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S Scala, … , M Tsokos, S E Bates

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Research Article

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Downregulation of *mdr*-1 Expression by 8-Cl-cAMP in Multidrug Resistant MCF-7 Human Breast Cancer Cells

Stefania Scala,* Alfredo Budillon,§ Zhirong Zhan,* Yoon S. Cho-Chung,‡ Jane Jefferson,‡ Maria Tsokos,‡ and Susan E. Bates*

**Medicine Branch and ‡Laboratory of Pathology, Division of Cancer Treatment; and §Laboratory of Tumor Immunology and Biology, Division of Cancer Biology, Diagnosis, and Centers; National Cancer Institute, Bethesda, Maryland 20892*

Abstract

8-Cl-cAMP, a site-selective analogue of cAMP, decreased *mdr*-1 expression in multidrug-resistant human breast cancer cells. A sixfold reduction of *mdr*-1 mRNA expression by 8-Cl-cAMP began within 8 h of treatment and was associated with a decrease in the synthesis of P-glycoprotein and with an increase in vinblastine accumulation. A reduction in *mdr*-1 expression after 8-Cl-cAMP treatment was also observed in multidrug-resistant human ovarian cancer cell lines. 8-Cl-cAMP is known to change the ratio between the two regulatory subunits, RI and RII, of protein kinase A (PKA). We observed that RIα decreased within 24 h of 8-Cl-cAMP treatment, that RIIβ increased after as few as 3 h of treatment, and that PKA catalytic activity remained unchanged during 48 h of 8-Cl-cAMP treatment. The results are consistent with the hypothesis that *mdr*-1 expression is regulated in part by changes in PKA isoenzyme levels. Although 8-Cl-cAMP has been used to differentiate cells in other model systems, the only differentiating effect that could be detected after 8-Cl-cAMP treatment in the MCF-7TH cells was an increase in cytokeratin expression. Evidence that the reduction of *mdr*-1 mRNA occurred at the level of gene transcription was obtained by measuring chloramphenicol acetyltransferase (CAT) mRNA in MCF-7TH cells transfected with an *mdr*-1 promoter–CAT construct prior to 8-Cl-cAMP treatment. Thus, 8-Cl-cAMP is able to downregulate *mdr*-1 expression and suggests a new approach to reversal of drug resistance in human breast cancer. (*J. Clin. Invest.* 1995. 96:1026–1034.) Key words: P-glycoprotein • protein kinase A • MDR • cAMP • reversal

Introduction

Advances in the treatment of metastatic breast cancer with standard dose cytotoxic therapy have been limited during the last 15 yr. Breast cancer is not inherently refractory to chemotherapy, since most women respond initially but later relapse and treatment failure occurs. Although multiple drug resistance mechanisms probably account for this, P-glycoprotein has been cited as playing a role in resistance in breast cancer (1–3). The product of the *mdr*-1 gene, P-glycoprotein, mediates multidrug resistance by enhanced outward transport of drugs of natural product origin (4). P-glycoprotein has been demonstrated in human breast cancer cells selected in vitro for drug resistance (5), and the protein has been observed in breast cancer samples (1, 2, 6). Expression is typically found in previously treated breast cancers and less often in untreated breast cancer (2, 3, 7). The present studies were undertaken to determine whether P-glycoprotein expression could be modulated in multidrug-resistant human breast cancer cells.

Studies of the regulation and function of P-glycoprotein have demonstrated that the *mdr*-1 gene can be induced in various model systems. Beginning with the observation of increased *mdr*-1 in regenerating rat liver (8), multiple studies have demonstrated increased expression in response to various agents or environmental stimuli (9–13). In contrast, decreased *mdr*-1 expression is seldom reported. Abraham et al. demonstrated a decreased *mdr*-1 level in a cell line transfected with a mutant regulatory subunit of protein kinase A (PKA)[1] (14). Likewise, Kim reported a decrease in *mdr*-1 levels after treatment of P388 resistant cells with the PKA inhibitor H-87 (15). Okabe-Kado et al. reported a 50% decrease in MRK-16 binding and an enhancement of drug sensitivity after treatment of multidrug-resistant K562/VCR leukemic cells with erythroid differentiation factor, one of four agents which induced erythroid differentiation in the cells (16). Schecter et al. reported that xenografts of a mammary adenocarcinoma cell line had a lower level of *mdr*-1 expression than was observed in the cells cultured in vitro (17). Taken together, these experiments demonstrate that the *mdr*-1 gene can be regulated by diverse agents but give little clue to the mechanism whereby this regulation occurs.

The finding that the differentiating agents sodium butyrate and retinoic acid increased levels of *mdr*-1 in cell lines derived from colon cancer, kidney cancer, and neuroblastoma (9, 10) (unpublished results) gave rise to the hypothesis that differentiating agents could alter *mdr*-1 expression in a cancer cell toward the level found in the tissue of origin. Thus, in neuroblastoma and in colon and kidney cancer cell lines, the addition of differentiating agents could increase *mdr*-1 because the normal adrenal, colon, and kidney express *mdr*-1/P-glycoprotein. We proposed that where normal tissue expression is low, as in the normal ovary or breast (6, 18–20), the effect of some differentiating agents on malignant cells could be to decrease levels of *mdr*-1. We found that 8-Cl-cAMP, a differentiating agent in human leukemic cells (21), reduced *mdr*-1 expression in a multidrug-resistant human breast cancer subline.

8-Cl-cAMP is a site-selective analogue of cAMP which has been demonstrated to induce differentiation in several model

Address correspondence to Susan E. Bates, M.D., Medicine Branch, NCI, Building 10, Room 12N226, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-0795; FAX: (301) 402-0172.

