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Resistance and cross-resistance of the IgM immunocytoma in the LOU/M Wsl rat for cisplatin, carboplatin, and iproplatin

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Summary. We investigated the antitumor activity of *cis*-diammine[1,1-cyclobutanedicarboxylato]platinum(II) (CBDCA, JM8) and *cis*-dichloro-*trans*-dihydroxybis(isopropylammine)platinum(IV) (CHIP, JM9) for the *cis*-DDP-sensitive and -resistant IgM immunocytoma in the LOU/M Wsl rat. The optimal dose for the antitumor effect of *cis*-diamminedichloroplatinum (cis-DDP) in this tumor model is 1 mg/kg body weight. In order to determine the dose range for antitumor activity of JM8 and JM9, tumor-bearing rats were treated i.p. (twice weekly) with 2, 4, 8, 16, or 32 mg/kg JM8 or with 2, 4, or 8 mg/kg JM9. The maximal antitumor activity of JM8 was found at a dose of 4–8 mg/kg and that of JM9, at 4 mg/kg. Doses of 16 or 32 mg/kg JM8 did not increase the antitumor activity. Recurrence of tumors was observed in JM8- and JM9-treated rats. It was demonstrated that these relapses during treatment with JM8 or JM9 involved tumor cell populations almost completely resistant against therapy with the respective drugs. The growth of *cis*-DDP-resistant tumors was not influenced by the analog JM9 (4 and 8 mg/kg). Only a high dose of JM8 (32 mg/kg) caused growth retardation of the *cis*-DDP-resistant IgM subline. The JM8-resistant tumor was resistant to treatment with *cis*-DDP (1 and 2 mg/kg). The JM9-resistant tumor was also resistant to this treatment (1 mg/kg); however, at a dose of 2 mg/kg *cis*-DDP, growth retardation of the tumor occurred. We conclude that *cis*-DDP, JM8, and JM9 induce resistance in the IgM immunocytoma tumor system; tumors resistant for *cis*-DDP were not sensitive to the treatment with JM8 or JM9. Although JM9 reacts in vitro distinctly differently with DNA than *cis*-DDP and JM8, no differences were found in the induction of Pt resistance. In this study tumor cells were readily made resistant, which allows us to study in more detail the induction of (cross-) resistance by *cis*-DDP, JM8, and JM9.

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Abbreviations used: Cisplatin or *cis*-DDP, *cis*-diamminedichloroplatinum(II); CBDCA, carboplatin, or JM8, *cis*-diammine[1,1-cyclobutanedicarboxylato]platinum(II); CHIP, iproplatin, or JM9, *cis*-dichloro-*trans*-dihydroxybis(isopropylammine) platinum(IV); TNO-6, *cis*-1,1-diaminomethylcyclohexaneplatinum(II)sulfate; IgM/*cis*-DDP, IgM immunocytoma resistant to treatment with *cis*-DDP; IgM/JM8, IgM immunocytoma resistant to treatment with JM8; IgM/JM9, IgM immunocytoma resistant to treatment with JM9

Introduction

The rationale in the search for new platinum analogs is to find compounds with a greater clinical antitumor effectiveness, a decreased (nephro)toxicity, an improved solubility, and a synergism in combination therapy [7]. Preferably these new compounds should also lack cross-resistance with *cis*-diamminedichloroplatinum(II) (cisplatin, *cis*-DDP). Among the second generation platinum drugs, *cis*-diammine[1,1-cyclobutanedicarboxylato]platinum(II) (CBDCA, carboplatin, or JM8) and *cis*-dichloro-*trans*-dihydroxybis(isopropylamine)platinum(IV) (CHIP, iproplatin, or JM9) have been compared with *cis*-DDP. These analogs show antitumor activity comparable to that of *cis*-DDP and reduced (nephro-) toxicity in animal models [3, 13, 17, 18, 39] as well as in clinical practice [4, 8, 11, 24, 35, 40]. Another favorable characteristic is their improved solubility [17, 38].

