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TOXICITY OF CISPLATIN AND HYDROXYMALONATODIAMMINE PLATINUM (II) TOWARDS MOUSE BONE MARROW AND B16 MELANOMA IN RELATION TO DNA BINDING *IN VIVO*

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Abstract—The antitumour selectivity of cisplatin and hydroxymalonatodiammine platinum II ($Pt[OHmal(NH_3)_2]$), a second generation platinum drug, was evaluated in C57Bl/Cbi mice bearing advanced intramuscular B16 melanoma. At maximally tolerated doses, $Pt[OHmal(NH_3)_2]$ produced greater inhibition of growth of the advanced B16 melanoma than did cisplatin. Comparison of dose–response curves for survival of B16 lung colony-forming cells and bone marrow stem cells treated *in vivo* indicated that selective killing of B16 cells was achieved with $Pt[OHmal(NH_3)_2]$, whereas cisplatin was relatively nonselective. The extent of reaction of platinum with DNA at doses producing measurable levels of survival in tumour and marrow *in vivo* was similar to values previously observed in cultured cells treated with cisplatin *in vitro*. Studies of the amount of platinum bound to DNA in tumour and marrow following administration of the two drugs revealed that the improved selectivity of $Pt[OHmal(NH_3)_2]$ was associated with a selective increase in the amount of platinum bound to tumour DNA, relative to cisplatin. In addition, platinum lesions produced in DNA by $Pt[OHmal(NH_3)_2]$ appeared to be more effective in producing tumour cell killing than those produced by cisplatin. No significant excision of total DNA-bound platinum from tumour was observed up to 48 hr after administration of either drug.

The antitumour agent cisplatin is a coordination complex of platinum which is particularly active against advanced testicular cancer in man [1]. The dose-limiting toxicities seen in clinical use of this drug are renal damage and induction of severe nausea and vomiting [2]. The agent is active against a broad spectrum of experimental neoplasms in rodents; however, gastrointestinal, hematopoietic and renal toxicities are observed following administration of sublethal doses [3].

A number of congeners of cisplatin have been synthesized in an attempt to discover complexes with less toxicity and improved therapeutic efficacy. The effect of variations in the amine ligands, as well as the leaving groups, coordinated to the metal atom on antitumour activity have been studied. Cleare *et al.* described the synthesis and chemical and biological properties of a series of platinum complexes [4]. Several of the more promising compounds were complexes containing dicarboxylate ligands as leaving groups in place of chloride ions; such complexes were kinetically inert to substitution reactions *in vitro*, relative to the parent compound. Active antitumour agents containing dicarboxylate leaving groups included hydroxymalonatodiammine platinum (II) ($Pt[OHmal(NH_3)_2]$), 1,1-cyclobutanedicarboxylatodiammine platinum (II), and malonato-1,2-diamminocyclohexane platinum (II). Bradner *et al.* [5] reported that $Pt[OHmal(NH_3)_2)]$ showed good activity against several solid tumours in mice; this complex was the only one shown to be clearly superior to cisplatin against the B16 melanoma. $Pt[OHmal(NH_3)_2]$, as well as other complexes containing dicarboxylate ligands, appeared to be less nephrotoxic than cisplatin [6].

A number of studies of the action of platinum drugs on cultured mammalian cells *in vitro* have produced evidence to argue that damage to DNA, and the replication of DNA on a damaged template, are the factors responsible for the cytotoxic effects of platinum complexes [7]. Such studies do not of course directly address the question of the mechanism of antitumour action of these drugs *in vivo*, nor do they address the critical question of the basis for selectivity of these agents. Selective antitumour activity *in vivo* depends upon a number of factors, including physiological disposition of active forms of drug to tumour and host tissue, and the relative susceptibility of molecular targets in tumour and host cells. In these studies, we assessed the antitumour selectivity of two platinum drugs. Cytotoxicity was quantitated *in vivo* and related to DNA interaction, to enable comparison with previous *in vitro* studies and to evaluate the role of pharmacologic and molecular factors in selective tumour inhibition.

