doxorubicin hydrochloride, chlorambucil and 5-FU were purchased from Sigma–Aldrich. Gill's haematoxylin, DPX mountant and aquamount were obtained from BDH. The 1% eosin solution was obtained from RA Lamb. The monoclonal antibromodeoxyuridine (Bu20a) was provided by the Leukaemia Research Fund Immunodiagnostics Unit, Nuffield Department of Clinical Laboratory Sciences, University of Oxford.

2.2. Cell lines, culture and MCTS production

DLD-1 human colon carcinoma cells were obtained from Dr. Roger Phillips (University of Bradford, UK) and grown as monolayer cultures in RPMI-1640 medium with glutamax and supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin (100 IU mL^{-1} and 100 mg mL^{-1}, respectively).

MCTS of DLD-1 cells were grown using the liquid overlay technique [22] in 96-well tissue culture plates. The 96-well plates were given a 100 μL base-coat of 0.75% (w/v) agar that had been prepared in RPMI-1640 medium. Freshly trypsinised DLD-1 cells taken from exponentially growing cultures were overlaid on solid agar base-coats at a density of 1×10^3, 4×10^3 or 2×10^4 cells per 200 μL RPMI-1640 medium (supplemented as described for monolayers). The cells were kept still for 24 hr (37°, 5% CO_2) after which the plates were transferred to a Titramax 100 (Heidolph Instruments) and shaken at 300 rpm in a tissue culture incubator. MCTS could be routinely cultured under these conditions for up to 7 days, with replacement of medium at 3-day intervals. Growth of MCTS was assessed by measurement of the tissue diameter using a calibrated eye-piece graticule (100 pts per 10 mm) (Pyser-SGI).

2.3. Synthesis of platinum(II) and (IV)-complexes

Full details describing the synthesis of the platinum(IV)-complexes [$PtCl_2$(en)], [$PtCl_4$(en)], *cis*,*trans*-[$PtCl_2(OAc)_2$-(en)] and *cis*,*trans*-[$PtCl_2(OH)_2$(en)] have previously been described by Ellis *et al.* [16]. The synthesis of cisplatin is based on that previously described by Boreham *et al.* [23]. The chemical structures of each platinum complex used in this investigation are found in Fig. 1.

2.4. Cytotoxicity measurements in cell monolayers and MCTS

DLD-1 cells were seeded at a density of 10^3 cells per well in a 96-well tissue culture plate in 100 μL RPMI-1640 medium and left to attach for 24 hr. Drugs were prepared in medium at twice the desired concentration and 100 μL aliquots added to each well. The cells were incubated in the presence of drug for 72 hr and the number of viable cells determined using the MTT assay. The absorbance of the formazan crystals produced was recorded at 550 nm in a SpectraMAX 250 spectrophotometer (Molecular Devices). Cell viability was determined as the drug concentration (IC_{50} value) that reduced the absorbance to 50% of that in the untreated control group. Platinum derivatives were stored as 50 mM stock solutions in 100 mM KCl and administered to cells in the concentration range 10^{-7} to 3.10^{-4} M. All organic cytotoxic drugs were stored as 50 mM stocks in DMSO and the final solvent concentration added to cells was less than 0.1%. Doxorubicin and vinblastine were added to cells in the range 10^{-12} to 3×10^{-6} M, whilst chlorambucil and 5-FU were used at 10^{-9} to 3×10^{-3} M.

Cytotoxicity of drugs in MCTS was determined by measuring the amount of cellular outgrowth from the tissue following or during drug exposure. MCTS were grown from a starting density of 4×10^3 DLD-1 cells per well of agarose coated 96-well plates for 48 hr prior to drug addition. Drugs were then added to the wells and the MCTS were incubated for a further 72 hr. Following incubation the MCTS were transferred to "uncoated" 96-well tissue culture plates and washed two times in 200 μL drug free medium. The MCTS were incubated for a period of 72 hr to allow cellular outgrowth from the tissue—this procedure is referred to as the "post-dosage" outgrowth assay. An alternative procedure employed involved transfer of the MCTS to 96-well tissue culture plates following the initial 48 hr establishment period. Outgrowth in the presence of cytotoxic drugs was then allowed—this procedure is referred to as the "concomitant" outgrowth assay. Following the incubation period, the medium was aspirated and replaced with 150 μL of 5 g L^{-1} methylene blue in 50% (v/v) methanol to fix and stain cells. The wells were washed three times in phosphate buffered saline and the radial outgrowth measured using a graduated microscope eye-piece graticule. The radius of the MCTS was subtracted from the measurements and the degree of outgrowth was expressed as a percentage of that obtained in the absence of drug treatment. The drug concentrations used were identical to those for the monolayer cytotoxicity assays described above.

Drug effects on the growth of MCTS were achieved by measuring tissue diameter daily using a calibrated microscope eye-piece graticule (100 pts per 10 mm) (Pyser-SGI). MCTS, obtained from a seeding density of 4×10^3 cells per well in a 96-well plate, were established

for 48 hr and drugs incubated over a period of 5 days during which the measurements were taken.

2.5. Routine histological procedures

To enable histological assays, MCTS were harvested, washed in PBS and fixed in neutral buffered formalin (pH 7.0). The MCTS were transferred to cut-off sections of 1 mL round-bottom cryogenic vials (Nalgene), which acted as casting moulds for embedding. The MCTS were allowed to settle, formalin was removed manually and the casting moulds filled with melted 2% (w/v) agar in 4% (v/v) formaldehyde. The agar was allowed to set and subsequently removed from the casting moulds, placed in a tissue culture cassette (RA Lamb) and routinely processed (Histopathology Department, John Radcliffe Hospital, Oxford, UK). The processed MCTS (in agar) were then embedded in paraffin wax, 5 μm sections were cut and haematoxylin and eosin staining of sections was done according to standard protocols [24].

2.6. Proliferation status of MCTS: the BrdU incorporation assay

The cellular proliferation status of cells in MCTS was determined by measurement of 5-bromo-2′-deoxy-uridine (BrdU) incorporation in DNA using a peroxidase-based immunohistochemical assay. MCTS were incubated with BrdU (10 μM) for 12 hr prior to harvesting and histological preparation. The paraffin-embedded tissue sections were completely de-waxed and re-hydrated with PBS. Antigen retrieval was achieved by placing slides in 1 M HCl at 60° for 10 min and then washing twice in PBS. Endogenous peroxidase activity was inhibited with Peroxidazed 1 (BioCarta), washed thoroughly with PBS and covered with the mouse monoclonal anti-BrdU antibody Bu20a solution [25] for 45 min. The goat–anti-mouse, poly-HRP linked secondary antibody MACH2^{TM} (BioCarta) was added for 30 min and detection was achieved using the commercial DAB substrate-chromogen (DAKO).

2.7. Morphological evaluation of cell death (necrosis and apoptosis) in MCTS

For morphological examination, samples of MCTS were treated with various drugs for 0, 1, 12, 24, 48 and 72 hr and then fixed in 4% glutaraldehyde in 0.1 M phosphate buffer. Samples were post-fixed in osmium tetroxide, dehydrated in ethanol, treated with propylene oxide and embedded in Spurr's epoxy resin. For light microscopy, 1 μm sections were stained with Azure A. For electron microscopy, thin sections were cut and stained with uranyl acetate and lead citrate prior to examination in a Jeol 1200EX electron microscope.

