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Response of sensitive and resistant IgM immunocytomas to *cis*-diamminedichloroplatinum(II) does not correlate with the platination level or with the formation or removal of DNA adducts

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Abstract An IgM immunocytoma cell line sensitive to *cis*-diamminedichloroplatinum(II) (CDDP) and a subline with acquired resistance were grown in LOU/M rats. In a previous study with such rats that had been treated with a high dose of CDDP (10 mg/kg) the tumors did not show differences in cellular platinum content or DNA-adduct levels, either immediately after treatment or 24 h later. Recently, this high dose was found to overcome resistance. Therefore, the study was repeated with a 10-fold lower dose (1 mg/kg, i.v.). At 1 and 24 h after treatment, tumor and kidney tissue were assayed for cellular platinum (atomic absorption spectroscopy, AAS) and DNA platination (immunochemical detection of the four CDDP-DNA adducts). The results were compared with previous data. All tissues showed a linear response to dose with regard to platinum uptake as well as adduct formation, with no quantitative difference being seen between the tumors. Also the relative occurrence of the four adducts was very similar. Between 1 and 24 h, in tumors a substantial decrease occurred in both platinum content and adduct level; the kidneys showed little reduction, if any. At the lower CDDP dose a somewhat larger loss of platinum and removal of DNA adducts was observed for the resistant tumor, but these differences could be explained by "dilution", as this tumor continues to grow after low-dose treatment (about 20% within 24 h). Since the strong difference observed between the tumors in sensitivity to CDDP cannot be attributed to differences in CDDP uptake, efficiency of adduct formation, or repair capability, other mechanisms are held responsible.

Key words Platinum-DNA adducts · Resistance · Sensitivity · Immunocytoma

Abbreviations *CDDP* *cis*-diamminedichloroplatinum(II) · *CDDP-(DNA) adducts* reaction products of CDDP with nucleobases in DNA or in deoxy(oligo)-nucleotides · *Pt-G* Pt$(NH_3)_3$-dGMP · *PtAG* *cis*-Pt$(NH_3)_2$-d(pApG) · Pt-GG *cis*-Pt$(NH_3)_2$-d(pGpG) · *G-Pt-G* *cis*-Pt$(NH_3)_2$-d$(GMP)_2$ · *AAS* atomic absorption spectroscopy · *ELISA* enzyme-linked immunosorbent assay · *IgM-I* CDDP-sensitive parent tumor-cell line · *IgM/CDDP* CDDP-resistant tumor-cell line

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Introduction

cis-Diamminedichloroplatinum(II) (CDDP) has a remarkable chemotherapeutic potential against a variety of human neoplasms, especially testicular and ovarian tumors, but the frequent occurrence of resistance to the drug is a major clinical problem. Similar events occur in animal models such as the IgM immunocytoma in rats; after completely regressing following treatment with CDDP, in most animals the tumor has recurred [1, 2]. Sublines isolated from these recurrences, upon inoculation of rats, gave rise to tumors that were

insensitive to treatment, indicating that the resistance was not caused by alteration of the host but was a stable, genetic characteristic of the tumor cell.

The antitumor activity of CDDP is generally assumed to result from the interaction of the drug with DNA in the cell, mainly leading to the formation of interstrand and intrastrand cross-links. We have developed methods to establish the identity of the various adducts formed in DNA by isolation of the four nucleolytic digestion products: Pt-AG and Pt-GG, derived from intrastrand cross-links on neighboring bases in the sequences pApG and pGpG, respectively; Pt-G from CDDP monofunctionally bound to guanine; and G-Pt-G from intrastrand cross-links on the sequences pG(pX)-pG and/or interstrand cross-links connecting two guanines [3, 4].

Acquired resistance to CDDP has been attributed to decreased permeability of the cellular membrane [5, 6], reduced accumulation of drug within the cell [5, 7], and increase in the intracellular or intranuclear glutathione concentration [7–10]. These features would be expected to lead to a decreased formation of DNA adducts. Indeed, reduced adduct levels have been found in human small-cell carcinoma cell lines and human melanoma cell lines resistant to CDDP [10, 11]. Furthermore, resistance might be the result of a more effective removal of the harmful DNA modifications by cellular repair systems [12].

