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Regulation of the Stability of P-glycoprotein by Ubiquitination

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Running Title: **Ubiquitination and P-glycoprotein stability**

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistant or multidrug resistance; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; EGF, epidermal growth factor; PDGF, platelet-derived growth

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factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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ABSTRACT

Ubiquitination plays a crucial role in regulating protein turnover. Here we show that ubiquitination regulates the stability of the *MDR*1 gene product, P-glycoprotein, thereby affecting the functions of this membrane transporter that mediates multidrug resistance. We found that P-glycoprotein was constitutively ubiquitinated in drug-resistant cancer cells. Transfection of multidrug resistant cells with wild-type ubiquitin or treatment with an N-glycosylation inhibitor increased the ubiquitination of P-glycoprotein and increased P-glycoprotein degradation. MG-132, a proteasome inhibitor, induced accumulation of ubiquitinated P-glycoprotein, suggesting the involvement of the proteasome in the turnover of the transporter. Treatment of multidrug resistant cells with 12-*O*-tetradecanoylphorbol-13-acetate, a phorbol ester that increases the phosphorylation of P-glycoprotein through activation of protein kinase C, or substituting phosphorylation sites of P-glycoprotein by nonphosphorylatable residues did not affect the ubiquitination of the transporter. Enhanced ubiquitination of P-glycoprotein resulted in decrease of the function of the transporter, as demonstrated by increased intracellular drug accumulation and increased cellular sensitivity to drugs transported by P-glycoprotein. Our results indicate that the stability and function of P-glycoprotein can be regulated by the ubiquitin-proteasome pathway, and suggest that modulating the ubiquitination of P-glycoprotein might be a novel approach to reversal of drug resistance.

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INTRODUCTION

Drug resistance is a major impediment to successful cancer chemotherapy. Multidrug resistance (MDR) mediated by P-glycoprotein (P-gp), the product of the *MDR*1[1] gene, is believed to be one of the major causes of failure of cancer therapy. P-gp is a 150 – 180 kDa heavily glycosylated and phosphorylated plasma membrane protein that functions as a drug transporter. Overexpression of P-gp confers resistance to a variety of structurally and functionally diverse anticancer drugs such as paclitaxel, doxorubicin and vinblastine. Despite promising early studies showing that blocking of P-gp by pharmacological means could sensitize drug resistant cells, the ultimate goal of restoring drug sensitivity has met with limited success in clinic. Therefore, we and others began to elucidate the factors that control P-gp synthesis. For example, we demonstrated that several substances control the expression of *MDR*1 through activation of PLC, and that the transcriptional modulation of *MDR*1 expression by PLC is mediated by the Raf-MAPK pathway (Yang et al., 2001). Recently, we became interested in the factors that regulate P-gp stability.

P-gp, at steady state, is located in the plasma membrane and undergoes endocytosis and recycling (Kim et al., 1997). Experimentally induced alterations in trafficking of P-gp can change the steady-state distribution of the transporter thereby affecting the MDR phenotype (Kim et al., 1997). Proteasome inhibitors can decrease the activity of P-gp by preventing its maturation and localization in the plasma membrane (Loo and Clarke, 1999). These studies indicate that reducing the content of P-gp within the plasma membrane by perturbing its subcellular localization, inhibiting its synthesis,

or facilitating its degradation might be effective approaches to modulating MDR in cancer cells.

P-gp is a relatively stable protein with a half-life of 14-17 h (Muller et al., 1995); its immature, core-glycosylated or glycosylation-deficient forms have much shorter half-lives (approximately 3 h) (Loo and Clarke, 1994; Loo and Clarke, 1999). N-glycosylation was shown to contribute to the stability of P-gp (Schinkel et al., 1993), and inhibiting glycosylation reduced membrane-associated P-gp and altered the MDR phenotype (Kramer et al., 1995). In addition, a protease-sensitive site was found in the first extracellular loop near the consensus glycosylation sites of P-gp, and proteolytic enzymes were shown to play an important role in proper P-gp folding (Loo and Clarke, 1998).

