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Evaluation of *cis*-diamminedichloroplatinum(II) combined with metoclopramide or sodium thiosulfate on L1210 leukemia in vitro and in vivo

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Summary. A microtiter assay was developed to monitor cytotoxic activity of drugs alone and in combination at varying ratios on a single plate. Combinations of metoclopramide or sodium thiosulfate with *cis*-diamminedichloroplatinum(II) (cisplatin) were evaluated in vitro and in vivo for cisplatin cytotoxicity to murine leukemia L1210. The in vitro assay indicated that metoclopramide did not interfere with cisplatin-induced cytotoxicity and confirmed previously reported inhibition of cisplatin activity by sodium thiosulfate. The drug combinations were also evaluated in vivo for antitumor activity and the results of these studies corroborated the in vitro results.

Introduction

Cis-Diamminedichloroplatinum(II) (cisplatin) has demonstrated antitumor activity against a variety of murine tumors and human malignancies [9]. Two of the major toxicities associated with cisplatin therapy are nephrotoxicity and emesis. There is interest in controlling these toxicities through the use of protective agents in conjunction with cisplatin therapy. Sodium thiosulfate was investigated as an inhibitor of cisplatin-induced nephrotoxicity, but was found to inhibit cisplatin activity as well as nephrotoxicity [7, 11]. Metoclopramide has been shown to be effective clinically in reducing the emetic effects of cisplatin without apparent reduction in the therapeutic effect [5, 6].

Recently we have reported a method of evaluating drug combinations in vitro in a microtiter cytotoxicity assay [1]. In the present study we evaluated the effects of metoclopramide on the in vitro cytotoxicity of cisplatin against murine leukemia L1210 cells. Sodium thiosulfate in combination with cisplatin was included in these studies as a positive control. Cisplatin alone and in combination with metoclopramide or sodium thiosulfate was also evaluated in vivo for antitumor activity against L1210. This study has demonstrated the ability to evaluate drug combinations in vitro by microtiter techniques which have been proven to be rapid, sensitive, and cost-effective [2, 4].

Materials and methods

Cell culture. L1210 murine leukemia cells were obtained from DBA/2 mice carrying an ascitic form of the tumor and passaged in vitro as suspension cultures in Dulbecco's minimal essential medium containing 2 m*M* L-glutamine, 25 μ*M* 2-mercaptoethanol, penicillin/streptomycin (10 units/ml and 10 μg/ml, respectively), and 10% horse serum (enriched DMEM). Cells were maintained in logarithmic growth at 37 °C in a 5% CO_2, balance air, high-humidity incubator.

Cytotoxicity assay. Each well of a 96-well V-bottom microtiter plate (Costar, #3896) received 0.15 ml enriched DMEM. Cisplatin (50 μl 1 mg/ml in saline from bulk supplies, Bristol Laboratories, Syracuse, NY) was added to wells A3 through A12 and serially diluted from row A through row F by serial transfer of 50 μl with a Cetus pro/pette (Cetus #85001-01011). Metoclopramide (50 μl 0.5 mg/ml in saline, A. H. Robbins, Richmond, Va) or sodium thiosulfate (50 μl 12.5 mg/ml in saline, Fisher Scientific, Rochester, NY) was added to column 3, rows A through H and serially diluted from column 3 to column 10, by serial transfer of 50 μl with the Cetus pro/pette. This dilution scheme provides on a single plate two columns of no-drug controls (columns 1 and 2), two columns of cisplatin-only titrations (columns 11 and 12), two rows of metoclopramide – or sodium thiosulfate – only titrations (rows G and H, columns 3 through 10), and 48 wells of drug combinations at varying ratios and concentrations. Following dilution, 0.1 ml cell suspension (1×10^7/ml in enriched DMEM) was added to each well with the Cetus pro/pette and plates were incubated at 37 °C, 5% CO_2, balance air in an incubator for 2 days. Assays were performed in duplicate.

Staining and evaluation. A modification of the method of Parish was used to quantitate viable cells following drug incubation [8]. Briefly, microtiter plates were centrifuged at 800 *g* for 5 min, and 0.15 ml medium supernatant was removed. Each well received 50 μl 0.04% Neutral Red Stain in HBSS and was mixed by pipetting. Plates were incubated for 20 min at 37 °C, 5% CO_2 and stopped by centrifugation and aspiration. Cell pellets were washed once with 0.1 ml cold 0.9% saline, centrifuged, and aspirated. Stained cell pellets were lysed with 0.2 ml 0.1 *M* acetic acid/ethanol (1:1, v:v) and mixed well; optical densities were then determined at 540 nm on a Dynatech microtiter plate reader. Cisplatin cytotoxicity (IC_{50}) in the presence and absence of the second compound was determined by linear regression analysis of optical density data.