cis-DDP interacts with the nucleophilic sites on the DNA, resulting in DNA-interstrand [23, 25, 30, 41], DNA-intrastrand [10, 12, 25], and DNA-protein cross-links [10, 41]. The DNA-intrastrand cross-link is the predominant DNA adduct (60%) formed [12], whereas the DNA-interstrand cross-link accounts for about 1% of the platination sites on the DNA [25, 29]. Although it is generally thought that *cis*-DDP exerts its antitumor effect by DNA interaction, the correlation between the cytotoxicity of the drug and the above-mentioned DNA interactions is still uncertain [19, 31, 36].

DNA lesions produced by JM8 seem to be similar to those induced by *cis*-DDP. Using the DNA alkaline elution technique on *cis*-DDP- and JM8-exposed L1210 cells, Micetich et al. [21] have found that JM8 formed interstrand cross-links and DNA-protein cross-links similar to those formed by *cis*-DDP. However, the kinetics of lesion formation and lesion frequencies were different [21]. Knox et al. [16] have shown that *cis*-DDP and JM8 reacted to the same overall extent and induced the same proportion of interstrand cross-links at equitoxic doses in Chinese hamster cells. In contrast to that induced by JM8, the major type of damage observed after the reaction of JM9 with DNA in vitro were DNA breaks and interstrand cross-links [22]. However, cellular DNA breakage and interstand cross-linking have not been demonstrated [22].

It has been suggested that the effect of the interaction of platinum drugs with DNA depends on the efficiency and activity of the cell's excision repair system [26, 28] act-

ing on DNA lesions. In addition, besides the mechanism of DNA repair, alterations in membrane permeability [14, 34, 37], amino acid transport [33], or metallothionein induction [1] were also found to be associated with resistance to *cis*-DDP. There are only a few studies in which cross-resistance between *cis*-DDP and its analogs JM8 and JM9 have been investigated. In most studies with the L1210 *cis*-DDP-resistant cell line, cross-resistance has been observed [3, 9, 32]. There is some clinical evidence that JM8 may be active in cancers which do not respond well to *cis*-DDP [35] or which have become resistant to *cis*-DDP therapy [8, 11].

For clinical practice it is very important to have indications as to whether JM8 and JM9 can bypass *cis*-DDP resistance. In the past we have shown [15] that cross-resistance *to cis*-DDP and *cis*-1,1-diaminomethylcyclohexane platinum(II)sulfate (TNO-6) occurs in the IgM immunocytoma in the LOU/M Wsl rat, a finding which was different from the results previously obtained in the L1210 model [32]. In the present study, we investigated whether JM8 and JM9 could induce tumor regression in the *cis*-DDP-resistant IgM immunocytoma in the LOU/M Wsl rat. Furthermore, we were interested in knowing whether a difference in DNA interaction in vitro between JM8 and JM9, which has been suggested by Mong et al. [22], would result in a difference in induction of Pt resistance.

Materials and methods

Animals. Breeding pairs of LOU/M Wsl rats and the transplantable IgM immunocytoma of LOU/C Wsl origin were kindly provided by Dr. H. Bazin (Catholic University, Louvain, Belgium) [2]. Animals were bred under specified pathogen-free conditions at the National Institute of Public Health and Environmental Hygiene, Bilthoven, the Netherlands. Female rats weighing 160–190 g and 10–16 weeks of age were used. The animals were maintained according to accredited procedures in our facility and enjoyed uniformly good health at the beginning of the studies.

Tumor model. LOU/M Wsl rats were inoculated s.c. on the left flank with 2×10^4 IgM immunocytoma cells in 0.5 ml Iscove's modified Dulbecco's medium (IMDM, Grand Island Biological Co., Europe B.V., Hoofddorp, the Netherlands). Details on the tumor model have previously been described elsewhere [15]. In short, animals inoculated with 2×10^4 cells developed a palpable tumor after 14–17 days, which grew to a diameter of 25–35 mm within a further 6–7 days. By that time the tumor had metastasized to the regional lymph nodes and micrometastases in the liver could be detected. The growth of the tumor was measured twice weekly with vernier callipers and expressed as the mean value of three perpendicular measurements.