It is now appreciated that ubiquitination, a reaction in which ubiquitin molecules are covalently ligated to substrate proteins via isopeptide bonds formed through its C-terminal glycine to the ε-amino group of lysine residues, plays a crucial role in degrading certain membrane proteins. For example, ubiquitination was found to be required for degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) by the proteasome (Ward et al., 1995). A number of other plasma membrane proteins, such as the epithelial Na^+ channel (Staub et al., 1997), epidermal growth factor receptor (Galcheva-Gargova et al., 1995) and platelet-derived growth factor receptor (Mori et al., 1992), are ubiquitinated before proteasomal or lysosomal degradation.

Despite a previous study showing that inhibition of calpain-mediated proteolysis caused the accumulation of ubiquitinated P-gp (Ohkawa et al., 1999), little is known

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about the factors that regulate P-gp ubiquitination or the impact of the modification on P-gp function. Therefore, the aim of this study was to explore the role of ubiquitination in the stability of P-gp, the factors that control the process, and the possible effect on the function of the transporter. We demonstrate here for the first time that the stability of P-gp is regulated by the ubiquitin-proteasome pathway in MDR cancer cells. Furthermore, we show that inhibition of N-glycosylation enhances the ubiquitination and degradation of P-gp, thereby reducing the function of the multidrug transporter.

MATERIALS AND METHODS

Cell Lines and Culture. The MDR human breast cancer cell lines, MCF-7/AdrR and MCF-7/BC-19, and their parental, sensitive line, MCF-7, were kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). They were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. MCF-7/AdrR was developed by step-wise selection (Cohen et al., 1986) and MCF-7/BC-19 is a *MDR*1-transfected MCF-7 cell line (Yu et al., 1991). The MDR human oral carcinoma line KBV-1 (Ueda et al., 1986) and the sensitive parental line KB3-1, and murine NIH3T3 fibroblasts and their *MDR*1 transfectants, N3V2400 (wild-type P-gp), N4V600 and N5V2400 (phosphorylation-defective mutants of P-gp), were provided by Dr. Michael Gottesman of the National Cancer Institute (Bethesda, MD), and were grown in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum under the identical conditions described above. For KBV-1, 1 µg/ml of vinblastine was added in the medium for the maintenance of the MDR phenotype. For N3V2400, N5V2400 and N4V600, 2400 ng/ml and 600 ng/ml of vincristine were added in the medium respectively. Human ovarian carcinoma cell lines, A2780 and A2780Dx5, were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY) and were grown in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum under the identical condition as described above except that for A2780Dx5, 2 µM of doxorubicin was added to the medium for the maintenance of the MDR phenotype (Alaoui Jamali et al., 1989). All cultures were

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checked routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after three months and new lines obtained from frozen stocks.

Antibodies and Reagents. Polyclonal antibody mdr (Ab-1), recognizing human P-gp, was purchased from Oncogene Research Products (Boston, MA), and C219 anti-P-gp monoclonal antibody was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Polyclonal anti-ubiquitin antiserum was purchased from Sigma-Aldrich, Inc. (Saint Louis, MO), and monoclonal anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Additional reagents were purchased from the following sources: Ubiquitinated Protein Enrichment Kit, Calbiochem-Novabiochem (San Diego, CA); Tunicamycin, Sigma-Aldrich, Inc. (Saint Louis, MO); Lipofectamine 2000, Invitrogen Life Technologies, Inc. (Carlsbad, CA); Protein-A Sepharose CL-4B, Amersham Pharmacia Biotech (Piscataway, NJ); MG132, Calbiochem (San Diego, CA); ECL Western blotting analysis kit and [^{35}S]-methionine (Ci/mmol), Amersham (Arlington Heights, IL).

Immunoprecipitation and Immunoblotting. Confluent cells in 150-mm dishes were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), scraped off the dishes and pelleted at 800 x g for 10 min. Cell pellets were then lysed in cold TNT buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 10 mM iodoacetamide, 1 mM PMSF and 1% aprotinin) for 45 min with occasional rocking. The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 x g for 40 min at 4°C. Identical amounts (2 mg

protein) of precleared cell lysates were immunoprecipitated with 1 µg mdr (Ab-1) or anti-ubiquitin polyclonal antibodies by overnight incubations at 4°C after adjusting the volumes to 0.5 ml with cold NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mM PMSF and 1% aprotinin). The immune complexes were precipitated with Protein-A Sepharose CL-4B and washed three times with TNT buffer, once with NET buffer and once with PBS.

