**Iododeoxyuridine Chemosensitization of cis-Diamminedichloroplatinum(II) in Human Bladder Cancer Cells**

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**ABSTRACT**

We investigated the effect of iododeoxyuridine (IdUrd) exposure on cis-diamminedichloroplatinum (CDDP) cytotoxicity in the human bladder cancer cell line 647V. Following a 48-h incubation with 2–20 μM IdUrd, a 1-h exposure to 0–120 μM CDDP produced a dose-dependent increase in CDDP cytotoxicity as measured by clonogenic survival. IdUrd exposure of 2, 5, 10, and 20 μM prior to CDDP resulted in dose-modifying factors at 10% survival of 1.2, 1.6, 2.0, and 3.5, respectively. The increase in CDDP cytotoxicity appears to be associated with the level of DNA thymidine replacement in DNA by IdUrd over the range of 13–36%.

Atomic absorption spectrophotometric analysis of DNA extracted from 647V cells showed that IdUrd substitution did not affect the total amount of platinum bound to the DNA or the persistence of the bound platinum over a 24-h period post-CDDP exposure versus control cells. IdUrd, unlike thymidine, was found to form two monofunctional adducts with CDDP both in vitro and in vivo. IdUrd was also found to form a mixed bifunctional adduct with deoxyguanosine (dGua) and CDDP in vitro. 1H NMR analysis of purified IdUrd-Pt and IdUrd-Pt-dGua adducts confirmed the identity of these adducts. High pressure liquid chromatography analysis of [3H]IdUrd-labeled 647V DNA digests exposed to CDDP showed the presence of two monofunctional adducts. Unlike the free solution production of adducts in vitro, the predominant adduct formed was not IdUrd-Pt. Results using [3H]IdUrd-labeled 647V DNA suggests that this adduct is 5-Pt-deoxyuridine. We were not able, however, to detect the presence of the bifunctional adducts IdUrd-Pt-dGua or dGua-Pt-IdUrd. This was most likely due to the extremely low proportion of mixed bifunctional adducts produced in vivo. Nonetheless, these results suggest that IdUrd DNA incorporation may enhance CDDP cytotoxicity through the increase of available sites for Pt adduct formation. A Phase I clinical trial of this approach is planned.

**INTRODUCTION**

CDDP3cept has become an integral part of multidrug regimens for treating a wide variety of solid tumors including bladder, ovarian, testicular, and head and neck carcinomas (1–3). Studies suggest CDDP acts as a bifunctional alkylating agent, exerting its cytotoxic effect through interactions with DNA (4–8). The reaction of CDDP with DNA results in altered DNA conformation (9, 10), inhibition of DNA replication and repair (11–13), and RNA synthesis (14, 15). Enhanced CDDP sensitivity has been demonstrated by depleting glutathione levels using buthionine sulfoximine (16–18). Additionally, CDDP sensitivity has been increased by inhibition of DNA synthesis and repair by agents such as aphidicoline (18, 19), arabinofuranosyl-cytosine (20), and hydroxyurea (21).

IdUrd, a dThd analogue (22), has been used in clinical trials as a radiosensitizer with encouraging results in some poorly radiosensitive tumors including high grade gliomas and unresectable sarcomas (23, 24). The radiosensitizing effect of IdUrd on mammalian cells in vitro is highly associated with the amount of IdUrd incorporated into DNA (25–28). IdUrd is an interesting radiosensitizer in that its effect is directly related to the level of DNA incorporation, where the highly electronegative halogen atom greatly increases the cross-sectional area available for trapping of radiation-produced electrons (29, 30).

The in vitro chemosensitization by IdUrd has also been observed for several different drugs including Adriamycin, melphalan, CDDP (31), and bleomycin (32). Bromodeoxyuridine, another dThd analogue, has also been shown to modulate CDDP cytotoxicity in vitro (33). However, the mechanism of halopyrimidine chemosensitization of CDDP has not been reported previously. Since the site of action of the above mentioned chemotherapeutic agents are mediated through DNA damage and repair, it is possible that IdUrd chemosensitization of CDDP may result from direct IdUrd-CDDP interactions in the DNA of IdUrd-treated cells.

