Preclinical Antitumor Evaluation of Bis-acetato-ammine-dichloro-cyclohexylamine Platinum(IV): an Orally Active Platinum Drug

Lloyd R. Kelland, George Abel, Mark J. McKeage, Mervyn Jones, Phyllis M. Goddard, Melanie Valenti, Barry A. Murrer, and Kenneth R. Harrap


ABSTRACT

The cytotoxicity of a novel platinum(IV) complex, bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV) (JM216), has been evaluated in vitro against a panel of human tumor cell lines (predominantly ovarian) representative of models of intrinsic and acquired resistance to cisplatin. In addition, the activity of JM216 administered by the p.o. route has been determined in vivo using the murine ADJ/PC6 plasmacytoma and four human ovarian carcinoma xenograft lines. In vitro, against seven human ovarian carcinoma cell lines, JM216 showed similar cytotoxicity and pattern of cytotoxicity to cisplatin (mean 50% inhibitory concentrations of 3.5 μM for cisplatin and 1.7 μM for JM216). The cytotoxicity of JM216 was more dependent on the duration of drug exposure than that of cisplatin, suggesting that extended split-dosing rather than a single bolus administration might be a more appropriate schedule in patients. Using six pairs of acquired cisplatin-resistant and parent human tumor cell lines (four ovarian, one testicular, and one cervical) JM216 exhibited non-cross-resistance (resistance factor of <1.5) in three whereas tetraplatin exhibited partial or full cross-resistance in all six pairs. Notably, in two of the acquired cisplatin-resistant lines (41McisR and HX/155cisR) where JM216 retained activity, resistance has previously shown to be due primarily to reduced platinum uptake. In vivo, following p.o. administration using the cisplatin-sensitive murine ADJ/PC6 plasmacytoma, JM216 showed antitumor selectivity far superior to that observed for either cisplatin, carboplatin, or tetraplatin. Across four human ovarian carcinoma xenografts of widely differing sensitivity to cisplatin and carboplatin, JM216 exhibited p.o. activity, broadly comparable to that observed for i.v. administered cisplatin and carboplatin and markedly superior to i.p. administered tetraplatin. These antitumor properties suggest that JM216 provides a structural lead to platinum complexes which may circumvent transport-determined acquired resistance to cisplatin and is a suitable candidate as an orally administrable platinum complex for phase I clinical trial.

INTRODUCTION

The introduction, some 20 years ago, of cisplatin into the clinical treatment of cancer has resulted in dramatic improvements in the response rates for some tumor types, notably testicular and ovarian carcinoma (1–5). Since then, platinum drug development has proceeded in two broad directions aimed at either modulation of the particularly toxic side effects of cisplatin (especially nephrotoxicity, but also gastrointestinal effects) or circumvention of cisplatin resistance in tumors.

Since 1971, around 20 platinum complexes have entered clinical trial (see Refs. 6 and 7 for recent reviews). However, of these, only one, carboplatin, has received worldwide registration and acceptance.

Carboplatin is undoubtedly able to offer patients platinum-based chemotherapy with a more acceptable level of morbidity, with myelosuppression being dose-limiting (8, 9). However, the results of both randomized cisplatin versus carboplatin and cross-over studies (particularly in advanced ovarian cancer) strongly suggest that the two agents are effective against essentially the same tumor population (10–14).

While the major long-term objective of our platinum-based drug discovery program is to broaden the clinical spectrum of activity of the established drugs, cisplatin and carboplatin, by circumventing resistance, we also aim to facilitate further patient comfort and convenience during chemotherapy. The success of carboplatin is testimony to the importance of addressing patient quality of life in the treatment of cancer. An additional strategy we have used (in conjunction with Bristol-Myers Squibb) is to develop a p.o. platinum drug formulation. This could represent a clinical advantage in terms of ease of administration (particularly in patients who could not be treated systemically) and allow the possibility of treatment on an outpatient basis, thus reducing substantially hospitalization costs. Moreover, a p.o. administered compound might provide differing pharmacological and pharmacokinetic properties and thereby exhibit a different spectrum of antitumor activity to the conventional platinum drugs.

