

# Active Efflux of Daunorubicin and Adriamycin in Sensitive and Resistant Sublines of P388 Leukemia<sup>1</sup>

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## ABSTRACT

Significantly decreased uptake and retention of daunorubicin (DAU) and Adriamycin (ADR) have been reported in sublines of P388 leukemia resistant to these anthracyclines. We studied the effects of inhibitors of oxidative phosphorylation on uptake and retention of DAU and ADR in order to characterize the transport process for these anthracyclines and to clarify further the alteration in resistant cells. In glucose-free medium, uptake of DAU and ADR was accelerated by metabolic inhibitors to a greater extent in resistant cells than in sensitive cells. Under these conditions, drug uptake was similar in sensitive and resistant cells. When glucose was added to the incubation medium in the presence of 2,4-dinitrophenol, efflux of DAU from both sensitive and resistant cells was observed. Net efflux of ADR was also observed with the resistant cells, whereas glucose markedly inhibited 2,4-dinitrophenol-stimulated uptake of ADR by the sensitive cells. Furthermore, in sensitive and resistant cells preloaded with ADR and DAU, efflux of the drugs was inhibited by the addition of 2,4-dinitrophenol. These results suggest that there is an active outward transport mechanism for anthracyclines in P388 leukemia cells and that enhanced activity of this efflux process renders cells highly resistant to the cytostatic and cytotoxic effects of ADR and DAU.

## INTRODUCTION

One of the serious problems in chemotherapy of cancer is that tumor cells are able to acquire resistance to initially effective cytotoxic agents. In most instances in which the mechanism of resistance has been elucidated in experimental systems, resistance is conferred by either a change in the activity of a specific enzyme or an alteration in the cell membrane (3, 9). In the former instance, changes in the levels of anabolic, catabolic, or target enzymes have been observed for a number of antimetabolites. In the latter, changes in specific transport mechanisms or general membrane biochemistry have been shown to render cells less permeable to a variety of cytotoxic agents.

In anthracycline-resistant sublines of P388 leukemia, as shown in an earlier paper (11), reduced uptake of DAU<sup>3</sup> and ADR was observed. Similar changes have been shown to be associated with resistance to anthracyclines in sublines of

Ehrlich carcinoma (6-8, 18) and Chinese hamster ovary cells (1, 2, 15). In resistant P388 leukemia cells, diminished retention of ADR and DAU was shown to correlate with resistance to these agents (11). In addition, diminished retention was observed with actinomycin D (10) and emetine (5), to which the anthracycline-resistant cells are cross-resistant. Danø (8) and Skovsgaard (17, 18) have characterized an active outward transport mechanism for anthracyclines in Ehrlich carcinoma cells and have shown enhanced efflux in resistant sublines. The studies reported herein indicate that active outward transport of anthracyclines also occurs in P388 leukemia cells and that a remarkable degree of resistance is conferred by enhanced activity of this efflux mechanism. Preliminary results of this study have appeared elsewhere (12).

## MATERIALS AND METHODS

**P388/ADR.** This ADR-resistant subline of P388 leukemia was developed by repeating *in vivo* treatment of parental P388 cells with ADR as described previously (13, 14). Although drug treatment was discontinued after 50 transplant generations, no change in drug sensitivity has been observed over 2 years of continuous serial transplantation of these cell lines.

**Chemicals.** Tritium-labeled DAU and ADR (specific activities, 27.1 and 27.4  $\mu\text{Ci}/\text{mg}$ , respectively) were kindly donated by Farmitalia (Milan, Italy) through Meiji Seika Kaisha, Ltd. (Tokyo, Japan) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), respectively. [<sup>14</sup>C]Jara-C and [<sup>3</sup>H]MTX (specific activities, 47 mCi/mmol and 16 Ci/mmol, respectively) were purchased from Daiichi Chemical Co., Tokyo, Japan, and The Radiochemical Centre, Amersham, England, respectively. These chemicals were completely pure chemically and radiochemically by thin-layer chromatography. In addition, no decrease in radioactivity of these chemicals after lyophilization following 1 hr incubation at 37° was observed. DNP, sodium azide, oligomycin, and valinomycin were purchased from Sigma Chemical Co. (St. Louis, Mo.), and the solution of these inhibitors was prepared immediately before use.

