Comparison of Cellular Accumulation and Cytotoxicity of Cisplatin with That of
Tetraplatin and Amminedibutyratodiacloro(cyclohexylamine)platinum(IV)
(JM221) in Human Ovarian Carcinoma Cell Lines

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ABSTRACT

We have compared the cellular accumulation and cytotoxicity of three platinum compounds in a panel of five human ovarian carcinoma cell lines. The cell lines, which were established from both untreated and pretreated patients, showed a wide range in sensitivity to cisplatin and other platinum drugs. The panel consisted of two sensitive (41M, CH1), one in vitro acquired resistant (PXN/94) with moderate sensitivity, and two intrinsically resistant (SKOV-3, HX/62) cell lines. The cisplatin 2-h concentration of drug required to inhibit cell growth by 50% compared with vehicle treated control cells (IC50 values) for these cell lines were in the following order: CH1 < 41M < PXN/94 < SKOV-3 < HX/62. None of the cell lines showed saturation of platinum accumulation (per mg protein) at 2 h after exposure to cisplatin concentrations of up to 500 μM. The highest cellular platinum accumulation was observed in the sensitive 41M cell line which was established from an untreated patient. The lowest accumulation was found in the intrinsically resistant HX/62 cell line. The rate of platinum accumulation at an equimolar concentration of cisplatin was 41M > SKOV-3 > CH1 > PXN/94 > HX/62. The relationship between drug accumulation and cytotoxicity was evaluated by comparing 2-h IC50 values with platinum accumulation following exposure to both equimolar and equitoxic doses of the agent. The results suggest that reduced drug accumulation may play a partial role in the mechanism of intrinsic resistance to cisplatin in one cell line (SKOV-3) and a major role in another (HX/62), where reduced accumulation is attributable to reduced uptake rather than enhanced efflux. Decreased drug accumulation may also contribute significantly to the lower sensitivity of the PXN/94 cell line to cisplatin. Interestingly, both the PXN/94 and the sensitive CH1 cell lines, which were established from patients pretreated with platinum drugs, showed reduced drug accumulation relative to the 41M cell line. Cellular accumulation of tetraplatin and JM221 [(ammine)dibutyratodiacloro(cyclohexylamine)platinum(IV)], a novel platinum(IV) dicarboxylate complex exhibiting enhanced cell cytotoxicity compared to cisplatin, was examined. Comparison with platinum accumulation from cisplatin suggests that the increased cytotoxicity of tetraplatin and JM221 may be related to their increased accumulation. Significantly both agents are more lipophilic than cisplatin, which may account partially for their improved uptake in cisplatin resistant cells.

INTRODUCTION

Over the past two decades cisplatin has proved to be a very effective anticancer drug, particularly in the treatment of ovarian and testicular tumors (1, 2). However, a number of tumors are unresponsive (intrinsically resistant) and others develop resistance after an initial response (2, 3). The second generation analogue, carboplatin, which exhibits markedly reduced side effects, has a spectrum of clinical activity similar to that of cisplatin (4–7). Various mechanisms have been implicated in cellular resistance to platinum drugs including reduced drug accumulation, enhanced inactivation, reduced DNA damage, and enhanced repair of platinum-DNA adducts (for reviews see Refs. 8–10). The majority of the evidence for the involvement of these mechanisms has come from the use of sensitive and acquired resistant sublines of murine and human tumor models. In general the acquired resistant sublines have been established by in vitro exposure to cisplatin.

We are attempting to develop a new generation of platinum analogues which will overcome both intrinsic and acquired resistance to cisplatin and exhibit a broader spectrum of clinical activity. To assist in these objectives we have established a number of in vitro and in vivo human tumor models with a wide range of sensitivity to cisplatin. The mechanisms of cisplatin sensitivity/resistance in these models are being characterized. Decreased drug accumulation resulting from reduced uptake has been reported previously to be at least partially responsible for acquired resistance to cisplatin in several cell lines (9, 11–19). The precise mechanism by which cisplatin enters cells is unclear; both passive diffusion and carrier mediated mechanisms have been implicated (see Ref. 10). In the present study we have examined the relationship between drug accumulation and cisplatin cytotoxicity in five human ovarian carcinoma cell lines. These were established from tumors of both untreated and pretreated patients and exhibited a wide range of sensitivity to cisplatin. The role of efflux in drug accumulation was investigated. We explored whether the enhanced cytotoxicity of tetraplatin and JM221, compared with cisplatin, is attributable to the enhanced accumulation of these agents in some cell lines (see Fig. 1 for structures). Tetraplatin is currently undergoing phase II clinical evaluation, while JM221 is an example of a novel class of platinum(IV) dicarboxylate compounds which are more lipophilic (20) and exhibit considerably greater cytotoxic activity than cisplatin in all five cell lines (21).

