Cancer Chemother Pharmacol (1993) 32: 419–424

Cancer Chemotherapy and Pharmacology

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Analysis of the cytotoxic interaction between cisplatin and hyperthermia in a human ovarian carcinoma cell line

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Received: 1 September 1992/Accepted: 28 April 1993

Abstract. Expression of the heat-shock protein HSP-60 is associated with poor survival in patients with ovarian carcinoma. We examined both the nature of the interaction between hyperthermia and cisplatin (DDP) using the human ovarian carcinoma cell line 2008 and the effect on this interaction of the induction of the heat-shock response. The nature of the interaction was assessed using median-effect analysis. Despite the observation that 45°C hyperthermia increased the intracellular uptake of the DDP analog [^{3}H]-*cis*-dichloro(ethylenediamine)platinum(II) (DEP) during a 1-h exposure by 155% ± 5% ($P = 0.02$), median-effect analysis indicated only cytotoxic additivity (combination index at the level of 50% cell kill, 0.96 ± 0.25). When cells were first exposed to hyperthermia for various periods and then allowed to incubate at 37°C for 4 h to allow induction of the heat-shock genes before being treated with DDP for 1 h, there was a very small degree of antagonism between hyperthermia and DDP (combination index at 50% cell kill, 1.11 ± 0.04). Our results indicate that DDP and hyperthermia interact only in an additive manner against this human ovarian carcinoma cell line and that the induction of heat-shock proteins by hyperthermia does not significantly antagonize the activity of DDP.

This work was supported by grant CH-377 from the American Cancer Society and grant 100-R041 from Bristol-Myers Squibb. This work was conducted in part by the Clayton Foundation for Research – California Division. Dr. Howell is a Clayton Foundation investigator

Abbreviations: CI, combination index; DDP, cisplatin; DEP, *cis*-dichloro(ethylenediamine)platinum(II); IC_{50}, concentration or time producing 50% inhibition of colony formation; PBS, phosphate-buffered saline

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Introduction

The interaction between the cytotoxic effects of cisplatin (DDP) and hyperthermia has been the subject of many reports [1, 3, 11, 14, 16, 17, 24], but the nature of the interaction remains controversial in part because, in most cases, the techniques used to analyze the interaction have not been appropriate [4]. Although in vivo the addition of hyperthermia to DDP therapy may increase the tumor response and improve the therapeutic outcome, this by itself does not establish that the interaction is truly synergistic at the cellular level. Analysis of the nature of the interaction at the cellular level is complicated by the observation that both modalities are independently cytotoxic and have complex dose-response curves; under these conditions, approaches such as determining that the product of the surviving fractions for the two modalities used alone is greater than the observed surviving fraction when the two are used together are not appropriate [4, 5, 8].

Interest in the nature of the interaction between hyperthermia and DDP has been given new impetus by the discovery that DDP can induce expression of one of the heat-shock protein genes, hsp60, and that human ovarian carcinoma cells selected in vitro for 11-fold resistance to DDP overexpress the hsp60 message 1.7- ±0.16-fold and the HSP60 protein 3.8- ±0.45-fold [20]. These cells also demonstrate a mild degree of thermotolerance in that the duration of 45°C hyperthermia required to reduce clonogenic survival by 50% is increased 1.3-fold. In addition, expression of the hsp60 gene in human ovarian carcinomas in vivo demonstrates striking tumor-to-tumor variation and is a highly significant prognostic indicator of survival in patients treated with platinum drug-containing regimens [21]. Patients whose tumors express high levels of hsp60 mRNA respond poorly to the platinum drugs and have much shorter survival than patients whose tumors express low levels.

