

# Level of protein kinase C activity correlates directly with resistance to adriamycin in murine fibrosarcoma cells

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In this report, we demonstrate a direct correlation between protein kinase C (PKC) activity and adriamycin (ADR) resistance in mouse fibrosarcoma cells. PKC activity was measured in four murine UV-2237M fibrosarcoma cell lines that differed in the degrees to which they expressed resistance to ADR, which is an inhibitor of PKC. A comparison of the four cell lines revealed a positive correlation between the level of PKC activity and resistance to ADR. Incubation of the cells with the PKC inhibitor H-7 produced a partial reversal of ADR resistance. Taken together, these results suggest a role for PKC in the mechanism of ADR resistance.

Protein kinase C; Adriamycin; Resistance correlation; Enzyme activity

## 1. INTRODUCTION

Protein kinase C, a  $\text{Ca}^{2+}$ - and phospholipid-dependent protein kinase, is implicated in the regulation of cell growth, since it plays a critical role in the signal transduction mechanisms of certain growth factors and other mitogens, and because it is specifically activated by phorbol ester tumor promoters [1,2]. Adriamycin (ADR) is an antineoplastic drug that appears to exert its biological effects through multiple biochemical mechanisms. ADR intercalates DNA, perturbs cell membranes and inhibits calmodulin. In addition, ADR is a PKC inhibitor [3]. ADR can be used to select cells with a multidrug resistance phenotype

[4,5]. MDR cells are resistant to a broad range of structurally unrelated anticancer drugs [6,7]. MDR has been associated with a net decrease in the intracellular accumulation of drugs and with overexpression of a membrane-associated p170 glycoprotein [6,7]. It was recently reported that an MDR human breast cancer cell line expressed elevated levels of PKC activity [8]. In this report, we demonstrate a direct correlation between ADR resistance and PKC activity in four murine fibrosarcoma cell lines which differ in their sensitivities to ADR. We also report a partial reversal of ADR resistance in these cells with the PKC inhibitor H-7.

## 2. MATERIALS AND METHODS

### 2.1. Tumor cell lines

The parental UV-2237 fibrosarcoma was induced in a C3H/Hen (mammary tumor negative) mouse by chronic exposure to ultraviolet-B radiation [9]; the UV-2237M line was isolated from spontaneous lung metastases of the parental line [10]. Both lines are sensitive to the cytotoxic effects of ADR. UV-2237M-ADR<sup>R</sup> cells and UV-2237M-ADR<sup>RR</sup> cells were selected from the UV-2237M cells by their capacities to proliferate in the presence of 1  $\mu\text{g}/\text{ml}$  [4] and 10  $\mu\text{g}/\text{ml}$  ADR, respectively. UV-2237M-rev cells were derived from UV-2237M-ADR<sup>R</sup> cells subsequent to growth for 4 months in the absence of ADR [11]; they exhibited an intermediate level of resistance to

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*Abbreviations:* CMEM, Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, vitamins and L-glutamine; DEAE, diethylaminoethyl; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; MDR, multidrug resistant; PDBu, phorbol dibutyrate; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C; PS, phosphatidylserine

ADR. UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup> were maintained in the continual presence of 1  $\mu\text{g}/\text{ml}$  and 10  $\mu\text{g}/\text{ml}$  ADR, respectively.

## 2.2. Reagents

[ $\gamma$ -<sup>32</sup>P]ATP was purchased from Amersham Corp. (Arlington Heights, IL). Protein concentrations were determined with the BioRad protein assay solution (BioRad, Richmond, CA). ADR was purchased from Adria (Columbus, OH). All other reagents were from Sigma (St. Louis, MO).

