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Activity of two platinum-linked phosphonic acids against autochthonous rat colorectal cancer as well as in two human colon-cancer cell lines

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Summary. Two new platinum-containing phosphonate compounds, *cis*-diammine[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum(II) (AMDP) and *cis*-cyclohexane-1,2-diamine[nitrilotris(methylphosphonato)(2-)-O^1, N^1]platinum(II) (DADP) were investigated in acetoxymethylmethylnitrosamine-induced autochthonous colorectal rat adenocarcinoma in vivo as well as in two human colon-cancer cell lines (SW707 and SW948) in vitro. In the in vivo model, the two compounds were given i. v. at doses of 8 and 13 mg/kg as well as p. o. at 16 and 26 mg/kg twice a week for 10 weeks, respectively. AMDP produced more intensive toxicity at both doses but showed higher antitumour activity only following i. v. administration. On the other hand, DADP caused significant tumour-growth inhibition after both modes of application, but as it produced only low toxicity, its use should be favoured. The in vitro assays were performed using two cell lines derived from human colorectal adenocarcinomas. According to the microculture tetrazolium test (MTT) AMDP (IC_{50}, 34 and 59 μM in SW707 and SW948, respectively) was more effective than DADP (IC_{50}, 412 and 660 μM in SW707 and SW948, respectively) in inhibiting cell growth. Based on cell counts AMDP (IC_{50}, 8 and 11 μM in SW707 and SW948, respectively) and DADP (IC_{50}, 266 and 285 μM in SW707 and SW948, respectively) showed more intensive antiproliferative efficacy as determined by the Coulter Counter method vs the MTT assay. The promising activities of these new platinum-linked phosphonic acids in autochthonous rat colorectal carcinoma and in human colorectal cancer cell lines warrant further investigations of compounds of this class to elucidate their role in the treatment of colorectal cancer.

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Introduction

Colorectal cancer, one of the most frequently occurring malignancies [10], is considered to be highly resistant to the currently available chemotherapeutic agents [7, 34]. In fact, despite the numerous efforts made in recent years to improve the median survival of advanced colorectal cancer patients using chemotherapy, only 5-fluorouracil and mitomycin C have shown some, albeit very limited, activity against this disease [12]. *cis*-Diamminedichloroplatinum(II) (cis-DDP) [29] has shown therapeutic efficacy in a wide spectrum of human tumours such as testicular and ovarian cancers [14, 26], but it exerts only minimal activity against advanced colorectal carcinoma [9, 19, 21, 31]. The latter result has been paralleled in an experimental model, whereby cis-DDP showed no antineoplastic efficacy in acetoxymethylmethylnitrosamine (AMMN)-induced rat colorectal carcinoma [3, 6]. Other metal complexes such as ruthenium and titanium compounds have shown promising antitumour activity in autochthonous colorectal cancer therapy in rats [4, 6, 13]. In view of the chemical similarities between ruthenium and platinum derivatives, which belong to the same class of metal complexes, this work was extended by the synthesis of new *cis*-configurated platinum compounds linked with phosphonic acids. The promising antitumour activity of platinum phosphonates against rat osteosarcoma in vivo and in vitro [15, 17] encouraged us to investigate these compounds in colorectal cancer models. The chemically induced rat colorectal cancer was chosen because of differences in its characteristics as compared with those of transplanted tumours, such as orthotopic occurrence, intact histology, original tumour-host interaction and slow growth kinetics [2, 3]. Besides this in vivo trial, the platinum-linked phosphonic acids were investigated in vitro in two human colon-cancer cell lines (SW707 and SW948) using the microculture tetrazolium test (MTT) and cell counting by Coulter Counter. In this report, we describe the adoption of the MTT assay to measure the antineoplastic activity of therapeutic agents in colon tumour cells and to compare the activity of these compounds in vitro with their efficacy in the in vivo model.

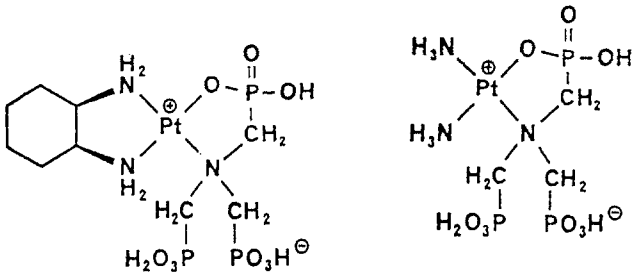


Fig. 1. Structures of AMDP (*right*) and DADP (*left*)

Materials and methods

Substances. The following compounds were tested both in vivo and in vitro: *cis*-diammine[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum(II) (AMDP) and *cis*-cyclohexane-1,2-diamine[nitrilotris(methylphosphonato)(2-)-O^1,N^1]platinum(II) (DADP; Fig. 1). Both substances were diluted at appropriate concentrations in sterile water (Ampuwa).