To measure the antitumor activity of JM8 and JM9, a range-finding study was done based on the equitoxic doses previously described by Prestayko et al. [27]. For JM8 two experiments were carried out, and for JM9 the range of antitumor activity was determined in one experiment.

Pt-resistant IgM immunocytoma cells. Rats with a growing parent IgM immunocytoma developing resistance to JM8 or JM9, as indicated by regrowth of the tumors during continued treatment, were killed and the tumors on the flank were aseptically extirpated. Tumors were disaggregated with 0.25% trypsin, and single-cell suspensions in IMDM with 20% heat-inactivated fetal calf serum and 20% dimethyl sulfoxide (Hicol B.V., Oud Beijerland, the Netherlands) were stored in liquid nitrogen at a concentration of $2-5 \times 10^6$ cells/ml. In previous experiments [15] IgM immunocytoma cells resistant to *cis*-DDP had been isolated. Prior to inoculation, the cells were thawed, washed, and diluted at a concentration of 4×10^4 cells/ml plain medium.

Drugs. cis-Diamminedichloroplatinum(II) (*cis*-DDP) was kindly provided by Dr. H. Meinema (Institute of Applied Chemistry, TNO, Utrecht, the Netherlands) and Dr. D. de Vos (Pharmachemie B. V., Haarlem, the Netherlands). *cis*-Diammine[1,1-cyclobutanedicarboxylato]platinum(II) (CBDCA, carboplatin, or JM8) and *cis*-dichloro-*trans*-dihydroxybis(isopropylammine)platinum(IV) (CHIP, iproplatin, or JM9) were kindly provided by Bristol-Myers Research Laboratories (Syracuse, NY). For experiments with *cis*-DDP and JM9, the drug was dissolved in 0.2% NaCl/4.2% mannitol; JM8 was dissolved in 5% mannitol. The drugs were given i.p. twice weekly.

Statistical analysis. Differences in tumor growth were evaluated for their statistical significance with the Student's *t*-test (two-sided).

Results

Antitumor activity

Figure 1 shows that a dose of 4 and 8 mg/kg JM8 exerted maximal antitumor activity on day 14. However, despite prolongation of treatment, the tumors recurred in all treatment groups. This antitumor activity was comparable with the activity of *cis*-DDP at 1 mg/kg. In the first experiment with JM8 recurrence of the tumors was determined in all animals. In rats treated with 8, 16, or 32 mg/kg JM8, recurrence of the tumors was observed on day 18 in 3/6, 4/6 and 6/6 animals, respectively. Rats without a relapse on day 18 either died or were killed because of their bad condition due to toxicity on approximately day 23 after the start of therapy.

At a dose of 2 mg/kg, JM9 caused only tumor growth inhibition. At a dose of 4 and 8 mg/kg, antitumor activity was similar to that of *cis*-DDP at 1 mg/kg. In this experiment, 5/6 and 4/6 animals showed recurrence of the tumor in groups treated with 4 and 8 mg/kg JM9, respectively, whereas the other three animals were killed on day 23 of therapy.

Development of resistance to JM8 and JM9

Experiments were carried out to assess whether the observed recurrences of the IgM immunocytoma during treatment with JM8 or JM9, as observed in the previously mentioned experiments (Fig. 1), were due to alterations in the host or to the development of drug resistance in the tumor cells. After regrowth of the tumors, cells of individual tumors were isolated for this purpose. Cells of four sublines referred to as IgM/JM8 or IgM/JM9 were inoculated s.c. in nontreated rats. After 15 days, rats inoculated with IgM/JM8 or IgM/JM9 developed tumors with a diameter of 12.3 ± 0.7 mm and 16.6 ± 0.3 mm (mean ± SE), respectively. As no individual differences were observed in

