Cancer Chemother Pharmacol (1986) 16: 269–272

Cancer Chemotherapy and Pharmacology

In vivo toxicity of phenothiazines to cells of a transplantable tumor*

Shirley Lehnert

Department of Radiation Oncology, Montreal General Hospital, 1650 Cedar Ave, Montreal H3G, 1A4, Canada

Summary. The toxicities of three phenothiazines, promazine, chlorpromazine, and trifluoperazine, towards cells of a mouse fibrosarcoma were quantified by means of an in vitro assay of clonogenic cell survival.

For all three drugs cell kill was proportional to the amount of drug injected. Following injection of equimolar (0.2 m*M*/kg) amounts, cell survival was progressively reduced for a period of at least 48 h. On the basis of cell survival at 48 h after administration the ranking of the drugs for cytotoxicity, in ascending order, was trifluoperazine, chlorpromazine, promazine.

A period of acute hypoxia prior to processing of the tumor did not enhance the toxicity of any of the drugs, and no change in the size of the hypoxic fraction of the tumor was seen 24 h after the injection of chlorpromazine. On this basis it was concluded that there was no evidence of enhanced toxicity of drugs for either chronically or acutely hypoxic tumor cells.

A reduction in the number of clonogenic tumor cells per gram of tumor was largely the result of a fall in the number of viable cells recovered from the tumor. The plating efficiency of surviving cells remained constant or was only slightly depressed.

Introduction

The phenothiazine neuroleptics, of which chlorpromazine is the protoype, are widely used for their antipsychotic, tranquillizing and antiemetic effects. A number of reports have also described cytotoxic and radiosensitizing activities of chlorpromazine which, in some cases, were specific to hypoxic cells. Thus for bacterial systems, chlorpromazine (0.1 m*M*) has been found to be toxic to hypoxic. *E. coli* B/r but not to cells exposed to the drug under aerated conditions [19]. In mammalian cells, Lehnert [16] used an in vitro screening system to determine the relationship between the chemical structures of a number of phenothiazines and their activity as cytotoxic and radiosensitizing agents. It was found that factors influencing the degree of interaction of each drug with the cell membrane also influenced its cytotoxic and radiosensitizing activity. The toxicity of some of the drugs was enhanced under hypoxic conditions.

In terms of antitumor activity, chlorpromazine has been reported to be toxic to sarcoma 180A of mice growing as a solid tumor but not as the ascites form of the same tumor [20]. Belkin and Hardy [3] found that a high dose of chlorpromazine inhibited the growth of a transplantable sarcoma in mice, and Kruger [16] found that chlorpromazine increased the survival time of leukemic mice. George et al. [11] reported that injection of chlorpromazine directly into a mouse fibrosarcoma delayed growth of the tumor and that the period of growth delay was increased if the tumor was rendered hypoxic by clamping.

The reported specificity of the cytotoxic and radiosensitizing effect of chlorpromazine for hypoxic cells is of particular interest, since hypoxia is known to protect cells against the cytotoxic effects of radiation [12] and of cell-cycle-dependent chemotherapeutic agents [2]. Many solid tumors are characterized by the presence of a significant fraction of hypoxic cells [21], and the desirability of developing drugs which specifically target or sensitize hypoxic cells has long been recognized [1].

The aims of this investigation were, first, to quantitate, on the basis of single cell survival, the cytotoxicity of phenothiazines to a solid tumor system treated in vivo; second, to use the assay system to compare the effects of three readily available phenothiazines; and third, to determine whether the drugs are selective for hypoxic tumor cells.

Materials and methods

Animals and tumors. The KHT fibrosarcoma used in these experiments was grown in male C3H mice weighing approximately 25 g. Tumors were implanted IM by injection of 2.5×10^5 cells in a volume of 0.05 ml into the distal portion of the gastrocnemius muscle of one hind leg. Tumors were treated at 8–9 days after implantation, when the tumor was 0.45–0.55 g. Tumor weights were determined as previously described [17].