Immunoprecipitated proteins were eluted with Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, and 0.001% bromphenol blue) and resolved by SDS-PAGE. Transfer of proteins to nitrocellulose was carried out by the method of Towbin et al. (40). The blots were incubated in blocking solution consisting of 5% milk and 0.1% bovine serum albumin in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with anti-P-gp, anti-ubiquitin or anti-β-actin monoclonal antibodies. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham).

Preparation of Cytosolic and Membrane Fractions. Membrane and cytosolic fractions were prepared in homogenization buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM iodoacetamide, 0.2 mM PMSF, 20 µM ALLN), as previously described (VanSlyke and Musil, 2002). Briefly, cells plated in 150-mm culture dishes were grown to 95% confluence, and then homogenized in homogenization buffer. Membrane and cytosolic fractions were separated by centrifugation at 100,000 x g for 60 min at 4^{o}C.

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Plasmids and Transfection. Plasmids PCW7 (wild-type ubiquitin) and PCW8 (ubiquitin K48R mutant) were kindly provided by Dr. Ron Kopito (Stanford University, CA). Cells were transfected with the plasmids using LipofectAMINE 2000 (Invitrogen™ Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Briefly, 90% confluent cells grown in 100-mm dishes were washed twice with serum-free medium before the addition of 25 µg plasmid DNA and 60 µl LipofectAMINE 2000 in 3 ml serum-free media. After 4 h incubation at 37^{o}C, 7 ml medium containing 10% fetal bovine serum were added. Incubations were continued at 37^{o}C for another 48 h.

Metabolic Labeling and Pulse-chase Analysis. Metabolic labeling was performed as previously described (Muller et al., 1995). Briefly, MCF-7/AdrR cells were plated in 100-mm dishes and grown to 60~70% confluence in complete growth medium, then [^{35}S]-methionine (10 µCi/ml) was added. After 48 h incubation, the cells were washed and incubated in complete growth medium containing 450 µg/ml of methionine. The labeled proteins were chased at 0, 6 h, 12 h, 24 h and 48 h. P-gp levels were determined by immunoprecipitation with an anti-P-gp antibody, mdr (Ab-1), and resolved by 7% SDS-PAGE. The gels were fixed with 10% methanol/5% glycerol, dried, and exposed for 5 days to X-ray film with an intensifying screen at -70^{o}C.

Measurement of Doxorubicin Accumulation. Control or ubiquitin- or tunicamycin-treated cells were incubated with 25 µM of doxorubicin for 2h. At the end of incubation, cells were washed three times with PBS and observed under a fluorescence

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microscope with 100X magnification (Nikon ECLIPSE TE200, Nikon Inc., Melville, NY).

Assay of Drug Sensitivity. Cells grown in 96-well plates were transfected with PCW7 or control vector. Twenty-four h later, various concentrations of drug were added and the cells were incubated at 37^{o}C in a humidified 5% CO_2 atmosphere for another 72 h. At the end of incubation, the viability of cells was determined using Promega's CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI).

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RESULTS

Ubiquitination of P-gp and its Effect on P-gp Turnover.

To investigate whether or not P-gp was ubiquitinated in MDR cells, cell lysates were immunoprecipated with an anti-P-gp antibody followed by immunoblotting with an anti-ubiquitin antibody, or by a reciprocal approach. As shown in Fig. 1A and B, ubiquitinated P-gp was found in all MDR cell lines tested, including the *MDR*1 transfectant, MCF-7/BC19, and drug-resistant lines whose expression of P-gp was induced by step-wise selection with chemotherapeutic drugs (MCF-7/AdrR, KBV-1 and A2780Dx5). Ubiquitinated P-gp was also detected using polyubiquitin-affinity beads comprised of a GST-fusion protein containing a ubiquitin-associated sequence (Chen and Madura, 2002; Chen et al., 2001) conjugated to glutathione-agarose, then immunoblotting with an anti-P-gp antibody (Fig. 1C). Ubiquitination of P-gp was only detected in the plasma membrane fraction (Fig. 2).