MATERIALS AND METHODS

Chemicals. Cisplatin and JM221 were obtained from Johnson Matthey Technology Centre, Reading, United Kingdom. Tetraplatin was a generous gift from Dr. M. Wolpert-Defilippis (National Cancer Institute, Bethesda, MD).

Cell Lines and Assessment of Cytotoxicity. The biological details of the five cell lines used in this study have been described previously (22). Table 1 summarizes the prebiopsy treatment and response of the patients from whom the cell lines were established. All five cell lines grew as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Imperial Laboratories, Andover, United Kingdom). The culture conditions and cytotoxicity assay have been described previously (23). Briefly, cells (0.5–1.0 × 10^6/well) seeded in microtiter
plates were allowed to attach for 20–24 h and were then exposed to drugs, in quadruplicate wells, for 2 or 96 h. After a 2-h exposure the medium was removed and each well was washed consecutively with 200 μl of PBS and medium at 37°C. The cells were then incubated in fresh medium for 96 h before assessing cytotoxicity by the sulforhodamine B assay (23).

Platinum Drug Accumulation. Cells (1–4 x 10⁶) in exponential growth phase were exposed to either a single concentration (for details see “Results”) of a platinum derivative for various times up to 2 h or to various concentrations (range, 0–100 μM) of the agent for 2 h. Immediately after exposure the medium was aspirated and the cells were washed with ice-cold PBS (3 × 25 ml). The cells were then scraped, harvested in 0.5 ml PBS, and sonicated (Soniprep 150, MSE) at 4°C. The cell sonicates were analyzed for protein content according to the method of Lowry et al.(24) and for platinum by flameless atomic absorption spectrometry (Perkin-Elmer model 1100B and HGA700 atomizer). For protein determination, 50 μl of cell sonicate were incubated with 200 μl of 1 N sodium hydroxide overnight at 37°C before analysis. Between 20 and 80 μl of each sample, depending on the concentration of platinum, were analyzed for platinum in duplicate. A maximum volume of 40 μl was introduced into the graphite furnace and volumes greater than 40 μl were analyzed by two injections interspersed with drying and charring steps of the heating program. The platinum concentration in the samples was determined from a calibration curve prepared using platinum solutions in 0.2% nitric acid. The recovery of added platinum from the samples ranged between 80 and 95%. Cellular platinum concentrations were expressed as nmol/mg protein.

To determine the loss of accumulated platinum, cells (1–4 x 10⁶) were treated with cisplatin for either 30 or 120 min. The medium was then aspirated and the cells were washed with 2 × 8 ml of medium at 37°C and incubated in fresh medium for various times (0–120 min). The washing procedure time of 90 s was added on to the efflux time. The medium was again aspirated and the cells were washed with ice-cold PBS (3 × 25 ml), before processing the cells and analyzing for platinum and protein content as described above.

Statistical Analysis. The statistical significance was determined by t test for grouped data.

RESULTS

The cell lines used in these studies exhibited a wide range of sensitivity to cisplatin (Table I). The ranges of panel IC₅₀ values following 2-h and 96-h continuous exposure were 16- and 120-fold, respectively, the order of sensitivity being: CH1 > 41M > PXN/94 > SKOV-3 > HX/62. Thus the cytotoxicity gain for extended exposure for intrinsically sensitive cells was 3-fold greater than that for intrinsically resistant cells.

Cisplatin Accumulation. Following exposure of 41M and HX/62 cells to 100 μM cisplatin, platinum accumulation was linear over 60 min for the former (r = 0.993) and over 120 min for the latter (r = 0.994) (see Fig. 2). Moreover, between 40 and 120 min of exposure, platinum accumulation in the HX/62 cell line was lower [3.04 ± 0.88-fold (SD)] than in the 41M cell line.