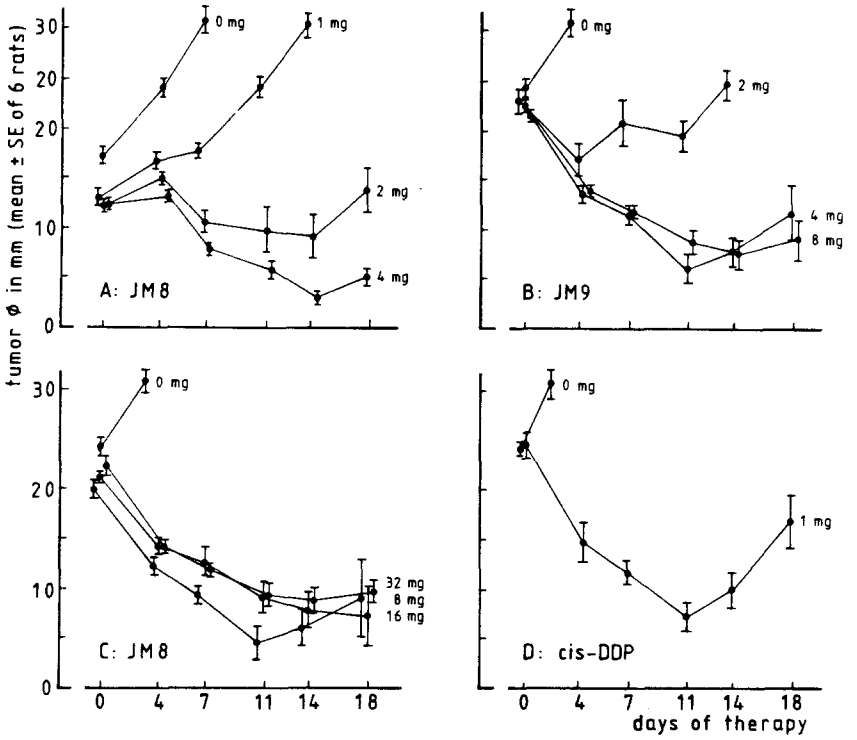

Fig. 1. Antitumor effect of JM8, (**A** and **C**) JM9 **(B)** and *cis*-DDP **(D)** against the IgM immunocytoma in the LOU/M rat. Tumor cells (2×10) were inoculated s.c. and treatment was started when the tumors had reached a size between 10 and 25 mm. Drugs were inoculated i.p. twice weekly. The dose is expressed per kg body weight. Tumor growth is presented as mean diameter ± SE of six rats

tumor growth kinetics or sensitivity to therapy between the various sublines of IgM/JM8 or IgM/JM9, only the data of one subline each of IgM/JM8 and IgM/JM9 are presented in Fig. 2. In Fig. 2A the growth kinetics of IgM/JM8 are similar to those of the parent IgM tumor. A dose of 8 mg/kg JM8 induced regression of the parent IgM tumor. However, this dose did not induce tumor regression or tumor growth inhibition in rats bearing the IgM/JM8-resistant tumor. Only a high dose of 32 mg/kg JM8 induced a slight but statistically significant tumor growth retardation on day 7 and day 10 ($P < 0.05$) compared with tumor growth in nontreated rats. Figure 2B shows similar