The characteristic features of necrosis and apoptosis were identified by electron microscopy. This information was then used to identify necrotic and apoptotic cells in light microscopy sections. This method was used to obtain counts on the number of apoptotic cells (dark staining picnotic cells) per spheroid.

2.8. Data analysis

Drug toxicity in monolayers and MCTS was quantified using non-linear regression of the general dose–response equation [26]:

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

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

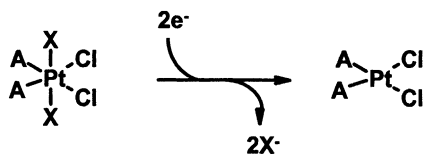
The viable cell number at each drug concentration was expressed as a percentage of the MTT absorbance at 550 nm obtained in the control wells (i.e. no added drug).

Statistical comparisons between mean values of two parameters were done using the Student's *t*-test. Where multiple ($\geq$3) comparisons were done, an ANOVA was used with a Tukey's *post-hoc* test. In either case, a P value <0.05 was considered statistically significant.

3. Results

3.1. Drug cytotoxicity in monolayer cultures of DLD-1 cells

The drugs used in the subsequent investigations were classified as either organic or platinum based on the latter shown in Fig. 1 and further divided according to the platinum oxidation state (II or IV). The "parent" compound for the synthesis of Pt(IV) derivatives is *cis*-[$PtCl_2$(en)] (En), a Pt(II)-complex with a structural analogue of cisplatin (Fig. 1). The difference between the two compounds lies in the ethane bridge between the amines in En. The inert Pt(IV)-complexes are activated upon reduction to the more reactive Pt(II) by endogenous biomolecules:



The propensity for reduction is dependent on the axial (X) group according to the general sequence: Cl > OAc > OH. All of the platinum complexes display neutral charge at physiological pH values. A comparison of the potencies of all the compounds to produce cytotoxicity in a simple monolayer system is shown in Table 1. The organic compounds vinblastine ($\mathrm{IC}_{50} = 1.7 \pm 0.7$ nM) and doxorubicin ($\mathrm{IC}_{50} = 89 \pm 20$ nM) were the most potent drugs tested in DLD-1 monolayer cultures with potencies

Table 1
Cytotoxicity of organic, Pt(II) and Pt(IV) compounds in monolayer and MCTS cultures of DLD-1 cells

Drug class	Specific drug	Monolayer	Spheroids	
			Concomitant	Post-dosage
Organic	Doxorubicin (nM)	89 ± 20	12 ± 4	60 ± 24
	Vinblastine (nM)	1.7 ± 0.7	7.0 ± 3	50 ± 38
	5-FU (μM)	3.5 ± 0.4	1.9 ± 0.7	4.5 ± 3.2
	Chlorambucil (μM)	55 ± 12	1.2 ± 1.1	2.6 ± 2.0
Pt(II)	Cisplatin (μM)	14 ± 4	8.4 ± 2.5	13 ± 5
	En (μM)	9.7 ± 1.8	24 ± 10	12 ± 4
Pt(IV)	EnCl (μM)	42 ± 4	52 ± 9	54 ± 13
	EnOAc (μM)	57 ± 8	75 ± 16	47 ± 9
	EnOH (μM)	26 ± 7	118 ± 31	50 ± 7

DLD-1 cells were grown either as monolayer or MCTS cultures as described in the materials and methods. Monolayers were exposed to various concentrations of drug (1 nM–100 μM) for a 72 hr period. Cytotoxicity in MCTS was determined by an outgrowth assay using one of two different drug exposure protocols: (i) outgrowth measured in the presence of drug for 72 hr—"concomitant", and (ii) a 72 hr incubation in the presence of drug followed by a recovery period to establish outgrowth—"post-dosage". Viable cells were determined by the MTT assay for monolayers whilst outgrowth from MCTS was measured on methylene blue stained cells. The drug potencies were determined by non-linear regression of the dose–response equation and the values represent IC_{50} values (mean ± SEM) from three to five independent observations.

approximately 1000-fold greater than observed for the antimetabolite 5-FU ($IC_{50} = 3.5 \pm 0.4$ μM) and the alkylating agent chlorambucil ($IC_{50} = 55 \pm 12$ μM). The Pt(II) drugs cisplatin ($IC_{50} = 14 \pm 4$ μM) and En ($IC_{50} = 9.7 \pm 1.8$ μM) did not differ in cytotoxic potency; however, their efficacy also lay in the micromolar range. The Pt(IV) compounds displayed significantly lower cytotoxicity than the parent agent En, however, the order of potency to produce cytotoxicity in DLD-1 monolayers (EnOH > EnCl > EnOAc) did not correlate precisely with their propensity for reduction. Determination of log *P* values for the platinum complexes[2] indicates that the order of hydrophobicity (EnOAc > EnCl > cisplatin = En > EnOH) displayed a poor correlation with the potency to produce cytotoxicity in DLD-1 monolayers. Exposure to drugs was standardised to 72 hr in order that Pt(IV) compounds could be allowed sufficient time to be activated.

3.2. Characterisation of MCTS morphology and cellular proliferation status

The MCTS system was used to produce a model for the 3-D cellular organisation inherent to solid tumours. The MCTS were uniform, compact structures and a stable 3-D structure was generated in less than 24 hr. A necrotic core only developed in MCTS of size greater than approximately 400 μm diameter and after culturing for several

