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Cyclophosphamide and *cis*-Dichlorodiammine Platinum (II)

Nonempiric Scheduling to Spare Dose-limiting Tissues in the Rat

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Summary. *Fractional incorporation (FI) of ^{3}H-thymidine (i.e., the proportion of total tissue ^{3}H incorporated into DNA) has been used as a parameter for judging temporal scheduling of cyclophosphamide and cis-dichlorodiammine platinum (DDP). Differences in the recovery times of FI following a dose of 100 mg CY/kg, between tumor (> 12 days), gut (2–3 days), and bone marrow (4 days) suggested a basis for a normal tissue-sparing drug regimen when administering double-agent CY-DDP therapy. When 100 mg CY/kg and 8 mg DDP/kg were administered simultaneously or when the doses were separated by 1 day the survival was 0/10 or 1/10, respectively. However, when the doses were separated by 4 days all rats survived. This 4-day interval was considered to allow time for gut and bone marrow recovery prior to a second insult. Such factors appear crucial to the survival of the animal. These three combinations were similar in their antitumor effect, giving a greater than additive response. Cyclophosphamide was more myelotoxic than DDP, but DDP showed greater gut toxicity. The recovery of bone marrow cellularity was delayed by 2 days compared with FI. Peripheral white blood cell counts returned to normal after a further 2-day delay.*

Introduction

It is well known that the optimal scheduling of antineoplastic drugs can make the difference between success and failure in the treatment of many tumors [14]. A single dose of CY has been shown to protect mice from a second insult with either γ-irradiation [8] or busulphan [9], provided that the CY dose is given 2–4 days prior to the second insult. This protection was considered to be mediated by enhanced hemopoietic recovery, possibly through an unspecified humoral factor or factors. In addition, it has been shown that two doses of CY given 5 days apart were less toxic to bone marrow stem cells [1] than the equivalent single dose.

This study has attempted a rational scheduling of a combination of CY and DDP, normal tissue-recovery data based upon FI studies [4, 16, 18–20] being used to decide optimum treatment times. This combination has been employed in certain other cases, and synergistic effects upon mouse L1210 [2, 3, 24] and reticulum-cell sarcoma [10] have been reported. Both drugs are thought to exert their cytotoxic effect through alkylation of macromolecules, especially DNA; thus this study was set up to consider combined alkylation potential as related to tumors and normal cell cytotoxicity. Renal toxicity of the DDP was responsible for animal deaths only at very high doses (16 mg/kg). In August rats, the tissues that were dose-limiting for these drugs were intestinal mucosa and bone marrow, both of which exhibit anaplasia and DNA synthesis inhibition after single doses of either CY [18, 20] or DDP [7, 17].

The BICR A15 kidney carcinoma has been found to differ in its response, as demonstrated by FI, from intestinal mucosa and bone marrow after a single dose of 100 mg CY/kg [5, 18]. The data presented consider the implications of these recovery times in relation to the scheduling of subsequent doses of DDP, while concomitantly comparing tumor response with the biochemical and clinical responses of bone marrow and intestinal mucosa.

Materials and Methods

Animals and Tumor Type

Inbred male and female August rats aged 4–6 months were used. A serially passaged, nonmetastasizing rat carcinoma of renal origin

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(BICR A15/IF +1) was maintained SC in the flanks of female rats. Experiments were carried out on tumor-bearing rats approximately 2 weeks after implantation, when tumors were around 1 cm^3.

Injections

Cyclophosphamide (WB Pharmaceuticals, Bracknell, Berks., England) was dissolved in sterile water to a concentration of 25 mg/ml; DDP was prepared as a fresh solution by dissolving in warm isotonic saline (final concentration 2 mg/ml). Both drugs were administered by IP injection to lightly anesthetized rats.

Toxicity and Antitumor Studies

Rats were weighed routinely every morning before and after drug treatment. Groups of ten rats (5 male, 5 female) were used for each dose. Deaths were recorded as days after initial injection. Moribund animals were killed and it was assumed that natural death would have occurred the same day. Tumor volumes were calculated from the formula $V = d^3/6$, where d is the mean of two tumor diameters measured with vernier calipers. Tumor growth delay times were calculated as the difference between the times required for the treated and the control tumors to reach twice the pretreatment volume.