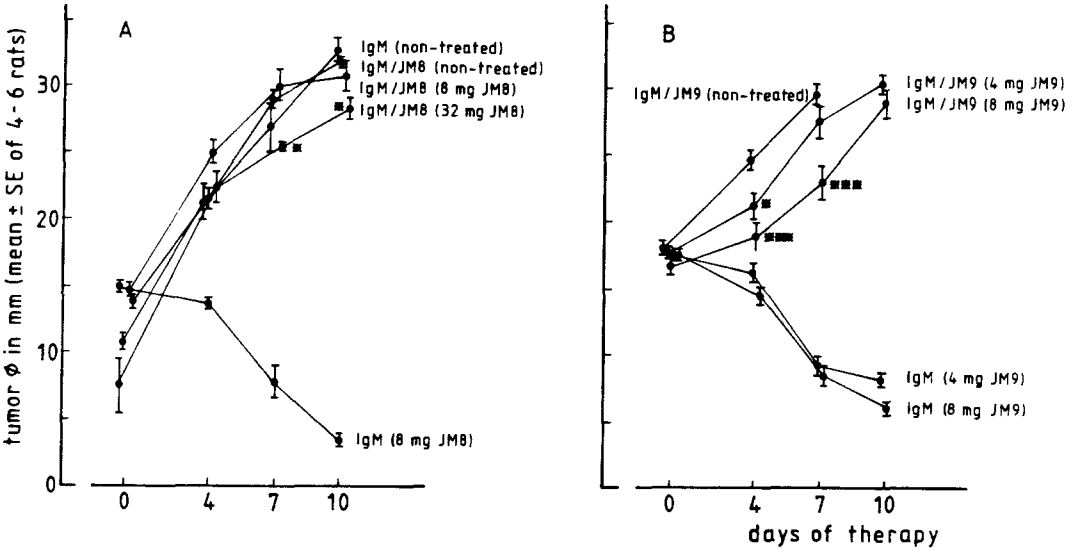

Fig. 2. Effect of JM8 and JM9 on tumors grown from inoculated cells that had been isolated from recurrences during treatment with JM8 or JM9, referred to as IgM/JM8 and IgM/JM9, respectively. Tumor cells (2×10^4) were inoculated s.c. in nontreated rats. Treatment was started when the tumors had eached a size of 12.3 ± 0.7 mm and 16.6 ± 0.3 mm (mean diameter ± SE), respectively. Drugs were given i.p. twice weekly. **A**: antitumor activity of JM8 on the parent IgM immunocytoma and the IgM/JM8 tumor. **B**: antitumor activity of JM9 on the parent IgM immunocytoma and the IgM/JM9 tumor. Differences in tumor growth relative to nontreated IgM/JM8 **(A)** or nontreated IgM/JM9 **(B)** were analyzed by Student's *t*-test (two-sided). * $P < 0.05$; *** $P < 0.001$