Little information is available on the factors that regulate expression of hsp60 or on the actual role that the product of this gene plays in determining cellular sensitivity to DDP. However, one way that hsp60 can reliably be

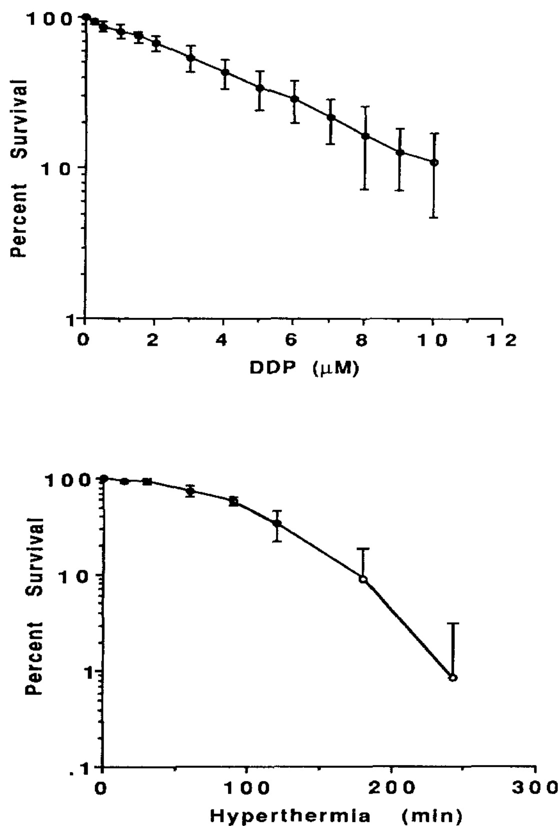

Fig. 1. The survival of 2008 cells as a function of the concentration of DDP during a 1-h exposure (*upper panel*) and the duration of treatment with 45°C hyperthermia (*lower panel*). Data are expressed as percentages of survival as compared with control cultures containing no drug. Each data point represents the mean of 8 (*upper panel*) or 4 (*lower panel*) separate experiments performed with triplicate cultures; bars indicate the SD

induced is with heat shock. This adds a new dimension to the issue of the nature of the interaction between hyperthermia and DDP, since it raises the possibility that heat shock serves to induce a gene that may confer DDP resistance.

We undertook a detailed analysis of the interaction between hyperthermia and DDP against a human ovarian carcinoma cell line using the technique of median-effect analysis to determine the nature of the interaction in a cell line in which DDP has been shown to induce hsp 60 [20] and in which the biochemical pharmacology of the DDP analog DEP has been well defined [9, 15].

Materials and methods

Cells and culture conditions. Experiments were conducted using the human ovarian carcinoma cell line 2008, which was established from a patient with a serous cystadenocarcinoma of the ovary who had received no prior platinum-based chemotherapy and who had no unusual clinical or histologic characteristics [10]. Cells were maintained in logarithmic growth in RPMI 1640 medium supplemented with 5% heat-inactivated bovine calf serum and 2 m*M* L-glutamine without antibiotics, and they were carefully equilibrated with humidified 5% CO_2 in air. Cultures were routinely tested for mycoplasma by using the GenProbe kit (GenProbe Inc., San Diego, Calif.) and were negative throughout these experiments.

Cytotoxic exposures and colony-forming assays. Cells growing in the log phase were harvested by trypsinization, washed, and then plated onto 60-mm plastic tissue-culture dishes (Corning Glass Works, New York) in triplicate at a density of 350 cells/dish. Cells were allowed to attach overnight and then exposed to 45°C hyperthermia either alone or in combination with DDP for varying periods and at varying concentrations as indicated below. Following hyperthermia and/or DDP exposure, the cells were washed once with fresh media and then incubated at 37°C for 10 days, at which point the dishes were washed, fixed in methanol, and stained with Giemsa dye in methanol. Hyperthermic conditions were created by placing cultures in an incubator prewarmed to 45°C; the pH of the media did not change significantly during the hyperthermic exposure. Clusters of >50 cells were scored as a colony; the cloning efficiency of 2008 cells in the absence of cytotoxic treatment was 47.2% ± 5.9% (n = 21). The nature of the interaction between DDP and heat shock was determined by median-effect analysis [8]. Levels of statistical significance were determined with a two-tailed paired Student's t-test.

