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The antitumor activity of the platinum complex D-17872 is associated with tumor cell differentiation

Hans Rainer Maurer[1], Christine Echarti[1], Rainer Voegeli[2], Jörg Pohl[2], Peter Hilgard[2]

[1] Institut für Pharmazie der Freien Universität Berlin, Kelchstrasse 31, W-1000 Berlin 41, Germany
[2] Department of Experimental Cancer Research, ASTA Medica AG, Weismüllerstrasse 45, W-6000 Frankfurt am Main 1, Germany

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Abstract. The novel cisplatin analogue D-17872 was studied for its anticancer activity using in vivo and in vitro preclinical models. The compound at the sublethal dose of 215 mg/kg (ca. 50% of the approximate LD_{50}) induced no nephrotoxic effect strong enough to increase the blood urea level in rats. It had good in vivo antitumor efficacy against murine P388 (max. ILS: D-17872 132%, cisplatin 55%) and L1210 leukemia (max. ILS: D-17872 43%, cisplatin 38%), L5222 leukemia of the rat (max. ILS: D-17872 163%, cisplatin 163%) and murine B16 melanoma. Activity against P388 leukemia substantially exceeded that of cisplatin. Moreover, the M5076 reticulum cell sarcoma implanted into the subrenal capsule and the DMBA-induced mammary tumor of the rat were inhibited by D-17872 to a greater extent than by cisplatin (min. T/C: D-17872 –3%, cisplatin 11%). Using clonogenic microassays, D-17872 was active in vitro against a variety of human and rodent tumor cell lines, albeit at higher concentrations than cisplatin (IC_{50} values: D-17872 2.6–12.7 μmol/l, cisplatin 0.13–0.42 μmol/l). Apart from its cytotoxic action it was able to induce in vitro differentiation of the human HL-60 and K562 and of the murine M1-T22 cell lines, while cisplatin induced differentiation only in the HL-60 cell line. Thus D-17872 exhibited a pharmacological and toxicological profile different from that of the parent compound. The results suggest that induction of differentiation contributes to the antineoplastic efficacy of this novel cisplatin derivative.

Introduction

After the discovery of its antitumor activity in 1969 [19] *cis*-diamminedichloroplatinum (cisplatin) has become one of the most widely used drugs in the treatment of cancer. Its importance derives largely from its ability to cure patients with tumors of the testes and to induce complete responses in patients with tumors of the ovary. It also shows therapeutic efficacy in prostate, uterus, bladder, and in head and neck cancer [9, 13].

The remarkable antitumor effects coincide, however, with marked toxic side effects, the most important of which are emesis and nephrotoxicity [10]. This gave rise to the search for derivatives which would combine the anticancer efficacy of the parent compound with less toxicity [8]. The first second-generation platinum drug, carboplatin, has since been introduced for the treatment of malignancies. Its spectrum of antitumor activity is not different from that of cisplatin, but it induces only minor nephrotoxicity or none at all [5]. In contrast to cisplatin, however, it has strong hematological side effects, the most prominent being a depression of platelets [24].

A recent chemical approach to cisplatin analogue development led to the synthesis of compounds where the ammine ligands were replaced by benzylethylenediamines [3, 4]. We have selected one of these compounds (D-17872, Fig. 1), because of its high activity in our primary screening model, the P388 murine leukemia, for further studies, and report here on its antitumor activity against a number of experimental rodent tumors in vivo. In addition, some of its biological effects related to the antitumor activity were investigated in vitro. Our particular interest focussed on the induction of tumor cell differentiation. The cell lines employed in these studies have previously been demonstrated to possess differentiation markers, inducible by standard agents such as DMSO and dexamethasone [17].

Materials and methods

Animals. Mice of both sexes and 6–8 weeks of age and female rats were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, Germany, and from Möllegaard Breeding Center, Ejby, Denmark. The ani-

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Correspondence to: R. Voegeli

mals were kept under specific-pathogen-free (SPF) conditions, were fed with a standard pellet diet (Altromin 1324) ad libitum and had unrestricted water supply (acidified to pH 3).