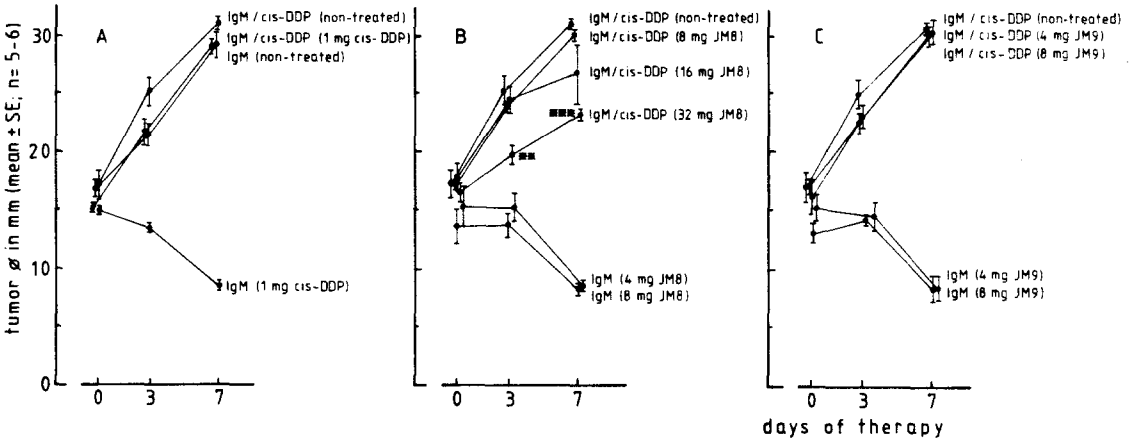

Fig. 3. Effect of JM8 and JM9 on the *cis*-DDP-resistant IgM immunocytoma (IgM/*cis*-DDP) in the LOU/M rat. Tumor cellls IgM and IgM/*cis*-DDP (2×10^4) were inoculated s.c. in nontreated rats. Treatment was started when the tumors had reached a size of 16.0 ± 2.4 mm (mean diameter ± SE). Drugs were given i.p. twice weekly. **A**: antitumor activity of *cis*-DDP against the parent IgM immunocytoma and the IgM/*cis*-DDP tumor. **B**: antitumor activity of JM8 against the parent IgM immunocytoma and the IgM/*cis*-DDP tumor. **C**: antitumor activity of JM9 against the parent IgM immunocytoma and the IgM/*cis*-DDP tumor. Differences in tumor growth relative to nontreated IgM/*cis*-DDP were analyzed by Student's *t*-test (two-sided). ** $P < 0.01$; *** $P < 0.001$

results with the IgM/JM9-resistant subline. Increasing the dose of JM9 resulted in tumor growth inhibition on day 3 and day 6 ($P < 0.001$) compared with the nontreated IgM/JM9 tumors. However, although tumor growth retardation was assessed in comparison with nontreated tumors, all tumors showed progressive growth.

Cross-resistance

We investigated whether JM8 and JM9 were active against the *cis*-DDP-resistant IgM tumor subline referred to as IgM/*cis*-DDP. For this purpose rats were inoculated s.c. with the parent IgM cells or with the IgM/*cis*-DDP cell line. When the tumors reached a size of 16.0 ± 2.4 mm (mean diameter ± SE) the treatment with JM8 or JM9 was started. Figure 3A shows that the growth of the parent IgM tumor and the IgM/*cis*-DDP tumors was almost identical. Figure 3B illustrates that treatment using JM8 (4 and 8 mg/kg) of rats with the parent IgM immunocytoma caused tumor regression comparable to that of *cis*-DDP (1 mg/kg). However, JM8 induced no tumor regression in the *cis*-DDP-resistant tumor, even at a high treatment dose of 32 mg/kg, where only slight tumor growth retardation ($P < 0.001$) was observed. Figure 3C shows that JM9 (4 and 8 mg/kg) was extremely active in rats bearing the parent IgM immunocytoma. However, these doses were inactive against the *cis*-DDP-resistant cell line.

In a similar experiment, tumor cells of JM8- and JM9-resistant sublines, designated IgM/JM8 and IgM/JM9, were inoculated s.c. When the tumors had reached a diameter of 13.1 ± 0.6 (mean diameter ± SE), rats were treated with JM8 (8 mg/kg) or with *cis*-DDP (1 or 2 mg/kg). Figures 4A and B show that treatment with *cis*-DDP did not result in regression of the IgM/JM8 or IgM/JM9 tumors; the high dose of *cis*-DDP (2 mg/kg) induced a significant ($P < 0.001$) growth retardation only in the JM9-resistant IgM/JM9 tumor.

Discussion

Drug resistance is a major obstacle to a successful chemotherapy of cancer. In this study we examined the development of resistance to *cis*-DDP and two *cis*-platinum analogs JM8 (carboplatin, CBDCA) and JM9 (iproplatin, CHIP), which have shown promising results in préclinical and clinical studies. Drug resistance can be the result of different mechanisms. Even from the beginning, some tumors do not respond to antineoplastic agents, and others initially show a good response but will eventually regrow and are then resistant to the originally effective drug [20]. The IgM immunocytoma in the LOU/M Wsl rat, used in this study, shows acquired resistance upon continued platinum treatment ([15], Fig. 1).

