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COMPARATIVE STUDY OF INTRACELLULAR CALCIUM AND ADENOSINE 3′, 5′-CYCLIC MONOPHOSPHATE LEVELS IN HUMAN BREAST CARCINOMA CELLS SENSITIVE OR RESISTANT TO ADRIAMYCIN®: CONTRIBUTION TO REVERSION OF CHEMORESISTANCE

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Abstract—Multidrug resistance (MDR) corresponds to the cross-over resistance of tumour cells to structurally unrelated cytotoxic chemotherapeutic drugs. One of the mechanisms causing this resistance is the enhanced expression of a transmembrane drug efflux pump P-glycoprotein (P-170). Reversal of P-glycoprotein-associated MDR has received much attention in recent years. In experimental cell lines, P-170 and the glutathione redox cycle seem to contribute to this phenomenon; P-170 may be inactivated by calcium and calmodulin antagonists and the glutathione redox cycle altered by buthionine sulphoximine (BSO). Treatment of human MCF-7 breast cancer cells with chemosensitizers (CS), such as verapamil, trifluoperazine or BSO, for 72 hr resulted in an enhanced sensitization of cells to Adriamycin, trifluoperazine being the most potent compound in the reversion of chemoresistance. In these Adriamycin sensitive or resistant cells, treated or not by the CS, the possible role of calcium and cyclic adenosine monophosphate (cAMP) in mediating the reversion of chemoresistance to Adriamycin was investigated. It was found that intracellular calcium was approximately 2-fold higher in resistant than in sensitive cells, the opposite was true for cAMP. Modifications in calcium and cAMP levels were observed in MCF-7 resistant cells after treatment with verapamil and BSO; trifluoperazine had no effect on these two parameters. These results seemed to rule out any implication of calcium and cAMP levels in the contribution of these three chemosensitizers in the mechanisms of reversion of chemoresistance to Adriamycin.

Key words: calcium; cyclic adenosine monophosphate; Adriamycin; chemoresistance; chemosensitizers; cells

Molecular and cellular mechanisms underlying MDR† have been investigated in cell lines simultaneously resistant to unrelated cytotoxic agents, such as anthracyclines and Vinca alkaloids. The most consistent finding has been the overexpression of a 170 KDa transmembrane glycoprotein, referred to as P-170, encoded by the mdr-1 gene. This P-glycoprotein functions as an energy-dependent, rapid drug efflux pump [1–3]. Alternative mechanisms have been described. For example, the Adriamycin-selected MDR MCF-7 human breast cancer cell line has been shown to possess increased glutathione peroxidase and transferase activities [4, 5]. Several investigators have also discovered alterations in the levels of protein kinase C activity in drug resistant lines, as well as increased amounts of calcium in some Adriamycin-resistant cell lines [6–12]. Adriamycin is an antineoplastic drug that appears to exert its biological effects through multiple biochemical mechanisms, e.g. DNA intercalation, generation of reactive oxygen species, stabilization of the topoisomerase II–DNA cleavage complex and damage to the plasma membrane [13–16]. The chemoresistance to this anticancer drug can be reversed by various CS [1, 17–19]. Certain calcium influx blockers and calmodulin inhibitors efficiently inhibit the Adriamycin efflux function of tumour cells, especially resistant tumour cells [20, 21]. Photolabelling studies have shown that verapamil binds specifically to P-170, and possibly inhibits its drug efflux effect [22]. These drugs could also act by modulating intracellular calcium exchange inhibiting the active efflux of drugs. Other CS, such as BSO, a specific inhibitor of γ-glutamylcysteine synthetase, cause a marked decrease in cellular glutathione, thus decreasing the activity of glutathione-dependent detoxifying enzymes [23, 24]. Modulation by BSO may significantly alter the clinical toxicity of many drugs to normal tissues, as well as to tumour cells. This can lead to cellular damage (swelling, membrane disintegration, etc.) [25, 26].