Synthesis and characterisation of platinum phosphonato complexes. The synthesis of the two compounds was realised by the following methods. First, the platinum complexes used as educts were activated by silver nitrate. The resulting solution with containing aquo complexes was then placed on an ion exchanger to remove the nitrate. The resultant highly reactive solution was thereafter reacted with phosphonic acid. According to this scheme AMDP and DADP were prepared. Both compounds were characterised by elemental analyses and various spectroscopic methods, including [^{31}P]-nuclear magnetic resonance (NMR) and [^{13}C]-NMR. Their purity was confirmed by high-performance liquid chromatographic (HPLC) investigations.

Description of in vivo trials. A total of 100 male Lewis rats (Zentralinstitut für Versuchstierkunde, Hannover, FRG) were purchased at a weight of 140–160 g and thereafter kept under conventional conditions: 2 rats per Makrolon III cage, tap water and Altromin pellets ad libitum. Colorectal carcinomas were induced by the administration of a fresh 0.2% solution of AMMN [28, 35] in physiological saline. A dose of 2 mg/kg was given intrarectally once a week for 10 weeks using a rectal tube, the tip of which was inserted up to the colonic flexure. At 5 weeks after the 10-week induction period, the animals were anesthesised i.p. with 300 mg/kg chloral hydrate diluted in 0.9% NaCl. Endoscopic examination was performed carefully using a pediatric bronchoscope (Olympus BF type 4C2, Olympus Optical Co., Tokyo) [23, 25]. Animals with evident tumours were randomly allocated to treatment and control groups.

Treatment was started following the endoscopic diagnosis of tumours, which was carried out at weeks 5, 7 and 9 after the induction period. The therapy with platinum-linked bisphosphonates was carried out for 10 weeks. The doses were chosen according to their acute toxicity in mice as determined by the lethal dose in 50% of the experimental group (LD_{50}: AMDP, 300 mg/kg; DADP, 600 mg/kg) and to prior experiments in an in vivo rat osteosarcoma model [17, 18]. The doses and routes of administration of AMDP and DADP are listed in Table 1. At the end of treatment, the animals were killed and dissected and the last 20 cm of the gut was removed, opened and weighed. The volume of each tumour was estimated by measuring three diameters according to the formula $a \times b \times c/2$. Statistical analysis was performed according to the Kruskal-Wallis test [11]. The mortality in the individual groups was compared using Fischer's exact test [30].