## 2.3. Isolation of PKC activity from UV-2237M, UV-2237M-rev, UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup> cells

Cell lines were maintained on plastic in 10% CMEM, and the media for the maintenance of the UV-2237M-ADR<sup>R</sup> and the UV-2237M-ADR<sup>RR</sup> cell lines contained 1  $\mu\text{g}/\text{ml}$  and 10  $\mu\text{g}/\text{ml}$  ADR, respectively. ADR was removed from the medium of the cells 24 h before they were harvested for the isolation of PKC. Subconfluent cells (approx.  $3 \times 10^6$ ) were harvested with buffer A containing 0.1% Triton X-100 (20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, 10  $\mu\text{g}/\text{ml}$  leupeptin, 0.25 mM PMSF, 25  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor, 0.1% Triton X-100, pH 7.5). All subsequent procedures were done at 4°C. Cell suspensions were stirred for 1 h and then centrifuged at  $13\,800 \times g$  for 15 min. Supernatants were loaded onto 0.5-ml DEAE-Sephacel columns equilibrated in buffer A. The columns were washed with 3 ml buffer A, and PKC was then eluted with 2 ml of 0.2 M NaCl in buffer A.

In experiments designed to determine PKC activities in cytosolic and particulate cell fractions, the procedure described above was followed with the following modifications. Cells were scraped into buffer A without Triton X-100. The cells were manually homogenized with a glass homogenizer (50 strokes) and then spun at  $100\,000 \times g$  for 1 h. The supernatant contained the cytosolic fraction. The pellet was resuspended in buffer A containing 0.1% Triton X-100 (5 ml), stirred for 2 h, and then centrifuged for 15 min at  $13\,800 \times g$ . The supernatant represented the particulate fraction. Cytosolic and particulate fractions were chromatographed on DEAE-Sephacel.

## 2.4. Assay of PKC activity

Levels of PKC activity were determined using the DEAE-purified cell extracts. PKC activity was measured as previously described [12], by subtracting the phosphotransferase activity between [ $\gamma$ -<sup>32</sup>P]ATP and histone III-S observed in the presence of 1 mM Ca<sup>2+</sup> from the activity observed in the presence of 1 mM Ca<sup>2+</sup> plus 30  $\mu\text{g}$  PS/ml.

## 2.5. Assay for in vitro growth inhibition by ADR and effects of H-7

Cultures of the UV-2237M cell lines in their exponential growth phase were harvested by a brief exposure to 0.25% trypsin/0.02% EDTA (w/v). Single-cell suspensions with a viability of >95% (trypan blue exclusion) were seeded into 96-well microculture plates at 2500 cells/well and incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For PKC inhibitor (H-7) studies, the cells were incubated with various concentrations (1–100  $\mu\text{M}$ ) of H-7 (or HA1004) for 60 min prior to the addition of 5  $\mu\text{g}/\text{ml}$  ADR. Control cultures were incubated in medium with or without H-7 but without ADR. Additional controls consisted of cells incubated with ADR, but

without H-7. After 48 h, cell proliferation was determined by a modification of a colorimetric tetrazolium procedure [13]. Briefly, at the end of the assay period, 50  $\mu\text{l}$  of tetrazolium dye at 5 mg/ml in phosphate-buffered saline was added to each well, and cells were incubated for 2 h at 37°C. After reaction of the dye with mitochondrial dehydrogenase in the viable cells, the dye and media were removed, dimethyl sulfoxide was added to each well, and the absorbance at 590 nm was measured with a microplate scanning spectrophotometer.

## 3. RESULTS

### 3.1. Cytostatic effects of ADR

We first examined the sensitivities of the four UV-2237M cell lines to cytostatic effects of ADR. The data of fig.1 show that parental UV-2237M cells were highly sensitive to the cytostatic effects of ADR. In contrast, resistance to ADR was greatest in UV-2237M-ADR<sup>RR</sup> and UV-2237M-ADR<sup>R</sup> cells, and intermediate in UV-2237M-rev cells.

### 3.2. Levels of PKC activity in UV-2237M, UV-2237M-rev, UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup> cell lines

Next we determined whether a correlation existed between levels of PKC activity and the ADR resistance phenotype of cell lines derived from UV-2237M cells. Table 1 shows that cells highly resistant to ADR (UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup>) had twice as much PKC activity as ADR-sensitive cells (UV-2237M). In addition, cells with intermediate resistance (UV-2237M-rev) had elevated levels of PKC activity relative to the drug-sensitive cells (UV-2237M) but less PKC activity than that found in the highly resistant cell lines (UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup>). The data indicate that acquisition of the resistance phenotype correlates with elevated PKC activity and that partial loss of the resistance phenotype observed in UV-2237M-ADR<sup>R</sup> cells that were grown in the absence of ADR for 4 months (UV2237M-rev) correlates with reduced PKC activity.