Compounds. The compounds were synthesized at the University of Regensburg by Professor Brunner and by ASTA Medica. Cisplatin was dissolved in normal saline and D-17 872, which is not readily soluble in aqueous media, was suspended in 0.5% tragacanth (Sigma Chemicals) for injection. For tissue culture studies the compounds were dissolved in RPMI 1640 medium (cisplatin) or DMSO (D-17 872, final concentration 0.2%), and then diluted appropriately with RPMI 1640 medium.

To prepare a saturated aqueous solution of D-17 872, 5 mg was added to 2 ml of RPMI 1640 medium and sonicated in an ultrasonic bath (Bandelin, Berlin, 35 kHz) for 2 h at room temperature. Undissolved material was removed by filtration through 0.45- and 0.22-μm cellulose acetate membranes. The final solution contained 14 mg/l of Pt (determined by atomic absorbtion chromatography), equivalent to 7.2×10^{-5} mol/l D-17 872. Retinoic acid (RA; Sigma, St. Louis) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) were used as positive controls for HL-60 cell differentiation induction at 4×10^{-7} mol/l and 1.6×10^{-9} mol/l final concentrations, respectively. For K-562 cell differentiation induction 3.6×10^{-6} mol/l cytosin-arabinoside (Ara-C; Sigma) served as positive control. In experiments using M1-T22 cells 4×10^{-7} mol/l dexamethasone (Sigma) was included as a reference. RA and dexamethasone were dissolved and diluted in absolute ethanol (final concentration 0.2%), Ara-C was dissolved and diluted in culture medium, TPA in acetone (final concentration 0.2%).

Acute toxicity. The approximate LD_{50} was determined with minimal animal numbers. Two male CD2F1 mice per dose group received the test compound by i. p. injection. The animals were observed for clinical signs of toxicity over a period of 28 days.

Blood urea nitrogen. D-17 872 was administered i. p. and cisplatin i. v. to Sprague-Dawley rats (four animals per group). Blood samples were taken from the retroorbital plexus on the day before and from the 4th to the 7th day after treatment. Urea was determined by standard methods (Boehringer Mannheim test kit urea).

Transplantable tumors. Murine leukemias P388 and L1210: Six male CD2F1 mice per group were inoculated i. p. with 10^6 P388 or 10^5 L1210 freshly harvested leukemia cells. The test substances were administered i. p. the following day. The survival time was recorded. Results are given as percent increase in life span (ILS) compared with the control group.

M5076 sarcoma: A tumor fragment of approx. 1 mm^3 was implanted under the renal capsule of female B6C3F1 mice. The animals were then randomized into treatment groups of 6 animals and control groups of 10 animals (negative control treated with solvent, positive control treated with cisplatin at 3.2 mg/kg per day). The animals received daily i. p. treatment for 5 days and were killed on day 6. Tumor size was measured on day 0 and day 6 in situ (two diameters) in ocular micrometer units using a stereoscopic microscope equipped with an ocular micrometer.

B16 melanoma: Ten female C57Bl/6J mice were inoculated subcutaneously with 5×10^5 tumor cells. Daily treatment i. p. was started the following day. The animals were killed after 21 days, and the tumor was then resected and weighed.

L5222 rat leukemia: Freshly harvested cells (10^6) were injected i. p. into six female BD IX rats (230–290 g) per group. The animals were treated once on day 5. The survival time was recorded. Results are given as percent increase in life span (ILS) compared with the control group.

Dimethyl-benz(a)anthracene-induced mammary tumor of the rat: Experiments were performed as described earlier [11]. In short, mammary tumors were induced by gavage of 20 mg dimethyl-benz(a)anthracene (DMBA) to female Sprague-Dawley rats 50 days after birth. As soon as the tumor area had reached 100 mm^2 the animals were randomized into treatment groups of 9 animals and treatment (once a week) was started.

Error probabilities were determined using the Wilcoxon two-sample rank sum test in all animal experiments if not stated otherwise.