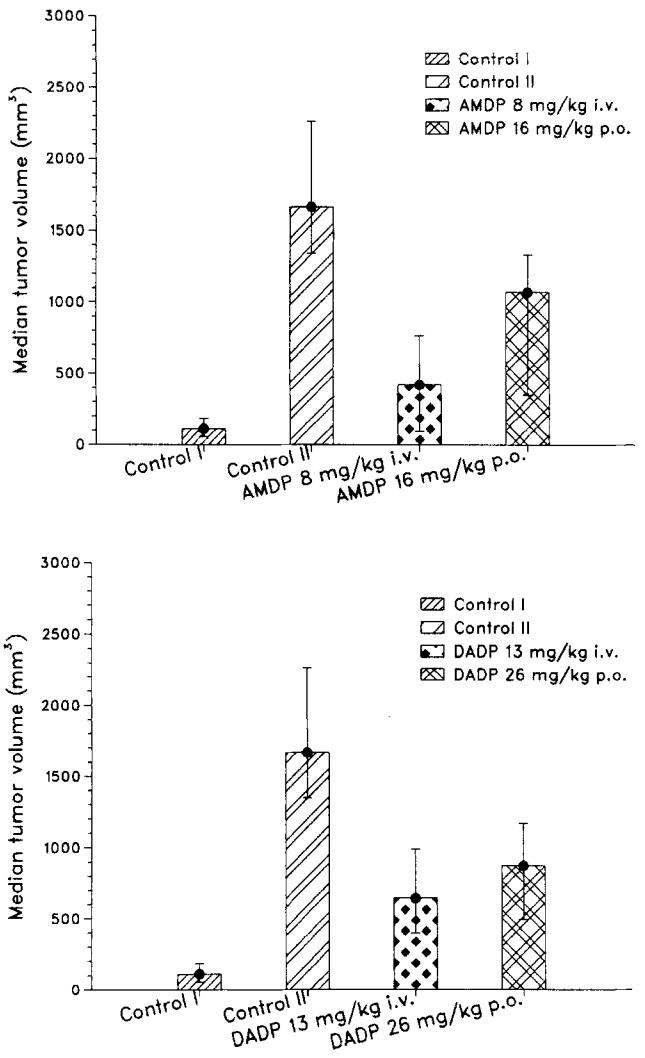


Fig. 2. Therapeutic efficacy of AMDP (*top*) and DADP (*bottom*) against AMMN-induced colorectal carcinoma in the Lewis rat. The *bars* represent the T/C × 100 values obtained. *Controls I and II* show the median tumor volume measured in untreated tumor-bearing animals before and after treatment, respectively

Table 1. Scheme of treatment with AMDP and DADP

Group number	Compound	Animals (*n*)	Administration route	Single dose		Weekly dose[a] (mg/kg)	Total dose[a] (mg/kg)
				(mg/kg)	(mmol/kg)		
2	Control	20	–	–	–	–	–
3	AMDP	15	i.v.	8	0.015	16	160
4	AMDP	15	p.o.	16	0.030	32	320
5	DADP	15	i.v.	13	0.021	26	260
6	DADP	15	p.o.	26	0.042	52	520

[a] Administration period, 10 weeks

Table 2. Anticancer activity of AMDP and DADP

Group number	Animals (*n*)	Treatment mode	Median colon weight[a] (g)	Median tumor volume[b] (mm^{-3})	T/C%[c]	Median number of tumors
1[d]	10	–	2.8 (2.3–3.3)	110.2 (54–183.5)	6.6	3.5 (1–6)
2[e]	20	–	4.2 (3.4–4.7)	1665.5 (1343–2264)	100	13 (9–15)
3	15	AMDP i.v. 2×8 mg/kg	2.75* (2.2–3.2)	420.5* (94– 764.5)	25.2	7* (4–9)
4	15	AMDP p.o. 2×16 mg/kg	3.8 (2.9–4.4)	1068.5 (351–1332)	64.1	7.5 (3–12)
6	15	DADP i.v. 2×13 mg/kg	3.7 (3.1–4.4)	639.5* (394–987.5)	38.3	9 (6–10)
7	15	DADP p.o. 2×26 mg/kg	4.5 (3–5.4)	872.2* (493–1168)	52.3	8.5* (5–11)

Values in parentheses represent the 95% confidence limits
[a] Weight of the distal colorectum 20 cm proximal to the anal region
[b] Calculated using the equation a×b×c/2
[c] Ratio of increases in median tumor volume in the treated groups to those in controls
[d] Control at the beginning of treatment
[e] Control after the termination of therapy
* Difference significant relative to control (P <0.05)

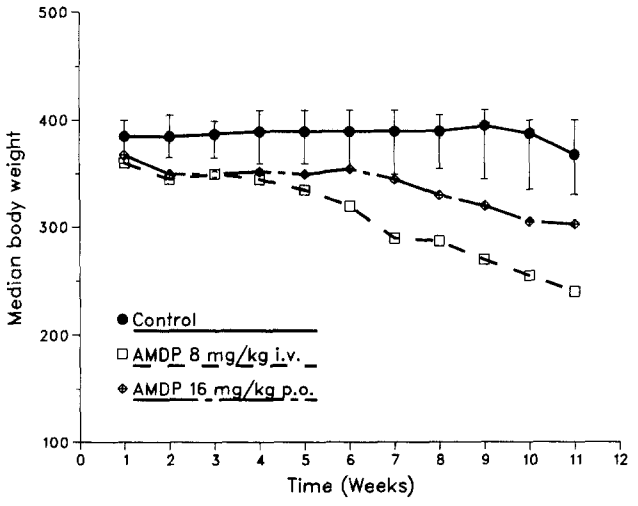
Table 3. Parameters of toxicity following therapy with AMDP and DADP