A brief clinical study revealed that carboplatin given p.o. resulted in severe gastrointestinal side effects and poor absorption (15). Our chemistry initiative has focused upon complexes designed to be neutral and more lipophilic than cisplatin/carboplatin, i.e., ammine/amine (so called "mixed amine") platinum(IV) dicarboxylates of general formula [Pt(IV)Cl₂(OCOR)₂NH₄(RNH₂)] where R₁ and R₂ may be aliphatic, alicyclic, or aromatic. Some of the chemical, pharmacological, and antitumor properties of this novel class of platinum complex have been reported previously (16–19) and show that, as a class, they may contain suitable candidate drugs for p.o. administration.

This study reports the in vitro and in vivo antitumor activity of JM216 (R = c-C₆H₄; R₂ = CH₃) against murine (ADJ/PC6 plasmacytoma and L1210 leukemia) and human tumor (mainly ovarian carcinoma) models both sensitive and resistant to cisplatin. Comparison has been made with cisplatin, carboplatin, and tetraplatin (Ormaplatin) which is currently in phase I clinical trial (20).

MATERIALS AND METHODS

In Vitro Studies

Cell Lines. Seven "parent" human ovarian carcinoma cell lines have been used. SKOV-3 (21) and OVCAR-3 (22) were obtained from the American Type Culture Collection. A2780 was kindly provided by Dr. T. Hamilton, (Fox Chase Cancer Center, Philadelphia, PA). Establishment details and biological properties of the remaining four lines (HX/62, PXN/94, CHI, and 41M) have been described previously (16–19) and show that, as a class, they may contain suitable candidate drugs for p.o. administration.

In addition, six pairs of human tumor cell lines (parent line and derived subline with acquired resistance to cisplatin) have been used: 41M/41McisR; CH1/CH1cisR; A2780/A2780cisR; OVCAR-3/OVCAR-3cisR (all ovarian); GCT27/GCT27cisR (testicular nonseminomatous germ cell); and HX155/HX155cisR (cervical carcinoma). The parent 41M, A2780, GCT27, and HX/155 lines were all derived from previously untreated patients. Establishment details of 41M/41McisR, CH1/CH1cisR, A2780/A2780cisR, and GCT27/GCT27cisR have been described previously (Refs. 24–26, respectively). The
derivation of the parent HX/155 cell line has also been previously reported (27). Acquired resistance to cisplatin was developed in the OVCAR-3 and HX/155 cell lines as described previously for the 41M and CHI cell lines (24). Briefly, cells were exposed to increasing concentrations of drug (starting at approximately 10% inhibitory concentration) over a 12-18-month period. Typically, cells were exposed at each concentration three times, after which the concentration was doubled. Exposure was continuous over 3 days; the drug was then removed, and the cells were exposed again when normal growth had resumed.

All lines grew as monolayers in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Imperial Laboratories, Andover, United Kingdom), 50 μg/ml of gentamicin, 2.5 μg/ml of amphotericin B, 2 mM L-glutamine, 10 μg/ml of insulin, and 0.5 μg/ml of hydrocortisone in 10% CO₂/90% air. Cells were periodically checked and found to be free of Mycoplasma; parent lines were used from passage 25 to 60.

**Platinum Drugs.** Cisplatin, carboplatin, and JM216 were synthesized by and obtained from the Johnson Matthey Technology Centre (Reading, Berkshire, United Kingdom). Tetraplatin was kindly provided by Dr. M. Wolpert-Defilippes (National Cancer Institute, Bethesda, MD). The structures of these agents are shown in Fig. 1.

