Cancer Chemother Pharmacol (1994) 34: 302–306

Cancer Chemotherapy and Pharmacology

Thermal enhancement of drug uptake and DNA adducts as a possible mechanism for the effect of sequencing hyperthermia on cisplatin-induced cytotoxicity in L1210 cells

Shinji Ohno[1], Zahid H. Siddik[2], Yuichiro Kido[2], Leonard A. Zwelling[2], Joan M. C. Bull[1]

[1] Department of Internal Medicine, The University of Texas Medical School, Houston, TX 77225, USA
[2] Department of Medical Oncology, M. D. Anderson Cancer Center, Houston, TX 77030, USA

Received: 23 April 1993/Accepted: 11 March 1994

Abstract. An optimal scheduling of hyperthermia and *cis*-diammine-dichloroplatinum(II) (cisplatin) may increase the therapeutic gain of the combination of these two modalities. In this study, intracellular platinum accumulation, total platinum binding to DNA, and DNA interstrand cross-links (ISC) were assayed to investigate the molecular mechanisms responsible for the effect of sequencing hyperthermia on the thermal enhancement of cisplatin-induced cytotoxicity in mouse leukemia L1210 cells. Simultaneous treatment with heat (41.5° C, 60 min) and cisplatin produced maximal cell killing with a 4-fold decrease in the 50% growth-inhibitory concentration (IC_{50}) of the platinum complex. Super-additive cell killing was also shown when cells were exposed to heat before cisplatin treatment, whereas no thermal enhancement in cisplatin-mediated cytotoxicity was observed in cells given heat after exposure to cisplatin. These results correlated with the degree of formation of ISC observed in cells following various treatments. A 2- to 3-fold increase in ISC formation was observed in cells given heat before or during cisplatin exposure, whereas heat after cisplatin treatment did not alter either the formation or the reversal of ISC as compared with cisplatin alone. The increased ISC formation was associated with an increase in intracellular platinum accumulation and total platinum binding to DNA in cells given heat before or during cisplatin exposure. These data, showing that hyperthermia potentiates cisplatin cytotoxicity by increasing drug uptake and the formation of DNA adducts without inhibiting the repair of DNA lesions, demonstrate the potential utility of sequencing hyperthermia combined with cisplatin as a clinical anticancer therapy.

This work was supported by National Cancer Institute grants, R01-CA-43090, R01-CA-41581, R01-CA-50380, R01-CA-40090, and American Cancer Society-CH-324E. S. Ohno and Y. Kido are on leave from the Department of Surgery II, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Correspondence to: Joan M. C. Bull, University of Texas, Health Science Center at Houston, Division of Oncology, P. O. Box 20708, Houston, TX 77225, USA

Key words: Hyperthermia – Cisplatin – DNA adducts

Introduction

Cisplatin is a commonly used antineoplastic agent with proven efficacy against a variety of human malignancies [7, 25]. Hyperthermia increases the cytotoxicity of cisplatin in vitro [3, 9] and in vivo [1, 16]. Increasing attention has been given to this combination as a powerful anticancer therapy. However, simultaneous application of these two modalities increases drug-mediated toxicity to normal tissue in human as well as in experimental animals, limiting the clinical utility of this combination [5, 13, 22].

A number of studies demonstrate that the antitumor effect is greatest when tumors are exposed simultaneously to cisplatin and heat [12, 21]. A synergistic effect has been produced by various sequences and intervals between cisplatin exposure and hyperthermia [6, 24]. Previously, we reported that varying the sequence between whole-body hyperthermia and cisplatin modulated the therapeutic gain in rats bearing fibrosarcoma [2]. Whole-body hyperthermia enhanced the cytotoxicity of cisplatin to both normal tissue and tumor, whereas the relative degree of this enhancement was modified by the sequence and interval between the two modalities. These data suggest that an optimal scheduling of cisplatin with heat could improve the therapeutic gain by reducing normal tissue injury while maintaining enhanced antitumor activity. For the clinical application of sequencing hyperthermia combined with cisplatin, it may be useful to understand the molecular basis causing the different effects observed using various timing and sequences in combinations of the two modalities.