DDP accumulation. Uptake of DDP was investigated using the DDP analogue [^{3}H]-DEP, which was synthesized and purified according to the method of Eastman [12]. The biochemical pharmacology of DEP is similar to that of DDP in both DDP-sensitive and DDP-resistant cells [15] and in response to modulators that alter accumulation [9], and its use permits more precise measurements to be made. Log-phase 2008 cells were seeded into 60-mm tissue-culture dishes, allowed to attach overnight, and then treated for 1 h with 5 μM [^{3}H]-DEP (5 μCi/ml) in the presence or absence of 1 h hyperthermia at 45°C. Cells were exposed to DEP either concurrently with hyperthermia or after a delay of 0–12 h following hyperthermia, during which time the cells were maintained at 37°C. After DEP exposure, the medium was aspirated and the cells were washed rapidly four times with 4°C phosphate-buffered saline (PBS). Then, 1 ml of 1 *N* NaOH was added and the cells were allowed to digest overnight. An aliquot was removed for the determination of protein content by the method of Bradford [7]. Cellular [^{3}H]-DEP accumulation was determined by liquid scintillation counting of the digested material after neutralization with an equal volume of 1 *N* HCl.

Results

Interaction between DDP and hyperthermia

Figure 1 (upper panel) shows the clonogenic survival of 2008 cells as a function of concentration following a 1-h exposure to DDP alone. The dose-response curve was very nearly log-linear over the survival range examined, and the IC_{50} was 3.1 ± 0.8 μ*M* (n = 8). Temperatures of <43°C caused minimal cytotoxicity in 2008 cells, even on prolonged exposure. A temperature of 45°C was chosen to permit an adequate degree of cytotoxicity and induction of HSP60 with durations of exposure short enough to allow analysis of the effect of sequential exposures. Figure 1 (lower panel) shows the clonogenic survival of 2008 cells as a function of the duration of exposure to 45°C hyperthermia. The 2008 cells proved to be unusually resistant to hyperthermia, requiring 107 ± 7 min (n = 4) to produce 50% kill.

Figure 2 shows that a 1-h exposure to 2.5 μ*M* DDP reduced survival to 62.0% ± 2.7%, whereas a 1-h exposure to 45°C hyperthermia resulted in a survival of 73.9% – 3.9%. When cells were exposed to both 2.5 μ*M* DDP and 45°C hyperthermia concurrently for 1 h, the survival was significantly lower, being only 18.8% ± 3.2% (P <0.004). The enhanced cell kill produced by exposure to both cytotoxic modalities persisted when the 1-h exposure to DDP began immediately following the 1 h of hyperthermia

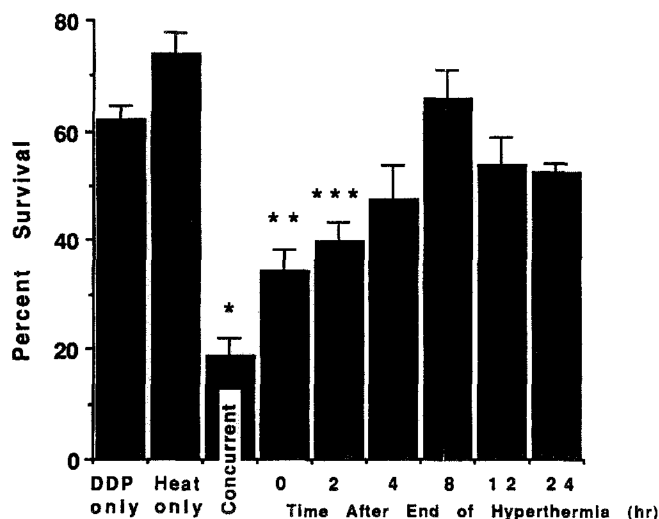

Fig. 2. The effect of 1 h of 45° C hyperthermia on the sensitivity of 2008 cells to a 1-h exposure to 2.5 μM DDP. Data are expressed as percentages of survival as compared with control cultures. Cells were exposed to 1 h of either DDP or hyperthermia alone, to both modalities concurrently, or to 1 h of hyperthermia followed by 1 h of DDP, with increasing intervals at 37° C being interspered between the two treatments. Each column represents the mean of 3 experiments performed with triplicate cultures; bars indicate the SD. * $P = 0.004$, ** $P = 0.01$, *** $P = 0.02$ vs the percentage of survival of the cells treated with DDP alone