The effect of variation in cisplatin concentration on platinum accumulation in the five human ovarian carcinoma cell lines is shown in Fig. 3. Cellular platinum accumulation per mg protein after 120 min exposure to cisplatin appeared to be linear up to at least 50 μM drug concentration in all five lines. The sensitive 41M cell line contained the highest platinum levels at all cisplatin concentrations examined (P < 0.05 versus all cell lines compared); the linear regression equation for the cellular platinum levels versus cisplatin concentration curve had a slope for the accumulation versus cisplatin concentration curve was 1.5-, 2.0-, 3.1-, and 6.8-fold lower for the SKOV-3, CH1, PXN/94, and HX/62 cell lines, respectively. There was no significant difference in protein content per cell volume between the cell lines; 41M, 0.27 ± 0.08; CH1, 0.38 ± 0.10; PXN/94, 0.28 ± 0.17; SKOV-3, 0.21 ± 0.03; and HX/62, 0.27 ± 0.19 mg protein/μl cell volume (SD, n = 3). Cell volume was measured using a Coulter Counter coupled to a multichannel analyzer. The relationship between drug accumulation and cytotoxicity was evaluated by comparing 2-h IC₅₀ values with platinum accumulation following exposure to both equimolar and equitoxic concentrations of the drug. The cellular platinum levels

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<th>Table 1 Patient prebiopsy treatment and cisplatin IC₅₀ values relating to cell lines used in uptake studies</th>
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*IC₅₀ values were determined following 2-h or 96-h continuous exposure to the drug using sulforhodamine B protein assay (mean ± SD, n = 4–7).*
were 0.0066 ± 0.0014 and 0.28 ± 0.046 MM, (SD, n = 3 and 5), exposure to tetraplatin than cisplatin (12.3 versus 61 pmol/mg resistance (HX/62) cell line. The 96-h IC50 values for JM221 dichloro platinum(IV) complexes, of general formula c,t,c,- to 100 MM cisplatin for various times before and 5), compared to 19.1 ± 5.2 μM (SD, n = 4) for cisplatin (ratio, 10.7). To determine whether increased drug sensitivity in all five cell lines compared to cisplatin (21). The result of cellular platinum accumulation following 2 h exposure to various concentrations of cisplatin, the highest platinum levels were observed in one of the most sensitivity to cisplatin and other platinum drugs (22). The 2-h IC50 for tetraplatin in this cell line was 1.78 μM (mean of two separate experiments), compared to 19.1 ± 5.2 μM (SD, n = 4) for cisplatin (ratio, 10.7). To determine whether increased drug accumulation underlies the greater cytotoxicity of tetraplatin we have examined cellular platinum accumulation following 2 h exposure to various concentrations of the two drugs. The results (Fig. 5) show that cellular platinum concentration was linear up to 100 μM tetraplatin (linear regression equation, y = 0.015x - 0.014; correlation coefficient, r = 0.999) and cisplatin (y = 0.004x - 0.009; r = 0.998) concentration. The slope for the tetraplatin accumulation curve was 4.2-fold greater than that for cisplatin. At equitoxic concentrations (2-h IC50) of the two drugs cellular platinum levels were 4.9-fold lower after exposure to tetraplatin than cisplatin (12.3 versus 61 pmol/mg protein).

Accumulation of JM221 versus Cisplatin. Recently we have reported that a series of alkylamine-ammine-dicarboxylato-dichloro platinum(IV) complexes, of general formula c,t,c,-[PtCl2(OCOR)2NH2(RNH2)], has a markedly enhanced cytotoxicity in all five cell lines compared to cisplatin (21). The mechanisms involved in the increased cytotoxicity are unknown. Since these compounds are more lipophilic than cisplatin (20) we have examined if increased cytotoxicity was associated with an increased cellular accumulation of these drugs. JM221 was used as an example of this novel class of compounds and we compared the cytotoxicity and accumulation of this agent with that of cisplatin in a sensitive (CH1) and a resistant (HX/62) cell line. The 96-h IC50 values for JM221 were 0.0066 ± 0.0014 and 0.28 ± 0.046 μM, (SD, n = 3 and 5), in the CH1 and HX/62 cell line, respectively. These values were 16- and 45-fold lower than that for cisplatin in the two cell lines. The cellular platinum accumulation (after 2 h exposure) was linear up to 25 μM and 10 μM JM221 concentration in the CH1 and HX/62 cell line, respectively, whereas the rate of incorporation decreased (Fig. 6). The platinum levels were 66-100-fold greater in the CH1 and 130-220-fold greater in the HX/62 cell line (P < 0.001) after exposure to JM221 (below 25 μm) than to cisplatin. The sensitive CH1 compared with the resistant HX/62 cell line accumulated 1.4-2-fold greater levels of platinum after exposure to JM221.