**In Vitro Culture.** Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Grand Island Biological Co.), 10  $\mu\text{M}$  2-hydroxyethyl disulfide (Aldrich Chemical Co., Inc., Milwaukee, Wis.), and kanamycin (100  $\mu\text{g}/\text{ml}$ ; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) was used as culture medium.

Cells were collected from tumor-bearing BALB/c  $\times$  DBA/2 F<sub>1</sub> mice 5 days after transplant and were suspended in the culture medium to cell density of  $5 \times 10^4$  cells/ml. Varying concentrations of DAU and ADR were added, followed by incubation in a CO<sub>2</sub> incubator at 37° for 1 or 48 hr. In the case of 1 hr exposure, cells were centrifuged at  $185 \times g$  for 5 min

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<sup>3</sup> The abbreviations used are: DAU, daunorubicin; ADR, Adriamycin; ara-C, 1- $\beta$ -D-arabinofuranosylcytosine; MTX, methotrexate (4-amino-N<sup>10</sup>-methylpteroylglutamic acid); DNP, 2,4-dinitrophenol; HBSS, Hanks' balanced salt solution; IC<sub>50</sub>, 50% inhibition concentration of cell growth.

after 1 hr incubation with the drug, resuspended in fresh complete medium, and then cultured.

After 48 hr culture, 4 ml of 0.25% trypsin-calcium, magnesium-free Ringer's buffer solution were added to each tube containing 1 ml of cell suspension and then incubated for 5 min at 37° to deaggregate cells. Cells were counted in a Model ZB1 Coulter counter.

**Uptake and Retention Measurement.** HBSS (pH 7.4) with or without glucose (1000 mg/liter) was prepared and used for these experiments. When metabolic inhibitors were used, cells were suspended in glucose-free HBSS. Under these conditions, incubation time was restricted to 50 min, since thereafter an increase was evident in cells incapable of excluding trypan blue.

Cells were harvested 7 days after transplant, washed 3 times with centrifugation, and suspended in HBSS (final cell density,  $10^8$  cells/ml). Cells were preincubated for 10 min before the addition of metabolic inhibitor or tritium-labeled antibiotic. In efflux experiments, cells were preloaded with isotopically labeled ADR or DAU during a 20-min incubation, washed 3 times with cold medium, and resuspended in fresh HBSS.

For measurement of the radioactivity of the cells, triplicate 1-ml aliquots were withdrawn at appropriate time points and added to centrifuge tubes with 4 ml of cold 0.9% NaCl solution, followed by 2 washes with cold 0.9% NaCl solution and centrifugation ( $600 \times g$ , 5 min, 4°). After the tubes were placed upside down on the filter paper for 3 to 5 hr to remove the washing solution, cells were solubilized in 0.5 ml of Protosol (New England Nuclear, Boston, Mass.), and 15 ml of liquid scintillation cocktail (Econofluor; New England Nuclear) were added. The radioactivity was determined in a Beckman Model LS-355 liquid scintillation counter with 52% counting efficiency for tritium.

## RESULTS

**Difference in Drug Sensitivity *In Vitro* between P388/S and P388/ADR cells.** In a previous paper (11), sensitivity of P388/ADR and P388/S (the parental drug-sensitive P388 leukemia) to ADR or DAU was compared by exposure to the drugs *in vitro* for 1 hr followed by determination of the surviving fraction by *in vivo* bioassay. In this study, differences in drug sensitivity between 1 hr exposure and 48 hr exposure to ADR or DAU were determined with respect to growth inhibition rather than to cytotoxicity. P388/S and P388/ADR cells taken directly from animals grew well in Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum supplemented with  $10 \mu\text{M}$  2-hydroxyethylidisulfide.