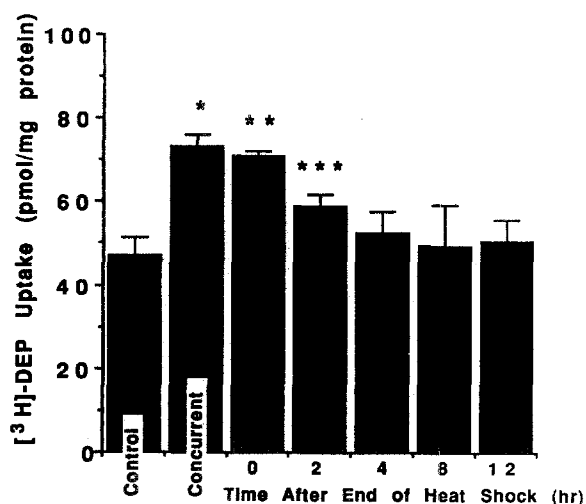

Fig. 3. The effect of hyperthermia on the uptake of [^{3}H]-DEP. The 2008 cells were incubated at 45° C for 1 h and exposed to 5 μM [^{3}H]-DEP for 1 h either concurrently or following increasing intervals during which the cells were maintained at 37° C. Each column represents the mean of 3 experiments performed with duplicate cultures; bars indicate the SD. * $P = 0.02$, ** $P = 0.005$, *** $P = 0.03$

(P <0.01) and when there was a 2-h delay between the end of the hyperthermia and the start of the DDP exposure (P <0.02). However, the enhanced cytotoxicity resulting from exposure to both modalities waned as the time between the 1 h of hyperthermia and the 1 h of DDP exposure was further increased, and the combination was no longer significantly more cytotoxic when there was a delay of 4 h or more. The combination with an 8-h delay produced a survival of 65.9%±5.1%, which was approximately the same as that resulting from exposure to DDP alone, which was 62.0%±2.7%.

Several interpretations of these data are possible. One is that there was a synergistic effect when the two cytotoxic modalities were applied in close temporal relation to each other and that this synergy was lost as the interval between the hyperthermia and the DDP exposures was increased. Another is that either the greater cytotoxicity of the combination simply reflected an additive effect or that with increasing time between the two exposures, cellular defenses were activated that resulted in antagonism at the later time points. To resolve these possibilities, we examined the effect of hyperthermia on the cellular accumulation of the DDP analog [^{3}H]-DEP.

Effect of hyperthermia on the intracellular accumulation of DEP

Figure 3 shows that during a 1-h exposure to [^{3}H]-DEP, the 2008 cells accumulated 47.2±4.0 pmol DEP/mg protein (n = 3) at 37° C and 73.3±2.8 pmol DEP/mg protein (n = 3) at 45° C, representing a 155%±5% increase in DEP uptake (P = 0.02). A 1-h exposure to 45° C hyperthermia also increased [^{3}H]-DEP uptake by 151%±4% (P = 0.005) and 125%±6% (P = 0.03) when [^{3}H]-DEP uptake was measured during the 1 h immediately following the hyperthermia and when cells were incubated for 2 h at 37° C between the end of the hyperthermia and the beginning of the [^{3}H]-DEP exposure, respectively. However, when the interval was increased to 4 h or more, the preceding hyperthermic exposure no longer had a significant effect on [^{3}H]-DEP uptake. The ability of a 1-h exposure to 45° C hyperthermia to increase [^{3}H]-DEP uptake correlated well with its ability to sensitize 2008 cells to a 1-h DDP exposure (Fig. 1). These results identify an effect on drug uptake as one potential mechanism underlying the interaction between hyperthermia and DDP.