A few years ago, Fichtinger-Schepman et al. [13] compared the CDDP-sensitive immunocytoma IgM-I with a subline selected for resistance at 1 mg/kg CDDP (IgM/CDDP). In tumors grown in LOU/M rats, platinum content and DNA-adduct levels were determined at 1 and 24 h after a single 10-fold higher dose of CDDP (10 mg/kg, i.v.), but no difference was detected. Recently, however, it appeared that such a high dose would overcome the resistance, as it resulted in a degree of sensitivity of IgM/CDDP tumor growth to CDDP that was comparable with that of the IgM-I tumor. It was therefore considered possible that after administration of a lower dose of CDDP, different results would be obtained for IgM-I and IgM/CDDP tumor cells with regard to formation or removal of adducts.

In this study, data were obtained for the platination levels and the DNA-adduct formation and removal observed in the parent and resistant tumors after the treatment of tumor-bearing rats with a dose of CDDP that in the animal discriminates between sensitive and resistant immunocytomas. In addition to the tumors, the kidneys were also assayed for the content of CDDP-DNA adducts; this organ is known to attain well-measurable adduct levels and may be used as a reference organ. Equal levels and type of DNA-adduct formation in the kidneys of tumor-bearing and control animals demonstrate that observed differences in tumors are entirely due to intrinsic properties of the tumors themselves and not to some other influence.

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). Animals were bred under specified pathogen-free conditions at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. Female rats weighting 160–190 g and aged 10–16 weeks were used.

Tumor model

LOU/M Wsl rats were inoculated s.c. on the left flank with either IgM-I or IgM/CDDP cells derived from CDDP-sensitive or CDDP-resistant immunocytomas (2×10^4 IgM immunocytoma cells in 0.5 ml medium). Details on the tumor model have been described elsewhere [1]. Rats inoculated with 2×10^4 cells developed a palpable tumor after 12–14 days, with rapid growth occurring thereafter. The tumor size was measured with Vernier callipers and was expressed as the mean value of three perpendicular measurements. For a three-dimensional approach the depth of the tumor was determined after lifting of the skin including the tumor growing s.c.

Experimental design

Tumors were grown to about 15 mm in diameter and the animals were treated with a single dose of 1 or 10 mg/kg CDDP. After treatment the decline in tumor diameter was determined for sensitive and resistant tumors. For determination of platinum content and CDDP-DNA adducts, at 1 and 24 h after the treatment, rats were euthanized and tissues were collected. About 0.5 g each of kidney and tumor tissue was collected and kept frozen at $-20\,^{\circ}C$ until analysis of the platinum concentration by atomic absorption spectroscopy (AAS). For CDDP-DNA adduct measurements, cell suspensions of the tumors and kidneys were prepared by gentle pressing of fragments of the fresh (i.e., nonfrozen) tissues through a 60-mesh stainless-steel wire screen in saline. The cells were washed with saline and centrifuged, and the pellets were stored at $-70\,^{\circ}C$. All isolations were performed at $4\,^{\circ}C$.

Platinum determinations in tissues

Platinum was determined by AAS with electrothermal atomization in a graphite furnace in a model 451 video atomic absorption spectrometer (Instrumentation Laboratory, Inc., Wilmington Mass.). The tissue samples were analyzed after enzymatic digestion and modification of the final matrix [14]. The results were expressed in milligrams of platinum per kilogram of wet tissue.

DNA isolation and determination of adducts

The procedure used for isolation of DNA from the cell pellets of the tissue samples was similar to that previously described for white blood cells [4]. The method included the inactivation with NH_4HCO_3 of the still reactive coordination site of monofunctionally bound CDDP [15, 16]. The isolated DNAs were enzymatically digested to the unmodified mononucleotides dCMP. dAMP, dTMP, and dGMP and the platinum-containing (di)nucleotides and were subsequently chromatographed as described elsewhere [3]. The quantitation of these platinum products, present at identified

