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Modification of methyliminodiacetato-*trans*-*R*,*R*-1,2-diamminocyclohexane platinum(II) pharmacology using a platinum-specific monoclonal antibody*

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Summary. Platinum complexes are extremely active chemotherapeutic agents. A murine monoclonal antibody designated 1C1 was developed that binds to the third-generation platinum complex methyliminodiacetato-*trans*-*R*,*R*-1,2-diamminocyclohexane platinum(II) (MIDP). Competitive enzyme-linked immunosorbent assay (ELISA) shows that antibody 1C1 binds preferentially to the 1,2-diamminocyclohexane (DACH) side-chain of the platinum complex, although non-DACH-containing platinum complexes can compete for binding at high concentrations. When tested against MCF-7 breast carcinoma cells, the 1C1-MIDP complex caused 50% growth inhibition at 0.63 μg Pt/ml, whereas MIDP alone caused 50% growth inhibition at a concentration of 0.16 μg Pt/ml. Pharmacokinetic studies in rats using [^{3}H]-MIDP showed that the drug was cleared triphasically from plasma, with elimination-phase half-lives ($t_{1/2}$) of 1.2, 10.2, and 243 min for α, β, and γ phases, respectively. The MIDP-1C1 complex was cleared with longer half-lives of 5, 26, and 291 min, respectively. The overall clearance rate from plasma of the MIDP-1C1 complex was 10-fold lower than that of MIDP alone (0.37 vs 3.01 ml/kg × min). Tissue concentrations of [^{3}H]-MIDP 3 h after administration showed that 1C1 antibody prevented MIDP distribution to most organs and dramatically reduced [^{3}H] concentration in the intestine, liver, kidney, heart, and skeletal muscles. Studies are under way to determine the relative therapeutic activity of the 1C1 antibody-MIDP complex and assess whether the 1C1 antibody may be useful for antibody-directed delivery of platinum complexes to tumors.

Introduction

Cisplatin [cis-diamminedichloroplatinum(II)] was the first inorganic antitumor complex to be used clinically. This drug, which still serves as the parent-drug complex with which newer platinum derivatives are compared, has shown impressive antitumor activity in clinical trials against a variety of human tumors [3, 7, 16, 23]. The biological effects of platinum complexes include damage to mitochondria, altered calcium metabolism, and disruption of microfilament organization [1, 15]. However, the major antiproliferative effects of platinum complexes are cytostatic in nature because of specific damage to DNA [26] as well as direct metabolic damage that follows the pattern of classic heavy-metal poisoning, probably caused by interaction of the platinum complex with proteinaceous sulfhydryl groups, resulting in vital enzymatic disruption [2]. It has been postulated that the sulfhydryl reactivity of platinum complexes may be the basis for platinum nephrotoxicity [21]. In spite of impressive antitumor properties, however, the utility of platinum complexes has been severely compromised by their profound and dose-limiting toxicities, which include nephrotoxicity, nausea and vomiting, ototoxicity, and neurotoxicity [31].

In attempts to improve the therapeutic index of this class of metal compounds, over 1,000 platinum complex analogs have been synthesized. In particular, the 1,2-diamminocyclohexane platinum derivatives demonstrate impressive antiproliferative activity. One agent in the series, methyliminodiacetato-*trans*-*R*,*R*-1,2-diamminocyclohexane platinum(II) (MIDP), appears to be the most active of the leading, relatively nontoxic antitumor platinum complexes. MIDP [14] (Fig. 1) has been shown to be curative in mice against L1210 leukemia and B16 melanoma using a multidose regimen. Additionally, at equieffective cisplatin doses, MIDP demonstrates no renal toxicity in animal models [25]. Furthermore, cells resistant to the parent cisplatin complex have been shown to retain sensitivity to MIDP [14].

