Effect of Hypoxia and Acidosis on the Cytotoxicity of Four Platinum Complexes at Normal and Hyperthermic Temperatures

Terence S. Herman, Beverly A. Teicher, and Laura S. Collins

ABSTRACT

The cytotoxicities of cis-diaminedichloroplatinum(II) (CDDP) and of three recently developed dichloro complexes of bivalent platinum with radiosensitizing ligands [1,2-diamino-4-nitrobenzene]dichloroplatinum(II) (Plato), trans-bis(2-amino-5-nitrotetrazole)dichloroplatinum(II) (Plant), and trans-bis(2-nitroimidazole)dichloroplatinum(II) (NIPt) were evaluated at 37°C, 42°C, and 43°C at normal pH, at pH 6.45, and under normally oxygenated and hypoxic conditions in EMT6 cells in vitro. For CDDP, marked hyperthermic sensitization to the drug was evident in normally oxygenated cells, but hypoxic cells showed essentially no sensitization to the cytotoxicity of CDDP at elevated temperature at normal pH. Low pH further increased the cytotoxicity of CDDP toward normally oxygenated but not hypoxic cells at 37°C and 42°C. At 43°C, however, low pH increased the cytotoxicity of CDDP toward both normally oxygenated and hypoxic cells, restoring nearly the full sensitizing effect of hyperthermia on CDDP cytotoxicity in the hypoxic cells. Plato was much more cytotoxic toward hypoxic than normally oxygenated cells under all culture conditions. At normal pH, hyperthermia increased the cytotoxicity of Plato in both hypoxic and normally oxygenated cells. At low pH, however, the cytotoxicity of Plato was inhibited at all temperatures and in both normally oxygenated and hypoxic cells. Plant was also more toxic to both normally oxygenated and hypoxic cells at elevated temperatures at normal pH. In contrast to Plato, however, Plant became much more cytotoxic toward hypoxic cells and showed increased cytotoxicity in normally oxygenated cells at low pH. Hyperthermia, however, did not further increase the rate of cell killing by Plant at low pH. NIPt, at the concentrations tested, was essentially nontoxic to cells at normal pH at 37°C. Hyperthermia significantly increased the killing of hypoxic cells by NIPt under both normal and low pH conditions, but little cytotoxicity was noted for NIPt in normally oxygenated cells under any culture conditions.

These results demonstrate that pH and the level of oxygenation of cells significantly affect the cytotoxicity of drugs at both normal and elevated temperatures. This sort of investigation may help delineate optimum drugs for use against environmentally determined subpopulations of cells within tumors.

INTRODUCTION

Several clinical studies (1–3) have now shown that the addition of local hyperthermia to radiation therapy can substantially improve tumor control. Recently, the Stanford hyperthermia group (4) has reported that two heat treatments were as effective as six heat treatments when used during a course of fractionated radiation. This result lends further credence to the hypothesis that local hyperthermia when used in conjunction with radiation is acting to increase tumor control, predominantly by killing a radioresistant subpopulation of cells within the tumor, probably hypoxic cells within an acidic environment (5). This is because exposure to temperatures of 42–43°C results in only about a 20–30% increase in cell killing of normally oxygenated cells in vitro when the heat treatment is given in close temporal proximity to radiation doses of 200–300 cGy (1, 5, 6). The relatively small radiosensitizing effect of only two heat fractions could not by itself, therefore, account for the striking increase in tumor cell killing which must be occurring in patients in order to improve complete regression rates so dramatically.

