The Effect of Phenobarbital on Cyclophosphamide Antitumor Activity¹

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SUMMARY

We have used the spleen colony assay system and survival duration studies in male DBA/2 mice with P388 leukemia to study the effects of microsomal enzyme induction by phenobarbital on the antileukemic activity and bone marrow toxicity of cyclophosphamide. Phenobarbital drinking water (0.5 mg/ml) was given for 7 days prior to cyclophosphamide (10 to 200 mg/kg i.p.). Average daily phenobarbital intake per mouse was 1.25 mg (equivalent to 4 mg/kg/day human dosage). Dose-response curves with and without phenobarbital pretreatment showed a constant 90% (1-log) reduction in the toxicity of cyclophosphamide to leukemic colony-forming units, whereas enzyme induction had no effect on the toxicity of the drug to normal bone marrow colony-forming units. Parallel survival studies confirmed the 1-log diminution in the antileukemic activity of cyclophosphamide in phenobarbital-pretreated mice. This phenobarbital-induced change in the antitumor activity of cyclophosphamide appears explainable on a pharmacokinetic basis. The Friedman and Boger assay for plasma alkylating metabolites showed that the reduction in the area under the plasma metabolite curve caused by enzyme induction exactly predicted the observed reduction in cyclophosphamide antitumor effect.

INTRODUCTION

Cyclophosphamide is a major anticancer drug with activity against a wide variety of human and animal neoplasms (16). This drug is inactive in vitro, requiring hepatic microsomal enzymes to convert it to its active forms (4, 6, 14). A number of routine drugs (e.g., sedatives, narcotics, antibiotics, etc.) may increase or decrease hepatic microsomal enzyme activity and thereby alter cyclophosphamide metabolism (7, 19). Phenobarbital, a microsomal enzyme inducer, has been extensively studied by a number of investigators concerning its potential interaction effects on cyclophosphamide metabolism. This drug and barbiturate-type hypnotics are used extensively in the supportive care of the cancer patient. The literature concerning the interactions of phenobarbital with cyclophosphamide in mouse tumor model systems is confusing and controversial. Hart and Adamson (13) suggested that phenobarbital pretreatment of tumor-bearing mice had no significant effect on cyclophosphamide antileukemic activity. Sladek (19), using Walker 256 carcinosarcoma, also showed that phenobarbital had no effect on cyclophosphamide activity. Field et al. (8) and Garattini et al. (11) have, in contrast, demonstrated definite decreases in the mean survival times of tumor-bearing mice treated with cyclophosphamide following phenobarbital enzyme induction. These workers suggested that the pharmacokinetics of cyclophosphamide were changed by prior phenobarbital exposure (i.e., decreased total cyclophosphamide alkylating metabolites) (10). We have used the mouse spleen colony assay system to determine the effects of phenobarbital enzyme induction on the in vivo activity of cyclophosphamide against leukemic and normal bone marrow stem cell growth potential. We have supplemented these data with survival time studies in tumor-bearing mice and with pharmacokinetic analyses of plasma cyclophosphamide-alkylating metabolites in the presence or absence of phenobarbital enzyme induction.

MATERIALS AND METHODS

Mice. Six- to 8-week old male DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine) weighing approximately 25 g were used in these experiments.

Mouse Tumor Line. P388 lymphocytic leukemia (3) was supplied by Dr. John Harris (Department of Radiobiology, University of California, San Francisco, Calif.) and serially transplanted as an ascites tumor at weekly intervals (10⁶ cells every 7 days in Medium 199; Grand Island Biological Co., Grand Island, N. Y.). It was selected for these studies because it more accurately predicts clinical efficacy of anticancer drugs than other mouse tumor lines (20).

Chemotherapeutic Agents. Phenobarbital sodium in parenteral form (Invernex, Chagrin Falls, Ohio) was brought to a final concentration of 0.5 mg/ml of solution by the addition of distilled water. Cyclophosphamide (Mead Johnson and Co., Evanston, Ill.) in powder form was dissolved in sterile water in the desired concentration. Pentobarbital sodium was used in its parenteral form (Abbott Laboratories, North Chicago, Ill.). SKF 525A,² a microsomal enzyme

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inhibitor (Smith, Kline and French, Philadelphia, Pa.), was prepared for parenteral use by dissolution in sterile water (2, 9, 18).

The Administration Schedule of Chemotherapeutic Agents. The mice were divided into 4 groups of 5 animals. On Day 0, Groups 2 and 4 were started on phenobarbital drinking water; Groups 1 and 3 stayed on a regular acid water (17) diet. On Day 7, Groups 3 and 4 received cyclophosphamide i.p. at 10- to 200-mg/kg dosages in a constant volume of 0.01 ml/g body weight. An additional set of mice (Group 4) received SKF 525A (70 mg/kg i.p.) 1 hr before cyclophosphamide at 20 mg/kg. All groups were sacrificed on Day 10, and their femurs were assayed for leukemic or normal bone marrow spleen colony-forming units. Mice averaged 2.5 ml/day of either acid water or phenobarbital drinking water; therefore, daily total phenobarbital dosage averaged 1.25 mg or approximately 50 mg/kg.