The interaction of cisplatin with DNA has been implicated as the major cytotoxic action of the drug, and the formation of DNA interstrand cross-links (ISC) has been correlated with both in vivo and in vitro cytotoxicity [4, 26]. Hyperthermia has been shown to increase ISC in cultured

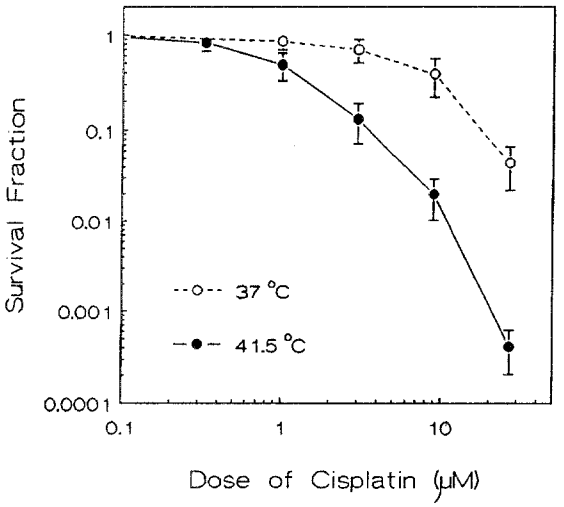

Fig. 1. Survival of L1210 cells after treatment with cisplatin at either 37° C or 41.5° C for 1 h. Data are presented as mean values ± SE (*bars*) for three independent experiments

cells when simultaneously combined with cisplatin [15]. To investigate the molecular mechanism that might account for the interaction between heat and cisplatin at various sequencing and timing, we examined cisplatin-induced ISC together with intracellular platinum accumulation and total platinum binding to DNA using cultured L1210 cells.

Materials and methods

Cell cultures. L1210/0 cells were grown in fresh RPMI 1640 (supplemented with penicillin, streptomycin, 1 m*M* L-glutamine, and 10% heat-inactivated fetal bovine serum). Cell cultures were kept at 37° C in a humidified incubator with a mixture of 95% air and 5% CO_2. Under these conditions, the cells have a doubling time of approximately 12 h. All experiments were done with cells in the exponential growth phase.

Drug treatment. Cisplatin, obtained from Johnson Matthey Research Centre (Reading, UK), was dissolved in sterile saline (0.9% NaCl) and diluted to the appropriate concentration with complete medium immediately prior to its use. Exponentially growing L1210 cells were resuspended in the medium at pH 7.4 and the cell density was adjusted to 5×10^5 cells/ml. The drug treatment was carried out in plastic tubes. Cells were exposed to cisplatin for 60 min at 37° C or 41.5° C. The cells were then centrifuged and washed twice in phosphate-buffered saline (PBS) and then resuspended in growth medium. In the sequential treatment studies, the cells were exposed to either cisplatin or heat for 1 h, rinsed with PBS, and exposed to the other agent for 1 h, with a variable interval being interposed between the exposures.

Hyperthermia. For hyperthermia, tubes were immersed in a thermostatically controlled circulating water bath maintained at 41.5° C ± 0.1° C by a Haake model E 12 circulator/heater. Temperatures in the water bath were measured using multiple thermometers and displayed on a YSI model 49 TA digital telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) connected to a YSI model 4002 12-channel switch box [16].

Survival assay. Exponentially growing L1210 cells were treated and washed as described above and then suspended in medium. A 1-ml aliquot of cells was suspended in 3 ml of medium containing 0.1% Noble agar in closed tubes, and colony formation was scored after 11–14 days of incubation. The colony-forming efficiency of untreated cell growth varied between 80% and 90%.