Treatment with local hyperthermia and radiation therapy is not always successful, however, especially when tumors are large, radiation tolerance is limited, or achievable tumor temperatures are low (2). For this reason we have proposed adding selected, systemically administered drugs to local hyperthermia and radiation therapy in an effort to improve local tumor control and we have begun studying this approach in the clinic. After a review of the pertinent preclinical and clinical literature, we concluded that CDDP or misonidazole was probably the best drug to use weekly with local hyperthermia and daily fractionated radiation therapy (5, 7). One of the reasons why we suggested these drugs for study was that CDDP (8, 9) has been shown to become more cytotoxic to acidic cells at elevated temperature, and misonidazole (10, 11) has been shown to become more cytotoxic to acidic, hypoxic cells at elevated temperature, so that their use in combination with local hyperthermia should add to the thermal killing of the radioresistant subpopulation in tumors (i.e., hypoxic cells at low pH). In addition, both drugs have been shown to have significant radiosensitizing properties (12–14). The cytotoxicity of CDDP, however, had not been tested at elevated temperatures in a cellular model system which was both acidic and hypoxic. Since both conditions would be expected to occur in the poorly vascularized portions of tumors (15–17), we undertook to study the effect of these culture conditions on hyperthermic sensitization to CDDP in cells in vitro. In addition, our laboratory has prepared platinum-nitroimidazole, -nitrothiazone, and -nitrobenzene coordination complexes which have been shown to be radiosensitizing agents (18). We have also tested the cytotoxicity of these drugs under these same culture conditions.

These in vitro studies were conducted in an attempt to define promising drugs for in vivo study in combination with radiation and hyperthermia. We believe it is likely that the best drugs for use in trimodality protocols will have significant radiosensitizing properties and will interact positively with the cytotoxicity of hyperthermia across the different environmentally determined regions present within tumors. We thought, therefore, that it was quite important to test the cytotoxicities of potential agents at normal and clinically achievable hyperthermic temperatures, at normal and acidic pH, atoxic and hypoxic conditions, and under a combination of these parameters. We recognized that rigorous statistical analysis would be difficult because of the multiple variables studied, but we thought that
it would, nonetheless, be important to define large differences in cytotoxicity.

MATERIALS AND METHODS

Drugs. cis-Diaminedichloroplatinum(II) and potassium tetrachlo-
roplatinate were gifts from Drs. Donald Picker and Michael Abrams
(Johnson Matthey Inc., West Chester, PA). The platinum complexes,
Plant, Plato, and NIPt, were prepared in our laboratory by the reaction of 0.024 mol of potassium tetrachloroplatinate in 10–25 ml of purified
water with either 0.049 mol of the organic ligand 2-nitroimidazole, 2-
enzymatically to produce single cell suspensions, and plated out for in
excised and tumor cells were mechanically disaggregated, then treated
to grow to a volume of 100–200 mm³, at which time the tumors were
stocks were injected s.c. into the flanks of male BALB/c mice obtained
alternate in vivo and in vitro passages in a manner similar to thatreported by Gerner et al. (22). Briefly, IO6 EMT6 cells from frozen
and was obtained from her laboratory. This line was maintained by
EMT6 tumor cell line was originally developed by Rockwell et al. (23)
and was used for the study of hypoxia (19-21) and heat effects in vitro (22). The
(24, 25). Following treatment, suspensions of known cell numbers were
plated in plastic Petri dishes and allowed to grow in a 37°C incubator under standard culture conditions for 8–10 days. After this time inter-
macroscopic colonies were stained with crystal violet in methanol
containing 3.7% formaldehyde and were counted manually. Each experi-
ment was repeated 3–5 times and each data point per experiment
represents the results of 3 different dilutions of cells plated in triplicate.

RESULTS

The structures of the various platinum-containing drugs studied
in these experiments are shown in Fig. 1. NIPt and Plant have nitro-containing rings bound to platinum in a trans-configura-
tion, while Plato is a cis complex, as is CDDP.

The various panels in Fig. 2 show the survival of EMT6 cells
exposed for 1 h to CDDP at the concentrations indicated at 37°C, 42°C, or 43°C at pH 7.40 or pH 6.45 and under normally
oxygenated or hypoxic conditions. Fig. 2A shows that the
cytotoxicity of CDDP towards EMT6 cells at 37°C at pH 7.40
is concentration dependent and is not affected by the level of
oxygenation of the cell culture. Fig. 2, B and C, shows, as has
previously been reported (24, 27), that exposure of normally oxygenated cells at pH 7.40 to CDDP at 42°C or 43°C markedly
increased the cytotoxicity of the drug (approximately 2 decades
of increased cell killing after exposure of normally oxygenated
cells to 5 μM CDDP at 42°C for 43°C versus 37°C). In contrast,
however, both B and C of Fig. 2 also show, unexpectedly, that

