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Effects of phorbol esters on doxorubicin transport systems

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When neoplastic cells are selected for resistance to a single anti-tumor agent, the resulting sublines are sometimes cross-resistant to a variety of other agents of apparently unrelated structure. This phenomenon is termed "multi-drug resistance" (MDR*), and it has been described in several reports and recent reviews [1–5]. Cells exhibiting the MDR phenotype have several characteristics: a new membrane "P-glycoprotein" having a molecular weight of approximately 170 kD; cross-resistance to anthracyclines, actinomycin D, Vinca alkaloids and certain other natural or synthetic products; and an outward drug-transport system which limits accumulation of cytotoxic agents. Outward transport may be mediated by the 170 kD glycoprotein which bears a close resemblance to transport proteins in bacterial systems [6, 7]. This transport system can be inhibited by a variety of drugs including calcium-channel antagonists [8–11] and calmodulin inhibitors [12, 13].

A suggestion that phosphorylation of "P-glycoprotein" is involved in the maintenance of MDR was provided by *in vitro* studies [14–16]. While P-glycoprotein phosphorylation can be promoted by calcium-channel antagonists, calmodulin inhibitors and phorbol esters, sites of phosphorylation can differ, depending on the stimulus [17]. In one cell line, treatment with phorbols resulted in decreased phosphorylation of the 170 kD glycoprotein [18]. The role of phorbol ester-induced promotion of P-glycoprotein phosphorylation is not yet clear. Treatment of one drug-sensitive tumor cell line with a phorbol ester promoted vincristine resistance but there was no phorbol-induced alteration in drug responsiveness in a vincristine-resistant subline [19].

In this study, we examined the effects of several phorbol esters on anthracycline transport and cytotoxicity in the P388 murine leukemia cell line and an anthracycline-resistant subline, P388/ADR. The properties of these cell lines have been described before; P388/ADR cells exhibit the pertinent characteristics of the MDR phenotype [20, 21].

Cells were grown in sealed flasks using Fischer's medium (GIBCO, Grand Island, NY) supplemented with 10% horse serum. [^{14}C]DNR (30 Ci/mol) was provided by the Division of Cancer Treatment, NCI. [20-^{3}H(N)]-Phorbol,12-13-dibutyrate (550 Ci/mol) was purchased from New England Nuclear, Boston, MA. The 4α- and 4β-phorbol didecanoate esters were obtained from the Sigma Chemical Co., St. Louis, MO. Incubations were carried out in growth medium buffered with 20 mM HEPES at pH 7.2.

Cell cultures were exposed to 3–200 nM concentrations of different phorbol esters for 10 min to 24 hr, at 37°, and then suspended at a density of 7 mg (wet weight)/ml in growth medium buffered with 20 mM HEPES at pH 7.4. This medium contained the same levels of phorbol esters as were present during the first incubation. The cells were then treated with 0.1 μM [^{14}C]DNR for 0.3 to 90 min at 37°. In some studies verapamil (5–20 μM) was present during the second incubation. Drug exodus studies were carried out after 90-min incubations; cells were suspended in fresh medium (containing phorbol esters if these were present in the first incubation), and intracellular anthracycline concentrations were measured over an additional 60 min at 37°. Cytotoxicity of DNR was measured by a clonogenic assay [22] after a 2-hr exposure to phorbol esters, followed by a 1-hr treatment with specified anthracycline levels. Colonies were counted after 7 days. Cloning efficiency of P388 cells was approximately 80%.

We observed a 40% inhibition of steady-state DNR uptake by P388 cells upon treatment with 4β-phorbol ester for 120 min (Table 1). This effect was not seen after shorter (10–30 min) or longer (8–24 hr) incubations with the phorbol ester. The optimal phorbol concentration was 10 nM; we observed no effect at 3 nM, and no additional effects on DNR uptake were observed when the phorbol concentration was increased. Treatment with phorbol for 10 min to 24 hr had no effect on DNR accumulation by P388/ADR. The (inactive) 4α-phorbol ester did not alter the time-course of DNR accumulation by either cell line. Inhibition of DNR uptake by the 4β-phorbol ester (in P388 cells) was not reversed by 5–25 μM verapamil (Table 1).

Treatment with 10 nM 4β-phorbol ester for 2 hr protected P388 cells from the cytotoxic effects of a 1-hr exposure to DNR (Table 2). But when the time of exposure to DNR was increased to 24 hr, the 2-hr phorbol treatment did not alter DNR toxicity (data not shown).