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; MCF, Michigan Cancer Foundation; MDR, multidrug resistance; PKA, protein kinase A.

systems (21–23). The differentiating effect of 8-Cl-cAMP is thought to be mediated by changes in the levels of the regulatory subunits of cAMP-dependent PKA. PKA exists in the holoenzyme state as four subunits, two catalytic and two regulatory (24). Two classes of PKA (type I and type II) are composed of distinct regulatory subunits, I and II, and a common catalytic subunit. In the normal setting, cAMP binds to two sites on the regulatory subunits and results in dissociation of mainly type I PKA, releasing the catalytic subunit from the regulatory subunit (RI), thereby activating the enzyme for phosphorylation of various proteins. 8-Cl-cAMP binds to the two sites on RI with a higher affinity than cAMP and results in activation of the type I PKA, with subsequent downregulation of both the protein and mRNA for RI (22, 25–28). 8-Cl-cAMP also binds, with a high affinity, one of the two sites of RII, but binds the other poorly. This results in an increase of type II PKA holoenzyme. Moreover, RII (or PKA type II) is translocated into the nucleus within 10 min of addition of 8-Cl-cAMP, where transcription of different genes may be affected (25).

We examined the effect of 8-Cl-cAMP on PKA activity, *mdr*-1 expression, P-glycoprotein synthesis, and vinblastine accumulation in MCF-7TH cells.

Methods

Cell lines and cell culture conditions. The MCF-7 human breast cancer cell line was obtained from Marc Lippman (Georgetown University, Washington, D.C.), having been frozen in its 66th passage (29). MCF-7TH cells are a multidrug-resistant subline isolated from parental MCF-7 cells by intermittent exposure to 1×10^{-6} M Adriamycin (Adria Labs, Columbus, OH). The cells were a gift from Tom Hamilton (Fox Chase Cancer Center, Philadelphia, PA); verification of their MCF-7 origin was made by Ward Peterson (Cell Culture Identification Service, Children's Hospital of Michigan, Detroit, MI). The cells were maintained in 200 ng/ml Adriamycin and have a classical multidrug-resistant phenotype, with decreased drug accumulation and overexpression of the *mdr*-1 gene. Both the parental cells and the drug-resistant subline were maintained in DME (GIBCO BRL, Gaithersburg, MD) plus 10% FCS, glutamine, and gentamicin (25 μg/ml). Other cell lines studied were ovarian cancer cells derived by selection of parental A2780 and 1847 cells in Adriamycin (30). These cells were maintained in RPMI (GIBCO) plus 10% FCS, and penicillin and streptomycin.

Cells were treated with 8-Cl-cAMP (Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) in the logarithmic phase of growth, because the effects of 8-Cl-cAMP were blunted if cells were treated during the plateau phase of growth. In addition, certain serum lots were found to blunt the 8-Cl-cAMP effect, due to higher adenosine deaminase levels (Y. Cho-Chung, unpublished observations). Studies were routinely carried out in heat-inactivated FCS.

Cytotoxicity assays. The cytotoxicity of Adriamycin, 8-Cl-cAMP, and 8-Cl-adenosine (from Biolog Life Science Institute, San Diego, CA) was evaluated in MCF-7TH cells seeded at 2,000 cells/well in 96-well plates. Cells were treated 24 h later, incubated for 2, 3, and 6 d, and then fixed with trichloroacetic acid, stained for 30 min with 0.4% (wt/vol) sulforhodamine B (SR B; Sigma Chemical Co., St. Louis, MO) and dissolved in 1% acetic acid. Plates were washed in 1% acetic acid, and the remaining dye extracted with 10 mM Tris for determination of optical density at 564 nM by a plate reader (Bio Rad Laboratories, Richmond, CA), as previously described (31). The percentage of growth inhibition was calculated according to the formula $(T_{drug} - T_o)/(T_c - T_o) \times 100$; for $T_{drug} < T_o$, the formula was $(T_{drug} - T_o)/T_o \times 100$, where T_{drug} represents the optical density of the treated cells; T_o, the optical density of cells harvested at the time treatment was started (T_o); and T_c, the optical density of the control cells.

RNA extraction, electrophoresis, and blotting. Total cellular RNA was extracted from cells by homogenizing in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. Total RNA was electrophoresed in 1% agarose, 6% formaldehyde gels, which were subsequently stained with 2 μg/ml ethidium bromide to allow inspection of the quantity and quality of RNA (32).

Northern blot and RNase protection analysis. For Northern blot analysis, a 1.4-kb cDNA containing sequences from the middle third of *mdr*-1/Pgp subcloned in a pGEM vector was used for riboprobe synthesis. Hybridization was performed as previously described (9). For RNase protection, we utilized a 1-kb genomic sequence from the 5′ end of the gene subcloned into pGEM-3 and linearized with PvuII (33). The 1-kb PstI fragment contains a promoter region of the human *mdr*-1/Pgp gene. As a control for RNA loading, an antisense molecule (cRNA) was synthesized from p36B4 using T7 polymerase (34). Because it is not affected by estrogen, 36B4 has been utilized as a control by investigators in studies of mRNA regulation in MCF-7 cells (35). Hybridization and RNase digestion were performed as previously described (9). Quantitation was performed using a densitometer (Fotodyne, New Berlin, WI).

Quantitative polymerase chain reaction. A quantitative PCR assay was utilized as previously described (36, 37). Briefly, reverse transcription of 1-μg total RNA was initiated with random primers using M-MTLV reverse transcriptase. The resulting cDNA was diluted 2,000-fold and amplified 30 cycles using β-tubulin–specific 5′ primer (nucleotides 342–360, TTCTGTCCTGGATGTGGTA) and 3′ primer (nucleotides 521–539, AAGTGTCTGACACCGTGGT) (38). The PCR products were electrophoresed in agarose-NuSieve (FMC Bioproducts, Rockland, ME) gels and then stained with ethidium bromide for densitometric analysis.

Immunoprecipitation and metabolic labeling. Untreated cells or cells treated with 8-Cl-cAMP were labeled as previously described (39) with 1 mCi of [^{35}S]methionine (New England Nuclear Research Co., Boston, MA) in 4 ml of methionine-free DME without serum for 16 h. 8-Cl-cAMP was added 72 h before harvesting the cells and continued during cell labeling. After harvesting cells in RIPA buffer (1% Triton X-100, 0.1% $NaDodSO_4$, 1% sodium deoxycholate, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.2), immunoprecipitation was carried out with a polyclonal antibody raised against recombinant protein fragments (40); the product was analyzed on a 7% polyacrylamide gel.