Measurement of DNA Synthesis

A dose of 50 μCi of thymidine-6-3H (TdR), 24 Ci/mmol (Radiochemical Centre, Amersham) was injected IP 1 h prior to death by cervical dislocation. After death, the tumors, tibias and 10-cm lengths of jejunum were excised and immediately frozen on dry ice. DNA was extracted by a modification of the Schmidt-Thannhauser technique previously described [5, 20] and the incorporation of TdR into the DNA was expressed as FI:

$$\text{FI} = \frac{^3\text{H in DNA}}{\text{Total tissue } ^3\text{H}}.$$

Serum Creatinine

Blood samples were collected from rats killed by exsanguination via left ventricular puncture.

Kidney function was monitored by colorimetric assay of serum creatinine levels with alkaline picrate solution [12].

Peripheral White Blood Cell Counts

Samples (20 μl) of whole blood were diluted with 10 ml Isoton. Red blood cells were lysed by the addition of 0.1 ml Saponin (2% w/v). After clearing, the samples were counted on a model F Coulter Counter.

Bone Marrow Cellularity

A syringe was used to suck the marrow from a femur into 10 ml Isoton. After serial dilution and lysis of red blood cells with 0.1 ml Saponin, total nucleated cell counts were estimated on a model F Coulter Counter.

Results

Overt Toxicity

Administration of drugs, recording of animal weights, and tumor measurements were carried out at the same time each day to avoid any complications arising from variation in diurnal rhythm.

Table 1 shows the effect of single and double doses of the two drugs upon the survival of non-tumor-bearing rats. At the LD_5 values of 100 mg CY/kg and 8 mg DDP/kg a single dose of DDP caused greater weight loss than CY. When these doses were administered simultaneously massive weight loss occurred during the first 5 days and all the animals died. A similar effect was apparent when the DDP administration was delayed for 1 day (0 + 1). At 0 + 2 the weight loss was less than that observed following a single dose of 8 mg DDP/kg and 40% of the animals survived. When the drugs were separated by 4 days (0 + 4) there was little overt toxicity and minimal weight loss, and all animals survived. In addition to weight loss, sick animals had buccal, nasal, and ocular hemorrhages and blood in the urine and feces, and appeared more susceptible to infections of the lung and submaxillary lymphatics. Postmortem revealed heavy intestinal hemorrhage.

Table 1. Toxicity studies

Drug dosage (mg/kg)				% Mean maximum weight loss	Day of lowest weight[a]	Number of survivors at 4 weeks	Mean day of death[a]
Day 0	Day 1	Day 2	Day 4				
CY 50	–	–	–	1.0	–	10/10	–
CY 100	–	–	–	1.9	9	9/10	8
DDP 4	–	–	–	7.3	5	10/10	–
DDP 8	–	–	–	12.2	5	10/10	–
CY 100 + DDP 8	–	–	–	28.3	5	0/10	7
CY 100	DDP 8	–	–	21.9	8	1/10	8
CY 100	–	DDP 8	–	10.9	6	4/10	6
CY 100	–	–	DDP 8	8.7	8	10/10	–

[a] Based upon time after day 0

Table 2. Serum creatinine levels[a]

	Hours after CY dose (100 mg/kg)					
	24	48	72	96	120	
100 CY (Day 0)	177 ± 16	81 ± 18	93 ± 9	47 ± 16	220	
100 CY (0), 4 DDP (1)	185 ± 20	67	61 ± 11	135 ± 14	236 ± 31	
100 CY (0), 4 DDP (2)	169 ± 15	115 ± 10	377 ± 140	200 ± 15	184 ± 20	
100 CY (0), 4 DDP (4)	175 ± 18	111 ± 12	101 ± 8	165 ± 21	184 ± 83	
4 DDP (0)	110 ± 27	173 ± 37	157	154 ± 10	–	
8 DDP (0)	189	189 ± 36	299 ± 73	220 ± 41	–	
	Hours after CY dose (100 mg/kg)					
	144	168	192	216	240	264
100 CY (Day 0)	–	141 ± 78	236 ± 110	–	–	–
100 CY (0), 4 DDP (1)	189 ± 31	251 ± 94	–	–	–	–
100 CY (0), 4 DDP (2)	323 ± 50	139 ± 10	–	254 ± 22	161 ± 26	154 ± 31
100 CY (0), 4 DDP (4)	110 ± 28	189 ± 42	299 ± 47	267 ± 70	–	–
4 DDP (0)	–	–	–	–	–	–
8 DDP (0)	–	–	–	–	–	–