Median-effect analysis of the interaction between DDP and heat shock

The exact nature of the interaction between hyperthermia and DDP cannot be determined from the data presented in Fig. 2 since both agents are independently cytotoxic. To determine whether the interaction was synergistic, additive, or even antagonistic, we used the mathematically rigorous technique of median-effect analysis [8]. Median-effect analysis permits calculation of the combination index (CI). CI values of >1 indicate antagonism, a value of 1 indicates additivity, and values of <1 indicate synergy. The magnitude of the difference from a value of 1 reflects the magnitude of the synergy or antagonism.

Figure 4 shows the CI plots resulting from experiments in which hyperthermia and DDP were combined in a ratio of 1 : 1.8, which was the ratio of their individual IC_{50} values in units of hours and micromoles/liter, respectively. Median-effect analysis using a 1-h exposure to DDP and vary-

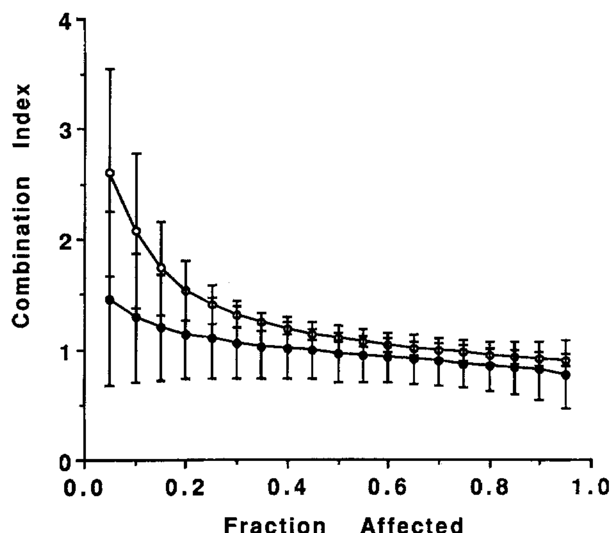

Fig. 4. CI for 2008 cells as a function of the fraction affected for the interaction between hyperthermia and DDP. CI values of <1 indicate synergy, and values of >1 indicate antagonism. *Filled circles*, Concurrent exposure; open circles, hyperthermia followed 4 h later by a 1-h exposure to DDP. Each curve represents the mean of 3 experiments performed with triplicate cultures; bars indicate the SD

ing periods of concurrent exposure to hyperthermia yielded CI values very close to 1 over the 2-log range of cell kill examined, indicating only additivity. The CI value at the level of 50% cell kill was 0.96 ± 0.25 ($P > 0.05$). In a second series of experiments, we examined the nature of the interaction under conditions where cells were first exposed to hyperthermia for various periods and then incubated at 37°C for 4 h to allow more extensive induction of the heat-shock genes before being treated with DDP for 1 h. Figure 4 shows that under these conditions there appeared to be a very small degree of antagonism between hyperthermia and DDP. The CI value at the level of 50% cell kill was 1.11 ± 0.04, which was not significantly different from a value of 1 ($P > 0.05$).

Discussion

DDP has significant antitumor activity against a broad range of human malignancies [29, 37] and is one of the most widely used chemotherapeutic agents, either as a single agent or in combination. Interactions between DDP and hyperthermia have been reported by several investigators. Hyperthermia has been shown to increase the cellular uptake of DDP [1, 32], the amount of DNA cross-linking [18, 24], and the cytotoxic action of DDP in vitro [3, 14, 16, 17, 24]. Elevated temperature also alters the pharmacokinetic profile of DDP in vivo [11, 30, 36]. A number of investigators have reported that hyperthermia increases the activity of DDP in vivo in animal models as well [1, 2, 22, 25], and these reports have served as the basis for initiation of clinical trials at several centers [31]. However, very little information is available about the actual nature of the interaction between DDP and hyperthermia at the cellular level using techniques such as isobologram [4] or median-effect analysis [8] that can rigorously distinguish between synergy, additivity, and antagonism.