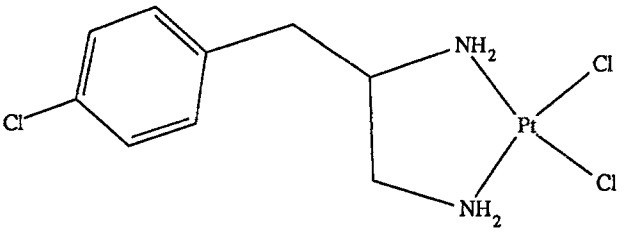

Fig. 1. D-17 872, chemical formula

Cells. HL-60 human promyelocytic leukemia cells (ATCC CCL 240) [1] were routinely cultivated in RPMI 1640 Medium (Biochrom, Berlin) supplemented with 20% heat-inactivated fetal calf serum (FCS; Paesel, Frankfurt).

K-562 human erythroleukemic cells [14] and KB human squamous epithelial carcinoma cells (ATCC CCL 17) [1] were grown in RPMI 1640 medium containing 10% FCS.

M1-T22 mouse myeloblastic leukemia cells [12, 22] were maintained in Earle's Minimum Essential Medium (MEM; Biochrom) enriched with twice the normal concentration of amino acids and vitamins and with 10% heat-inactivated calf serum (CS; Biochrom).

P388 and L1210 mouse leukemia cells (ATCC CCL 46 and CCL 219) [1] were kept in RPMI 1640 medium with 10% heat inactivated horse serum (HS; Boehringer Mannheim).

B16 mouse melanoma cells (obtained from G. Atassi, NCI Brussels) were kept in RPMI 1640 medium supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, non-essential amino acids, and 20% FCS.

Fresh mouse bone marrow cells were obtained by flushing the femur of male C57/Bl mice with normal saline.

HL-60 cell differentiation assay. Cultures (2 ml) were set up in RPMI 1640 medium with 10% FCS, containing 2×10^5 cells/ml and 4 μl of appropriate test agent dilutions. Negative controls containing 4 μl solvent without test agent were also included. After incubation for 7 days at 37° C in a fully humidified atmosphere containing 7.5% CO_2, samples were withdrawn for cell counts using a Sysmex Microcell Counter model CC-108 and differentiation induction was assessed morphologically by differential counts on May-Grünwald-Giemsa-stained cytospin preparations (cytocentrifuge from Shandon, Frankfurt), by cytochemical determination of α-naphthylacetate esterase activity [25] using kit no. 91-A from Sigma, and by nitro blue tetrazolium (NBT) reduction as described by Collins et al. [7]. Differential counts were always performed on 200 cells per slide. When HL-60 cells were induced to differentiate by 4×10^{-7} mol/l retinoic acid or 1.6×10^{-9} mol/l TPA, respectively (positive controls), the following percent values were determined under identical assay conditions: morphology 71/86, cell growth 47/2, NBT reduction 89/86, unspecific esterase 14/73. That is to say that RA induced 71% and TPA 86% of cells to differentiate to granulocytes or macrophages. The percentage of cell growth was calculated from the formula:

$$\frac{\text{No. of treated cells at end of incubation} - \text{No. of cells at start}}{\text{No. of untreated cells at end of incubation} - \text{No. of cells at start}} \times 100$$

K-562 cell differentiation assay. Cultures were set up as described for HL-60 cells, but containing only 5×10^4 cells/ml, and incubated at 37° C and 7.5% CO_2 for 4 days. Erythroid cell differentiation induction was monitored by benzidine staining as described by Rowley et al. [20]. Cell counts were performed as for HL-60. When K562 cells were induced to differentiate by 3.6×10^{-6} mol/l Ara-C (positive control), the following percent values were determined under identical assay conditions: cell growth 74%, benzidine positive cells 65% (cf. method for HL-60).