Alkaline elution assay. ISC formation was assayed by the technique of alkaline elution with proteinase K as described previously [10, 27]. Exponentially growing L1210 cells were radiolabeled with 0.05 μCi [2-^{14}C]-thymidine/ml (New England Nuclear, Boston, Mass.) for 24 h. L1210 cells that were used as internal standards were radiolabeled with 0.1–0.2 μCi [^{3}H]-thymidine/ml (New England Nuclear). Following treatment with 54 μ*M* cisplatin for 1 h, cells were washed twice in ice-cold PBS. [^{14}C]- and [^{3}H]-labeled L1210 cells were subjected to 300 rad X-irradiation and deposited on membrane filters. Filter elution was performed as described previously [10, 27]. The levels of [^{14}C]- and [^{3}H]-DNA radioactivity remaining on the filter and in the eluted fractions were determined by counting in a Beckman LS 5800 liquid scintillation spectrometer, and the retention of label on the filter as a function of the time of elution was calculated. The DNA ISC frequency in rad equivalents was calculated by the equation

$$\mathrm{ISC} = \left\{[(1-r_0)\,/\,(1-r)]^{-1/2}-1\right\} \times 300 \text{ rads},$$

where r and r_0 are the relative retention of [^{14}C]-DNA in treated and control cells, respectively. Cells exposed to heat alone for 1 h were used as the control for the treatment of heat combined with cisplatin. For the sequencing treatment, cells were rinsed after exposure to drug or heat and were then immediately exposed to the other treatment.

Intracellular cisplatin accumulation. Cells growing exponentially were treated with 54 μ*M* cisplatin for 1 h at 37° C or 41.5° C or at 37° C after heat exposure. Cells were washed twice in ice-cold PBS and pelleted. The pellets were solubilized with 25 μl hyamine hydroxide at 55° C for 3 h and acidified with 475 μl 0.1 *N* HCl, and the platinum content was determined by flameless atomic absorption spectrophotometry (FAAS; Varian, model AA300/GTA-96) and the technique of standard addition as described previously [18].

Total platinum binding to DNA. High-molecular-weight DNA was isolated from cell pellets following drug incubation (see above) according to standard procedures [11]. Briefly, the pellets were lysed at 37° C overnight using the extraction buffer [10 m*M* TRIS, 100 m*M* ethylenediaminetetraacetic acid (EDTA), 20 μg RNase/ml, 0.55% sodium dodecyl sulfate (SDS), pH 8.0] and then treated with proteinase K (100 μg/ml, 50° C, 3 h), and the DNA was extracted with phenol three times, precipitated with ethanol, and dissolved in H_2O (200 μl). The DNA content was assessed by absorption at 260 nm, and its purity was judged to be acceptable when the ratio of absorbance values determined at 260 and 280 nm was ≥1.8. The amount of Pt in the sample was determined by FAAS.

Statistical analysis. Student's *t*-test was used to determine the significance of the difference between the groups. When the *P* value was less than 0.05, the statistical difference was considered to be significant.

Results

The survival of exponentially growing L1210 cells exposed to various concentrations of cisplatin at 37° C or 41.5° C is shown in Fig. 1. In this cell line, exposure to heat alone at 41.5° C for 1 h reduced survival to 94% ± 4.7% (SE). Cells exposed to cisplatin and heat simultaneously showed a greater reduction in survival as compared with those treated with cisplatin at 37° C. The 50% growth-inhibitory (IC_{50}) doses as determined by linear regression analysis were 5.6 μ*M* at 37° C and 1.4 μ*M* at 41.5° C.

Figure 2 shows the effects of sequencing on the interaction of cisplatin (27 μ*M*) and heat. Exposure of cells at 41.5° C in the absence of cisplatin produced little, if any cell killing. Hyperthermic potentiation of cisplatin cytotoxicity was maximal when both modalities were given