MATERIALS AND METHODS

Drugs. Cisplatin and $Pt[OHmal(NH_3)_2]$ were kindly supplied by Dr Michael Cleare of the Johnson Matthey Research Centre (Reading, U.K.).

Animals and drug treatment. Four- to six-week-old male C57Bl/Cbi strain were used in these studies. C57Bl/Cbi mice of either sex of varying ages were

used for recipients in bone marrow spleen colony assays or B16 lung colony formation assays. The mice were maintained in plastic cages with chopped balsa wood bedding, on a standard laboratory diet (No. 1 diet, BP Nutrition, U.K. Ltd.). A 12-hr light–dark cycle (0700–1900 lights on) was maintained and drugs were administered between 1000 and 1400. Cisplatin was injected intraperitoneally in 0.9% NaCl (0.02 ml/g body wt). Pt[OHmal$(NH_3)_2$] was administered intraperitoneally as a suspension in a 2.0% aqueous solution of methylcellulose (0.01 ml/g body wt). Controls were injected with vehicles only.

Tumour. The B16 melanoma used in these studies was provided by Dr T. Stephens of the Insitute of Cancer Research. The tumour was maintained in serial passage by intramuscular injection of a 1:10 dilution of a tumour brei into the gastrocnemius muscle of the mice. Experiments were generally performed 10–12 days after inoculation of tumour cells, when the tumours were 100–400 mg in weight.

Spleen colony formation assay. The assay for bone marrow spleen colony forming cells (CFU-S) was performed essentially as described by Till and McCulloch [8]. Twenty-four hours after injection of vehicle or drugs, mice were sacrificed, femoral marrow was harvested, and the cell suspension diluted with serum-free F10 Ham's tissue culture medium. An aliquot of marrow containing about 10 viable CFU-S was injected into the lateral tail vein of mice given 850 rads of whole body irradiation 2 hr previously. Eight days later, recipient mice were sacrificed. The spleens were removed and fixed in Bouin's solution, and colonies on the spleen surface were counted under low power magnification. Femurs from control mice contained approximately 5000 CFU-S.

Inhibition of tumour growth. Groups of 6–8 tumour-bearing mice were injected with vehicle or various doses of cisplatin or Pt[OHmal$(NH_3)_2$]. The tumour diameters were then measured using calipers, and tumour volumes were estimated using the calibration curve technique [9]. Experiments were terminated when size of the tumour approached 1 g; at least two volume doublings were observed.

Lung colony formation assay. The lung colony formation assay was performed as described by Steel and Adams [10]. Mice were treated with vehicle or various drug doses, and were sacrificed 24 hr later. The tumours were removed, dissected with crossed scalpels, and treated with trypsin (0.25% in phosphate-buffered saline) and DNAase I (50 μg/ml) for 10 min. The first tumour digest was discarded, and the remaining tumour fragments were then digested with the trypsin–DNAase mix for 10 min. Cells and fragments were harvested by low speed centrifugation, then resuspended and filtered through a glass wool column to remove debris. The single cell suspension was adjusted to an appropriate density and injected intravenously into recipient animals along with 1.0×10^6 plastic microspheres and 10^6 B16 feeder cells (previously treated with 7.5 Krad ionizing irradiation). Twenty-one days later, recipient mice were sacrificed, their lungs were removed and fixed in Bouin's solution, and tumour colonies on the surface were counted under low power magnification. Control tumours yielded approximately 5 colonies/1000 cells injected.

