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Interaction of platinum complexes of thiazin and xanthene dyes with hyperthermia*

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Summary. In an attempt to develop platinum-containing drugs for use with hyperthermia that would be relatively nontoxic at 37° C but would become very cytotoxic at 42° or 43° C, several nuclear dyes were complexed to the tetrachloroplatinum(II) dianion ($PtCl_4$) at a ratio of 2:1. The cytotoxicity of $PtCl_4$ complexes of three thiazin dyes (thionin, azure B, and methylene blue), the xanthene dye pyronin Y, and the thiazole dye thioflavin was examined in exponentially growing euoxic and hypoxic EMT6 cells in vitro at 37°, 42°, and 43° C and at pH 7.40 and 6.45. Of the thiazin dye complexes, the cytotoxicity of Pt(methylene blue)$_2$ was most enhanced at hyperthermic temperatures. Both Pt(pyronin Y)$_2$ and Pt(thioflavin)$_2$ also became markedly more cytotoxic at 42° and 43° C at pH 6.45 vs pH 7.40. In vivo tumor excision assays in the FSaIIC fibrosarcoma showed that with each of the thiazin dye-platinum complexes, hyperthermia enhanced cell kill [most effectively on Pt(methylene blue)$_2$] but was not dose-modifying. For both Pt(pyronin Y)$_2$ and Pt(thioflavin)$_2$, however, administration of 43° C, 30-min hyperthermia to the tumor immediately after i.p. drug injection was dose-modifying. Tumor growth delay studies in the FSaIIC tumor system demonstrated that, as with the in vitro studies, Pt(pyronin Y)$_2$ and Pt(methylene blue)$_2$ were most enhanced by hyperthermia [tumor growth delay increased by 4.8- and 3.0-fold, respectively, vs only 1.3-fold for cisplatin (CDDP)]. Examination of intracellular platinum levels after exposure of EMT6 cells to 25 μM of drug for 1 h at 37° and 42° C and at pH 7.40 and 6.45 showed that each platinum-dye complex achieved platinum levels that were 100–600 times higher at 37° C and pH 7.40 than those obtained using CDDP. The platinum levels for each drug dropped markedly when exposure took place at pH 6.45. Exposure at 42° C only moderately increased platinum levels in cells exposed to these drugs. Thus, for several of these drugs the level of cytotoxicity observed was in great part independent of the intracellular platinum levels achieved. Pt(pyronin Y)$_2$ is an effective drug for use with hyperthermia, and further studies using this combination with and without radiation are under way.

Introduction

The combination of cisplatin (CDDP), local hyperthermia, and radiation has proved to be a practical and reasonably successful treatment for solid tumors in our clinic [10], but we feel that better agents can be developed specifically for these trimodality protocols. An ideal drug for this use would become highly cytotoxic in the presence of hyperthermia [9] and would serve as a powerful radiosensitizing agent across all the different environmentally determined regions present within tumors.

Our laboratory has been studying new complexes of the platinum tetrachlorodianion with positively charged nuclear and mitochondrial dyes [7, 8, 16, 20–24, 28], which we designed as antitumor agents for use alone or in conjunction with hyperthermia and/or radiation. In this report, we describe the effect of hyperthermia on the cytotoxicity of five complexes of the platinum tetrachlorodianion using three thiazin dyes, thionin, azure B, and methylene blue, the xanthene dye pyronin Y, and the thiazole dye thioflavin in both in vitro and in vivo systems.

Materials and methods

Drugs. Thionin, azure B, methylene blue, pyronin Y, and thioflavin were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Potassium tetrachloroplatinate was a gift from Drs. Donald H. Picker and Michael J. Abrams, Johnson Matthey, Inc. (West Chester, Pa). The platinum complexes of tetrachloroplatinate and each of the dyes were prepared in our laboratory by reaction of potassium tetrachloroplatinate with a slight molar excess of the dye at a ratio of 2 mol dye: 1 mol potassium tetrachloroplatinate in water at room temperature to form $PtCl_4(dye)_2$. The precipitated complexes were washed with ice-cold water, methanol, and

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diethyl ether, then used for experiments [1, 16]. Elemental analysis of the complexes for C, H, N, Cl, and Pt was carried out by Galbraith Laboratories (Knoxville, Tenn.) and found to be in agreement with calculated values within 0.5%. These complexes are hereafter referred to as $Pt(dye)_2$ [i.e., $Pt(thionin)_2$].

Cell culture. EMT6 mouse mammary tumor cells have been widely used for the study of hypoxia [12–14] and heat effects in vitro [3]. The EMT6 tumor cell line was originally developed by Rockwell et al. [15]. Cultures were maintained in exponential growth in Waymouth's medium (I. S. I. Corp., Chicago, Ill.) supplemented with 15% newborn calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, NY). The doubling time of these cultures, growing at 37° C in an atmosphere comprising 5% CO_2/95% air, was 16–19 h. In vitro plating efficiencies of control cultures were 65%–80%.

Heat treatments. Exponentially growing cells were exposed to a temperature of 37°, 42°, or 43° C for 1 h. Heating was accomplished in a Plexiglas water tank, with a continuous inflow and outflow system monitored by a water-temperature controller (Braun Thermomix 1460; Braun Instruments) [6]. Cells underwent heating in sealed plastic flasks (Falcon Plastics) containing 5 ml complete medium. Water temperature could be maintained at ± 0.10° C (SD).

Production of hypoxia. To produce hypoxia, the plastic flasks, containing exponentially growing monolayers in complete medium plus serum, were fitted with sterile rubber septa and exposed to a humidified, continuously flowing atmosphere of 95% N_2/5% CO_2 for 4 h at 37° C as previously reported [18, 19]. Parallel flasks were maintained in 95% air/5% CO_2. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia.

Alterations in pH. The pH of the medium was adjusted using a sodium bicarbonate ($NaHCO_3$)/5% CO_2 buffer system [4]. For medium with serum, the lowest pH that could be achieved with 5% CO_2 in the absence of $NaHCO_3$ was 6.43 ± 0.01. The reduced solubility of CO_2 at 40°–45° C increased the actual pH to 6.45 as measured by a bioprobe combination pH electrode (Orion Research, Cambridge, Mass.). For experiments, the original bicarbonate-buffered medium (pH 7.40) was replaced by media without $NaHCO_3$, and flasks were either purged with 95% air/5% CO_2 4 h before heating for normally oxygenated conditions or gassed with 95% N_2/5% CO_2 for 4 h at 37° C for hypoxic experiments, as stated above.