Group number	Animals (*n*)	Treatment mode	Median body weight (g)		Change in median body weight (%)[a]	Mortality
			Therapy week 1	Therapy week 10		
2[b]	20	–	385 (360–400)	387.5 (335–400)	+0.6	0 (0)
3	15	AMDP i.v. 2×8 mg/kg	360 (350–370)	255 (230–295)	–19	1 (6%)
4	15	AMDP p.o. 2×16 mg/kg	367.5 (340–390)	305 (250–355)	–17	3 (20%)*
6	15	DADP i.v. 2×13 mg/kg	375 (355–430)	370 (330–405)	–2	1 (6%)[d]
7	15	DADP p.o. 2×26 mg/kg	365 (345–395)	340 (290–375)	–7	2 (13%)[e]

Values in parentheses represent the 95% confidence limits
[a] Difference between the final and the initial body weight divded by the initial body weight ×100 yields the percentage of change in the initial body weight
[b] Control after the termination of therapy
[d] 1 death due to pneumonia
[e] 1 death due to tumor perforation and 1 due to technical failure
* Significant difference vs control (p = 0.036) according to Fischer's exact test

Cell lines. For in vitro experiments, the two human colon-cancer cell lines SW707 and SW948 were used. They were derived from patients with colorectal cancer and were provided by the Tumor Bank of the Institute of Experimental Pathology, German Cancer Research Center (Heidelberg, FRG). Their characteristics were as follows; SW707 and SW948 were derived from well-differentiated adenocarcinomas of the rectum and colon, respectively, with modal chromosome numbers of 47 for SW707 and 76 for SW948. Microscopically, SW707 cells showed no microvesicular bodies in contrast to SW948 cells. The latter cell line synthesised considerably more carcinoembryonic antigen (CEA) and produced more mucus than SW707 cells [20]. Both cell lines were routinely checked for their mucus production to exclude the possibility that fibroblasts had overgrown the carcinoma cells. Moreover, they were tested for mycoplasma contamination and proved to be negative. The cell lines were grown as monolayer cultures in MEM medium (Gibco) supplemented with 10% heat-inactivated (57°; 40 min) fetal calf serum, streptomycin (100 µg/ml), penicillin (100 IU/ml) and L-glutamine (2 µmol/ml; all from Serva, Heidelberg, FRG). The cells were maintained in a humidified atmosphere (5% CO_2/95% air) at 37° C.

MTT test. Monolayer cell cultures were trypsinised and single-cell suspensions were obtained by repeated pipetting. The percentage of viability was determined by the trypan-blue exclusion test. A final concentration of 2×10^4 cells/ml medium was prepared, and then 1 ml was added to each well of a 24-well culture plate. On the day following the plating of the cells, the test compounds were added. The MTT assay was performed after the cells had been incubated with the test substances for 5 days. On the 5th day of incubation, cell counting by Coulter Counter was performed. AMDP was evaluated at doses of 19, 38 and 190 µM, whereas DADP was tested at levels of 165, 330, 660, 1320 and 2640 µM.



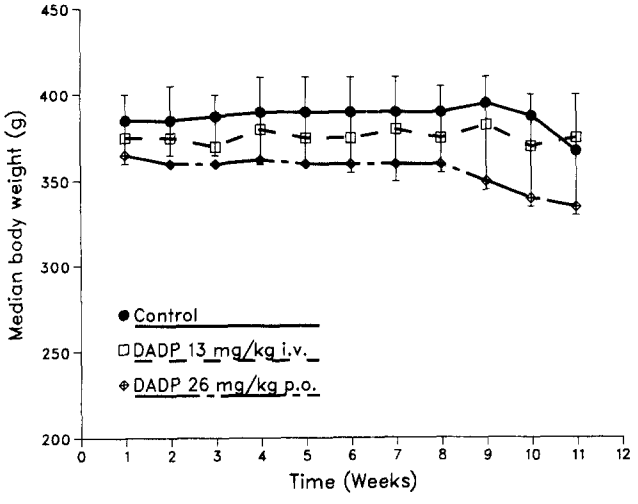
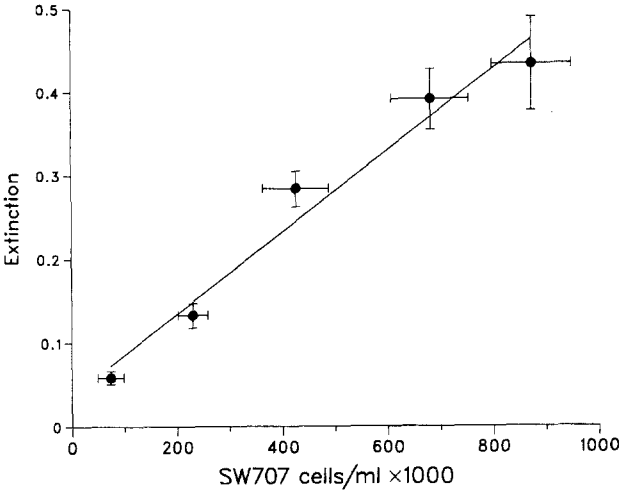