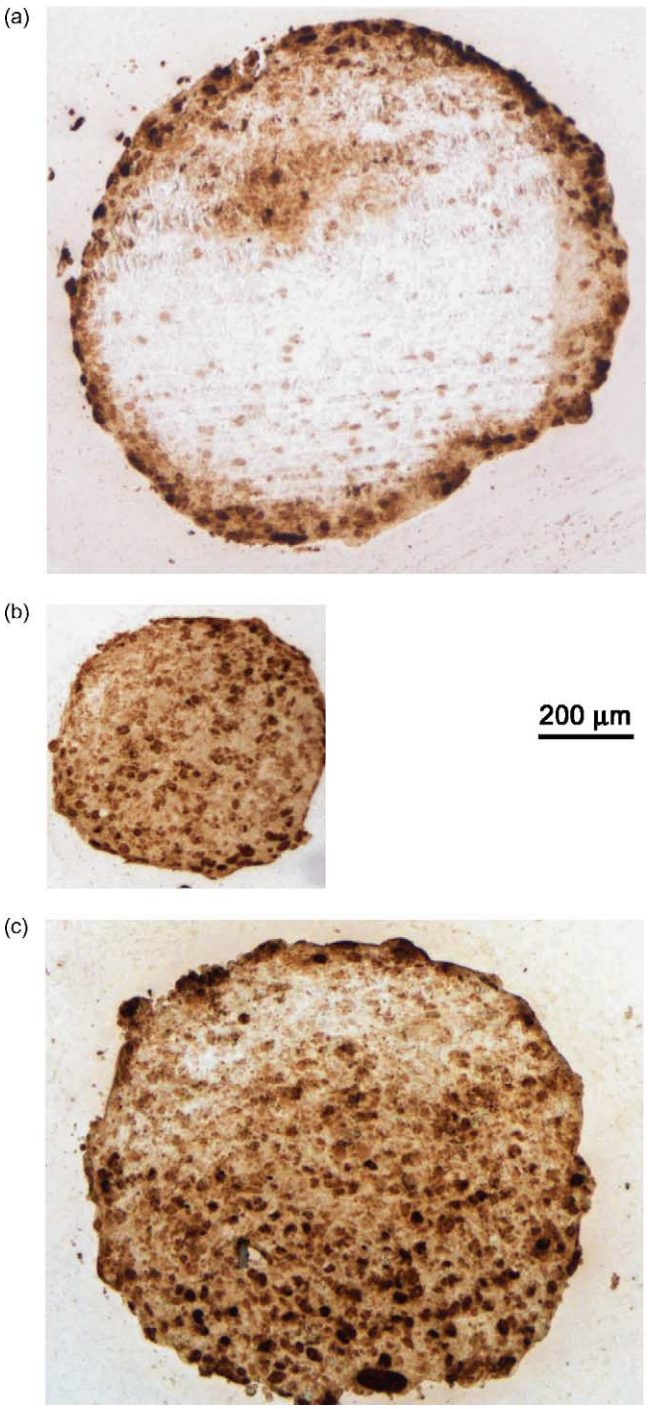


Fig. 2. The effects of anticancer agents on proliferating cell number in MCTS. Intermediate ($d \approx 350$ μm) and large ($d \approx 600$ μm) MCTS were incubated for 72 hr in the presence of organic and Pt-based anticancer drugs. BrdU (10 μM) was added to the MCTS 12 hr prior to harvesting and fixing in formalin. Immunohistochemistry with the Bu20a antibody was used to detect the location of BrdU incorporation into drug-treated spheroids. (a) Untreated large MCTS, (b) intermediate MCTS incubated in the presence of 30 μM cisplatin, (c) large MCTS incubated with 3 μM doxorubicin. The bar corresponds to 200 μm.

days. Proliferating cells are identifiable in the untreated MCTS (Fig. 2a) by the presence of darkly stained nuclei, indicative of incorporation of the thymidine analogue 5′-BrdU into cells during the S-phase of the cell cycle.

[2] Hall M, Amjadi S, Zhang M, Beale P, Hambley T, manuscript in preparation.

These large spheroids displayed proliferating cells restricted to the outer two to three cell layers, indicating that the majority of cells are in a quiescent state, whilst MCTS with diameters of approximately 100 μm displayed proliferating cells throughout (data not shown).

3.3. Cytotoxicity of organic, Pt(II) and Pt(IV) compounds in MCTS cultures of DLD-1 cells

The cytotoxicity of organic and platinum-based drugs in DLD-1 MCTS ($d \approx 350$ μm) was determined by measurement of cellular outgrowth following attachment to tissue culture plates. Similarly to the monolayer cultures, a 72 hr incubation period was chosen to ensure adequate reduction of the Pt(IV) compounds and allow for sufficient drug penetration within the solid tissue for all drugs. Two drug incubation strategies were employed: (i) ''concomitant'', during which the spheroids were incubated with drugs during the 72 hr outgrowth stage or (ii) ''post-dosage'', wherein MCTS were incubated with drugs for 72 hr prior to initiation of the outgrowth phase in the absence of any drug.

Of the organic compounds tested, vinblastine displayed significantly lower potency to produce cytotoxicity in MCTS compared to monolayer cultures, regardless of the incubation strategy (Table 1). This phenomenon has been termed ''multicellular'' resistance, but was not observed for doxorubicin, 5-FU or chlorambucil. In fact, the potency of chlorambucil was actually significantly increased in MCTS compared to monolayer cultures of drug ($P < 0.05$, ANOVA).

The Pt(II) compounds cisplatin and En displayed almost identical potencies to produce cytotoxicity in MCTS and monolayer cultures of DLD-1 cells. In addition, drug administration during or prior to the outgrowth phase did not influence production of cytotoxicity by these two highly reactive platinum species. As expected, the Pt(IV) compounds exhibited lower potency to produce cytotoxicity in MCTS compared to the Pt(II)-complexes. However, a similar lack of difference in potency to elicit cytotoxicity on MCTS outgrowth compared to monolayer growth was observed for the Pt(IV) compounds EnCl and EnOAc. EnOH, the compound with lowest potential for activation to the reactive Pt(II)-species displayed a significantly lower potency in MCTS culture compared with monolayers (Table 1). The IC_{50} value was increased 2-fold from the concentration observed in monolayer culture (26 ± 7 μM) when drug was administered prior to outgrowth (50 ± 7 μM, $P < 0.05$) and 4.5-fold when added during the outgrowth stage (118 ± 31 μM, $P < 0.05$).

3.4. Effects of drugs on MCTS size

The outgrowth assay may be considered a specific measure of effects on the outer proliferative layers of the MCTS. A more global assessment of the cytotoxic efficacy of drugs on MCTS was determined by measurement of solid tissue growth. To achieve this, MCTS were incubated for 5 days in the presence of drug and a daily measurement of diameter undertaken. Untreated MCTS displayed growth during this time period reaching diameters of 480 ± 11 μm, from an initial dimension of $d = 349 \pm 7$ μm (Fig. 3). MCTS incubated with chlorambucil or 5-FU grew in a manner undistinguishable from the untreated controls until days 4 and 5 when the increase in diameter was retarded (Fig. 3a). In contrast, the effects of vinblastine and doxorubicin were apparent after a 48 hr exposure and manifest as a reduction on overall diameter (Fig. 3a), reflecting an increase in cell death and cell shedding from the MCTS. The gross architecture was consistent with this effect and the MCTS displayed a sensitivity to shear forces (data not shown).

Cisplatin caused a marginal initial drop in MCTS diameter over the first 48 hr; however, there was no further alteration in size over the course of measurements (Fig. 3b). En produced a more significant fall in MCTS diameter by 48 hr ($d = 304 \pm 17$ μm, $P < 0.05$ vs. untreated control) followed by recovery to $d = 339 \pm 8$ μm by 96 hr. However, the recovery was not complete and growth appeared to stagnate by the end of the drug incubation period. A significant reduction in growth of MCTS compared to untreated controls was only evident at 72 hr incubation with the most readily reduced Pt(IV)-species, EnCl (Fig. 3c). The effect of this compound was similar to that produced by vinblastine and doxorubicin as evidenced by the continued reduction in diameter. In comparison, EnOH produced a measurable difference from untreated MCTS after 96 hr incubation and EnOAc only after 120 hr (Fig. 3c). It should be noted that a reduction in MCTS diameter following extended incubation in drug is reflective of cell shedding caused by cell death subsequent to the initiation of a cytotoxic response.

