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BIOCHEMICAL PHARMACOLOGY OF PENCLOMEDINE (NSC-338720)

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Abstract—Penclomedine (PEN) is a synthetic pyridine derivative that has been selected for clinical development based on its activity against human and mouse breast tumors implanted in mice. Its mechanism of action was unclear, and we were interested in determining its mechanism of cytotoxicity *in vitro* and *in vivo*. We found chromosome breaks, gaps, and exchanges in P388 ascites cells from BD_2F_1 mice treated with 200 mg/kg PEN. Maximal observed damage occurred 24 hr after drug administration. Alkaline elution indicated only limited DNA strand breaks and interstrand cross-linking. *In vitro*, PEN (75 μg/mL) inhibited RNA and DNA syntheses almost completely. In addition, incubation of [^{14}C]PEN with rat liver S-9 fraction in the presence of calf thymus DNA resulted in the stable transfer of radioactivity to DNA. Addition of butylated hydroxytoluene, a free radical scavenger, to the incubation mixture inhibited the binding of drug to DNA, implicating free radicals as the ultimate reactive species. These data suggest that PEN can be metabolized to free radical, DNA-reactive products, and that its cytotoxicity is due to chromosomal damage produced by monofunctional alkylation. As an alternate mechanism, the ability of PEN to inhibit cellular dihydroorotate dehydrogenase was explored. Although PEN is an inhibitor of this enzyme in cells *in vivo*, *in vitro*, and in isolated cell sonicates, HPLC analyses of ribonucleotide triphosphate pools in P388 cells showed that all triphosphates had increased, especially UTP. Addition of uridine to the cell culture failed to prevent PEN-mediated cytotoxicity, suggesting that inhibition of *de novo* pyrimidine biosynthesis was not likely to be an important mechanism of action of this drug. These data suggest that PEN is activated in cells to a free radical that binds DNA.

Key words: penclomedine; biochemical pharmacology; mechanism of action; chromosomal aberrations; dihydroorotate dehydrogenase; breast tumors

PEN‡ is a lipid-soluble, highly substituted 2-methylpyridine derivative (Fig. 1) with a distinctive spectrum of antitumor activity against a panel of murine tumors, particularly breast tumors [1]. The drug is now in Phase I trial. PEN demonstrated antitumor activity against s.c. implanted mouse $CD8F_1$ mammary adenocarcinoma and renal capsule implanted human MX-1 mammary carcinoma after i.p. administration [1]. PEN was active when given orally against several s.c. implanted tumors including advanced $CD8F_1$, MX-1, advanced human MCF-7 breast adenocarcinoma, early mouse 16/C breast adenocarcinoma, and advanced human H-82 small cell lung carcinoma [1, 2]. It was equally active against advanced intracerebrally implanted MX-1 mammary tumor when given i.v., p.o., and i.p. [2]. P388 cell lines with acquired resistance to the alkylating agents melphalan, cyclophosphamide, and carmustine were also resistant to PEN, but were not cross-resistant to antimetabolites and DNA intercalators [2]. These data suggested that the drug is likely to be an alkylating agent.

After i.v. administration to mice, PEN is cleared rapidly from plasma, mainly by metabolism; only a small amount of the parent drug is recovered in the urine [3]. The bioavailability of PEN after p.o. administration to mice is less than 5% of that after i.v. injection. Murine hepatic microsomes readily transform PEN by both oxidative and reductive pathways [3]. When [^{14}C]PEN is incubated with activated mouse microsomes in the presence of calf thymus DNA, radioactivity is irreversibly bound to both protein and DNA [4]. It has been proposed that the binding of PEN may result from the homolytic cleavage of chlorine from the trichloromethyl group by reductive microsomal metabolism [3, 4].

Because of the selective antitumor activity of PEN against breast tumors and its ability to cross the blood–brain barrier, it is a prime candidate for clinical study. The present study was initiated to investigate the basis for the unique properties of PEN, in particular its mechanism of cytotoxicity and the role that metabolism plays in causing DNA and chromosomal damage.

MATERIALS AND METHODS

Materials

PEN [(3,5-dichloro-2,4-dimethoxy-6-trichloromethyl)-pyridine], [*trichloromethyl*-^{14}C]PEN (17.6 mCi/mmol), and formulated PEN (7.5 mg/mL in a 10% oil/lecithin/water emulsion) were supplied by the Pharmaceutical Resources Branch (Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer

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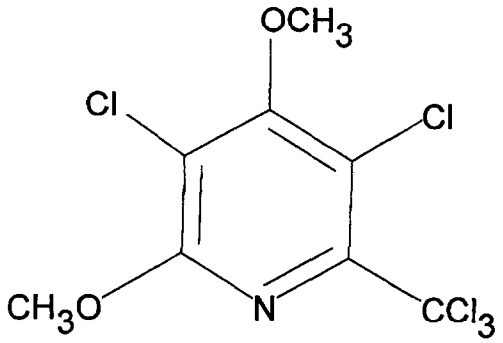
‡ Abbreviations: PEN, penclomedine; BHT, butylated hydroxytoluene; DBT, 3,4-dichlorobenzenethiol; DHO-DH, dihydroorotate:ubiquinone oxidoreductase (EC.1.3.3.1), dihydroorotate dehydrogenase; and BrdU bromodeoxyuridine.



PENCLOMEDINE

Fig. 1. Structure of penclomedine [(3,5-dichloro-2,4-dimethoxy-6-trichloromethyl)pyridine].

Institute). [5-^{3}H]Uridine (22.5 Ci/mmol) was obtained from New England Nuclear, and [*methyl*-^{3}H]thymidine was obtained from Amersham. All solvents were HPLC grade. Aroclor-1254-activated rat liver S-9 supernatant fractions were obtained from Microbiological Associates. Male BD_2F_1 mice (20–25 g) were obtained from Charles River Associates and were housed under controlled lighting and humidity conditions. Animals were allowed free access to food and water, and their treatment conformed to all care and use standards.