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† Abbreviations: MDR, multidrug resistance; CS, chemosensitizers; BSO, buthionine sulphoximine; cAMP, adenosine 3′, 5′-cyclic monophosphate; MCF-7/adr, MCF-7 Adriamycin-resistant cell line; MCF-7/S, MCF-7 sensitive cell line; FCS, foetal calf serum; Indo-1 AM, Indo-1 acetoxy-methyl; BCA, bicinchoninic acid assay, DT, doubling time

Verapamil is a calcium-channel blocker which has proved to be efficient in malignant lymphomas, multiple myeloma and acute myeloid leukemias [27–29]. This calcium ion antagonist has significant effects on membrane ion flux [30]. It modulates the slow calcium-dependent inward current and is currently used in the treatment of supraventricular tachyarrhythmias and angina pectoris [31]. Verapamil may retard the active outward transport of the drug, modify membrane permeability to a variety of compounds by affecting the cell membrane structure and increase oxygen and ATP utilization; it may also reduce intracellular pH and interfere with calcium-regulating mechanisms [1, 22, 32]. Intracellular calcium homeostasis is normally maintained by the concerted operation of cellular transport and compartmentalization [33, 34]. Impairment of these processes during cell injury can result in enhanced calcium influx, release of calcium from intracellular stores and inhibition of calcium extrusion at the plasma membrane [35, 36]. The inhibition of calmodulin, the most important high-affinity calcium-binding protein present in considerable amounts in all eukaryotic cells, also modulates the slow calcium-dependent inward current [37]. Calmodulin inhibitors, such as phenothiazines and, in particular, trifluoperazine, can stabilize membranes by impairing calcium fluxes and are currently used in reversion of chemoresistance to Adriamycin [1, 38].

To understand how the cell response was modified and which key processes were altered by Adriamycin-induced chemoresistance, it was useful to compare second messenger levels in sensitive and resistant cells. The major second messengers that operate in eukaryotic cells are cAMP and calcium ions [39, 40]. These two signalling systems interact at protein kinase and protein phosphatase sites, regulate cellular processes, such as contraction and secretion, and play a specific role in mitogenesis [27]. To explore the reversion of chemoresistance to Adriamycin, the possible involvement of cAMP and calcium as intracellular second messengers in the transmembrane signal transduction of two resistant cell lines (MCF-7, a human acquired resistant breast carcinoma cell line and MIP-101, a colon carcinoma cell line resistant *de novo* to Adriamycin) expressing P-170 glycoprotein, was investigated.

MATERIALS AND METHODS

Cell culture. The MCF-7/adr and MCF-7/S breast cancer cell lines were a gift from Dr K. Cowan (Bethesda, U.S.A.). The MIP-101 human colorectal cell line was a gift from Dr P. Thomas (Harvard Medical School, Boston, MA, U.S.A.). The DLD-1 cell line was purchased from ATCC (CCL 221). MIP-101 and DLD-1 are poorly differentiated colon adenocarcinoma cell lines. Cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% FCS, 2 mM glutamine, 100 UI/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained at 37° in a humidified atmosphere of 95% air : 5% CO_2.

Subcultures of breast and colon cancer cells were achieved by the use of 0.25% trypsin plus 0.10% versene.

To study the growth rates of resistant (MCF-7/adr and MIP-101) and sensitive (MCF-7/S and DLD-1) cells, 15,000 cells/cm^2 were seeded in 24-well plates. After detachment by trypsinization, Coulter-counter cell counts were determined daily without refeeding. DT were determined during the log phase of the growth.

Drugs. Adriamycin was supplied by Farmitalia Carlo Erba (Milan, Italy) and P-Glyco-CHECK C 219 by Centocor Diagnostics (U.K.). Streptavidin-fluorescein was purchased from Boehringer (Mannheim, Germany), Indo-1-AM from Sigma (St Louis, MO, U.S.A.) and pluronic F-127 from Calbiochem, (San Diego CA, U.S.A.).

Chemosensitivity testing. Log-phase cultures were treated with Adriamycin in the presence and absence of three CS: verapamil, trifluoperazine and BSO. The cells were plated at 75×10^3 cells/mL in triplicate in a 6-well plate. After incubation, drugs were added to the desired final concentration and the cells were incubated for 72 hr. Adriamycin was added at increasing concentrations (from 10^{-7} to 10^{-4} M for the Adriamycin resistant cells and from 2.5×10^{-8} to 2.5×10^{-6} M for the sensitive cells). Treatments were performed for 72 hr with non-toxic concentrations of 22.2 μM verapamil, 4 μM trifluoperazine or 200 μM BSO for the MCF-7 cells and with non-toxic concentrations of 22.2 μM verapamil, 6 μM trifluoperazine or 200 μM BSO for the colon carcinoma cells. In control and treated cultures, cells were counted in a hemocytometer and cell viability was determined by Trypan blue dye exclusion.