**Assessment of Cytotoxicity.** Platinum drugs were dissolved immediately before use in 0.9% saline or water (for carboplatin) at 1 mM or 500 μM (for JM216). Cytotoxicity was then assessed using the sulforhodamine B assay as described previously (19, 24, 28). Briefly, single viable cells were seeded into 96-well microtiter plates (at concentrations between 5 x 10⁴ to 1 x 10⁵/well in 200 μl growth medium) and allowed to attach overnight. Agents were then added to quadruplicate wells. Unless otherwise stated, exposure was for 96 h. Where the effect of time of exposure of agents was being determined, at the end of the appropriate exposure period, plates were washed with prewarmed phosphate-buffered saline and then with growth medium. Fresh growth medium (200 μl) was then added to each well and the cells were incubated to 96 h after initial addition of the drug.

Basic amino acid content/well was then determined using 0.4% sulforhodamine B, obtained from Sigma Chemical Co., Ltd (Poole, United Kingdom), dissolved in 1% acetic acid.

**Fig. 1.** Structures of the platinum complexes studied.

**In Vivo Studies**

**Tumor Lines.** Two murine tumor models have been used: the solid ADJ/PC6 plasmacytoma and the ascitic L1210 leukemia plus their respective variants with acquired resistance to cisplatin. The L1210 tumor was included merely to provide a comparative measure of antitumor activity with historical data for other antitumor drugs. Details of the generation of the acquired resistant lines and the growth of the tumors (ADJ/PC6 in syngeneic female BALB/c mice and L1210 in DBA₂ mice) have been described previously (29).

In addition, four human ovarian carcinoma xenografts have been used: PXN/109T/C; SKOV-3; OVCAR-3, and HX/110. These lines (grown s.c. in female BALB/c nude mice) were chosen from our previously described panel of 16 ovarian carcinoma xenografts to encompass the broad range in responsiveness observed to the reference platinum drugs cisplatin, carboplatin, and iproplatin [(CHIP: JM9; and cis-dichloro-trans-dihydroxy-cis-bis(isopropy-lamine) platinum (IV)] (30). PXN/109T/C (which was derived from the CHI cell line) is comparably sensitive to all three reference drugs, SKOV-3 (again derived from the cell line) is refractory to all three drugs, OVCAR-3 (also derived from the cell line) shows a differential sensitivity to iproplatin, and HX/110 shows a differential sensitivity to cisplatin and carboplatin. We have previously shown, using eight companion human ovarian carcinoma lines, an excellent in vitro versus in vivo correlation (r = 0.88) in cisplatin cytotoxicity/responsiveness (30).

**Assessment of Antitumor Activity**

**ADJ/PC6.** This was performed as described previously (29). Briefly, 20 days post-s.c. implantation of 1-mm³ tumor fragments, animals bearing comparably sized tumors were randomized (3/dose level and 10 vehicle-only controls) and drugs were administered as a single dose (i.p. or by p.o. gavage as indicated) as sonicated suspensions in arachis oil. With the p.o. gavage procedure, drug was essentially administered directly into the stomach through the usage of a specialized wide-gavage needle thus ensuring reliable dosing and eliminating any regurgitation of drug. Ten days later, tumors were dissected out and the weights of control and treated groups were compared.

Fig. 1. Structures of the platinum complexes studied.
Antitumor activity has been defined in terms of a "therapeutic index," the ratio of the 50% lethal dose to ED90. L1210. This was also performed as described previously (29). Briefly, 5 mice/treated group and 10 untreated controls were implanted i.p. with 1 × 106 cells. Drugs were administered (i.p. or by p.o. gavage in arachis oil) on days 1, 5, and 9 postimplantation. JM216 was administered at 1, 2, 4, 8, 16, and 32 mg/kg by the i.p. route and 8, 16, 32, 64, 128, and 256 mg/kg by the p.o. route. Activity has been assessed in terms of increase in life-span although it is important to note that animals were killed painlessly at the onset of moribundity rather than being allowed to die from the effects of tumor or drug-induced toxicity.