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\text{cis-}
\begin{align*}
\text{NH}_3\text{PtCl}_2
\end{align*}
\text{Cl}_2
\text{cis-diaminedichloroplatinum(II)}
\text{(CDDP)}
\]

\[
\text{NH}_3\text{Pt}(\text{2-nitroimidazole})\text{Cl}_2
\text{DI(2-nitroimidazole) dichloroplatinum(II)}
\text{(NIPt)}
\]

\[
\text{NH}_3\text{Pt}(\text{2-amino-5-nitrothiazole})\text{Cl}_2
\text{DI(2-amino-5-nitrothiazole) dichloroplatinum(II)}
\text{(Plant)}
\]

\[
\text{NH}_3\text{Pt}(\text{1,2-diamino-4-nitrobenzene})\text{Cl}_2
\text{DI(1,2-diamino-4-nitrobenzene) dichloroplatinum(II)}
\text{(Plato)}
\]

Fig. 1. Structure and nomenclature for various platinum(II) complexes.
at normal pH at 42°C or 43°C the cytotoxicity of CDDP is dependent on the oxygenation of the cell cultures and that these hyperthermic temperatures at normal pH do not significantly sensitize the hypoxic EMT6 cells to CDDP cytotoxicity. At 42°C (Fig. 2B), the survival of normally oxygenated cells is very steeply concentration-dependent and as low as a concentration as 5 μM CDDP for 1 h kills approximately 2.0 decades of cells, whereas in hypoxic cells, 10 times this CDDP concentration (50 μM) for 1 h kills only about 2.5 decades of cells. This level of survival in hypoxic cells at normal pH is not different from the killing observed with CDDP at this concentration at 37°C at normal pH. Slightly more cytotoxicity due to CDDP is evident at 43°C than at 42°C in both hypoxic and normally oxygenated cells at normal pH, but again in hypoxic cells the survival curve at 43°C over the dose range of CDDP tested shows only slightly more cytotoxicity than is seen in either the hypoxic or normally oxygenated cells at 37°C. Thus, it appears that the mechanism(s) by which hyperthermia sensitizes cells to CDDP at normal pH are significantly inhibited under hypoxic conditions. Fig. 2, D, E and F, shows the concentration-dependent survival curve for CDDP at pH 6.45 at 37°C, 42°C, and 43°C, respectively, for normally oxygenated versus hypoxic cells. At 37°C and pH 6.45, normally oxygenated cells are significantly more sensitive to CDDP cytotoxicity than are hypoxic cells. For instance, at a concentration of 25 μM, over 3 decades of normally oxygenated cells are killed as compared with only about 1 decade of hypoxic cells. In addition, in comparison to the survival curve for CDDP at normal pH, at pH 6.45 significantly more normally oxygenated cells are killed at a given concentration of CDDP. For instance, at 25 μM about 3 decades of cell killing occurs in normally oxygenated cells at pH 6.45, while at pH 7.40 only about 1.5 decades of normally oxygenated cells are killed. In contrast, the concentration-dependent survival of the hypoxic cells at pH 6.45 is essentially the same as the survival seen in both hypoxic and normally oxygenated cells at 37°C at pH 7.40. Thus, whatever mechanism is responsible for the increase in cytotoxicity of CDDP at pH 6.45 at 37°C in normally oxygenated cells, hypoxic conditions appear to prevent its expression.

Fig. 2, E and F, demonstrates that at 42°C and 43°C at pH 6.45, CDDP becomes significantly more toxic to hypoxic cells than at pH 7.40. For instance, after exposure to 50 μM CDDP at 42°C at pH 7.4 (Fig. 2B), only approximately 2.5 decades of hypoxic cells are killed. In contrast, at pH 6.45 at 43°C (Fig. 2F), as little as 10 μM CDDP for 1 h kills approximately 3.5 decades of hypoxic cells over and above the cytotoxicity due to 43°C under these conditions. Thus, when exposure to CDDP occurs under acidic conditions, hypoxic cells are significantly sensitized to the cytotoxicity of the drug by temperature elevation (especially at 43°C). Heat alone cytotoxicity increased by about 0.3 decade in normally oxygenated cells at pH 6.45 following exposure to 42°C for 1 h and by about 0.4 decade at pH 6.45 in normally oxygenated cells following 43°C for 1 h as compared with the cytotoxicity of these temperatures at pH 7.40. Heat alone cytotoxicity was further increased at pH 6.45 under hypoxic conditions by approximately 0.3 decade at 42°C and by about 0.4 decade at 43°C.