To prove microsomal enzyme induction, control and phenobarbital pretreated mice underwent sleep duration studies following pentobarbital administration. Pentobarbital, used in a dosage of 45 mg/kg, caused acid water-fed mice to sleep between 25 and 45 min. Those animals pretreated with phenobarbital either did not sleep or rarely slept beyond 5 to 8 min following pentobarbital injection.

LCFU Assay. Our assay for LCFU was adapted from that of Bruce and van der Gaag (5) and was carried out in the following way (Chart 1). On Day 5, 10⁶ P388 cells were injected into the tail vein of each control and phenobarbital-pretreated mouse. In the appropriate groups, cyclophosphamide was injected i.p. 2 days (Day 7) after tumor cell injection (when the i.p. P388 tumor has a 93+% growth fraction ) (12). On Day 10 the mice were sacrificed, femurs were isolated, and bone marrow cells were washed out with Medium 199. Appropriate dilutions of the femoral bone marrow cells were injected in 0.2-ml volumes into the tail veins of groups of 15 to 20 recipient mice. Nine days later these mice were sacrificed, spleens were removed and fixed in Bouin’s solution, the macroscopic LCFU were counted using a dissecting microscope, and the fraction of surviving LCFU per femur (as compared to the controls) was determined.

NCFU Assay. NCFU were assayed in a way similar to that for LCFU except for the following (Chart 2). Three days after therapy with cyclophosphamide i.p. the mice were sacrificed, and appropriate dilutions of normal femoral bone marrow cells were injected i.v. into groups of 15 to 20 whole-body-irradiated (750 rads from a 250-kv source) mice.

Mean Survival Time Studies. Groups of 7 to 10 DBA/2 mice were given 10⁶ P388 ascites cells i.v. in a volume of 0.2 ml, and the mean survival times of each of the control and treatment groups were calculated by observing for death at 12-hr intervals. The administration schedule of chemotherapeutic agents was similar to that for the LCFU and NCFU assays.

Statistical Analysis. A simple t test was used to determine statistical significance between different experimental groups. Dose-response curves were constructed on the basis of least-squares regression analysis using the Wang 2200 computer program.

Determination of Cyclophosphamide-alkylating Metabolites. Phenobarbital-pretreated and untreated mice were given cyclophosphamide (200 mg/kg i.p.). This dosage was selected for the pharmacokinetic studies because it could be sensitively and reproducibly assayed and because others had previously used it in their mouse studies of cyclophosphamide (8). At each time point after cyclophosphamide administration, groups of 7 mice were sacrificed by decapitation and their blood was collected in iced, heparinized centrifuge tubes. The blood samples were centrifuged at 2000 rpm for 10 min at 0°, and the resulting 2.5 to 3.5 ml plasma (for each group of 7 mice) were immediately separated and frozen at −20°.

Cyclophosphamide metabolites in the mouse plasma samples were assayed using a modification of the method of Friedman and Boger (10). Two ml of each plasma sample were placed into a centrifuge tube along with 2 ml of 4%
perchloric acid. The samples were centrifuged at 2000 rpm for 10 min at 0°. One-mI aliquots of the resulting supernatants were transferred to clean test tubes to which were added, in succession, 1 ml of 2 M sodium acetate, pH 5.5, and 0.5 ml of 5% 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, Wis.) in acetone. These mixtures were then heated at 100° for 25 min and thereafter cooled in ice for 20 min. To release the colored chromophore for absorbance determinations, each sample was treated as follows: (a) 2 ml of acetone, 5 ml of ethyl acetate, and 2 ml of 1 N NaOH were added; (b) samples were immediately mixed for 20 sec on a Vortex mixer; (c) samples were spun for 20 sec in a tabletop centrifuge; and (d) an aliquot of the upper phase of each sample was placed in a 1-cm quartz cell and absorbance was read on a Beckman Kintrac spectrophotometer at 540 nm 80 sec after addition of the NaOH.

Because cyclophosphamide is inactive in vitro, it was necessary to construct a standard curve using nitrogen mustard in human plasma. Standard curve points were determined in quadruplicate. The absorbances of the standards and the μg/ml plasma of nitrogen mustard equivalents were determined.

RESULTS

Phenobarbital drinking water pretreatment changed neither the total LCFU or the total NCFU per femur from those of the untreated control mice; however, phenobarbital prior to cyclophosphamide (10 to 30 mg/kg) caused a constant 1-log reduction in cyclophosphamide antileukemic stem cell effect. Chart 3 shows the dose-response curve with respect to the fraction of surviving LCFU per mouse for cyclophosphamide alone and phenobarbital plus cyclophosphamide at dosages of 10 to 30 mg/kg. In order to determine whether this decrease in cyclophosphamide antileukemic activity resulted from an alteration in microsomal enzyme activity, we treated the mice with SKF 525A (70 mg/kg i.p.) 1 hr prior to cyclophosphamide in both the control and phenobarbital-pretreated groups. As shown in Chart 4, SKF 525A completely reversed the phenobarbital-induced decrease in cyclophosphamide antileukemic activity. There was no statistically significant difference in the number of spleen colonies per femur for cyclophosphamide alone and cyclophosphamide following phenobarbital plus SKF 525A.