3.5. Effects of drugs on BrdU incorporation within MCTS

The vast majority of currently used anticancer drugs interfere with specific aspects of cell replication and thus proliferating cells represent their biological target. As shown in Fig. 2, MCTS with a diameter greater than approximately 300 μm contain a proliferating cellular pool that is largely restricted to the outer layers. What is the consequence to the MCTS of cytotoxic damage produced by anticancer drugs on this sensitive cell population? Figure 2 demonstrates the effects of 72 hr drug treatment on the incorporation of BrdU into large ($d \approx 600$ μm) and intermediate ($d \approx 350$ μm) MCTS. The striking effect of both 10 μM cisplatin and 3 μM doxorubicin was to cause a marked increase in BrdU incorporation as shown in Fig. 2b and c, respectively. In contrast to the profile shown for untreated MCTS, BrdU incorporation is no longer highly localised to cells in the periphery and is clearly visible throughout the entire tissue following a 72 hr incubation

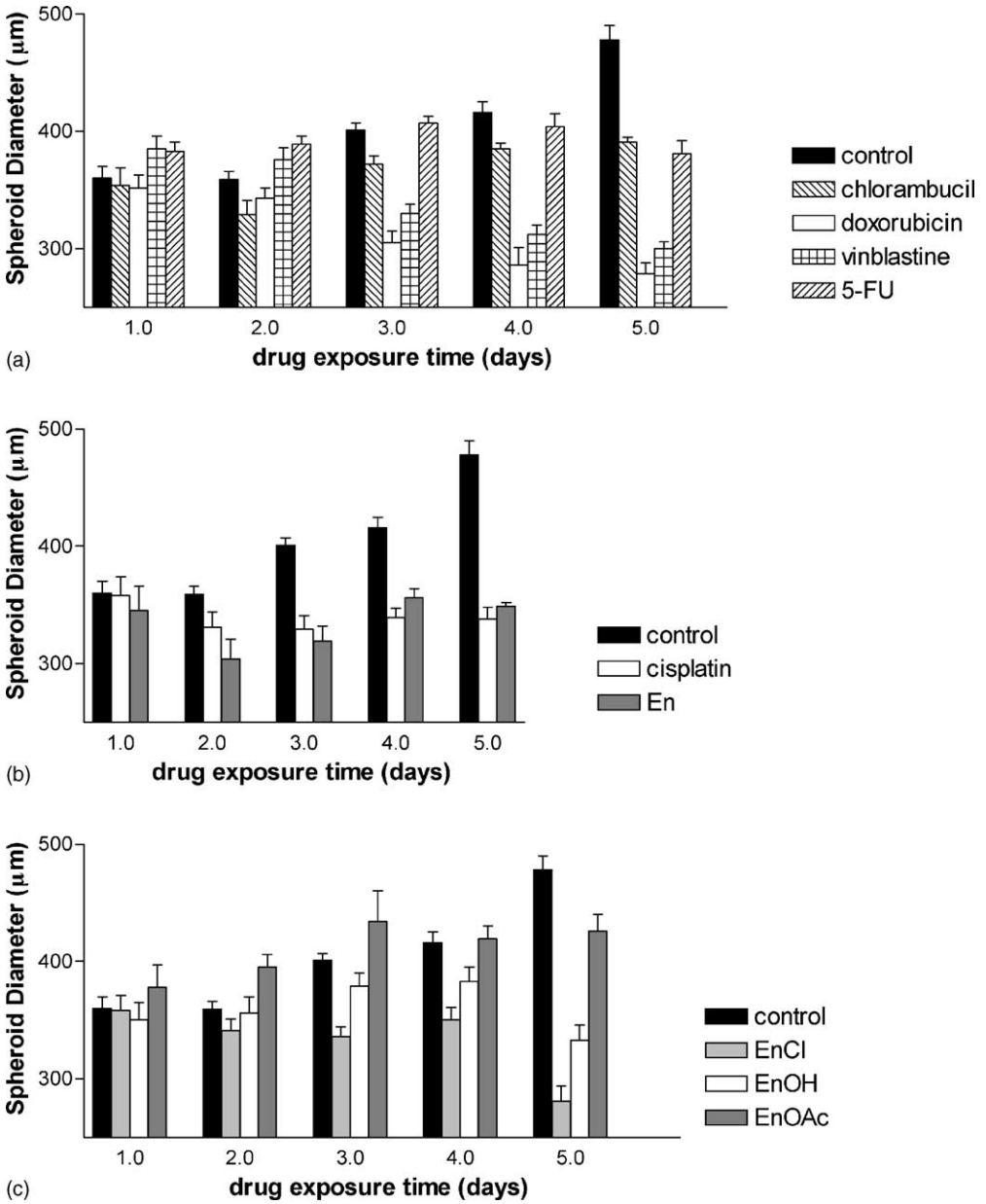


Fig. 3. Effects of organic, Pt(II) and Pt(IV) cytotoxic agents on the growth of MCTS composed of DLD-1 cells. MCTS with a diameter of approximately 350 μm were incubated in the presence of drug for a period of 5 days during which the diameter was measured daily using an eyepiece graticule. (a) Effects of chlorambucil (30 μM), doxorubicin (3 μM), vinblastine (3 μM) and 5-FU (30 μM) on MCTS diameter. (b) The Pt(II) compounds cisplatin and En were added to spheroids at concentrations of 30 μM. (c) The Pt(IV) derivatives EnCl, EnOH and EnOAc were all added at concentrations of 100 μM. The concentrations of all drugs added were based on the minimal amount required to completely inhibit outgrowth. Diameters were measured for 10 independent spheroids and the results are expressed as mean ± SEM.

with drug. An identical effect was observed in both sizes of MCTS for each of the drug classes examined. The increased BrdU incorporation is likely to result from drug-induced recruitment of cells to the proliferating pool and unscheduled DNA synthesis resulting from DNA repair pathways.

3.6. Morphological evaluation of necrosis and apoptosis in MCTS

Initial electron microscopic observations were used to characterise the nature of cell damage in control and treated spheroids. The early stages of apoptosis were identified by peripheral condensation of the chromatin in otherwise intact cells (Fig. 4a). This was followed by fragmentation of the nucleus and cell cytoplasm into a number of apoptotic bodies (Fig. 4b). Many of these appear more electron dense than unaffected neighbouring cells (Fig. 4c). These apoptotic bodies were located between cells but many were phagocytosed by adjacent cells, where the cell fragments underwent degeneration within phagolysosomes (Fig. 4d). In the later stages, numerous apoptotic bodies were observed being released from the surface of