Fig. 3 shows the results of exposure to Plato under the various treatment conditions. Fig. 3A presents the survival curve for this drug at 37°C and pH 7.40. As previously reported (18), Plato is significantly more cytotoxic to hypoxic than to normally oxygenated cells (about 2 decades more cytotoxic to hypoxic cells than to normally oxygenated cells at 500 μM). Fig. 3, B and C, shows that the cytotoxicity of Plato toward both hypoxic and normally oxygenated cells at normal pH significantly increased at 42°C and 43°C. The increase in cytotoxicity seen at 42°C and 43°C at pH 7.40 is in the range of 1.0–2.5 logs over the doses of Plato investigated, and the degree of hyperthermic sensitization toward Plato was similar for normally oxygenated and hypoxic cells so that the relatively greater killing by Plato of hypoxic cells was preserved at 42°C and 43°C.

For Plato, unlike CDDP, exposure of cells to the drug at pH...
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6.45 resulted in less cell killing for a given concentration of the drug. At 37°C and pH 6.45, although hypoxic cells were still more readily killed by Plato than normally oxygenated cells, exposure at 500 μM killed only approximately 1.5 decades of hypoxic cells while exposure to this concentration for 1 h at 37°C at pH 7.40 killed approximately 2.5 decades of hypoxic cells. Exposure of cells to Plato at 42°C or 43°C at pH 6.45 also resulted in significantly less cell killing under both normally oxygenated and hypoxic conditions over the dose range studied than was achieved by this drug at these temperatures at pH 7.40.

Fig. 4 depicts the survival of EMT6 cells exposed to Plato under various conditions. The pattern of cytotoxicity for this drug was distinct from either CDDP or Plato. Plato was slightly more cytotoxic to hypoxic than normally oxygenated cells at 37°C (about 0.4 decade more cell killing after exposure to 500 μM Plato in hypoxic than in normally oxygenated cells). Fig. 4, B and C, demonstrates that Plato also kills more cells at 42°C and 43°C at normal pH than at 37°C (about 1 decade more at 42°C and about 1.5 decades more at 43°C after exposure to 500 μM Plato). Hyperthermic sensitization to Plato was slightly greater in normally oxygenated than hypoxic cells because at 42°C and 43°C the concentration-dependent survival curves for normally oxygenated and hypoxic cells are essentially the same, unlike at 37°C where hypoxic cells were more sensitive to the drug than are normally oxygenated cells.

For Plato, acidic conditions significantly increased cell killing by the drug in both hypoxic and normally oxygenated cells, although the effect was greater in hypoxic cells. For instance, at 37°C, 100 μM killed only about 0.7 decade of hypoxic cells at pH 7.40, but this concentration killed 1.5 decades of hypoxic cells at pH 6.45 and at 37°C. At 42°C and 43°C, more cells were killed by Plato under acidic conditions, but essentially all of the increase in cell killing could be accounted for by greater direct thermal cytotoxicity at pH 6.45. Thus, the slopes of the survival curves were virtually identical for the drug in hypoxic and normally oxygenated cells at pH 6.45 at 37°C, 42°C, or 43°C.

Fig. 5 presents the survival data for cells exposed to NIPt under the various culture conditions. The concentrations of NIPt studied were essentially nontoxic toward either normally oxygenated or hypoxic cells at 37°C and pH 7.40. NIPt became slightly more cytotoxic to hypoxic but not normally oxygenated cells at pH 7.40 at 42°C and 43°C (about 1 decade of increased cell killing of hypoxic cells after exposure to 500 μM NIPt at 42°C and about 0.5 decade of increased cell killing in hypoxic cells by this concentration of NIPt at 43°C after correcting for heat alone cytotoxicity, as compared with the cytotoxicity of NIPt at 37°C).

