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PRELIMINARY COMMUNICATIONS

MODE OF ACTION OF CALCIUM ANTAGONISTS WHICH ALTER ANTHRACYCLINE RESISTANCE

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In transplantable tumor cell lines, a common mode of resistance to several natural products, including the anthracyclines, the Vinca alkaloids and actinomycin D, involves an outward transport process which limits drug accumulation to sub-lethal levels (1-5). This transport system can be inhibited, and drug responsiveness promoted, by calcium antagonists of two structural classes: papaverine analogs, e.g. verapamil, and dihydropyrimidines, e.g. nitrendipine (6-9). This report describes studies on the two classes of calcium antagonists named above. The data indicate that verapamil is a substrate for the outward transport system and promotes anthracycline accumulation via competition for exodus. Nitrendipine is not a substrate for this outward transport system and may act via a 'chaotropic' mechanism.

Methods: Patterns of drug responsiveness of the P388 murine leukemia and an adriamycin resistant subline (P388/ADR) have been described (10). Both cell lines were grown in Fischer's Medium supplemented with 10% horse serum and 1 μM mercaptoethanol. [^{14}C]-Daunorubicin (DNR), labeled at position 14 (31 Ci/mole), was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH; [^{3}H]-verapamil (60 Ci/mmol) and [^{3}H]-nitrendipine (70 Ci/mmole) were purchased from the New England Nuclear Corp. Purity of labeled compounds was >99% by chromatographic assays carried out as specified by the suppliers. These employed silica G TLC plates; solvents were butanol:acetic acid:water (5:1:1) for verapamil, chloroform:acetone (99:1) for nitrendipine, and chloroform:methanol:acetic acid (88:9:3) for DNR.

Exponentially-growing cells were collected by centrifugation and suspended in 1 ml portions of Fischer's medium with 20 mM HEPES (pH 7.2) replacing sodium bicarbonate. In some experiments, glucose (normally 1 mg/ml) was omitted, and 10 mM NaN_3 added. Incubations were carried out at 37°, at cell densities of 2 x 10^6/ml (7 mg/ml wet weight). Calcium antagonists were added at specified concentrations, followed 5 min later by labeled DNR, verapamil or nitrendipine (total volume of additions <20 μl). Incubations were terminated by centrifugation (500 x g, 30 sec) and cell pellets were washed once with cold 0.9% NaCl. Intracellular accumulation of radioactivity was determined by liquid scintillation counting. Drug levels are reported in terms of μmoles/kg cells (wet weight).

Table 1. Drug Accumulation Studies*

Cell Line	Medium	Additions	Transport substrate		
			DNR	VER	NTR
P388	glucose	--	80	30	750
P388/ADR	glucose	--	16	6.2	740
P388	NaN_3	--	84	33	730
P388/ADR	NaN_3	--	80	27	750
P388	glucose	VER	81	31	745
P388/ADR	glucose	VER	32	13	740
P388	glucose	NTR	80	32	750
P388/ADR	glucose	NTR	31	13	750

*Accumulation units: μmoles drug/kg cells. Cells were incubated in medium containing 1 mg/ml glucose or 10 mM sodium azide (Column 2), for 30 min at 37° with 2 μM [^{14}C]-DNR, or for 5 min with 3 μM [^{3}H]-verapamil (VER) or 50 μM [^{3}H]-nitrendipine (NTR). Non-labeled drugs (3 μM verapamil or 50 μM nitrendipine) were also present as specified in Column 3. These results represent the mean of 5 experiments in which we observed a range of ±10% of values shown.

For drug exodus studies, cells were loaded with labeled drugs in glucose-free medium containing sodium azide to (reversibly) inhibit outward transport. The cells were then suspended in fresh medium (containing glucose) at 37° and loss of label monitored. Drug biotransformations were examined using methanol homogenates of cells previously incubated with labeled drugs, with the TLC systems described above.

Results and Discussion: Studies on drug transport are summarized in Table 1. In the presence of glucose, the steady-state accumulation of DNR was impaired in P388/ADR (column 4). This impairment was not observed in glucose-free medium containing sodium azide. In medium containing glucose, the addition of 3 μM verapamil or 50 μM nitrendipine doubled the steady-state DNR accumulation by P388/ADR cells. Neither replacement of glucose with sodium azide nor addition of the calcium antagonists affected the accumulation of DNR by P388 cells.