Cell survival assay. At various times after drug treatment, tumor-bearing mice were sacrificed by cervical fracture or nitrogen asphyxiation, tumors excised, and a single-cell suspension prepared by the enzymic method of Thomson and Rauth [22]. The number of viable cells per gram of tu-

* The investigations reported in this paper were supported by the National Cancer Institute of Canada and by the Cancer Research Society Inc. of Montreal

Offprint requests to: S. Lehnert

Table 1. Phenothiazines

Name	R_1	R_2	Mean lethal dose
Promazine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	–H	225 mg/kg, IP [9]
Chlor-promazine	$-CH_2-CH_2-CH_2-N(CH_3)_2$	–Cl	120 mg/kg, IP 260 mg/kg PO [4]
Trifluo-perazine	$-CH_2-CH_2-CH_2-N$ (piperazine) $-CH_2$	$-CF_3$	424 mg/kg PO [8]

Table 2. Surviving fraction of clonogenic cells at 48 h after drug administration

	Drug Concentration (m*M*)		
	0.10	0.20	0.40
Promazine	0.16	0.017	0.0025
Chlorpromazine	0.36	0.090	0.040
Trifluoperazine	0.37	0.190	–

Surviving fraction was calculated from the mean of two or more determinations at each dose level. Control value for clonogenic cells per gram was $1.5 \pm 0.37 \times 10^6$ (mean ± SEM), calculated from values obtained for ten tumors the same size as those in the drug-treated groups

mor was determined by hemocytometer counts of Trypan-Blue-excluding cells. Appropriate numbers of cells were plated in semisolid medium for assay of colony-forming ability by the in vitro agar plating procedure originally described by Thomson and Rauth [22] and modified by Hill et al. [13]. Clonogenic cells per gram of tumor were calculated as the product of the number of viable cells per gram of tumor and the plating efficiency of those cells. The surviving fraction of clonogenic cells was the ratio of clonogenic cells per gram for treated tumors to clonogenic cells per gram for control tumors of the same size processed in parallel with the drug-treated group.

Estimation of the hypoxic fraction. The size of the hypoxic fraction was determined by comparison of the survival curves of tumors irradiated in situ in air-breathing and nitrogen-asphyxiated mice, a method originally described by Van Putten and Kallman [23]. Straight lines were fitted to data points by linear regression analysis for radiation doses between 15 and 30 Gy plotted on log linear coordinates. The hypoxic fractions were calculated from the displacement of the terminal portions of the survival curves for air-breathing and nitrogen-asphyxiated animals.

Drugs. Details of the drugs used in this study are shown in Table 1. They were selected on the basis of availibility, wide clinical use, and range of pharmacological activity. Drugs were dissolved in saline for IP injection at a concentration ensuring that the volume injected was always 0.1 ml. Control mice received injections of saline, and their tumors were processed in parallel with those in drug-treated groups.

Chlorpromazine and trifluoperazine were obtained from Sigma Chemical Co. and Promazine, from Wyeth Ltd.

Results

Table 2 shows the effect of injections of various concentrations of the three drugs on the surviving fraction of clonogenic cells per gram of tumor. Tumors were assayed 48 h after drug injection. For all three drugs the reduction in clonogenic cells was proportional to the concentration of drug injected over the range of dose levels studied. A concentration of 0.2 m*M*/kg was chosen for a study of the effects of equimolar quantities of the three drugs on tumor cell survival at various times after injection. This dose fell well below the (MLD) for all three drugs, but was within the range of drug dosage where tumor cell response was proportional to amount injected.

Figure 1 shows changes in numbers of viable cells recovered per gram of tumor, plating efficiency, and clonogenic cells per gram for KHT tumors at various times after injection of equimolar amounts of promazine, chlorpromazine or trifluoperazine. Tumors were excised and processed immediately after sacrifice by cervical fracture (aerated) or were processed approximately 15 min after nitrogen asphyxiation, i.e., after a period of hypoxia. It had been found in previous experiments with a cytotoxic reserpine derivative that a brief period of hypoxia prior to removal of the tumor significantly reduced the surviving fraction of clonogenic cells [17]. No reduction in the surviving fraction of phenothiazine-treated cells was induced by a period of acute hypoxia, and the data points representing survival of aerated and hypoxic cells fell on the same curve for all three drugs studied.

The yield of clonogenic cells per gram of tumor is the product of the number of viable cells per gram of tumor and the plating efficiency of those cells. It is apparent that the reduction in the number of clonogenic cells per gram resulted largely from a reduction in the number of cells recovered from the tumor, while the plating efficiency of these cells remains virtually constant (promazine, trifluoperazine) or declines only slightly (chlorpromazine). The number of clonogenic cells per gram of tumor continued to fall for at least 48 h after drug administration. In terms of the fraction of clonogenic cells surviving 48 h after injection the three drugs can be ranked in increasing order of toxicity as trifluoperazine, chlorpromazine, and promazine (surviving fractions of 0.19, 0,06, and 0.016 respectively.

