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PRELIMINARY COMMUNICATION

INITIAL MECHANISTIC STUDIES WITH MERBARONE (NSC 336628)

David A. Cooney, Joseph M. Covey, Gil J. Kang, Maha Dalal,
James B. McMahon and David G. Johns

Laboratory of Pharmacology and Experimental Therapeutics
Developmental Therapeutics Program
Division of Cancer Treatment
National Cancer Institute, NIH
Bethesda, Maryland 20205
U.S.A.

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The structure of merbarone [5-(N-phenylcarboxamido)-2-thiobarbituric acid] (NSC 336628) is shown in Fig. 1. This compound exhibits curative activity in L1210 leukemia and significant activity in several other murine tumors, when administered by either the intraperitoneal or oral routes (1). In view of this unusual activity, we have instituted studies into the mechanism of action of this structurally novel molecule.

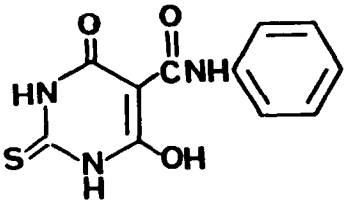

Figure 1: Merbarone [5-(N-phenylcarboxamido)-2-thiobarbituric acid].

Exposure of logarithmically growing cultures of L1210 cells, cultivated in RPMI 1630 medium, to graduated concentrations of merbarone (1-100 μM) resulted in progressive inhibition of proliferation. The median inhibitory concentration (IC_{50}) was 10 μM (range 9-13 μM, Fig. 2a). Cloning experiments revealed that a 16 hr exposure to 15 μM merbarone produced a 1 log decrease in viability (Fig. 2b), higher concentrations being progressively more cytocidal. A 2 hr exposure to this concentration produced no decrease in cloning efficiency; however, a 2 hr exposure to 50 μM merbarone resulted in 36% cell kill. Preliminary pharmacokinetic studies established that a single intraperitoneal injection of 2-^{14}C-merbarone (14.7 mCi/mmole) at its optimal therapeutic dose (50 mg/kg) (1) produced plasma concentrations of drug-derived radioactivity which approximated 10 μM for at least 4 hours (Fig. 2c). This result establishes that the concentrations of merbarone used *in vitro* can be achieved *in vivo*.

Cytofluorometric analysis revealed accumulation of drug-treated cells late in S-phase. This effect was detectable at 5 μM, and was pronounced at merbarone levels of 10 μM and higher. Parallel studies of the influence of merbarone on macromolecular synthesis revealed