**Human Ovarian Carcinoma Xenografts.** Mice bearing comparably sized tumors (typically of 8 mm largest diameter) were randomized into treatment groups (6 animals) or control groups (10 animals). Drugs were administered in 0.9% saline (or sonicated in arachis oil for JM216) at previously determined maximum tolerated doses (approximately 10% lethal dose) on days 0, 7, 14, and 21. Tumors were measured weekly until they doubled their starting volume and responses were assessed as described previously (30–32). Briefly, the two longest tumor diameters (a and b) were measured, tumor volume (V) was calculated according to the equation:

\[ V = \frac{a \times b^2}{2} \]

and volumes were normalized to their respective day 0 values. Growth delays (the difference in time required for control and treated tumors to double in volume) were then determined. Approximate control tumor volume doubling times (in days) were 7.2 for PXN/1097/C. 6.2 for SKOV-3, 17.3 for OVCAR-3, and 8.7 for HX/110.

**RESULTS**

**In Vitro Cytotoxicity.** Cytotoxicity determinations for cisplatin, carboplatin, tetraplatin, and JM216 against a panel of seven human ovarian carcinoma cell lines are shown (Fig. 2). Across the seven lines, JM216 showed a similar cytotoxicity and cell line ranking to cisplatin; mean IC50 values (μM) were cisplatin, 3.5 (range 12.6 to 0.11); carboplatin, 26.3 (range 70 to 1.34); tetraplatin, 3.2 (range 16.7 to 0.16); and JM216, 1.7 (range 4.6 to 0.084). As noted previously (23), the PXN/94 cell line shows a striking differential sensitivity to tetraplatin; this property was not apparent with JM216. A comparison of patterns of response across the panel has been performed using Spearman rank analysis. From such an analysis, a high statistically significant correlation coefficient (r) for a given pair of compounds is indicative of a similar pattern of response across the seven cell lines whereas a low, nonsignificant coefficient indicates that the two compounds are acting in different ways. Calculated coefficients were as follows: cisplatin/carboplatin, 0.929 (P < 0.01); cisplatin/tetraplatin, 0.536 (P > 0.05); cisplatin/JM216, 0.857 (P = 0.01); carboplatin/tetraplatin, 0.536 (P > 0.05); carboplatin/JM216, 0.964 (P < 0.01); and tetraplatin/JM216, 0.464 (P > 0.05).

Fig. 3 shows the effect of alterations in the time of exposure on cytotoxicity (in terms of IC50) for cisplatin, carboplatin, tetraplatin, and JM216 for the relatively cisplatin-resistant SKOV-3 cell line and the cisplatin-sensitive 41M cell line, respectively. For the SKOV-3 cell line, cisplatin and JM216 were markedly more cytotoxic than tetraplatin and carboplatin whereas, with the 41M cell line, cisplatin was the most potent at each exposure time point. Calculated time dependency indices (IC50 2-h exposure followed by 94 h culture without drug/IC50 96-h exposure) were as follows: cisplatin, 12.2 for SKOV-3 and 12.6 for 41M; carboplatin, 14 for SKOV-3 and 31.5 for 41M; tetraplatin, 28 for SKOV-3 and 10.6 for 41M and JM216, 28.6 for SKOV-3, and 37.7 for 41M. While cytotoxicity was essentially unchanged for 96-versus 24-h exposure for both cisplatin and tetraplatin (mean time dependency indices across the two cell lines of 1.1 and 1.2, respectively), for JM216, the IC50 following a 96-h drug exposure was a mean of 1.8-fold lower than that obtained after a 24-h drug exposure. Similarly, for carboplatin, the mean 96-h/24-h IC50 comparison value was 2.

**Activity of JM216 against Cisplatin-acquired Resistant Cell Lines.** Fig. 4 shows resistance factors (IC50 acquired resistant line/IC50 parent line) for six pairs of human tumor cell lines (parent and variant with in vitro-derived acquired resistance to cisplatin) for cisplatin, carboplatin, tetraplatin, and JM216. Carboplatin exhibited cross-resistance in all six pairs of lines. Defining non-cross-resistance as a resistance factor of less than 1.5, tetraplatin showed at least partial cross-resistance in all pairs; for the OVCAR-3 and HX/155 lines, resistance to a similar level as that observed for cisplatin itself was observed. JM216, however, exhibited non-cross-resistance in three of the six resistant lines (41M, OVCAR-3, and HX/155) and only partial cross-resistance in the remainder.