Measurement of platinum bound to B16 bone marrow DNA in vivo. At various times following injection of cisplatin or Pt[OHmal$(NH_3)_2$], mice were sacrificed, and tumours were removed and frozen in liquid nitrogen. The tumours were homogenized using a Teflon pestle into a solution (PAS solution) containing 6% w/v sodium *p*-aminosalicylate, 1% w/v NaCl, 1% triisopropyl naphthalene sulphonate, and 6% w/v sec-butanol in water, and then DNA was extracted as described previously [11]. Femoral marrow was harvested from groups of 6–10 mice by flushing the PAS lysis solution through the femurs; DNA was then extracted as above. The amount of platinum bound to melanoma or marrow DNA was determined using flameless atomic absorption spectroscopy, as described previously [11].

RESULTS

Inhibition of tumour growth by cisplatin or Pt[OHmal$(NH_3)_2$]

A number of experiments were performed to measure inhibition of growth of B16 melanoma by the two platinum drugs. The results of one experiment utilizing maximally tolerated single doses of the two agents are shown in Fig. 1. Cisplatin, even at toxic doses, only produced modest inhibition of the growth of advanced B16 melanoma, whereas Pt[OHmal$(NH_3)_2$] consistently produced tumour growth delay even at sublethal doses. In no instances were cures of the advanced tumour observed.

Effect of cisplatin or Pt[OH*mal*$(NH_3)_2$] *on tumour lung colony forming cells or bone marrow CFU-S*

The above finding, that greater inhibition of tumour growth could be achieved by maximally tolerated doses of Pt[OHmal$(NH_3)_2$] than by maximally tolerated doses of cisplatin, suggested that greater selectivity of tumour cell killing was achieved with Pt[OHmal$(NH_3)_2$]. To explore further the question of selectivity in a more quantitative fashion, the survival of tumour lung colony forming cells was measured at several doses of each agent and compared to that of bone marrow CFU-S. The results for cisplatin are shown in Fig. 2 and those for Pt[OHmal$(NH_3)_2$] are shown in Fig. 3. It can be seen that cisplatin does not achieve selective killing of tumour cells; indeed, the D_{37} for B16 lung colony forming cells was approximately 11 mg/kg and that for bone marrow CFU-S was 5 mg/kg. In contrast, for a given dose of Pt[OHmal$(NH_3)_2$] more tumour cells are killed than are bone marrow CFU-S, the D_{37} for B16 being approximately 20 mg/kg and that for the CFU-S being 40 mg/kg. It should be noted that toxicity of these agents to bone marrow is not necessarily dose limiting in this system; marrow is merely an example of a proliferating host cell population that is sensitive to both agents wherein toxicity may be quantitated. The fact that the same enhanced selectivity of Pt[OHmal$(NH_3)_2$] was observed in both tumour growth inhibition assays (above and Ref. 5) and in the lung colony assay argues that the improved selectivity here is the result of enhanced tumour inhibition rather than an artefact of either assay.

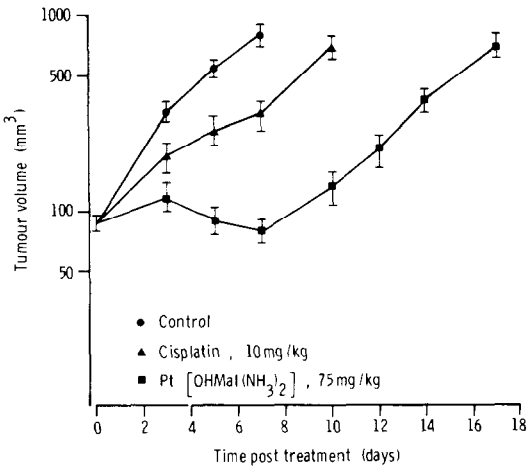

Fig. 1. Inhibition of growth of intramuscular B16 melanoma by maximum tolerated doses of cisplatin or $Pt[OHmal(NH_3)_2]$. Approximate LD_{10} doses of each drug were administered; values are means ± S.E. of 10–12 tumour measurements. ●——●, control; ▲——▲, cisplatin (10 mg/kg); ■——■, $Pt[OHmal(NH_3)_2]$ (75 mg/kg).