M1-T22 cell differentiation assay. Cells were seeded at 2×10^5 cells/ml in enriched MEM as described above in the presence or absence of test agent and incubated at 37° C and 5% CO_2 for 2 days. Morphological differentiation was scored by differential counts on May-Grünwald-

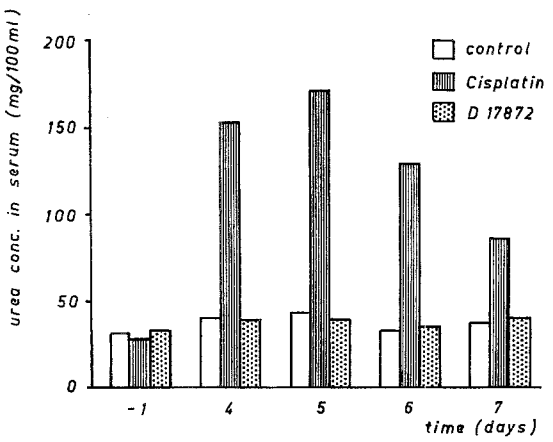

Fig. 2. Blood urea levels in rats treated with a single dose of D-17 872 (215 mg/kg i.p.) or cisplatin (3.2 mg/kg i.v.)

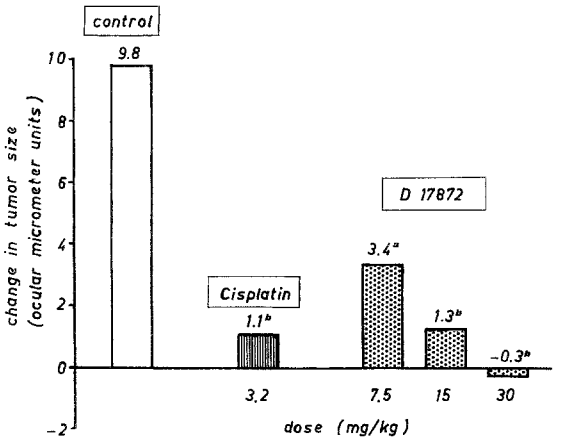

Fig. 3. Growth of M5076 reticulum cell sarcoma in the subrenal capsule assay. Doses are daily doses given for five days. [a] $P < 0.05$; [b] $P < 0.01$

Table 1. Effects of D-17 872 and cisplatin on rodent leukemia lines in vivo. The substances were given as a single dose i.p. on day 1 (P388, L1210) or day 5 (L5222) after i.p. tumor transplantation. Results are given as percent median increase of life span ILS

Tumor cell line	D-17872		cisplatin	
	dose (mg/kg)	ILS (%)	dose (mg/kg)	ILS (%)
LD_{50}	460		13	
P388 murine leukemia	100	105[b]		
	147	114[b]	3.2	55[b]
	215	132[b]		
L1210 murine leukemia	147	43[b]	3.2	38[b]
L5222 rat leukemia	10	119[b]	1	163[b]**
	46.4	125[b]	2.15	106[b]*
	215	163[b]	4.64	144[b]

[b] $p < 0.01$/* 1/6 survivors/** 2/6 survivors

Giemsa-stained cytospin preparation. The percentage of phagocytic cells was determined as outlined by Okabe et al. [16] using polystyrene latex beads from Serva (Heidelberg, Germany) average diameter 1 µm. Cells were counted as above. When M1-T22 cells were induced to differentiate by 4×10^{-7} mol/l dexamethasone (positive control) the following percent values were determined under identical assay conditions: morphology 13%, cell growth 60%, phagocytic cells 55% (cf. method for HL-60).

Clonogenic microassays. Cells were seeded in their appropriate medium (see above), except that the serum concentrations were raised as follows: P388 and L1210, 15% HS; B 16, K-562, 15% FCS; HL-60, 25% FCS. Cultures were solidified with 0.18% agar. Appropriate test agent dilutions were added prior to seeding 4×10^3 cells/ml of HL-60 or 2.4×10^3 cells/ml of the other tumor lines into glass capillaries as described earlier [15]. Mouse granulocyte-macrophage (GM) colonies were grown from bone marrow cells in IMDM medium (Boehringer Mannheim) containing 33% (v/v) of saline and supplemented with 2 mM of L-glutamine, 20% HS, 10% mouse lung conditioned medium as a source of colony-stimulating factor, prepared as described by Sheridan and Metcalf [21], and 0.18% agar. Cells were seeded at 1×10^5/ml. All cultures were incubated at 37° C and 7.5% CO_2 in a fully humidified atmosphere for 7 days. Colonies (aggregates of more than 40 cells) were counted under a dissecting microscope. From the three colony counts per test point the median ± the mean absolute deviation from the median (MAD) were determined and the percentage of the colony number achieved in untreated controls calculated. IC_{50} values were read from dose–response curves.