**In Vivo Antitumor Activity.** Table 1 summarizes antitumor and toxicity data obtained using the murine ADJ/PC6 plasmacytoma following either i.p. or p.o. single-dose administration. With cisplatin, carboplatin, and tetraplatin both toxicity (50% lethal dose) and particularly antitumor efficacy (ED90) were reduced by the p.o. route as compared to the i.p. route resulting in lower therapeutic indices (ED90 values were 40-, 16.5-, and 56-fold higher for p.o. versus i.p. administration for cisplatin, carboplatin, and tetraplatin, respectively). With JM216, however, while toxicity was reduced 10-fold upon p.o. administration, ED90 values were similar for both routes of administration.

JM216 was also evaluated against the cisplatin-resistant variant of the ADJ/PC6 tumor (29). Following p.o. gavage, although the ED90 value was 180 mg/kg (around 30-fold higher than that observed in the parent tumor) a small therapeutic index (2.2) was achieved. JM216 did not show appreciable activity against the murine L1210 and cisplatin-resistant leukemia ascites tumors; maximum percentage increases in life spans were 41 and 11.4% for i.p. administration against parent and cisplatin-resistant tumors, respectively, and 2 and 21% for p.o. administration against parent and resistant tumors, respectively.

**Activity against Human Ovarian Carcinoma Xenografts.** The antitumor efficacy of the four platinum compounds (cisplatin and carboplatin administered by i.v. injection, tetraplatin administered i.p., and JM216 administered by p.o. gavage) against four human ovarian carcinoma xenografts is shown in Fig. 5. Growth delays are presented from a single parallel experiment. None of the agents were particularly effective against the highly platinum-resistant SKOV-3 tumor (maximum growth delay of 3 days with JM216). Against the remaining three tumor lines, JM216 exhibited...
The development of 1,2-diaminocyclohexane-containing platinum complexes including tetraptalin and oxaliplatin (1,2-diaminocyclohexane oxalato platinum) (which has recently undergone phase I testing in France; Ref. 32) originated largely through the demonstration of their activity against cisplatin-resistant murine L1210 and P388 leukemia cell lines (33, 34). Spearman rank analysis of the cytotoxicity data revealed that tetraptalin, but not JM216, elicits a completely

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DISCUSSION

A novel ammine/amine platinum (IV) dicarboxylate, JM216, a platinum coordination complex designed for p.o. administration, has been evaluated in a number of in vitro and in vivo preclinical tumor models. Although both tetraptalin (currently in phase I trial in the United States) and JM216 possess bulky lipophilic amine ligands (1,2-diaminocyclohexane and ammine/cyclohexylamine, respectively) the 2 agents exhibit individually distinct in vitro and in vivo antitumor properties. In terms of in vitro cytotoxicities this was most clearly apparent with the PXN/94 and SKOV-3 cell lines. While tetraptalin was less potent than cisplatin in the SKOV-3 cell line (3.8-fold), it was dramatically more potent in the PXN/94 cell line (19-fold). JM216, however, was between 2.5- and 2.8-fold more potent than cisplatin in both cell lines. At least part of the enhanced potency of tetraptalin in the PXN/94 cell line appears to be attributable to a 4.2-fold increase in intracellular accumulation compared to cisplatin (31).

Studies using six pairs of parent and cisplatin-resistant cell lines of human origin revealed shared cross-resistance between cisplatin and carboplatin in all six lines. These in vitro data thus reflect clinical observations in advanced ovarian cancer of shared cross-resistance between the two drugs (11-13).
different pattern of response to cisplatin and carboplatin. Nevertheless, it is encouraging that (in the six pairs of human tumor lines used in this study) JM216 showed a superior circumvention of cisplatin resistance than tetraplatin in three of the lines (41M, OVCAR-3, and, particularly, the cervical carcinoma line HX/155).