Toxicity in relation to DNA binding in vivo

Antitumour selectivity can result from a number of factors, including drug disposition and differences in drug action at the molecular level. Considerable evidence from studies of cultured cells *in vitro* has indicated that the cytotoxic effects of cisplatin are due to interaction of the drug with DNA. To determine whether or not the enhanced antitumour selectivity of $Pt[OHmal(NH_3)_2]$ could be related to DNA

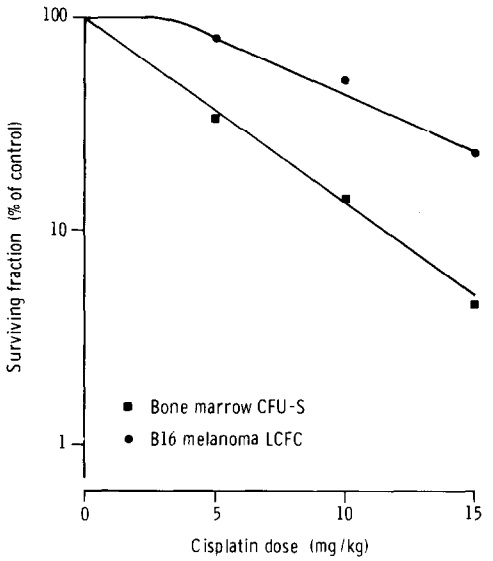

Fig. 2. Survival of B16 lung colony forming cells or bone marrow CFU-S 24 hr after cisplatin treatment. Data are the means of at least two determinations from tumour or marrow samples pooled from 2–3 treated animals. ●——●, B16 lung colony forming cells; ■——■, bone marrow CFU-S.

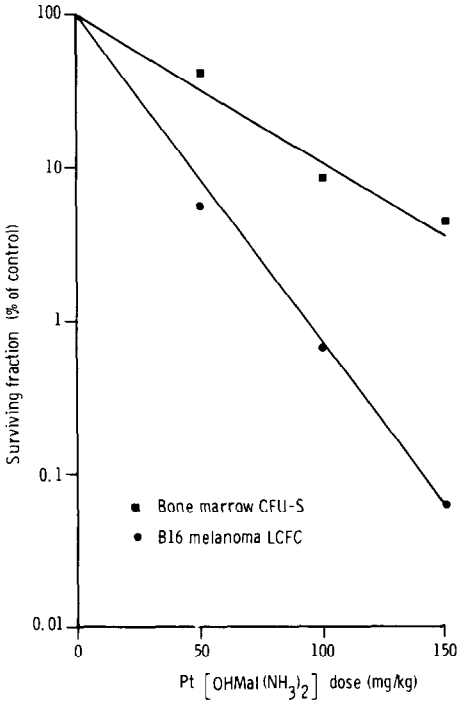

Fig. 3. Survival of B16 lung colony forming cells or bone marrow CFU-S 24 hr after $Pt[OHmal(NH_3)_2]$ treatment. Data are means of at least two determinations from tumour or marrow samples pooled from 2–3 treated animals. ●——●, B16 lung colony forming cells; ■——■, bone marrow CFU-S.

binding, the amount of platinum bound to tumour and marrow DNA was determined 24 hr following administration of cisplatin or $Pt[OHmal(NH_3)_2]$ to mice. Results for cisplatin are shown in Fig. 4; those for $Pt[OHmal(NH_3)_3]$ in Fig. 5. Administration of either agent resulted in greater binding of platinum to tumour DNA compared to bone marrow DNA.