In this study, we evaluated the effect of IdUrd incorporation into the DNA of 647V cells on CDDP cytotoxicity. We observed that CDDP cytotoxicity was associated with the level of IdUrd incorporation into DNA. Analysis of DNA samples by atomic absorption spectroscopy showed no significant difference in the total amount of Pt bound to DNA between control and IdUrd-substituted DNA. IdUrd was found to produce two monofunctional Pt adducts plus the mixed bifunctional adduct IdUrd-Pt-dGua in vitro. Only the monofunctional adducts were detected from [3H]IdUrd or [125]IdUrd-labeled DNA extracted from 647V cells.

**MATERIALS AND METHODS**

**Drugs and Enzymes** Platinol, a clinical preparation of CDDP used for cell survival assays, was generously donated by Bristol Myers Squibb Co. (Evansville, IN). CDDP for in vitro assays, IdUrd, dGua, dThd, Proteinase K, DNase I, alkaline phosphatase, and phosphodiesterase were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media and reagents were obtained from GibCO-BRL (Grand Island, NY). [3H]IdUrd was obtained from Moravek Biochemicals (Brea, CA). [125]IdUrd was purchased from DuPont NEN Research Products (Boston, MA).

**Clonogenic Survival Assay.** The human bladder carcinoma cell line, 647V, was maintained in MEM+ (34). The 647V cell line has a 24-h doubling time and a plating efficiency of 40–60%. Exponentially growing 647V cells were exposed to CDDP for 1 h (0–120 μM) following a 48-h incubation (~2 population doublings) with 0–20 μM IdUrd. The treated 647V cells were then suspended in MEM+ following trypsinization. Two hundred to 200,000 cells were inoculated into 60-mm cell culture dishes (Costar Corp., Cambridge, MA) containing 4 ml MEM+. The dishes were incubated for 4 h at 37°C to allow the cells to attach. CDDP (0–120 μM) was then added, and the cells were incubated at 37°C for 1 h. The medium was then replaced with drug-free MEM+ and the dishes were incubated for 9–10 days at 37°C in a humid 5% CO2 atmosphere. The dishes were fixed and stained using 0.5% crystal violet in methanol-acetic acid (3:1, v/v). Colonies containing >50 cells were scored.

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3 The abbreviations used are: CDDP, cis-diamminedichloroplatinum(II); IdUrd, 5-ioodeoxyuridine; dGua, deoxyguanosine; dAde, deoxyadenosine; dThd, thymidine; HPLC, high pressure liquid chromatography; TCA, trichloroacetic acid; Pt, platinum; MEM+, α-minimal essential medium containing 25 mg 4-(2-hydroxyethyl)-1-piperazinethane-sulfonic acid (pH 7.4) and supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 1 mM L-glutamine, and 26 mM NaHCO3; DMF, dose modifying factor; NMR, nuclear magnetic resonance; dUrd-Pt, 5-Pt-deoxyuridine.
Each survival point was plated in triplicate with a minimum of two replicate experiments performed for each treatment condition. The DMF of IdUrd on CDDP cytotoxicity was determined at 10% survival by:

\[
DMF = \frac{SF_{10\%\text{CDDP}}}{SF_{10\%\text{MEM + CDDP}}}
\]

where \(SF\) = surviving fraction.

**Digestion of DNA to Nucleosides.** A portion of cells used for the clonogenic survival assay was saved to determine the percentage of dThd replacement where \(SF\) = surviving fraction. CDDP adduct was checked by HPLC analysis of the pooled fractions. No contamination from the reaction mixtures of CDDP, IdUrd, and dGua by HPLC using solvent phase B.

At —20°C. CDDP adduct formation was analyzed by HPLC using mobile phase A, which consisted of 100 mM sodium acetate (pH 7.45) containing 7% (v/v) acetonitrile and 0.1 mg/ml aprotease, 1.4 mg/ml phosphodiesterase. The mixture was incubated for 2 h at 37°C. Debris was removed by centrifugation for 10 min at 1000 \(\times\) g. The supernatant was centrifuged through Millipore Ultrafree-MC filtration units (Millipore, Bedford, MA) and the filtrate was stored at —20°C until analysis by HPLC.