Murine monoclonal antibodies to tumor-associated surface antigens have been shown to localize within human tumors after systemic administration in man [9, 10, 19]. This powerful property of monoclonal antibodies has been successfully exploited to provide selective tumor targeting of drugs, toxins, and radionuclides [5, 20, 24, 30]. Antibody targeting of platinum complexes represents the next logical step in imparting improved selectivity and tumor specificity to cytotoxic platinum drugs. However, chemical modification of platinum complexes and coupling to monoclonal antibodies can lead to a rapid loss of antiproliferative activity [6, 17]. Because of their labile chemical nature, platinum complexes are therefore not well-suited for chemical coupling directly to tumor-targeting antibodies. However, the active platinum complex MIDP contains a stable cyclohexane ligand (Fig. 1)

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Abbreviations: MIDP, methyliminodiacetato-*trans*-*R*-*R*-1,2-diamminocyclohexane platinum(II); *MTT*, 2,5-diphenyltetrazolium bromide thiazolyl blue

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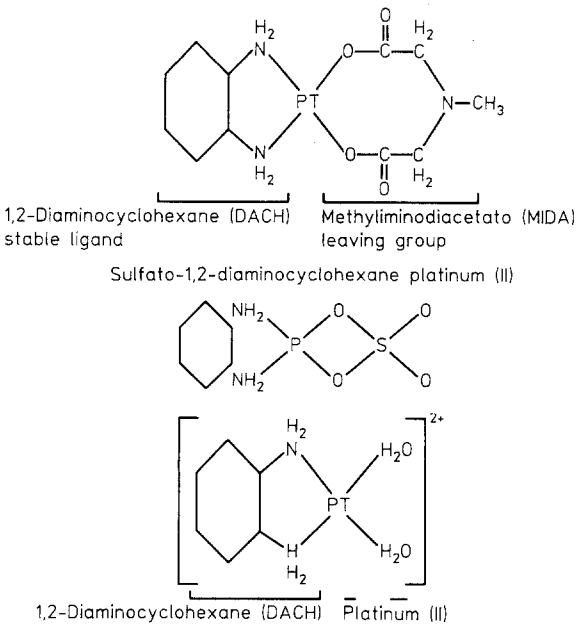

Fig. 1. Structure of platinum complexes (methyliminodiacetato-1,2-diaminocyclohexane platinum(II); MIPP)

that may be used advantageously as an antibody-binding site, thereby enabling its reversible binding to a monoclonal antibody without inactivation of this agent by structural modification.

In this report we describe the development of a murine monoclonal antibody that binds to platinum complexes. The specificity and characteristics of this antibody are described, as well as the utility of this reagent for selective alteration of the in vivo pharmacology of MIDP.

Materials and methods

MIDP was synthesized as previously described [14]. An analog labeled with [^{3}H] in the diamminocyclohexane-ring position (specific activity = 0.402 mCi/mg) was synthesized for use in comparative binding studies. Calf-thymus DNA and MTT were purchased from Sigma Chemical Co. (St. Louis, Mo). Phosphate-buffered saline (PBS) was purchased from GIBCO Laboratories (Grand Island, NY). Spectra/Por dialysis tubing (M. W. C. O. 12–14,000) was obtained from Spectrum Medical Industries, Inc. (Los Angeles, Calif). Aquasol scintillant was obtained from New England Nuclear, Inc.

Preparation of anti-platinum murine monoclonal antibodies. MIDP bound to a solid support was used for mouse immunization. DNA-sepharose (Sigma Chemical Co.) was washed twice with distilled water and briefly centrifuged to form a pellet. MIDP (50 μg) was dissolved in 1 ml distilled H_2O and applied to the DNA-cellulose pellet. The solution was incubated with shaking for 24 h at room temperature. The MIDP/DNA-cellulose product was washed three times with distilled water.