Detection of P-glycoprotein. The cell lines were pelleted by centrifugation, then deposited as a monolayer cell smear on slides. The slides were incubated with the mouse monoclonal antibody C-219; this antibody recognizes an internal epitope. The cells were rinsed and incubated with biotinylated sheep anti-mouse second antibody or with biotinylated horse anti-mouse second antibody. The streptavidin-fluorescein-Blue Evans complex, was then applied. Slides were then covered with antifading and immediately observed under a Zeiss Axiophot microscope. Control slides were treated similarly except that the primary antibodies were omitted [42].

Glutathione measurement. Total intracellular glutathione (reduced and oxidized glutathione) levels were measured using HPLC and electrochemical detection [43].

Intracellular calcium measurements $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was estimated from Indo-1 fluorescence by the ratio method using single wavelength excitation (350 nm) and dual emission (405 and 480 nm) [44]. The optical system was developed around a Nikon diaphot inverted microscope equipped with epifluorescence optics and a 75 W xenon lamp. Neutral density filters that reduced the intensity of illumination by factors 64 or 128 were routinely used. Cells were grown on glass coverslips and treated for 72 hr with the three CS. They were then loaded with 5 μM of the penetrant Indo-1 AM from a stock solution of 1 mM in dimethylsulphfoxide containing 3% pluronic F-127.

Loading was performed at 37° for 75 min in a bathing solution containing 20 mM HEPES, 5.5 mM sucrose, 130 mM NaCl, 5.4 mM KCl, 0.9 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4. The cells were then washed and left for a further 75 min period in the bathing solution to allow ester hydrolysis to reach completion.

The ratio, R, of emitted fluorescence at 405 and 480 nm was used to calculate the $[Ca^{2+}]_i$ concentration (expressed in nM) according to the equation of Grynkiewicz *et al.* [44]:

$$[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min}/R_{max} - R)$$

where R_{min} and R_{max} are the fluorescent ratios obtained in the virtual absence of calcium [by the addition of 10 μM ionomycin plus 5 mM ethylene glycol-*bis*(β-aminoethyl ether) *N*, *N*, *N'*, *N'*-tetraacetic acid] and at the saturating concentration of calcium (by the addition of 10 μM ionomycin plus 5 mM $CaCl_2$), respectively. β is the ratio of the 480 nm signals in the absence and at the saturating concentration of calcium. For the dissociation constant, K_d, a value of 250 nM was assumed [44]. An average of 30 different cell measurements were assessed for one $[Ca^{2+}]_i$ estimation.

Cell surface determination. Cells were loaded with 5 μM Fluo-3 dye (Fluo-3 AM; Sigma, France) as described for Indo-1 AM. This fluorescent dye excited at 490 nm has a single emission wavelength at 510 nm. Cells were visualized using a microscope video camera system connected with an Argus-10 image processor (Hamamatsu, Japan).

cAMP Concentration determination. After a 72 hr treatment, the cells were harvested by trypsinization and sedimented by centrifugation at 100 *g* for 10 min. The pellet was resuspended in PBS and, after an additional centrifugation, the supernatant was removed. The cells were resuspended in 500–1000 μL PBS. This solution was applied to a minicolumn (Amprep-Amersham) previously conditioned with 2 mL methanol and 2 mL water. The column was washed once with 3 mL methanol. The cAMP fraction was eluted with 3 mL 0.1 M HCl and collected. This fraction was lyophilized and its cAMP content measured by a competitive binding assay (Amersham Cyclic AMP Assay Kit).

Protein measurement. Total protein determinations were carried out, following sonication of cells, by the BCA, using a kit from Pierce [45].

RESULTS

Cell characteristics

The MCF-7/adr cells were significantly larger than MCF-7/S cells (438 ± 159 and 307 ± 102 μm^2, respectively) ($P < 0.01$, $N = 50$, Student's test), whereas no modification was observed in the colon carcinoma cell lines (207 ± 53 and 204 ± 54 μm^2 for MIP-101 and DLD-1, respectively).