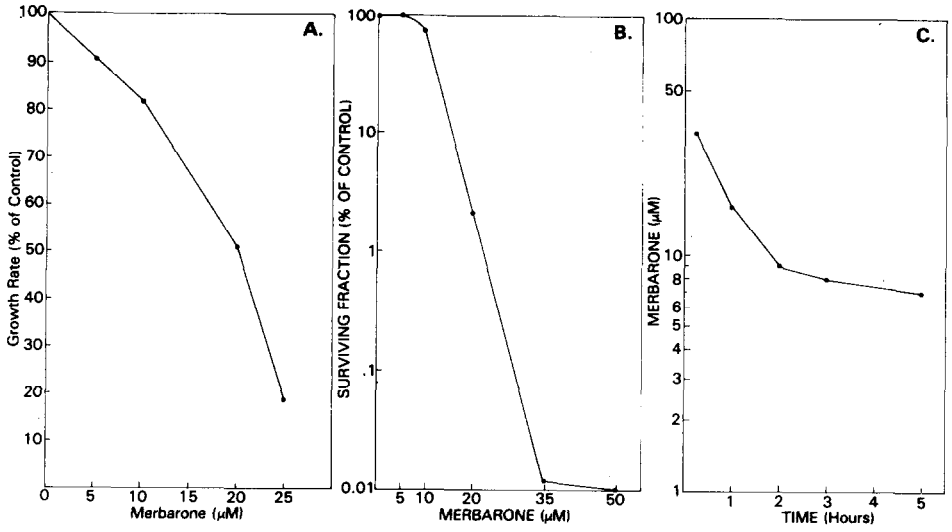

Figure 2a (left panel): Antiproliferative effect of merbarone on L1210 cells in culture. Logarithmically growing L1210 cells (RPMI 1630 medium; initial cell count 2.5 x 10^5 cells/ml) were exposed for 24 hr at 37° to merbarone at the concentrations indicated. DMSO (1%) was present in all treated and control cultures. Figure 2b (center panel): Effect of merbarone on the colony-forming ability of L1210 cells. Logarithmically growing L1210 cells (initial cell count 10^5 cells/ml) were exposed to merbarone at the concentrations indicated for 16 hr at 37°. The cells were then washed 3 times in fresh medium and the concentration was adjusted to 10^5 cells/ml. Cloning was carried out in RPMI 1630 medium supplemented with 20% fetal bovine serum and gentamicin, at a final agar concentration of 1 mg/ml. Colonies were counted at 2 weeks, and the results shown are averages of triplicate samples. Figure 2c (right panel): Plasma clearance of merbarone. 2-^{14}C-Merbarone (SA 14.7 mCi/mmole) was administered intraperitoneally to a BDF_1 mouse at a dose-level of 50 mg/kg. Blood samples (20-50 μl) were taken from the tail vein into heparinized vessels and ^{14}C-radioactivity determined in 5 μl aliquots of plasma.

that a 2 hr exposure to the drug failed to inhibit nucleic acid or protein synthesis significantly, and that even an 18 hr exposure to 25 μM merbarone reduced the rate of DNA synthesis by only 30% (Table 1). Transformation of human lymphocytes in the presence of 20 μM merbarone did not lead to prominent chromosomal aberrations.

Mechanistic studies with merbarone were initially guided by its hybrid structure - a conjugate of thiobarbituric acid and aniline, joined in amide linkage. Thiobarbituric acid has two well-defined biochemical properties: its reactivity with dialdehydes, notably malonaldehyde (2), and its ability to inhibit the membrane enzyme, γ-glutamyl transpeptidase (3). Merbarone, tested at 100 μM under published conditions (2,3), failed to function as a substrate or inhibitor in either system. Aniline is known to react with erythrocytes, producing methemoglobinemia (4). Merbarone incubated in vitro with human red cells (10-100 μM) failed to produce methemoglobin. Parenthetically, it is relevant that merbarone was also nitrobenzyl pyridine-negative (5) and thus is unlikely to function as an alkylating species - at least in its native state. The drug also was incapable of inhibiting the respiration of intact L1210 cells to a significant degree in vitro (at concentrations up to 50 μM) and so is unlikely to function as a direct respiratory inhibitor.

The pyrimidine moiety of merbarone prompted studies of its possible role as an antimetabolite. However, exposure of L1210 cells to 10 μM drug for 24 hr failed to engender significant perturbation of their nucleotide or amino acid pools; a 5 hr exposure to 5 and 10 μM merbarone also was without influence on the cellular content of PRPP. In addition, none of the customary preformed purines or pyrimidines (singly, and in combination) or amino acids, tested at their maximally tolerated concentrations, proved capable of reversing, even partially, merbarone's cytotoxicity, thus making it unlikely that the drug behaves as a classical antimetabolite.

Table 1. Effect of merbarone on incorporation of radiolabeled precursors into DNA, RNA and protein

Merbarone concentration (μM)	Exposure time (hr)	Cell density (Cells/ml)	Incorporation (% of control) of TdR	UR	L-valine
0	2	3.1×10^5	100	100	100
10	2	3.0×10^5	132	86	115
25	2	2.9×10^5	82	76	158
0	18	6.3×10^5	100	100	100
10	18	4.7×10^5	123	168	118
25	18	3.1×10^5	70	273	183

L1210 cells in logarithmic growth at 37° in RPMI 1630 medium (initial cell count 3.0×10^5 cells/ml) were exposed to the indicated concentrations of merbarone for 2 or 18 hr, after which they were pulsed with 10 μCi of ^{3}H-thymidine (SA 6.7 Ci/mmol), uridine (SA 27 Ci/mmol) or L-valine (SA 10 Ci/mmol) for 30 min. The cell suspensions were then collected on saline-soaked glass fiber filters, washed once with normal saline, three times with cold 10% trichloroacetic acid and twice with absolute ethanol. The filters were then counted and the incorporation of precursors expressed as nCi incorporated/10^6 cells/hr. In the 2 hr control cells, 73.7, 93.7 and 4.8 nCi of labeled thymidine, uridine and L-valine were incorporated per 10^6 cells per hr, respectively. The 18 hr control cells incorporated the same precursors at rates of 68.0, 26.0 and 3.1 nCi/10^6 cells/hr.