On 1 hr exposure to DAU, the  $\text{IC}_{50}$  was found to be  $2.8 \times 10^{-5}$  mM for P388/S and  $3.0 \times 10^{-3}$  mM for P388/ADR (Chart 1). On a 48-hr continuous exposure to DAU, growth of P388/S cells was inhibited by 50% at  $7.9 \times 10^{-10}$  mM, whereas the  $\text{IC}_{50}$  for P388/ADR cells was  $4.8 \times 10^{-4}$  mM. Thus, the index of resistance of P388/ADR to the growth-inhibitory effects of DAU was 107 for 1 hr exposure and  $6 \times 10^5$  for 48 hr exposure. This is due to the fact that the  $\text{IC}_{50}$  for P388/S was 4 orders of magnitude for continuous as opposed to acute drug exposure, whereas there was only a 6-fold decrease in  $\text{IC}_{50}$  for P388/ADR cells when going from 1 hr to 48 hr exposure. Similar results were observed for ADR in P388/S and P388/ADR cells (Chart 2). The index of resistance to ADR was even

more dramatically affected by increasing exposure time (59-fold resistance for 1 hr exposure and  $5.9 \times 10^{10}$ -fold resistance for 48 hr exposure).

**Effect of Metabolic Inhibitors on Transport of ADR and DAU.** The effects of inhibitors of mitochondrial electron transport and/or oxidative phosphorylation including DNP, sodium azide, oligomycin, and valinomycin on the uptake of DAU were studied in P388/S and P388/ADR cells suspended in glucose-free HBSS (Table 1). These metabolic inhibitors did not inhibit but instead stimulated the uptake of DAU by both cell lines. DAU uptake was enhanced to a much greater extent in P388/ADR cells than in sensitive cells. This stimulation was not evident in medium containing glucose. Thus, the influence of addition of glucose to cell suspensions in the presence of DNP was studied (Chart 3). The enhanced rate of DAU uptake in the presence of DNP is evident in Chart 3. It is noteworthy that the rate of DAU uptake and steady-state levels achieved in sensitive and resistant cells are essentially identical in the absence of glucose and the presence of DNP. The addition of glucose at 20 min resulted in a net efflux of DAU from both cell lines, even though DAU was still present in the medium. After 30 min, intracellular DAU levels approached those of cells incubated

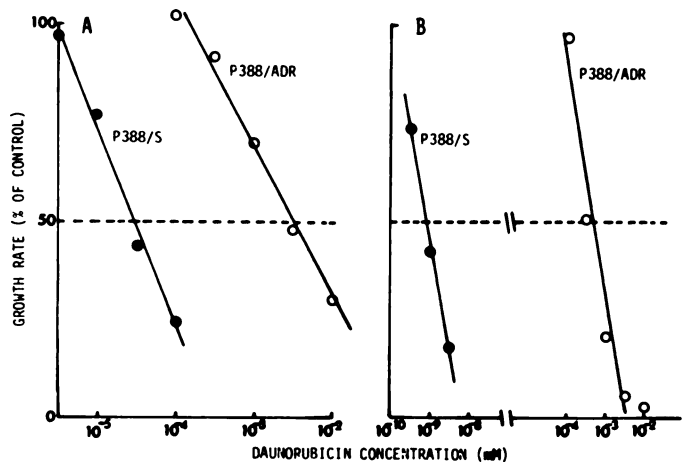


Chart 1. Growth inhibition of ADR-sensitive and -resistant P388 leukemia cells by exposure (1 or 48 hr) to DAU. A, 1 hr exposure; B, 48 hr exposure. ---, level of  $\text{IC}_{50}$ .

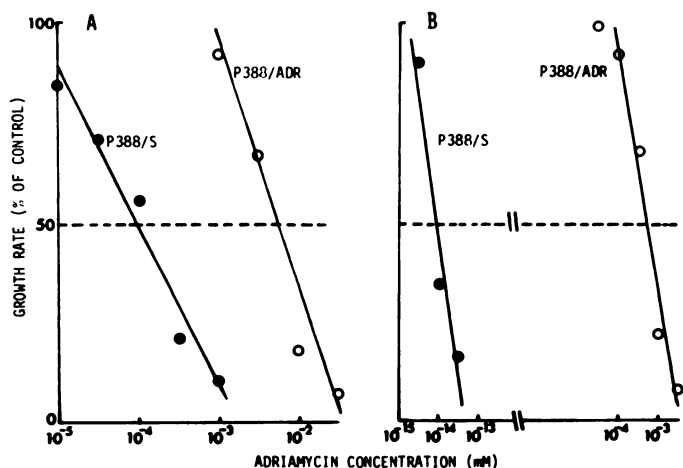


Chart 2. Growth inhibition of ADR-sensitive and -resistant P388 leukemia cells by exposure (1 or 48 hr) to ADR. A, 1 hr exposure; B, 48 hr exposure. ---, level of  $\text{IC}_{50}$ .