Calculation of the combination index as reported by Chou and Talalay [8] for mutually exclusive drugs is one of several that provides a formal mathematic criteria for assessing the nature of the interaction between two cytotoxic modalities [4]. The data presented in this paper bring into sharp focus the need for such a formal analysis. When hyperthermia and DDP were applied to the 2008 cells concurrently, there was clearly a greater cell kill than when either agent was used alone, and the extent of interaction appeared to vary with time after the completion of the hyperthermia exposure. In addition, hyperthermia enhanced the cellular uptake of [^{3}H]-DEP, providing a reasonable mechanistic basis for a possible synergistic interaction. However, when the interaction was subjected to median-effect analysis, it turned out that it was simply additive; there was no suggestion of synergy at all.

Wang et al. [34] reported that hyperthermia and DDP did interact synergistically against another human ovarian carcinoma cell line when a somewhat different dose schedule was used that incorporated a 1-h exposure to both DDP and hyperthermia with variation in the temperature rather than the duration of exposure to a fixed temperature as used in our study. However, close examination of the CI plots presented by Xu and Alberts [35] show that, in fact, over the most reliable portion of the curve there was significant antagonism, and at the level of 50% cell kill there was simple additivity consistent with the results obtained with the 2008 cells line. In addition, no statistical evaluation of the CI plot was undertaken, and the CI value varied from 0.3 to 2 over the first 2 logs of cell kill, which in itself limits the cogency of any claim for synergy [26].

The extent of thermal enhancement of DDP cytotoxicity depends on the sequence or interval between the exposure to hyperthermia and the administration of DDP [19, 23, 33]. Cytotoxicity has been reported to be greatest when DDP and hyperthermia are applied simultaneously or within a short interval in vitro [6, 33] and in vivo [2, 23, 28]. Our results, which showed that the greatest cell kill was obtained with concurrent exposure, are consistent with these previous reports. The observation that a prior exposure to hyperthermia became less and less effective at sensitizing cells to DDP with increasing time between the hyperthermia and the DDP exposures raised the question of whether induction of the heat-shock response in 2008 cells was protecting them against subsequent DDP cytotoxicity. This possibility was reinforced by the finding that expression of the 60-kDa heat-shock protein (hsp60) gene was associated with a DDP-resistant phenotype [13, 20, 21]. This gene was induced maximally at 2–4 h after the end of heat exposure at 45°C [20]. However, when the interaction between DDP and hyperthermia was examined by median-effect analysis under conditions where a 4-h period was allowed between the end of the hyperthermic exposure and the start of a 1-h DDP exposure, there was only a minimal degree of antagonism. This indicates that the heat-shock response does not offer significant protection against DDP cytotoxicity within 4 h; it is possible that it does so at later time points.

It should be emphasized that the analysis in this report was carried out on dispersed cells, and it is quite possible that the nature of the interaction between hyperthermia and DDP is different in cells growing as a mass in vivo, where cell-to-cell and cell-to-matrix interactions are more extensive. Likewise, it should be emphasized that therapeutic benefit may be gained by combining DDP and hyperthermia even when they are not synergistic at the cellular level; clinical advantage may be realized even when two treatments are merely additive (or even antagonistic) if the therapies have nonoverlapping host toxicities. However, this circumstance has sometimes been inappropriately described as being reflective of synergy at the cellular level [5]. The interaction of DDP and hyperthermia at the clinical level is likely to be more complex than at the cellular level since, for example, hyperthermia is likely to alter such parameters as the plasma pharmacokinetics of DDP, and appropriate assessment of synergy at the clinical level is substantially more complex. The response of tumor cells in vivo will be influenced by such factors as drug metabolism, tumor vasculature, drug-resistant cell subpopulations, and side effects. In fact, despite the observation that the greatest antitumor effect occurs with the simultaneous application of hyperthermia and DDP in vitro, an improvement in the therapeutic gain was not observed with simultaneous treatment schedules but rather with sequential administration of the two modalities when consideration was given to normal tissue injury, including nephrotoxicity [2, 27].

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