To determine the effect of ubiquitination on P-gp stability, we transiently transfected the MDR MCF-7 cells with a wild-type ubiquitin plasmid, PCW7, or a dominant-negative ubiquitin PCW8 (K48R mutant), in which the invariant lysine at position 48 was replaced by arginine (Finley et al., 1994). The lysine 48 is the site of isopeptide linkage to other ubiquitin molecules and is required for the generation of multiubiquitin chains that mark proteins for degradation (Ciechanover and Schwartz, 1998; Ward et al., 1995). The K48R ubiquitin mutant (PCW8) produces ubiquitin chain-termination and accumulation of incompletely ubiquitinated proteins that are not targeted for proteasomal degradation (Ward et al., 1995; Yu and Kopito, 1999). Transfection of wild-type ubiquitin increased accumulation of ubiquitinated P-gp (Fig.

3B) as well as other ubiquitinated cellular proteins (Fig. 3A). This increase in ubiquitination was accompanied by a reciprocal decrease in P-gp (Fig. 3A and B). In contrast, transfection of the dominant-negative mutant of ubiquitin caused an increase in P-gp content (Fig. 3C), indicating that disruption of P-gp ubiquitination decreased its degradation. Neither wild-type nor dominant-negative ubiquitin affected the content of β-actin, a protein whose monoubiquitination does not trigger proteolysis (Ball et al., 1987; Hicke, 2001).

Inhibition of Glycosylation Increases Ubiquitination and Turnover of P-gp.

Since N-glycosylation is linked to P-gp stability (Schinkel et al., 1993), we studied whether or not ubiquitination of P-gp was influenced by its glycosylation state. Tunicamycin, a compound that inhibits the formation of N-linked oligosaccharide chains (Elbein, 1987), increased the ubiquitination of P-gp (Fig. 4). In pulse-chase experiments, the degradation of P-gp in tunicamycin-treated cells ($t_{1/2}$: ~4 h) was approximately 3-fold faster than that in vehicle-treated cells ($t_{1/2}$: ~12 h) (Fig. 5). These data provide a clear link between glycosylation, ubiquitination, and degradation of P-gp.

Phosphorylation does not Affect P-gp Ubiquitination.

The ubiquitination of certain plasma membrane proteins is regulated by phosphorylation (Hicke et al., 1998; Marchal et al., 1998). Since P-gp is also phosphorylated (Aftab et al., 1994), we sought to determine whether the ubiquitination state of P-gp was controlled by phosphorylation. Cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is known to increase the phosphorylation

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of P-gp through activation of protein kinase C (Aftab et al., 1994; Fine et al., 1988). Fig. 6A shows that treatment of MCF-7/AdrR cells with TPA did not affect the level of P-gp ubiquitination. We also compared the ubiquitination of P-gp in NIH3T3 cells transfected with wild-type or phosphorylation-defective P-gp mutants. N3V2400 cells express wild-type P-gp, whereas N4V600 cells express a mutant P-gp in which serines at positions 661, 667, 671, 675 and 683 were replaced by nonphosphorylatable alanine residues. N5V2400 cells express the mutant carrying aspartic acid residues at the respective positions to mimic permanently phosphorylated serine residues (Germann et al., 1996). N3V2400, N4V600 and N5V2400 lines express similar levels of P-gp, but N4V600 and N5V2400 exhibit no detectable levels of P-gp phosphorylation (Germann et al., 1996). As shown in Fig. 6B, the ubiquitination of P-gp in these three cell lines was identical. These experiments demonstrate that the phosphorylation state of P-gp had no effect on its ubiquitination.

The Proteasome is involved in the Degradation of P-gp.

