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Short Communication

BEHAVIOR OF *N*-ACYLATED DAUNORUBICINS IN MDR1 GENE TRANSFECTED AND PARENTAL CELLS

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Abstract—The substrate specificity of the P-glycoprotein (P-170), a multidrug transporter, was studied using *N*-acylated daunorubicin derivatives and four MDR1 cDNA transfected cell lines. Results showed that *N*-acetyl-daunorubicin is a substrate, but the longer fatty acid derivatives, *N*-octanoyl and *N*-dodecanoyl daunorubicins, are not. This conclusion was reached by flow cytometric drug uptake assay, cell proliferation assays, and confocal microscopy. It was concluded that the longer fatty acid derivatives interact with plasma membranes in a way that affected P-glycoprotein function.

Key words: multi-drug resistant cancer cells; *N*-acyl-daunorubicins; cell proliferation; fluorescence; confocal microscopy

Certain N-acyl-Ds§ have been shown to have antitumor activity against P388 leukemia in mice [1,‖]. We investigated the behaviors of two of these N-acyl-Ds, N-C8-D and N-C12-D, along with that of N-acetyl-D with respect to P-glycoprotein, the product of MDR1 gene-expressing cells. Overexpression of P-glycoprotein is one cause of resistance to chemotherapeutic agents by cancer cells [2, 3]. P-glycoprotein decreases intracellular concentration of these agents, possibly by facilitating active efflux [4, 5]; however, the mechanism by which this active efflux occurs is not well known [6]. Speculation that substrates of P-glycoprotein need to be lipophilic and pumped out of the plasma membrane remains to be clarified [7, 8]. We have used the above-mentioned N-acyl-Ds to investigate this possibility.

Materials and Methods

Cells and proliferation assay. Four cell lines, leukemia L1210, lymphoma L5178Y, murine fibroblast NIH3T3 and human melanoma FEMX, were transfected with a recombinant MDR1 retroviral vector (pHa MDR1/A) as described by published protocols [8–10]. Cells expressed P-glycoprotein as assessed by MRK16-FITC labeling and flow cytometry (not shown). Cells were seeded at 0.25×10^6 cells/mL in 24-well plates, and proliferation was assessed after 24 hr of incubation at 37° and 5% CO_2 by cell counting using a Coulter counter. Proliferation was also assessed after 48 and 72 hr. Results of these time points gave an assessment of proliferation comparable to that of the 24-hr assay. Ten minutes after CsA was added, D or its derivatives were added in DMSO, dissolved to concentrations not to exceed 0.2% (v/v) in the medium; this amount of DMSO had no effect on cell growth. Concentrations of D, N-acyl-Ds and CsA were selected by preliminary experiments to obtain clear differences with the number of applied cells in both the proliferation and the drug uptake assays. D and CsA were of FDA standards; preparation and characterization of N-acyl-Ds were described earlier [11].

Flow cytometric drug uptake assay and confocal microscopy. Uptake of drugs (1 μg/mL) by the parental and MDR1 cells was assessed by flow cytometry as described earlier, using a 488 nm excitation wavelength and a FACScan instrument (Becton Dickinson) [8]. When R123 was assayed together with N-acyl-D, fluorescence compensation was applied so that the fluorescence intensity of D was equal to that of the cell-autofluorescence in the FL-1 channel. Confocal microscopic studies were performed with a BioRad MR C 600 confocal microscope, and a 63x (N.A. = 1.4, oil) phase contrast objective, essentially as described earlier [7]. The Z axis position was fixed so that the optical section clearly transected the nuclei in phase contrast. Fluorescence was excited by the use of a 2 W air-cooled argon ion laser at 488 nm, and a 10% neutral density filter. Images were acquired by accumulating 25 scans using the Fast Photon Counting setup. Subsequently, the corresponding phase contrast images were acquired by accumulating 20 Kalman averaged images with PMT2.