In previous experiments with *cis*-DDP [15], the maximal antitumor activity in rats bearing the IgM immunocytoma has been obtained at a dose of 1 mg/kg *cis*-DDP. In this study a similar antitumor activity was found for both JM8 and JM9 at a dose range of 4–8 mg/kg (see Fig. 1). From the results it seems that the IgM immunocytoma in the LOU/M rat is more sensitive than the L1210 leukemia cell line to treatment with JM8. Rose et al. [32], who used the L1210 leukemia cells in BDF_1 mice, have found a 20-fold difference between the optimal doses of *cis*-DDP and JM8. For the optimal treatment of the osteosarcoma $C_{22}LR$, ten times more JM8 than *cis*-DDP was needed [18]. The optimal dose we determined for JM9 agrees well with the results previously reported by others [17, 39]. Prestayko et al. [27] have also found an optimal dose for JM9 that was 4–8 times higher than that of *cis*-DDP in the murine L1210 leukemia model.

As can be seen from our data in Fig. 1, clinically complete regression of the tumor occured. However, in most of the rats regrowth of the tumor was observed. A high dose of JM8 (32 mg/kg) or JM9 (8 mg/kg) could not prevent the recurrence of the tumor. The results presented in Fig. 1C show that at a dose higher than 8 mg/kg JM8 the antitumor activity did not increase. Cells of tumors recurring after treatment with JM8 or JM9 were isolated and transplanted in nontreated rats, which were subsequently treated with JM8 or JM9, respectively, after tumor growth. The results presented in Fig. 2A and 2B show that the tumors were almost completely resistant to treatment with JM8 or JM9.

cis-DDP and JM8 have produced comparable lesions in DNA, such as intrastrand cross-links and a low percentage of interstrand and DNA-protein cross-links [16, 21]. However, it has been demonstrated in vitro that the major types of damage induced by JM9 were interstrand cross-links and DNA-breaks [22]. Apparently, these differences in DNA interaction demonstrated in vitro are not relevant for the induction of Pt resistance in vivo, as both JM8- and JM9-resistant tumors were frequently observed in our experiments. In addition, Fig. 3 shows that the *cis*-DDP-resistant subline is also resistant to treatment with JM8 and JM9. Only JM8 was used at a high dose level, which induced growth retardation. Similarly, IgM/JM8 and IgM/JM9 tumors can be considered resistant to treatment with *cis*-DDP, although the IgM/JM9 tumors showed growth retardation after treatment with the high, toxic doses (2 mg/kg) of *cis*-DDP (Fig. 4). As *cis*-DDP is the drug commonly used in patients, we were mainly interested in the effect of JM8 and JM9 on *cis*-DDP-resistant immunocytoma sublines; therefore, we did not reciprocally test JM8 and JM9 on the resistant tumors IgM/JM8 and IgM/JM9.

From our results it may be concluded that, at least in the IgM immunocytoma tumor system in the rat, cross-resistance cannot be circumvented by analogs of *cis*-DDP. In this tumor system in the LOU/M rat it may be likely that resistance against platinum compounds is based on a mechanism that occurs relatively easily after platinum exposure and is not related to the molecular structure of the analogs. On the other hand, considering the high drug doses used in our study, extremely high doses of JM8 may overcome *cis*-DDP-resistance (Fig. 3 B). Similarly, indications were found that high doses of *cis*-DDP are active against JM9-resistant tumors (Fig. 4). Thus, platinum resistance may partly be a dose-related phenomenon. However, in patients a Pt dose exceeding the maximally tolerated dose will be required, which may emphasize the importance of drug targeting. Resistance may then be abolished by high, local drug concentrations, due to selective drug delivery accompanied by low systemic concentrations.