As noted above, no difference was seen between the response of aerated cells and that of cells exposed to a brief period of acute hypoxia prior to processing of the tumor. However, in the experiment concerned it is possible that the period of hypoxia was not sufficiently long to cause significant sensitization of the cells. To determine the effect of a prolonged period of hypoxia on drug toxicity an experiment was performed in which the size of the fraction of chronically hypoxic cells in the tumor was measured in

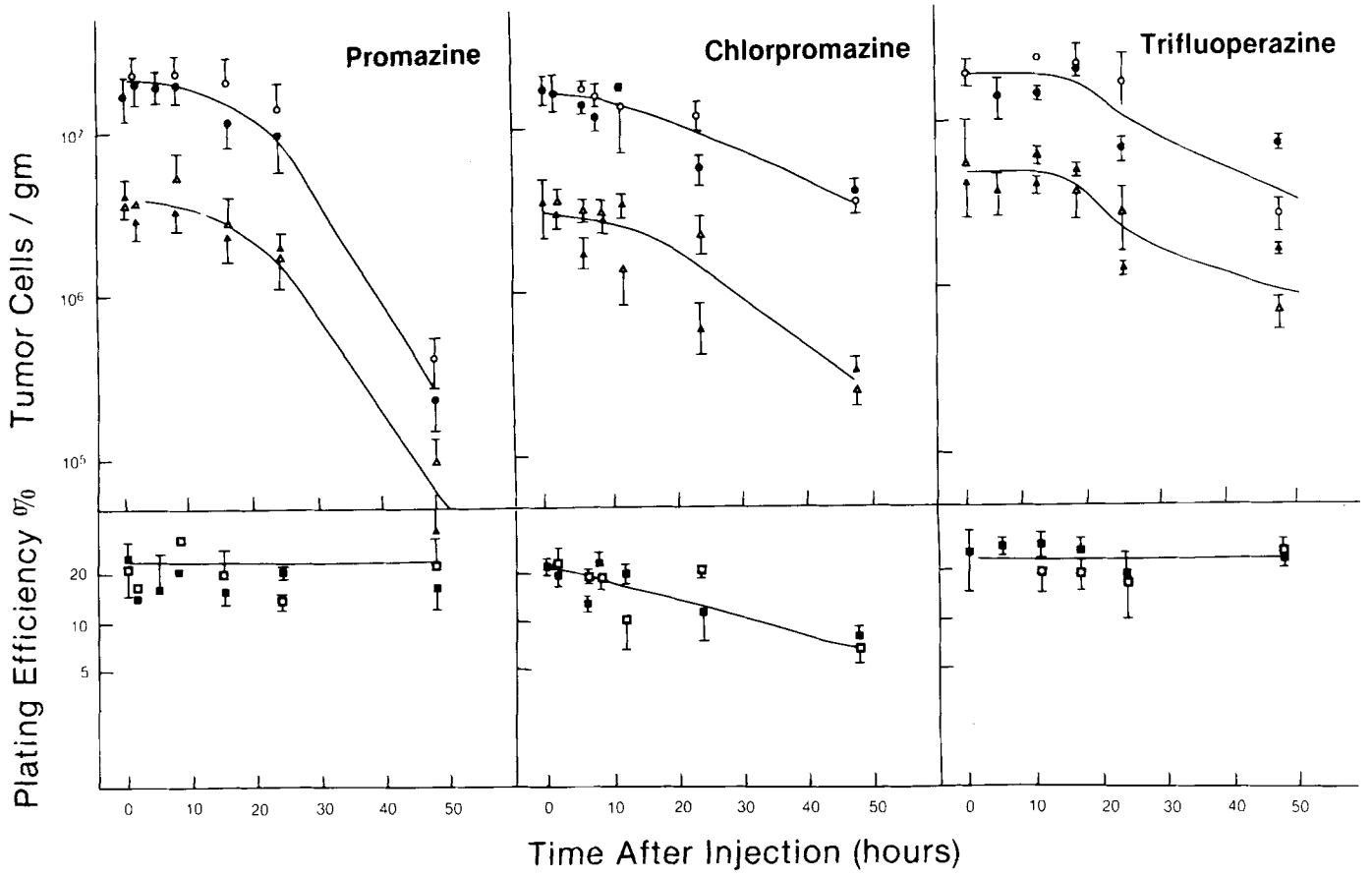

Fig. 1. Cells recovered per gram of tumor (○———○), plating efficiency (□———□), and clonogenic cells per gram of tumor (———) for KHT tumors at various times after injection of promazine, chlorpromazine of trifluoperazine. *Open symbols* (○, □, △) represent data from mice sacrificed by cerical fracture whose tumors were processed immediate after *Closed symbols* (●, ■, ▲), data from mice sacrificed by nitrogen asphyxiation 15 min before removal and processing of the tumor. Each *data point* represents the mean of values from 3–4 mice. *Bars*, SEM