Octanol-water partition of drugs. Partition coefficients for D, N-acetyl-D, and N-C8-D were determined in *n*-octanol and KH_2PO_4-NaoH, 0.05 M, pH 5.8, 7.0 and 7.8 buffers, at 25°. The concentration of drugs was 0.1 mM, and the distribution in the two phases was determined spectrophotometrically at 234 nm. Results were expressed as percent of distribution of the compounds in the octanol phase.

Results and Discussion

The antiproliferative activities of the N-acyl-Ds and D were compared in suspension-grown L1210 cells. Cells exposed to different concentrations of the compounds were counted after 24 hr. Typical results (N = 3) are shown in Fig. 1. Higher concentrations of D and N-acetyl-D (IC_{50} = 120 and >10,000 nM, respectively) were needed to inhibit the growth of L1210 MDR cells than were needed to inhibit the growth of the parental cells (IC_{50} = 35 and 1200 nM, respectively). In contrast, N-C8-D and N-C12-D suppressed the growth of both of these cells at equal concentrations (IC_{50}, N-C8-D = 500, N-C12-D parental = 4500, and MDR = 5500 nM, with no significant

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§ Abbreviations: D, daunorubicin; N-acyl-D, *N*-acyl-daunorubicin; N-C8-D, *N*-octanoyl-daunorubicin; N-C12-D, *N*-dodecanoyl-daunorubicin; MDR, multi-drug resistance; FITC, fluorescein isothiocyanate; CsA, cyclosporin A; and R123, rhodamine-123.

‖ Aszalos A, unpublished results. NCI evaluation in the P388 lymphocytic leukemia models. Example: D, T/C = 176 (1 mg/kg); N-dodecanoyl-D, T/C = 158 (12 mg/kg).

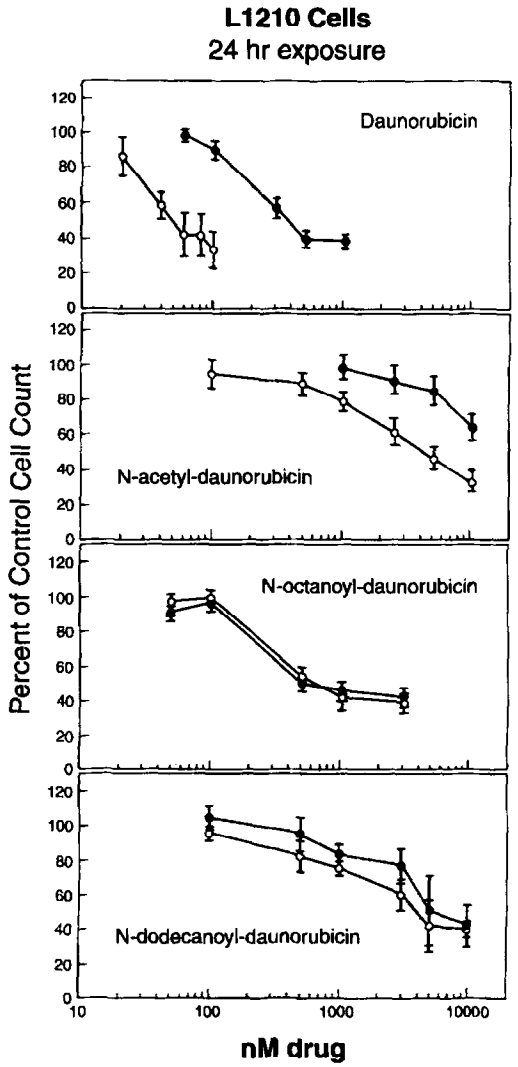

Fig. 1. Antiproliferative effect of daunorubicin (D) and N-acyl-Ds in L1210 MDR (●) and parental (○) cell cultures. The initial cell concentration was 0.25 × 10^6/mL. The maximum concentration of DMSO (<0.2%, v/v) had no effect on cell growth. A typical experiment (N = 3) is shown; error bars represent the SD of the quadruplicate measurements for each concentration.