Monoclonal antibodies to the platinum complex MIDP were prepared according to standard procedures. Briefly, spleen cells derived from BALB/c mice that had been immunized with MIDP/DNA-sepharose complex were fused with a myeloma cell line essentially as described by Kohler and Milstein [18]. Following fusion, cells were plated into 96-well microliter plates. For enzyme-linked immunosorbent assay (ELISA) studies, 50 μl calf-thymus DNA (Sigma, 100 μg/ml) in distilled H_2O was added to each well of a 96-well plate and dried overnight. Then, 50 μl MIDP in H_2O (100 μg/ml) was added to each well and incubated overnight at 37° C. The plates were washed three times with PBS, hybridoma supernatant was applied, and a standard ELISA assay was carried out to determine the presence of murine antibody bound to the wells. Those hybrids producing antibodies of appropriate specificity were subcloned by limiting dilution to insure monoclonality. Hybridoma $1C1H_2A5$ (designated 1C1) was selected based on its ability to secrete an antibody that binds to platinum complex; this antibody belongs to the IgM subclass. Culture supernatants were harvested from antibody-producing cells and concentrated 10-fold using an Amicon concentrator (XM-30 membrane), and the resulting concentrate was further purified by both S-300 gel permeation and DEAE ion-exchange chromatography [8]. Antibody thus prepared was approximately 80% pure as analyzed by polyacrylamide gel electrophoresis.

Complex of antibody $1C1H_2A5$ and [^{3}H]-MIDP. A 1-μg sample of purified 1C1 in 1 ml PBS was incubated with 2 μg [^{3}H]-MIDP (total cpm, 1×10^6) for 24 h at 4° C. The samples were then dialyzed for 24 h at 4° C against 2 l PBS. Aliquots of dialysate were removed for [^{3}H] analysis by their addition to 10 ml Aquasol scintillant. Radioactivity was determined using a Packard scintillation spectrometer (Model 1500). To assess [^{3}H]-MIDP, free or bound to antibody 1C1, an aliquot of dialysate was subjected to HPLC on a Waters system consisting of two M600A pumps, a Wisp 710B sample injector, a 720 system controller, a data module, and a model 421 variable-wavelength UV detector. Chromatographic separation of [^{3}H]-MIDP from [^{3}H]-MIDP antibody complex was accomplished using a Varian TSK 3000 SW gel-permeation column (1.2 × 60 cm). The samples were eluted with 0.2 *M* Na_2HPO_4 at a flow rate of 0.5 ml/min. The column effluent was collected into 0.5-ml fractions using a Gilson (FC-80) fraction collector, and [^{3}H] was assessed in the eluate by counting a 250 μl aliquot of each fraction in 10 ml Aquasol.

In vitro effect of antibody 1C1 on the antiproliferative activity of MIDP. To determine whether MIDP bound to the 1C1 antibody retained antiproliferative activity, the MIDP/antibody complex was formed by incubation of 2 mg purified antibody with 50 μg MIDP in PBS at 4° C for 24 h. Free MIDP was removed by dialysis against 1 l PBS for 24 h at 4° C.

An MIDP stock solution (10 mg/ml in PBS) was then prepared and both solutions were sterile-filtered. Aliquots of both solutions were removed for platinum analysis by atomic absorption spectrometry [28]. The solutions were adjusted to equivalent platinum concentrations and either MIDP or the MIDP/antibody complex was added to log-phase MCF-7 cells that had been plated at 5,000 cells/well for 24 h. After treatment, the plates were incubated for 72 h at 37° C (5% CO_2). Relative cell number was assessed using the MTT assay as previously described [11].