Immunoblotting. Cells were homogenized in 250 mM sucrose, 10 mM Tris pH 7.5, 1% aprotinin, and membrane protein obtained for immunoblotting as previously described (41). Samples (20 μg) were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The blots were blocked for 2 h with 3% BSA in TTBS (0.1% Tween 20, mM Tris-HCl, pH 7.5, 150 mM NaCl) prior to incubating for 1 h in a 1:2,000 dilution of the primary polyclonal anti-Pgp antibody, 4007 (40). After washing with TTBS, the blots were incubated for 1 h in a 1:4,000 dilution of donkey anti–rabbit horseradish peroxidase–conjugated secondary antibody. Immunoreactive protein was detected using an enhanced chemiluminescence method (ECL; Amersham Corp., Arlington Heights, IL).

Immunohistochemical staining. For determination of keratin, cells were plated on poly-L-lysine–coated slides in quadriperm plates, treated with 8-Cl-cAMP for 72 h, and fixed according to the antibody manufacturer's directions. Keratin was stained using monoclonal anti-cytokeratin peptide 18 antibody (Sigma Chemical Co.). Bound antibody was detected using the ABC immunoperoxidase technique (42).

Drug accumulation assay. Drug accumulation and efflux studies were performed in six-well dishes as previously described by Fojo et al. (43).

Immunoprecipitation. Immunoprecipitation of RIIβ was performed by the method previously described (21). Briefly, proteins (30 μg) in cell lysates were photoaffinity labeled with ^{32}P-8-N_3-cAMP (ICN Biochemicals, Irvine, CA) and incubated with monospecific anti-RIα or anti-RIIβ antibody (kindly provided by Dr. S. D. Park, Seoul National University, Seoul, Korea) at a dilution of 1:10, in the presence of 1% SDS, first for 10 min at 37°C and then for 16 h at 4°C. The reactions

were then incubated with an equal volume of a 1:2 slurry of Protein A Sepharose in PBS for 60 min at 23°C. Proteins bound to Protein A Sepharose were recovered by centrifugation and washing the pellet 2 times with PBS, released by heating, resolved by electrophoresis in SDS 10% polyacrylamide gels, and blotted onto nitrocellulose paper. Radioactivity was detected by autoradiography using Kodak X-Omat film (Eastman Kodak Co., Rochester, NY).

c-AMP–dependent protein kinase assay. PKA was measured in cell pellets that were lysed and dounced before the assay was performed with ATP, [γ-^{32}P] ATP (ICN Biochemicals), $MgCl_2$, kemptide (Sigma Chemical Co.) and Tris—with and without protein kinase inhibitor or cAMP (both from Sigma Chemical Co.) (21). The incubation mixture was spotted on phosphocellulose filters and washed with phosphoric acid; then the radioactivity was measured. The PKA ratio is defined as the ratio of activity measured in the absence and in the presence of 8 μM cAMP. Since cAMP fully activates PKA, this ratio is a measure of how much PKA is activated relative to the total amount of PKA present.

DNA transfection and analysis of transient transfections. Transient transfection of vector DNA was accomplished by the lipofectin method (GIBCO BRL). The MDR promoter–CAT (Chloramphenicol acetyltransferase) construction was kindly provided by Ken Cowan (National Cancer Institute, Bethesda, MD). MDRCAT contains 4.7 kb of genomic sequence from *mdr*-1, upstream of the transcription initiation site (−4741 to +286), in the plasmid vector SVOCAT (44). This vector (30 μg) was cotransfected with 10 μg of pGL2–control vector (Promega Corp., Madison, WI) containing the luciferase reporter gene under the SV40 promoter and enhancer sequence. MCF-7TH cells were transfected for 2 h in OPTIMEM media (GIBCO BRL). The transfection media was then removed, and DME supplemented with 10% heat-inactivated FBS was added. The cells were allowed to recover for 8 h before a 48-h treatment with 5 and 25 μM 8-Cl-cAMP began. Cell extracts were prepared according to the manufacturer's directions (Luciferase Assay System; Promega), and the reactions were initiated by addition of 100 μl of luciferase assay reagent. The luciferase activity was determined for each sample by integration of luminescence signals over 20 s using a luminometer (Monolight model 301; American Instruments Co., Silver Spring, MD). The luciferase assay was performed on one-third of the sample. The remainder was harvested in GTC for RNA isolation, and CAT expression was detected by RNase protection with a protected fragment of 250 bp from psv2CAT subcloned in PGEM7ZF+ (Promega) and linearized with BamHI.

Results

We investigated the effect of 8-Cl-cAMP, a site-selective analogue of cAMP, on *mdr*-1 expression in multidrug-resistant

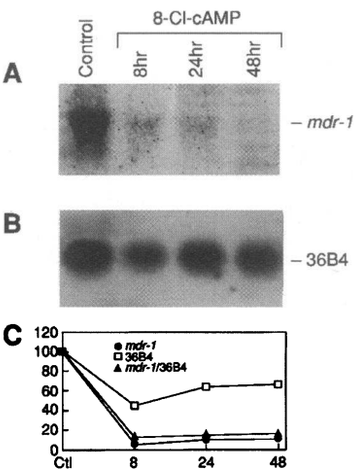


Figure 1. Time course of *mdr*-1 expression after 8-Cl-cAMP treatment in MCF-7TH cells. (*A*) Northern analysis of *mdr*-1 expression in 8-μg total RNA from MCF-7TH cells treated for 8, 24, and 48 h with 8-Cl-cAMP, 5 μM. (*B*) Hybridization signal for 36B4, control gene. (*C*) Plot of densitometric analysis of *mdr*-1 and normalization to 36B4. Results are expressed as a percentage of control levels, which are assigned a value of 100%.