### 3.3. Reversal of the ADR resistance phenotype in UV-2237M-rev and UV-2237M-ADR<sup>R</sup> cells by the PKC inhibitor H-7

H-7, an isoquinolesulfonamide, is an active-site inhibitor of PKC [14]. In order to assess the significance of elevated PKC activity in the ADR

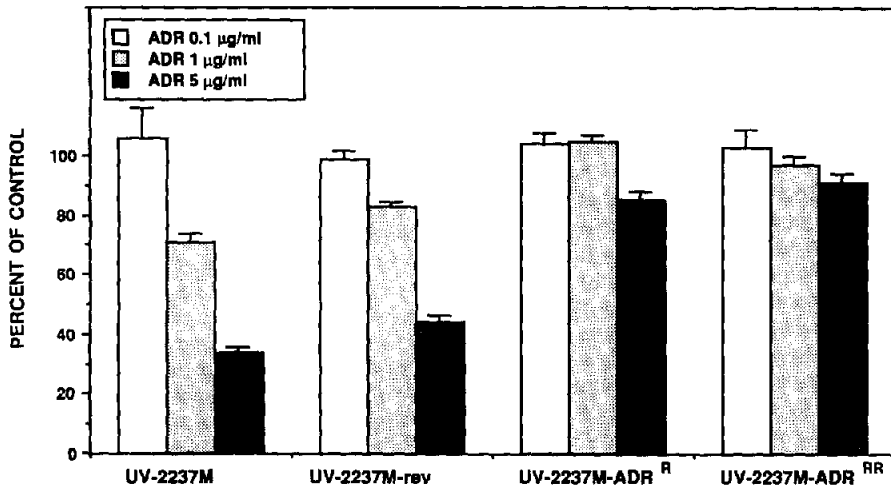


Fig.1. Cytostatic effects of ADR on various UV-2237M cell lines. Effects of the indicated concentrations of ADR on UV-2237M, UV-2237M-rev, UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup> cells were determined. Each bar represents the average (± SD) of triplicate determinations.

resistance phenotypes of UV-2237M-rev and UV-2237M-ADR<sup>R</sup> cells, we tested the capacity of H-7 to reverse the resistance phenotype. H-7, at the concentrations shown in fig.2, did not produce cytostasis. However, H-7 increased the sensitivity of UV-2237M-rev and UV-2237M-ADR<sup>R</sup> cells to ADR (fig.2). HA1004, a structural analogue of H-7 and a weak PKC inhibitor [14], failed to increase the ADR sensitivity of either UV-2237M-rev or UV-2237M-ADR<sup>R</sup> cells at concentrations from 10 to 100 µM (not shown). H-7 was more potent in

reversing the resistance phenotype of the UV-2237M-rev cells that that of the UV-2237M-ADR<sup>R</sup> cells. These data indicate that the levels of PKC activity in UV-2237M-rev and UV-2237M-ADR<sup>R</sup> cells correlate inversely with the capacity of H-7 to reverse resistance to ADR.

4. DISCUSSION

In this study, we examined whether expression of

Table 1  
Levels of PKC activity in murine fibrosarcoma cell lines exhibiting different sensitivities to adriamycin

Cell line	Total activity	Background activity	PKC activity	% particulate
UV-2237M	1069 ± 50	445 ± 50	624	73 ± 16
UV-2237M-rev	1449 ± 73	498 ± 61	951	65 ± 17
UV-2237M-ADR <sup>R</sup>	1670 ± 90	173 ± 13	1497	50 ± 8
UV-2237M-ADR <sup>RR</sup>	1218 ± 69	68 ± 24	1150	-