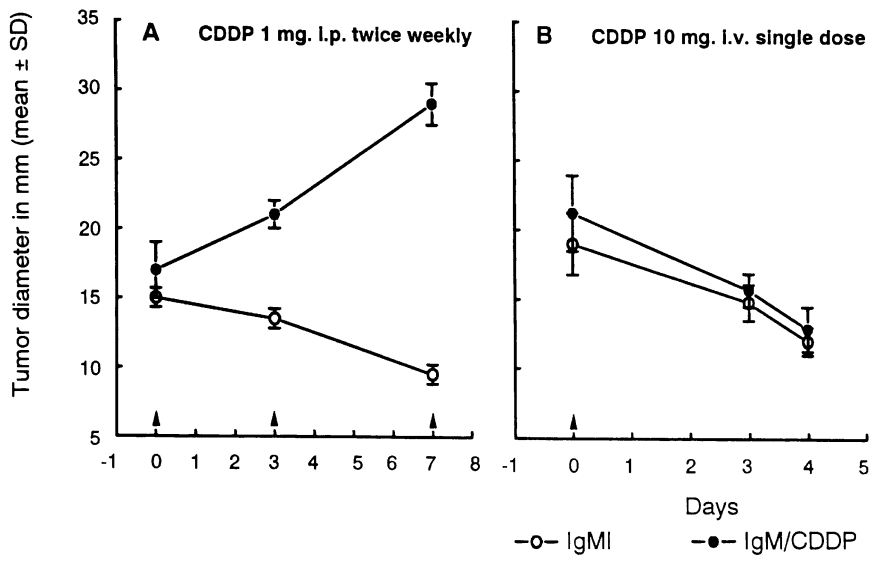

Fig. 1A, B Growth response of CDDP-sensitive and CDDP-resistant IgM tumors (IgM-I and IgM/CDDP, respectively) in response to CDDP treatment. After tumors had grown to a diameter of 10–25 mm, administration of CDDP (*arrowheads*) was started and tumor growth was followed. **A** Based on data taken from Steerenberg et al. [2]

positions in the eluate of the Mono Q column, was performed with immunochemistry techniques by the use of three specific antisera raised against synthetic platinum (di)nucleotides. In this method the dilutions of the fractions giving 50% inhibition of antibody binding in the competitive ELISA were determined and used to calculate the amounts of CDDP-DNA digestion products by comparison with inhibition curves obtained with reference compounds. Each fraction was measured in at least two independent ELISAs in four dilutions each [3, 4]. In this way, adduct levels of 0.1 fmol/μg DNA can be detected when DNA samples of about 350 μg are analyzed.

Statistical analysis

All statistical calculations were performed according to Student's two-sided *t*-test.

Results

To investigate whether IgM-I and IgM/CDDP tumors might behave differently with respect to DNA-adduct formation and repair when rats were subjected to a mild CDDP treatment, a single i.v. dose of 1 mg/kg was given to tumor-bearing LOU/M rats. This low dose is known to discriminate between the two tumors with respect to growth, in contrast to the 10-fold higher dose, as is illustrated in Fig. 1. Rats were killed at 1 h after CDDP treatment to determine the DNA-adduct formation, whereas DNA samples taken from animals killed after 24 h were analyzed to determine the rapair of the adducts. Table 1 presents the total platination levels determined with AAS in tumor tissue and in kidneys at 1 and 24 h after CDDP injection. For purposes of comparison, earlier data obtained with 10 mg/kg CDDP were included. As no significant difference was found between kidney platination levels in IgM-I and IgM/CDDP tumor-bearing rats, the results obtained in these tissues were pooled. A comparable experiment was performed in tumor-free rats. These results are also shown in Table 1.

Table 1 Platinum concentrations measured with AAS

Tissue	CDDP[a]	n[b]	Time after CDDP treatment	
			1 h	24 h
Kidney	1	6[c]	2.5 ± 0.5[d]	2.3 ± 0.4
	1	12	2.2 ± 0.4	2.0 ± 0.3
	10	6	26 ± 4	27 ± 3
IgM-I	1	7	0.5 ± 0.1	0.3 ± 0.1
	10	4	5.9 ± 1.2[e]	4.0 ± 0.4[f]
IgM/CDDP	1	8	0.5 ± 0.1[g]	0.3 ± 0.1
	10	3	5.2 ± 0.4	2.7 ± 0.5[h]

[a] Dose in mg/kg given i.v.; the 10-mg/kg data were taken from a previous paper by Fichtinger-Schepman et al. [13]
[b] Number of animals
[c] Non-tumor-bearing rats
[d] Mean values ± SD for platinum concentrations measured with AAS, expressed in mg/kg of wet tissue
[e] Data on 3 animals
[f] Significantly different ($P < 0.05$) from the 1-h concentration
[g] Data on 6 animals
[h] Significantly different ($P < 0.01$) from the 1-h data and from the 24-h data on the IgM-I tumors ($P < 0.02$) according to Student's two-sided *t*-test