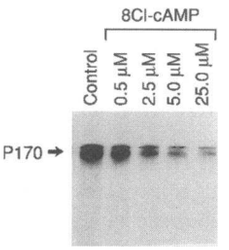


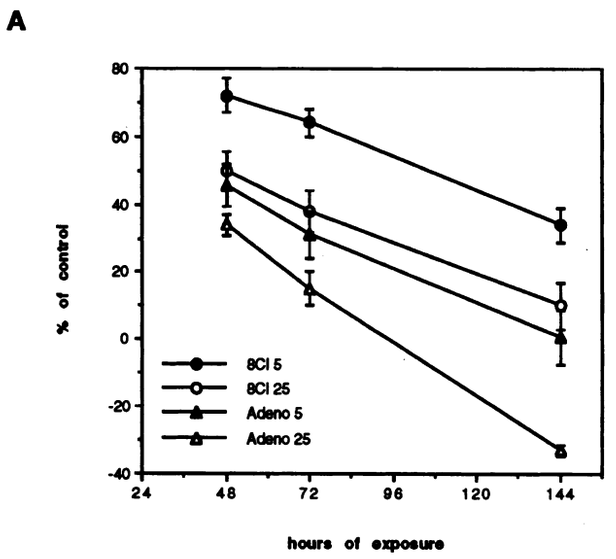
Figure 2. Dose-response relationship of 8-Cl-cAMP in MCF-7TH cells. [^{35}S]-Methionine–labeled MCF-7TH cells were treated with varying concentrations of 8-Cl-cAMP for 72 h prior to immunoprecipitation. [^{35}S]Methionine labeling was carried out for the final 16 h of the 8-Cl-cAMP treatment, and the latter was present during the labeling period.

MCF-7TH cells. 8-Cl-cAMP resulted in a marked decrease in *mdr*-1 expression by Northern analysis. To determine the rapidity of the reduction, time course studies were performed after 8-Cl-cAMP treatment, shown in Fig. 1. A reduction in *mdr*-1 expression ranging from 5- to 10-fold could be observed as early as 8 h after treatment with 5 μM 8-Cl-cAMP and persisting over 24 and 48 h of treatment (Fig. 1 *A*). The decrease in *mdr*-1 can be compared to that of the control gene 36B4 (Fig. 1 *B*) (35).

The decrease in *mdr*-1 expression was accompanied by a decrease in P-glycoprotein synthesis that was dose dependent, as shown in Fig. 2. [^{35}S]methionine labeling of cells was examined after 72 h treatment with 8-Cl-cAMP, in doses ranging from 0.5 to 25 μM. Decreased synthesis was seen at all doses, with greater inhibition observed at higher doses of 8-Cl-cAMP.

The dose-dependent decrease in Pgp synthesis was accompanied by a decrease in cell growth. Although this observation has been reported associated with the differentiating effect of 8-Cl-cAMP, some have ascribed the growth inhibition seen with 8-Cl-cAMP to toxic effects of its adenosine metabolite (45, 46). Thus, to evaluate whether 8-Cl-adenosine could be mediating the reduction in *mdr*-1 expression, cell growth assays and Northern analysis were performed. Cell growth assays in parental MCF-7 and MCF-7TH cells showed significant growth inhibition with both 8-Cl-cAMP and 8-Cl-adenosine. Fig. 3 illustrates the growth of MCF-7TH (*A*) and MCF-7 (*B*) cells treated with 8-Cl-cAMP (5 and 25 μM) and 8-Cl-adenosine (5 and 25 μM) for 48 and 72 h, and 6 d. 8-Cl-adenosine was much more of a growth inhibitor than was 8-Cl-cAMP at the three different time points in both cell lines. Northern analysis again demonstrated the reduction in *mdr*-1 m-RNA expression after 48 h of treatment with 5 and 25 μM 8-Cl-cAMP, but no reduction in *mdr*-1 expression after treatment with 25 μM 8-Cl-adenosine (Fig. 4 *A*). Thus, the metabolite 8-Cl-adenosine may account for some of the growth inhibition observed in MCF-7TH cells but cannot account for the regulation of *mdr*-1 expression. Although a modest reduction in expression of the control gene, 36B4, was also observed after 8-Cl-cAMP treatment, no modulation was seen in the expression of a second control gene, β-tubulin. Fig. 4 *B* shows the results of a separate experiment in which *mdr*-1 was reduced fivefold by 25 μM 8-Cl-cAMP, while β-tubulin levels were unchanged. In addition, the effects of the P-glycoprotein antagonist, verapamil (Knoll Pharmaceuticals, Whippany, NJ), and of the nonspecific protein kinase inhibitor, H-7 (Seikagaku America, Inc., Rockville, MD), were examined (47). Neither agent downregulated *mdr*-1 expression.

After demonstrating downregulation of *mdr*-1 mRNA levels by 8-Cl-cAMP, we evaluated drug transport by vinblastine accumulation and efflux studies. Because of the long half-life of Pgp (72 h) (39), functional studies were performed after 5 and 10 d of treatment with 5 μM 8-Cl-cAMP. 8-Cl-cAMP increased ^{3}H-vinblastine accumulation 2.5- and 3.5-fold, respectively, less



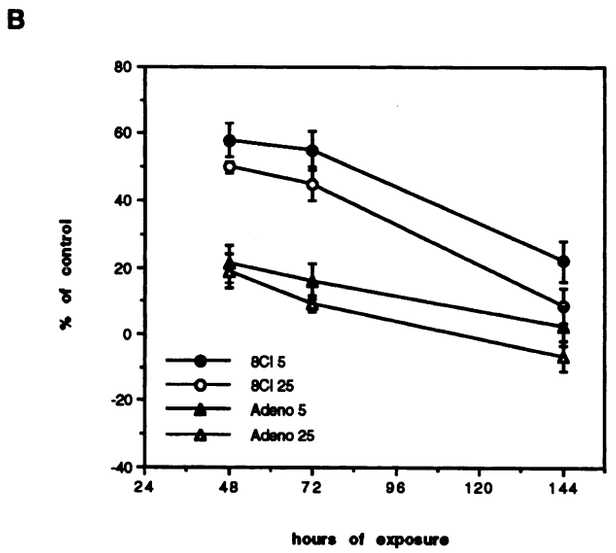


Figure 3. Cell growth assays with 8-Cl-cAMP and 8-Cl-adenosine. Cells were plated at 2,000 cells/well in 96-well plates the day before treatment with the concentrations shown. After 48 h, 72 h, and 6 d incubation, the cells were fixed with 1% trichloroacetic acid and the results assayed using sulforhodamine B (31). Results are shown as a percentage of untreated control±SD. (*A*) MCF-7TH cells; (*B*) MCF-7.