To assess if P-gp was subject to proteasomal or lysosomal degradation, we treated the drug-resistant MCF-7 cells with MG132, monensin or NH_4Cl, then assayed the ubiquitination of P-gp. Treatment with the proteasome inhibitor MG132 increased both P-gp and its ubiquitinated form in MCF-7/AdrR cells (Fig. 7A), whereas the lysosome inhibitors, monensin and NH_4Cl, had no effect on the content of P-gp or its ubiquitinated form (Fig. 7B). These experiments indicate that the proteasome, but not the lysosome, is involved in the P-gp turnover.

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Effect of Ubiquitination on P-gp Function.

We also studied the effect of ubiquitination on the function of P-gp and drug sensitivity. Fig. 8A shows that as compared to controls, transfection of MCF-7/AdrR cells with the wild-type ubiquitin construct, PCW7, enhanced the intracellular accumulation of doxorubicin, a P-gp-transportable chemotherapeutic drug. Tunicamycin, an inhibitor of N-glycosylation that increased ubiquitination and degradation of P-gp (Fig. 4), also increased intracellular doxorubicin accumulation (Fig. 8B). In addition, the sensitivity of MDR MCF-7 cells to another P-gp-transportable drug, vinblastine, was increased by transfection with wild-type ubiquitin (Fig. 9). In contrast, ubiquitin transfection did not alter the sensitivity to a non-P-gp substrate drug, hydroxyurea, in MDR cancer cells (Fig. 9).

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DISCUSSION

In the current study we investigated the role of ubiquitination in the stability and function of P-gp, an ATP-dependent drug transporter that mediates resistance to a variety of structurally and functionally diverse chemotherapeutic agents. We found that P-gp is ubiquitinated (Fig. 1), and that increasing ubiquitination of P-gp by transfection with wild-type ubiquitin or by treatment with the N-glycosylation inhibitor, tunicamycin, increases P-gp degradation (Fig. 3A, B, Fig. 4, and Fig. 5), reduces the function of the protein (Fig. 8), and selectively increases the sensitivity of MDR cells to P-gp transportable cytotoxic drugs (Fig. 8 and 9). These results indicate that modification of P-gp via ubiquitination can affect the stability and activity of this drug transporter, and suggest the potential for circumvention of P-gp-mediated drug resistance by modulating the ubiquitin pathway.

Covalent modification of cellular proteins with ubiquitin is associated with the regulation of diverse cellular processes including stress response, oncogenesis, transcription, protein turnover, organelle biogenesis, DNA repair, and cell-cycle control (Peters et al., 1998). Although most of proteins subjected to ubiquitin conjugation identified to date are cytoplasmic or nuclear, the ubiquitination of membrane proteins has been reported recently. For example, receptors for epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor were found to be ubiquitinated and ubiquitination was shown to participate in cell-surface signaling (Mori et al., 1992; Stang et al., 2000). The role of ubiquitination in endocytosis and degradation of the yeast ATP binding cassette transporters has also been reported (Egner and Kuchler, 1996; Kolling and Hollenberg, 1994). Here, we demonstrate, for the first time, that

ubiquitination of the human multidrug transporter, P-gp, a member of the ATP-binding cassette family, is involved in regulating its turnover and function. It would be of interest to find whether other human multidrug transporters, such as multidrug resistance-associated protein (MRP), breast cancer resistance protein (BCRP) and lung cancer resistance protein (LRP), are ubiquitinated, since these ABC transporters share many similarities (Gottesman et al., 2002).

Increases in ubiquitination by transfection with wild-type ubiquitin leads to a decrease in the content of P-gp (Fig. 3A and B), whereas introduction of dominant-negative ubiquitin results in an increase in P-gp content (Fig. 3C). These data suggest that modification by ubiquitin is important for degradation of P-gp. We also found that degradation of P-gp appears to be mediated through the proteasomal rather than lysosomal pathway. This conclusion is derived from the results using the proteasome inhibitor, MG 132, which increases the accumulation of P-gp and the ubiquitinated form of the transporter (Fig. 7A) and the lysosome inhibitors, monensin and NH_4Cl, which do not even at high concentrations (Fig. 7B). This result is consistent with previous observations that the proteasome is involved in degradation of the misfolded, core-glycosylated or glycosylation-deficient mutant of P-gp (Gribar et al., 2000; Loo and Clarke, 1998). For example, Loo and Clarke found that proteasome inhibitors prevented degradation of the proteolytic digestion products of P-gp (Loo and Clarke, 1998). Gribar et al. showed that treatment with proteasome inhibitors increased glycosylation-deficient P-gp (Gribar et al., 2000). Therefore, the ubiquitinated forms of P-gp may represent immature or misfolded forms of the transporter. Our result differs from that of Ohkawa et al. who used low concentration of lactacystin as proteasome inhibitor (Ohkawa et al.,