Drug treatments. Exponentially growing cells were exposed to varying concentrations of $Pt(thionin)_2$, $Pt(azure\ B)_2$, $Pt(methylene\ blue)_2$, $Pt(pyronin\ Y)_2$, or $Pt(thioflavin)_2$ in T-25 flasks for 1 h at 37°, 42°, or 43° C. Non-drug treated controls were handled identically. Drugs were prepared in sterile PBS immediately before use and added to the flasks in a small volume (50–100 μl). Addition of the drug solution did not significantly alter the pH of the culture.

Cell viability measurements. Cell viability was measured by the ability of single cells to form colonies in vitro, as described previously [18, 19]. Each experiment was repeated 3–5 times, and each data point per experiment represented the results of 3 different dilutions of cells plated in triplicate.

Platinum determinations. Solutions of CDDP, K_2PtCl_4, and each of the $Pt(dye)_2$ complexes were prepared in media without serum. The final concentration of all seven solutions was confirmed by flameless atomic absorption spectrophotometry [7]. EMT6 cells in exponential growth were trypsinized with a 1:1 0.25% trypsin: EDTA solution and centrifuged at 500 *g* for 4 min. The cell pellets were resuspended in media containing 15% newborn calf serum at a concentration of approximately 4×10^6 cells/ml for 1 h. Tubes containing the drugs in media were preheated in water baths at 37° and 42° C. The cells were added to the drug-containing media at each temperature and were incubated for 1 h at a concentration of 1.15×10^6 cells/ml of 25 μ*M* drug solution.

After incubation, the cells were placed on ice and washed four times with 0.9% PBS to remove extracellular drug. The final washings were determined by atomic absorption spectrophotometry to have below-detectable levels of platinum. The final cell pellet was sonicated, and the mass of intracellular platinum was determined by atomic absorption spectrophotometry.

Flameless atomic absorption spectrophotometry. By this procedure [8], platinum from a 15-μl sample injection volume was atomized from the walls of pyrolytically coated graphite tubes. A Perkin Elmer model 2380 atomic absorption spectrophotometer was used in conjunction with a Perkin Elmer model 400 graphite furnace to measure the absolute mass of platinum in the cell samples [2, 7]. Each measurement was made in triplicate in three independent experiments.

Tumor. The FSaII fibrosarcoma [11] adapted for growth in culture (FSaIIC) [17] was carried in C3H/He male mice (Jackson Laboratory, Bar Harbor, Me.). For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted intramuscularly into the legs of 8- to 10-week-old C3H/He male mice.

Tumor growth delay experiments. When the tumors were approximately 100 mm^3 in volume, treatment was initiated. In those groups receiving the drug, either CDDP (5 mg/kg), $Pt(thionin)_2$ (100 mg/kg), $Pt(azure\ B)_2$ (100 mg/kg), $Pt(methylene\ blue)_2$ (100 mg/kg), $Pt(pyronin\ Y)_2$ (100 mg/kg), or $Pt(thioflavin)_2$ (1 mg/kg) in 0.9% phosphate-buffered saline (PBS) (0.2 ml) was injected i.p. as a single dose. In the groups undergoing hyperthermia, heat was locally delivered as a single dose to the tumor-bearing limb by immersion in a specially designed Plexiglas water bath at 44° C, which enabled the centers of tumors to reach 43° ± 0.2° C as measured by a digital readout thermistor (Sensortech Inc., Clifton, NJ) placed into the center of the tumor in selected control animals as previously described [5]. No anesthetic was used. Hyperthermia was delivered immediately following i.p. injections. The progress of each tumor was measured thrice weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the number of days taken by each individual tumor to reach 500 mm^3 as compared with untreated controls. Each treatment group had seven animals, and the experiment was repeated three times. Days of tumor growth delay represent the mean ± SE for the treatment group as compared with controls.

Tumor excision assay. When the tumors were approximately 100 mm^3 in volume (about 1 week after tumor cell implantation), the animals were injected i.p. with various doses of either $Pt(thionin)_2$, $Pt(azure\ B)_2$, $Pt(methylene\ blue)_2$, or $Pt(pyronin\ Y)_2$ at 100, 250, or 500 mg/kg or with $Pt(thioflavin)_2$ at 0.5, 1, or 2 mg/kg either alone or immediately followed by hyperthermia (43° C, 30 min), as described above, to the tumor-bearing limb. Mice were sacrificed and soaked in 95% ethanol 24 h after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised under sterile conditions and single-cell suspensions were prepared as previously described [25]. The untreated tumor cell suspensions had a plating efficiency of 10%–16%. The results are expressed as the surviving fraction ± SE of cells from treated groups as compared with untreated controls [5].

Results

The structures of the three thiazin dyes thionin, azure B, and methylene blue as well as those of the xanthene dye pyronin Y and the thiazole dye thioflavin are shown in Fig. 1. The thiazin molecules represent a structural series with increasing methylation of the amino groups. Pyronin Y is the tetramethylamino xanthene, which may be compared with methylene blue, the thiazin with the same substitution pattern. Thioflavin has a five-member heterocycle similar in structure to the six-member heterocyclic ring of the thiazin molecules. These five dyes, each of which bears