[a] Control = 152 ± 17 (n = 8). Levels are expressed as μmol creatinine/liter serum, and each value is the mean ± SD from four animals

Normal Tissue Toxicity

Kidney. Table 2 shows the levels of serum creatinine in the plasma of control and drug-treated, non-tumor-bearing rats. The efficiency of glomerular excretion in the kidney is inversely proportional to the circulating serum levels of creatinine. Normal creatinine levels in non-tumor-bearing rats were found to be 152 μmol/liter. In this case, a 50% reduction in kidney function would be expected to raise the creatinine level to 300 μmol/liter. In only a few instances was this level reached after treatment, and in all cases the levels returned to normal within 10 days. Since normal kidney function is maintained at extraction efficiencies as low as 25%, such increases in creatinine levels were not considered critical.

Bone Marrow. Figure 1 compares the recovery rates of the total nucleated marrow cell counts (TNC) and peripheral white blood cell counts (WBC) with that of the FI after a single dose of 100 mg CY/kg. The drug caused an initial suppression of FI to 20% of control at 48 h but normal values were regained after 4 days. The TNC level reached a nadir on day 3 and had recovered by day 6.

Single doses of DDP depressed FI, TNC, and WBC transiently in a dose-dependent fashion (Table 3). A CY dose of 100 mg/kg (Fig. 1), which is an LD_5 dose (equivalent to 8 mg/kg DDP), caused a higher degree of myelosuppression.

When 4 mg DDP/kg was administered 1 day after 100 mg CY/kg (Fig. 2a), all three bone marrow parameters were suppressed. Fractional incorporation was reduced to 30% of the control level and took 7 days to recover; TNC was reduced to less than 10% of control and had regained control levels by day 9; WBC were depressed and remained so for the observed time period.

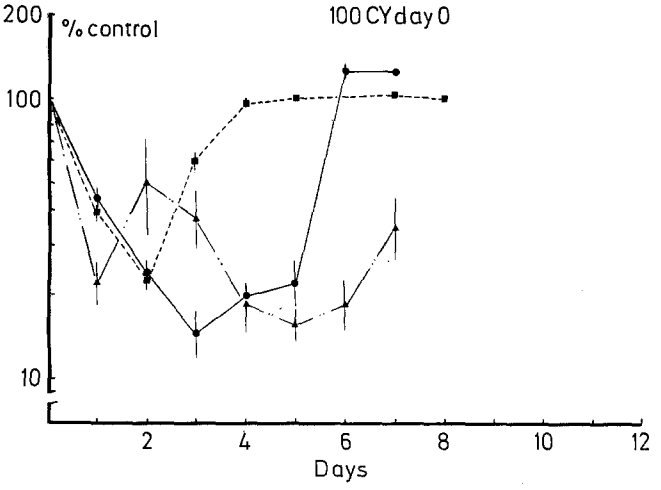

Fig. 1. The effect of 100 mg CY/kg on parameters of bone marrow proliferation. *Ordinate* is a log scale of % of control values (see Table 3). Each point is the mean ± SD of four experiments. FI values are from four animals ± SD. ■, FI; ●, TNC; ▲, WBC

At the least toxic dose when the drugs were given 4 days apart, FI in the bone marrow had returned to normal before the DDP dose was given. The second drug caused a slight depression of FI, which returned to normal 3 days later, i.e., 7 days after the initial treatment. The TNC reached a nadir of 15% of the control level on day 3. The DDP delayed recovery by 2 days, i.e., until day 8. The WBC followed a similar pattern to before. In terms of