At 37°C, significantly more normally oxygenated and hypoxic cells were killed at pH 6.45 by NIPt. After exposure to 500 μM NIPt, for instance, approximately 0.8 decade of additional cytotoxicity was measured in normally oxygenated cells and just over 1 decade of increased cell killing in hypoxic cells at pH 6.45 as compared to pH 7.40. As indicated in Fig. 5, more cells were killed at 42°C at pH 6.45 by NIPt, but essentially all of the increase in cell killing could be accounted for by the increase in direct thermal cytotoxicity at pH 6.45. In contrast, at 43°C in hypoxic but not normally oxygenated cells, significantly more cell killing was accomplished by NIPt at pH 6.45. Thus, after exposure of cells to 250 μM NIPt at pH 6.45 at 43°C, for instance, approximately a 0.3 decade of increased cell killing was observed over and above that due to exposure to 43°C at pH 6.45 in hypoxic cells as compared with the killing by exposure to this concentration of the drug for 1 h at pH 6.45 in hypoxic cells at 37°C.

DISCUSSION

These studies were undertaken in an effort to model various environmentally determined, physiological conditions which

Fig. 4. Survival curve of exponentially growing normally oxygenated (○) and hypoxic (□) EMT6 cells exposed to the concentrations of Plato indicated at 37°C, 42°C, or 43°C at pH 7.40 (A-C) and pH 6.45 (D-F). The survival value plotted on the y-axis represents heat alone killing at the conditions indicated. Points, means of 3 independent determinations ± SEM (bars).

Fig. 5. Survival curve of exponentially growing normally oxygenated (○) and hypoxic (□) EMT6 cells exposed to the concentrations of NIPt indicated at 37°C, 42°C, or 43°C at pH 7.40 (A-C) and pH 6.45 (D-F). The survival value plotted on the y-axis represents heat alone killing at the conditions indicated. Points, means of 3 independent determinations ± SEM (bars).
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should be present in tumors undergoing treatment with anticancer drugs in conjunction with hyperthermia. We are searching for drugs capable of causing important degrees of increased cytotoxicity in conjunction with hyperthermia across various environmental conditions. Each of the four drugs studied became more cytotoxic at elevated temperatures under some but not all culture conditions. CDDP showed by far the largest degree of hyperthermic sensitization, but for reasons which are not obvious, hypoxia obviated much of this effect in cells exposed to CDDP and hyperthermia at normal pH. This effect of hypoxia, however, was substantially lessened under low pH conditions, especially at 43°C. Since it is likely that low pH conditions will coincide with hypoxic regions within tumors (15–17), CDDP should be a good drug to use clinically with hyperthermia against this tumor subpopulation, especially if temperatures above 43°C can be obtained in the tumor in these relatively underperfused areas. Cellular resistance to CDDP has been reported to be associated with a reduction in drug uptake (28–30), and increased protein and/or non-protein thiol levels within cells (28, 31, 32) in various laboratory investigations. In addition, the induction of hypoxia in cells may alter several cellular properties such as thiol levels, membrane properties, and DNA repair capabilities (33, 34). Any one or more than one of these factors may be responsible for the relative lack of hyperthermic sensitization to CDDP observed in hypoxic cells at normal pH.

We have previously reported that the cytotoxicity of CDDP in EMT6 cells at 37°C was unaffected by hypoxia (25) and these results were confirmed in the present study. The mechanism by which cells are sensitized to CDDP by hyperthermia, however, does appear to be oxygen-dependent. Previous mechanistic studies have examined the interaction between CDDP and hyperthermia (35, 36). In general, these studies have found an increase in DNA cross-linking by CDDP at elevated temperature. We have also examined the mechanisms responsible for the CDDP-hyperthermia interaction. In work conducted in human cell lines in vitro to be published separately, we found no significant increase in CDDP level within cells at elevated temperature, but we also found significant increases in DNA cross-linking at 42°C and 43°C. Whether the net increase in DNA damage observed, however, is due to an increase in the kinetics of the CDDP-DNA reaction and/or inhibition of DNA repair at elevated temperatures is currently unknown.