Table 1

**Effect of metabolic inhibitors on uptake of [<sup>3</sup>H]DAU by ADR-sensitive and -resistant P388 leukemia cells**

Cells were preincubated with metabolic inhibitor in glucose-free HBSS for 10 min, and then a small quantity of [<sup>3</sup>H]DAU solution (final concentration,  $1.8 \times 10^{-3}$  mM) was added, followed by 30 min incubation. "Control" was incubated in normal HBSS.

Metabolic inhibitors	DAU uptake (pmol/10 <sup>6</sup> cells)	
	P388/S	P388/ADR
Control	286 ± 8 <sup>a</sup> (100) <sup>b</sup>	83 ± 4 (100)
Sodium azide, 10 mM	312 ± 24 (109)	279 ± 14 (336)
DNP, 1 mM	372 ± 7 (130)	383 ± 3 (461)
Oligomycin, 0.01 μg/ml	426 ± 14 (149)	545 ± 8 (657)
Vallinomycin, 10 <sup>-5</sup> mM	398 ± 21 (139)	478 ± 16 (576)

<sup>a</sup> Mean ± S.D.

<sup>b</sup> Numbers in parentheses, percentage of control.

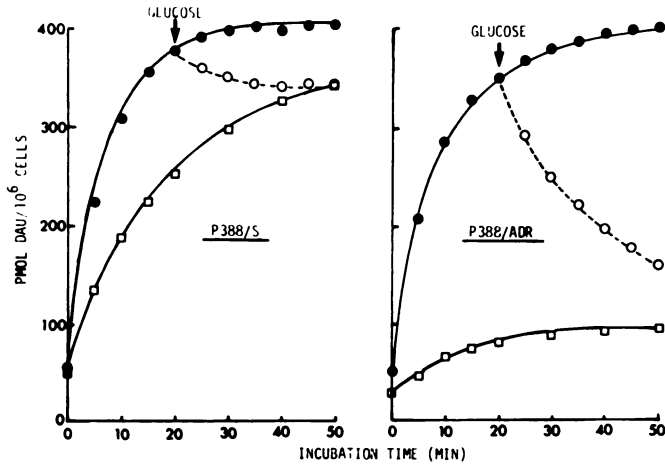


Chart 3. Reversing effect of glucose on increased uptake of [<sup>3</sup>H]DAU by DNP in ADR-sensitive and -resistant P388 leukemia cells. Cells were incubated in HBSS (□) or glucose-free HBSS with 1 mM DNP (●) for 10 min, and then [<sup>3</sup>H]DAU ( $1.8 \times 10^{-3}$  mM) was added. ○, uptake level after glucose (final concentration 1 mg/ml) was added. Each point is the mean of 3 determinations with coefficient of variation less than 10%.

without DNP. The rate of DAU efflux induced by the addition of glucose was much greater in P388/ADR cells than in P388/S cells.

Similar results were obtained with the uptake of ADR (Chart 4). Enhancement of ADR uptake by DNP was more remarkable in the resistant cells than in the sensitive ones. Addition of glucose at 20 min induced net efflux of ADR only in the resistant cells, and the ADR uptake approached those observed in the absence of DNP. In the experiment of Chart 4, an ADR concentration of  $6.0 \times 10^{-4}$  mM was used. When the ADR concentration was  $1.7 \times 10^{-2}$  mM, however, the addition of glucose slowed the rate of uptake by the resistant cells as well as the sensitive ones without resulting in net efflux of ADR.

To examine the specificity of this phenomenon, the influence of DNP on uptake of [<sup>14</sup>C]ara-C and [<sup>3</sup>H]MTX by P388/ADR cells was observed. In the presence of DNP and the absence of glucose, uptake of ara-C was completely inhibited and MTX uptake was suppressed to a greater extent compared to the normal uptake level in the absence of DNP. These results indicate that active efflux mechanism demonstrated in these cells is specific to some particular compounds.

