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SHORT COMMUNICATIONS

Synergism between 4-hydroperoxycyclophosphamide and cisplatin: importance of incubation sequence and measurement of cisplatin accumulation

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In 1971 Speer and coworkers reported that cyclophosphamide and cisplatin are synergistic in an *in vivo* model of mouse leukemia [1]. Several reports followed supporting this observation [2–4]. In an attempt to find improved *in vitro* purging methods for autologous bone marrow transplantation, we recently reported that combinations of 4-hydroperoxycyclophosphamide (4-HC), an analog of activated cyclophosphamide, and cisplatin have cytotoxic synergism in two human leukemia cell lines (K-562 and Raji) as determined by an *in vitro* clonogenic assay [5–7]. This synergism is present at various molar ratios of the drugs. We now report that the synergism is sequence-dependent: it was only present when the cells were exposed to 4-HC first, being more pronounced at high levels of inhibition of colony formation. When the sequence of incubation was reversed, the drugs were antagonistic. To determine whether the synergism observed was associated with a net increase in total cellular cisplatin levels, platinum levels were measured in leukemic cells using flameless atomic absorption spectroscopy (FAAS). We found that 4-HC pretreatment did not affect significantly cellular platinum levels.

Materials and methods

Drug–effect assays. The cells and the drugs were handled as previously described [7]. K-562 cells (blastic chronic myelogenous leukemia) [8] were incubated with the drugs (4-HC, 1 hr; cisplatin, 4 hr) in RPMI 1640 (GIBCO, Chagrin Falls, OH) supplemented with 10% fetal calf serum (FCS) at 37°; the incubation cell concentration was 1×10^5 cells/mL. The cells were incubated sequentially with the drugs with one cell wash between the incubations. Next, the cells were plated in soft agar and incubated for 1 week, and their survival, assayed by their ability to form colonies in agar, was reported as a percentage of untreated controls. Drug interactions were quantitated using the multiple drug–effect analysis method [9].

Measurement of platinum levels. The cells (1×10^6 cells/mL) were incubated in RPMI 1640 + 10% FCS with 4-HC (0 or 100 μM) for 1 hr, washed once with RPMI 1640, and then incubated with graded concentrations of cisplatin (0–100 μM) for 4 hr. Next, the cells were washed three times with cold phosphate-buffered saline. Cell viability (determined by trypan blue exclusion) was at all times 90% or above. Platinum levels were measured by graphite furnace atomic absorption spectroscopy (Perkin Elmer, model 503, Norwalk, CT) as described previously with minor modifications [10]. The cell pellets (10^7 cells) were brought up to 1 mL with distilled water. The samples were then sonicated and 20 μL of the suspension was injected in an HGA-2100 graphite furnace (Perkin Elmer) for platinum measurement. The atomic absorption spectrophotometer was used to monitor the platinum line at 265.9 nm, with a slit band width of 0.7 nm. The furnace was programmed to execute a 120-sec drying stage at 98°; a 30-sec charring (ashing) stage at 1500°; and an 8-sec atomizing stage at 2700°. Nitrogen gas was used to purge the furnace at a flow rate of 30 mL/min in the "interrupt flow" mode. The limit of detection was approximately 0.4 ng (3-fold difference signal–noise ratio). The biological matrix caused no interference: the overall recovery of platinum was 100%.

Results and discussion

In Table 1 we report the percentage of clonable cells after exposure to graded concentrations of the drugs alone and in combination at a fixed (10:1) molar ratio of 4-HC:cisplatin. The data in Table 1 were analyzed by the multiple drug–effect analysis method using a computer program [11], and the calculated combination index (CI) values, determined by solving the multiple drug-effect equation for mutually exclusive drugs, are reported in Table 2 (the equation for mutually non-exclusive drugs was also solved yielding slightly different values for CI, but the general trend was unchanged). The synergism ($CI < 1$) was only present when the cells were exposed to 4-HC first. Reverting the incubation sequence resulted in antagonism ($CI > 1$). Also, when the cells were exposed to 4-HC first, the synergism was most pronounced at high levels of cloning inhibition, an important fact since, for cancer treatment, it is important to operate at high cloning inhibition to achieve complete eradication of tumor cells. Using a cell survival assay did not permit us to determine whether the apparent antagonism at low levels of cloning inhibition was due to changing of a single interaction as the concentration of drugs was varied or due to changing of the net effect of several interactions between the two drugs at different levels of inhibition. Sequence-dependent interactions have already been described for other anticancer agents. For instance, combinations of methotrexate and 5-fluorouracil are synergistic only when cells are exposed to methotrexate first [12]. The present results with 4-HC and cisplatin are previously unreported, however, and are of prime importance to designing optimal purging methods for *in vitro* bone marrow cleansing in the treatment of hematologic cancers. In addition, because it is difficult to extrapolate *in vitro* results to *in vivo* situations, similar studies on the sequential interactions between cyclophosphamide and cisplatin need to be undertaken in *in vivo* models of cancers. Indeed, in all previous *in vivo* studies, the two drugs have been used simultaneously [1–4].

Studying the mechanism(s) of interaction between anticancer agents is necessary to design improved therapeutic regimens. Since the synergism described here was sequence-dependent, it is conceivable that 4-HC altered the cells in such a way that more cisplatin was associated with them. Indeed, alkylating agents have been reported to alter the function and integrity of the plasma membrane [13]. Thus, testing the effect of 4-HC on the cellular accumulation of cisplatin is a logical first step in trying to understand the mechanism(s) of the synergism between these drugs.