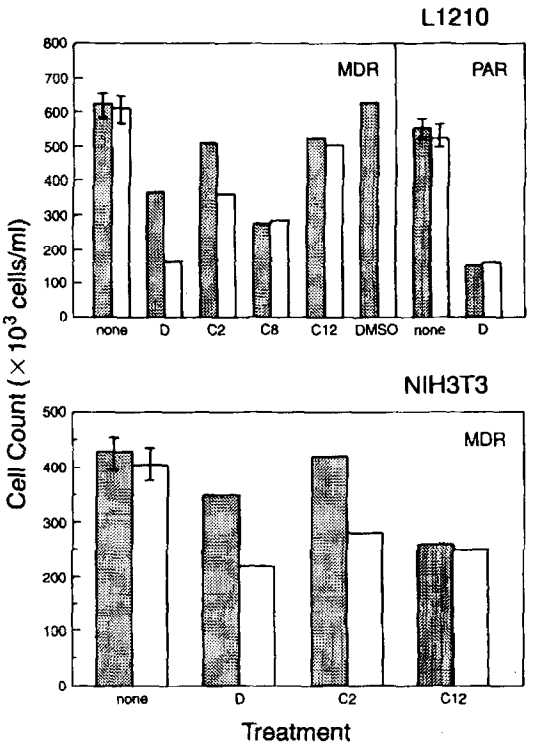

Fig. 2. Influence of CsA on the antiproliferative effects of D and N-acyl-Ds. L1210 or NIH3T3 cells were seeded at a 0.25 × 10^6/mL concentration and treated with D (0.2 μM), N-acetyl-D (C2, 5 μM), N-C8-D (C8, 3 μM), N-C12-D (C12, 2 μM), CsA (1 μg/mL) or the solvent, DMSO (max. 0.2%, v/v). CsA was added 10 min before the other drugs. The 24-well plates were incubated for 24 hr at 37° and 5% CO_2, and proliferation was determined by cell counting. Dark bar: drug alone; open bar: drug plus CsA. One typical experiment out of three is shown; each data point was obtained in quadruplicate. Maximum SD was less than ±5%. Typical SD bars are indicated for this experiment and for each untreated cell culture.

difference between the last two IC_{50} values). These results indicate that D and N-acetyl-D are substrates of P-glycoprotein, but N-C8-D and N-C12-D are not. Similar conclusions were drawn for the N-acyl-Ds from proliferation studies of the adherently grown FEMX cells (not shown). A contrasting conclusion for N-acetyl-D was reached by Friche *et al.* [12], who used a different cell line (Ehrlich ascites tumor) and measured drug accumulation rather than cell proliferation.

We also performed cell proliferation assays with selected concentrations of D and N-acyl-Ds in the presence and absence of the known P-glycoprotein blocker CsA (1 μg/mL) [8]. CsA blocks P-glycoprotein and can prevent the efflux of substrates such as D and N-acetyl-D from MDR cells, thereby increasing the antiproliferative effects of these drugs. CsA does not modify the uptake of nonsubstrate drugs. Figure 2 indicates, as expected, that CsA (1.0 μg/mL) increased the antiproliferative effects of D and N-acetyl-D, but not that of N-C8-D or N-C12-D in transfected L1210, FEMX (not shown) and NIH3T3 cells.

We investigated the substrate behavior of D and the N-acyl-Ds by our flow cytometric drug accumulation assay using the four cell lines described above [8]. Table 1 shows that D and N-acetyl-D accumulated to a higher concentration (expressed as fluorescence level) in the parental cells than in the MDR cells. In contrast, N-C8-D and N-C12-D accumulated in MDR and parental cells about equally. When cells are pretreated with CsA (1 μg/mL), D and N-acetyl-D also accumulated in the MDR and parental cells about equally, except for N-acetyl-D in the NIH3T3 cells. Thus, these flow cytometric studies support results of the proliferation assay and indicate that N-C8-D and N-C12-D are not substrates of P-glycoprotein. The observation that CsA increases sensitivity of the cells to drug-substrates, as with N-acetyl-D (Figs. 1 and 2), without altering drug-substrate accumulation (Table 1) was described earlier [13].