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In vivo assay. DBA/2 and (BALB/c × DBA/2 F_1 (CDF_1) mice were used for these studies. Food and water were provided ad libitum. The L1210 leukemia was maintained in ascitic form in DBA/2 mice as described previously [10]. Experiments were initiated by implanting 10^6 L1210 cells i. p. into CDF_1 mice weighing 18–22 g each. One day after tumor implantation the mice, in groups of six per dose, were treated i. p. with one of the following: cisplatin, metoclopramide, sodium thiosulfate, or cisplatin in combination with one of the other drugs. Groups treated with combinations received cisplatin first, then the other drug immediately afterwards. A saline-treated tumor-control group was included. Selected doses of the combinations were administered in non-tumor-bearing mice to determine toxicity. Cisplatin was dissolved in saline and the other drugs in water. The concentrations of the drug solutions were adjusted according to the average weight of each experimental group (18–22 g, determined on day 0), so that the correct dose was administered in a volume of 0.5 ml/mouse. The doses of cisplatin and sodium thiosulfate were based on previous reports [7, 10], whereas the metoclopramide doses were multiples (2×, 4×, and 8×) of the usual optimal dose (8 mg/kg) of cisplatin.

Antitumor drug activity was determined based on the % T/C using the median survival time (MST) of the drug-treated (T) mice divided by the MST of saline-treated tumor-control (C) mice × 100. The experiment was terminated on day 30. Mice dying on or before day 6 were excluded from the calculations.

Results

Cytotoxic concentrations of metoclopramide and sodium thiosulfate to L1210 cells in vitro were determined in preliminary experiments. Concentrations of metoclopramide and sodium thiosulfate for the drug combination assay were then chosen to include one toxic concentration and seven nontoxic concentrations extending 4 logs below the toxic concentration. The results of the in vitro studies are summarized in Table 1. The cytotoxic activity of cisplatin to L1210 cells was not altered by the presence of nontoxic concentrations of metoclopramide. In contrast, sodium thiosulfate was found to inhibit the cytotoxic activity of cisplatin in a concentration-dependent manner. The concentration of cisplatin required to kill 50% of L1210 cells was increased 40-fold (1.6 μg/ml to 65.2 μg/ml) at the highest nontoxic concentration of sodium thiosulfate evaluated. Figure 1 compares the effects of increasing concentrations of metoclopramide and sodium thiosulfate on cisplatin cytotoxicity, Metoclopramide did not significantly alter cisplatin cytotoxicity, whereas sodium thiosulfate was inhibitory in a dose-dependent manner.

Table 1. In vitro cisplatin cytotoxicity to L1210 cells in the drug combination assay

Compound	Concentration (μg/ml)	Cisplatin IC_{50} (μg/ml) ± SEM
None	–	1.6 ± 0.3
Metoclopramide	125	Toxic[a]
	31.25	0.9 ± 0.1
	7.8	1.5 ± 0.1
	2.0	0.8 ± 0.1
	0.49	1.2 ± 0.1
	0.12	1.5 ± 0.2
	0.03	1.6 ± 0.1
	0.008	1.4 ± 0.1
Sodium thiosulfate	3125	Toxic[a]
	781.3	65.2 ± 15.2
	195.3	10.0 ± 1.8
	48.8	4.0 ± 0.9
	12.2	1.4 ± 0.1
	3.0	1.6 ± 0.2
	0.8	2.4 ± 0.4
	0.2	1.6 ± 0.2

[a] Metoclopramide or sodium thiosulfate alone was toxic to L1210 cells at this concentration

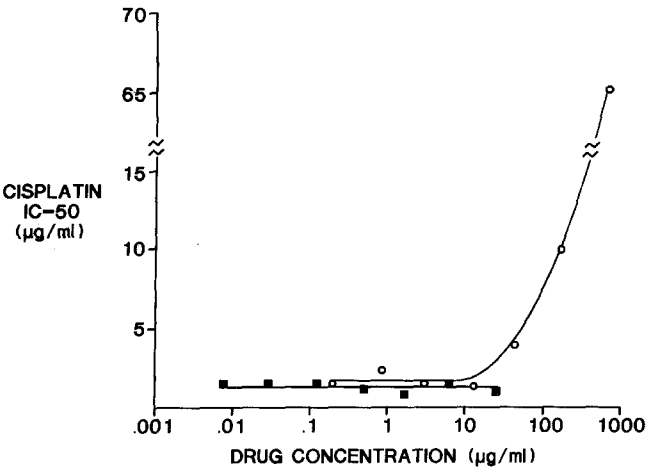

Fig. 1. Concentration of cisplatin (μg/ml) required to kill 50% of L1210 cells in vitro with increasing concentrations of metoclopramide (■) or sodium thiosulfate (○)

The results of the in vivo studies are summarized in Table 2. Cisplatin was effective, resulting in a T/C of 180% at the optimal dose of 8 mg/kg. Metoclopramide and sodium thiosulfate had no antitumor activity and caused no weight loss. The antitumor effects of cisplatin, 6 and 8 mg/kg, in combination with metoclopramide were comparable to those of cisplatin alone at these doses. Cisplatin at 10 mg/kg was less well tolerated in combination with metoclopramide than when given alone. Sodium thiosulfate reduced the peak T/C values obtained with cisplatin and shifted the optimal dose to higher values. This effect of sodium thiosulfate agrees with results reported by Howell and Taetle [7]. Sodium thiosulfate also reduced the cisplatin induced loss of body weight in the present study.