1999). Degradation of membrane proteins by either proteasomes or lysosomes, or by both, has been observed. For example, CFTR, an ABC transporter, is degraded by the proteasome (Ward et al., 1995), and Ste2p, a G protein-coupled plasma membrane receptor, is degraded in the lysosme (Hicke and Riezman, 1996). Both the proteasome and the lysosome are involved in the degradation of the epithelial Na^+ channel (Staub et al., 1997). In addition, degradation of membrane proteins by calpain has also been reported (Ohkawa et al., 1999; Salamino et al., 1994; Zaidi and Narahara, 1989).

The ubiquitination-proteasome system is known to be involved in degradation of short-lived, mutant or misfolded proteins. P-gp is a relatively stable protein with a half-life of 14-17 h (Muller et al., 1995). Inhibition of N-glycosylation increases the ubiquitination (Fig. 4), decreases the stability (Fig. 5), and reduces the function (Fig. 8B) of P-gp. Immature, core-glycosylated or glycosylation-deficient P-gp has a shorter half-life of approximately 3 h (Gribar et al., 2000; Loo and Clarke, 1994; Loo and Clarke, 1999). In addition, Loo and Clarke demonstrated that mutant forms of P-gp that are unable to fold into the mature forms are rapidly degraded (Loo and Clarke, 1994). Based on the analysis of deletion mutants, Schinkel et al. proposed that N-glycosylation contributes to proper routing or stability of P-gp (Schinkel et al., 1993). Furthermore, inhibiting N-glycosylation of P-gp has been shown to decrease drug resistance in cancer cells overexpressing the transporter (Kramer et al., 1995). These data suggest that core-glycosylated, immature or glycosylation-deficient P-gp are the targets of ubiquitination-proteasome system, and inhibition of N-glycosylation can decrease the stability of P-gp by increasing ubiquitination/proteasome-mediated degradation. Ubiquitinated P-gp is only detected in the plasma membrane fraction (Fig. 2), probably due to that the majority

of cellular P-gp is located on the cell surface, and the intracellular P-gp pool may represent newly synthesized, properly folded protein (Kim et al., 1997; Ohkawa et al., 1999).

The ubiquitin-mediated turnover of P-gp has important functional consequences. For example, transfection of wild-type ubiquitin increased the ubiquitination and degradation of P-gp (Fig. 3B), and also restored intracellular accumulation of and sensitivity to drugs transported by P-gp (Fig. 8 and 9). Although expression of a dominant-negative ubiquitin increased P-gp abundance (Fig. 3C), this did not detectably increase activity of the already active transporter (data not shown).

Using a web-based algorithm, PESTfind, we did not find in P-gp any possible PEST sequence, a region usually considered to control the ubiquitination and degradation of short-lived proteins (Kornitzer et al., 1994; Marchal et al., 1998). This might account for the relative stable nature of P-gp. However, a PEST sequence is not always required for protein ubiquitination and degradation (Chen and Clarke, 2002; Van Antwerp and Verma, 1996). For example, the PEST sequence was found to play no significant role in ubiquitination and degradation of CFTR (Chen and Clarke, 2002). What trigger(s) the ubiquitination of membrane proteins is currently unclear. Although P-gp contains no PEST sequences, there are 85 lysine residues for potential ubiquitination. Unfortunately, mapping of critical ubiquitination site(s) on P-gp through systematic mutation seems unrealistic, since mutation of a ubiquitinated lysine typically results in modification of a second non-phsyiological site (Hou et al., 1994).