**HPLC Analysis.** HPLC analysis of nucleosides and Pt-nucleoside adducts was performed using a Waters 600E solvent delivery system on a 3.9 \(\times\) 300-mm Bondapak C18 reversed-phase column (Waters Associates, Milford, MA). Peak elution was monitored by UV absorbance using a Waters model 490e UV detector. Data analysis was performed using a Waters Model 745 Data Module against authentic nucleoside standards.

IdUrd DNA incorporation was determined using mobile phase A, which consisted of 100 mM sodium acetate (pH 5.45) containing 7% (v/v) acetonitrile at a flow rate of 2 ml/min. Peaks were detected at 288 nm. The percentage of dThd replacement was calculated using:

\[
\% \text{dThd replacement} = \frac{\text{nmol of IdUrd}}{\text{nmol of IdUrd} + \text{nmol of dThd}}
\]

CDDP adducts were resolved using a modification of the method of Eastman (36). The solvent system for this analysis (mobile phase B) consisted of a 20-min linear 5–25% acetonitrile gradient in 100 mM ammonium acetate (pH 5.5) at a flow rate of 1 ml/min. Peaks were monitored by UV absorption at 254 nm.

**Platinum Binding in Intact Cells.** Exponentially growing 647V cells were incubated for 48 h in 0 or 10 \(\mu\)M IdUrd prior to a 1-h exposure to 100 \(\mu\)M CDDP. The cellular DNA was extracted at 0–24 h post-CDDP removal according to the method of Gross-Bellard et al. (37). Quantitation of total DNA-associated Pt was determined using a Varian SpectrAA20 atomic absorption spectrophotometer (Varian Associates, Sunnyvale, CA) with O2 background correction and GTA graphite tubes. The Pt content was expressed as pg Pt/\(\mu\)g DNA (38).

**Reaction of CDDP with Deoxyribonucleosides in Vitro.** Solutions (2.5 mM) of CDDP, IdUrd, and dGua were made in 20 mM NaClO4 (pH 5.5). Five hundred \(\mu\)l of CDDP and IdUrd or dGua were mixed and incubated at 37°C. The reaction mixture was sampled at various time points (1–96 h) and stored at —20°C. Mixed bifunctional adduct formation was accomplished by incubating 1 ml of 2.5 mM dGua plus 1 ml of 2.5 mM CDDP together for 24 h at 37°C. IdUrd (1 ml of 2.5 mM solution, pH adjusted to 5.5 with 0.1 N HCl) was then added to 200 \(\mu\)l of reaction mixture with incubation continuing at 37°C. Samples (100 \(\mu\)l) were taken at 24-h intervals to 96 h and stored at —20°C. CDDP adduct formation was analyzed by HPLC using mobile phase B.

**Identification of CDDP Adducts.** The individual adducts were purified from the reaction mixtures of CDDP, IdUrd, and dGua by HPLC using solvent system B. Fractions were collected and immediately frozen. The purity of each adduct was checked by HPLC analysis of the pooled fractions. No contaminating peaks were detected. The pooled fractions were lyophilized and redissolved in D2O. Structures of the various adducts were determined by proton NMR spectroscopy.

**In Vivo CDDP Adduct Formation.** The 647V cells (2 \(\times\) 10^6) growing in 75-cm^2 cell culture flasks were incubated for 24 h at 37°C in 10 ml of MEM + containing 10 \(\mu\)M [\(^{3}H\)]IdUrd (specific activity, 500 dpm/pmol) or 10 \(\mu\)M \(^{125}\)IIdUrd (specific activity, 4000 cpm/pmol). CDDP was then added to the culture flasks at a final concentration of 100 \(\mu\)M, and the flasks were incubated for 1 h at 37°C. The medium was then removed, and the dishes were washed twice with 10 ml of MEM+. Ten ml of MEM+ was added to the flasks, and the incubation continued at 37°C. Cells were mechanically harvested 6 h post-CDDP exposure. The DNA was digested to nucleotides and Pt adducts were analyzed as described above. HPLC separation of adducts was performed using solvent system B. Fractions (300 \(\mu\)l) were collected. The \(^{3}H\)-labeled fractions were mixed with 10 ml of Poly-Fluor scintillation cocktail (Packard Instruments, Meriden, CT) and counted using a Beckman LS-6000 scintillation spectrophotometer (Beckman Instruments, Fullerton, CA). \(^{125}\)I-labeled fractions were counted using a Model 5820 Gamma Counter (Packard Instruments).