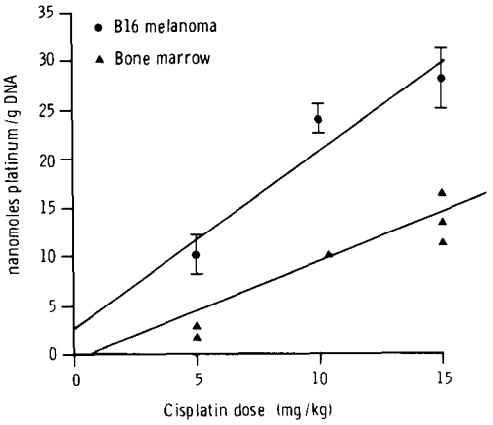

Fig. 4. Binding of platinum to B16 melanoma DNA or marrow DNA 24 hr after cisplatin treatment. Tumour values are means ± S.E. of determination on three samples of tumour pooled from 2–3 animals; marrow values are separate determinations on femoral marrow pooled from 10 mice. ●——●, B16 melanoma; ▲——▲, bone marrow.

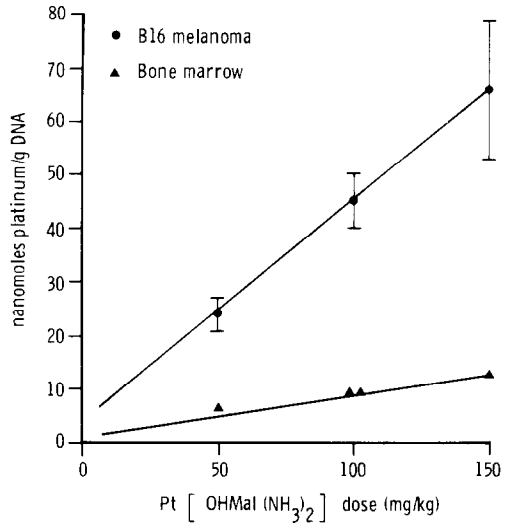

Fig. 5. Binding of platinum to B16 melanoma DNA or bone marrow DNA 24 hr after Pt[OHmal(NH_3)$_2$)] treatment. Tumour values are means ± S.E. of determinations on three samples of tumour pooled from 2–3 mice; marrow samples are separate determinations on femoral marrow pooled from 10 mice. ●——●, B16 melanoma; ▲——▲, bone marrow.

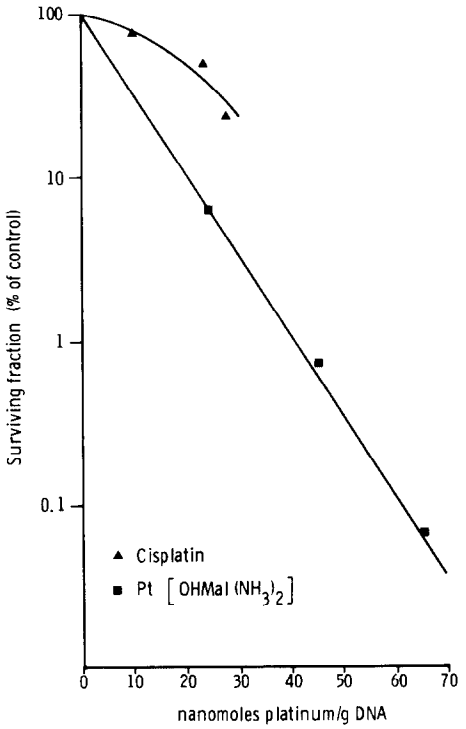

Fig. 6. Survival of B16 melanoma lung colony forming cells vs platinum bound to DNA 24 hr after cisplatin treatment. Data from Figs. 2–5 were replotted to show extent of reaction of platinum with DNA at measured levels of cell killing for both drugs. ▲——▲ Cisplatin; ■——■ Pt[OHmal(NH_3)$_2$].

At any given dose of cisplatin, binding to tumour DNA was roughly twice that of marrow. For any given dose of Pt[OHmal(NH_3)$_2$], binding to tumour DNA was four to five times higher than DNA binding in marrow. Thus Pt[OHmal(NH_3)$_2$] administration selectively produced a greater degree of DNA damage in the B16 melanoma compared to cisplatin.