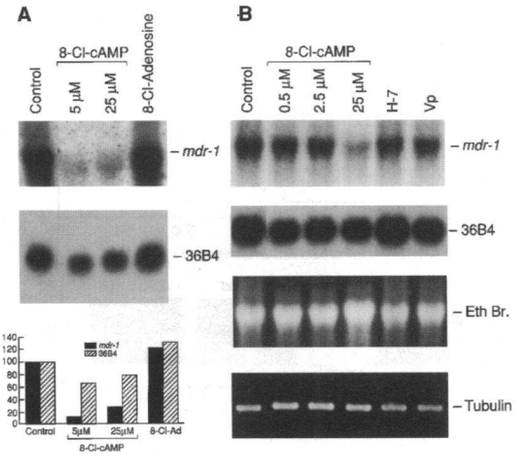


Figure 4. Expression of *mdr*-1 following treatment with 8-Cl-cAMP, 8-Cl-adenosine, H-7, and verapamil. (*A*) Northern analysis of *mdr*-1 after 8-Cl-cAMP and 8-Cl-adenosine treatment of MCF-7TH cells. 8 μg total RNA from control cells, and cells treated for 48 h with 5 or 25 μM 8-Cl-cAMP or 25 μM 8-Cl-adenosine were analyzed by Northern hybridization for *mdr*-1 expression and for expression of a control gene, 36B4. Densitometric analyses of the two are shown, with the results shown as a percentage of control levels, which were assigned a value of 100%. (*B*) Northern analysis of *mdr*-1 and 36B4 expression and analysis of β-tubulin expression by quantitative PCR. The ethidium bromide–stained 28S ribosomal RNA band from the gel used in the Northern analysis is shown for comparison. Cells were treated with the indicated doses of 8-Cl-cAMP, with 10 μM H-7, and with 10 μM verapamil for 48 h.

than the sixfold increase observed after incubation in verapamil (Fig. 5 *A*). Fig. 5 *B* demonstrates that *mdr*-1 mRNA continues to be downregulated after 10 d of 8-Cl-cAMP treatment. However, the incomplete reversal of accumulation in *A* suggests that Pgp persists despite the downregulation of *mdr*-1. This suggestion was confirmed by immunoblotting, which demonstrated reduction to only 25% of control levels of Pgp after 10 d of treatment with 8-Cl-cAMP (Fig. 5 *B*). To confirm that the increase in vinblastine accumulation was due to decreased efflux, cells were first loaded with ^{3}H-vinblastine for 45 min, after which efflux was measured over a 10-min period. In control cells, 50% of the drug present in the cells after a 1-h loading period was effluxed in the first 10 min, compared with the 40% level in cells treated with 8-Cl-cAMP and the 32% level in cells treated with 10 μg/ml verapamil (Fig. 5 *C*). The reduction in P-glycoprotein function after 8-Cl-cAMP paralleled the decrease in expression and synthesis observed after 8-Cl-cAMP treatment. In addition, the effectiveness of 8-Cl-cAMP in reversing Adriamycin resistance was evaluated by cytotoxicity assay. However, the results were difficult to analyze in the presence of the growth inhibition resulting from 8-Cl-cAMP treatment. Adriamycin cytotoxicity could not be demonstrated when the cells were growth inhibited, which is consistent with descriptions by other investigators regarding the lack of effect of Adriamycin in quiescent cells (48–50).

To evaluate whether the 8-Cl-cAMP treatment was associated with cellular differentiation, as reported by others, electron microscopy plus immunohistochemical staining with a variety of markers were performed (Fig. 6). An electron microscope (Phillips 201; Phillips Technologies, Cheshire, CT) was used to examine parental MCF-7 cells and treated and untreated MCF-7TH cells. In both the parental MCF-7 cells and the untreated drug-resistant subline, features of differentiation such as attachments, microvilli, and organelles were observed. However, parental MCF-7 cells (Fig. 6 *A*) appeared somewhat more differentiated than the drug-resistant subline (Fig. 6 *B*), as evidenced by better orientation and more well-developed attachments in the MCF-7 cells. By light microscopy, MCF-7 parental cells also appeared more differentiated than the MCF-7TH cells, with circular structures suggestive of gland formation present in the parental (Fig. 6 *C*) but not in the resistant subline (Fig. 6 *D*). Immunohistochemical analysis of cytokeratin 18 showed higher levels in the parental (Fig. 6 *E*) than in the resistant

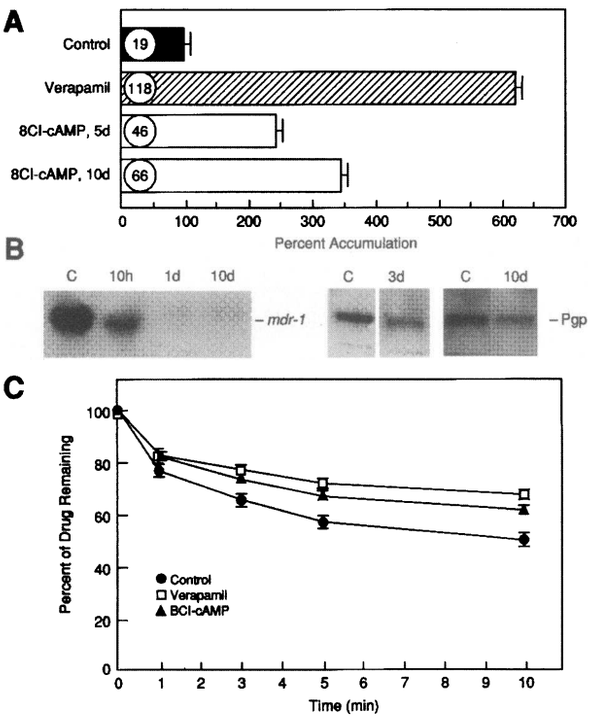


Figure 5. Drug accumulation and efflux in response to 8-Cl-cAMP treatment of MCF-7TH cells. (*A*) This section demonstrates the accumulation of tritiated vinblastine in the MCF-7TH cells. The control cells are depicted with black bars and are assigned a value of 100%. The actual femtomoles of drug accumulated per 10^5 cells are noted on each bar of the graph. The accumulation after 8-Cl-cAMP rises to 250% and 350% of control, with increasing duration in 8-Cl-cAMP, but does not rise to the level achieved after verapamil (600%). The error bars present the mean, ±SD. Northern analysis following a 10-d 8-Cl-cAMP treatment is found on the left side of (*B*). Immunoblot analysis following a 3- and 10-d treatment with 8-Cl-cAMP is shown on the right. (*C*) This section demonstrates the drug efflux in untreated control cells (●) and in cells after 72-h treatment with 8-Cl-cAMP (▲). For comparison, efflux in untreated cells was also measured in the presence of 5 μg/ml verapamil (□). After loading cells with ^{3}H-vinblastine for 45 min, the media was exchanged and the amount of ^{3}H-vinblastine that appeared in the media was measured. The graph plots the amount of drug remaining in the cells at each time point as a percentage of that present after the initial loading in ^{3}H-vinblastine.