When the results of the AAS determinations were compared for the low and the high dose of CDDP, in general an approximately 10-fold difference was observed in the platinum levels, for kidneys as well as tumors, suggesting a linear dose-response relationship. With regard to the kidney values, the tumor-bearing rats did not appear to differ from the tumor-free animals. In agreement with earlier observations [13], no reduction in platination levels occurred in this organ between 1 and 24 h after injection. Furthermore, the

Table 2 CDDP-DNA adduct levels determined in kidneys and tumors

Tissue	CDDP[a]	Time[b]	n[c]	CDDP-DNA adducts (fmol/μg DNA)				
				Pt-G	Pt-AG	Pt-GG	G-Pt-G	Σ adducts[d]
Kidney	1	1	6[e]	1.5 ± 1.2[f]	8.4 ± 3.4	21 ± 4	9.6 ± 1.8[g]	41 ± 5[g]
	1	24	6[e]	0.4 ± 0.2	5.4 ± 3.3	22 ± 9	6.6 ± 2.2[g]	31 ± 11[g]
	1	1	12	1.7 ± 0.6	6.2 ± 2.1	19 ± 5	5.2 ± 3.2[h]	32 ± 7[h]
	1	24	12	0.4 ± 0.3	2.9 ± 1.5	18 ± 5	3.6 ± 2.6[h]	24 ± 6[h]
	10	1	6	46 ± 16	51 ± 14	247 ± 63	75 ± 7	419 ± 85
	10	24	2	58 ± 17	51 ± 6	193 ± 36[i]	53 ± 11	383 ± 87
IgM-I	1	1	7	0.5 ± 0.3	1.4 ± 0.6	3.5 ± 0.7	1.8 ± 0.8[i]	7.2 ± 1.9[i]
	1	24	7	0.2 ± 0.2	0.5 ± 0.2	2.0 ± 0.5	1.0 ± 1.1	3.7 ± 1.7
	10	1	4	3.6 ± 2.2	11 ± 6	38 ± 6	14 ± 7	67 ± 19
	10	24	4	1.1 ± 0.7	7.2 ± 3.9	34 ± 12	9.4 ± 3.8	52 ± 18
IgM/CDDP	1	1	7	0.7 ± 0.7	1.6 ± 0.4	3.8 ± 1.4	1.3 ± 0.8	7.3 ± 2.5
	1	24	8	0.1 ± 0.3	0.4 ± 0.2[j]	1.4 ± 0.5	0.3 ± 0.2	2.2 ± 1.1[j]
	10	1	3	2.3 ± 0.5	13 ± 4	40 ± 13	16 ± 4	71 ± 16
	10	24	3	2.2 ± 1.8	8.4 ± 5.2	25 ± 18	7.2 ± 4.1	43 ± 26

[a] Dose in mg/kg given i.v.; the 10-mg/kg data were taken from a previous paper by Fichtinger-Schepman et al. [13]
[b] Time (h) after CDDP injection
[c] Number of animals
[d] The difference between 1 and 24 h at the low dose of CDDP is significant for kidneys in tumor-bearing animals ($P < 0.02$), IgM-I ($P < 0.01$) and IgM/CDDP ($P < 0.001$) according to Student's two-sided t-test
[e] Non-tumor-bearing rats
[f] Mean values ± SD
[g] Data on 5 animals
[h] Data on 10 animals
[i] Data on 6 animals
[j] Data on 7 animals

data shown in Table 1 do not indicate a significant difference in total platinum concentration between the sensitive and the resistant tumors, either at the higher or the lower dose of CDDP, in the samples taken at 1 h after treatment. In the 24-h samples, lower platination levels were found, with reductions amounting to roughly 40%. At 10 mg CDDP/kg a significantly greater decrease occurred in the IgM/CDDP tumor (48%) as compared with the IgM-I tumor (32%; $P < 0.02$).

The determination of CDDP-DNA adducts involves enzymatic digestion of the DNA followed by chromatographic isolation of the platinum-containing fragments. All four digestion products known could be detected and quantitated, albeit with poor accuracy due to the low levels. The results are summarized in Table 2 together with those obtained in experiments with 10 mg/kg CDDP [13]. Since no difference in adduct formation in the kidneys was found between IgM-I and IgM/CDDP tumor-bearing animals, the results were pooled in the table.