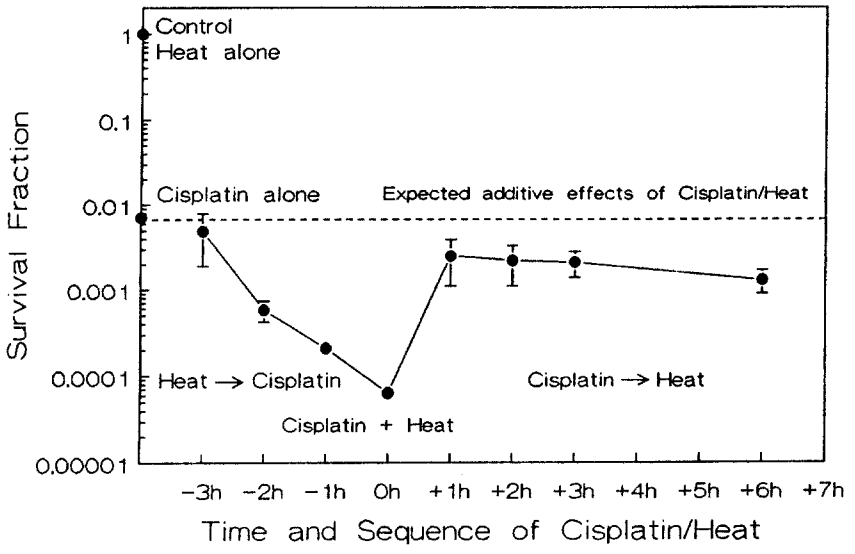

Fig. 2. Effects of sequencing on heat and cisplatin interaction in L1210 cells. Cells were exposed to heat (41.5° C, 1 h) before, during or after treatment with 27 μ*M* cisplatin for 1 h. Data are presented as mean values ± SE (*bars*) for three independent experiments

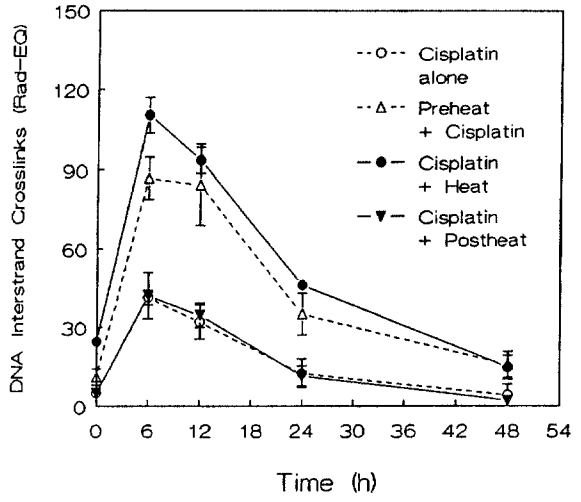

Fig. 3. DNA ISC formation and repair as a function of time after treatment of cells with 54 μ*M* cisplatin for 1 h combined with different sequencing heat. Cells were exposed to heat simultaneously with cisplatin or immediately before or after treatment with cisplatin. Data are presented as mean values ± SE (*bars*) for three independent experiments

simultaneously. When cells were exposed to heat 1 or 2 h before being treated with cisplatin, a synergistic cell killing was observed, whereas there was no augmentation of the effect of cisplatin when the cells were heated 3 h before drug exposure. In contrast, heat after exposure to cisplatin caused little decrease in the survival fraction as compared with cisplatin treatment alone.

The degree of formation and repair of DNA cross-links as a function of time after drug treatment with a various sequencing heat treatment is shown in Fig. 3. When treatment with 54 μ*M* cisplatin was carried out at 37° C, the maximal fraction of cross-links occurred at 6 h and then decreased, with most of the cross-links disappearing by 24 or 48 h. When cells were exposed to heat before or during cisplatin treatment, significantly greater ISC was produced, whereas no difference in the degree of ISC formation was observed in cells given heat after cisplatin treatment as compared with those exposed to cisplatin at 37° C. Essentially, the kinetics of the formation and reversal of ISC was similar in cells treated with cisplatin with a various sequencing of heat.

Table 1 summarizes the effect of sequencing between heat and cisplatin on intracellular platinum accumulation, total platinum binding to DNA, and the peak ISC value shown in Fig. 3. The former two parameters were measured just after exposure to cisplatin. The increased ISC mirrored an increase in intracellular platinum accumulation and total platinum binding to DNA. The maximal levels of intracellular platinum accumulation and total platinum binding to DNA were observed in cells given heat during cisplatin exposure, and these values were also greater in cells exposed to heat immediately before cisplatin treatment as compared with those given cisplatin alone.