In vivo pharmacokinetics and tissue distribution of [^{3}H]-MIDP and [^{3}H]-MIDP/antibody complex. To compare the in vivo pharmacology of [^{3}H]-MIDP to that of [^{3}H]-

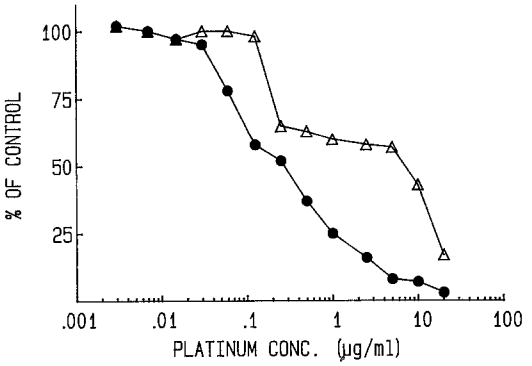

Fig. 2. Competitive ELISA assay of 1C1 cisplatin and MIDP. A fixed amount of 1C1 antibody was added to various concentrations of either MIDP or cisplatin. After incubation, the antibody-platinum mixture was added to MIDP-coated plates and a standard ELISA was carried out. These curves indicate that both cisplatin and MIDP compete with plate-bound MIDP for binding to the antibody. △ Cisplatin; ● MIDP

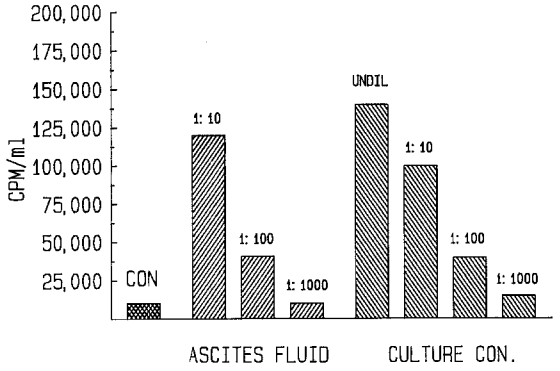

Fig. 3. Equilibrium dialysis of antibody 1C1 against [^{3}H]-MIDP. Antibody 1C1 obtained from either ascites or cultured cells was added to a fixed amount of [^{3}H]-MIDP. After incubation, the Pt/antibody complex was dialyzed to remove free [^{3}H]-MIDP. Aliquots were counted to determine [^{3}H]-MIDP bound to the antibody compared with a concentration-matched, irrelevant IgM control

MIDP/antibody complex, male Fischer rats weighing 250–260 g were lightly anesthetized with sodium thiopental. Plastic cannulae were surgically inserted into both the femoral vein and the femoral artery. A bolus dose of 1×10^6 cpm of either [^{3}H]-MIDP in PBS or [^{3}H]-MIDP/antibody complex (preincubated for 18 h at 4° C and dialyzed for 24 h against PBS to remove free MIDP) was given over 25 s via the venous catheter. Blood samples (0.5 ml) were withdrawn at various times from the arterial catheter. After each blood withdrawal, 0.5 ml PBS was given via the venous catheter to maintain fluid balance.

The blood samples were centrifuged at 12,000 *g* in a Beckman microfuge for 10 min. The plasma was decanted, and duplicate 50 µl aliquots of plasma were added to 10 ml Aquasol for determination of radioactivity. At 180 min after injection, the animals were sacrificed by cervical dislocation. Samples of various organs were removed and weighed, and 0.5-g samples were oxidized in a Packard model C-306 sample oxidizer to determine [^{3}H] content.