In the exponential growth phase the average DT of MCF-7/adr were significantly higher (DT = 34 hr 05 min ± 2 hr 09 min) than in MCF-7/S cells (DT = 29 hr 30 min ± 1 hr 40 min) ($P < 0.01$, $N = 6$, Student's test). The population DT was also significantly longer in the MIP-101 resistant cells (DT = 22 hr 05 min ± 1 hr 19 min) than in DLD-1 sensitive cells (DT = 19 hr 36 min ± 1 hr 23 min) ($P < 0.01$, $N = 6$, Student's test).

Table 1. Enhancement of Adriamycin cytotoxicity by verapamil, trifluoperazine and BSO in a mammary carcinoma cell line (MCF-7) and in a colon carcinoma cell line (MIP-101 and its sensitive counterpart DLD-1)

	Ratio of chemoresistance	
Chemosensitizer	MCF-7 cells	DLD-1 and MIP-101 cells
None	200	10
Verapamil	55	2.5
Trifluoperazine	29	5
BSO	44	5

These values are the means of three experiments with SD less than 10% of the reported values.

Circumvention of Adriamycin resistance in MDR cells

The sensitivity of MDR positive and sensitive cells was evaluated by cell counting in a hemocytometer and viability was determined by Trypan blue dye exclusion. The Adriamycin IC_{50} were 50 μM for MCF-7/adr and 0.25 μM for MCF-7/S, indicating an approximate 200-fold resistance, and 1 μM for MIP-101 and 0.1 μM for DLD-1 cells, indicating an approximate 10-fold resistance.

Treatment of cells with non-toxic concentrations of verapamil, trifluoperazine or BSO resulted in an increased sensitivity of MDR cells to drugs such as Adriamycin. These three CS partially overcame Adriamycin resistance in the two cancer cell lines. The ratios of resistance were 55, 29 and 44, respectively, compared to 200 for untreated MCF-7/adr cells and 2.5, 5 and 5, respectively, compared to 10 for MIP-101 untreated cells (Table 1).

Detection of P-170

Tumour cell expression of glycoprotein P-170 associated with MDR was estimated using the monoclonal antibody C-219. P-170 was observed in MCF-7/adr but no immunoreaction was detected using the MCF-7/S cells, the controls showed no immunofluorescence (data not shown). MIP-101 cells, resistant *de novo* to Adriamycin, showed an intense membrane immunoreaction to C219 and there was also some staining of the membrane in the DLD-1 cells [42], this being due to the fact that P-glycoprotein plays an important physiological role in these tissues. The basal levels of P-170 expression were not modified by using the three CS, were unrelated to the action of verapamil, trifluoperazine or BSO, and were not associated with any consistent change in potentiation of Adriamycin sensitivity.

Glutathione levels

No significant modification was observed between the values of reduced and oxidized glutathione in sensitive and resistant MCF-7 cells. The values of reduced glutathione (expressed in nmol/mg protein)

Table 2. Content of calcium and cAMP in Adriamycin sensitive (MCF-7/S and DLD-1) and resistant (MCF-7/R and MIP-101) cell lines

	MCF-7/S	MCF-7/R	DLD-1	MIP-101
Calcium (nM)	117.18 ± 4.22	232.49 ± 15.30	100.84 ± 3.39	335.39 ± 7.90
cAMP (pmol/mg protein)	112.42 ± 10.78	18.74 ± 2.72	132.33 ± 11.64	44.34 ± 3.46

Values are the means ± SD of at least 35 determinations for calcium and at least three separate experiments for cAMP. The values were significantly different for sensitive and resistant cells ($P < 0.01$, Student's t-test).

were 44.50 ± 3.91 and 45.02 ± 3.93 in MCF-7/S and MCF-7/adr cells, respectively; the values of oxidized glutathione under the same conditions were 1.32 ± 0.49 and 1.47 ± 0.65, respectively. Verapamil and BSO, in contrast to trifluoperazine, induced an important reduction in reduced glutathione in sensitive and resistant cells (residual value of ±5% of the initial value). The values of reduced glutathione (expressed in nmol/mg protein) were 52.89± 5.10 and 89.72 ± 8.97 in DLD-1 and MIP-101 cells, respectively; the values of oxidized glutathione under the same conditions were 4.49 ± 1.49 and 4.55 ± 1.21, respectively [42]. The level of reduced glutathione was lowered by the three chemosensitizers in the colon carcinoma cells.