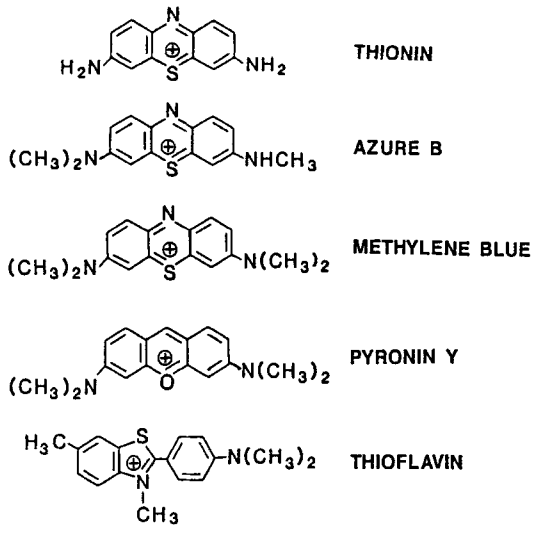

Fig. 1. Structure of the free dyes

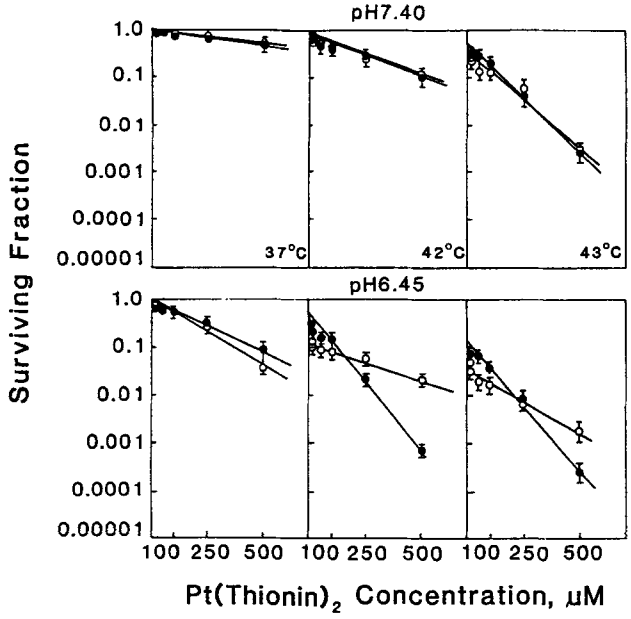

Fig. 2. Survival curve of exponentially growing, normally oxygenated (●) and hypoxic (○) EMT6 cells exposed to the concentrations of Pt(thionin)$_2$ indicated at 37°, 42°, or 43° C and at pH 7.40 and 6.45. The survival value plotted on the y-axis represents heat killing alone under the conditions indicated. Points represent the means of 3 independent determinations ± SEM (bars)

Table 1. Concentrations of the free dyes that produced 1 log of kill of EMT6 cells under the various treatment conditions

Dye	Dye concentration, μ*M*											
	pH 7.4						pH 6.45					
	37°C		42°C		43°C		37°C		42°C		43°C	
	Oxic	Hypoxic	Oxic	Hypoxic	Oxic	Hypoxic	Oxic	Hypoxic	Oxic	Hypoxic	Oxic	Hypoxic
Thionin	500	>500	500	250	250	250	500	>500	200	175	150	150
Azure B	450	>500	250	>500	250	>500	>500	>500	250	500	75	50
Methylene blue	100	260	50	125	50	75	150	120	100	120	100	110
Pyronin Y	25	75	20	75	10	50	125	150	7	20	5	7
Thioflavin	10	150	10	50	5	25	5	25	1	5	0.5	0.25
K_2PtCl_4	500	>500	400	450	200	250	>500	>500	>500	>500	200	300

Experimental details were the same as for the platinum(dye)$_2$ complexes

a diffuse, positive charge, form tight ion-pair complexes with the platinum tetrachlorodianion at a ratio of 2:1 in a manner analogous to that of rhodamine-123 [16, 20, 23, 24], fast black [5], and other positively charged dyes [16, 21, 23, 24].

At normal pH (pH 7.40) and 37°C, Pt(thionin)$_2$ is not very cytotoxic, killing only approximately 50% of normally oxygenated and 40% of hypoxic EMT6 cells on exposure to a 500 μ*M* concentration of drug for 1 h (Fig. 2). The cytotoxicity of the free dye is shown in Table 1. When the temperature during drug exposure was further elevated to 43°C at pH 7.40, there was a marked increase in the cytotoxicity of Pt(thionin)$_2$. Exposure to 500 μ*M* Pt(thionin)$_2$ for 1 h at 43°C and pH 7.40 resulted in the kill of approximately 2.5 log of EMT6 cells, whether they were normally oxygenated or hypoxic. Pt(thionin)$_2$ was more cytotoxic when the extracellular pH was reduced to pH 6.45. At pH 6.45 and 37°C, exposure to a concentration of 500 μ*M* for 1 h killed approximately 1 log of normally oxygenated EMT6 cells and approximately 1.5 logs of hypoxic EMT6 cells.

When the temperature during drug exposure was increased to 42°C, there was a marked increase in the cytotoxicity of Pt(thionin)$_2$ toward normally oxygenated EMT6 cells, whereas there was a much smaller change in the cytotoxicity of the drug toward hypoxic EMT6 cells. At

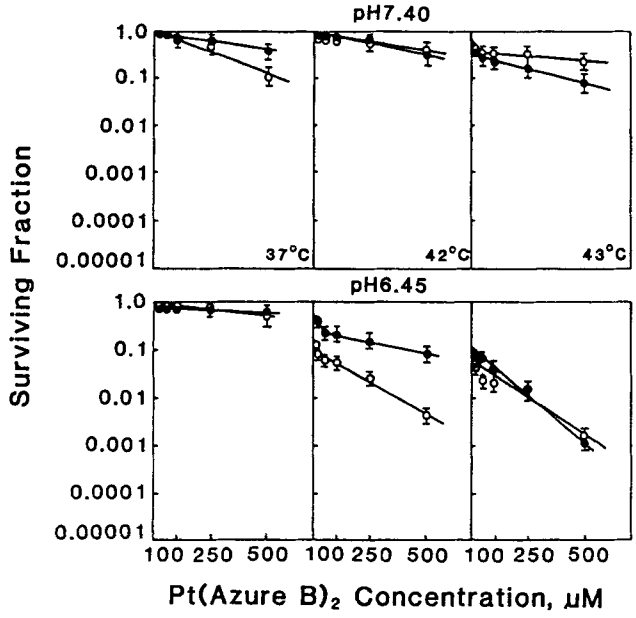

Fig. 3. Survival curve of exponentially growing, normally oxygenated (●) and hypoxic (○) EMT6 cells exposed to the concentrations of Pt(azure B)$_2$ indicated at 37°, 42°, or 43° C and at pH 7.40 and 6.45. The survival value plotted on the *y*-axis represents heat killing alone under the conditions indicated. Points represent the means of 3 independent determinations ± SEM (bars)

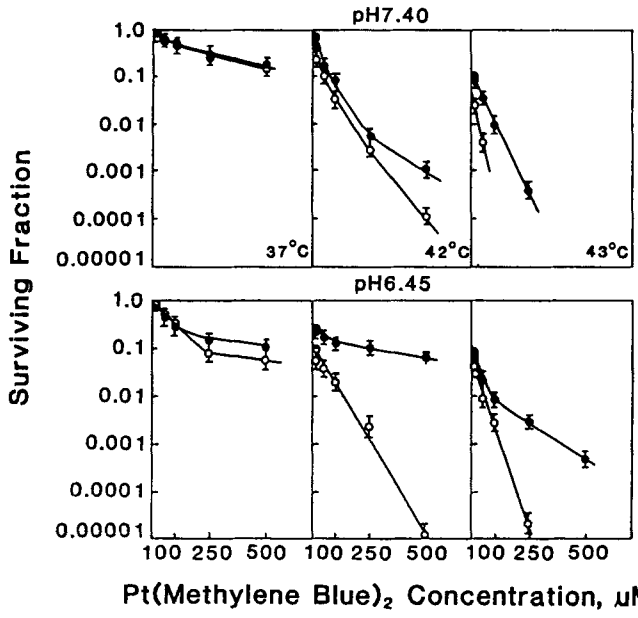

Fig. 4. Survival curve of exponentially growing, normally oxygenated (●) and hypoxic (○) EMT6 cells exposed to the concentrations of Pt(methylene blue)$_2$ indicated at 37°, 42°, or 43° C and at pH 7.40 and 6.45. The survival value plotted on the *y*-axis represents heat killing alone under the conditions indicated. Points represent the means of 3 independent determinations ± SEM (bars)