The present study examines the relationship between drug accumulation and the cytotoxicity of three platinum coordination complexes in human ovarian carcinoma cell lines. Following 2 h exposure to various concentrations of cisplatin, the rate of platinum accumulation at an equimolar concentration of cisplatin was 41M > SKOV-3 > CH1 > PXN/94 > HX/62. Interestingly, the other sensitive cell line (CH1), which was established from a patient who had received prior cisplatin and carboplatin therapy only, showed a 2-fold lower platinum accumulation than the 41M cell line after exposure to equimolar concentrations of cisplatin. However, the CH1 cells appeared to be very sensitive to platinum, accumulating 5.9-fold less platinum than the 41M cells at equitoxic (2-h IC50) concentrations of cisplatin. The mechanism(s) which determine the increased sensitivity of the CH1 cells to cisplatin is presently being investigated.

The PXN/94 cell line accumulated 3.1-fold less platinum than the 41M cell line following exposure to cisplatin; this reduction was similar to the difference in sensitivity of the two cell lines to the drug (2-h IC50 ratio, PXN/94:41M, 3.7). Moreover, at equitoxic concentrations of the drug the two cell lines accumulated similar amounts of platinum. These results imply that reduced drug accumulation may play a significant role in the reduced cisplatin sensitivity of the PXN/94 relative to the 41M cell line. Since both the CH1 and the PXN/94 cell lines were established from patients pretreated with platinum containing therapy it is possible that reduced drug accumulation represents an early response in the development of acquired resistance in vivo. However, direct comparison with cell lines...
CELLULAR ACCUMULATION AND CYTOTOXICITY OF PLATINUM DRUGS

Fig. 4. Loss of accumulated platinum from cells preincubated with cisplatin for 30 min (A) and 120 min (B). The sensitive 41M (□) and the relatively resistant HX/62 (●) cells were preincubated with 50 μM and 100 μM cisplatin, respectively. At the end of the incubation period the cells were washed and incubated in fresh medium for various times before determining the retained cellular platinum levels. The values represent mean ± SD (bars) of triplicate determinations in 2 separate experiments.

Fig. 5. Accumulation of platinum in the PXN/94 cell line after 2 h exposure to cisplatin (△) and tetraplatin (●). The values represent mean ± SD (bars) of triplicate determinations in 2 separate experiments. SD was less than the symbol size where not indicated.

established prior to treatment is not possible to support this hypothesis. Reduced drug accumulation has been postulated as an early phenotypic change in the development of low levels of acquired resistance to cisplatin in murine cells in vitro (25) and in the human ovarian carcinoma xenograft established from the 2008 cell line (26).

The two most resistant cell lines, SKOV-3 and HX/62, were classed as intrinsically resistant to platinum drugs because they were established from the tumors of patients who had received non-platinum based therapy (22). However, the possibility that these cell lines represent acquired resistance phenotypes cannot be ruled out. Comparison of the platinum accumulation in these two cell lines with that in the 41M cell line indicates that reduced drug accumulation plays a significant role in the intrinsic resistance to cisplatin in the HX/62 but only a partial role in the SKOV-3 cell line. Both the HX/62 and SKOV-3 cell lines required higher platinum levels (2.2- and 4.3-fold, respectively) than the 41M cell line for equivalent inhibition of cell growth. This may be related partly to the fact that the resistant cell lines are tetraploid whereas the sensitive 41M cell line is diploid (22).

However, the involvement of other factors such as increased inactivation of the drug by interaction with cellular thiols, or reduced DNA binding and increased repair of DNA damage in the reduced sensitivity of these cell lines to platinum drugs cannot be discounted. Indeed, we have previously shown 2-4-fold higher levels of glutathione in these two cell lines compared to the sensitive 41M and CH1 cell lines (23). This appears to be reflected by the formation of higher levels of platinum-glutathione adducts after cisplatin exposure in the two intrinsically resistant cell lines, compared to the sensitive (CH1) cell line (27). It is not known whether reduced drug accumulation in the HX/62 and PXN/94 cell lines is accompanied by reduced formation of DNA-platinum adducts which are believed to be responsible for the antitumor activity of cisplatin (28). Reduced DNA platination resulting from reduced drug uptake has been observed in some murine and human cells (12, 16, 19, 29) but not in others (30, 31). However, these latter cell lines showed no correlation between drug sensitivity and total DNA platination.