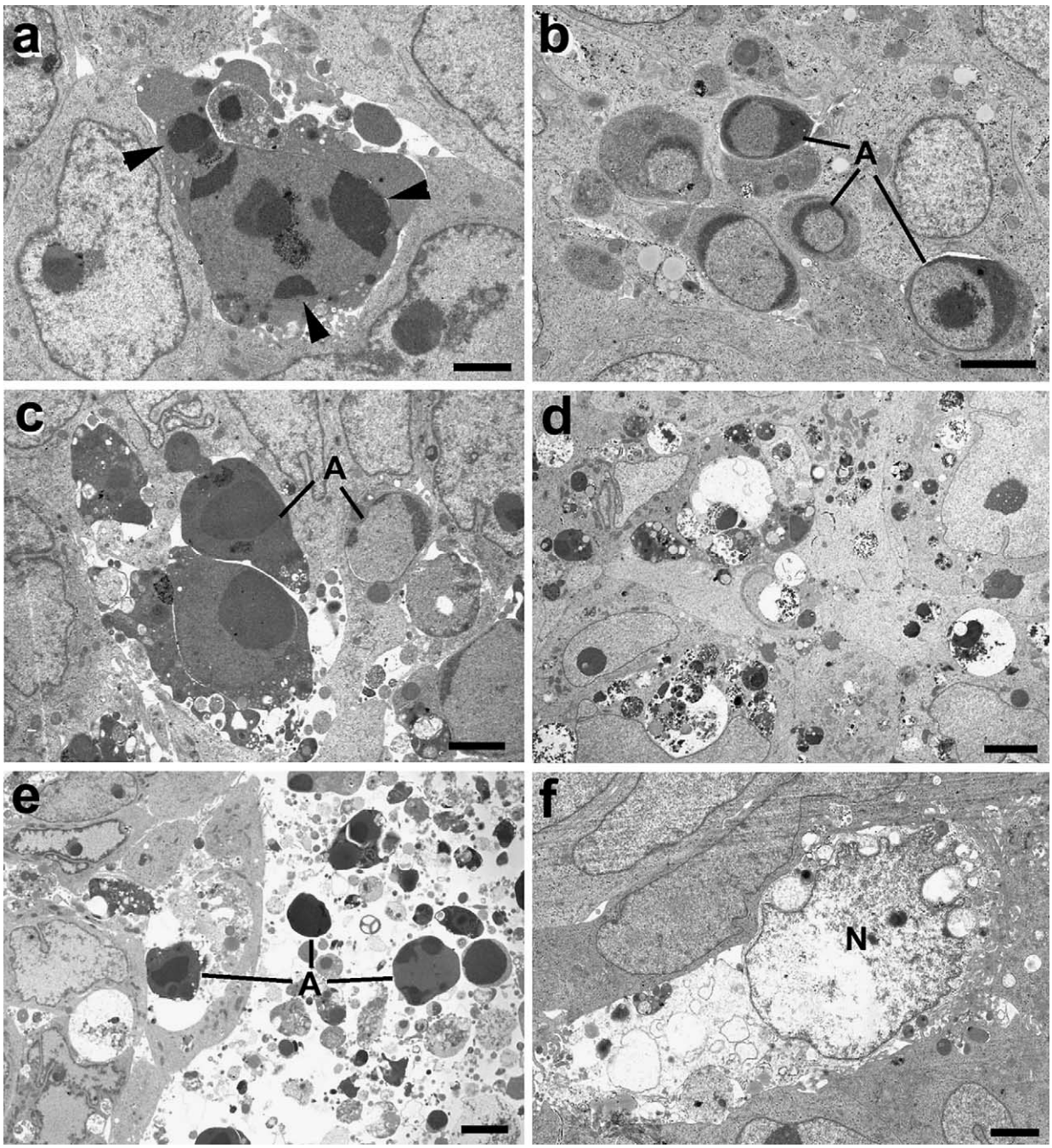


Fig. 4. Electron micrographs illustrating of various stages in cell death in MCTS. (a) Early stage of apoptosis in which the nucleus exhibits large peripheral clumps of chromatin (arrowheads). The cell cytoplasm is intact although the cell is rounding up. The drug treatment was 12 hr incubation with 3 μM cisplatin. Bar: 2 μm. (b) Later stage with the apoptotic cell fragmented into a number of apoptotic bodies (A) many containing nuclear fragments with peripherally condensed chromatin. The MCTS had been incubated with EnCl (30 μM) for 12 hr. Bar: 2 μm. (c) An example showing apoptosis bodies (A) from two cells at slightly different stages. The apoptotic bodies in the centre exhibited increased electron density. The tissue had been incubated for 24 hr in cisplatin (3 μM). Bar: 2 μm. (d) Late stage showing many cell fragments within and between cells. Many of these show evidence cytoplasmic degeneration within phagolysosomes of adjacent intact cells. Images taken following 72 hr incubation with 30 μM EnCl. Bar: 5 μm. (e) Part of the periphery of a spheroid at a late stage showing numerous apoptotic bodies (A) being released from the surface and also be between intact cells. The drug treatment was 48 hr incubation with 3 μM cisplatin. Bar: 5 μm. (f) A cell undergoing necrosis is illustrated in which the lucent cytoplasm is associated with degeneration of the organelles. The intact nucleus (N) is also lucent and lacks the peripherally condensed chromatin characteristic of apoptosis. The MCTS had been treated with EnCl (30 μM) for 24 hr. Bar: 2 μm.

the MCTS (Fig. 4e). Very few cells appeared to undergo necrosis characterised by the loss of cytoplasmic integrity in the absence of nuclear changes (Fig. 4f). While the incidence of apoptosis varied between samples the process was similar in all instances.

Two of the drugs produced unique morphological changes that have not previously been reported. In spheroids treated with doxorubicin there were unique changes in the nuclear morphology that exhibited a progression from the outer cells to the centre of the spheroid. There were