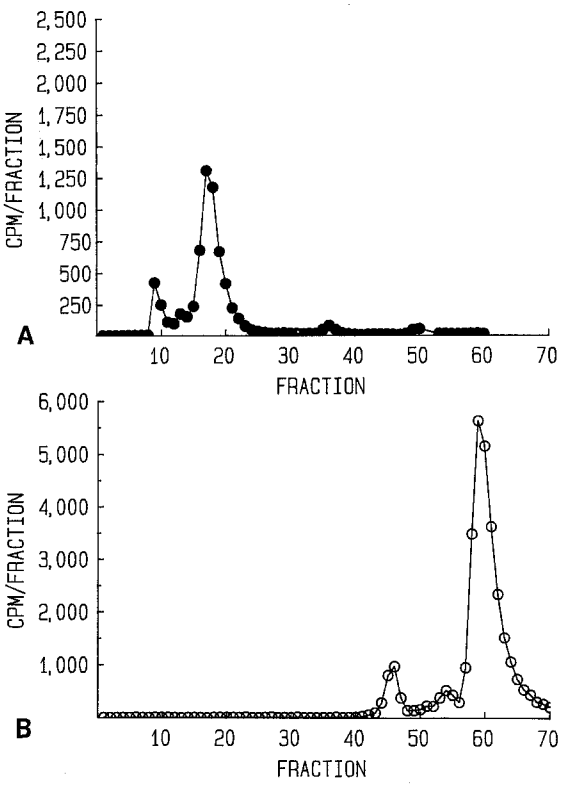

Fig. 4. A An aliquot of the [^{3}H]-MIDP/antibody dialysate from the previous experiment was chromatographed on a TSK 3000SW gel-permeation HPLC column. Aliquots of the effluent were collected and counted. As shown, all of the [^{3}H] label migrated as antibody-bound complex; there was no free [^{3}H]-MIDP. **B** Gel-permeation chromatogram of [^{3}H]-MIDP standard

Results

The third-generation platinum complex MIDP (Fig. 1) is composed of a stable ligand, 1,2-diamminocyclohexane (DACH), in a coordinate-covalent complex containing the platinum ion and a leaving group, methyliminodiacetato (MIDA), that aquates in solution, yielding DACH-Pt^{+2}, $(H_2O)_2$, the active alkylating agent [13]. Antibodies against the MIDP complex were raised against either the stable ligand or the platinum-ion portion of the complex. Coupling of the platinum complex to larger carrier molecules (such as DNA) was carried out for hybridoma generation and for use as a matrix support for binding to 96-well plates in antibody screening studies. Approximately 100 hybrids (of 1,000 original wells) were found to produce antibodies and were further cloned and subcloned by limiting dilution. The screening criteria for selection of hybrid clones were: (a) ELISA positive for DACH-Pt-DNA, unreactive against unmodified DNA; (b) ELISA positive for MIDP; and (c) ability to immunoprecipitate [^{3}H]-MIDP.

Several clones were developed that passed the screening criteria. Final selection based on immunoprecipitation delineated an IgM-class antibody designated $1C1H_2A_5$. Antibody 1C1 was chosen for further study. To determine whether antibody 1C1 recognizes the DACH side-chain alone or the entire Pt-DACH molecule, a competitive ELISA was carried out using samples containing equal amounts of platinum, either cisplatin or the MIDP analog preincubated with antibody 1C1 (4° C for 2 h). The an-

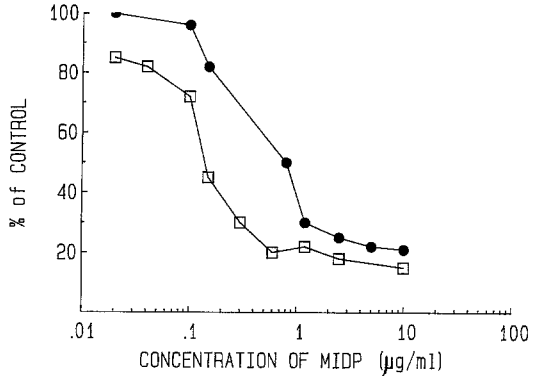

Fig. 5. Antiproliferative effect of MIDP-antibody and MIDP against MCF-7 cells. MIDP or MIDP/antibody complex was added to log-phase MCF-7 cells in 96-well plates and incubated for 72 h at 37° C (5% CO_2). Growth inhibition of 50% with MIDP alone occurred at 0.2 μg Pt/ml, whereas the complex required a concentration of 0.8 μg Pt/ml for the same effect. ● Antibody + MIDP; □ MIDP alone