In the non-tumor-bearing mice, the combination of cisplatin at 8 and 10 mg/kg with metoclopramide (64 mg/kg) caused lethality in many mice (7/11) by day 30. The cisplatin and sodium thiosulfate combination was nonlethal.

Discussion

Anticancer drugs are often administered in combination with other types of drugs to modulate the toxicity of the anticancer agent. The use of metoclopramide to reduce the emetic effects of cisplatin is one example of such combinations. These combinations are often evaluated in the clinic with little or no preclinical information regarding poten-

Table 2. Effect of metoclopramide and sodium thiosulfate on the activity of cisplatin against L1210 leukemia in vivo

Drugs	Dose i.p. (mg/kg per inj)	MST (days)	% T/C	Av. Wt. (g) Day 5	Survivors Day 5 (30)
Cisplatin	12	7	93	−5	6/6
	10	13	173	−5.1	6/6
	8	13.5	180	−4.2	6/6
	6	11.5	153	−3.3	6/6
Metoclopramide	64	7	93	2	6/6
Sodium thiosulfate	1200	6.5	87	1.7	6/6
a) Cisplatin + b) Metoclopramide	a) 12+b) 64	7	93	−4.7	5/6
	a) 10+b) 64	8	107	−4.9	6/6
	a) 8+b) 64	14	187	−4.7	6/6
	a) 6+b) 64	12	160	−3.2	5/6
	a) 12+b) 32	7	93	−4.6	5/6
	a) 10+b) 32	7.5	100	−5.2	5/6
	a) 8+b) 32	12	160	−2.8	6/6
	a) 6+b) 32	12	160	−4.2	5/6
	a) 12+b) 16	9	120	−5	6/6
	a) 10+b) 16	9.5	127	−5.1	6/6
	a) 8+b) 16	12	160	−4.8	6/6
	a) 6+b) 16	12	160	−4.7	6/6
a) Cisplatin + b) Sodium thiosulfate	a) 12+b) 1200	10.5	140	−1.8	6/6
	a) 10+b) 1200	10	133	−1.4	6/6
	a) 8+b) 1200	8.5	113	−1.7	6/6
	a) 6+b) 1200	8	107	−0.1	6/6
	a) 12+b) 800	11	147	−2.4	6/6
	a) 10+b) 800	11	147	−2.3	6/6
	a) 8+b) 800	9.5	127	−2	6/6
	a) 6+b) 800	8.5	113	−0.2	6/6
Control	Saline	7.5	100	2.3	10/10
Non-tumored control mice					
a) Cisplatin + b) Metoclopramide	a) 10+b) 64	7	–	−4.5	5/5 (1)
	a) 8+b) 64	>18	–	−3.4	6/6 (3)
a) Cisplatin + b) Sodium thiosulfate	a) 10+b) 800	>27	–	−2.5	6/6 (6)
	a) 8+b) 800	>27	–	−1.5	6/6 (6)

Tumor inoculum: 10^6 ascites cells i.p.
Host: CDF_1 female mice
Treatment: All on day 1 only, combinations given simultaneously
MST: Median survival time
% T/C: (MST treated/MST control) × 100
Criteria: % T/C ≥ 125 = significant activity

tial effects on the efficacy of the anticancer agent. The present studies were intended to evaluate the usefulness of a mammalian-cell-based microtiter cytotoxicity assay to analyze such drug combinations [1, 2].

The in vitro and in vivo activity of cisplatin against murine leukemia L1210 cells alone and in combination with metoclopramide or sodium thiosulfate were analyzed. Sodium thiosulfate is a known inhibitor of cisplatin [7, 11], as was confirmed in the microtiter assay as well as in vivo. The in vitro drug combination microtiter assay indicated that metoclopramide did not interfere with cisplatin cytotoxicity, which was also corroborated by the in vivo study.

Microtiter assays in general provide a rapid, sensitive, cost-effective system [2, 4]. In addition to the assays previously described for anchorage-dependent cells [2, 4], the methodology for evaluation of suspension cells in a microtiter system is presented in this study. A major advantage of this drug combination microtiter cytotoxicity assay is that duplicate rows of no-drug controls, drug A only and drug B only titrations are available on the same plate as the drug combination. Additionally, the quantitative nature of the assay allows analysis by linear regression or even more sophisticated techniques such as the median effect analysis recently described by Chou and Talalaly [3]. Furthermore, it is possible to perform in vitro scheduling experiments with this drug combination assay using anchorage-dependent cells. The second drug can be added and titrated hours after the first drug. This drug combination assay greatly expands the utility of microtiter-based cytotoxicity evaluations. Additional drug combinations need to be evaluated to determine the scope and limitations of this assay method.

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