42° C and pH 6.45, exposure to 500 μ*M* Pt(thionin)$_2$ resulted in the kill of approximately 3 logs of normally oxygenated EMT6 cells and nearly 2 logs of hypoxic EMT6 cells. Increasing the temperature during drug exposure to 43° C at pH 6.45 produced about 3.5 logs of kill of normally oxygenated EMT6 cells and almost 3 logs of kill of hypoxic EMT6 cells on treatment with 500 μ*M* Pt(thionin)$_2$ for 1 h.

The cytotoxicity of Pt(azure B)$_2$ was not effectively enhanced by hyperthermia at pH 7.40 and either 42° or 43° C in normally oxygenated or hypoxic cells (Fig. 3). The killing of hypoxic EMT6 cells by 500 μ*M* Pt(azure B)$_2$ for 1 h decreased 5-fold when the temperature during drug exposure increased from 37° to 42° C at pH 7.40. Even at 43° C and pH 7.40, there was 2-fold less kill of hypoxic EMT6 cells by 500 μ*M* than was seen at 37° C with the same drug treatment. At pH 6.45 and 37° C, Pt(azure B)$_2$ was also not very cytotoxic, killing 50% of normally oxygenated or hypoxic EMT6 cells at a concentration of 500 μ*M*. Exposure to 500 μ*M* for 1 h at 42° C and pH 6.45 resulted in the kill of approximately 1 log of normally oxygenated EMT6 cells and of approximately 2.5 logs of hypoxic EMT6 cells. Increasing the temperature during drug exposure to 43° C at pH 6.45 produced almost 3 logs of kill of either normally oxygenated or hypoxic EMT6 cells treatment with 500 μ*M* Pt (azure B)$_2$ for 1 h.

At normal pH (pH 7.40) and 37° C, Pt(methylene blue)$_2$ was only moderately cytotoxic (Fig. 4). There was a marked increase in the cytotoxicity of Pt(methylene blue)$_2$ toward both normally oxygenated and hypoxic EMT6 cells at elevated temperatures. At 42° C and pH 7.40, exposure to 500 μ*M* for 1 h killed approximately 3 logs of normally oxygenated EMT6 cells and approximately 4 logs of hypoxic EMT6 cells. When the temperature during drug treatment was further increased to 43° C at pH 7.40, exposure to 250 μ*M* Pt(methylene blue)$_2$ killed 3.5 logs of normally oxygenated EMT6 cells, and exposure to 100 μ*M* killed nearly 4.5 logs of hypoxic EMT6 cells. Pt(methylene blue)$_2$ was slightly more cytotoxic at pH 6.45 and 37° C than at pH 7.40 and 37° C.

Increasing the temperature during drug exposure to 42° C at pH 6.45 produced only a small increase in the cytotoxicity of Pt(methylene blue)$_2$ in normally oxygenated EMT6 cells. However, there was a marked increase in its cytotoxicity toward hypoxic EMT6 cells, such that exposure to 500 μ*M* killed nearly 5 logs of hypoxic cells. Exposure to 500 μ*M* Pt(methylene blue)$_2$ for 1 h at 43° C and pH 6.45 killed approximately 3.5 logs of normally oxygenated EMT6 cells, whereas exposure to 250 μ*M* under the same conditions killed nearly 5 logs of hypoxic EMT6 cells. Overall, however, at 43° C Pt(methylene blue)$_2$ was more cytotoxic to cells under normal pH conditions (pH 7.40) than under acidic conditions (pH 6.45).

Pt(pyronin Y)$_2$ was moderately cytotoxic at normal pH (pH 7.40) and 37° C, killing nearly 2 logs of normally oxygenated EMT6 cells and nearly 1 log of hypoxic EMT6 cells on treatment with 500 μ*M for 1 h (Fig. 5). Exposure to 250* μ*M* Pt(pyronin Y)$_2$ at 42° C killed approximately 3 logs of normally oxygenated EMT6 cells, whereas exposure to 500 μ*M* killed approximately 2 logs of hypoxic EMT6 cells.

Increasing the temperature during drug exposure to 43° C at pH 7.40 did not significantly change the cytotoxicity of Pt(pyronin Y)$_2$ toward normally oxygenated EMT6 cells compared with that seen at 42° C but did increase the

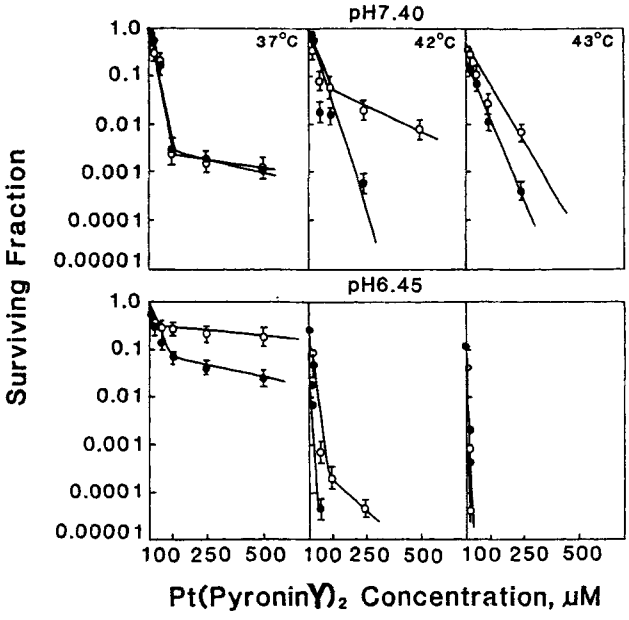

Fig. 5. Survival curve of exponentially growing, normally oxygenated (●) and hypoxic (○) EMT6 cells exposed to the concentrations of Pt(pyronin Y)$_2$ indicated at 37°, 42°, or 43° C and at pH 7.40 and 6.45. The survival value plotted on the *y*-axis represents heat killing alone under the conditions indicated. Points represent the means of 3 independent determinations ± SEM (bars)