Results

The approximate LD_{50} of D-17 872 after i.p. treatment of CD2F1 mice was 460 mg/kg and that of cisplatin was 13 mg/kg. Rats treated with the sublethal dose of 215 mg/kg of D-17 872 i.p. showed no increase in BUN whereas in rats treated with cisplatin (3.2 mg/kg i.v.) BUN rose sharply showing the known nephrotoxicity of this compound (Fig. 2).

The effect of D-17 872 in three rodent leukemia models is shown in Table 1. The maximal ILS achievable with D-17 872 in the treatment of P388 leukemia-bearing mice exceeded that of cisplatin by a factor of 2. The flat dose–response relationship indicated that even the single dose of 100 mg/kg yielded the near-maximal therapeutic effect. In the treatment of L1210 leukemia-bearing mice treatment-with D-17 872 resulted in a significant prolongation of survival comparable to the effect of cisplatin.

Treatment of rats bearing the L5222 leukemia with D-17 872 resulted in a 160% ILS, while with cisplatin the maximal ILS was comparable but, in contrast to treatment with D-17 872, cures were achieved.

D-17 872 had substantial efficacy in the treatment of solid tumors. The growth of B16 melanoma was significantly inhibited by D-17 872. Compared with cisplatin, however, its activity was less (cisplatin 1 mg/kg per day ×9: 66%, D-17 872 6.8 mg/kg per day ×6: 49% growth inhibition). The growth of the M5076 reticulum cell sarcoma implanted into the subrenal capsule was inhibited in a dose-related manner by treatment with D-17 872. With the highest dose tested no tumor growth was detected (Fig. 3). This dose was less than 10% of the LD_{50} per day, whereas the cisplatin dose was about 25% of the LD_{50} per day.

D-17 872 was also effective in the treatment of the autochthonous DMBA-induced mammary carcinoma. The growth curve over the period of 21 days is shown in Fig. 4. Treatment with D-17 872 significantly inhibited tumor growth, whereas cisplatin had only minor activity in this

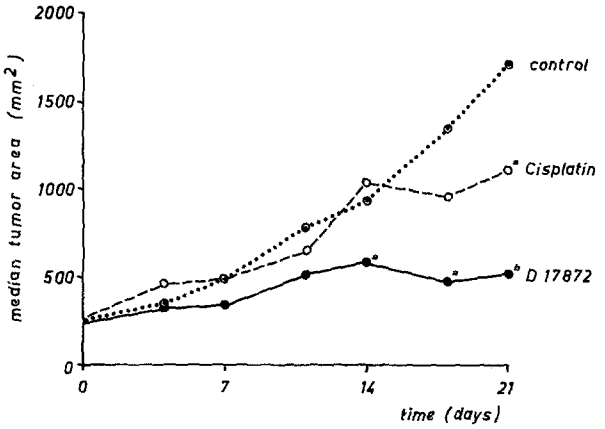

Fig. 4. Growth of DMBA-induced mammary tumors treated with cisplatin (1.47 mg/kg per week) or D-17872 (46.4 mg/kg per week) for 3 weeks. [a] $P < 0.05$; [b] $P < 0.01$

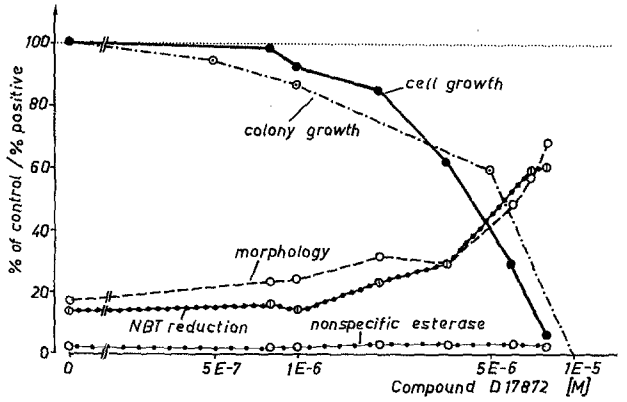

Fig. 5. Effect on growth and differentiation markers of the human promyelocytic leukemia cell line HL-60 by D-17872 (dissolved in 0.2% DMSO)