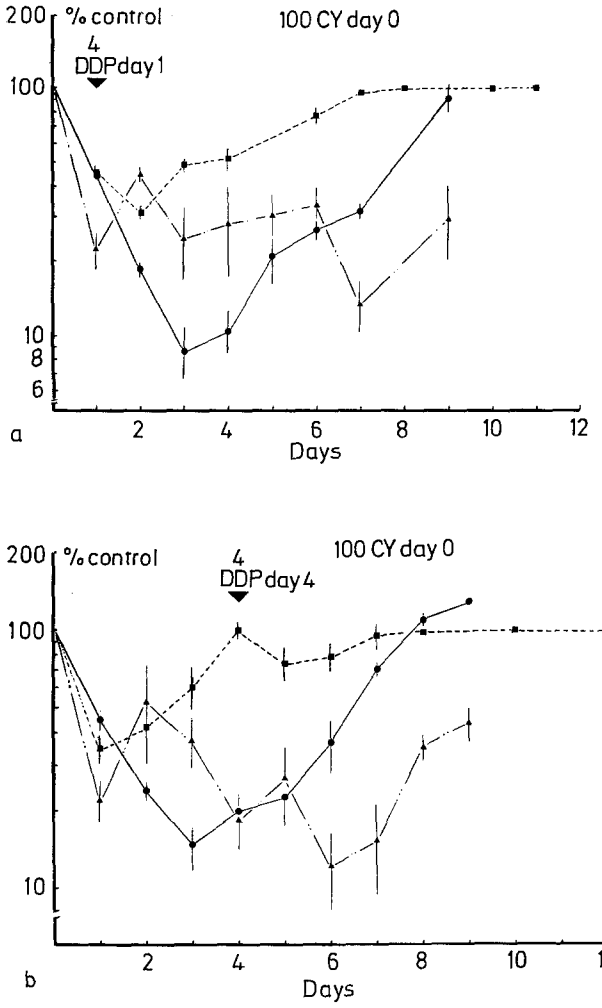

Fig. 2a and b. The effect of CY-DDP combinations on parameters of bone marrow proliferation. **a** 100 mg CY/kg on day 0, 4 mg DDP/kg on day 1; **b** 100 mg CY/kg on day 0, 4 mg DDP/kg on day 4. *Ordinate* is a log scale of % of control values for each parameter. Each point is the mean ± SD of four experiments. FI values are from four animals ± SD. ■, FI; ●, TNC; ▲, WBC

Table 3. Effects of a single dose of DDP on FI, TNC, and WBC[a]

DDP dose		Hours after DDP		
		24	48	72
4 mg/kg	FI[b]	89	95	101
	TNC	95.4 ± 9	92.8 ± 4	118 ± 5
	WBC	126 ± 11	135 ± 32	91.4 ± 4
8 mg/kg	FI[b]	80	82	97
	TNC	84.7 ± 8	88.4 ± 11	96 ± 14
	WBC	76.8 ± 15	108 ± 30	110 ± 2

[a] Control values: FI, 92%; TNC, 6.8 × 10^7/femur; WBC, 4.6 × 10^7/ml blood. Results are expressed as percentage of control

[b] FI was recorded in the pooled marrow of four animals

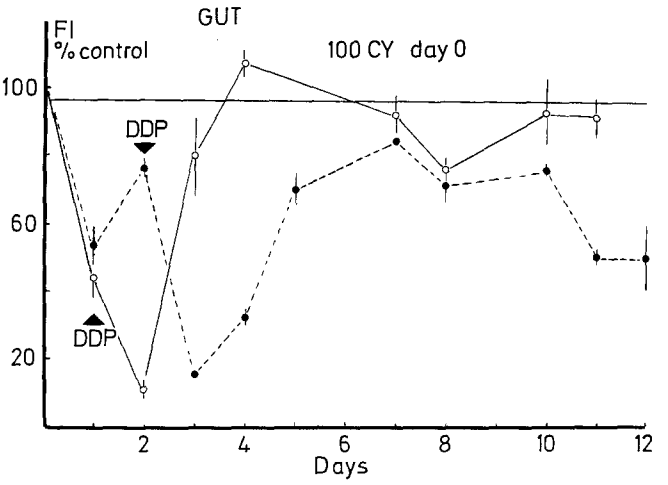

Fig. 3. The effect of CY-DDP combinations on DNA synthesis in intestinal mucosa. ○, 100 mg CY/kg on day 0, 4 mg DPP/kg on day 1; ●, 100 mg CY/kg on day 0, 4 mg DDP/kg on day 2. Each point is the mean ± SE of four experiments. *Horizontal line* is lower-limit SE for control. *Ordinate* is % of control FI (control value = 76 ± 4)