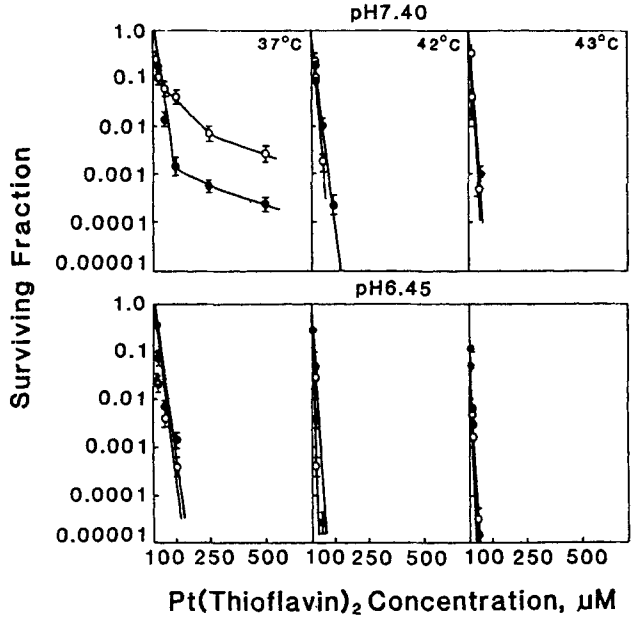

Fig. 6. Survival curve of exponentially growing, normally oxygenated (●) and hypoxic (○) EMT6 cells exposed to the concentrations of Pt(thioflavin Y)$_2$ indicated at 37°, 42°, or 43° C and at pH 7.40 and 6.45. The survival value plotted on the *y*-axis represents heat killing alone under the conditions indicated. Points represent the means of 3 independent determinations ± SEM (bars)

Table 2. Intracellular levels of platinum after exposure to various platinum complexes at 37° C or 42° C for 1 h

EMT6 cells were exposed to 25 μ*M* drug for 1 h. Platinum levels were measured by flameless atomic absorption spectrophotometry. Data are shown as ng Pt/10^6 cells ± SE

Treatment	pH 7.40		pH 6.45	
	37°C	42°C	37°C	42°C
CDDP	0.95 ± 0.13	1.17 ± 0.21	1.06 ± 0.08	1.16 ± 0.12
K_2PtCl_4	0.18 ± 0.06	0.20 ± 0.04	0.21 ± 0.11	0.24 ± 0.12
Pt(thionin)$_2$	306 ± 42	373 ± 53	280 ± 28	355 ± 43
Pt(azure B)$_2$	637 ± 121	760 ± 130	10 ± 2	17 ± 2
Pt(methylene blue)$_2$	202 ± 32	287 ± 46	176 ± 24	437 ± 51
Pt(pyronin Y)$_2$	649 ± 108	767 ± 127	102 ± 15	147 ± 22
Pt(thioflavin)$_2$	116 ± 18	124 ± 16	54 ± 13	60 ± 12

sensitivity of hypoxic EMT6 cells to this drug, such that exposure to 250 μ*M* killed nearly 2 logs of cells. At elevated temperature and pH 6.45, Pt(pyronin Y)$_2$ became markedly more cytotoxic to both normally oxygenated and hypoxic EMT6 cells. Exposure to a concentration of 50 μ*M* for 1 h at 42° C and pH 6.45 killed almost 4.5 logs of normally oxygenated EMT6 cells, whereas while exposure to a concentration of 100 μ*M* killed approximately 3.5 logs of hypoxic EMT6 cells. Increasing the temperature during drug exposure to 43° C resulted in an additional, marked increase in the cytotoxicity of Pt(pyronin Y)$_2$ toward both normally oxygenated and hypoxic EMT6 cells.

At normal pH (pH 7.40) and 37° C, Pt(thioflavin)$_2$ was more cytotoxic to normally oxygenated EMT6 cells than toward hypoxic EMT6 cells (Fig. 6). There was a 1-log differential in cell kill by Pt(thioflavin)$_2$, depending on the cellular oxygenation condition, such that 500 μ*M* killed slightly more than 3.5 logs of normally oxygenated EMT6 cells, and the same treatment killed slightly more than 2.5 logs of hypoxic EMT6 cells.

Increasing the temperature during drug exposure to 42° C had a very marked effect on the cytotoxicity of this drug toward both normally oxygenated and hypoxic EMT6 cells. Increasing the temperature during drug exposure to 43° C produced a moderate increase in the cytotoxicity of Pt(thioflavin)$_2$ toward cells under both oxygenation conditions. Exposure to 50 μ*M* for 1 h at 43° C and pH 7.40 killed approximately 3 logs of normally oxygenated EMT6 cells and approximately 3.5 logs of hypoxic EMT6 cells. At 37° C and pH 6.45, treatment with 100 μ*M* for 1 h killed almost 3 logs of normally oxygenated EMT6 cells and nearly 3.5 logs of hypoxic EMT6 cells. A concentration of 50 μ*M* Pt(thioflavin)$_2$ at 42° C and pH 6.45 killed 4.5 logs of normally oxygenated EMT6 cells in a 1-h treatment, whereas 25 μ*M* killed approximately 3.5 logs of hypoxic EMT6 under the same conditions. Increasing the tempera-

Table 3. Growth delay of the FSaIIC fibrosarcoma produced by combinations of $Pt(dye)_2$ or CDDP with heat

Treatment group	Tumor growth delay, days[a]
43° C, 30 min[b]	1.4 ± 0.7
CDDP (5 mg/kg)	4.4 ± 0.9
CDDP →Heat	5.9 ± 1.1
$Pt(thionin)_2$ (100 mg/kg)	3.1 ± 0.5
$Pt(thionin)_2$ →Heat	4.6 ± 0.7
$Pt(azure\ B)_2$ (100 mg/kg)	2.6 ± 0.5
$Pt(azure\ B)_2$ →Heat	5.6 ± 0.9
$Pt(methylene\ blue)_2$ (100 mg/kg)	3.6 ± 0.6
$Pt(methylene\ blue)_2$ →Heat	10.9 ± 1.2
$Pt(pyronin\ Y)_2$ (100 mg/kg)	2.0 ± 0.5
$Pt(pyronin\ Y)_2$ →Heat	9.5 ± 1.1
$Pt(thioflavin)_2$ (1 mg/kg)	1.3 ± 0.3
$Pt(thioflavin)_2$ →Heat	3.9 ± 0.7

[a] Tumor growth delay is the difference in the number of days for the treated tumors to reach 500 mm^3 compared with untreated control tumors. The data presented represent the means of 14 animals ± SE

[b] On day 1 of treatment, heat was locally delivered as a single dose to the tumor-bearing limb by emersion in a water bath at 44° C, which enabled the tumors to reach 43° C

Drug was injected in PBS as a single i. p. dose

ture further during drug exposure to 43° C at pH 6.45 produced only a small additional increment in the cytotoxicity of $Pt(thioflavin)_2$ toward cells under either condition of oxygenation.