Distribution of D, N-acetyl-D and N-C8-D was studied between pH 5.8, 7.0 and 7.8 buffers and octanol. Results indicated that the octanol phase contained 20, 38 and 65% of D at pH 5.8, 7.0 and 7.8, respectively, and close to 100% of N-acetyl-D and N-C8-D at all three pH values. These distributions indicate that hydrophobicity alone does not determine substrate behavior, since N-acetyl-D but not N-C8-D was shown to be a substrate of P-glycoprotein in our MDR cell lines (Table 1).

Confocal microscopic studies using the NIH3T3 transfected and parental cell lines indicated that both D and N-acetyl-D are substrates, and that N-C12-D is not (Fig. 3). Concentrations of 1 μg/mL of these compounds at identical microscopic condi-

Table 1. Fluorescence intensities of daunorubicin and *N*-acyl-daunorubicin-treated parental and MDR cells

Drug (1 μg/mL)	Daunorubicin			N-Acetyl-D			N-Octanoyl-D			N-Dodecanoyl-D		
CSA (1 μg/mL)	–	+	*Ratio*	–	+	*Ratio*	–	+	*Ratio*	–	+	*Ratio*
L5 (par)	39.34	46.91	*1.19*	ND*	ND		66.47	74.53	*1.12*	54.52	55.47	*1.02*
L5 (MDR)	16.53	37.38	*2.26*	ND	ND		53.83	57.61	*1.07*	46.42	45.63	*0.98*
Ratio	*0.42*	*0.80*	*1.90*				*0.81*	*0.77*	*0.95*	*0.85*	*0.82*	*0.97*
L1210 (par)	17.44	21.57	*1.24*	19.34	32.88	*1.70*	54.50	ND		58.66	59.77	*1.02*
L1210 (MDR)	11.95	27.47	*2.30*	9.4	31.25	*3.32*	48.38	ND		58.76	59.21	*1.01*
Ratio	*0.69*	*1.27*	*1.86*	*0.49*	*0.95*	*1.96*	*0.89*			*1.00*	*0.99*	*0.99*
FEMX (par)	46.75	44.41	*0.95*	71.38	92.81	*1.30*	98.86	101.65	*1.03*	76.09	82.04	*1.08*
FEMX (MDR)	29.02	65.83	*2.27*	41.00	92.84	*2.26*	89.91	108.09	*1.20*	59.03	68.71	*1.16*
Ratio	*0.62*	*1.48*	*2.39*	*0.57*	*1.00*	*1.74*	*0.91*	*1.06*	*1.17*	*0.78*	*0.84*	*1.08*
3T3 (par)	151.64	160.3	*1.06*	49.3	61.11	*1.24*	75.95	79.32	*1.04*	88.04	90.33	*1.03*
3T3 (MDR)	19.34	36.97	*1.91*	28.83	35.35	*1.23*	72.08	68.65	*0.95*	69.95	81.48	*1.16*
Ratio	*0.13*	*0.23*	*1.81*	*0.58*	*0.58*	*0.99*	*0.95*	*0.87*	*0.91*	*0.79*	*0.90*	*1.14*

Fluorescence intensities of daunorubicin (D) and N-acyl-D-loaded MDR and parental (par) cells were obtained by flow cytometry, according to the protocol described in Materials and Methods and in Ref. 8. Ratios of fluorescence intensities of par/MDR and ± CsA are shown, and the number in the bottom right corner of each panel is the ratio of these ratios. Shown is one set of typical data, N = 2.

* ND = not done.