Attempts to determine whether merbarone might bind to DNA were complicated by its affinity for conventional cellulose dialysis tubing, and a variety of other polymeric materials. However, when nucleic acids were extracted from L1210 cells exposed to 100 μM [^{14}C]merbarone for 3 hours, only negligible radioactivity was associated with these macromolecules. The drug also appeared not to displace the intercalating compound ethidium bromide from calf thymus

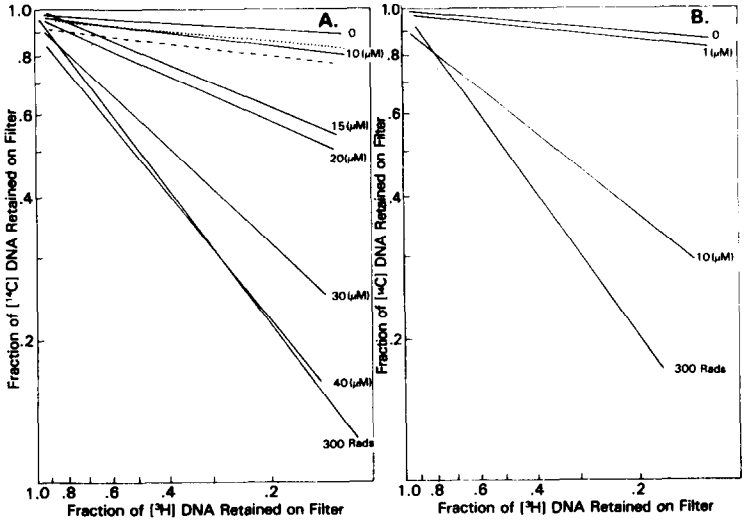

Figure 3: Effect of merbarone on the alkaline elution profile of DNA from L1210 cells. Panel A (left): L1210 cells grown in RPMI 1630 medium were labeled for 18 hr with ^{14}C-thymidine (0.02 μCi/ml), then washed and resuspended in fresh medium for 1 hr. The prelabeled cells were then treated with merbarone at the indicated concentrations (μM) for 16 hr. At the end of the drug exposure period, cells were washed with fresh medium and aliquots of ca. 10^6 cells resuspended in PBS were layered onto polycarbonate filters (0.8μ). Alkaline elution was carried out according to the procedure of Kohn et al. (6). Untreated internal standard cells, labeled with ^{3}H-thymidine and exposed to 300 rads of γ-irradiation at 0°C, were added to each sample and carried through the alkaline elution procedure (---: thiobarbituric acid control, 40 μM; ···: formanilide control, 40 μM). Panel B (right): cells were labeled with ^{14}C-TdR and treated with merbarone simultaneously. Curves represent the mean of 2-4 determinations.

DNA, as determined by fluorescence spectrometry. However, when L1210 cells were exposed to merbarone (10-50 μM) for 16-24 hr and their DNA examined by alkaline elution (6), significant numbers of dose-related DNA single-strand breaks (SSB) were demonstrable (Fig. 3). SSB were produced in both [^{14}C]thymidine pre-labeled cells (Fig. 3A) and in cells exposed to [^{14}C]thymidine and merbarone simultaneously (Fig. 3B), although the latter protocol resulted in higher SSB frequencies. Proteinase-K did not materially alter the elution pattern of DNA damaged by merbarone, suggesting that the SSB are not protein-associated. Moreover, initial experiments demonstrated no formation of DNA-protein crosslinks by this compound.

In order to examine the rate at which cells repaired the merbarone-induced lesions in their DNA, alkaline elution analyses were conducted at representative time-points after removal of the drug. These experiments demonstrated that about 24 hr were required for the repair of 50% of the lesions.

Although it is not clear from these experiments whether merbarone itself, or a metabolite, produces the DNA damage observed by alkaline elution, this damage may well be associated with the therapeutic action of the drug inasmuch as it is seen at comparatively low concentrations of merbarone.

Recently, a number of non-alkylating antitumor drugs have been shown to produce SSB (7-11). Some are incorporated into DNA (FUdR, Ara-C, thioguanine) while others are not (adriamycin, methotrexate). Several mechanisms have been proposed to explain these findings, including glycosylase-mediated formation of apyrimidimic sites (FUdR), interactions with repair processes (thioguanine, methotrexate) and free-radical mediated DNA damage (adriamycin). It is presently unclear if merbarone, an agent which does not itself appear to interact with DNA, produces DNA lesions (perhaps via a reactive metabolite in vivo) by any of these mechanisms. It will remain for future studies to pursue the lines of investigation suggested by the data reported herein.

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