Cell culture

P388 murine lymphocytic leukemia, MX-1 human breast carcinoma, MCF-7 human breast adenocarcinoma, and H82 human small cell lung cancer cells were grown in RPMI-1640 medium (Whittaker) containing 10% fetal bovine serum (FBS, Hyclone), 2 mM glutamine (Gibco), and 1% penicillin/streptomycin/neomycin (PSN) antibiotic mixture (Gibco) in an incubator at 37° under 5% CO_2 and 100% relative humidity. 2280P Human Epstein-Barr virus transformed lymphoblastoid cells were grown in RPMI-1640 medium with 20% FBS, and M5076 fibrosarcoma cells were grown in RPMI-1640 with 10% horse serum (Atlanta Biologics) under similar conditions as above.

Inhibition of cell growth

The growth inhibitory activity of PEN was assessed in P388, MX-1, MCF-7, H82, and M5076 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [5]. Drug was dissolved in DMSO and added to cell cultures in a minimal volume so that the final DMSO concentration was below 0.2%. Cell growth was assessed after 24, 48 and 72 hr.

Chromosomal damage

BD_2F_1 mice bearing P388 ascites were administered PEN (200 mg/kg, i.p.) or saline. Ascites were obtained through an i.p. tap at 4, 8, 12 and 24 hr after drug injection and placed in ice. The specimens were resuspended in RPMI 1640 medium containing 0.025 μg/mL Colcemid, incubated for 1.5 hr, treated for 10 min with 0.075 M KCl, and fixed in methanol:glacial acetic acid (10:1, then 3:1); drops of cell suspension were placed on wet microscope slides. Following Giemsa staining, metaphases were scored for chromosome gaps, breaks, and exchanges for each time point (two mice/time point). To determine the timing of chromosomal aberration formation, BrdU was administered to animals 30 min before PEN to label those cells in S phase at the time of treatment; the rest of the experiment was carried out as described above. Mitotic spreads were stained with an anti-BrdU antibody to determine when the labeled cells reached mitosis after PEN treatment [6].

Alkaline elution

BD_2F_1 mice bearing P388 as ascites were administered 75 μCi [*methyl*-^{3}H]thymidine (83 μCi/mmol), i.p., to label DNA. After 18 hr, mice were administered PEN (200 mg/kg in 10% oil/lecithin/water emulsion, i.p.) or saline. Ascites cells were obtained at 1, 4, 8 and 24 hr after PEN administration and analyzed for DNA interstrand cross-links, DNA single-strand breaks, and endonucleolytic cleavage (an indicator of apoptosis), as previously described [7–9].

Cell cycle analysis

Cell cycle distributions of samples were determined using flow cytometry as previously described [6]. Aliquots of control and PEN-treated 2280P lymphoblastoid cells were washed twice in PBS, fixed in 70% ethanol/30% PBS, incubated at 37° with RNase (EC 3.1.27.5, 100 μg/mL), and then stained with propidium iodide. DNA content was determined by quantitative flow cytometry using a FacScan Analyzer (Becton Dickinson) equipped with a 15-mW argon ion laser set at 488 nm. The accuracy of the analyses was checked with calibrated fluorescent beads, and the intensity was controlled with chicken erythrocytes.

Alteration of macromolecular synthesis

The effects of PEN on macromolecular synthesis were determined in cell culture by measuring the incorporation of [*methyl*-^{3}H]thymidine (1 μCi, 2.9 ng) and [5-^{3}H]uridine (1 μCi, 10.8 ng) into DNA and RNA using the method of Bennett *et al.* [10]. Concentrations of PEN ranging from 25 to 75 μg/mL were incubated with P388 cells for 4 hr before the addition of radiolabeled precursors. One hour after the addition, cells were chilled in an ice bath, centrifuged, and then rapidly washed in ice-cold buffered physiological saline. The washed cell pellets were dissolved in 0.1% sodium lauryl sulfate, and 0.2 mL of 2 N NaOH was added per mL of solution. The samples were incubated overnight at 37°, neutralized with 4 N HCl, washed with cold ethanol, and filtered. The filters were dried and incubated overnight in 1 ml of Soluene (Packard Instrument Co.). Radioactivity was determined by liquid scintillation counting.

HPLC determination of PEN

To analyze for PEN in biological systems, an HPLC assay was developed that quantitated [^{14}C]PEN and metabolites. This method was adapted from that reported by Reid *et al.* [3]. Samples (0.5 mL) were extracted with 4 mL of diethyl ether, and the organic layers were transferred to a conical tube containing 100 μL of butanol. The samples were concentrated under nitrogen until 100 μL of butanol remained in the tube. A 100 μL aliquot of the mobile phase (acetonitrile:water; 85:15, v/v) was added. PEN was chromatographed on a C18 IBSil octa-

decyl HPLC column (250 mm × 4.5 mm, 5 μm; Phenomenex). Fractions (1 mL) of the eluant were collected, and their radioactivity was determined by liquid scintillation counting.