Drug resistance in the cell can develop through various mechanisms. Alterations in the cell membrane can result in a decreased intracellular concentration of the drug [14, 34]. The degree of toxicity especially of metallic compounds is also dependent on the presence and amount of metallothionein [1]. Depending on the characteristics of the tumor cell, one or more of the mentioned mechanisms might be involved. Most studies on *cis*-DDP resistance have been carried out with L1210 leukemia cells. Studying various Pt derivatives, Burchenal et al. [5, 6] have found that the Pt compounds showing a lack of cross-resistance with *cis*-DDP were various derivatives of 1,1-diaminocy clohexane-Pt, 1,2-diaminocycloheptane-Pt, and, to a somewhat lesser extent, 1,2-diaminocyclopentane-Pt compounds. Rose et al. [32] have shown that JM8 and diammine 2-ethylmalonato Pt(II) (JM10) were inactive against the L1210/*cis*-DDP-resistant cell line, but all TNO Pt analogs, including TNO-6, showed similar activity against the parental and the *cis*-DDP-resistant L1210 leukemia cell line. However, in the IgM immunocytoma model, TNO-6 had no effect on the *cis*-DDP-resistant subline [15].

In two clinical trials it has been observed that JM8 has some effect on *cis*-DDP-resistant tumors [8, 11]. The results of additional clinical studies will become available in the near future. Hopefully, this will make it possible to determine which of the laboratory tumor models may have a predictive value. In the meantime, tumor models with drug-resistant sublines may give us some insight into the mechanism of acquired drug resistance.

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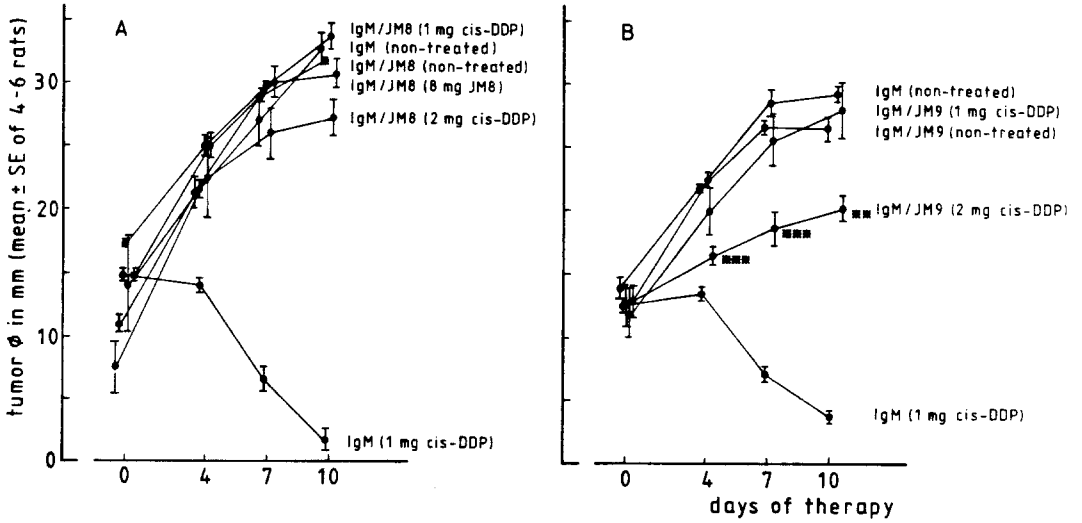

Fig. 4. Effect of *cis*-DDP on the JM8- and JM9-resistant IgM immunocytomas (IgM/JM8, IgM/JM9) in the LOU/M rat. Tumor cells (IgM/JM8 and IgM/JM9, 2×10^4) were inoculated s.c. in nontreated rats. Treatment was started when the tumors had reached a mean size of **(A)** 13.0 ± 0.7 mm and **(B)** 12.7 ± 0.4 mm. Drugs (*cis*-DDP and JM8) were given i.p. twice weekly. **A**: antitumor activity of *cis*-DDP against the parent IgM immunocytoma and the IgM/JM8 tumor. **B**: antitumor activity of *cis*-DDP against the parent IgM immunocytoma and the IgM/JM9 tumor. Differences in tumor growth relative to nontreated IgM/JM9 were analyzed by Student's *t*-test (two-sided). ** $P < 0.01$; *** $P < 0.001$

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