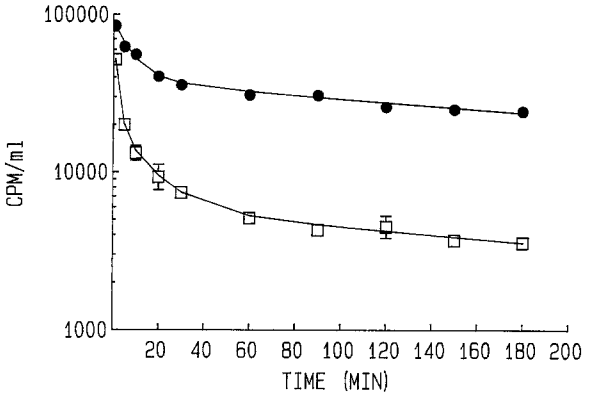

Fig. 6. Plasma clearance of [^{3}H]-MIDP and [^{3}H]-MIDP/antibody complex in the anesthetized rat. Equivalent doses of [^{3}H] label were injected into each group (3 rats/group) as outlined in Preliminary studies. Shown are the means ± SEM of single determinations on three animals. *Solid lines* represent the calculated curves from exponential regression analysis (NONLIN) of the data points. In both cases, the data closely fit (r^2 = 0.96) a triphasic model for clearance. Pharmacokinetic analyses are shown in Table 1. ● Antibody + [^{3}H]-MIDP; □ [^{3}H]-MIDP

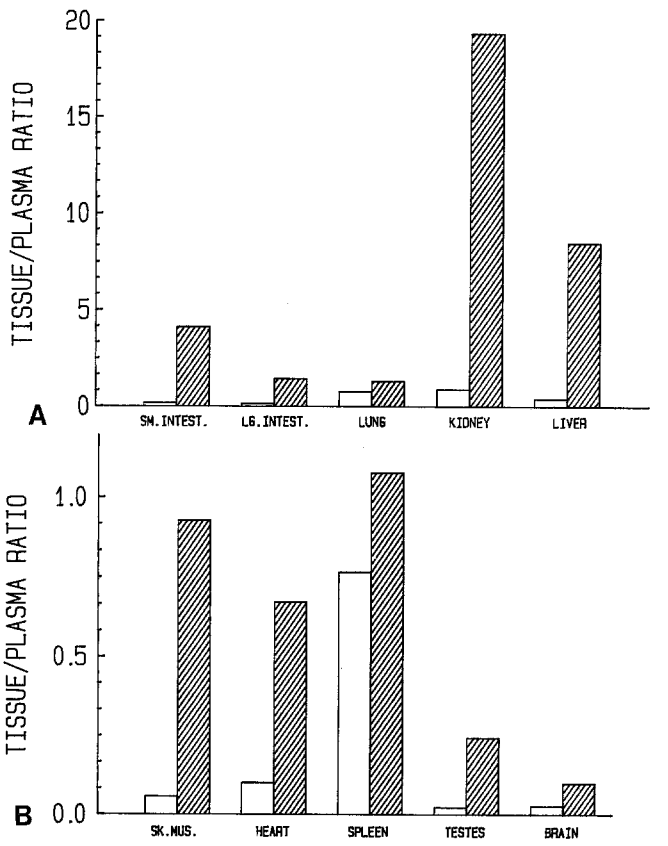

Fig. 7 A, B. Comparative tissue distribution of [^{3}H]-MIDP and [^{3}H]-MIDP/antibody complex in the rat. As shown, 180 min after i. v. injection, the major sites of distribution of [^{3}H]-MIDP/antibody complex, in decreasing order, were kidney >liver >sm. intest. □ Antibody + [^{3}H]-MIDP; ▨ [^{3}H]-MIDP

tibody-platinum mixtures were then assessed by ELISA for binding to an MIDP-coated 96-well plate. As shown in Fig. 2, both MIDP and cisplatin can compete for antibody binding to MIDP-coated plates. However, the concentration of MIDP required to compete for 50% inhibition of binding was substantially lower than that required for cisplatin competition (0.2 vs 10 μg/ml). This suggests that the DACH side-chain of the MIDP molecule may be preferred for recognition by the antibody and that the platinum complex itself can also be recognized by this antibody.