PEN binding to calf thymus DNA

The binding of [^{14}C]PEN to calf thymus DNA was assessed in the presence of microsomes prepared by ultracentrifugation of a commercial Aroclor-1254 activated rat S-9 fraction, using literature procedures [11, 12]. The reaction mixture (2 mL) contained potassium phosphate (160 mM, pH 7.8), microsomal protein (1.75 mg/mL), EDTA (1 mM), magnesium chloride (3 mM), glucose-6-phosphate (3 mg/mL), glucose-6-phosphate dehydrogenase (0.25 U/mL), calf thymus DNA (1 mg/mL) and 1–10 μCi of [^{14}C]PEN (final concentration 0.28 μM in 20 μL ethanol). The reaction was started by the addition of NADP (1.25 mM) and NADH (1 mM), and incubated at 37° for 60 min with shaking. The reaction was terminated by the addition of 2 mL of chloroform:isoamyl alcohol:phenol (24:1:25, by vol., "CIP"). The mixture was centrifuged at 2000 *g* for 10 min, and the aqueous layer was removed. This DNA extraction was repeated. The combined aqueous washes were extracted twice with an equal volume of ethyl acetate:diethyl ether (1:1, v/v). The aqueous solution was incubated with RNase A (100 μg/mL) and RNase T1 (50 U/mL) for 45 min at 37°, followed by protease (500 μg/mL) for 30 min. The reaction mixture was extracted again with "CIP" and then twice with ethyl acetate:diethyl ether (1:1, v/v). The solution was made 0.1 M in NaCl, and the DNA was precipitated by addition of 5 mL of ice-cold ethanol. DNA was pelleted by centrifugation at 2000 *g* for 30 min and washed with 4 mL of ice-cold ethanol until the radioactivity in the ethanol was reduced to at least 100 cpm. The DNA was dissolved in 1 mL of 15 mM citrate/150 mM NaCl, and the radioactivity was determined by liquid scintillation counting. The DNA content was determined by absorbance spectroscopy at 260 nm. A control contained boiled microsomes (100°, 5 min). In addition, BHT (0.01%, w/v) was added to determine the involvement of free radicals [13]. The oxygen dependence of the binding of PEN to DNA was determined by bubbling nitrogen into a cold incubation mixture for 5 min [14].

Binding of PEN cellular DNA

After [^{14}C]PEN (3 μCi) was incubated with 5.2×10^6 P388 cells for 1 hr at 37°, DNA was isolated, and the radioactivity covalently bound to DNA was determined as described above for microsomal activated binding. A control was prepared by incubating cells for 1 hr at 37° and then adding [^{14}C]PEN.

Trapping of electrophilic alkylating intermediates

[^{14}C]PEN (20 μg, 0.062 μmol) was incubated with the S-9 supernatant fraction as described above in the presence of DBT (110 μg, 0.62 μmol) [15, 16]. The DBT was dissolved in 10 μL DMSO and added to the incubation mixture before the S-9 fraction. The reaction mixture was incubated at 37° for 30 min with shaking, then extracted with diethyl ether, and analyzed by HPLC as described above. Concurrent controls included systems lacking the S-9 fraction, PEN, DBT, or the NADPH system.

Inhibition of DHO-DH activity

DHO-DH activity was determined using the procedure of Peters *et al.* [17]. For the *in vivo* studies, BD_2F_1 mice containing P388 cells were administered 200 mg/kg PEN (i.p.). After 1 hr, ascites fluid was removed, and cells were isolated, washed twice with 0.9% saline, and counted. The cells were added to 0.1 M Tris-HCl, pH 8.0 (10^8 cells/17.6 mL buffer), and lysed by sonication. The enzyme reaction mixture consisted of 0.9 mL of the whole cell extract and 31.5 μL of 30 mM L-dihydroorotic acid. The reaction was shaken at 37° for 30 min and terminated by addition of 180 μL of 16% trichloroacetic acid. The reaction was chilled on ice for 20 min, and the denatured protein was removed by centrifugation. After neutralization with 2 mL alamine-Freon (1:4, v/v), the reaction was centrifuged and the aqueous phase removed. The orotic acid in the aqueous phase was determined by HPLC (column: Whatman Partisil SAX; solvent: 10 mM KH_2PO_4, 10 mM KCl, pH 4.0; flow rate: 1.4 mL/min; detector: UV, 282 nm). Enzyme activity was expressed as nanomoles orotic acid formed per hour per milligram protein. For the *in vitro* studies, P388 cells were incubated with 0, 10, 20, 40, and 80 μg/mL PEN for 1 hr. Cell extracts were prepared, and orotic acid was determined as described above.

To determine DHO-DH activity in cell sonicates, approximately 10^8 cells were added to 17.6 mL of 0.1 M Tris-HCl, pH 8.0, and lysed by sonication as above. Concentrations of PEN ranged from 4 to 16 μg/mL. The L-dihydroorotate concentrations were 2–15 μg/mL. The determination of DHO-DH activity by direct measurement of orotic acid using HPLC was the same as above.

Uridine rescue

P388 cells were incubated for 72 hr with PEN (2.5 to 120 μg/mL) in the presence of uridine (1 mM); cell growth was measured by the MTT assay.

Ribonucleotide triphosphate pools

To determine the effects of PEN on cellular nucleotide pools, P388 cells were incubated with PEN (20 and 80 μg/mL). Aliquots of cells were removed at 1, 4, and 18 hr, and extracted with $HClO_4$; then ribonucleotide triphosphate pools were analyzed by anion exchange HPLC, as previously described [18].

Lipid peroxidation

Lipid peroxidation was determined by measurement of malondialdehyde using the method of Buege and Aust [13]. One milliliter of the whole cell lysate from above (~0.1 mg protein) was mixed thoroughly with 2 mL TCA–TBA–HCl reagent [the reagent contains 15% (w/v) trichloroacetic acid and 0.375% (w/v) thiobarbituric acid dissolved in 0.25 N HCl]. The mixture was heated for 15 min in a boiling water bath, then cooled, and the precipitate was removed by centrifugation at 1000 *g* for 10 min. The absorbance was read at 535 nm against a blank that contained all the reagents except the lipid sample. Malondialdehyde was determined using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

RESULTS

In vitro *cytotoxicity and cell cycle specificity*

Inhibition of cell growth by PEN was determined in five cell lines of mouse and human origin. The IC_{50}

values (μg/mL) for 72-hr exposures were: P338, 7.0; MX-1, 5.7; M5076, 5.3; MCF-7, 7.4; and H82, 8.6. Similar to alkylating agents, no cell cycle specificity was observed when PEN was incubated with 2280P lymphoblastoid cells (data not shown).