Fig. 3. Median body weight after treatment of Lewis rats with AMDP (*top*) and DADP (*bottom*) for AMMN-induced colorectal carcinoma



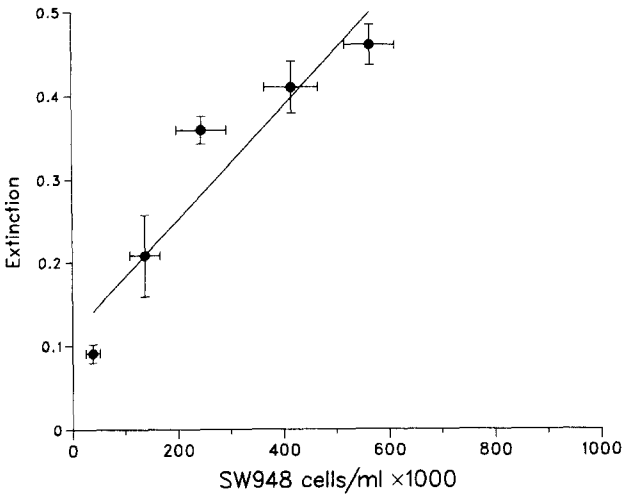


Fig. 4. Correlation of extinction as estimated using the MTT assay and cell counts (Coulter Counter) in cell lines SW707 (*top*) and SW948 (*bottom*)

According to a previously described procedure [24], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Munich, FRG) was dissolved in phosphate-buffered saline (Oxoid Ltd., Basingstoke, England) at 5 mg/ml and filtered through a 0.22-µm filter (Millipore, Molsheim, France), after which 100 µl of this MTT solution was added to each well (0.1 ml/ml medium). After incubation of the plates with MTT for 1 h at 37° C, the medium was discarded and 500 µl isopropanol (0.2 ml 0.04 *N* HCl in 10 ml isopropanol) was added to all wells to stop the enzyme reaction. Within 1 h of this addition, the extinction of the coloured formazan derivative dissolved in acid-isopropanol was determined on a Flow Multiscan MC plate reader at a wavelength of 540 nm (reference wavelength, 690 nm).

Results

The treatment of AMMN-induced colorectal carcinoma with AMDP brought about higher antitumour efficacy than did treatment with DADP following both routes of administration (Table 2). In fact, both i.v. and oral administration of AMDP caused a significant reduction in median tumour volumes as indicated by the T/C% value (Table 2, Fig. 2). Its i.v. administration also significantly inhibited the median number of tumours, suggesting remarkable antineoplastic activity (Table 2). AMDP also considerably reduced the body weight of mice treated by the i.v. and the oral routes of administration by 19% and 17% respectively, but mortality was significantly increased only in the orally treated group (20%; $P = 0.036$; Table 3, Fig. 3).

The i.v. and oral administration of DADP significantly decreased the median tumour volume (Table 2, Fig. 2) but at the same time caused distinctly less toxicity than did AMDP as shown by the moderate loss of body weight (2% and 7%, respectively; Table 3, Fig. 3) and by the insignificantly increased mortality (6% and 13%, respectively; Table 3). It should be noted that the three deaths observed in DADP-treated groups were not caused by the treatment (Table 3).

In vitro, the doubling time of cell lines SW707 and SW948 was 24 and 33 h, respectively (Fig. 4). AMDP almost completely inhibited cell proliferation at the highest

