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ESTROGEN-BRIDGED PURINES: A NEW SERIES OF ANTI-TUMOR AGENTS WHICH ALTER CELL MEMBRANE PROPERTIES

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An estrogen-bridged adenine derivative was equitoxic to both the P388 murine leukemia and an adriamycin-resistant subline, P388/ADR. The drug rapidly altered several P388 and P388/ADR membrane properties resulting in impaired nucleoside transport and increased membrane hydrophobicity. Resistance to anthracyclines in P388/ADR is associated with an operational barrier to drug retention which was reversed by exposure to the estrogen-bridged adenine derivative. These results suggest further exploration of the estrogen-bridged purines as chemotherapeutic agents.

The estrogen-bridged purines represent a new series of prospective anti-tumor agents whose syntheses has been described (1). An initial characterization of one such drug, an estrogen-bridged adenine derivative (EBA) shown in (Fig. 1), is described here. The results have interesting implications with regard to chemotherapy of anthracycline-resistant tumors.

MATERIALS AND METHODS

Cell lines The P388 murine leukemia and an adriamycin-resistant subline (P388/ADR) were obtained by Dr. R.K. Johnson, Arthur D. Little Corp. Cambridge, MA. The derivation of P388/ADR and its drug responsiveness pattern has been described (2). These cell lines were maintained in cell culture using Fischer's medium supplemented with 10% horse serum and 1 uM mercaptoethanol.

Drugs. EBA was made up in DMSO and stored at 20°. Thymidine, uridine and cycloleucine, labeled with ^{14}C, were obtained from New England Nuclear Corp., Boston, MA. All were diluted with carrier to obtain 3-5 mM stock solutions containing approx. 5000 counts/min/ul. [^{14}C]-Daunorubicin (31 Ci/mole, labeled at carbon-14) was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH. Purity was >99% by TLC assay.

Growth studies. Drug toxicity studies were carried out by addition of graded amounts of EBA to cell cultures for 1 hr. The cells were collected, washed and diluted into a mixture of growth medium + 2% agar. Colonies of control vs. treated tubes were counted after 7 days. At an LD_{50} drug concentration, there was a 50% decrease in cell viability.

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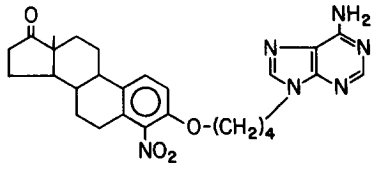

Fig. 1: Structure of the methylene-bridged estrogen derivative of adenine (EBA) used in these studies.

Uptake studies. These were carried out at cell densities of 5 x 10^6/ml (7 mg/ml, wet weight) in HEPES-buffered MEM-Eagle's medium (GIBCO, Grand Island, NY). Cells were treated with 3 uM EBA for 5 min at 37°, resuspended in fresh medium and worked up as specified below. Accumulation of labeled substrates was monitored by liquid scintillation counting.

1. To measure incorporation of nucleosides into nucleic acid, cells were incubated with 30 uM labeled uridine or thymidine for 5 min, collected by centrifugation, and washed twice with 0.2 M perchloric acid.

2. Nucleoside transport was measured at 10° to minimize transformation into nucleotides and incorporation into nucleic acid (4). Cells were incubated with 30 uM [^{14}C]-uridine for 5 min, collected by centrifugation and washed with a $HgCl_2$-NaI solution to prevent subsequent loss of intracellular nucleoside (4).

3. Accumulation of daunorubicin was measured at 37° over 30 min in media containing 0.3 ug/ml of labeled drug. Cells were collected by centrifugation, washed once with cold 0.9% NaCl, and intracellular radioactivity measured.

4. Cycloleucine uptake was measured after 5 min incubations at 37° in medium containing 10 uM of this non-metabolizable amino acid. Cells were washed with 0.9% NaCl at 0° and intracellular radioactivity measured. Data are reported in terms of the distribution ratio achieved: intracellular/extracellular concentration.

5. Studies on daunorubicin exodus were carried out using cells loaded with labeled drug as outlined above, but in glucose-free medium containing 10 mM NaN_3, to temporarily inhibit drug exodus. The drug-loaded cells were suspended in fresh medium at 37° containing specified levels of EBA and the loss of label monitored over 30 min.