Measurement of basal calcium and cAMP levels

A fluorescent dye like Indo-1, whose fluorescence changes on complexing calcium ions, could be loaded into cells as the AM ester. Indo-1 has high selectivity for Ca^{2+} over H^+ and Mg^{2+} and the ratio dye output is independent of dye concentration, thereby making the measurements relatively insensitive to dye leakage and changes in cell shape and volume. Under the conditions in this study, autofluorescence of cells was negligible. The levels of $[Ca^{2+}]_i$ in the two different cell lines (MCF-7 and MIP-101 cells) were approximately 2–3-fold higher in resistant than in sensitive cells. In contrast, cAMP levels in these different cells, as measured by radioimmunoassay, were significantly lower in resistant than in sensitive cells (Table 2).

Effect of chemosensitizers on calcium and cAMP levels

The three CS induced significant, but conflicting, modifications in calcium levels in both types of Adriamycin-resistant cells. The MCF-7/adr cells treated with BSO or verapamil tended to decrease their calcium levels to values close to those of the MCF-7/S cells, the effect of trifluoperazine was very weak (Fig. 1A). In the MIP-101 cells, the effect of verapamil was the same as in the MCF-7/adr cells, and trifluoperazine was nearly as efficient as this calcium-channel blocker. The effect observed with BSO was particular: the calcium level increased in the DLD-1 and the MIP-101 cells but the MIP-101/DLD-1 ratio tended to decrease, thus contributing to reversion of chemoresistance (Fig. 1B).

When considering the effect of the CS on the cAMP level, the modifications observed were distinctly different. BSO was the only CS which significantly modified the cAMP values of MCF-7/S and MCF-7/adr cells (significant modification, $P < 0.01$, Student's test) (Fig. 2A). No significant modifications were observed in the DLD-1 and MIP-101 cells (Fig. 2B).

DISCUSSION

An experimental mechanism of resistance to cytotoxic agents extensively studied in recent years is P-170 glycoprotein associated MDR, an energy-dependent multidrug efflux pump.

A variety of agents have been shown to be capable of reversing MDR *in vitro* and in animal models [1]. Such so-called CS are believed to function by inhibiting P-170 activity or by modifying glutathione metabolism, resulting in increased intracellular concentrations of the cytotoxic agents.

In recent years various CS have been evaluated. This group has studied verapamil, trifluoperazine and BSO.

Due to the inter-relations existing between calcium and cAMP, and the effects of these second messengers on protein kinases, the study of these parameters was particularly interesting [41, 45, 46]. Phosphorylation of certain forms of the inositol 1,4,5 triphosphate receptor by protein kinase A may regulate the release of calcium from the endoplasmic reticulum, while protein kinase A has been reported to up-regulate certain calcium-activated potassium channels and to down-regulate others in the brain. Adenylate cyclase, the catalytic protein that converts ATP to cAMP, plays a critical role in the signal transduction cascade of a number of fundamental hormones and neurotransmitters.

The MCF-7 Adriamycin-resistant cell line, in contrast with its sensitive counterpart, expressed the P-170 glycoprotein, detected by immunofluorescence (data not shown). Whereas the fluorescence intensity of DLD-1 sensitive cells was slightly higher than that of control antibody, that of MIP-101 cells, resistant *de novo* to Adriamycin displayed a definite increment in fluorescence, indicating overexpression of P-glycoprotein in these resistant cells [42].

Data reported here clearly demonstrate a sensitization effect of verapamil, trifluoperazine and BSO in both human breast and colon carcinoma cell lines expressing P-170 glycoprotein. These compounds, added at non-toxic doses, induced a partial reversion of the process of chemoresistance