With the 41M pair of lines, where the major mechanism responsible for resistance to cisplatin is decreased uptake (24), the non-cross-resistance observed with JM216 appears to correlate with enhanced uptake in the resistant line relative to cisplatin (35). For example, following exposure to cisplatin (25 μM × 2 h) platinum uptake in the resistant line was only 18% of that observed in the parent line. However, following exposure to JM216 (again, 25 μM × 2 h) platinum uptake was 37% higher in the resistant compared to the sensitive line. Moreover, similar findings have been found for the HX/155 and HX/155cisR lines (36). In the resistant line, platinum uptake was 42% of that observed in the parent line following a 2-h exposure to cisplatin and it was 55% following tetraplatin, whereas, following exposure to JM216, there was no significant difference in uptake. The mechanisms underlying resistance in the OVCAR-3 pair of lines are, at present, unknown. Interestingly, in two cisplatin-resistant lines, CH1cisR (24) and GCT27cisR (26), in which enhanced DNA repair of platinum-DNA adducts has been shown to be mainly responsible for the resistance, tetraplatin exhibited slightly lower resistance factors than JM216 (CH1 pair, 2.9 versus 4; GCT27 pair, 1.9 versus 2.9). With the A2780 pair of lines, in which resistance has been shown to be due to decreased accumulation (37), increased intracellular glutathione (25) and enhanced DNA repair (38), tetraplatin and JM216 showed similar levels of partial cross-resistance. It is noteworthy that both tetraplatin and JM216 exhibit complete non-cross-resistance in vitro against L1210 cells with acquired resistance to cisplatin (39). Overall, these data suggest that JM216 provides a structural lead to platinum complexes which may circumvent transport-determined resistance to cisplatin.

The results of experiments investigating the effect of exposure time on cytotoxicity might have relevance in guiding dose scheduling in patients. These data suggest that the antitumor activity of JM216 might be more dependent on exposure time than for either cisplatin or tetraplatin. Therefore, it would appear beneficial (from an antitumor viewpoint) to maintain adequate plasma levels of JM216 for longer periods than for cisplatin or tetraplatin through the usage of split dosing over several days rather than using a single bolus administration. If these findings are repeated in vivo then a p.o. preparation would appear to be better suited (in terms of ease of administration and hospitalization costs) than i.v. therapy for delivering such schedules.

In summary, JM216 exhibits comparable in vitro cytotoxicity to cisplatin and encouraging activity against some cisplatin-resistant human tumor lines. In vivo, following p.o. dosing of mice bearing the murine ADJ/PC6 plasmacytoma, JM216 demonstrated markedly superior antitumor efficacy to cisplatin, carboplatin, and tetraplatin. This is a tumor model which we have used previously in preclinical studies identifying carboplatin as a viable alternative to cisplatin (40). Across four human ovarian carcinoma xenografts of varying sensitivity to cisplatin (and carboplatin) JM216 exhibited p.o. activity broadly comparable to i.v. administered cisplatin and carboplatin. The activity of JM216 was markedly superior to i.p. administered tetraplatin.

Toxicological studies performed with JM216 in rodents indicate properties reminiscent of carboplatin rather than cisplatin (41). Specifically, JM216 administered p.o. at maximum tolerated doses (200 mg/kg) exhibited no decrease in glomerular filtration rate (as measured by 14C-inulin clearance) relative to controls (42). In rodents, myelosuppression was the dose-limiting toxicity; no hepatotoxicity was evident and gastrointestinal effects were less than those observed with cisplatin and carboplatin. These antitumor properties and favorable toxicological properties exhibited by JM216 suggest that JM216 represents a suitable candidate as an p.o. administrable platinum complex for phase I clinical trial.

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