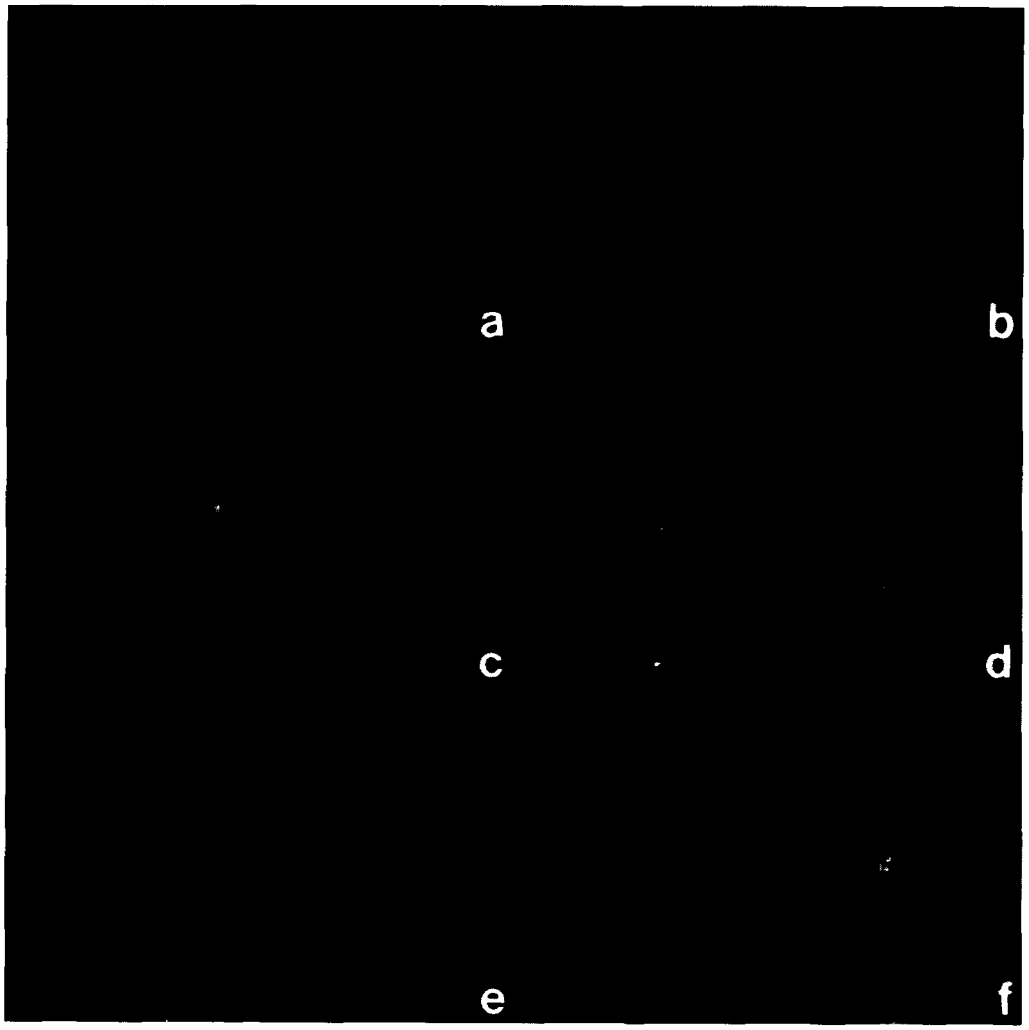

Fig. 3. Typical confocal microscopic pictures of 1 μg/mL daunorubicin (a and b), N-acetyl-daunorubicin (c and d) and N-dodecanoyl-daunorubicin (e and f) stained parental cells (a, c and e) and NIH3T3-vMDR cells (b, d and f). Pictures were taken as described in Materials and Methods and in Ref. 7. Identical optical settings were used for parental and MDR cells treated with the same drug. However, pictures were intensity-normalized to high and low fluorescence intensities.

tions showed (Fig. 3, e and f) similar distributions of N-C12-D in parental and MDR1 transfected cells. In contrast, D (Fig. 3, a and b) and N-acetyl-D (Fig. 3, c and d) accumulated to a higher concentration (fluorescence intensity) in parental cells than in MDR1 cells. Less N-acetyl-D than D accumulated in both cell lines, which is in agreement with the IC_{50} values, as shown above. One can observe accumulation of D in the nucleus, but not of N-acetyl-D and N-C12-D, since these last two compounds are not DNA intercalators [1]. Also, localization of the positively charged D in organelles (lysosomes), and greater distribution of the neutral N-acyl-D derivatives is in agreement with the octanol-buffer distribution pattern, as discussed above.