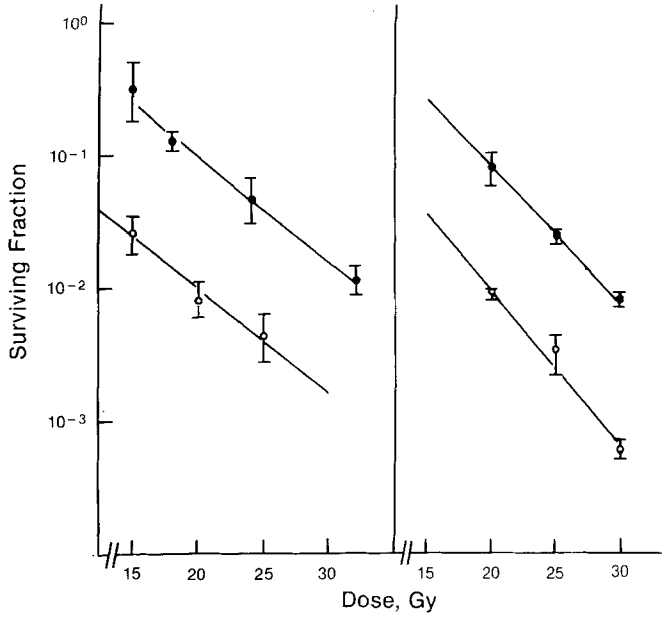

Fig. 2. Radiation survival curves for KHT tumor cells irradiated in situ and assayed in vitro. ○———○, mice irradiated while air breathing: ●———●, mice 15 min after sacrifice by nitrogen asphyxiation. Each *data point* represents mean of 3–4 assays; *bars*, SEM, *Panel A*, saline-injected mice; *Panel B*, 24 h after injection of chlorpromazine

control and drug-treated tumors 24 h after administration of chlorpromazine. Results of this experiment are shown in Fig. 2. Calculation of the size of the hypoxic fraction from the ratio of radiation survivals of aerated and hypoxic cells gave values of 11% for control tumor and 10% for experimental tumors at 24 h after chlorpromazine injection. If aerated and hypoxic cells had different sensitivities to chlorpromazine, prolonged exposure of a mixed tumor cell population to the drug would have resulted in a change in size of the hypoxic fraction of the tumor. Thus, on the basis of the results of this experiment it can be concluded that the toxicity of chlorpromazine towards cells of the KHT tumor is not enhanced under conditions of chronic hypoxia.

Discussion

The antitumor effect of chlorpromazine has been reported elsewhere [3, 11, 15, 20]. The present paper describes results of the first investigation in which toxicity was quantitated on the basis of single cell survival and the effects of several phenothiazines were compared.

All three drugs tested substantially reduced the surviving fraction of clonogenic cells, the degree of the reduction excluding the possibility that toxicity could have been confined to hypoxic cells. In fact, it was not possible to demonstrate that any of the drugs showed enhancement of

toxicity under hypoxic conditions. This observation is in contrast to findings made using an in vitro screening system of cytotoxicity [16]. In this system, while no difference was seen between the toxicity of promazine towards aerated and hypoxic cells, hypoxia produced some enhancement of the toxic effect of chlorpromazine and trifluoperazine. Results of in vivo studies also differed from in vitro findings in terms of the relative toxicity of the drugs studied. In the in vitro system promazine was the least toxic drug and trifluoperazine, the most effective.

The findings were also at variance with a previous report of the effect of chlorpromazine on a solid tumor treated in vivo, where cells were found to be sensitized to the drug by hypoxia [11]. In that case, however, the drug was injected directly into a clamped tumor, a situation not comparable with the more conventional method of drug administration used in this study.

For all three drugs studied it is apparent that a fall in the number of viable cells recovered from the tumor is the major factor responsible for the reduction in the number of clonogenic cells, and the proliferative capacity of surviving cells remains constant or is only slightly depressed. Cytotoxic agents whose primary target is DNA (e.g., ionizing radiation, adriamycin) cause an immediate loss of the proliferative potential of the cell but produce no rapid change in visible indices of viability, such as ability to exclude Trypan Blue or resist lysis. Thus, it seems unlikely that the genetic material of the cell is the major site of action of the phenothiazines.

For all three drugs, tumor cell numbers in treated mice continued to fall for at least 24 h after drug injection. This prolonged period over which the effects of the drugs persist may reflect a slow rate of clearance of the drug from the tumor; however, apart from some studies on the concentration of chlorpromazine by melanomas [6], little is known of the rate of accumulation and clearance of phenothiazines by tumor tissue.