**RESULTS**

The effect of a 48-h pretreatment of 647V cells with IdUrd on CDDP cytotoxicity following a 1-h exposure, as assayed by clonogenic survival, is presented in Fig. 1. IdUrd increased 647V cell sensitivity to CDDP cytotoxicity in a dose-dependent manner, which parallels the increase in IdUrd incorporation into 647V DNA. Table 1 compares IdUrd exposure with dThd replacement and enhanced CDDP cytotoxicity as measured by the DMF. dThd replacement ranged from 13.6% at 2 \(\mu\)M IdUrd to 36.1% replacement at 20 \(\mu\)M IdUrd with corresponding DMF values of 1.2 and 3.5, respectively.

IdUrd incorporation into the DNA of 647V cells did not affect the total amount of Pt bound to the purified DNA or the persistence of DNA-bound CDDP in 647V cells in vivo out to 24 h post-CDDP exposure (Fig. 2).

IdUrd was found to form one major monofunctional adduct with CDDP when incubated in vitro for 96 h at 37°C as measured by HPLC (Fig. 3A). Peaks are tentatively identified as: IdUrd, elution time, 15.5 min; IdUrd-Pt, elution time, 5.8 min; peak U1, minor unknown, elution time, 8.9 min; and peak U2, minor unknown. The rate of
Table 1. IdUrd dose, percentage of dThd replacement, and DMF, 10%.
The relationship of IdUrd exposure to dThd replacement and CDDP cytotoxicity is illustrated. The DMF was measured at 10% survival.

<table>
<thead>
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<th>IdUrd (µM)</th>
<th>Thymidine replacement (%)</th>
<th>DMF (10% survival)</th>
</tr>
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<tr>
<td>0</td>
<td>0</td>
<td>1.00</td>
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<tr>
<td>2</td>
<td>13.6 ± 2.0</td>
<td>1.18</td>
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<tr>
<td>5</td>
<td>21.7 ± 2.5</td>
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<td>10</td>
<td>31.7 ± 2.0</td>
<td>2.00</td>
</tr>
<tr>
<td>20</td>
<td>36.1 ± 2.0</td>
<td>3.47</td>
</tr>
</tbody>
</table>

IdUrd-Pt adduct formation in vitro is very slow relative to dGua-Pt formation and is similar to that of deoxycytidine-Pt formation (36). 1H NMR analyses of the purified IdUrd-Pt and IdUrd are presented in Table 2. Platination of IdUrd resulted in an upfield shift in the signal from the H(6) proton from 8.21 ppm for IdUrd to 8.01 ppm in the IdUrd-Pt adduct. Peaks U1 and U2 were not produced in sufficient amounts to permit 1H NMR analysis.

The reaction rate for the dGua-Pt adducts (1/2 = 3 h) is faster than for IdUrd-Pt formation as reported by Eastman (36) with a nearly complete reaction occurring by 24 h. An HPLC chromatogram of dGua-Pt adducts following 24-h incubation at 37°C is presented in Fig. 3B. The peaks are identified as: dGua-Pt-dGua, elution time, 14.1 min; dGua-Pt, elution time, 6.0 min; and Pt-dGua-Pt, elution time, 4.8 min.

Fig. 4 shows the HPLC analysis of the time course of adduct formation resulting from the incubation of IdUrd to a mixture of dGua-Pt adducts in a 5:1 ratio. Both IdUrd-Pt and the peak tentatively identified as IdUrd-Pt-dGua (elution time, 17.1 min) are formed over the course of 96 h. 1H NMR analysis of the purified mixed bifunctional adduct (IdUrd-Pt-dGua) reveals an upfield shift of the H(6) proton to 8.02 ppm on the IdUrd molecule plus a downfield shift from 8.08 ppm for the H(8) proton of dGua to 8.33 ppm for Pt-dGua-Pt (Table 2). The shift in spectrum to 8.02 ppm was identical with that of the pure IdUrd-Pt adduct, whereas the magnitude of the shift is less for IdUrd-Pt-dGua (8.33 ppm) than for pure dGua-Pt (8.49 ppm). The reduced shift may be the result of a long distance or a steric effect by the IdUrd moiety.