In order to study more directly the effect of energy deprivation on the efflux of ADR and DAU from the sensitive and

resistant cell lines, cells were preloaded with the labeled drugs and resuspended in glucose-free medium containing DNP (Chart 5). It is apparent that efflux is inhibited by DNP in the absence of glucose in both cell lines. Efflux did occur during the first 5 min (10 min for DAU in P388/S) in glucose-free, DNP-containing medium inasmuch as depletion of ATP stores would not be instantaneous. The greater efflux of ADR and DAU from P388/ADR cells is evident in Chart 5.

**DISCUSSION**

In a previous study (11), we demonstrated that resistance to ADR and DAU in sublines of P388 leukemia was associated with decreased uptake and decreased retention of these anthracyclines. Evidence suggested that resistance was corre-

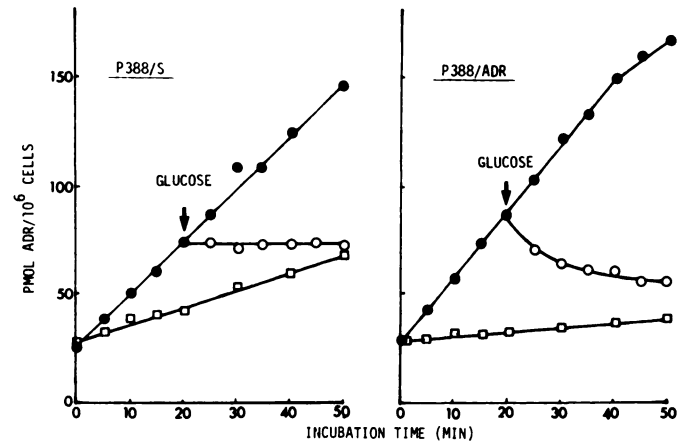


Chart 4. Reversing effect of glucose on increased uptake of [<sup>3</sup>H]ADR by DNP in ADR-sensitive and -resistant P388 leukemia cells. Cells were incubated in HBSS (□) or glucose-free HBSS with 1 mM DNP (●) for 10 min, and then [<sup>3</sup>H]ADR ( $6.0 \times 10^{-4}$  mM) was added. ○, uptake level after glucose (final concentration, 1 mg/ml) was added. Each point is the mean of 3 determinations with coefficient of variation less than 10%.

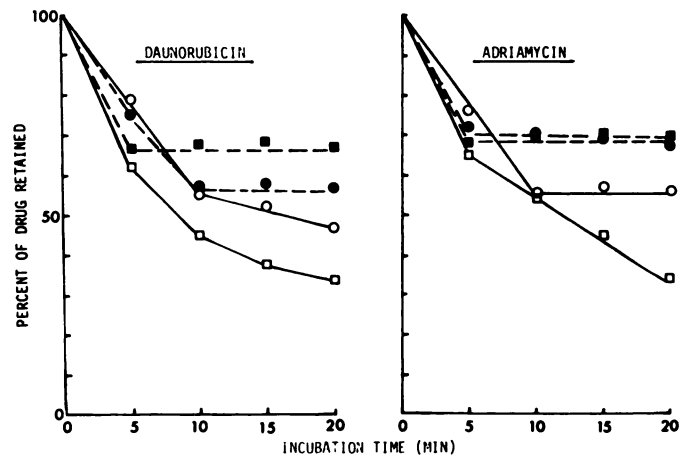


Chart 5. Inhibitory effect of DNP on efflux of [<sup>3</sup>H]DAU and [<sup>3</sup>H]ADR from ADR-sensitive and -resistant cells. Cells of P388/S (○, ●) and P388/ADR (□, ■) were preloaded with [<sup>3</sup>H]DAU ( $1.8 \times 10^{-3}$  mM) or [<sup>3</sup>H]ADR ( $1.7 \times 10^{-2}$  mM) for 20 min. The cells were washed and resuspended in either HBSS (□, ○) or glucose-free HBSS with 1 mM DNP (■, ●) followed by incubation for 20 min at 37°. Percentage of retention was obtained with the ratio of the amount of retained drug to that of original preloaded drug. (DAU by P388/S and P388/ADR, 190 and 72 pmol/10<sup>6</sup> cells; ADR by P388/S and P388/ADR, 440 and 186 pmol/10<sup>6</sup> cells, respectively). Each point is the mean of 3 determinations with coefficient of variation less than 10%.