The results of B16 survival and binding studies are combined in Fig. 6. It can be seen that the survival curves are different even when normalized for the amount of DNA bound *in vivo*; there is a shoulder on the survival curve for cisplatin that is not present on the curve for Pt[OHmal(NH_3)$_2$]. Table 1 shows the amount of platinum bound to tumour or marrow DNA at doses of cisplatin or Pt[OHmal(NH_3)$_2$] yielding 37% survival of CFU-S or B16 cells. At equitoxic doses to marrow CFU-S, the overall Pt-DNA binding in marrow was similar for the two drugs. Marrow would appear to be more sensitive to DNA-bound platinum than are B16 melanoma cells, comparing the amount bound at equitoxic doses of either drug.

Lastly, as shown in Fig. 6, platinum lesions introduced in B16 DNA by Pt[OHmal(NH_3)$_2$] appeared to be more effective in killing tumour cells than those produced by cisplatin.

Studies of the time course of platinum DNA binding following administration of either drug are shown in Fig. 7. Following cisplatin administration, the amount of platinum bound to CNA reached a maximum rapidly and did not decline significantly during the 48 hr following treatment. Similar results were obtained for bone marrow DNA (data not shown). After Pt[OHmal(NH_3)$_2$] administration, Pt binding to DNA reached a maximum more slowly. The slower rate of reaction seen with Pt[OHmal(NH_3)$_2$]

Table 1. Amount of platinum bound to marrow and tumour DNA at doses of cisplatin or Pt[OHmal(NH_3)$_2$] producing 37% survival of CFU-S or B16 lung colony forming cell (LCFC)

	CFU-S		B16 LCFC	
	D_{37} (mg/kg)	Amount bound (nmole/g)	D_{37} (mg/kg)	Amount bound (nmole/g)
Cisplatin	5	4	11	22
Pt[OHmal(NH_3)$_2$]	40	5	20	12.5

The amount of platinum bound to total marrow or tumour DNA at doses yielding 37% survival of CFU-S or B16 LCFC was estimated from data in Figs. 2–5.

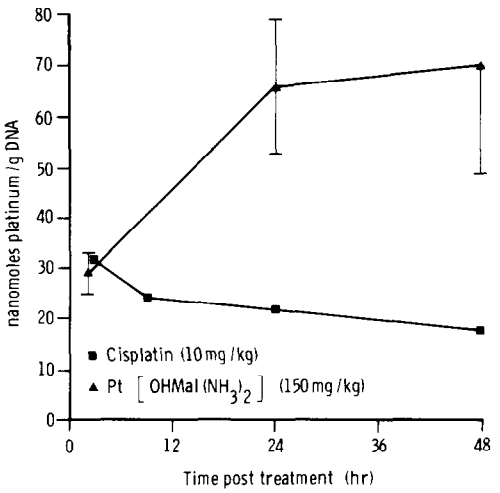

Fig. 7. Binding of platinum to B16 melanoma DNA at various times after treatment with cisplatin (10 mg/kg) or Pt[OHmal(NH_3)$_2$] (150 mg/kg). Values for cisplatin are means from determination on at least two pooled tumour samples; values for Pt[OHmal(NH_3)$_2$] are means ± S.E. from three pooled samples. ■——■, cisplatin; ▲——▲, Pt[OHmal(NH_3)$_2$].

may have been related to the slow release of this relatively insoluble compound, or its relatively low reactivity. Again, no evidence of significant loss of DNA-bound platinum was seen over the time period studied.