In general, the cytotoxicity of the free dyes was much less effected by hyperthermic temperatures during drug exposure. Table 1 shows the dye concentrations that produced 1 log of cell kill under the various treatment conditions.

Intracellular platinum levels were measured following exposure of normally oxygenated EMT6 cells to 25 μ*M* CDDP, K_2PtCl_4, or each of the five $Pt(dye)_2$ complexes for 1 h at pH 7.40 or 6.45 and at 37° or 42° C (Table 2). After exposure to 25 μ*M* CDDP under each of the conditions tested, approximately 1 ng platinum was found in 1×10^6 EMT6 cells. There was a trend toward a slightly higher level of platinum content in the EMT6 cells after exposure of the cells to CDDP at 42° C, but this effect was not statistically significant. As has previously been described [15], platinum in the form of K_2PtCl_4 enters cells poorly. There was no difference in the amount of platinum from K_2PtCl_4 found in the EMT6 cells at either pH or at the different temperatures. At pH 7.40 and 37° C, much more platinum was found in EMT6 cells after exposure to each of the platinum-dye complexes than was found with either CDDP or K_2PtCl_4. Platinum levels from $Pt(azure\ B)_2$ or $Pt(pyronin\ Y)_2$ amounted to >600 ng/1 × 10^6 cells, whereas the lowest levels measured were from $Pt(thioflavin)_2$, at about 100 ng/1 × 10^6 cells for the same treatment.

All of these platinum complexes showed decreased levels of platinum in EMT6 cells when the extracellular pH was reduced to pH 6.45. The greatest changes occurred with $Pt(azure\ B)_2$ (a decrease of 64-fold) and $Pt(pyronin\ Y)_2$ (a decrease of 6.4-fold). The smallest changes occurred with $Pt(thionin)_2$ (a decrease of 1.1-fold) and $Pt(methylene\ blue)_2$ (a decrease of 1.15-fold), but these were not statistically significant. In all cases there was a trend toward increased levels of platinum in the cells with increased temperature (42° C) during drug exposure, but this difference only reached statistical significance with $Pt(methylene\ blue)_2$. The level of platinum obtained in 1×10^6 EMT6 cells exposed to 25 μ*M* $Pt(methylene\ blue)_2$ for 1 h at pH 6.45 increased about 2.5-fold from 176 ng at 37° C to 437 ng at 42° C ($P<0.01$).

The potential of each of the $Pt(dye)_2$ complexes as antitumor agents alone and in combination with local hyperthermia was tested in the FSaIIC fibrosarcoma by assessment of tumor growth delay (Table 3) and tumor cell survival (Fig. 7, 8). Tumor growth delay was measured after a single dose of drug given alone or immediately followed by hyperthermia (43° C, 30 min) locally to the tumor-bearing limb (Table 3). The hyperthermia treatment itself had only a small effect on tumor growth delay (1.4 days). CDDP (5 mg/kg) produced a growth delay of 4.4 days; with hyperthermia, the growth delay produced by CDDP increased to 5.9 days. A dose of 100 mg/kg was selected for initial single-dose testing of the $Pt(dye)_2$ com-

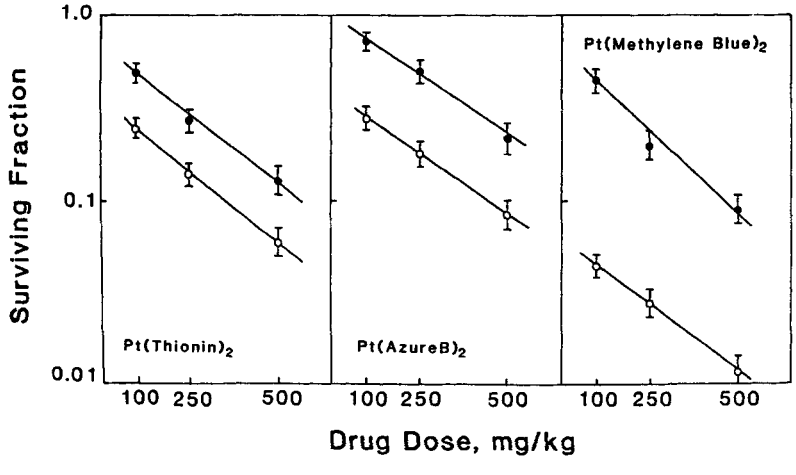

Fig. 7. Survival curve of FSaIIC cells from FSaIIC tumors treated with various doses of $Pt(thionin)_2$, $Pt(azure\ B)_2$, or $Pt(methylene\ blue)_2$ (●) with hyperthermia (43°C, 30 min) delivered immediately after drug administration or (○) without hyperthermia. The survival value for hyperthermia alone was 0.65 ± 0.05. The data represent the mean of 3 independent experiments (± SEM)

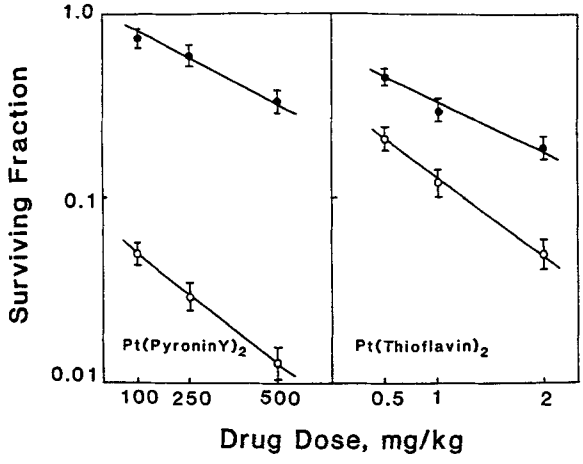

Fig. 8. Survival curve of FSaIIC cells from FSaIIC tumors treated with various doses of Pt(pyronin Y)$_2$, or Pt(thioflavin)$_2$ (●) with hyperthermia (43°C, 30 min) delivered immediately after drug administration or (○) without hyperthermia. The survival value for hyperthermia alone was 0.65 ± 0.05. The data represent the mean of 3 independent experiments ($\pm$ SEM)

plexes in vivo. Four of these complexes were well tolerated at this dose, but the maximum tolerated dose of Pt(thioflavin)$_2$ was 1 mg/kg. Among the platinum-thiazin complexes, the enhancement in tumor growth delay increased with increasing level of methylation of the dye molecule. The greatest enhancement in tumor growth delay was seen with the drug Pt(pyronin Y)$_2$, which produced little tumor growth delay itself (2 days), and was increased about 4.8-fold to 9.5 days with the addition of local hyperthermia to treatment with the drug. Treatment with Pt(thioflavin)$_2$ at the dose of 1 mg/kg was enhanced 3.0-fold when drug administration was followed by hyperthermia.