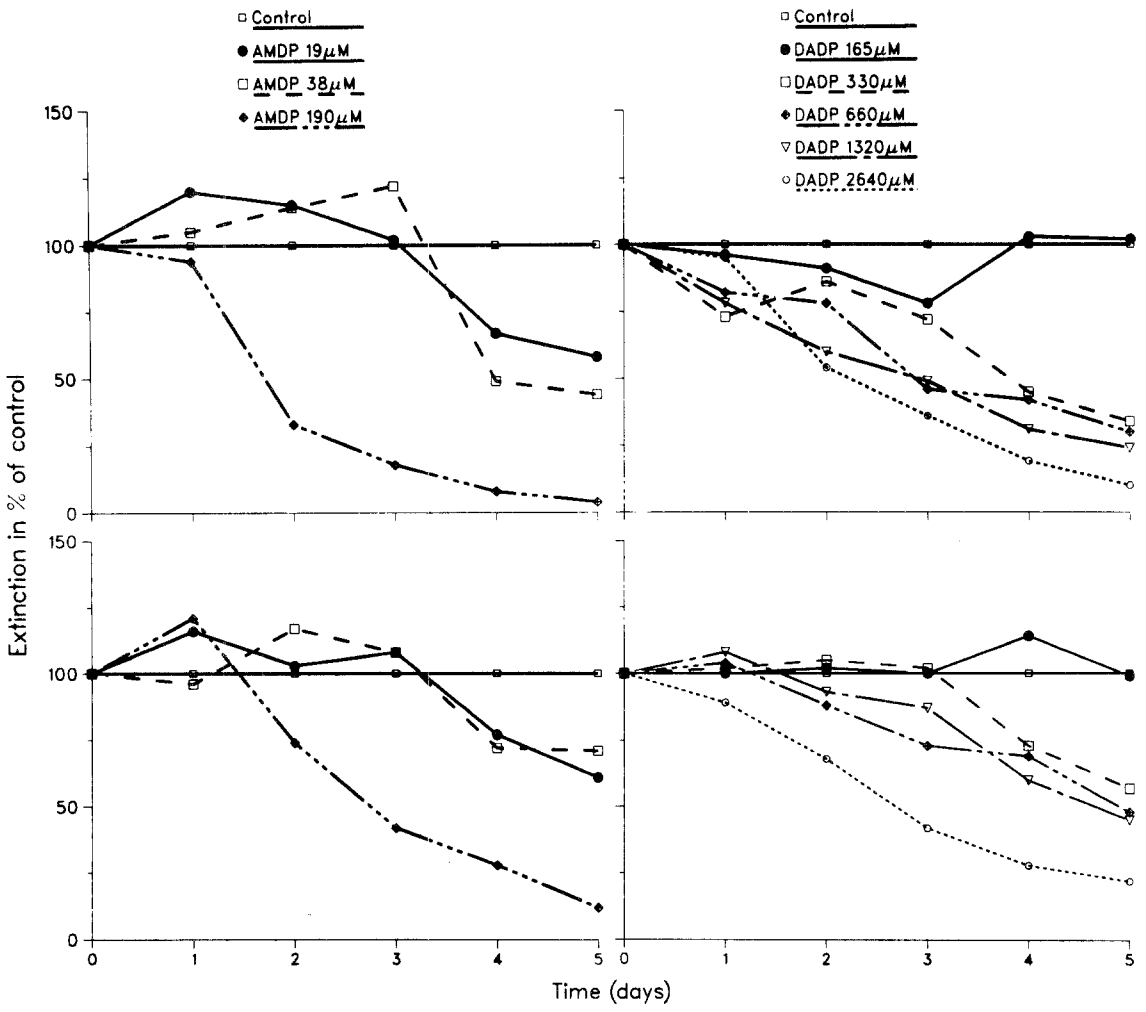


Fig. 5. Percentage of extinction relative to controls in the SW707 (*top*) and SW948 (*bottom*) cell lines after incubation with AMDP (*left panels*) and DADP (*right panels*)

concentration used (190 μM) in both of these human colon-cancer cell lines. The lower concentrations used showed measurable efficacy in inhibiting the growth of neoplastic cells (Fig. 5). The AMDP concentrations required to inhibit the growth of 50% of the cell population (IC_{50} values) were 34 and 59 μM in the SW707 and SW948 cell lines, respectively. DADP showed strong inhibitory activity at high concentrations only. Within the concentration range of 330–1320 μM, SW707 was more sensitive than SW948, but the highest concentration (2640 μM) was similarly effective in both cell lines (Fig. 5). The IC_{50} values estimated using the MTT assay were 412 and 660 μM in SW707 and SW948 cells, respectively. The determination of cell numbers by the Coulter Counter revealed antiproliferative activity for AMDP and DADP that was higher than that estimated using the MTT assay in both cell lines (Fig. 6). This is evident from the IC_{50} values of 8 and 11 μM found for AMDP and those of 266 and 285 μM found for DADP in SW707 and SW948 cells, respectively.