Chromosomal damage

Since PEN was suspected of being an alkylating agent based on its cross-resistance with known alkylating agents, we began our investigation by determining if the drug caused chromosomal damage. The administration of PEN to mice at the maximally tolerated dose of 200 mg/kg produced chromosome gaps, breaks, and exchanges. While the levels of aberration formation *in vivo* were variable, the damage was most pronounced 24 hr (the maximum time observed) after drug administration (Table 1). To further characterize the cell cycle determinants of aberration formation, animals were pretreated with BrdU just before PEN treatment to label cells in S phase at the time of drug treatment. Analysis of the post-treatment metaphases obtained after 1, 4, 8, and 24 hr using an anti-BrdU staining technique suggested that chromosomal damage required passage of the cells through S phase. The aberrations appeared to result from the inability of the cells to replicate past PEN-induced DNA lesions.

To investigate the nature of the DNA lesions that were translated into chromosomal aberrations, [^{3}H]thymidine-labeled P388 ascites cells were obtained from mice 1, 4, 8, and 24 hr after PEN administration (200 mg/kg, i.p.) and analyzed for DNA single-strand breaks and interstrand cross-links using alkaline elution. The data (Fig. 2) indicate that very limited single-strand DNA cleavage and interstrand cross-linking occurred. The overall extent of DNA damage was quite low. No evidence of apoptosis, as indicated by endonucleolytic cleavage, was found.

Inhibition of macromolecular synthesis

In vitro, PEN inhibited RNA and DNA syntheses in a concentration-dependent manner. At the lowest concentration used (25 μg/mL), there was little inhibition of RNA or DNA synthesis, but at concentrations greater than 35 μg/mL inhibition increased rapidly to a maximum of 98% at 75 μg/mL (Fig. 3).

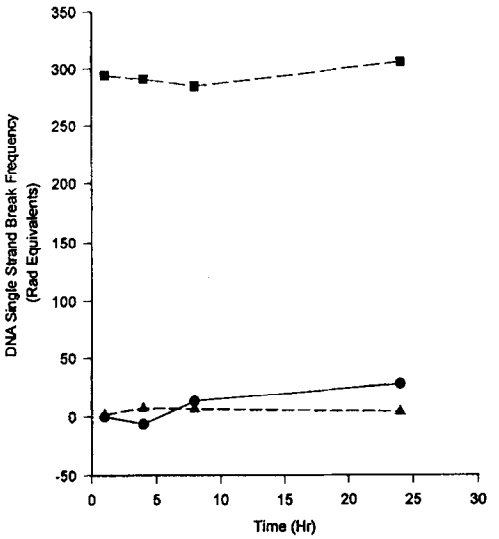


Fig. 2. DNA interstrand cross-links and DNA single-strand breaks in mice bearing P388 lymphocytic leukemia ascites treated with 200 mg/kg PEN,i.p. Key: (■) PEN with 300 rad, (●) PEN, and (▲) interstrand cross-links. Data are means of three experiments.

Binding of PEN to DNA

The effect of microsomal incubation of [^{14}C]PEN on the binding of radioactivity to DNA is shown in Fig. 4. The complete microsomal system was required for binding, as previously shown by Reid *et al.* [4]. The addition of BHT (0.01%, w/v), a radical scavenger, to the incubation mixture decreased binding significantly. A partial nitrogen atmosphere also inhibited the binding.

To determine if P388 cells could activate PEN to form reactive intermediates that bind irreversibly to DNA, radiolabeled PEN (3 μCi) was incubated with 5×10^6 P388 cells for 1 hr (Fig. 5). Binding of radiolabel to DNA occurred under these conditions. If PEN activation proceeds through free radical intermediates, as suggested by the microsomal experiments, PEN should alter the extent of lipid peroxidation in cells. Malondialdehyde, a by-product of membrane lipid peroxidation in cells [13], was measured in P388 cells treated with 10–80 μg/mL PEN (Fig. 6). Malondialdehyde formation decreased with increasing PEN concentration, indicating inhibition

Table 1. Chromosomal damage in P388 cells from mice treated with PEN (200 mg/kg)

Time (hr)	No. of cells	Gaps/cell	Breaks/cell	Exchanges/cell
Control-1	50	0.08	0.22	0.04
Control-2	50	0.04	0.08	0
4	50	0.08	0.06	0
4	30	0	0.07	0
8	80	0.05	0.05	0
8	80	0.15	0.17	0.02
12	21	0.24	0.05	0.05
12	81	0.22	0.72	0
24	80	0.17	0.85	1.27
24	22	0.41	1.68	0.32

Data are from two mice per point and represent mean values of 20–80 chromosome spreads per mouse per time point.

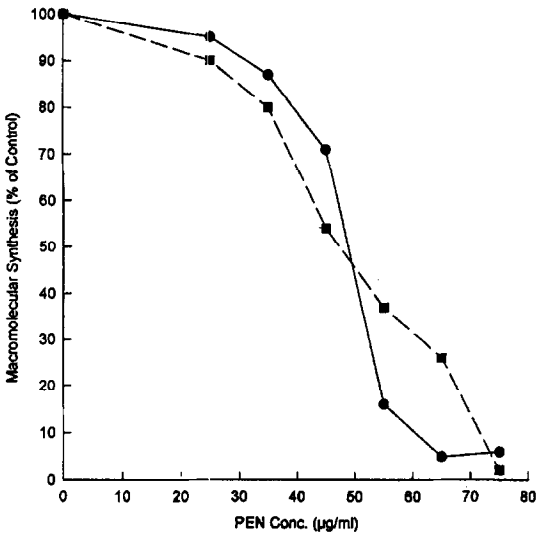


Fig. 3. Inhibition of macromolecular synthesis. P388 cells were incubated with 25–75 µg/mL of PEN for 4 hr before addition of either [*methyl*-^{3}H]thymidine or [5-^{3}H]uridine. After 1 hr, RNA and DNA were isolated, and incorporation was determined by liquid scintillation counting. Key: (●) RNA, and (■) DNA. Data are from two experiments.