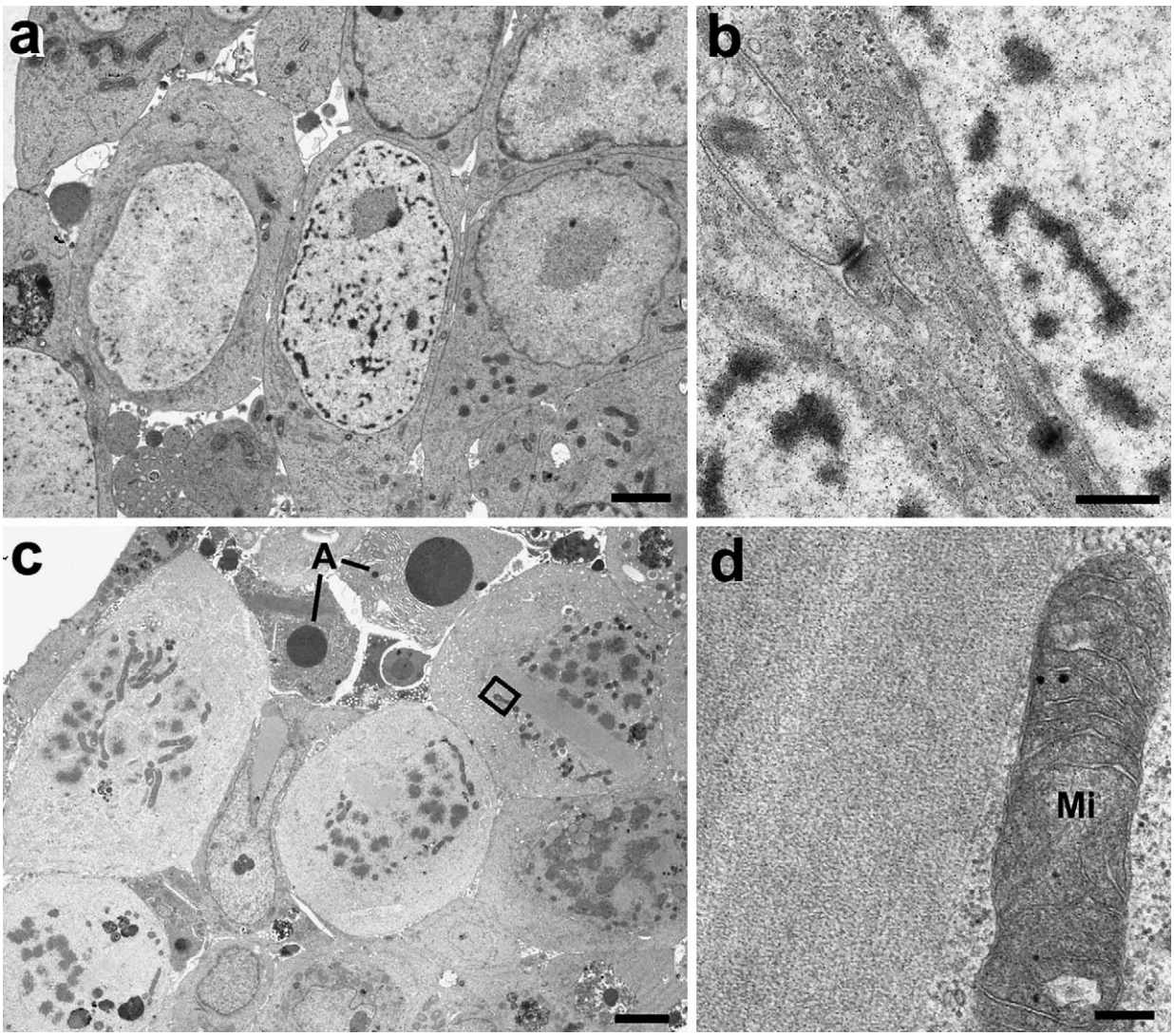


Fig. 5. Morphological alterations caused by vinblastine and doxorubicin in MCTS: electron microscopy. (a) Part of the periphery of spheroid treated with doxorubicin (3 μM) for 12 hr showing the progressive changes in nuclear morphology. The nucleus of the outer cells is electron lucent with little chromatin while the inner cell nucleus shows small clumps of dispersed chromatin typical of an interphase nucleus. The cell in the centre exhibits electron dense strands of material some of which forms a layer close to the nuclear membrane. Bar: 2 μm. (b) High magnification image (from panel a) showing the electron dense strand of material, which is not in contact with the intact nuclear membrane. In addition the cytoplasmic organelles and cell junctions are intact. Bar: 500 nm. (c) Part of a spheroid treated with (3 μM) vinblastine for 24 hr showing a number of cells in mitosis identified by the randomly orientated chromosomes and two apoptotic bodies. Bar: 5 μm. (d) Detail of the enclosed area in Fig 6c showing part of a large protein crystal and a mitochondrion. Bar: 200 nm.

changes in the electron dense chromatin distribution within the nuclei, (Fig. 5a). In many of the nuclei thin strands of electron dense material form toward the periphery of the nucleus but do not contact the nuclear membrane (Fig. 5b). These changes are totally different from those associated with apoptosis (Fig. 4a–e) and appeared to persist. It was not clear that these cells progressed directly to apoptosis. In the case of vinblastine there was a marked increase in the number of cells observed in mitosis. These were observed to contain randomly orientated chromosomes and lacked centrioles and the nuclear spindle (Fig. 5a). Many of these cells contained large protein crystals (Fig. 5a and b).

The light microscopy of the 1 μm sections gives improved resolution, which assists in the identification of both mitotic and apoptotic cells within the mass of interphase cells. In the control spheroids, the cells formed a tight mass of interconnected cells (Fig. 6a). The vast majority of cells were in interphase with only a few cells undergoing mitosis (3–5 mitotic cells per spheroid) and rare cells undergoing apoptosis (1–2 cells per spheroid). The mitotic and apoptotic cells were identified by the characteristic nuclear changes confirmed by electron microscopy. In treated spheroids, the incidence of apoptosis varied with duration of treatment and drug used. Applying the morphological criteria identified by electron microscopy it was possible to quantify the number of apoptotic cells per spheroid (Fig. 6b and c). In the early stages, when there were relatively few apoptotic cells (Fig. 6b), it is possible to accurately count the cells but in the later stages due to the fragmentation, degeneration

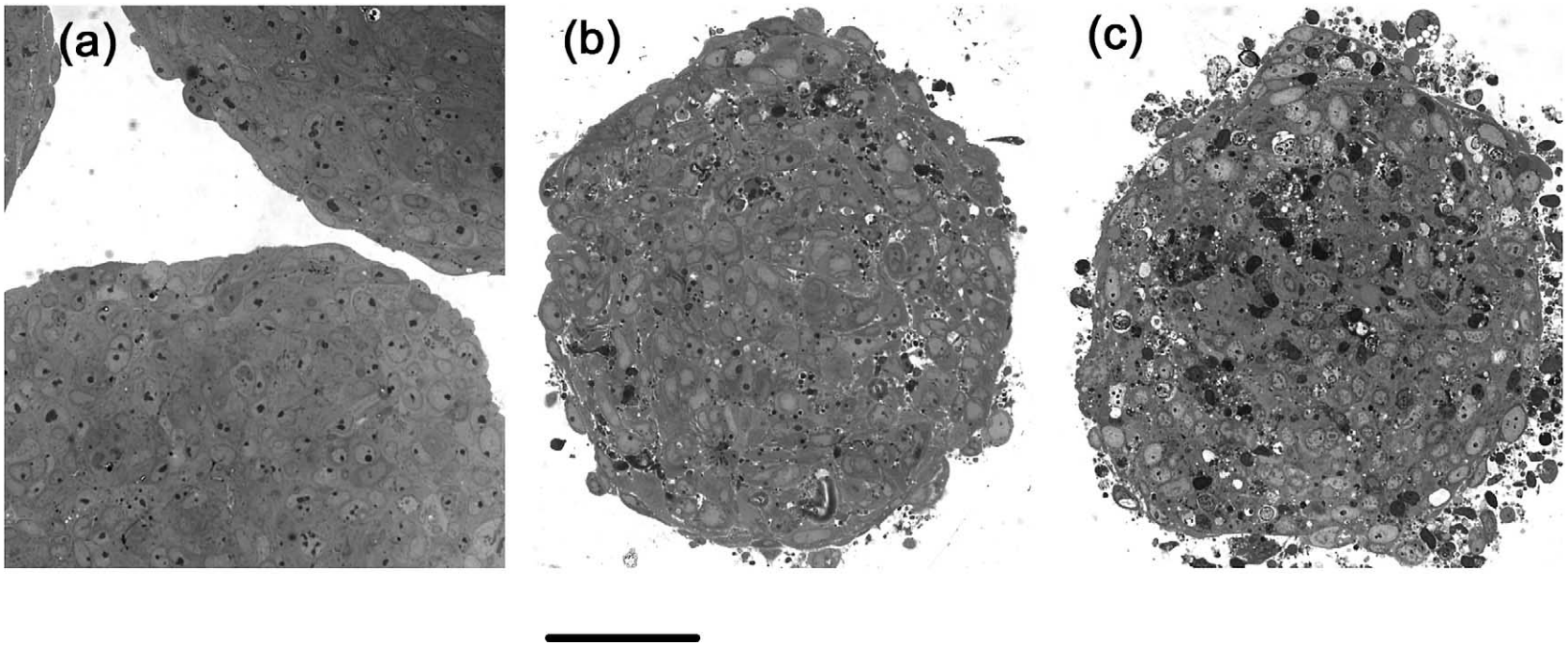


Fig. 6. Effects of organic anticancer drugs on cellular morphology in MCTS. (a) Control, untreated intermediate MCTS. The tissue is well compacted with no obvious apoptotic cells or regions of necrosis. (b) Morphology of MCTS that had been treated with cisplatin (10 μM) for 24 hr. Apoptotic cells had appeared, but were mainly localised to the periphery. (c) MCTS that had been incubated with 3 μM with doxorubicin for 72 hr. The appearance of apoptosis is widespread and necrotic regions are located at the periphery. MCTS were grown subsequent to seeding at a density of 4×10^3 cells per well of a 96-well plate. The MCTS were harvested, fixed in 4% glutaraldehyde, embedded in epoxy resin and 1 μm sections cut. Cellular morphology was stained using Azure A and nuclei stain dark. The bar corresponds to 100 μm.