The survival of FSaIIC tumor cells treated with the platinum-thiazin complexes alone or followed by treatment with hyperthermia is shown in Fig. 7. The tumor cell survival due to hyperthermia treatment alone was 0.65 ± 0.05. For each of these three drugs, the addition of hyperthermia to treatment with the drug produced an additional aliquot of tumor cell kill; that is, the tumor cell survival curves for the drug alone and the drug plus hyperthermia were essentially parallel. The pattern for increased tumor cell kill by the platinum-thiazin complexes in combination with hyperthermia was the same as that for tumor growth delay. Tumor cell kill by Pt(thionin)$_2$, Pt(azure B)$_2$ and Pt(methylene blue)$_2$ was increased about 1.9-fold, 2.6-fold and 9-fold, respectively, by the addition of hyperthermia to the drug treatment.

On the other hand, there was evidence of dose modification of Pt(pyronin Y)$_2$ and Pt(thioflavin)$_2$ by hyperthermia (Fig. 8). The tumor cell kill by Pt(pyronin Y)$_2$ at a dose of 100 mg/kg was enhanced about 14.6-fold by hyperthermia, whereas at a dose of 500 mg/kg Pt(pyronin Y)$_2$, tumor cell kill was enhanced 21.3-fold. Tumor cell kill by Pt(thioflavin)$_2$ at a dose of 0.5 mg/kg was enhanced about 2.1-fold by the addition of hyperthermia to drug treatment, whereas at the higher dose of 2 mg/kg Pt(thioflavin)$_2$, hyperthermia enhanced tumor cell kill about 3.8-fold. Overall, in the tumor-cell survival assay, Pt(pyronin Y)$_2$ showed the greatest enhancement of tumor cell kill by hyperthermia.

Discussion

Various physiological environments exist in solid tumor masses, which may effect the metabolic status of tumor cells and the actions of antitumor agents [9, 25, 26]. We are searching for drugs capable of becoming very cytotoxic in conjunction with hyperthermia across various environmental conditions. Thionin, azure B and methylene blue form a series of thiazin dyes with increasing methylation. Within the series of platinum-dye complexes formed by these three molecules, there is a trend toward increasing cytotoxicity to hypoxic EMT6 cells at acidic pH (pH 6.45) and hyperthermic temperatures with increasing methylation of the dye molecule. There is also a trend within this series of three complexes toward increasing cytotoxicity to normally oxygenated EMT6 cells at normal pH (pH 7.40) and elevated temperatures with increasing methylation of the dye molecule. Therefore, in vitro studies would predict that Pt(methylene blue)$_2$ would be the most effective of the Pt(thiazin)$_2$ complexes in vivo. This prediction was borne out in both tumor-growth delay and tumor-cell survival studies where the enhancement of hyperthermia on the effect of these drugs was observed with Pt(methylene blue)$_2$. It is interesting to note that Wang et al. [27] also found a positive interaction between methylene blue and hyperthermia.

The xanthene dye Pyronin Y is structurally most closely related to the thiazin dye methylene blue. The enhancement in cytotoxicity toward hypoxic EMT6 cells by Pt(pyronin Y)$_2$ at acidic pH (pH 6.45) with hyperthermia was even greater than that seen with Pt(methylene blue)$_2$ under the same conditions. The enhancement produced by hyperthermia of the cytotoxicity of Pt(pyronin Y)$_2$ toward normally oxygenated EMT6 cells at normal pH (pH 7.40) was at least as large as the effect of hyperthermia on the cytotoxicity of Pt(methylene blue)$_2$ toward EMT6 cells under the same conditions. In vivo, Pt(pyronin Y)$_2$ showed the largest differential enhancement of any of the drugs examined in this study, both in the tumor-growth delay assay and in the tumor-cell survival assay.

The platinum complex of the thiazole dye, thioflavin, was the most cytotoxic of the agents examined in this study. At normal pH (pH 7.40), the cytotoxicity of Pt(thioflavin)$_2$ showed a marked enhancement with hyperthermia, and, at acidic pH (pH 6.45), this dye complex was considerably cytotoxic under all of the conditions tested. In vivo, it was markedly more toxic than the other platinum complexes examined in this study; however, even at a dose of 1 mg/kg, there was a 3-fold enhancement in the tumor growth delay produced by this drug by hyperthermia.

One of the rationales for preparing these complexes was that by associating the negatively charged $PtCl_4$ with positively charged nuclear dyes, a neutral species would be formed that would enable more platinum to gain entrance into the cell. The data in Table 1 indicate that at normal pH

(pH 7.40) and temperature (37° C), about 100- to 600-fold more platinum enters cells in the form of these platinum-dye complexes than as CDDP at the same concentration and time of exposure. The platinum-dye complexes, however, are not as cytotoxic as CDDP, indicating that the platinum in this form is not as active or accessible in acting on critical intracellular target molecules.

In the series of thiazin-containing platinum-dye complexes at normal pH, the highest levels of platinum in cells were found with Pt(azure B)$_2$. Although it was not the most effective agent in this group with hyperthermia, Pt(azure B)$_2$ was the most effective radiosensitizer among this group of complexes, both in vitro [dose modifying factor (DMF)2.5 at a concentration of 100 μM] in hypoxic EMT6 cells and in vivo (DMF 2.1 at a dose of 100 mg/kg) in the FSaIIC fibrosarcoma [24]. Pt(thionin)$_2$ was also an effective radiosensitizer, but Pt(methylene blue)$_2$ was not [9]. Pt(pyronin Y)$_2$, which produced platinum levels in the cells comparable with those of Pt(azure B)$_2$, was a moderately effective radiosensitizer of hypoxic EMT6 cells in vitro (DMF 1.5 at a concentration of 100 μM) and, at the dose of 100 mg/kg, was also a moderately effective radiosensitizer of the FSaIIC fibrosarcoma in vivo (DMF 1.5) [24]. The platinum levels achieved in EMT6 cells with several of these agents decreased markedly when the extracellular pH was reduced to pH 6.45. In general, however, there was no corresponding decrease in cytotoxicity. The radiosensitizing potential of these drugs at pH 6.45 is currently under study.