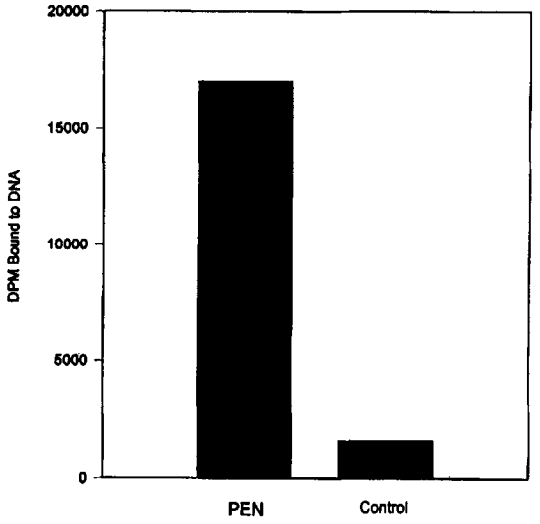


Fig. 5. Effect of incubation (1 hr at 37°) of [^{14}C]PEN (3 µCi) with 5 × 10^6 P338 cells on binding of radioactivity to DNA. The control was prepared by incubating the cells for 1 hr at 37°, and then adding radiolabeled PEN. Data are from duplicate analyses.

of lipid peroxidation by PEN. The decrease of malondialdehyde with increasing PEN paralleled the inhibition of DHO-DH in the same incubations.

If microsomal activation of PEN involved the formation of electrophilic intermediates that bind to DNA, the presence of a strong nucleophile would trap them. A nucleophile that has been used extensively to bind electrophiles is DBT. No DBT adducts were detected by HPLC when PEN was incubated with the S-9 fraction (data not shown).

Inhibition of DHO-DH activity

Because possible microsomal metabolites of PEN resemble potent inhibitors of DHO-DH, PEN inhibition of DHO-DH activity was evaluated in P388 ascites cells from mice treated with PEN (200 mg/kg), P388 cell sonicates, and intact P388 cells (10–80 µg/mL PEN, 1-hr incubation). DHO-DH activity in P388 ascites cells from mice treated with 200 mg/kg PEN was inhibited by 43% (data not shown). Unexpectedly, PEN also inhibited DHO-DH activity when incubated with P388 cells *in vitro* (Fig. 6) and with a crude cell sonicate (Table 2).

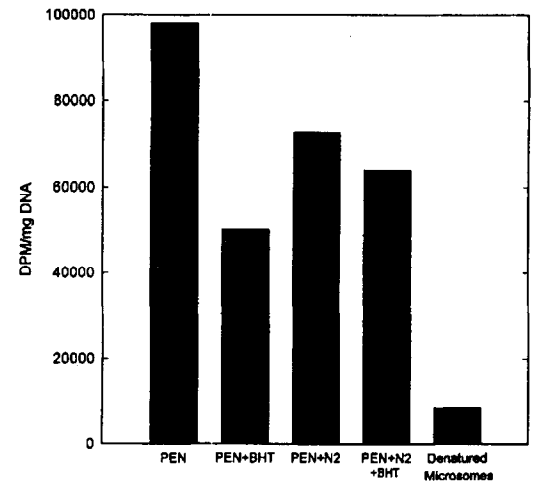


Fig. 4. Effect of microsomal metabolism of [^{14}C]PEN on binding of radioactivity to calf thymus DNA. PEN (0.28 µM) was incubated at 37° for 1 hr with activated rat S-9 liver fraction in the presence of an NADPH-generating system. The effects of the radical scavenger BHT (0.01%, w/v) and of bubbling nitrogen into the incubation mixture are shown. Microsomes were denatured by heating at 100° for 5 min. Results are from duplicate analyses.

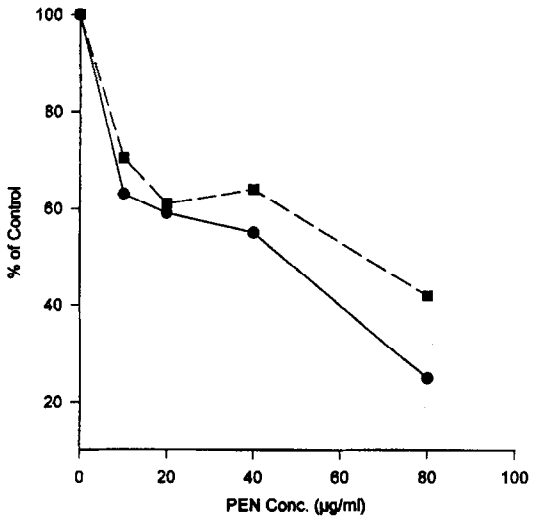


Fig. 6. Malondialdehyde formation and DHO-DH activity in P388 cells incubated with PEN. Malondialdehyde (■) was measured using the thiobarbituric acid assay, and DHO-DH activity (●) was determined by direct measurement of orotic acid using HPLC. The control values were: DHO-DH activity, 444 nmol/hr/mg protein; and malondialdehyde formation, 1.6 nmol/hr/mg protein. Results are from three experiments.