To determine the platinum-loading capacity of antibody 1C1 in solution, [^{3}H]-MIDP was added to various concentrations of DEAE-purified 1C1 antibody (obtained from either murine ascites or in vitro growth of hybrids) and incubated for 2 h at 4° C in phosphate buffer (0.05 *M*, pH 7.2). The samples were then dialyzed overnight (at 4° C) against a 1:1,000 volume of 0.05 *M* phosphate buffer. Samples of dialysate were then counted to determine total [^{3}H] content. As shown in Fig. 3, irrelevant-control murine IgM contained 10,000 cpm/ml, whereas a 1:10-diluted sample of concentration-matched 1C1 antibody contained 12-fold higher platinum concentration (120,000 cpm/ml). Analysis of the dialysate by size exclusion HPLC (60 × 1.2 cm TSK-3000 column) showed that after 24 h of dialysis, free [^{3}H]-MIDP remained (Figs. 4 A, B) and that all of the [^{3}H] migrated as a high-molecular-weight complex bound to the 1C1 antibody.

As a test to determine whether MIDP complex retained cytotoxic activity when bound to a monoclonal antibody, purified antibody 1C1 was incubated with MIDP for 2 h at 4° C. Unreacted MIDP was removed by dialysis overnight against two changes of phosphate buffer (0.05 *M*, pH 7.4). The MIDP/antibody complex was then sterile-filtered, and an aliquot was removed for platinum analysis by flameless atomic-absorption spectrophotometry. The remaining sample was added in quadruplicate to a 96-well plate containing breast carcinoma (MCF-7) cells in log-phase culture. An equivalent amount of MIDP alone was added in quadruplicate to the other half of the plate, and the cells were incubated for 72 h at 37° C in 5% CO_2. A tetrazolium dye-binding assay (MTT 85) was carried out to determine the number of metabolically active cells relative to control (untreated) wells. As shown in Fig. 5, the MIDP/antibody complex (containing no free MIDP) was effective in suppressing cell growth in vitro but was approximately 5-fold less active than free MIDP on an

equal-platinum basis. Nevertheless, these data suggest that when bound to a murine antibody, MIDP can still retain a substantial degree of cytotoxicity compared with the parent complex.

In this regard, we conducted a pharmacokinetic study of [^{3}H]-MIDP compared with [^{3}H]-MIDP bound to monoclonal antibody 1C1. A small amount of this IgM antibody was purified using DEAE chromatography and allowed to react with [^{3}H]-MIDP overnight at 4° C. Excess [^{3}H]-MIDP was removed by equilibrium dialysis for 24 h. Rats were anesthetized using sodium thiopental. Blood samples were taken from a small, indwelling venous catheter at various times after administration of either [^{3}H]-DACH-Pt/antibody complex. The clearance of [^{3}H]-MIDP itself from plasma fit a triphasic curve ($t_{1/2\alpha}$ = 1.24 min; $t_{1/2\beta}$ = 10 min; $t_{1/2\gamma}$ = 243 min). The plasma clearance for platinum/antibody complex also fit a triphasic curve for clearance ($t_{1/2\alpha}$ = 5.21 min; $t_{1/2\beta}$ = 26 min; $t_{1/2\gamma}$ = 291 min) but was substantially different from that of the free drug (Fig. 6, Table 1). The AUC for platinum/antibody complex was 7-fold higher than that found for the free drug alone, suggesting reduced tissue distribution of the antibody/drug complex. The tissue-disposition data confirms that the antibody prevented the platinum from distributing to the kidney, small intestine, and liver as well as to other organs (Figs. 7 A, B) and maintained the drug in circulation.