Discussion

Recently, a new class of metal complexes based on platinum linked to phosphonic acids was developed and characterised preclinically [16–18]. Some of these compounds possess remarkable activity in inhibiting the growth of tumour cells in vitro, i.e. in cell lines of lung metastases of osteosarcoma [14]. The effectivity of these new anticancer agents prompted us to investigate them in autochthonous colorectal cancer in the rat and – for a comprehensive evaluation as well as comparison of their antineoplastic activity – in two human colon-cancer cell lines. Of the two compounds investigated, AMDP showed higher antineoplastic efficacy after i.v. treatment in autochthonous colon cancer of the rat with regard to tumour-growth inhibition. The activity observed after i.v. treatment with DADP was discernibly lower. From a comparison of the median tumour volume in the i.v. and orally treated groups, it can be deduced that the bioavailability of both compounds is below 50% after oral administration. This can presumably be explained by the low absorption of the substances after oral administration. However, the toxicity of AMDP was higher than that of DADP, particularly after i.v. treatment;

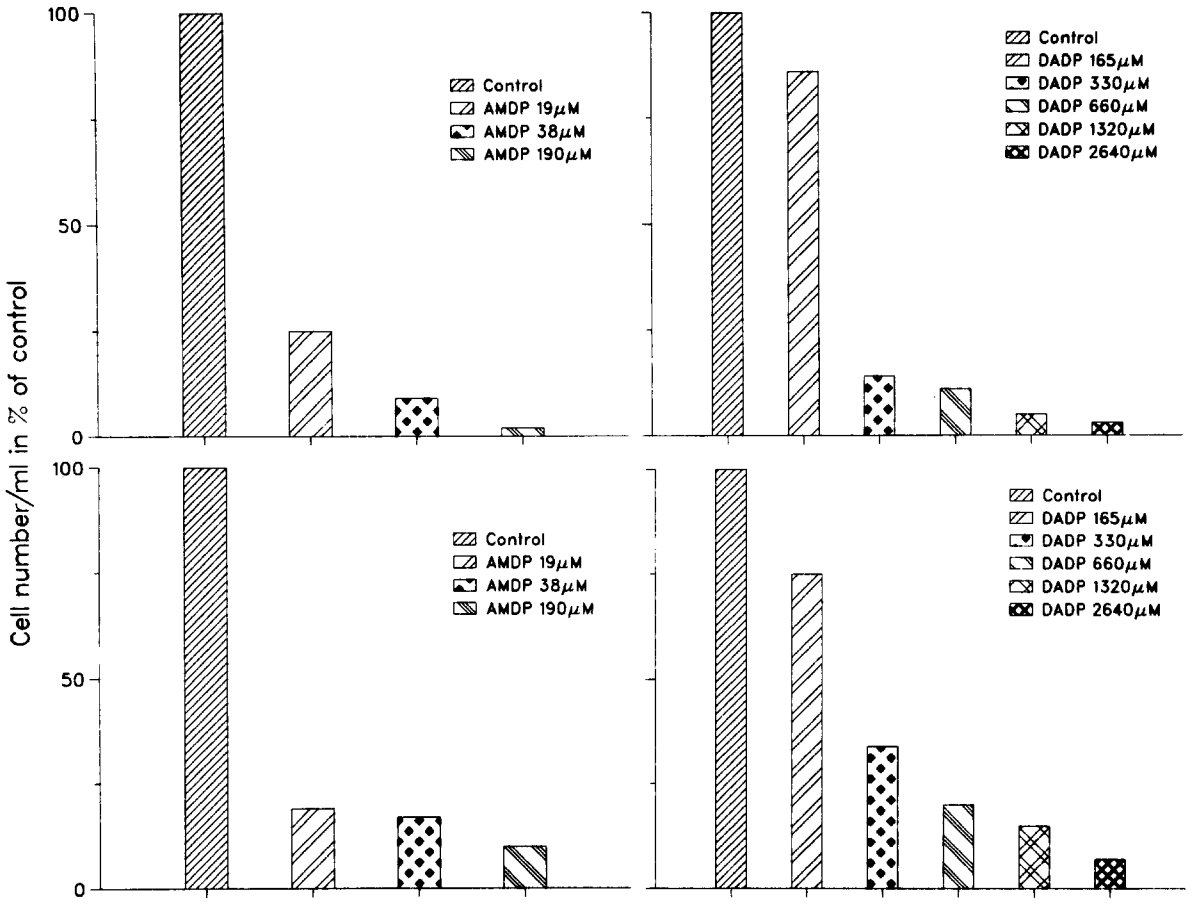


Fig. 6. Cell count expressed as a percentage of the control value as determined in the SW707 (*top*) and SW948 (*bottom*) cell lines after 5 days of incubation with AMDP (*left panels*) and DADP (*right panels*)

therefore, a comparison of the therapeutic ratios of AMDP and DADP clearly favours the latter compound.