Table 2. Effect of PEN on dihydroorotate dehydrogenase activity in P388 cell sonicates

	Dihydroorotate dehydrogenase activity (nmol orotic acid formed/hr/mg protein)	
	PEN (mM)	
DHO (mM)	0	0.025
0.0125	171 ± 22	134 ± 8
0.025	341 ± 55	253 ± 5
0.050	439 ± 34	409 ± 3
0.100	519 ± 57	529 ± 13

P388 cells were lysed by sonication and incubated with DHO with or without PEN for 10 min. Orotic acid was measured by HPLC. Values are means ± SD of 4–8 analyses.

There was a steep concentration-response for DHO-DH inhibition by PEN *in vitro* in the concentration range of 10–80 μg/mL. However, at 80 μg/mL PEN, a concentration that is highly lethal to cells and that almost completely inhibited macromolecular synthesis, there was only ~80% enzyme inhibition, suggesting that the primary mode of action of PEN may not be inhibition of *de novo* pyrimidine biosynthesis.

If the primary mechanism of action of PEN were inhibition of *de novo* pyrimidine biosynthesis, uridine should rescue cells from PEN toxicity, and pyrimidine ribonucleotide triphosphates should decrease [19]. The effect of 1 mM uridine on the IC_{50} values of PEN in various cell lines is shown in Table 3. Uridine did not rescue the cells from PEN cytotoxicity; in fact, PEN may be more toxic in the presence of uridine. Figure 7 shows the effect of PEN (20 μg/mL) on the ribonucleotide triphosphate pools of P388 cells. The concentrations increased rather than decreasing, as would be expected if pyrimidine biosynthesis were inhibited; UTP increased the most. By 18 hr, the purine nucleotides had returned to control levels, but the pyrimidine nucleotides remained elevated. These data strongly suggest that inhibition of *de novo* pyrimidine biosynthesis is not the major mode of cytotoxicity of PEN.

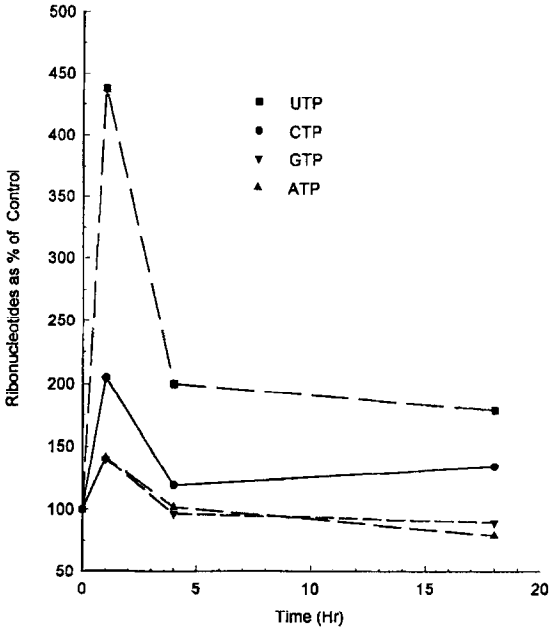


Fig. 7. Ribonucleotide triphosphate pools in P388 cells incubated with PEN (20 μg/mL). UTP, CTP, GTP, and ATP were determined by anion exchange HPLC. Control values (time 0), expressed as nmol/3 × 10^6 cells, were: UTP, 0.618; CTP, 0.473; GTP, 1.769; and ATP, 9.954. Data are from a single experiment.

DISCUSSION

PEN has been selected for phase I clinical trial based on its unique activity against a number of human and mouse tumors. However, its mechanism of action is still unknown. It has been suggested that PEN is an alkylating agent and requires hepatic microsomal metabolism for activation [3, 4], but the drug is also growth inhibitory to cells in culture. We have found that it causes chromosome gaps, breaks, and exchanges in P388 cells *in vivo* (Table 1), indicating interaction with DNA, consistent with alkylating activity. The maximum chromosomal damage was observed 24 hr post-treatment, suggesting that cells had to pass through S phase to translate alkylated lesions into chromosomal aberrations. Delayed activity is also characteristic of monofunctional alkylating agents [20]. Additionally, monofunctional alkylating agents can cause chromosomal breaks and deletions when the binding of these agents at specific sites cause

Table 3. Effect of uridine on PEN cytotoxicity

	IC_{50} Values		
	(μg/mL)	(mM)	(μg/mL)
		Additions	
Cell line	PEN	Uridine	PEN + Urd
P388	7.3 ± 1.3	>31.6	5.9 ± 1.9
MX-1	6.3 ± 0.9	23.5 ± 7.7	5.4 ± 0.6
M5076	5.3 ± 2.1	>31.6	3.0 ± 1.2
MCF-7	6.0 ± 2.2	ND*	ND
H82	8.8 ± 5.5	10.1 ± 3.5	5.2 ± 0.4

PEN (2.5 to 80 μg/mL) was dissolved in 0.2% DMSO. Cell number was determined at 72 hr using the MTT assay. The uridine concentration was 1 mM. Results are means ± SD of 3–9 experiments, each run in quadruplicate.

ND = not done.

base mispairing and physical impediments to DNA replication [21]. Our observation of the paucity of DNA interstrand cross-links (Fig. 2), as determined by alkaline elution, is again consistent with a monofunctional alkylating agent, although single-strand breaks were not similarly observed (Fig. 2), consistent with the modest activity of PEN in this tumor model [1].