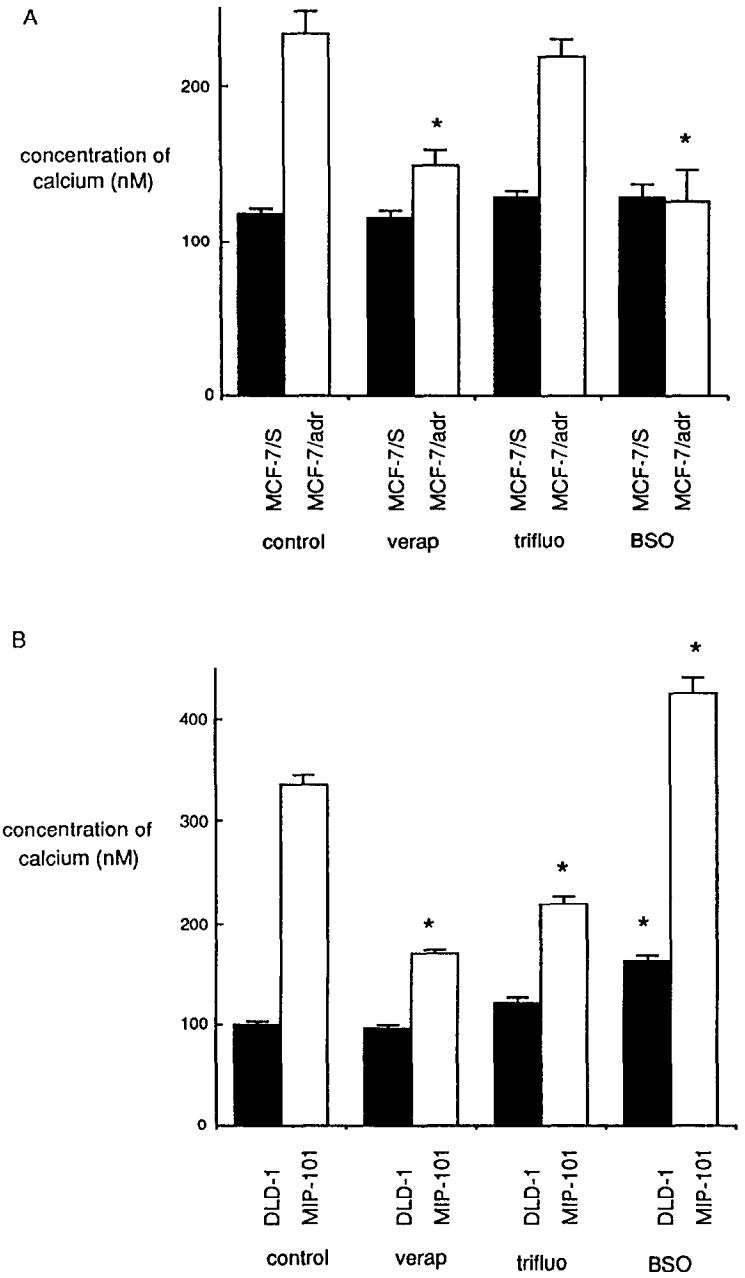

Fig. 1. (A) Effect of verapamil (verap), trifluoperazine (trifluo) or BSO on calcium levels in MCF-7 Adriamycin-resistant and sensitive cell lines. (B) Effect of verap, trifluo or BSO on calcium levels in MIP-101-resistant and DLD-1 sensitive cells. Cells were treated as described in Materials and Methods. Results are the means ± SD of at least 35 data. * Significantly different values from control ($P < 0.01$, Student's t-test).

to Adriamycin. In the MDR cells treated with verapamil, trifluoperazine and BSO, P-170 expression did not change from the Adriamycin-resistant untreated cells. The modification in sensitivity to Adriamycin over a 3 day period did not parallel P-170 expression.

As found by Tsuruo *et al.* [6], in leukemia and Chinese hamster ovary cells, a higher cellular calcium content in Adriamycin-resistant than in sensitive cells was observed. On the contrary, cAMP was significantly lower in the resistant than in the sensitive cells.