The steady-state verapamil concentration in P388 cells, loaded in medium containing 3 μM drug, was 30 μmoles/kg cells. This result was not altered by replacement of glucose with azide, nor by addition of nitrendipine or (carrier) verapamil. Under similar conditions, the steady-state accumulation of labeled verapamil by P388/ADR cells was 6.2 μmoles/kg, and drug accumulation was promoted by replacement of glucose with azide in the incubation medium, or by addition of nitrendipine or non-labeled verapamil (Table 1, column 5). When P388 cells containing 30 μmoles/kg of verapamil were suspended in fresh medium at 37°, 75% of the initial intracellular pool was rapidly lost, $T_{1/2}$ = 0.5 min. The remaining verapamil was tightly bound ($T_{1/2}$ > 60 min). In a similar study with P388/ADR cells, 90% of the accumulated drug was rapidly lost, $T_{1/2}$ = 0.1 min.

Nitrendipine accumulation was identical in P388 and P388/ADR cells. The steady-state was reached within 1 min of incubation at 37° (drug level = 1-100μM), a result not affected by replacing glucose with NaN_3, or by adding verapamil (10-100 μM). The intracellular/extracellular nitrendipine distribution ratio was approx. 15, at all extracellular drug levels examined

(1-100 μM). Suspension of nitrendipine-loaded cells (P388 or P388/ADR) in fresh medium resulted in loss of 75% of accumulated drug, $T_{1/2}$ = 1.5 min. No further exodus of label occurred during an additional 30 min, nor was this efflux pattern affected by altering the drug loading concentration over 5-100 μM, or by adding 5-100 μM non-labeled nitrendipine to the medium during exodus measurements.

In other studies, using cells pre-loaded with DNR in the presence of sodium azide, we found that the initial phase of drug exodus from P388/ADR cells had a $T_{1/2}$ of approx. 1.6 min; this value was increased to 4 min when 3 μM verapamil or 50 μM nitrendipine was present during exodus measurements. DNR was lost much more slowly from P388 cells ($T_{1/2}$ = 60 min), a result unaffected by the presence of either calcium antagonist.

We found no evidence of drug biotransformations in either P388 or P388/ADR cells, during 10-30 min incubations, except for a minor conversion of DNR (<20%) to daunorubicinol. The preferential exodus of DNR and verapamil from P388/ADR cells was therefore not related to drug metabolism to more readily exported products.

Several lines of evidence suggest that verapamil and DNR compete for an energy-dependent outward transport system in P388/ADR cells. When a steady-state was reached, P388/ADR cells had accumulated substantially less verapamil than did P388 cells. Verapamil accumulation by P388/ADR cells was promoted by incubation in glucose-free medium supplemented with sodium azide. In the presence of glucose, efflux of verapamil was more rapid from P388/ADR than from P388 cells. Similar results obtained with DNR, here (Table 1) and in other laboratories (1-5), have been interpreted to indicate the presence of an outward transport system in P388/ADR cells, associated with impaired DNR accumulation. We have also shown that verapamil promoted net DNR accumulation and slowed DNR exodus from pre-loaded P388/ADR cells. In a similar study, Skovsgaard used such criteria to establish that the DNR analog N-acetyldaunorubicin promoted DNR accumulation via competition for an outward transport system (11).

With regard to nitrendipine, we found no significant differences between P388 and P388/ADR cells when we measured steady-state drug accumulation or rates of drug loss from pre-loaded cells. These results indicate that nitrendipine is not a substrate for the enhanced outward drug transport system demonstrable in P388/ADR cells. The intracellular concentrations of verapamil and nitrendipine required to double steady-state DNR accumulation by P388/ADR cells were 6.2 and 740 μM/kg respectively. The high level required of the latter agent suggests that nitrendipine, and its structural analogs, may affect anthracycline transport via a 'chaotropic' mechanism.

In studies described in abstract form (12), to be published in detail elsewhere, we found that neither verapamil nor nitrendipine affected ^{45}Ca fluxes in P388 or P388/ADR cells, and that omission of Ca^{++} from incubation media did not affect kinetics of DNR transport by either cell line. These results, together with the present data, indicate that the effects of these calcium antagonists on DNR transport result from interactions with a membrane transport system, and not via modulation of calcium fluxes.

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