At cyclophosphamide dosages of 10 to 200 mg/kg, phenobarbital pretreatment did not significantly change the dose-response curve of cyclophosphamide for NCFU (Chart 5). The dose-response curves in each case are relatively flat with only a little more than a 1-log decrease in NCFU over a dosage range of 10 to 200 mg/kg.

Table 1 shows the mean survival time study results for tumor-bearing mice for phenobarbital alone, cyclophosphamide alone, and the combination of these drugs. Those groups of animals treated with phenobarbital or acid water and not cyclophosphamide had mean survival times of 5.5 days. At cyclophosphamide dosages of 20, 40, and 70 mg/kg, phenobarbital pretreatment clearly decreased survival duration with p values of less than 0.01 for each experiment.

As shown in Chart 6, phenobarbital prior to cyclophosphamide injection caused an earlier and higher peak plasma alkylating metabolite concentration, whereas cyclophosphamide alone was associated with a lower, later peak level and slower disappearance of these metabolites. The area under the plasma alkylating metabolite curve for cyclophosphamide used alone was 59 μg-hr/ml as opposed to 40 μg-hr/ml for the mice pretreated with phenobarbital. The plasma half-life of alkylating metabolites for the cyclophosphamide group was 20 min; for the phenobarbital plus cyclophosphamide group it was 14 min.
In Chart 7, the surviving LCFU dose-response curves for cyclophosphamide with and without phenobarbital pre-treatment have been compared to the relative area under their respective plasma alkylating metabolite curves. In that Brock et al. (4) have demonstrated linear pharmacokinetics at a wide dose range of i.p. cyclophosphamide in female rats, the total area under the cyclophosphamide and phenobarbital plus cyclophosphamide plasma alkylating metabolite curves at each dose level on the LCFU dose-response curve has been extrapolated from the experimentally derived areas at the 200-mg/kg dose of cyclophosphamide. The extrapolated dose-response curves for cyclophosphamide and phenobarbital plus cyclophosphamide become indistinguishable, demonstrating that the antileukemic effect of cyclophosphamide in this animal tumor model system can be related to the area under its plasma alkylating metabolite curve.

DISCUSSION

Using a mouse tumor model system for spleen colony assay and survival time studies, we have shown that phenobarbital pretreatment in amounts comparable to a standard daily human dosage markedly decreases the therapeutic ratio of the cyclophosphamide. The fact that the phenobarbital-induced decrease in cyclophosphamide antileukemic effect is not balanced by a decrease in toxicity to normal bone marrow stem cells makes it impossible to overcome this decrease in therapeutic ratio by increasing cyclophosphamide dosage.

Although our study results and those of Field et al. (8) and Garattini et al. (11) suggest that phenobarbital may interfere with the antitumor activity of cyclophosphamide in humans, it is impossible to extrapolate the results of such animal studies to the human situation. For example, Hart and Adamson (13), who used female BALB/c × DBA/2 F1 mice in their L1210 leukemia studies, and Sladek (19), who used Walker 256 carcinosarcoma in male and female rats, showed no change in cyclophosphamide effect following phenobarbital dosage. On the basis of the conflicting results of these papers, we can conclude that drug interaction studies in 1 animal species and gender or with a single tumor type may prove to be an inconclusive model for the prediction of drug interaction effects in humans.

We have used the mouse spleen colony assay system to quantitate drug interaction effects sensitively at the leukemic and normal bone marrow stem cell level. Adverse or efficacious drug interaction effects may be uncovered by this model system, whereas they could be overlooked using less sensitive models or the traditional mouse tumor mean survival time studies. Furthermore, the ability to contrast and compare the results of mouse spleen colony survival time studies creates a helpful new tool for evaluating potential anticancer drug-routine drug interaction effects.

We have shown that phenobarbital pretreatment does not decrease the toxicity of cyclophosphamide at a wide range of doses (e.g., 10 to 200 mg/kg) for normal bone marrow stem cells. In contrast Sladek (19) has shown that phenobarbital pretreatment causes an earlier and lower nadir of peripheral WBC following cyclophosphamide administration. A possible explanation of this discrepancy is that Sladek studied the effect of the phenobarbital exposure at only 1 dosage level of cyclophosphamide (i.e., 50 mg/kg). In addition, our studies dealt with the effect of this drug interaction at the critical stem cell level as opposed to the peripheral blood cell level.

Brock et al. (4) have shown that at a dose range of 15.6 to 62.5 mg/kg the pharmacokinetics of cyclophosphamide are definitely linear. If it is correctly assumed that this pharma-
Jao et al. (15) showed that phenobarbital pretreatment in humans had no quantitatively important effects on the distribution and renal excretion of cyclophosphamide; however, they used a radiolabeled cyclophosphamide in their drug assays which could, perhaps, account for the differences seen between our 2 studies. Studies are clearly warranted in patients to determine whether medications such as phenobarbital, which are commonly administered in the clinic, do significantly reduce the “area under the nitrobenzyl pyridine curve.”

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REFERENCES

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