subline (Fig. 6 *F*). After treatment of MCF-7TH cells with 8-Cl-cAMP, electron microscopy studies showed an increase in cytoplasmic filaments (not shown). Expression of cytokeratin 18 increased after treatment of the MCF-7TH cells with 8-Cl-cAMP (Fig. 6 *G*), confirming the findings by electron microscopy, while treatment of MCF-7 parental cells did not show significant changes. Other markers of differentiation, including expression of CA 15-3 (an antigen expressed by breast epithelial cells), HMFG (a polymorphic epithelial mucin antigen), and estrogen receptor, demonstrated no significant change in MCF-7TH cells after 72 h treatment with 8-Cl-cAMP (data not shown). Taken together, the findings are consistent with dedifferentiation in the drug-resistant cells, with a limited differentiation, at best, induced by 8-Cl-cAMP.

Previous studies have suggested that the effects of 8-Cl-cAMP are mediated by changes in the levels of the regulatory subunits of PKA (51). These studies have demonstrated an immediate translocation of RIIβ to the nucleus, followed by a decrease in RIα protein and mRNA (22, 25, 26, 51). Thus, we evaluated the levels of the RIα and RIIβ subunits by photoaffinity labeling followed by immunoprecipitation. As shown in Fig. 7, the RIα levels were markedly reduced after 24 h of 8-Cl-cAMP treatment, while the RIIβ levels increased within 3 h of treatment. Densitometric analysis showed that RIα decreased to 50% of the control level after 24 h treatment and RIIβ increased twofold compared with the control level after 6 h of 8-Cl-cAMP treatment.

To determine whether changes in the catalytic activity of PKA are involved in the effect of 8-Cl-cAMP, we examined phosphotransferase activity in cells during 8-Cl-cAMP treatment, using kemptide as a substrate in the presence and absence of a saturating concentration of cAMP and in the presence and absence of heat-stable protein kinase inhibitor (21). This method of assay gives accurate determination of the relative levels of dissociated catalytic subunit activity and total catalytic activity. The PKA activity ratio, the ratio of activity in the absence of added cAMP to that in the presence of cAMP, measures the degree of free catalytic subunit release. As shown in Fig. 8, no appreciable change in the PKA ratio during 48 h of 8-Cl-cAMP treatment was observed, while at 5 d of treatment, the ratio showed a 50% increase, consistent with an increase in free catalytic subunit. These results show that during the initial time of 8-Cl-cAMP treatment (up to 48 h) there was no increase in free catalytic subunit release.

To evaluate the generality of the effect of 8-Cl-cAMP in reducing *mdr*-1 expression, we studied multidrug-resistant ovarian cancer cells. We chose ovarian cells because studies in normal and malignant ovarian tissues have found low levels of *mdr*-1/Pgp, similar to the levels in the breast (19, 20). As shown in Fig. 9, decreased expression of *mdr*-1 was observed after 8-Cl-cAMP treatment of two multidrug-resistant sublines of the A2780 ovarian cancer cell line. In these cells, *mdr*-1 expression decreased 3-fold to 10-fold as demonstrated by RNase protection. Use of the cDNA complementary to the 5′ region of *mdr*-1 and the RNase protection methodology confirmed the specificity of the reduction observed by Northern analysis for the *mdr*-1 gene. The RNA was also evaluated by quantitative PCR, with reduction in *mdr*-1 expression seen after 8-Cl-cAMP treatment. The reduction again demonstrated the specificity of the findings for *mdr*-1, because the primers utilized were from the PVUII fragment previously shown to be specific for *mdr*-1 (52, 53).

Finally, the molecular basis of the reduction in *mdr*-1 expression was explored. MCF-7TH cells were transfected with the MDRCAT gene, and the *mdr*-1 promoter activity was assessed by evaluation of CAT mRNA levels. The MDRCAT vector, containing 4.7 kb of genomic sequence from *mdr*-1, including the promoter region, was cotransfected with the luciferase reporter gene under the control of SV40 promoter and enhancer sequences. After 24 h, cells were treated with 8-Cl-cAMP, 5 and 25 μM, for 48 h. Luciferase expression was measured in an aliquot of cells to control for the transfection efficiency achieved. RNA was harvested from remaining cells, and levels of CAT RNA were examined by RNase protection. The expression of CAT mRNA was normalized to 36B4 levels and to luciferase expression. The results demonstrated a decrease in *mdr*-1 promoter activity to 60% and 25% of control after treatment with 5 and 25 μM 8-Cl-cAMP, respectively (Fig.