Partitioning studies. These measurements were carried out using a two-phase system containing 5%(w/v) Dextran T-500 (Pharmacia, Piscataway, NJ, lot 78630), 4% (w/v) PEG molecular weight 6000 (Sigma Chemical Co., St. Louis MO), 140 mM NaCl, 10 mM sodium phosphate buffer pH 7.0 and 8%-esterified PEG palmitate (5). The concentration of the latter was 0.00006% in experiments involving P388 cells, and 0.0002% when P388/ADR cells were partitioned. This causes an approx. equal partitioning of both cell lines into the upper phase. The P388/ADR has an inherently less hydrophobic cell surface than does P388 (6). Adjusting the amount of the PEG-palmitate can correct for this difference (7). The partition coefficient (PC) = % total cells found in the upper phase after 20 min of settling at 22°.

RESULTS

Growth studies. A 60 min exposure to a 3 uM level of EBA decreased viability of P388 or P388/ADR cultures by 50%.

Alterations in transport and incorporation. Data are shown in Table 1. A 5 min exposure to 3 uM EBA inhibited incorporation of labeled

Table 1

EFFECT OF GRADED LEVELS OF EBA ON TRANSPORT AND BIOSYNTHESIS

Measurement	P388		P388/ADR	
	Control	+EBA	Control	+EBA
TdR incorporation into DNA	11.9[1]	3.8	12.2	4.1
UR incorporation into RNA	9.2[1]	4.3	8.1	3.3
UR uptake	11.5[2]	4.9	8.4	3.7
CL uptake	2.2[3]	2.1	2.3	2.4
DNR uptake	540[4]	550	160	400
PC	38[5]	65	11	35

The level of EBA was 3 uM. Replicate experiments yielded results within ±15% of numbers shown.

[1]Thymidine and uridine incorporation: nMoles/ul cell water.
[2]Total uridine uptake: nMoles/ul cell water at 10°.
[3]Cycloleucine accumulation (distribution ratio).
[4]Daunorubicin accumulation: pmoles/10^8 cells.
[5]Partition coefficient: % total cells in upper phase.

uridine or thymidine into nucleic acid by approx. 50%. Data are expressed in terms of nmoles incorporation/ul cell water to facilitate comparison with uridine transport measurements.

Other transport studies. A 5 min exposure of P388 or P388/ADR cells to EBA did not affect subsequent capacity of cells to carry out concentrative uptake of the non-metabolized amino acid cycloleucine. Accumulation of daunorubicin by P388/ADR cells was promoted >3-fold by EBA. This cell line exhibits impaired anthracycline accumulation (8-10), which is antagonized by EBA. Uptake of labeled daunorubicin by P388 cells was not affected by EBA. We also examined the effect of EBA on exodus of labeled daunorubicin from P388/ADR cells loaded with the drug as described in Ref. 8. Daunorubicin efflux was abolished by 30 uM EBA, and the exodus rate was decreased by 50% by 3 uM EBA.

Partitioning studies. A 5 min exposure of P388 or P388/ADR cells to EBA resulted in a increased number of cells partitioning into the upper phase (Table 1). Such a result has been interpreted to indicate increased hydrophobicity of the cell membrane (5).

DISCUSSION

In this study, we found EBA to be equitoxic to P388 cells and the adriamycin-resistant sub-line (P388/ADR). A brief exposure to a 3 uM level of this drug resulted in a 50% inhibition of incorporation of uridine or thymidine into nucleic acid; this effect was associated with inhibition of nucleoside transport. EBA did not affect concentrative uptake of the non-metabolized amino acid cycloleucine, but did promote retention of daunorubicin by the anthracycline-resistant P388/ADR cells. Impaired retention of anthracyclines has been associated with drug resistance (8-10). These transport data show that EBA is not simply a chaotropic agent, but causes selective alterations in cell membrane properties.

The lack of cross-resistance between EBA and anthracyclines, together with the observed promotion of anthracycline accumulation by P388/ADR suggest that combinations of estrogen-bridged purines and anthracyclines might produce additive toxicity, while suppressing development of anthracycline-resistant clones of tumor cells.

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