DISCUSSION

Antitumour selectivity

We have compared the antitumour selectivity of cisplatin to a second generation congener, Pt[OHmal(NH_3)$_2$], and we have examined the basis of this selectivity at the level of drug–DNA interaction. The findings have relevance to the pharmacology of the platinum drugs and to their mechanism of action. With regard to pharmacology of the platinum drugs, our results here confirm and extend those of Bradner *et al.*, who reported that Pt[OHmal(NH_3)$_2$] was superior to cisplatin against B16 melanoma [5]. The activity of this complex against solid tumours such as Lewis lung carcinoma and B16 melanoma, as well as its decreased nephrotoxicity, are of considerable interest. The low solubility of the agent presents a problem, but may not preclude oral administration. Several platinum complexes containing dicarboxylate ligands as leaving groups appear promising as second generation platinum drugs, including 1,1 cyclobutanedicarboxylatodiammine platinum (II), which is now undergoing clinical trial [12]. Cleare has noted the low reactivity of such complexes *in vitro*, and has postulated the existence of *in vivo* activation mechanisms [4]. In our studies, much higher doses of Pt[OHmal(NH_3)$_2$] were administered to achieve levels of Pt–DNA binding in marrow comparable to those seen with cisplatin. The enhanced Pt–DNA binding we observed with Pt[OHmal(NH_3)$_2$] in B16 melanoma might be due to more rapid activation of the drug in tumour cells, or to enhanced uptake of the parent compound. Further studies of the physiological disposition of dicarboxylate compounds and their active metabolites might shed light on this question.

Mechanism of drug action

Several points emerge from these studies concerning the mechanism of action of the platinum drugs. Firstly, the extent of DNA–platinum binding seen here at measurable levels of toxicity is of the same order as that seen in cultured cells *in vitro* at similar levels of cytotoxicity [7, 11, 13], at least in the case of cisplatin. Thus, the extent of reaction of the drug with DNA at pharmacologically relevant doses *in vivo* would be adequate to produce the same biological effects previously observed *in vitro*. This finding strengthens arguments concerning mechanisms of drug toxicity based upon studies of cultured cells. Secondly, although platinum binding to marrow DNA is similar at doses of cisplatin or Pt[OHmal(NH_3)$_2$] that are equitoxic to CFU-S, differences in the curves of tumour cell kill versus platinum bound to tumour DNA may indicate that not all platinum compounds produce DNA lesions of equal efficacy in killing all cells. For example, compounds varying in reactivity may produce different ratios of monofunctional to difunctional DNA adducts in different tissues. Further studies on the production of interstrand crosslinks by various complexes might elucidate the importance of such reactions.

There was little evidence for loss of total DNA-bound platinum adducts in the tumour, either with a dose of cisplatin yielding high levels of cell survival or with a dose of Pt[OHmal(NH_3)$_2$] yielding low levels of survival. Therefore these studies of the time course of DNA binding *in vivo* do not suggest that DNA excision repair plays a major role in determining the sensitivity of this particular tumour to platinum drugs, although some repair may occur at non-toxic doses, and the repair of specific lesions, such as interstrand crosslinks, has not been evaluated. In this regard, the apparent relative sensitivity of bone marrow cells to DNA-bound platinum compared to melanoma may relate not so much to excision repair as to the cellular capacity to synthesize DNA on a damaged template. Along these lines, Hill and Setlow compared synthesis of nascent DNA in u.v.-irradiated mouse melanoma cells to that in normal mouse fibroblasts in culture and found the tumour cells to be more proficient in synthesis of high molecular weight DNA following treatment [14]. Amongst cell types that excise damage only slowly, cellular sensitivity may be determined predominantly by capability of the replication machinery to cope with the damage.

Alternatively, the apparent greater sensitivity of marrow to DNA-bound platinum could reflect the fact that a lower proportion of marrow stem cells compared to tumour clonogenic cells are actively cycling. Stationary-phase cells are more sensitive in culture to DNA-bound platinum than are actively cycling cells [13]. Whereas only 5–10% of mouse CFU-S are in S-phase [15], the B16 melanoma, even when advanced, contains 30% cells in S phase [16].

Thus, these experiments have shown that

enhanced selectivity of Pt[OHmal(NH_3)$_2$] towards B16 melanoma could be correlated with the level of drug–DNA interaction. Furthermore, they illustrate the complex interplay of pharmacologic, molecular and possibly cell kinetic factors that determine the success of antitumour therapy *in vivo*.

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