'PKC activity' represents the pmol <sup>32</sup>P transferred to histone III-S/min per mg cellular extract protein in a Ca<sup>2+</sup>- and PS-dependent, PKC-catalyzed phosphotransferase reaction between [γ-<sup>32</sup>P]ATP and histone III-S. 'Total activity' represents the total phosphotransferase activity between histone III-S and [γ-<sup>32</sup>P]ATP observed in the presence of Ca<sup>2+</sup> and PS; and 'background activity' represents the phosphotransferase activity observed in the presence of Ca<sup>2+</sup>. PKC activity was calculated as the difference between total and background phosphotransferase activities. '% particulate' represents the percentage of the total PKC activity that was observed in the particulate fraction of the indicated cell line

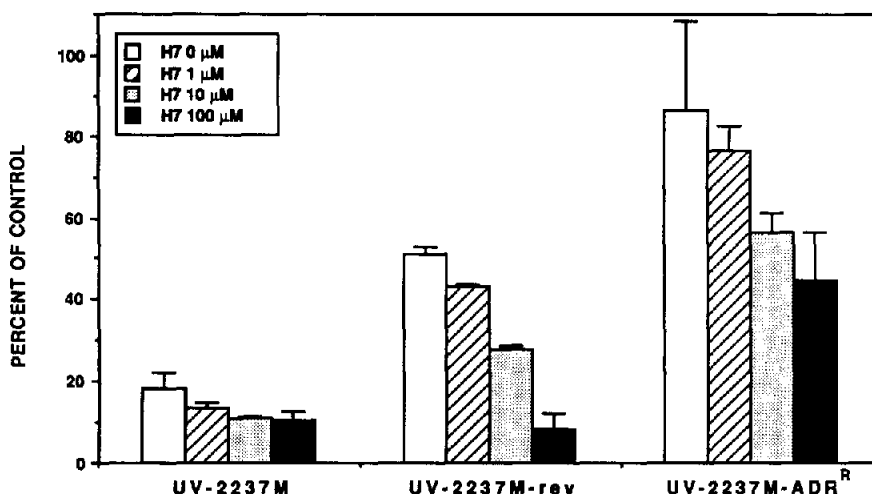


Fig.2. The effects of the PKC inhibitor H-7 on the ADR resistance phenotype. Each bar represents the average ( $\pm$  SD) of duplicate or triplicate determinations. Cells were incubated in the presence of  $5 \mu\text{g/ml}$  of ADR with various concentrations of H-7. The effects of adriamycin and H-7 on cell growth were expressed as the percentage of control and analyzed by the formula  $(B/A) \times 100$ , where A is the  $A_{590}$  observed with the control cells and B is the  $A_{590}$  associated with the treated cells.

ADR resistance was associated with elevated levels of PKC activity. Indeed, we observed that the highly resistant cell lines UV-2237M-ADR<sup>R</sup> and UV-2237M-ADR<sup>RR</sup> contained twice as much PKC activity as the parental ADR-sensitive UV-2237M cells, and that the partial reversal of the resistance phenotype by the removal of ADR for four months from the culture medium of the UV-2237M-ADR<sup>R</sup> cells was accompanied by a significant reduction in PKC activity. The level of PKC activity in the intermediately resistant cells was significantly greater than that observed in the drug-sensitive UV-2237M-parental cells. While the decreased level of PKC activity found in the UV-2237M-rev cells may reflect the selection of a subpopulation of relatively drug-sensitive cells, the data clearly demonstrate a positive correlation between the degree of resistance of ADR and the level of PKC activity in UV-2237M fibrosarcoma cells.

H-7 is a PKC inhibitor that acts at the active site of the enzyme, and HA1004 is a structural analog of H-7 that inhibits PKC only very weakly. We found that H-7, but not HA1004, could partially reverse the drug resistance phenotype of UV-2237M-ADR<sup>R</sup> and UV-2237M-rev cells. The extents to which H-7 reversed ADR resistance in these cell lines correlated inversely with their level of PKC activity. Our observations that ADR-resistant

cells have elevated levels of PKC activity and that a selective PKC inhibitor can partially reverse ADR resistance in these cells provide evidence that maintenance of ADR resistance is associated with expression of PKC.

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