Table 2. In vitro effects of cisplatin and D-17872 in clonogenic micro assays expressed as IC50 values (μmol/l, median of 3–5 experiments ± median absolute deviation from the median)

	D-17872	cisplatin	IC50 ratio: D-17872/cisplatin
Tumor cells:			
P388	4.8±0.6	0.17±0.01	28
L1210	2.6±0.4	0.13±0.04	20
B16	12.7±2.2	0.42±0.0	30
KB	3.3±1.9	0.18±1.05	18
HL-60	5.6±0.2	0.29±0.03	19
Normal cells:			
mouse GM-CFC	2.6±0.3	0.35±0.05	7

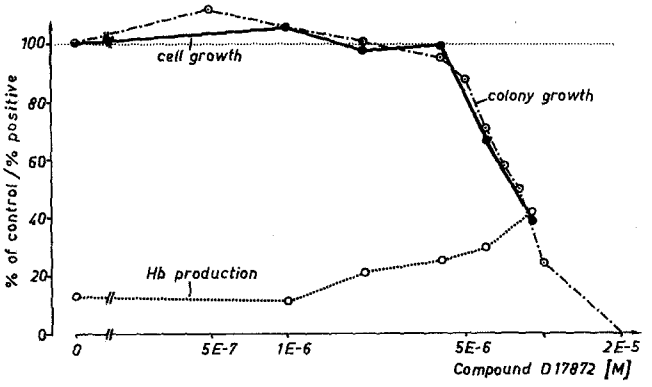

Fig. 6. Effect on growth and differentiation markers of the human erythroleukemic cell line K562 by D-17872 (dissolved in 0.2% DMSO)

tumor model, reaching the level of significance only at the last time point.

Thus, D-17872 had better antitumor efficacy than cisplatin in most of the in vivo test models, and was in addition less toxic. However, according to clonogenic microassays with the tumor cell lines P388, L1210, B16, KB and HL-60, D-17872 was 18- to 30-fold less inhibitory in vitro than cisplatin judging from the IC_{50} values (Table 2). Normal murine GM-CFC were inhibited at 7-fold higher concentrations. The in vivo effects of D-17872 might thus not only be due to mere tumor cell killing, but also to other mechanisms, such as induction of cell differentiation.

D-17872 was therefore tested for differentiation induction in the human promyelocytic leukemia cell line HL-60, the erythroleukemic cell line K-562, and the murine myeloblastic leukemia cell line M1-T22. HL-60 cell growth in liquid medium was significantly inhibited by concentrations above 2×10^{-6} mol/l of D-17872, dissolved in DMSO (Fig. 5). The dose–response curve was very similar to that obtained with the microclonogenic assay. The percentage of NBT-reducing cells increased 2-fold at 3.5×10^{-6} mol/l and 4-fold at higher concentrations. Morphologically, differentiated cells were similar to granulocytes. Only very few cells stained positively for α-naphthylacetate esterase, confirming that HL-60 cells were induced to differentiate along the granulopoietic pathway.

In contrast, when D-17872 was dissolved in aqueous medium, HL-60 cells were induced to differentiate into monocytic cells at concentrations as low as 1.2×10^{-9} mol/l: a 5-fold increase of monocyte-like cells and a 3-fold increase of unspecific esterase-positive cells were found.

Erythroid differentiation of K562 cells was induced by the same concentrations of D-17872 dissolved in DMSO as for HL-60 (Fig. 6). The percentage of hemoglobin-synthesizing cells gradually increased up to 3-fold at 9×10^{-6} mol/l of D-17872. Colony growth and cell growth in liquid medium were equally inhibited by concentrations above 5×10^{-6} mol/l. No significant difference was found when D-17872 was assayed in the absence of DMSO.