Microsomal metabolism is required for binding of PEN to DNA *in vitro* (Fig. 4). To determine the nature of the activated species, we investigated the effects of inhibitors. The addition of the antioxidant BHT (0.01%) to the incubation mixture inhibited binding of PEN to DNA by 50% (Fig. 4). Also, the introduction of nitrogen into the incubation mixture inhibited DNA binding (Fig. 4). On the other hand, we were unable to trap electrophilic alkylating species using DBT (data not shown). These data suggest the involvement of free radicals in the microsomal-mediated binding of PEN to DNA and the dependence of the binding on oxygen. In our experiments, the binding of the radiolabel to DNA appears to involve an organic free radical derived from PEN. However, these data do not preclude the additional contribution of oxygen free radicals to the cytotoxicity of PEN. Oxygen free radicals formed from organic free radicals can damage cell membranes and cellular macromolecules [22, 23]. In mammalian cells, the macromolecular damage is manifested as sister chromatid exchanges, DNA strand breaks, mutations, and malignant transformation [24], but whether PEN free radicals or oxygen free radicals (or both) are involved in PEN cytotoxicity is uncertain. This ambiguity is similar to that surrounding the mechanisms of action of quinoid antitumor agents [25]. A number of enzymes, mainly oxidoreductases, have been implicated in the activation of quinoid antitumor agents, but the nature of the activations is still controversial. Some investigators have proposed that the semiquinone free radicals that are formed by enzymatic reduction could irreversibly bind, probably covalently, to DNA. Another possibility involves redox recycling in which the semiquinone free radicals promote formation of superoxide, hydrogen peroxide, and finally hydroxyl radical [23, 25]. This latter process results in increased lipid peroxidation and oxygen-mediated DNA damage. In the case of PEN, lipid peroxidation decreases in P388 cells treated with PEN (Fig. 6), implying that redox recycling and oxygen free radicals are not involved.

Since there is no exposure to liver mixed-function oxidases, microsomal activation cannot explain why PEN is toxic to cells in culture (Fig. 5). Moreover, free radicals formed in the liver would be expected to be short-lived and would not yield cytotoxicity at distant sites. However, we found that P388 cells were also capable of activating PEN to a DNA-alkylating species (Fig. 5). Thus, *in vitro,* microsomal metabolism may not be required for DNA binding activity. *In vivo,* the requirement for oxygen in the microsomal-mediated binding to DNA and the detection of formaldehyde [3] may reflect the formation of active metabolites formed by oxidative demethylation. The demethylated metabolites may be transported throughout the body and activated in the target cells to alkylating species. This possibility is under investigation.

Because of the structural similarity of possible demethylated PEN metabolites to inhibitors of DHO-DH, we investigated the ability of the drug to inhibit this key enzyme in the *de novo* pyrimidine biosynthetic pathway. PEN inhibited DHO-DH in P388 ascites cells *in vivo,* in P388 cells in culture (Fig. 6), and in P388 cell sonicates (Table 2). However, *in vitro,* PEN never completely inhibited the enzyme, even at concentrations that totally prevented DNA and RNA syntheses and were lethal to cells. It would be expected that if inhibition of DHO-DH were the mechanism of PEN cytotoxicity, pyrimidine nucleotide triphosphate pools would decrease and uridine would rescue cells from toxicity. To the contrary, all the triphosphates increased (Fig. 7), while addition of uridine may have actually increased PEN toxicity (Table 3). The accumulation of ribonucleotide triphosphates presumably arose from the strong inhibition of DNA and RNA synthesis by PEN. Thus, the inhibition of *de novo* pyrimidine biosynthesis is unlikely to be the primary mechanism of action. But, since PEN does interact with DHO-DH as evidence by its inhibition of the enzyme, it may exert its action by competing with the co-substrates (ubiquinone and DHO) of DHO-DH to disrupt mitochondrial electron transport in a manner similar to naphthoquinones [26]. In the latter case, the inhibitors undergo one or two electron reductions. As shown in Fig. 6, malondialdehyde formation, and presumably lipid peroxidation, decreased in concert with inhibition of DHO-DH activity. This is reasonable since a by-product of the DHO-DH oxidation of DHO is the hydroxyl radical, which is primarily responsible for lipid peroxidation [27]. Inhibition of DHO-DH would result in decreased production of hydroxyl free radicals consistent with the concept that oxygen free radicals are not involved in the mechanism of action of PEN. Interestingly, the percent increase in lifespan of tumor-bearing mice increased with increased DHO-DH activity ($r = 0.999$) in a number of tumor cell lines [1, 28]. This is exactly opposite of what would be expected if PEN acted only as an inhibitor of DHO-DH and suggests that PEN may be activated by DHO-DH.

The data obtained in this investigation suggest that PEN is a monofunctional alkylating agent whose cytotoxicity is due to inhibition of DNA replication. The nature of the activation of PEN to an alkylating agent is still under investigation, but it may be similar to that of the quinoids and result from the enzymatic reduction of PEN that could occur during the disruption of electron transport processes. P388 was chosen to investigate the mechanism of action of PEN because it is a readily available, well-characterized tumor against which PEN has activity. There are also a number of variants of P388 that have resistance to specific classes of antitumor agents and that are cross-resistant to PEN [2]. We plan further studies of these cell lines to help establish the mechanism of action of PEN. In addition, the question of the selectivity of PEN towards breast tumors will be addressed in *in vivo* studies.

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REFERENCES

1. Plowman J, Harrison SD Jr, Dykes DJ, Paull KD, Narayanan VL, Tobol HK, Martin J and Griswold DP Jr, Preclinical antitumor activity of an α-picoline derivative, penclomedine (NSC 338720), on human and murine tumors. *Cancer Res* **49:** 1909–1915, 1989.
2. Harrison SD Jr, Plowman J, Dykes DJ, Waud WR and

Griswold DP Jr, Preclinical antitumor activity of penclomedine in mice: Cross-resistance, schedule dependence, and oral activity against tumor xenografts in brain. *Cancer Res* **51:** 1979–1983, 1991.