Discussion

The development of new and less toxic antitumor agents for therapeutic use is predicated, at least in part, on changes in the agent that favorably modify its pharmacology. Prolongation of plasma half-life, protection against enzymatic degradation, improving the disposition of drugs to target sites, and reducing the distribution of drug to sensitive toxicity sites are all of major concern in therapeutic design. Several investigators have attempted to modify the pharmacology of chemotherapeutic agents such as daunorubicin [22] and methotrexate [4] by covalently linking them to high-molecular-weight carriers. This enables the continuous release of the agents from the complex, thus providing sustained plasma drug levels and prolonged plasma half-lives.

Previous studies [27, 29] have also shown that monoclonal antibodies may be uniquely suited to the same purpose. Studies in our laboratory [27] and by other groups [29] have shown that the plasma pharmacokinetics and tissue disposition of biological response modifiers such as interferon and radioisotopes can be substantially modified by prior adsorption to monoclonal antibodies specific for these agents. Nonneutralizing antibodies to recombinant human leukocyte interferon (rIFNαA) were shown to prolong the in vivo half-life of rIFNαA biological activity. In vivo administration of an rIFNα/antibody complex to anesthetized rats resulted in a 3-fold increase in the plasma half-life and a 17-fold increase in the calculated AUC of interferon biological activity compared with the same dose of free interferon.

The use of a specific antibody to prolong the biological activity of rIFNαA and modify the plasma clearance and tissue distribution of MIDP is in marked contrast to classic antibody therapy, in which the basic function of the immunoglobulin is both to block the biological activity of the agent and to facilitate its removal from the body as an antigen/antibody complex. A recent study [12] has shown that $F(AB)_2'$ fragments of a monoclonal antibody reactive with digoxin can inactivate this agent when given to a patient suffering from digoxin overdose. From our studies it is clear that, by appropriate selection, specific antibodies may be used to either prolong or reduce the plasma concentration of biologically active therapeutic agents. Studies are now under way to determine whether this antibody-mediated increase in the plasma half-life of MIDP complex is translated into an improved therapeutic effect.

This method of modifying the pharmacology of MIDP may provide a distinct advantage over chemically coupling it to a carrier agent. Previous studies have suggested that chemical modification of platinum complexes may ablate platinum antitumor action. The use of platinum-specific antibodies such as 1C1 may eliminate the problem by providing a reversible protein linkage that may be exploited to prepare bifunctional antibody complexes capable of simultaneously binding both tumor antigens and platinum complexes. Such antibody complexes should provide specific, tumor-targeted delivery for platinum complex.

One concern in this study was that the affinity of platinum complex for the disulfide bonds in the antibody structure could result in permanently bound but inactive platinum complex. If this were the case, the MIDP/antibody complex would be inactive against cells in culture. The data in this study show that platinum can be successfully bound to a murine antibody and that a complex of Pt/antibody still retains a substantial degree of the cytotoxicity of the original platinum complex.

In summary, these studies demonstrate that antibody 1C1 binds platinum complex MIDP without substantially reducing its cytotoxicity. In addition, antibody 1C1 can dramatically change the pharmacology of MIDP complex, enabling it to circulate longer in plasma without being distributed to major sites of tissue toxicity. Studies are now underway to build a tumor-targeting complex for MIDP with antibody 1C1. Studies are also in progress to deter-

Table 1. Pharmacokinetic summary of [^{3}H] MIDP vs [^{3}H] MIDP-antibody in the anesthetized rat model

	Pharmacokinetic parameters					
	VD (ml)	$t_{1/2}$ (min)			CXT (μCi/ml × min)	CLP (ml/kg × min)
		α	β	γ		
[^{3}H] MIDP alone	42	1.2	10	243	1.09	3.01
[^{3}H] MIDP + antibody 1C1	29	5.2	26	291	7.23	0.37

mine whether changes in MIDP pharmacology with antibody 1C1 translate into improved therapeutic activity of the MIDP/1C1 complex compared with that of MIDP alone.

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