The different CS treatments did not induce the same effects on intracellular calcium. Verapamil significantly reduced calcium in both resistant cells to levels near to those found in sensitive counterparts. Verapamil is also, in the authors' opinion, the most efficient compound for the reversion of Adriamycin

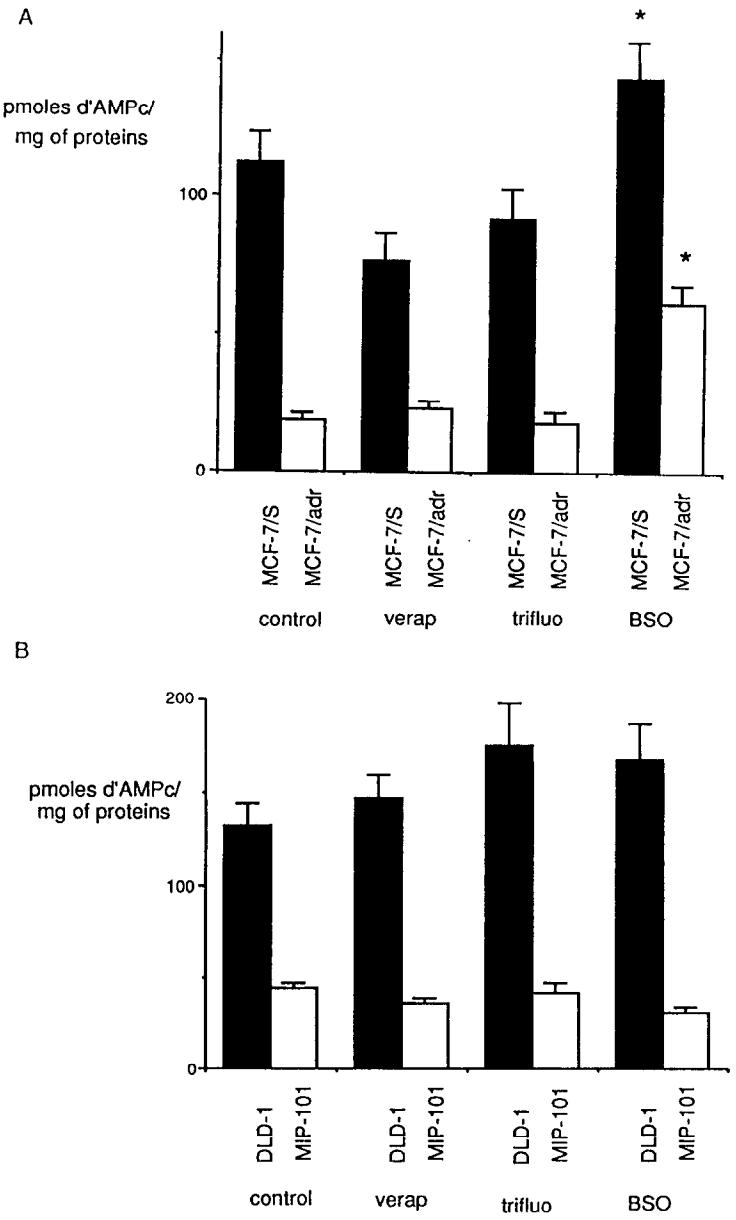

Fig. 2. (A) Effect of verapamil (verap), trifluoperazine (trifluo) or BSO on cAMP levels in MCF-7 Adriamycin-resistant and sensitive cell lines. (B) Effect of verap, trifluo or BSO on cAMP levels in MIP-101-resistant and DLD-1 sensitive cells. Cells were treated as described in Materials and Methods. Results are the means ± SD of at least three separate experiments, each in triplicate. * Significantly different values from control ($P < 0.01$, Student's t-test).

resistance in colon cancer cells. In contrast, trifluoperazine, the most important chemosensitizer in breast cancer cells, seemed to have a modest effect on the reduction of calcium levels in these cells. Furthermore, the effects of the three CS on the cAMP content of Adriamycin-resistant cells were not clear.

BSO treatment in the two resistant cell lines induced a very important depletion of glutathione. Conflicting results were obtained concerning its relation to calcium modification. As found in normal hepatocytes, during oxidative stress, a rise in the calcium level in colon carcinoma cells was observed but there was a decrease in breast cancer cells [48].

In conclusion, trifluoperazine, the most active CS in breast cancer cells, induced very slight differences in the second messenger levels, and verapamil, the most active CS in colon carcinoma cells, induced important modifications only in the calcium level. Thus, no clear relationship could be found between calcium and cAMP levels and reversion of chemoresistance to Adriamycin; therefore, these

results seemed to rule out any implication of calcium and cAMP modifications in the circumvention of the chemoresistance process. It is likely, therefore, that the clinical efficiency of verapamil may not be attributed to its role on calcium channels; the change in calcium level *per se* might not be the only event involved.

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