The clinical use of phenothiazine analogs is widespread and intensive. Several authors have commented on the possible effect of the prolonged use of chlorpromazine by patients with certain types of mental disorder on the incidence and response to treatment of malignant disease when it occurs among these patients [7, 21]. Results reported here indicate that the cytotoxic activity of the phenothiazines is not confined to chlorpromazine, but is characteristic of other widely prescribed phenothiazines.

In these experiments a relatively high concentration of drug was used to produce the observed level of tumor cell kill, and no specificity was demonstrable for hypoxic cells. In contrast, it has been reported that relatively low concentrations of chlorpromazine are effective in sensitizing hypoxic cells to radiation [4], chemotherapy [14] and hyperthermia [10]. These findings suggest that while phenothiazines do show a significant degree of cytotoxic activity when used alone, the cytotoxic potential of these drugs would be best exploited in combination with other treatment modalities.

Acknowledgement. I am indebted to David Ivancic for expert technical asistance.

References

1. Adams GE, Dewey DL (1963) Hydrated electrons and radiobiological sensitization. Biochem Biophys Res Commun 12: 473
2. Allison AC, Nunn JF (1968) Effects of general anesthetics on microtubules. A possible mechanism of anesthesia. Lancet II: 1326
3. Belkin M, Hardy WG (1957) Effect of chlorpromazine on Sarcoma 37. Science 125: 233
4. Bertsche U (1984) Induction and repair of X-ray damage in mammalian cell cultures treated with membrane active drugs. Br J Cancer 49 [Suppl VI]: 121
5. Buckley JP, Steenburg ML, Berry MH, Manian A (1973) Pharmacology of mono and disubstituted chlorpromazine metabolites. J Pharm Sci 62: 715
6. Fairchild RG, Greenberg D, Watts KP (1982) Chlorpromazine distribution in hamsters and mice bearing transplantable melanoma. Cancer Res 42: 556
7. Forrest JS, Forrest FM (1974) Letter to the Editor. Aggressologie 15: 34
8. Fowler PJ, Zirkle CL, Macko E (1977) Pharmacological evolution of Clomacran, a new potent psychotropic agent. Arzneimittelforsch 27: 866
9. Friebl H, Fick H, Reichle C (1954) Beziehungen zwischen der chemischen Konstitution und der pharmakologischen Wirkung einiger Phenothiazinderivate. Arzneimittelforsch 4: 171
10. George KC, Singh BB (1982) Synergism of chlorpromazine and hyperthermia in two mice solid tumours. Br J Cancer 45: 309
11. George KC, Srinavasan VT, Singh PB (1980) Cytotoxic effect of chlorpromazine and its interaction with radiation on a mouse fibrosarcoma. Int J Radiat Biol 38: 661
12. Gray LH, Conger AD, Ebert M (1953) The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 26: 638
13. Hill RP, Ng R, Warren BF, Bush RS (1979) Effects of intercellular contact on the radiation sensitivity of KHT sarcoma cells. Radiat Res 77: 182
14. Kanzawa F, Hoshi A, Kuretani K (1970) Relationship between antitumor activity and chemical structure in psychotropic agents. Gann 61: 529
15. Kruger S, Robinson GA, Schueler FW (1960) Antileukemic activity of rauwolfia alkaloids. Arch Intern Pharmacodyn 129: 125
16. Lehnert S (1983) A comparative study of the cytotoxic and radiosensitizing effect of phenothiazines towards aerated and hypoxic mammalian cells. Proceedings of the 7th International Conference on Radiation Research B6.
17. Lehnert S (1984) Interaction of diethylaminoreserpine with cells of a transplantable tumour in vivo. Br J Cancer 50: 847
18. Rassidakis NC, Kelepouris M, Goulis K, Kariossefidis K (1973) On the incidence of malignancy among schizophrenic patients. Aggressologie 14: 269
19. Shenoy MA, Singh BB (1980) Hypoxic cytotoxicity of chlorpromazine and the modification of response of *E. coli* B/r. Int J Radiat Biol 28: 519
20. Shenoy MA, Singh BB (1980) Cytotoxic and radiosensitizing effects of chlorpromazine hydrochloride in Sarcoma 180A. Ind J Exp Biol 18: 791
21. Thomlinson RH, Gray LH (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer 9: 539
22. Thomson JE, Rauth AM (1974) An in vitro assay to measure the viability of KHT tumor cells not previously exposed to culture conditions. Radiat Res 58: 262
23. Van Putten LM, Kallman RF (1968) Oxygenation status of a transplantable tumor during fractionated radiotherapy. J Natl Cancer Inst 40: 441

Received March 1, 1985/Accepted August 19, 1985