The patterns of cytotoxicity observed with the other drugs tested under the various culture conditions were different for each drug. For the cis-nitrobenzene-platinum complex, Plato, hypoxic cells are preferentially killed at both normal and elevated temperatures. At 42°C and 43°C at pH 7.40, significantly more of both hypoxic and normally oxygenated cells were killed than at 37°C. At pH 6.45, however, less cytotoxicity due to Plato was evident at both normal and hyperthermic temperatures. Additionally, analysis of the slope of the concentration-dependent survival curves revealed that at pH 6.45 no hyperthermic sensitization to the drug could be detected under either oxygenation condition. Because one of the main advantages to the use of hyperthermia in conjunction with radiation may be its ability to kill hypoxic, radioresistant cells at low pH, the failure of Plato to interact positively with hyperthermia under these conditions may indicate that Plato is not an optimum drug to test in trimodality protocols. Mechanistic studies with this drug are rudimentary, although the interaction of similar agents with DNA and radiation has been examined (37–39). We have found that Plato induces DNA cross-linking at 37°C in the alkaline elution assay, but we have not yet fully investigated the effect of the culture conditions tested here on intracellular drug levels or DNA damaging capabilities of Plato.

For the trans-nitrothiazole platinum complex Plant, exposure of cells to the drug at 42°C or 43°C did increase cell killing in both hypoxic and normally oxygenated cells at normal pH. In addition, at pH 6.45 hypoxic cells were significantly more sensitive to the drug at all temperatures tested. Unfortunately, normally oxygenated cells were far less sensitized to the drug by low pH, and 42°C or 43°C hyperthermia did not further increase the cell killing by Plant in either hypoxic or normally oxygenated cells at pH 6.45. These results suggest that Plant may be an important drug to use with radiation therapy, because we have previously found that this agent has significant radiosensitizing properties in hypoxic cells (18) and the present studies indicate that the drug is significantly directly cytotoxic toward hypoxic cells at low pH which may represent the radioresistant subpopulation within tumors. The failure of hyperthermia to further increase the cytotoxicity of Plant toward cells at low pH, however, may make it a less than optimum drug for use in protocols with hyperthermia. As with the other drugs tested, we have yet not investigated the mechanisms responsible for the difference in cytotoxicity observed with Plant at the various culture conditions studied.

For the trans-nitroimidazole platinum complex, NIPt, 42°C or 43°C hyperthermia minimally increased the killing of hypoxic cells at both normal pH and pH 6.45. At 43°C, especially, hypoxic cells at low pH became significantly more sensitive to NIPt. These findings are quite like those reported by Rajaratnam et al. (11) under similar culture conditions for the nitroimidazole drug misonidazole. We have previously shown that NIPt also has significant radiosensitizing properties (18) and we have found that this drug has very low toxicity in animals. The concentrations of NIPt investigated in the present studies were limited by the solubility of the drug in the culture medium. We therefore plan to test NIPt in trimodality protocols with hyperthermia and radiation in laboratory animals, because significantly more of this drug on a molar basis can be tolerated by animals than the other drugs tested.

Furthermore, since all of the new platinum complexes tested are much less toxic to laboratory animals than is CDDP and since their patterns of cytotoxicity in these culture conditions are sometimes complementary, it may be possible to combine two of the drugs tested with heat ± radiation in order to take advantage of these environmentally determined cytotoxic differences. For instance, CDDP, which is highly cytotoxic toward normally oxygenated cells at elevated temperatures regardless of pH but is less cytotoxic toward hypoxic cells except under culture conditions at 43°C, could be combined with Plant, which is more cytotoxic toward hypoxic cells at low pH at all temperatures studied.

One of the main findings of this group of experiments is that when the cytotoxic interaction between drugs and hyperthermia is assessed under various culture conditions which should be pertinent to environmentally determined subpopulations within tumors, large and sometimes unpredictable effects on cytotoxicity are then discernible. We intend to extend these studies to the investigation of other established anticancer drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea, mitomycin C, bleomycin, or Adriamycin, all of which have been shown to become more cytotoxic at elevated temperatures (39–41). In this way, we hope to be able to predict which drugs will be most appropriate to investigate in trimodality protocols with hyperthermia + radiation.
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