Comparison of the new data on the formation of the four DNA adducts (i.e., the 1-h values) with those obtained with 10 mg/kg CDDP demonstrates that in all cases the results are consistent with a 10-fold reduced level at the lower dose, indicating a linear dose-response relationship, also with respect to the formation of the individual adducts. Furthermore, these data show a strong similarity between the two tumors with regard to the levels as well as the relative occurrence (Table 3) of the adducts. With respect to the latter, the results show a fair level of agreement with the adduct distribution reported after administration of 10 mg/kg CDDP [13].

Again, the kidney values did not indicate any influence in terms of the presence of a tumor. With regard to the distribution of the adducts at 1 h after treatment with 1 mg/kg CDDP (Table 3), the kidney showed the same pattern seen in the tumors; at the higher dose, however, a somewhat higher percentage was reported for Pt-G in the kidney (11% versus ca. 5%) at the expense of Pt-AG (12% versus ca. 19%).

In general, the results obtained at 24 h after CDDP administration (Table 2) demonstrated a reduction in DNA-adduct levels as compared with the 1-h values. This reduction was less prominent for kidney tissues than for tumor samples, and it involved a larger fraction of the adducts in the animals receiving the lower dose of CDDP. In kidneys, total adduct levels dropped by about 10–25%, but only in the tumor-bearing rats, and after treatment with 1 mg/kg the reduction was significant. Overall, the IgM/CDDP tumors showed the strongest removal of adducts, with 70% being removed at the lower dose and about 40% being removed at 10 mg/kg CDDP, whereas the values

Table 3 Relative occurrence of CDDP-DNA digestion products in kidneys and tumors

Tissue	Time[a]	n[b]	Amount as % of total adducts			
			Pt-G	Pt-AG	Pt-GG	G-Pt-G
Kidney	1	5[c]	3.8 ± 2.8[d]	20 ± 10	52 ± 6	23 ± 5
	24	5[c]	1.2 ± 0.4	15 ± 8	63 ± 10	21 ± 2
	1	10	5.7 ± 3.1	18 ± 5	60 ± 10	16 ± 8
	24	10	1.6 ± 1.0	13 ± 6	71 ± 14	15 ± 11
IgM-I	1	6	6.5 ± 3.8	19 ± 7	51 ± 9	24 ± 9
	24	7	3.9 ± 6.6	15 ± 4	57 ± 13	24 ± 12
IgM/CDDP	1	7	9.3 ± 8.3	23 ± 7	52 ± 10	16 ± 8
	24	7	4.7 ± 8.2	18 ± 6	64 ± 16	14 ± 9

[a] Time (h) after CDDP injection (1 mg/kg given i.v.)
[b] Number of animals
[c] Non-tumor-bearing rats
[d] Mean values $\pm$ SD

recorded for IgM-I tumors amounted to about 50% and 20%, respectively. However, only the reductions noted after 1-mg/kg treatment were statistically significant (Table 2). Nonetheless, when the 24-h results recorded for the four adducts are considered, hardly any difference between the resistant and the sensitive tumor are seen, with the possible exception of the G-Pt-G values noted after a dose of 1 mg/kg (not significant, though).

Table 3 presents the relative occurrences of the four CDDP-DNA digestion products, expressed as percentages of the total DNA adducts detected in kidney and tumor DNAs after treatment with 1 mg/kg CDDP. The tumor data presented earlier for treatments with 10 mg/kg CDDP [13] showed corresponding results. The percentages shown in Table 3 for kidneys and tumors are strongly similar with respect to the distribution of the four products and, notwithstanding the above-noted relatively low G-Pt-G level found in IgM/CDDP tumors after 24 h, no significant difference can be detected. In all samples, Pt-GG was the main adduct, amounting to 51–60% of the total DNA platination after 1 h; after 24 h this percentage had increased and ranged between 57% and 71%.