lated with the decreased retention, and this conclusion was strengthened by observations that cross-resistance of the anthracycline-resistant sublines to actinomycin D and emetine was associated with decreased retention of these agents with little or no difference apparent in uptake (5, 10). The present studies in cells depleted of ATP by absence of glucose and addition of inhibitors of oxidative phosphorylation indicate that P388 cells have an active efflux mechanism for anthracyclines and that enhanced activity of this transport process in resistant cell lines apparently accounts for the observed decreases in uptake and retention of ADR and DAU.

The finding that uptake of the anthracyclines is strongly stimulated in sensitive and resistant P388 cells by metabolic inhibitors in the absence of glucose suggests that net uptake is determined by diffusion into the cells and active outward transport. Blockage of the active efflux mechanism by depletion of ATP results in enhanced uptake. The reversal of this stimulation by addition of glucose indicates that sufficient ATP can be generated by glycolysis in the presence of DNP to provide energy for active efflux process. Addition of glucose in the presence of DNP resulted in net efflux of DAU and ADR from P388/ADR cells even though these antibiotics were still present in the incubation medium (Charts 3 and 4).

The findings in P388 leukemia cell lines are in accordance with studies by Danø (8) and Skovsgaard (17, 18) of DAU transport in sensitive and resistant sublines of Ehrlich carcinoma. Active efflux of ADR (17) and DAU (8, 17, 18) by Ehrlich carcinoma cells was clearly demonstrated, and evidence was presented (8, 18) indicating that resistance to DAU was associated with enhanced efflux in DAU-resistant cells. Our studies indicate that enhanced active efflux of ADR as well as DAU is associated with anthracycline resistance in P388 leukemia sublines. Skovsgaard (18) found that, in addition to enhanced activity of the efflux transport mechanism, DAU-resistant Ehrlich carcinoma cells exhibited decreased uptake of DAU (unrelated to efflux) and lower affinity of intracellular binding sites. We have reported previously (11) that binding of DAU to nuclei isolated from P388/S and P388/ADR cells was similar. However, Skovsgaard (18) observed a biphasic curve for DAU binding to Ehrlich carcinoma homogenates with differences between sensitive and resistant cells evident only at high concentrations of DAU. The concentrations of DAU used in our nuclear binding experiments were below those at which Skovsgaard observed a difference in his studies. Thus, we cannot eliminate the possibility that decreased intracellular binding contributes to resistance in the P388 leukemia cell lines. As can be seen by comparing the uptake of DAU or ADR by P388/S and P388/ADR cells when efflux has been blocked by DNP in the absence of glucose (Charts 3 and 4), there is essentially no difference in uptake under these conditions. Thus, the decreased uptake of DAU unrelated to active efflux which was observed in DAU-resistant Ehrlich carcinoma cells (18) does not appear to be evident in ADR-resistant P388 leukemia cells.

A similar phenomenon has been reported (4, 16) in Chinese hamster ovary cells resistant to colchicine. Colchicine uptake was enhanced by potassium cyanide, and the enhanced uptake was reversed by addition of glucose to the culture medium.

These authors observed a correspondence between enhanced colchicine uptake and depletion of intracellular ATP. It was hypothesized that an energy-dependent drug permeability barrier accounted for these findings. Our evidence and the findings of Danø (8) and Skovsgaard (18) overwhelmingly support the conclusion that resistance to anthracyclines is associated with an enhanced active efflux process.

The broad pattern of cross-resistance of P388/ADR to a spectrum of structurally dissimilar DNA-binding agents, mitotic spindle poisons, and inhibitors of protein biosynthesis (5) raises the possibility that the efflux transport mechanism which handles anthracyclines is also involved in regulating intracellular levels of a variety of xenobiotics. Further studies to gain a better understanding of or to develop an inhibitor for this efflux transport mechanism could potentially lead to enhanced efficacy for a variety of agents and the capability to overcome resistance to anthracyclines and other drugs.

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