The effect of phorbol esters on DNR accumulation by P388 cells appears to derive from inhibition of uptake, rather than on activation of an outward transport system (Fig. 1). Even at the earliest time points measured, uptake of DNR in phorbol-treated P388 cells was impaired. Accumulation of DNR by phorbol-treated P388 cells was not enhanced by use of glucose-free medium containing 10 mM sodium azide, nor was DNR efflux from P388 cells altered by treatment with the phorbol ester (Fig. 1). In contrast, uptake of anthracyclines by P388/ADR cells was inhibited by azide, an effect traced to inhibition of energy-dependent outward drug transport [20, 21].

To examine the relative numbers of phorbol ester binding sites on P388 vs P388/ADR cells, we incubated cultures for 2 hr with 10 nM labeled phorbol dibutyrate. Binding was proportional to phorbol concentration and reached a steady-state at this time point. Under these conditions, the binding of labeled phorbol was 2-fold greater in P388 cells, but increasing the level of 4β-phorbol ester to 20 nM did not affect any result described above.

* Abbreviations: ADR, adriamycin; DNR, daunorubicin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and MDR, multi-drug resistance.

Table 1. Effect of phorbol esters (PE) on DNR transport

Substrate	DNR uptake (pmol/10^6 cells)			
	P388		P388/ADR	
	−PE	+PE	−PE	+PE
DNR	6.2 ± 0.4	3.6 ± 0.2	0.91 ± 0.1	0.74 ± 0.08
DNR + verapamil	5.7 ± 0.5	3.2 ± 0.2	5.0 ± 0.4	5.2 ± 0.3

Cells were incubated for 90 min with 0.1 μM labeled DNR + (if specified) 5 μM verapamil. Use of 10–25 μM verapamil did not alter the results shown. Where indicated (+PE), cells had been exposed previously to 10 nM 4β-phorbol ester for 2 hr at 37°. These experiments were carried out three times, and the numbers shown above represent the mean values ± SD.

Table 2. Effect of phorbol esters on DNR-induced cytotoxicity

DNR (M)	Surviving colonies (% control)	
	Untreated	Treated
2×10^{-7}	92 ± 3	96 ± 3
5×10^{-6}	12 ± 2	88 ± 4
2×10^{-6}	1.3 ± 0.5	9 ± 2

P388 cells were treated with 10 nM 4β-phorbol ester for 2 hr, and then with specified levels of DNR for 1 hr. Untreated cultures received no phorbol ester. Cultures were diluted so that the plates contained 75–125 colonies. Data represent percent control (no drugs) growth. Values are means ± SD, N = 5.

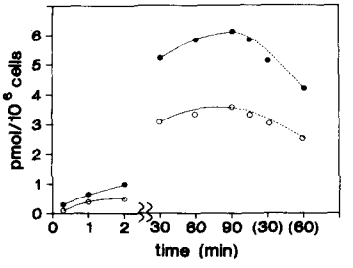

Fig. 1. Time–course of accumulation and exodus of labeled DNR in P388 cells at 37°. Left: control cells (●) and cells treated with 10 nM 4β-phorbol ester for 2 hr (○) were employed. Right: after 90 min of incubation, cells were suspended in fresh medium and loss of labeled drug was measured (dashed lines). Data shown represent the mean of three experiments (SD < 10% of results reported).

Just as calcium-channel antagonists and other agents can modify anthracycline transport by a variety of mechanisms [10], interactions between 4β-phorbol esters and anthracycline transport are diverse and can affect anthracycline responsiveness via different mechanisms. Although promotion of the phosphorylation of 170 kD glycoprotein in MDR cells by phorbol esters, calmodulin inhibitors and calcium-channel antagonists has been described, the site of these phosphorylations may be different [17]. Moreover, in some systems, treatment with phorbol esters can result in impaired phosphorylation of the 170 kD glycoprotein species [18]. The most common effect of phorbol ester treatment is the appearance of a transient increase in anthracycline resistance in drug-sensitive cell lines [19, 23 and this report], which can sometimes be reversed by verapamil [23], although not in the present case. While the phorbol esters mimic the enhanced membrane glycoprotein phosphorylation associated [15–17] with exposure of neoplastic cells to calcium-channel antagonists, we find a different consequence: impaired anthracycline influx in a drug-responsive cell line.

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