Drug efflux experiments with the HX/62 and 41M cell lines suggest that reduced drug uptake, rather than increased efflux, in the HX/62 cell line may explain its reduced sensitivity. Only
about 30% of the platinum was lost from either line over a 2-h period. These results confirm those reported for other cell lines (14, 16, 32, 33). Our results showed no saturation in platinum accumulation at 2 h after exposure to cisplatin concentrations up to 500 μM (data shown only up to 100 μM) in all five cell lines. In fact, platinum accumulation in three cell lines, 41M, CH1, and SKOV-3, increased exponentially after exposure to 50 μM cisplatin concentration. This may be due to drug induced damage to the plasma membrane although no uptake of trypan blue was observed after drug treatment. Lack of saturation of drug accumulation up to 3.3 mM in the 2008 human ovarian carcinoma cell line has been reported and it was suggested that this implied entry by passive diffusion (14). However, the mechanism(s) by which cisplatin enters cells remain unclear and both passive diffusion and carrier mediated transport may be involved (for review see Ref. 10). Although it is possible that both modes of entry have changed in resistant cells exhibiting reduced drug uptake, no changes in membrane lipid content or fluidity were reported in a resistant human ovarian carcinoma cell line (2008) showing reduced drug accumulation (34). Hence alteration in passive diffusion was discounted by these authors. We are currently investigating the possible changes in plasma membrane protein(s) in these and other cell lines exhibiting reduced drug accumulation as the major mechanism of resistance to cisplatin. Such changes have been reported in cisplatin resistant murine lymphoma (35) and human squamous carcinoma (36) cell lines. In the resistant lymphoma sublines expression of a glycoprotein (CP3P200) was found to correlate inversely with drug accumulation whereas in the squamous cell lines a membrane protein (SQM1) was reduced in a resistant subline showing decreased drug accumulation.

In a number of in vitro studies tetraplatin has been reported to be non-cross-resistant in cisplatin resistant cells or to elicit a completely different pattern of response to cisplatin and other platinum compounds (22, 37, 38). The PXN/94 cell line is more sensitive to tetraplatin compared to cisplatin (22). Our results suggest that the increased sensitivity of the PXN/94 and some other cell lines to tetraplatin compared to cisplatin may be partly due to the increased accumulation of tetraplatin and/or its metabolites and partly due to the inability of these cells to tolerate high levels of the platinum species present after tetraplatin exposure. It is widely believed that PtIV complexes are inert and need to be activated by reduction to the PtII compound (1,2-diaminocyclohexanedicarboxylplatinum(II)) in tissue culture medium and in cells; consequently very little intact drug is present intracellularly (27, 42, 43). Higher platinum levels after exposure to tetraplatin have been reported in both parent and cisplatin resistant human ovarian NIH-OVCAR cells (44).

Results of experiments comparing platinum accumulation after exposure to JM221, a novel platinum(IV) dicarboxylate complex, and cisplatin in a sensitive (CH1) and a resistant (HX/62) cell line indicated that increased drug accumulation may play a major role in the enhanced cytotoxicity of JM221 in these cell lines. We have reported similar results in the SKOV-3 cell line (21). The markedly enhanced platinum accumulation following JM221 exposure may be attributable to the fact that the compound is more lipophilic than cisplatin (20) and thus enters cells more readily by passive diffusion. Indeed, preliminary studies have shown that unlike tetraplatin, JM221 is very stable in tissue culture medium (t½ > 24h); hence it is likely to enter cells intact. However, once inside the cell, the compound is rapidly metabolized to several more polar species, including a PtII species JM118 [amminedichloro(cyclobexylamine)platinum(II)], which are retained more readily and are capable of binding to intracellular components.  

In summary, inasmuch as these cell lines were established from both untreated and pretreated patients, these studies suggest that reduced uptake of cisplatin may play a significant role in the mechanism of acquired and intrinsic resistance to this drug in vivo. Elucidation of the precise mechanism of transport of cisplatin in these cell lines may assist in the design of agents intended to circumvent transport determined resistance. Our data also indicate that cisplatin resistance resulting from reduced drug accumulation may be circumvented by analogues possessing increased lipophilicity.

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REFERENCES


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