When M1-T22 cells were exposed to D-17872 at concentrations above 1×10^{-6} mol/l, increasing numbers of giant cells, i.e. cells 2–4 times larger than control cells, were found in May-Grünwald-Giemsa-stained cytospin preparations (Fig. 7). Most of these cells, however, still looked like blast cells. Few cells exhibited morphological properties of macrophage-like cells (Fig. 8). The percent-

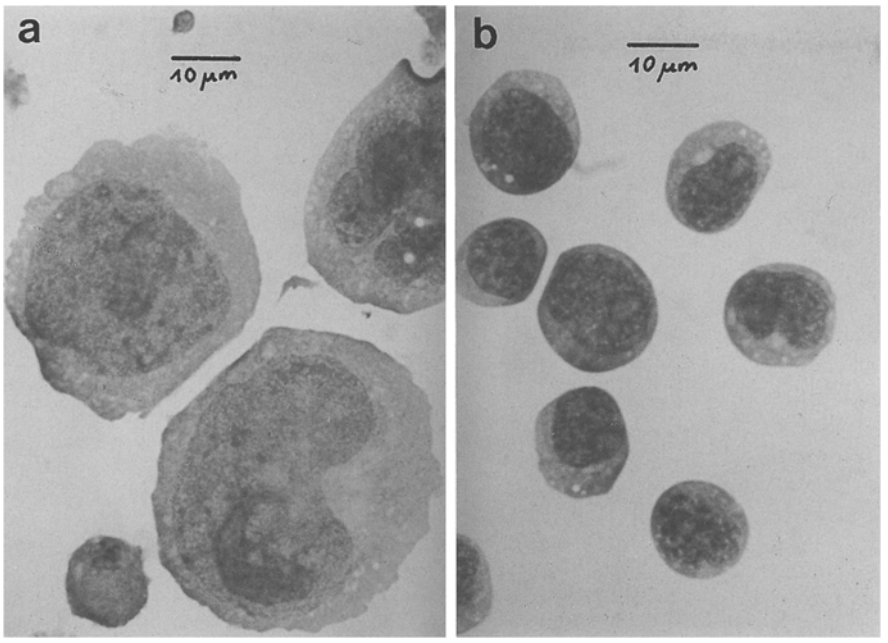

Fig. 7 a, b. Abnormal cells induced in cultures of M1-T22 by D-17872 (7×10^{-6} mol/l in 0.2% DMSO, d 3). **a** Treated; **b** untreated

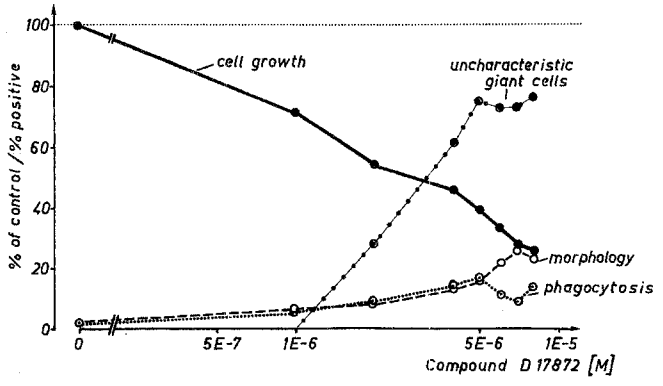

Fig. 8. Effect on growth and differentiation markers of the murine myeloblastic cell line M1-T22 by D-17872 (dissolved in 0.2% DMSO)

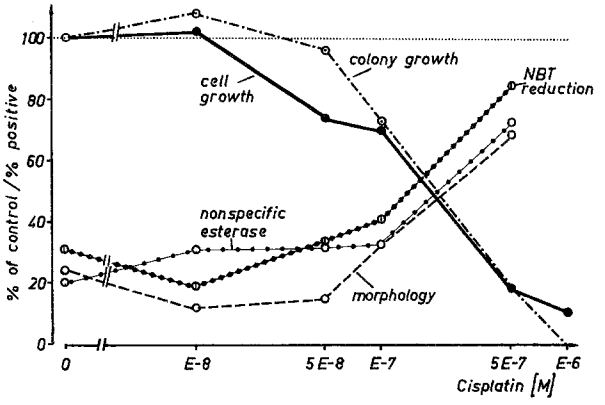

Fig. 9. Effect on growth and differentiation markers of the human promyelocytic leukemia cell line HL-60 by cisplatin

age of these cells reached 26% at 7×10^{-6} mol/l of D-17872. The maximal percentage of phagocytic cells, 17%, was found at 5×10^{-6} mol/l. Cell growth was increasingly inhibited by 1×10^{-6} mol/l and above.