In conclusion, of the five platinum-dye complexes examined in this study, Pt(pyronin Y)$_2$ appears to have the most favorable properties for use in combined modality therapy because it showed a marked increase in cytotoxicity, tumor growth delay, and tumor cell kill in combination with hyperthermia and was also a moderately effective radiosensitizer in vitro and in vivo [24]. Further studies are now under way with this drug.

References

1. Abrams MJ, Picker DH, Fackler PH, Lock CJL, Howard-Lock HE, Faggiani R, Teicher BA, Richmond RC (1986) The synthesis and structure of [(rhodamine 123)$_2$PtCl$_4$] · 4H$_2$O: the first tetrachloroplatinate(II) salt with anti-cancer activity. Inorg Chem 25: 3980
2. Drummer OH, Proudfoot A, Howes L, Louis WJ (1984) High-performance liquid chromatography determination of platinum(II) in plasma ultrafiltrate and urine: comparison with a flameless atomic absorption spectrophotometric method. Clin Chim Acta 136: 65
3. Gerner EW, Holmes PW, McCullough JA (1979) Influence of growth state on several thermal responses of EMT6/Az tumor cells in vitro. Cancer Res 39: 981
4. Gerweck LE (1977) Modification of cell lethality at elevated temperatures: the pH effect. Radiat Res 70: 224
5. Herman TS, Teicher BA (1988) Sequencing of trimodality therapy [*cis*-diamminedichloroplatinum(II)/hyperthermia/radiation] as determined by tumor growth delay and tumor cell survival in the FSaIIC fibrosarcoma. Cancer Res 48: 2693
6. Herman TS, Sweets CC, White DM, Gerner EW (1982) Effect of heating on lethality due to hyperthermia and selected chemotherapeutic drugs. J Natl Cancer Inst 68: 487
7. Herman TS, Teicher BA, Cathcart KNS, Kaufmann ME, Lee JB, Lee M (1988) Effect of hyperthermia on *cis*-diamminedichloroplatinum(II) and Pt(Rh-123)$_2$ in a human squamous carcinoma cell line and a *cis*-diamminedichloroplatinum(II) resistant subline. Cancer Res 48: 5101
8. Herman TS, Teicher BA, Chan V, Collins LS, Kaufmann ME, Loh C (1988) The effect of hyperthermia on the action of *cis*-diamminedichloroplatinum(II),rhodamine-123$_2$[tetrachloroplatinum(II)], rhodamine-123 and potassium tetrachloroplatinum in vitro and in vivo. Cancer Res 48: 2335
9. Herman TS, Teicher BA, Jochelson M, Clark J, Svensson G, Coleman CN (1988) Rationale for the use of hyperthermia with radiation therapy and selected anticancer drugs in locally advanced human malignancies. Int J Hyperthermia 4: 143
10. Herman TS, Jochelson MS, Teicher BA, Scott PJ, Hansen J, Clark JR, Gelwan LE, Molnar-Griffin BJ, Fraser SM, Svennson G, Bornstein BA, Coleman CN (1989) A phase I–II trial of cisplatin, hyperthermia and radiation in patients with locally advanced malignancies. Int J Radiat Oncol Biol Phys 17: 1273
11. Rice L, Urano M, Suit HD (1980) The radiosensitivity of a murine fibrosarcoma as measured by three cell survival assays. Br J Cancer 41 [Suppl 4]: 240
12. Rockwell S (1977) In vivo-in vitro tumor systems: new models for studying the response of tumors to therapy. Lab Anim Sci 27: 831
13. Rockwell S (1978) Cytotoxic and radiosensitizing effects of hypoxic cell sensitizers on EMT6 mouse mammary tumor cells in vivo and in vitro. Br J Cancer 37: 212
14. Rockwell S, Kallman RF (1973) Cellular radiosensitivity and tumor radiation response in the EMT6 tumor cell system. Radiat Res 53: 281
15. Rockwell SC, Kallman RF, Fajardo LF (1972) Characteristics of serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative. J Natl Cancer Inst 49: 735
16. Teicher BA, Holden SA (1987) Antitumor and radiosensitizing activity of several platinum-positively charged dye complexes. Radiat Res 109: 58
17. Teicher BA, Rose CM (1984) Perfluorochemical emulsion can increase tumor radiosensitivity. Science 223: 934
18. Teicher BA, Lazo JS, Sartorelli AC (1981) Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Res 41: 73
19. Teicher BA, Rockwell S, Lee JB (1985) Radiosensitivity by nitroaromatic Pt(II) complexes. Int J Radiat Oncol Biol Phys 11: 937
20. Teicher BA, Holden SA, Cathcart KNS (1987) Efficacy of Pt(Rh-123)$_2$ as a radiosensitizer with fractionated X-rays. Int J Radiat Oncol Biol Phys 13: 1217
21. Teicher BA, Herman TS, Kaufmann ME (1989) PtCl$_4$(Nile blue)$_2$ and PtCl$_4$(neutral red)$_2$: DNA interaction, cytotoxicity and radiosensitization. Radiat Res 120: 129
22. Teicher BA, Herman TS, Kaufmann ME (1989) Interaction of PtCl$_4$(fast black)$_2$ with superhelical DNA and with radiation in vitro and in vivo. Radiat Res 119: 134
23. Teicher BA, Herman TS, Kaufmann ME (1989) Platinum complexes of triaminotriphenylmethanes: interaction with DNA and radiosensitization. Cancer Lett 47: 217
24. Teicher BA, Herman TS, Kaufmann ME (1989) Cytotoxicity, radiosensitization and DNA interaction of platinum complexes of thiazin and xanthene dyes. Radiat Res 121: 187
25. Vaupel P, Frinak S, Bicher HI (1981) Heterogeneous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. Cancer Res 41: 2008
26. Vaupel P, Fortmeyer HP, Runkel S, Kallinowski F (1987) Blood flow, oxygen consumption, and tissue oxygenation of human breast cancer xenografts in nude rats. Cancer Res 47: 3496
27. Wang H, Shah V, Lanks KW (1987) Use of oxidizing dyes in combination with 2-cyanocinnamic acid to enhance hyperthermic cytotoxicity in L929 cells. Cancer Res 47: 3341
28. Wang Y, Herman TS, Teicher BA (1988) Platinum-dye complexes inhibit repair of potentially lethal damage following bleomycin treatment. Br J Cancer 59: 722