A comparison of the observed therapeutic activity with data previously obtained on cis-DDP, 5-fluorouracil, and other recently developed metal complexes that have been investigated in this model might be helpful in assessing the ranking of the two compounds used in the present study. Remarkably, cis-DDP showed no anticancer activity at a toxic dose that caused body-weight loss as well as insignificantly increased mortality [3]. Peroral administration of 5-fluorouracil effected a T/C% value of only 41% at the expense of 10% mortality and 10% body-weight loss [3]. Combined i.p. administration of 5-fluorouracil and leucovorin increased this anticancer activity only marginally [33]. In its chemosensitivity, the AMMN model used in our study parallels the effectiveness of 5-fluorouracil and cis-DDP in human colorectal cancer. It may therefore be assumed that agents with higher antitumour activity might be of value in the clinical setting. A newly synthesised ruthenium complex has also shown promising activity, surpassing the two platinum complexes in terms of T/C% ratio [4]. Despite their relative inferiority, the two platinum-based complexes deserve interest as model compounds, as the linking of platinum with phosphonic acids changes the activity profile of this noble metal from inactive to active in the present model.

The use of in vitro studies could complete and improve the understanding of results obtained in vivo and thus lead to a better evaluation of the antineoplastic activity of antitumour substances. The two cell lines used in this study were derived from well-differentiated human adenocarcinomas. They have largely been characterised in terms of biological features such as CEA production, chromosome number and mucus secretion [20]. Moreover, SW707 cells are known to be moderately sensitive to 5-fluorouracil [27]. On this basis, we expected to obtain results that would parallel those obtained using the in vivo model. The MTT test used to quantitate the response in vitro is a simple enzyme-activity-based assay that was introduced by Mosmann [24]. In this assay, a tetrazolium salt [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is reduced to a coloured formazan product by enzymes present in metabolically active, living cells [24]. As dead cells are unable to reduce MTT, the colour reaction is a measure of cell viability.

The in vitro trials demonstrated that AMDP was more effective than DADP in both cell lines. For DADP, an inhibitory effect on cell proliferation was observed only at relatively high IC_{50} values as estimated by the MTT assay. However, the more than 10-fold difference found in IC_{50} values between AMDP and DADP in vitro was not reflected by the maximally 2-fold difference in the doses that

were active in vivo. Thus, reliance on in vitro results alone would probably have led us to discard a compound that is superior to its congener in vivo. In regard to this problem, which is related to the predictivity of in vitro assays, it should be noted that the in vitro model represents a simple, uncomplicated system comprising one indicator organism and few metabolising systems, whereas the in vivo model is highly complex, consisting of many indicator organisms, metabolising systems and both passive and active elimination, not to mention numerous interactions involving humoural, cellular and nervous factors [32]. An assessment contrary to that of DADP was made for cis-DDP, which has produced effective inhibition of the proliferation of human colorectal cell lines, a result that was not predictive for human colorectal cancer in vivo [5]. Obviously, human carcinoma cells growing as a monolayer on uncoated plastic substrata are highly artificial systems that do not reflect the in vivo situation and are not related to the naturally developing cancers or to their tissues of origin. Such failures could perhaps be avoided if normal colorectal tissue were used together with neoplastic tissue to improve the evaluation of chemotherapy trials in vitro.

Apart from this qualitative problem, both AMDP and DADP were more efficacious as assessed by the Coulter Counter method in comparison with the MTT assay; thus, the latter method underestimated the activity of these compounds. This observation might be related to an increase in the metabolic activity of the cells induced by the two complexes and might be evidence of an induction of differentiation [1, 8]. Recently, the use of the MTT assay in estimating anticancer drug efficacy has been called into question by the demonstration that the MTT assay does not necessarily measure basal cytotoxicity [22]. This question will be addressed in future studies.

In conclusion, the promising activity of the new platinum-linked phosphonic acids in both autochthonous rat-colon cancer in vivo and human colon-cancer cell lines in vitro may be considered to represent progress in chemotherapy against resistant tumours such as colorectal cancer and warrants their investigation in future experimental and clinical trials.

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