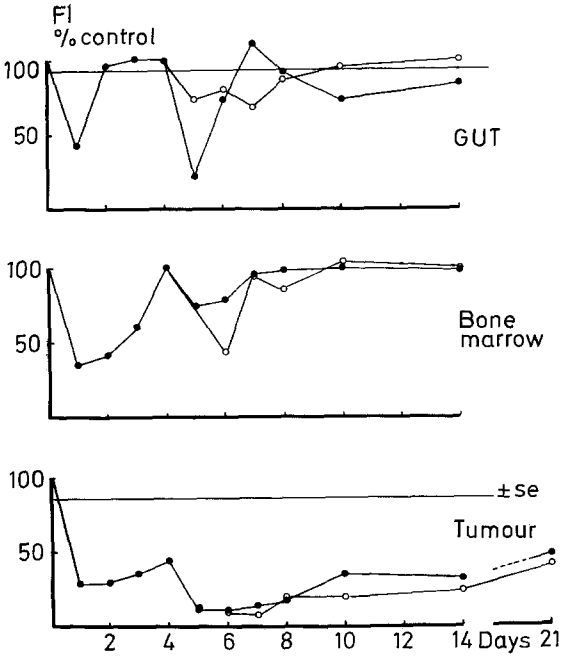

Fig. 4. The effect of CY-DDP and CY-CY combinations on DNA synthesis in gut, bone marrow, and tumor. ●, 100 mg CY/kg on day 0, 4 mg DDP/kg on day 4; ○, 50 mg CY/kg on day 4. Each point is the mean of four experiments. *Horizontal line* (gut and tumor) is lower-limit SE for control. *Ordinate* is % of control FI: gut, 76.7; bone marrow, 92; tumor, 41.2

the degree of suppression and the recovery time, the 4-day dose spacing was less toxic than the 1-day separation.

Intestinal Mucosa. When 4 mg/kg DDP was administered either 1 day or 2 days after 100 mg CY/kg, there were large decreases in the FI values (Fig. 3). In each case

Table 4. Tumour volume regressions

Drug dosage mg/kg[a]					Minimum post-treatment volume (% Day 0)	Day of maximal regression	Mean growth delay (days)
Day 0	Day 1	Day 2	Day 4	Day 7			
CY 50	–	–	–	–	85	6	9
CY 100	–	–	–	–	62	11	15
DDP 4	–	–	–	–	169[b]	4[b]	4[b]
DDP 8	–	–	–	–	75	8	10
CY 100 + DDP 4	–	–	–	–	50	14	27
CY 100	DDP 4	–	–	–	44	15	22
CY 100	–	DDP 4	–	–	54	14	21
CY 100	–	–	DDP 4	–	46	15	23
CY 100	–	–	–	DDP 4	32	20	26

[a] Each dose was tested in five rats (ten tumours)
[b] Tumour showed no volume regression at this dose

the FI values were suppressed to approximately 10% of control 1 day after the DDP. The decrease after day 8 in the 0 + 2 schedule is difficult to explain but did not cause any lethalities, all animals surviving 2 months after treatment.

Figure 4 illustrates the effect of the 0 + 4 schedule with either 4 mg DDP/kg or 50 mg CY/kg. These are equivalent half-LD_5 doses, and serve to compare the relative effect of a second dose of CY as against DDP. In the gut the DDP caused a greater depression in FI than did the CY dose. In both cases recovery was complete within 3 days.

Tumor Toxicity

The FI response to the least toxic schedule, 0 + 4, is shown in Fig. 4. A dose of 4 mg/kg DDP on the fourth day after 100 mg CY/kg, reduced the FI to less than 10% of control. Recovery was gradual and by day 21 the FI was only 50% of control. A similarly scheduled dose of 50 mg CY/kg resulted in a similar response.

Single doses of either drug have antitumor activity as measured by volume regressions (Table 4). However, the DDP per se caused only growth delay and no volume regression (minimum post-treatment volume 169% of day 0). The untreated tumor has a doubling time of between 2 and 3 days. After the toxicity studies (Table 1) the dose of DDP used in combination was lowered to ensure long-term survivors, although mortalities ensued when the drugs were administered simultaneously. All the combinations gave comparable growth delays and maximal volume depressions.

Animal Survival After High-dose DDP

A dose of 16 mg/kg DDP was found to be lethal to the rats, resulting in their death 4 days after treatment. Attempts to spare these animals by pretreatment with CY proved unsuccessful (Table 5). Deaths occurred around 4 days after DDP administration. Postmortem examination revealed small- and large-bowel hemorrhaging and kidney discoloration. Animals consumed no food and lost weight rapidly.