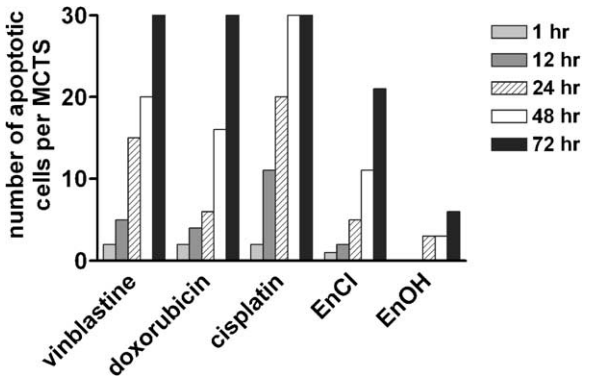


Fig. 7. Time-course of apoptosis progression in MCTS. Apoptotic cells were counted in sections of MCTS for 1, 12, 24, 48 and 72 hr of incubation with vinblastine (3 μM), doxorubicin (3 μM), cisplatin (30 μM), EnCl (100 μM) and EnOH (100 μM). Samples with extensive apoptosis, where quantitation was difficult were assigned a value of 30+ per spheroid.

and loss of apoptotic cells from the surface it was impossible to obtain accurate counts (Fig. 6c). These samples were identified as 30+ apoptotic cells per spheroid and the overall results quantified in Fig. 7.

Following 1 hr incubation the general appearances of MCTS where similar to that described for the control samples, irrespective of the drug used. The incidence of mitotic and apoptotic cells was also similar (3–5 mitotic and 2–4 apoptotic cells per spheroid). Following 12 hr of treatment, there were minor differences depending on the drug used. In MCTS treated with either cisplatin or doxorubicin, no mitotic cells were observed and there was a slight increased incidence of apoptosis (4–11 apoptotic cells per section). Vinblastine treatment produced a marked increase in the number of mitotic cells with a significant proportion of the cells observed in metaphase (33 mitotic cells per section). However, the number of apoptotic cells was unchanged at approximately 5 apoptotic cells per section. In MCTS treated with EnOH and EnCl, the general appearance at this stage of drug incubation and the incidence of mitotic and apoptotic cells was similar to that of the untreated controls.

There were more marked differences between the drugs used following a 24 hr drug treatment. In the case of cisplatin, there was an increased incidence of apoptosis (Figs. 6b and 7). At this point many of the apoptotic cells have fragmented into apoptotic bodies. By light microscopy, these appear as dense staining structures between and within the intact cells. There was no evidence of cells undergoing necrosis. In spheroids treated with doxorubicin, the appearances were similar to that observed at 12 hr with a small increase in apoptosis. However, the number of cells displaying distinctive nuclear changes was increased. In the case of vinblastine, there was a further marginal increase in the number of mitotic cells (38 per section) from that observed at 12 hr. There was also an increase in the number of apoptotic cells (15 per section). With EnOH and EnCl only minor differences were observed in the incidence of mitosis and apoptosis within the spheroids (Fig. 7).

Following 48 hr incubation, there was a marked increase in the number of apoptotic bodies in MCTS treated with cisplatin. The majority of viable cells contained apoptotic bodies and the spheroids were characterised by numerous dense cell fragments. Due to the fragmentation into apoptotic bodies it was difficult to calculate the exact number of apoptotic cells. A number of these show cytoplasmic breakdown similar to that seen in necrosis and late stage apoptosis. However, there were only rare examples of cells showing the characteristic features of necrosis. The major change in doxorubicin-treated cells was an increase in the number of apoptotic cells (Fig. 7). In the vinblastine-treated spheroids, the proportion of mitotic cells was further increased, as was the number of apoptotic cells and bodies. In the case of EnOH, there was still relatively little change in the morphological appearances by light or electron microscopy. However, for EnCl, there was a slight increase in the incidence of apoptosis (Fig. 7), to levels observed for cisplatin after 12 hr.

By 72 hr treatment, the effects for most drugs were quite dramatic. In the cisplatin, doxorubicin and vinblastine-treated MCTS; there was evidence of a reduction in size of the spheroids with a number of apoptotic bodies and a loss of the tight inner cellular connections (Fig. 6c). In addition, cells at the periphery appeared only loosely associated with the tissue and indicate that the decrease in MCTS volumes (Fig. 3) was due to shedding of cells. Samples at this stage of drug incubation were graded has having 30+ apoptotic bodies. Analysis using electron microscopy identified that many of the apoptotic bodies were within phagolysosomes and undergoing end stage degeneration. In the case of EnOH there was relatively little change from the controls except for a minor increase in the number of apoptotic cells (6 per spheroid). In contrast the samples treated with EnCl showed a marked increase in the number of apoptotic cells (20+ per spheroid), which was approaching the level seen in the other drug treatments. The effects of EnOAc were essentially similar to observations with EnOH.

In conclusion, cisplatin and vinblastine produced very marked cell death by 48 hr whereas changes produced by doxorubicin and EnCl reached a peak at 72 hr. The drugs EnOH and EnOAc were less effective, with little evidence of cell death even at 72 hr incubation, although mitosis had been inhibited. The majority of cell death, irrespective of the drug used was by apoptosis with no evidence of wide spread necrosis.

4. Discussion

The six co-ordinate Pt(IV)-complexes offer several benefits over the more frequently used Pt(II) agents [3,12,27] including increased stability in biological media which potentially causes less unwanted side-effects and

facilitates delivery of ''unreacted'' compound to the tumour site. The Pt(IV) pro-drugs are bioreductively activated to the Pt(II)-species by many intracellular molecules including glutathione [12]. In the present study the ability of several novel Pt(IV)-complexes to produce cytotoxicity has been characterised and compared to several established anticancer drugs including cisplatin. The axial groups were chosen in order to produce compounds with low, intermediate and high propensity for reduction to the active Pt(II)-species. Each of the Pt(IV)-complexes produced cytotoxicity in a colonic cancer cell line (DLD-1), albeit at a marginally lower potency than the Pt(II) compounds. The efficacy of Pt(IV)-complexes to produce cytotoxicity was also examined in a model system (MCTS) that reflects the hostile environment and cellular heterogeneity observed in solid tumours.