Discussion

How hyperthermia enhances the cytotoxicity of antitumor drug remains controversial. Some possibilities have been proposed as follows: increased drug uptake in cells [8], increased DNA lesions [15], alteration in drug metabolism [23], and inhibition of DNA repair by heat [14, 24]. Some or all of the above-mentioned mechanisms may be involved when cisplatin is given simultaneously with hyperthermia. Increased intracellular platinum contents were observed in cells simultaneously exposed to heat and cisplatin as compared with those given cisplatin alone a study involving cultured cells [21]. Meyn et al. [15] reported that increased formation of ISC was observed with a higher concentration of cisplatin at 37° C or 43° C, and the cells treated with the drug for 1 h at 43° C had a greater amount of DNA cross-links than did those treated with the drug at 37° C. No difference was observed in the repair of ISC between cells exposed to cisplatin at 37° C versus 43° C. In

Table 1. Intracellular platinum accumulation, total platinum binding to DNA, and ISC in cells exposed to cisplatin and heat at different schedules

	Intracellular platinum (ng Pt/10^7 cells)	Total platinum binding to DNA (ng Pt/mg DNA)	ISC[a] (rad Equiv.)
Cisplatin alone	19.7 ± 2.3[b]	20.2 ± 1.3	41.5 ± 2.9
Heat followed by cisplatin	30.0 ± 4.5	32.6 ± 3.9*	86.8 ± 8.0**
Simultaneous exposure	36.9 ± 5.3**	47.3 ± 3.2**	110.4 ± 6.6**

The cells were exposed to each drug or to heat for 1 h. Data represent mean values ± SE for three independent experiments

[a] Value obtained 6 h after exposure to cisplatin

* $P < 0.05$ as compared with cisplatin alone;

** $P < 0.01$ as compared with cisplatin alone

vivo studies by Teicher et al. [20] demonstrated that local hyperthermia (43° C, 30 min) simultaneously combined with cisplatin treatment increased ISC formation in tumor cells, which was reflected in an increased tumor growth delay in the mouse FSaII fibrosarcoma, as compared with treatment with cisplatin alone.

At normal temperature, the clinical use of cisplatin is limited by its side effects on normal tissue. Cisplatin-mediated normal-tissue toxicity is enhanced when treatment with the drug is combined with systemic hyperthermia, resulting in a limited therapeutic gain from this combination therapy [5, 13, 22]. The alterations of cisplatin pharmacokinetics under hyperthermic conditions may be responsible for the increased normal-tissue toxicity as well as the antitumor effect. Studying the effect of heat on cisplatin pharmacokinetics in normal dogs showed in vivo alterations of different pharmacokinetic parameters [17]. Tissue binding of free cisplatin was increased at elevated temperatures. Our pharmacology study showed that the drug concentration in normal tissues and tumor increased when cisplatin treatment was carried out during whole-body hyperthermia [19]. With respect to local hyperthermia, increased platinum levels were observed in normal tissues such as skin and muscle in mice [17].

For an increase in the therapeutic gain, an optimal scheduling of heat and cisplatin exposure seems to be important. In preclinical studies, the extent of thermal enhancement of cisplatin cytotoxicity depends on the sequence or the interval between the administration of cisplatin and heat [6, 12, 21, 24]. Our previous study demonstrated that hyperthermia given before or after cisplatin decreased normal-tissue toxicity without reducing the antitumor effect, resulting in an increased therapeutic gain [2].