Cisplatin, dissolved in aqueous solution, also induced HL-60 cells to differentiate to monocytic cells (Fig. 9). At 5×10^{-7} mol/l the percentage of both monocyte-like and unspecific esterase-positive cells increased almost 4-fold and of NBT-reducing cells about 3-fold. Cell and colony growth was inhibited by 80% at this concentration. However, K562 and M1-T22 cells were not induced to differentiate by cisplatin tested up to cytotoxic concentrations. No giant cells were observed.

Discussion

D-17872 was originally discovered in a broad screening program for cisplatin analogues. In comparison with cisplatin, D-17872 demonstrated an outstanding antitumor activity in the P388 leukemia and the M5076 sarcoma model. In the majority of the tumor models tested the efficacy of D-17872 was equal to or greater than that of cisplatin. Even in the DMBA-induced, autochthonous mammary tumor of the rat, which is only moderately sensitive to cisplatin, it caused marked retardation of tumor growth.

In the P388 model the ILS achievable with D-17872 was larger than 100% at less than 25% of the LD_{50}, cisplatin only effected an ILS of 55% at a dose equitoxic in regard to the LD_{50}. Furthermore, no gross evidence of nephrotoxicity was seen, even at the highest dose levels. In contrast to cisplatin, the BUN levels always remained within the normal range after D-17872 treatment.

In parallel with the acute toxicity in vivo, the effective concentrations in the clonogenic assays were 18- to 30-fold higher for D-17872 than for cisplatin.

Cisplatin, like most other cytostatic agents, has poor selectivity for cancer cells. As a result, its therapeutic range is narrow with considerable side effects at dose levels

necessary for antitumor activity. However, there have been repeated suggestions of additional effects of cisplatin contributing to its antitumor efficacy, e. g. immunostimulation [2, 6, 18], and it has also been suggested that cisplatin could induce a host immunologic reaction through alterations of tumor cell membranes [23]. Modifications of the cisplatin molecule could therefore lead to analogues with a different mode of action at the target cell, thus possibly increasing the selectivity for tumor cells relative to normal tissue.

Some anticancer drugs may cause morphological differentiation of tumor cells in culture, apart from their cytotoxic action, which in turn results in growth retardation. This effect has been described for several cytostatic drugs [17]. Our data suggest that the induction of differentiation may play a major role for the cytostatic activity of D-17872. This cisplatin analogue was able to induce differentiation in a broader range of cell lines than cisplatin which only showed an effect on HL-60 cells.

Although DMSO was used as solvent for D-17872 because of its poor water solubility, it was ruled out that the low concentrations of 0.2% affected the differentiation. When a stock solution of D-17872 in DMSO was added to HL-60 cell cultures, granulocytic differentiation was induced. However, monocytic cells were induced when stock solutions of both cisplatin and D-17872 in aqueous medium were used. Interestingly, D-17872 was about 100 times more effective than cisplatin under comparable assay conditions. This solvent phenomenon remains to be studied further, also whether the differentiation inducing properties of cisplatin are changed in the presence of DMSO, too. The low concentration of DMSO (0.2%) used as a solvent for D-17872 was by itself not able to induce differentiation in any of the cell lines we used, even HL-60 cells remaining unaffected.

We conclude from these studies that in vitro screening for potential cytostatic drugs should not rely solely upon clonogenic assays, even with a variety of tumor cells, but should include assays with other endpoints, such as differentiation induction to minimize false-negative results.

In summary, D-17872 is a new platinum analogue devoid of overt nephrotoxicity, which shows substantial antitumor activity in a variety of in vivo models. In addition to its cytotoxicity, it appears to induce tumor cell differentiation, which may contribute to its in vivo efficacy. Further analogue research is in progress with the goal of obtaining compounds that are better soluble in aqueous media and retain the unique pharmacological profile of D-17872.

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