Table 5. High-dose DDP treatment

Day 0	Day 4	4-week survivors	Mean day of death[a]
DDP 16	–	0/10	4
CY 25	DDP 16	0/10	9
CY 50	DDP 16	0/10	9
CY 100	DDP 16	0/10	7

[a] Based upon time after day 0

Discussion

The interactions of drugs used in combination can be crucial to the efficacy of such a combination in the treatment of many tumors. The data presented here have shown that CY, a bifunctional alkylating agent, and DDP, which is cytotoxic, at least in part, by 'platination' of G—C base pairs within DNA (see model proposed by Kelman et al. [6]), have exerted an additive or greater than additive antitumor effect when used at the doses shown. When administered individually neither CY [5, 18] nor DDP [16, 26] has been shown to localize preferentially within tumors. A possible explanation for this effect is that a combination of the two drugs affects the permeability of tumor cells, resulting in increased uptake of drug. We have shown previously that alkylation of nuclear targets is dependent upon the functional and structural characteristics of target chromatin [11, 15, 21–23]. These targets can be DNA, nuclear RNA, histone or nonhistone

proteins, all of which are involved in the regulation of nuclear structure and function. Differences in basic chromatin architecture between tumor and normal cells may affect a differential effect resulting in the observed response. Thus, an alternative explanation could propose that a preliminary interaction of one alkylating agent with chromatin may alter the 'dynamic configuration' of DNA, rendering it either more susceptible to subsequent alkylation or less able to repair resultant damage by excision/replacement or postreplicative mechanisms.

Irrespective of the mechanism of this drug interaction, having conceived that the two drugs in combination were useful in the treatment of this solid tumor, the therapeutic doses employed were found to be severely toxic to normal tissues when administered simultaneously. We have shown previously that both the bone marrow and the intestinal mucosa were extremely sensitive to both CY [18, 20] and DDP [17], and the sensitivity of both tissues was found to be dose-limiting in combination therapy. At the doses used, DDP has been shown to be more gut-toxic than CY, with the converse true for bone marrow toxicity. A relationship between recovery of DNA synthesis and the maturation of bone marrow elements is demonstrated. The 2-day delay between recovery of FI and TNC may be explained by considering the period of recovery following the cytocidal effect of the drug, and reflects the time taken for DNA-synthesizing precursor cells to produce the nondividing polymorphonuclear elements that constitute the greatest proportion of the TNC in the marrow. The appearance of these cells in the systemic circulation showed a further, not unexpected, delay.

The rapid weight loss, hemorrhagic gut, FI responses for intestinal mucosa, and recorded time of death following treatment indicated that lethalities occurred because of gut failure. Unlike bone marrow, there are no quasi-clinical parameters that facilitate comparison with FI values. However, we have shown evidence that gross microscopic recovery correlated well with recovery of FI following methotrexate treatment [19, 20]. Recovery of gut function appeared to be crucial to animal survival following treatment, and we have preliminary data to suggest that if FI values in gut are not restored to normal within 3–4 days animals will die through gut failure (unpublished data). This was an integral part of the rationale for scheduling the dose of DDP to allow an interval for recovery of both gut and bone marrow after the first dose of CY. The survival data presented support this rationale and concur with other findings [13] that DNA synthesis is a useful parameter on which to base chemotherapeutic regimens. However, the discovery that animal survival is highest when the second drug is administered after normal tissue recovery, as evaluated by FI, is contrary to the finding of these authors [13], who suggested that maximal survival was achieved by administering two doses of CY 12 h apart, i.e., giving the second dose at the nadir of bone marrow DNA synthesis. However, since this study has not considered doses separated by 12 h, increased survival at this time remains a possibility. Our findings are further supported by data already discussed [1, 8, 9] and by the fact that in mice a priming dose of CY is most successful in protecting against a second lethal dose of CY when administered 4 days before (JL Millar, personal communication). We were unable to repeat this observation in rats with a lethal dose of DDP. This may reflect the wide spectrum of toxicity of DDP, which precludes any protection by the humoral factor(s) previously postulated [8, 9].

Extrapolation of these data suggests that DDP in combination with CY (and possibly other alkylating agents) may produce favorable responses from human solid tumors. The nonempiric scheduling of these drugs should allow sufficient time between large therapeutic doses to permit normal tissue regeneration. Long-term kidney toxicity may be avoided by mannitol diuresis or prior protection with furosemide [25], and any bladder toxicity may be alleviated with high fluid output. The possibility of multiple repeated drug administration based upon these findings remains to be elucidated.

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