Histological characterisation revealed that in MCTS with diameter greater than 300 μm, only the outermost layers were actively proliferating. The proliferation of these cells is the source of outgrowth from the MCTS [28] and also the cell population most sensitive to chemotherapeutic regimes targeted against some aspect of DNA replication. All compounds tested were able to completely retard the growth of DLD-1 cells either from the outer layers of MCTS or in monolayer cultures. The potency to produce a cytotoxic response in 3-D cultures by the Pt(II) compounds was indistinguishable from that observed in monolayers. Similar potency in monolayers vs. 3-D culture systems have previously been reported for cisplatin using lung cancer cells [29]. The alkylating agent chlorambucil was the only compound observed to display greater potency in MCTS compared with monolayers and similar results have been reported for another DNA alkylating agent, Thiotepa [30]. The reasons for such observations are unclear and were in complete contrast to the mitotic inhibitor vinblastine, which demonstrated a significantly lower potency in MCTS; a phenomenon that has been termed ''multicellular resistance''. The potency of doxorubicin was unaffected in MCTS as determined by the outgrowth assay and is perhaps not surprising given the high localisation of this drug to outer layers of such tissue, due to its poor permeability in biological tissues. The Pt(IV)-complexes also displayed similar cytotoxicity in MCTS and monolayers, however, as expected, their potencies were lower than that observed for the active Pt(II)-species.

Characterisation of drug effects in terms of MCTS growth provided some discrimination between the classes of Pt-complex. Between 24 and 48 hr of drug exposure both Pt(II)-complexes produced a significant reduction, compared to the original MCTS diameter. This reduction is presumably caused by sloughing of necrotic/apoptotic cells from the outer layers and the time scale of this effect is similar to that previously reported for detachment of apoptotic cells from monolayer cultures [31]. A reduction in MCTS diameter was also observed for vinblastine and doxorubicin, although the effect continued over the duration of the investigation. In contrast, the reduction of MCTS size produced by the Pt(II)-complexes stabilised, and in the case of En appeared to recover. This response is consistent with the rapid production of cytotoxicity by Pt(II)-complexes and their short duration of action due to reductive metabolic inactivation by thiols and other endogenous biomolecules. The Pt(IV)-complex with highest propensity to lose axial groups, namely EnCl, retarded growth of MCTS initially and between 4 and 5 days of incubation produced a significant reduction in tissue diameter. The compound with lowest reduction potential, EnOH, produced a similar profile and time-course of effects on MCTS size, however, the magnitude was lower compared to EnCl. The delayed onset of effects by the Pt(IV)-complexes is consistent with the requirement for metabolic activation to produce the more reactive species. In contrast, EnOAc which is intermediate in reactivity between EnOH and EnCl did not produce a measurable effect on growth until 5 days of incubation. Therefore, although the axial moiety of the Pt(IV)-complexes delays the onset of action, the effects observed here are not absolutely predicted by the reduction potential of the compounds.

The drug-induced cytotoxicity and retardation/reduction in MCTS growth indicate that the outer proliferating layer of cells is indeed susceptible to anticancer agents. Regardless of the type of compound examined, a significant shift in the location of cells incorporating BrdU into DNA, which is indicative of increased DNA repair [32] and the recruitment of quiescent cells to the proliferating pool [21]. It is unclear what factors promote the establishment of quiescent cell populations within MCTS; however, several investigations demonstrate that hypoxia and glucose deficiency are not primary causative agents [20,33]. More likely causes include cell–cell, and cell–matrix interactions, growth factor availability and expression of growth factor receptors [20]. The ability to ''recruit'' cells from this quiescent population has previously been suggested [21] to act as a means of tumour re-growth following chemical insult and also potentially a source of resistance that depends not on genetic change but a time-dependent factor. The lack of dependence on class or mode of drug action suggests a primary response to stress induced by anticancer agents.

Unlike the generalised effects on proliferating cell number, the nature of cellular insult within MCTS varied considerably between the compounds investigated. Morphological data obtained in the present manuscript indicate that incubation with vinblastine leads to cells throughout the tissue progressing to metaphase during the first 24–48 hr incubation, consistent with its ability to disrupt microtubule formation [34]. Further prolonged incubation with vinblastine was associated with progression to widespread apoptosis. The mechanism of doxorubicin-mediated cytotoxicity in cancer cells is complex, although

the most likely mechanism is inhibition of topoisomerase II [35] and the ultimate effect is to elicit apoptosis [36]. Administration to MCTS rapidly caused nuclear damage that appeared as chromatin condensation and displacement to the periphery. This unique effect of the formation of dense strands of material within the nucleus differs from the well defined features of early cellular changes [37,38] in apoptosis and appeared to localise mainly to the outer cellular layers of the MCTS. Doxorubicin has been demonstrated to penetrate solid tissue masses slowly [39–41] and the initial distribution pattern is almost exclusively restricted at the periphery of MCTS [42]. Continued exposure of MCTS to doxorubicin resulted in the appearance of widespread general necrosis, presumably a result of the complex and multifaceted ability of this anthracycline to produce cellular damage.

Cisplatin produced deleterious effects on the morphology of MCTS cells within 24 hr and the damage appeared as apoptosis. The ability of cisplatin to elicit apoptosis in cancer cells has been well established [43,44] and this compound is able to penetrate solid tissue masses rapidly [45]. A prolonged exposure to drug resulted in progression of the damage to central areas of the MCTS rather than a significant widespread escalation of necrosis. This was in contrast to the increased damage observed with prolonged exposure to vinblastine and doxorubicin. This reduced long-term impact of cisplatin may be related to the inherent and well documented reactivity of this Pt(II) compound (for review see [12]). Inactivation of cisplatin is achieved by chelation with glutathione, ascorbate, metallothionein and other protein thiols (for review see [46]). The discrepancy between the time-course of effects between cisplatin and either vinblastine or doxorubicin were also consistent with data observed for MCTS growth kinetics (see above). Proliferating cells represent the cellular target for cisplatin and the progression to central areas of the MCTS is consistent with the recruitment of these cells from a quiescent state.

Did Pt(IV)-complexes produce similar morphological changes in MCTS that were described for the Pt(II)-complexes cisplatin and En? Short-term incubation (up to 24 hr) of MCTS with Pt(IV)-complexes failed to produce any measurable morphological changes. However, following prolonged exposure (72 hr) the most potent Pt(IV)-complex used, namely EnCl, caused cell death that was discernable from the untreated MCTS. Although the extent and distribution was significantly lower than that seen for Pt(II) compounds, the nature of the damage appeared similar. The propensity to produce cell death in solid tumours appeared to be related to reduction potential as evidenced by the further reduction in damage produced by the least reactive compound, EnOH. The delay in producing cellular damage is consistent with the requirement for cell-mediated activation of these compounds and a greater duration of action may also be indicated by the late onset of MCTS growth inhibition.

Thus, the novel Pt(IV) compounds are characterised by similar cytotoxicity profiles to the Pt(II) agents in a solid tumour environment, although with moderate reduction in potency. A significant benefit of the Pt(IV) compounds was the delayed onset of action, which is reflective of greater stability, even within the hostile intratumour environment.

Acknowledgments

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