Since the formation of Pt adducts is a rare event in vivo, we labeled the DNA of 647V cells with [3H]IdUrd prior to CDDP exposure to increase the detectability of IdUrd-Pt adducts. Fig. 5 shows the 3H elution profile (solid line) obtained from HPLC analysis of the DNA digests. Two [3H]IdUrd-Pt adducts were detected from the DNA
digests. The HPLC elution profile of [3H]IdUrd was quite different than expected based on the reaction of IdUrd and CDDP in solution. The elution profile shows that the predominant radioactive peak (other than IdUrd) eluted at the same time as the free solution reaction product U2. This was unexpected since only one IdUrd-Pt adduct was anticipated based on Eastman’s study of deoxynucleosides and CDDP (36) plus our own work on IdUrd-Pt. Since 1H NMR spectral data for

Table 2 1H NMR spectral data of deoxynucleosides and platinum adducts

<table>
<thead>
<tr>
<th>Deoxynucleoside-Pt adducts</th>
<th>ppm</th>
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</thead>
<tbody>
<tr>
<td>dGua</td>
<td></td>
</tr>
<tr>
<td>IdUrd</td>
<td></td>
</tr>
<tr>
<td>IdUrd-Pt</td>
<td></td>
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<tr>
<td>IdUrd-Pt-dGua</td>
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<tr>
<td>dGua-Pt</td>
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<tr>
<td>dGua-Pt-dGua</td>
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</table>

* Data obtained from Eastman (36).

this adduct were not available due to the small amount of adduct formed in vitro, we could not determine whether the Pt bound through O(4) or by displacement of I(S) on the pyrimidine ring. 125IdUrd was substituted for [3H]IdUrd to label the DNA in an attempt to elucidate a possible identity for this adduct. The radiolabel elution profile for 125IdUrd is also presented in Fig. 5 (dotted line). Normalization of peak height to IdUrd-Pt allowed direct comparison of the elution profiles. The loss of 125I from the area corresponding to U2 suggests that this Pt adduct is formed through the displacement of I(5), yielding dUrd-Pt. The putative structures of the two monofunctional adducts are presented in Fig. 6. The two other 125I detectable peaks (U3 and U4) are unidentified and do not correspond to any reaction products formed in vitro.

The radioactive HPLC elution profile for either radionuclide did not show the presence of either mixed bifunctional adduct, IdUrd-Pt-dGua, or dUrd-Pt-dGua. Since the amount of IdUrd-Pt or dUrd-Pt adducts formed was so small relative to the total amount of IdUrd incorporated in the DNA, the mixed bifunctional adduct IdUrd-Pt-dGua or dUrd-Pt-dGua may be present but below the level of detectability using this procedure.

DISCUSSION

In this study, we have demonstrated that a 48-h preexposure of 647V cells to 2–20 μM IdUrd enhanced the cytotoxicity of CDDP in a dose-dependent manner. Comparing clonogenic survival curves at 10% survival (Fig. 1), a dose modifying factor varied from 1.2 to 3.5 as IdUrd concentration increased from 2 to 20 μM (Table 1). Mancini and Greenberg (33) have recently demonstrated enhanced CDDP cytotoxicity in human glioma cells by treatment with bromodeoxyuridine, another thymidine analogue.

Studies by Eastman (36) showed that the rate of monofunctional adduct formation between CDDP and free nucleoside varied greatly (dGua > dAde > deoxycytidine, t 1/2 = 3, 20, and 72 h, respectively) whereas dThd did not form a CDDP adduct. While clonogenic survival assays show a dose-dependent chemosensitization of CDDP cytotoxicity by IdUrd, no difference was found in the total amount of Pt bound to the cellular DNA between IdUrd-substituted and unsub-
IdUrd CHEMOSENSITIZATION OF CISPLATIN

Fig. 6. Possible structures of the two IdUrd-CDDP adducts. IdUrd-Pt structure was determined by 1H NMR spectroscopy of the adduct purified by HPLC. The structure for dUrd-Pt is inferred from the loss of the 125I peak from the HPLC elution profile corresponding to the major 3H-labeled peak.

stituted DNA. This is not surprising considering the generally poor reactivity of pyrimidine deoxyribosides to CDDP.