Discussion

It is generally known that resistance to cytostatic agents is only relative and that resistance can be overcome by the use of high doses of drugs. In our model, IgM immunocytomas grown in LOU/M rats, a CDDP dose of 1 mg/kg leads to regression of the sensitive tumor, whereas no effect is seen on the resistant IgM/CDDP tumor (Fig. 1A). Treatment with 10 mg/kg, however, overcomes the resistance, with both tumors showing identical regression of about 50% within 4 days (Fig. 1B). In this tumor model, cross-resistance to other DNA-interactive drugs as well as several platinum analogues has been observed [1, 2]. Serial passage of tumors in vivo (and established cell liens in vitro) revealed that CDDP sensitivity and resistance are well preserved in the IgM-I parent tumor line and the IgM/CDDP tumor line, respectively (data not shown).

This study was initiated to determine whether the different sensitivities to CDDP with respect to tumor growth might be reflected in differences in drug uptake, DNA-adduct formation, or repair of these lesions. The data presented in Tables 1–3 demonstrate that qualitatively there was no real difference between kidney and tumor tissues or between the two tumor types. All three tissues contained well-measurable amounts of platinum and all known CDDP-DNA adducts were found to be present in very similar proportions, with Pt-GG occurring as the main product. After 24 h, partial removal of adducts appeared to have occurred, which generally resulted in a higher percentage of Pt-GG. This does not necessarily indicate a poorer repair of this DNA modification, since some formation may have occurred by the preferential conversion of monofunctionally bound CDDP (Pt-G) into Pt-GG.

A quantitative comparison of the data shows a higher content of platinum and of DNA-adduct levels in the kidney than in the tumor, which is in agreement with earlier results [13]. These kidney values were not affected by the presence or absence of the tumor in the animal body. In contrast to the tumors, the kidneys showed hardly any reduction in platinum content between 1 and 24 h after treatment; also, with regard to DNA adducts, removal was not very substantial.

As can be seen in Tables 1 and 2, there was no significant difference between the sensitive and the resistant tumors with respect to the platinum content or DNA-adduct levels detected at 1 h after treatment. This means that the CDDP resistance is not due to a lower availability of CDDP in the cell or to a decreased formation of the adducts. This obviates a role for differences in cell-membrane permeability, differences in accumulation or efflux of the drug, or differences in intracellular glutathione or metallothionein levels as

the cause of the CDDP resistance. At 24 h after treatment with a high or low dose of CDDP, both tumors showed a substantial decrease in platinum content, with no significant difference being observed between the tumors (Table 1). The DNA-adduct levels also showed a strong decrease, which as a percentage was clearly more prominent after treatment with 1 mg/kg than after the 10-fold higher dose (Table 2). This difference might well be attributable to a saturation of the cellular repair systems at the higher adduct levels. In this respect a distinction between the two tumors appeared to exist. Particularly at the lower CDDP dose, the resistant tumor showed a higher capacity for adduct removal. This difference was seen not only at the level of the four adducts taken together (70% versus 49% reduction) but also at the level of each of the individual adducts. However, it did not result in drastically lower adduct concentrations in IgM/CDDP tumors. The difference does not necessarily mean that the resistant tumor has a more efficient system of DNA repair. As Fig. 1A shows, the IgM/CDDP tumor continues to grow after treatment at 1 mg/kg, which may result in some 20% proliferation in 24 h. Provided that no inhibition of DNA synthesis occurs during this period, a drop in adduct level due to the dilution by newly synthesized DNA is to be expected. In conclusion, possibly the resistant tumor has a somewhat faster mode of DNA repair, but the difference appears to be very small and is not sufficient to explain the strong difference in sensitivity to CDDP.

Since more proficient DNA repair does not appear to be the principal reason for the resistance of IgM/CDDP tumors, other mechanisms must be responsible. In the assay used in the present study the level of CDDP adducts in total DNA is determined. Consequently, the data on adduct removal also apply to genomic DNA in general. The possibility exists, however, of preferential repair of specific genes [17, 18], which would remain unnoticed in our assay. One may therefore postulate that in IgM/CDDP cells, preferential repair occurs of genomic regions that are essential for survival, which enables these cells to complete replication of their genome [19]. On the other hand, the continuing growth of the resistant tumor in the presence of a multitude of DNA adducts might be taken to indicate an enhanced tolerance of unrepaired DNA adducts during the DNA-replication process, which would lead to less efficient cell killing and less inhibition of cell division, a phenomenon that is also found in other CDDP-resistant tumor cells [20, 21]. The precise mechanisms have not yet been defined but, in principle, such a tolerance could be caused by, e.g., an altered polymerase.

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