Our studies indicated that neutralization of the positive charge on D does not alter substrate behavior in MDR1 cells, but changes cellular distribution (Fig. 3) and antiproliferative activity (Fig. 1). Furthermore, we found that the larger chain-length derivatives, N-C8-D and N-C12-D, were not substrates of P-glycoprotein. Two explanations are possible for these findings: either high lipophilicity prevents transport of these compounds, or these D-derivatives are altering the physical properties of the plasma membrane, resulting in a modification of P-glycoprotein function. Since (a) N-acetyl-D is also highly lipophilic and is a substrate, (b) methoxymorpholinyl-D is also lipophilic, positively charged and not a substrate [14], and (c) deaminated doxorubicin is highly lipophilic and is a poor substrate [15], we conclude that lipophilicity is not an essential factor in P-glycoprotein substrate behavior. Similar conclusions can be drawn from studies on D derivatives by Lothstein *et al.* [16] and Friche *et al.* [12]. The second explanation, that N-C12-D or N-C8-D may alter P-glycoprotein functionality through membrane effects, was tested by assaying the uptake of the fluorescent substrate R123 in the presence of the nonsubstrate N-C12-D. Because of their different fluorescence emission spectra, these two compounds could be assayed simultaneously by flow cytometry using fluorescence compensation. As shown in Fig. 4, R123 uptake was increased greatly in the presence of N-C12-D in L1210 MDR and NIH3T3 MDR cells. Either no change or insignificant changes were observed in the parental cell lines under identical experimental conditions (not shown). R123 uptake was influenced more by N-C12-D in L1210 MDR than in NIH3T3 MDR cells. This difference can be explained by the fact that NIH3T3 MDR cells express about five times as much P-glycoprotein as L1210 cells and that the same concentration of P-glycoprotein blocker, N-C12-D, was used in the experiments with both cell lines.

We conclude that membrane intercalating nonsubstrates, such as N-C12-D, may inhibit P-glycoprotein function by exerting changes on this transmembrane pump directly or possibly through alteration in the physical status of the membrane.

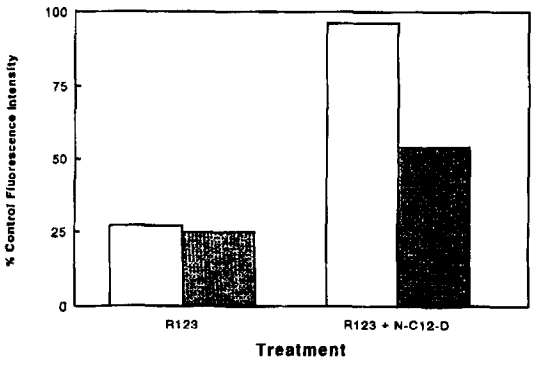

Fig. 4. Influence of N-dodecanoyl daunorubicin (N-C12-D) on the uptake of rhodamine-123 (R123) into L1210 and NIH3T3 cells. Controls (100%) are the fluorescence intensity (a.u.) of parental L1210 and NIH3T3 cells, treated with R123. Open bars = L1210 MDR, and shaded bars = NIH3T3 MDR cells. The flow cytometric uptake assay was carried out as described in Materials and Methods and in Ref. 8. N-C12-D was used at 1 μg/mL and R123 at 0.08 μM in both cell lines. Results of one typical experiment are shown (L1210 N = 3, NIH3T3 N = 2).

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