Figure 1 shows the results of platinum measurements using FAAS. We found no evidence of saturation of platinum accumulation by K-562 cells as extracellular cisplatin levels were increased within the range tested. This finding has been reported by others and believed to suggest that the cellular uptake of cisplatin is by passive diffusion [14–16]; however, the possibility of a carrier-mediated transport system with low affinity for cisplatin cannot be ruled out [17]. Of interest, after 4 hr of incubation at 37°, only 0.2% of total extracellular platinum was associated with the cells. Finally, it can be calculated from our previous studies [7] that, if the synergism were due entirely to increased cellular

Table 1. Effects of 4-HC, cisplatin, and their combination on colony formation

Drug	4-HC → Cisplatin: Concentration (μM)	4-HC → Cisplatin: % Cloning cells*	Cisplatin → 4-HC: Concentration (μM)	Cisplatin → 4-HC: % Cloning cells*
4-HC	6.25	61 ± 2	2.5	89 ± 0.5
	12.5	53 ± 4	7.5	72 ± 11
	20	15 ± 6	10	60 ± 6
	25	12 ± 6	12.5	40 ± 12
			25	3 ± 2
CDDP†	0.625	90 ± 2	0.625	92 ± 2
	1.25	76 ± 2	1.25	88 ± 6
	2	74 ± 3	2	77 ± 7
	2.5	66 ± 2	2.5	56 ± 6
	5	27 ± 1	5	24 ± 1
	10	3.5 ± 1.5	15	0.07 ± 0.02
Comb.‡	6.875	76 ± 3	5.5	89 ± 2
	13.75	37 ± 9	6.875	82 ± 2
	22	7 ± 4	13.75	20 ± 5
	27.5	0.5 ± 0.4	22	3 ± 1
			27.5	1 ± 0.5

* The means ± SEM of eight measurements are reported. The cells were exposed to 4-HC followed by cisplatin (left panel) or to cisplatin followed by 4-HC (right panel).
† CDDP, cisplatin.
‡ Comb., the sum of concentrations of 4-HC and CDDP at the combination ratio of 10:1.

Table 2. Combination index (CI) values for the combinations derived from inhibition of cloning data

	CI at following % cloning inhibition*			
	25	50	90	95
4-HC followed by cisplatin	1.67	1.27	0.74	0.61
Cisplatin followed by 4-HC	1.57	1.44	1.21	1.14

* CI values were determined by solving the multiple drug–effect equation for different degrees of cloning inhibition.

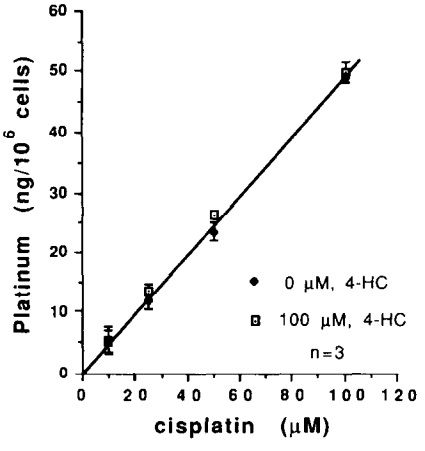

Fig. 1. Effect of 4-HC on cellular platinum levels of K-562 cells. The points ± 1 standard deviation represent the means of three measurements.

platinum levels, the increment would be approximately 5 ng/10^6 cells (a concentration resulting in a cell kill equivalent to the additional toxicity of the combination), and thus would be easily detectable by FAAS. We chose to incubate the cells with 100 μM 4-HC (a concentration of drug causing at least 99% inhibition in colony formation) because the synergism is most pronounced at high levels of cell kill. The cells were then exposed to various concentrations of cisplatin to test a series of molar ratios of the drugs: from a 1:1 to a 10:1 ratio (4-HC:cisplatin) since the synergism is present at various molar ratios [5–7]. The figure shows that the accumulation of cisplatin in human leukemic cells was the same whether the cells were pretreated with 4-HC or not. The slopes of the regression lines not being statistically different (power of t-test = 99%), only one regression line is represented.

In summary, our studies demonstrate that the synergism between 4-HC and cisplatin was sequence-dependent, being most pronounced at high levels of cloning inhibition. We also confirmed previous reports stating that the cellular accumulation of cisplatin does not appear to be saturable at concentrations of cisplatin ranging from 0 to 100 μM [14–16] and that only a small fraction of extracellular cisplatin is associated with the cells after 4 hr of incubation [18].

Moreover, 4-HC pretreatment did not change significantly platinum levels, therefore ruling out increased intracellular platinum as the principal mechanism responsible for the synergism between 4-HC and cisplatin.

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In vivo and *in vitro* effect of phenytoin on rat hepatic mixed function oxidases

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Phenytoin, a potent antiepileptic drug, is still widely prescribed during pregnancy for seizure control. A variety of congenital abnormalities, both in human [1, 2] and experimental animals [3, 4] have been observed following phenytoin treatment. Apart from prenatal exposure, young children are also exposed to phenytoin due to the use of this drug for the management of epilepsies and psychiatric ailments. Besides severe neurotoxicity, hepatotoxicity has also been observed in phenytoin treated individuals, though the fatal hepatic reactions are rare. Hepatocellular degeneration and/or necrosis associated with granulomas and cholestatic injury in treated human subjects has been reported [5, 6]. Developing children under the age of two are at higher risk of hepatic dysfunction who are being