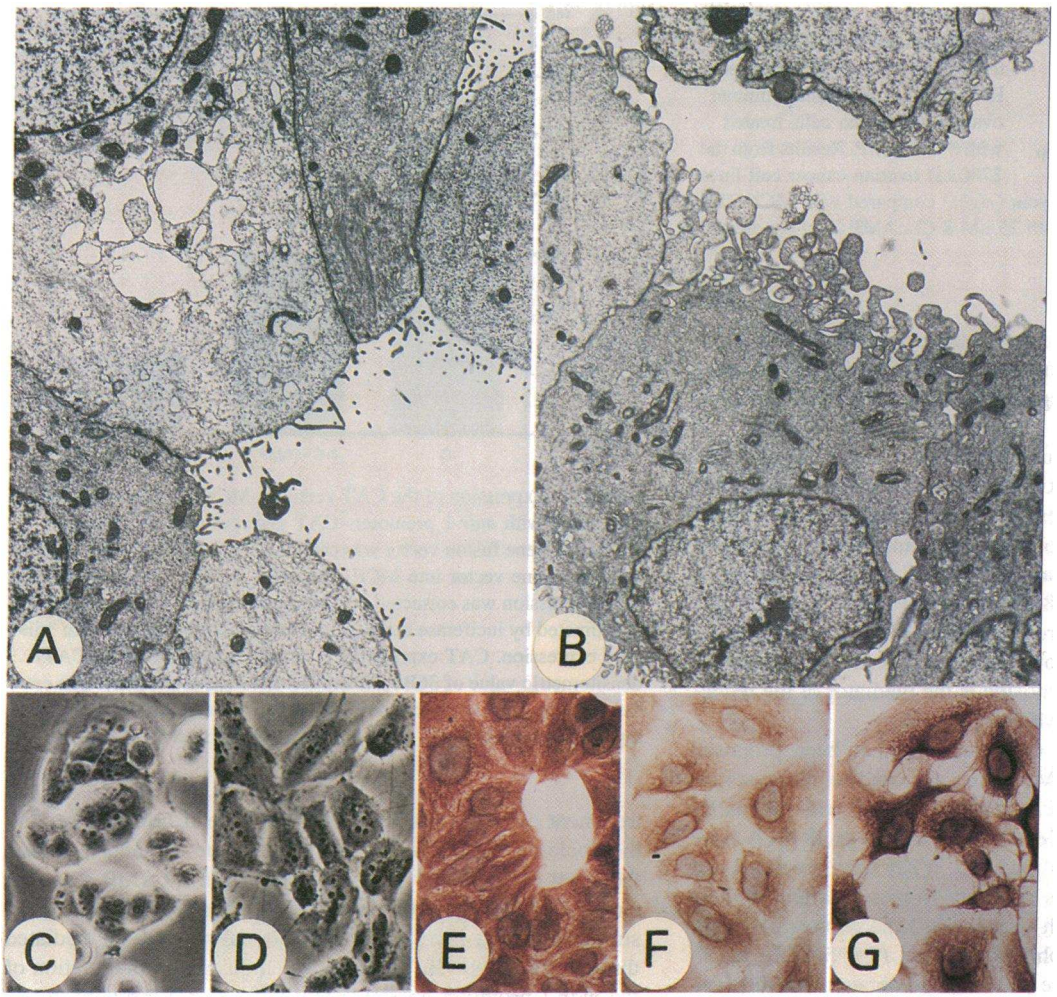


Figure 6. Photomicroscopy of MCF-7TH cells. (*A* and *B*) Electron microscopy to determine the level of differentiation in MCF-7TH cells was performed on cells cultured on slides and fixed in 2.5% glutaraldehyde. (*C* and *D*) Light microscopic appearance of the cells at 320×. (*E–G*) Immunohistochemical staining for cytokeratin 18 is shown in untreated MCF-7 (*E*) and MCF-7TH (*F*) cells, and in MCF-7TH cells treated with 8-Cl-cAMP (*G*) for 72 h prior to fixation in acetone. The cells were subsequently incubated in antibody for 1 h at 37°C, followed by an avidin-biotin–conjugated secondary antibody (42).

10). The MDR promoter–CAT construction, MDRCAT, contained 4.7 kb of genomic sequence from mdr-1, upstream of the transcription initiation site (44).

Discussion

Although potent P-glycoprotein antagonists have been introduced into clinical trials, their utility in reversing drug resistance in patients may be limited by effect on normal tissues. Thus, agents that could modulate the level or activity of P-glycoprotein itself could yield a separate or additive means of approaching the problem of clinical drug resistance.

In this work, we have described the effect of 8-Cl-cAMP on expression of the *mdr*-1 gene. We have demonstrated that mRNA expression is decreased in MCF-7TH cells within hours of treatment with 5 μM 8-Cl-cAMP, that the effect is dose dependent, and that synthesis of P-glycoprotein decreases in concert with the decrease in *mdr*-1 mRNA. These effects are manifested by an increase in drug accumulation and a decrease in drug efflux. We have not defined the mechanism by which 8-Cl-cAMP modulates *mdr*-1 expression, but it appears to be transcriptionally mediated. Several possibilities can be proposed for the effect of 8-Cl-cAMP on MCF-7TH cells: (1) the effect

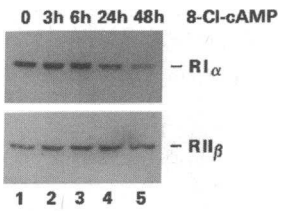


Figure 7. Photoaffinity labeling followed by immunoprecipitation of the regulatory subunits of PKA in MCF-7TH cells treated with 8-Cl-cAMP. Photoaffinity labeling with ^{32}P-8-N_3-cAMP followed by immunoprecipitation with monospecific anti-RIα and RIIβ antibodies, as described in Methods. Cells untreated (0) or treated for the indicated period of time with 8-Cl-cAMP (5 μM) were analyzed. Data represent one of two separate experiments that gave similar results.

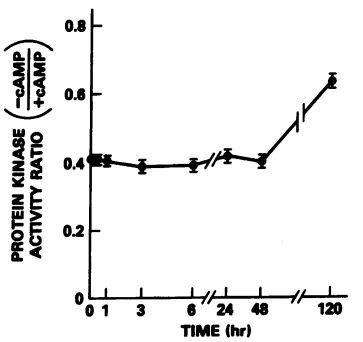


Figure 8. PKA in MCF-7TH cells during 8-Cl-cAMP treatment. Cells untreated or treated with 8-Cl-cAMP (5 μM) for indicated times were assayed for phosphotransferase activity, and the PKA ratio was calculated as described in the Methods. Data represent an average±SD of duplicate determinations from three separate experiments.