3. Reid JM, Mathieson DA, Benson LM, Kuffel MJ and Ames MM, Murine pharmacokinetics and metabolism of penclomedine [3,5-dichloro-2,3-dimethoxy-6-(trichloromethyl)-pyridine, NSC 338720]. *Cancer Res* **52:** 2830–2834, 1992.
4. Reid JM, Benson JW, Benson LM, Kuffel MJ and Ames MM, *In vitro* metabolic activation and metabolism of penclomedine (NSC 338720). *Proc Am Assoc Cancer Res* **33:** 510, 1992.
5. Mosmann T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55–63, 1983.
6. Pandita TK and Hittelman WN, Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells. *Radiat Res* **130:** 94–103, 1992.
7. Kohn KW, Ewig RA, Erickson LC and Zwelling LA, Measurement of strand breaks and cross-links by alkaline elution. In: *DNA Repair: A Laboratory Manual of Research Procedures* (Eds. Friedberg EC and Hanawalt PC), pp. 379–401. Marcel Dekker, New York, 1981.
8. Zwelling LA, Michaels S, Erickson LC, Ungerlieder RS, Nichols M, and Kohn KW, Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-*m*-anisidide and adriamycin. *Biochemistry* **20:** 6553–6563, 1981.
9. Wassermann K, Zwelling LA, Mullins TD, Silberman LE, Andersson BS, Bakic M, Acton EM and Newman RA, Effects of 3'-deamino-3'(3-cyano-4-morpholinyl)doxorubicin and doxorubicin on the survival, DNA integrity, and nucleolar morphology of human leukemia cells *in vitro*. *Cancer Res* **46:** 4041–4046, 1986.
10. Bennett LL Jr, Smithers D, Hill DL, Rose LM and Alexander JA, Biochemical properties of the nucleoside 3-amino-1,5-dihydro-5-methyl-1,4,5,6,8-pentaazaacenaphthylene (NSC-154020). *Biochem Pharmacol* **27:** 233–241, 1978.
11. Kramer RA, Cytochrome P-450 dependent formation of alkylating metabolites of the 2-chloroethylnitrosoureas MeCCNU and CCNU. *Biochem Pharmacol* **38:** 3185–3189, 1989.
12. Liu L, Daniels JM, Stewart RK and Massey TE, *In vitro* prostaglandin H synthase- and monooxgenase-mediated binding of aflatoxin B_1 to DNA in guinea-pig tissue microsomes. *Carcinogenesis* **11:** 1915–1919, 1990.
13. Buege JA and Aust SD, Microsomal lipid peroxidation. *Methods Enzymol* **52:** 302–310, 1978.
14. Mazel P, Experiments illustrating drug metabolism *in vitro*. *Fundamentals of Drug Metabolism and Drug Disposition* (Eds. La Du NB, Mandel HG and Way EL), pp. 546–582. Williams & Wilkins, Baltimore, 1971.
15. Preussmann R, Arjungi KN and Ebers G, *In vitro* detection of nitrosamines and other indirect alkylating agents by reaction with 3,4-dichlorothiophenol in the presence of rat liver microsomes. *Cancer Res* **36:** 2459–2462, 1976.
16. Kroeger-Koepke MB, Michejda CJ, Roller PP and Keefer LK, Use of 3,4-dichlorobenzenethiol as a trapping agent for alkylating intermediates during *in vitro* metabolism of nitrosamines. *Cancer Res* **45:** 2973–2975, 1985.
17. Peters GJ, Laurensse E, Leyva A and Pinedo HM, A sensitive, nonradiometric assay for dihydroorotic acid dehydrogenase using anion exchange high performance liquid chromatography. *Anal Biochem* **161:** 32–38, 1987.
18. Gandhi V, Danhauser L and Plunkett W, Separation of 1-α-D-arabinofuranosyl 2-fluoroadenine 5'-triphosphate in human leukemia cells by high performance liquid chromatography. *J Chromatogr* **413:** 293–299, 1987.
19. Bennett LL Jr, Smithers D, Rose LM, Adamson DJ and Thomas HJ, Inhibition of synthesis of pyrimidine nucleotides by 2-hydroxy-3-(3,3-dichloroallyl)-1,4-naphthoquinone. *Cancer Res* **39:** 4868–4874, 1979.
20. Ludlum DB, Molecular biology of alkylation: An overview. In: *Antineoplastic and Immunosuppressive agents.* Part II (Eds. Sartorelli AC and Johns DG), pp. 6–17. Springer, New York, 1975.
21. Shulman LN, The biology of alkylating agent cellular injury. *Hematol Oncol Clin North Am* **7:** 325–335, 1993.
22. Comporti M, Three models of free radical-induced cell injury. *Chem Biol Interact* **72:** 1–56, 1989.
23. Durse L, Covey JM, Collins C and Sinha BK, DNA damage, cytotoxicity and free radical formation by mitomycin C in human cells. *Chem Biol Interact* **71:** 63–78, 1989.
24. Weitberg AB and Corvese D, Hydroxy- and hydroperoxy-6,8,11,14-eicosatetraenoic acids induce DNA strand breaks in human lymphocytes. *Carcinogenesis* **10:** 1029–1031, 1989.
25. Bartoszek A and Wolf CR, Enhancement of doxorubicin toxicity following activation by NADPH cytochrome P450 reductase. *Biochem Pharmacol* **43:** 1449–1457, 1992.
26. O'Brien PJ, Molecular mechanisms of quinone cytotoxicity. *Chem Biol Interact* **80:** 1–41, 1991.
27. Lakaschus G, Kruger H, Heese D and Loffler M, Evidence from *in vitro* studies that dihydroorotate dehydrogenase may be a source of toxic oxygen species. *Adv Exp Med Biol* **309A:** 361–364, 1991.
28. Kensler TW and Cooney DA, Chemotherapeutic inhibitors of the enzymes of the *de novo* pyrimidine pathway. *Adv Pharmacol Chemother* **18:** 273–354, 1980.