In the current study, we examined the molecular mechanisms responsible for the effect of the sequencing hyperthermia on the thermal enhancement of cisplatin-mediated cytotoxicity. Heat had no effect on cell killing by itself. A dose of 41.5° C applied for 60 min is tolerated in vivo; that is, this heat treatment is physiologically relevant. The sequencing hyperthermia caused different effects on the cytotoxicity of cisplatin, and these results correlated with the degree of ISC formation produced in cells. The maximal degree of cell killing and the greatest ISC values were produced in cells simultaneously exposed to cisplatin and heat. Cells given heat before cisplatin showed significantly increased cell killing and ISC formation as compared with those exposed to cisplatin alone. This increased ISC formation was associated with an increase in intracellular platinum accumulation and total platinum binding to DNA. These data suggest that heat perturbs the integrity of the cell membrane and leads to an increase in intracellular platinum accumulation.

The effect of heat given before cisplatin depends on the interval between heat and cisplatin administration. When heat was applied 3 h before cisplatin treatment, no enhancement of cell killing was observed. This observation suggests that the altered permeability of the cell membrane might recover within 2 h after heat exposure. In contrast, heat after cisplatin exposure caused no effect on the cell killing or the ISC induced by cisplatin. Interestingly, heat did not influence the repair of DNA ISC. Even when cells were exposed to heat at 6 h after their exposure to cisplatin, at which time the peak ISC values were observed, no change in the kinetics of ISC formation was produced (data not shown). With regard to these data, the most likely explanation for the effect of heat on cisplatin-induced cell killing is that hyperthermia alters the permeability of the cell, resulting effectively in a higher intracellular platinum accumulation. Enhanced platinum binding to DNA and ISC formation are probable consequences of increased drug uptake.

Our data demonstrate that hyperthermia enhanced cisplatin-mediated intracellular platinum accumulation, total platinum binding to DNA, and ISC formation. These changes correlated with increased cytotoxicity of cisplatin. Furthermore, the biochemical pharmacology was modulated by sequencing heat and cisplatin. For the clinical application of sequencing combinations of hyperthermia and cisplatin, molecular and pharmacology studies need to be performed in normal tissues as well as tumors such that an optimal sequence for the maximal therapeutic gain can be determined.

Acknowledgements. The authors thank Elizabeth Altschuler for her excellent technical assistance.

References

1. Alberts DS, Peng YM, Chen G, Moon TE, Cetas TC, Hoeschele JD (1980) Therapeutic synergism of hyperthermia-*cis*-platinum in a mouse tumor model. J Natl Cancer Inst 65: 455
2. Baba H, Siddik ZH, Strebel FR, Jenkins GN, Bull JMC (1990) Increased therapeutic gain of combined *cis*-diamminedichloroplatinum(II) and whole body hyperthermia therapy by optimal heat/drug scheduling. Cancer Res 49: 7041
3. Barlogie B, Corry PM, Drewinko B (1980) In vitro thermotherapy of human colon cancer cells with *cis*-dichlorodiammineplatinum(II) and mitomycin C. Cancer Res 40: 1165
4. Bedford P, Walker MC, Sharma HL, Perera A, McAuliffe CA, Masters JRW, Hill BT (1987) Factors influencing the sensitivity of two human bladder carcinoma cell lines to *cis*-diamminedichloroplatinum(II). Chem Biol Interact 61: 1
5. Bull JMC (1984) A review of systemic hyperthermia. Front Radiat Ther Oncol 18: 171