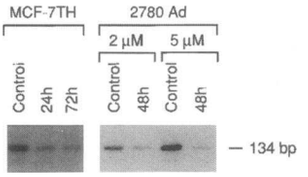


Figure 9. Effect of 8-Cl-cAMP on *mdr*-1 expression in RNase protection analysis of 30 μg of total RNA from drug-resistant human ovarian carcinoma cells treated with 8-Cl-cAMP. Results from the 2780AD ovarian cancer cell lines resistant to 5 and 2 μM Adriamycin (*right*) compared with MCF-7TH cells (*left*). Cells were treated with 25 μM 8-Cl-cAMP for the indicated periods of time.

of a toxic metabolite, (2) differentiation in parallel with a decrease in RI levels, (3) up- or downregulation of PKA activity, and/or (4) transcriptional regulation through the RII/PKA type II holoenzyme.

Two previous reports suggested that the activity of 8-Cl-cAMP is mediated by a toxic metabolite, 8-Cl-adenosine (45, 46). However, several lines of experimental evidence argue against a role for this metabolite in regulating *mdr*-1 expression. MDR promoter activity, assayed in MCF-7 cells, was decreased by 8-Cl-cAMP but not by 8-Cl-adenosine (54). In MCF-7TH cells, 8-Cl-adenosine is a growth inhibitor but has no effect on *mdr*-1 expression or promoter activity. These studies oppose the hypothesis that 8-Cl-adenosine is the mediator of the reduction in *mdr*-1 observed in MCF-7TH cells.

The second possibility, differentiation, has been proposed as a mechanism of 8-Cl-cAMP action in several model systems. Elevated levels of type I PKA have been linked with the malignant phenotype, whereas elevated levels of type II PKA have been linked with differentiation (51). It has been proposed that the phenotypical alterations resulting from regulation of these two subtypes occur through changes in gene expression rather than through changes in phosphorylation (25, 51). In MCF-7TH cells, the only evidence that could be obtained for differentiation was an increase in keratin expression. The higher level of keratin expression, which approximates that of parental MCF-7 cells, is consistent with differentiation of a ductal epithelial cell (55). The decreased expression of the RI subunit of PKA that results from 8-Cl-cAMP treatment in multiple model systems has been characterized as a differentiation marker (51). Thus, the reduced RI observed in MCF-7TH cells could be consistent with differentiation. Despite the less differentiated appearance of MCF-7TH cells relative to parental MCF-7 cells, no other differentiation markers were altered by 8-Cl-cAMP treatment.

A third possible mechanism for 8-Cl-cAMP action occurs through regulation of the catalytic activity of PKA. Our results showed that the amount of free C subunit remained unchanged during 48 h of 8-Cl-cAMP treatment. However, the suppression of *mdr*-1 expression was observed as early as 8 h post–8-Cl-cAMP treatment. Thus, it appears that the triggering mechanism of 8-Cl-cAMP leading to the suppression of *mdr*-1 expression does not involve up- or downregulation of the catalytic activity of PKA.

A fourth mechanism whereby 8-Cl-cAMP might alter *mdr*-1 expression occurs through the RIIβ subunit/PKA type II holoenzyme. Our data support this possibility: an increase in RIIβ subunit occurred as early as 3 h after 8-Cl-cAMP treatment; this increase preceded the suppression of *mdr*-1 expression that was detected at 8 h after 8-Cl-cAMP treatment. 8-Cl-cAMP renders the C subunit available for the RII subunit to form a PKA type II holoenzyme by downregulation of the RI subunit (27, 28). It has been shown previously that 8-Cl-cAMP

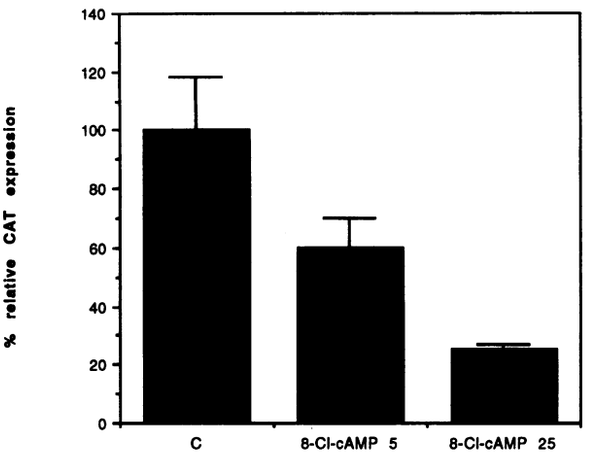


Figure 10. Expression of the CAT vector in MCF-7TH cells transiently transfected with *mdr*-1 promoter–CAT gene fusion vector. The MDRCAT gene fusion vector was cotransfected with the pGL2–control luciferase gene vector into MCF-7TH cells, as described in Methods. CAT expression was corrected for differences in transfection efficiency, as estimated by luciferase activity, and for loading compared with 36B4 gene expression. CAT expression = (densitometric value for CAT/densitometric value of 36B4)/(luminescence/mg of protein). The error bars represent the mean relative CAT expression from two out of four separate experiments, all of which gave similar results.

treatment enhances the level of PKA type II holoenzyme and the nuclear translocation of RII (PKA type II), and increases the level of a nuclear factor(s) that binds a cAMP response element (51). Thus, the action of 8-Cl-cAMP in the *mdr*-1 suppression may involve a transcriptional mechanism mediated through PKA type II. The results demonstrating modulation of the *mdr*-1 promoter are consistent with such a mechanism.

CRE consensus sequences have not been described in the *mdr*-1 promoter (44, 56–58). As suggested earlier, 8-Cl-cAMP may regulate a nuclear-binding protein that in turn downregulates *mdr*-1 expression. However, emerging data suggest that gene rearrangements have occurred proximal to the *mdr*-1 promoter in some *mdr*-1 overexpressing cell lines (T. Fojo, personal communication). If such a rearrangement occurred in the MCF-7TH cells, the effect may have been to bring *mdr*-1 under control of a cAMP response element. Sequencing of the region 5′ to *mdr*-1 is currently under way in these cells.

In conclusion, the data implicate the role of RIIβ subunit/PKA type II in regulating *mdr*-1 expression. The mechanism and sites of its action remain to be elucidated. These data are significant in that a new approach to reversing multidrug resistance is described in the use of 8-Cl-cAMP to inhibit expression of *mdr*-1/P-glycoprotein. A mechanism for decreasing *mdr*-1 expression would be a welcome addition to the therapeutic options for refractory breast cancer.

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