The initial CDDP-nucleoside adduct is produced through CDDP binding to the N7 position of a guanine base forming the monofunctional adduct. Subsequently, the monofunctional adduct reacts with a second nucleophile, forming intrastrand cross-links at dGua or dAde residues in the sequences dGua—Pt—dGua and dAde—Pt—dGua or less frequently, dGua—Pt—N—Pt—dGua or dAde—Pt—N—Pt—dGua, where N is any base. Less than 1% of the total adducts formed are interstrand cross-links between guanine in the opposite strands (39—41).

IdUrd was found to form two monofunctional adducts with CDDP, plus the bifunctional adduct IdUrd-Pt-dGua in vitro. These data suggest that dThd substitution by IdUrd in 647V DNA may provide additional sites for monofunctional adduct formation. The in vitro production of the mixed bifunctional adduct IdUrd-Pt-dGua suggests that IdUrd-substituted DNA may provide new sites for formation of either IdUrd-Pt-dGua or dUrd-Pt-dGua. This is significant since dThd does not form a Pt adduct (36).

Pinto and Lippard (10) have demonstrated inhibition of DNA synthesis at bifunctional adduct sites using a single strand DNA template and DNA polymerase I. Rice et al. (8) have also shown that intrastrand adducts introduce a sharp bend in the helix axis of the DNA resulting from the geometry of the CDDP complex bound to two adjacent guanine-N7 positions. This alteration in local structure may be responsible for the cytotoxicity of CDDP. Monofunctional adducts do not affect DNA replication and can be readily inactivated by sulfur-containing nucleophiles such as glutathione and metallothionein (17, 42) or repaired by cellular repair systems such as the uvrABC system of *Escherichia coli* (43) and the ERCC-1 gene in human systems (44). The replacement of dThd by IdUrd in DNA may thus provide additional sites for both the initial binding of CDDP and the formation of intrastrand cross-links, resulting in enhanced cytotoxicity.

While Bancroft et al. (45) have reported that *t*1/2 of bifunctional adduct formation takes about 2 h in *vitro* (45), we were not able to detect the presence of the mixed bifunctional adduct IdUrd-Pt-dGua over the course of 18 h post-CDDP exposure in *vitro* (data not shown). However, this does not exclude the existence of the mixed bifunctional adducts. *In vitro* experiments using solutions of IdUrd, dGua, and CDDP showed that IdUrd can form IdUrd-Pt, dUrd-Pt, and IdUrd-Pt-dGua. The lack of detectability of either [3H]IdUrd-Pt-dGua or [3H]IdUrd-Pt-dGua from 647V DNA may result from the small amounts of IdUrd-Pt that are actually present in vivo, combined with the even less frequent proportion of neighboring dGua residues available for formation of the specific mixed bifunctional adducts IdUrd-Pt-dGua and dUrd-Pt-dGua.

These results suggest that IdUrd chemosensitization of 647V cells to CDDP may be the result of the binding of CDDP to additional sites within the cellular DNA resulting from IdUrd replacement of dThd in the DNA of the cell. Although IdUrd-containing bifunctional adducts were not detected in *vivo*, the *in vitro* formation of the bifunctional adduct IdUrd-Pt-dGua suggests that the existence of this adduct and, by extension, dUrd-Pt-dGua, may occur in the cell.

On the basis of these observations, we are planning a Phase I trial of a 7-day continuous i.v. infusion of escalating doses of IdUrd prior to a fixed dose of CDDP at 100 mg/m2. In addition to the standard clinical toxicity criteria, biological assays, including the percentage of dThd replacement in circulating granulocytes and Pt adducts in lymphocytes using monoclonal antibodies, will be measured. Subsequent clinical trials using IdUrd and CDDP with radiation therapy are also planned since both drugs are potential radiosensitizers (46).

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REFERENCES


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