6. Douple EB, Jones EL, Kellog KC, Van Buren TA (1988) Treatment sequence effects of combined cisplatinum and hyperthermia in a murine tumor system. Proc 5th Int Symp Hyperthermia Oncol 3: 279
7. Durant JR (1980) Cisplatin: a clinical overview. In: Prestayko AW, Crooke ST, Carter SK (eds) Cisplatin, current status and new developments. Academic Press, New York, p 317
8. Hahn GM, Strande DP (1976) Cytotoxic effects of hyperthermia and adriamycin on Chinese hamster cells. J Natl Cancer Inst 37: 1063
9. Herman TS, Teicher BA, Cathcart KNS, Kaufmann ME, Lee JB, Lee MN (1988) Effect of hyperthermia on *cis*-diamminedichloroplatinum(II), (Rhodamine 123)$_2$[tetrachloroplatinum(II)], in a human squamous cell carcinoma line and a *cis*-diamminedichloroplatinum(II)-resistant subline. Cancer Res 48: 5101
10. Kohn KW, Erickson LC, Ewing RAG, Friedman CA (1976) Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15: 4629
11. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
12. Mella O, Dahl O (1985) Timing of combined hyperthermia and 1,3-bis(2-chloroethyl)-1-nitrosourea or *cis*-diamminedichloroplatinum in BD IX rats with BT4A tumors. Anticancer Res 5: 259
13. Mella O, Eriksen R, Dahl O, Laerum OD (1987) Acute systemic toxicity of combined *cis*-diamminedichloroplatinum and hyperthermia in the rat. Eur J Cancer Clin Oncol 23: 365
14. Meyn RE, Corry PM, Fletcher SE, Demetriades M (1979) Thermal enhancement of DNA strand breakage in mammalian cells treated with bleomycin. Int J Radiat Oncol Biol Phys 5: 1487
15. Meyn RE, Corry PM, Fletcher SE, Demetriades M (1980) Thermal enhancement of DNA damage in mammalian cells treated with *cis*-diamminedichloroplatinum(II). Cancer Res 40: 1136
16. Overgaard J, Radacic MM, Gran C (1991) Interaction of hyperthermia and *cis*-diamminedichloroplatinum(II) alone or combined with radiation in a C3H mammary carcinoma in vivo. Cancer Res 51: 707
17. Riviere JE, Page RL, Rogers RA, Change SK, Dewhirst MW, Thrall DE (1990) Non-uniform alteration of *cis*-diamminedichloroplatinum(II) tissue distribution in dogs with whole body hyperthermia. Cancer Res 50: 2075
18. Siddik ZH, Boxall FE, Harrap KR (1987) Flameless atomic absorption spectrophotometric determination of platinum in tissue solubilized in hyamine hydroxide. Anal Biochem 163: 21
19. Siddik ZH, Wondergem J, Strebel F, Alonso M, Burditt T, Bull JM (1989) Pharmacokinetics of cisplatin in rats receiving whole body hyperthermia. Proc Am Assoc Cancer Res 31: 551
20. Teicher BA, Pfeffer MR, Sotomayor EA, Herman TS (1991) Schedule dependent tumour growth delay, DNA cross-linking and pharmacokinetic parameters in target tissues with *cis*-diamminedichloroplatinum(II) and etanidazole with or without hyperthermia or radiation. Int J Hyperthermia 7: 773
21. Wallner DE, Li GC (1987) Effect of drug exposure duration and sequencing on hyperthermic potentiation of mitomycin-C and cisplatin. Cancer Res 47: 193
22. Wondergem J, Bulger RE, Strebel FR, Newman RA, Travis EL, Stephens LC, Bull JMC (1988) Effect of *cis*-diamminedichloroplatinum(II) on renal injury. Cancer Res 48: 440
23. Zakris EL, Dewhirst MW, Riviere JE, Hoopes PJ, Page RL, Oleson JR (1987) Pharmacokinetics and toxicity of intraperitoneal cisplatin combined with regional hyperthermia. J Clin Oncol 5: 1613
24. Zupi G, Badaracco G, Cavaliere R, Natali PG, Greco C (1984) Influence of sequence on hyperthermia and drug combination. Proc 4th Int Symp Hyperthermia Oncol 2: 429
25. Zwelling LA (1987) Cisplatin and new platinum analogues. In: Pinedo HM, Longo DL, Chabner BA (eds) Cancer chemotherapy and biological response modifiers. Elsevier, Amsterdam, p 71
26. Zwelling LA, Anderson T, Kohn KW (1979) DNA-protein and DNA interstrand cross-linking by *cis*- and *trans*-platinum(II) diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. Cancer Res 39: 365
27. Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M, Kohn KW (1981) Protein-associated DNA strand breaks in L1210 cells treated with the DNA intercalating agents 4'(9-acridinylamino)-methanesulfon-*m*-anisidide and adriamycin. Biochemistry 20: 6553