Ubiquitination of P-gp is not affected by phosphorylation. We found that neither treatment with a protein kinase activator (TPA) that is known to increase P-gp

phosphorylation, nor abolishment of phosphorylation by mutating phosphorylation sites have any effect on the ubiquitination level of P-gp (Fig. 6). These experiments suggest that ubiquitination of P-gp is independent of phosphorylation. Phosphorylation was reported to play a role in controlling ubiquitination of other plasma membrane proteins (Hicke et al., 1998; Marchal et al., 1998), but did not appear to affect ubiquitination of the yeast ABC transporter, Ste6 (Kolling, 2002).

In summary, our results demonstrate that the ubiquitination-proteasome system plays a role in the turnover of P-gp, thereby affecting the function of the drug transporter. Therefore, modification of P-gp via ubiquitin-proteasome pathway might represent a novel strategy for modulating MDR in cancer cells.

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Footnotes

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LEGENDS TO FIGURES

Figure 1. P-gp is ubiquitinated in MDR cancer cells. (A**)** Cell lysates (2 mg protein) were subjected to immunoprecipitation with 1 µg anti-P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-ubiquitin (upper panel) or anti-P-gp (C219) (lower panel) antibody, as described in "Materials and Methods". **(**B**)** The proteins immunoprecipitated with a polyclonal anti-ubiquitin antibody were immunoblotted with an anti-P-gp antibody. (C) Cell lysates were incubated with polyubiquitin affinity beads, then examined by immunoblotting with a monoclonal anti-P-gp antibody, C219. Results are representative of at least three similar experiments.

Figure 2. Subcellular distribution of the ubiquitinated P-gp. Crude membrane and cytosol preparations from the human MDR breast cancer cell line, MCF-7/AdrR, were subjected to immunoprecipitation with 1 µg anti-P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-P-gp (C219) or anti-ubiquitin antibody, as described in "Figure 1A". Results are representative of three similar experiments.

Figure 3. Effects of exogenous ubiquitin on ubiquitination of P-gp. MDR MCF-7 cells were transfected with wild-type ubiquitin (A and B) or mutant ubiquitin K48R (C). Forty-eight hours later, the cells were lysed, and the cell lysates were analyzed by immunoblotting (A and C) or immunoprecipitation followed by immunoblotting (B), as indicated. Results are representative of three similar experiments.

Figure 4. Effect of tunicamycin on ubiquitination of P-gp. MDR cancer cells were treated with tunicamycin (5 μg/ml) for 10 h, then cell lysates were prepared as described in "Materials and Methods". Equal amounts (2 mg) of cell lysates were immunoprecipitated with 1 μg anti-P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-ubiquitin (upper panel) or anti-P-gp (C219) (lower panel) antibody. Results are representative of three similar experiments.

Figure 5. Effect of tunicamycin on degradation rate of P-gp in MDR MCF-7 cells. MCF-7/AdrR cells were labeled with [^{35}S]-methionine (10 μCi/ml) for 48 h, then chased with an excess of cold methionine in the presence or absence of tunicamycin (5 μg/ml) for the indicated times. P-gp was immunoprecipitated with an anti-P-gp antibody, mdr (Ab-1), and analyzed by 7% SDS-PAGE and autoradiography, as described in "Materials and Methods". Results are representative of three similar experiments.

Figure 6. Phosphorylation does not affect ubiquitination of P-gp. (A) Cells were treated with 400 nM TPA for 10 h, then cell lysates were prepared as described in "Materials and Methods". Equal amounts (2 mg) of cell lysates were subjected to immunoprecipitation with 1 μg anti-P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-ubiquitin (upper panel) or anti-P-gp (C219) (lower panel) antibody. (B) Cell lysates (2 mg protein) prepared from parental NIH3T3 cells and from *MDR*1 transfectants expressing wild-type (N3V2400) or phosphorylation-defective mutants of P-gp were subjected to immunoprecipitation with 1 μg anti-P-gp

polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-ubiquitin (upper panel) or anti-P-gp (C219) (lower panel) antibody, as described in "Materials and Methods". Results are representative of two similar experiments.

Figure 7. Effect of the inhibitors of proteasome or lysosome on ubiquitination of P-gp. Cells were treated for 10 and 20 h with the proteasome inhibitor, MG132 (10 μM), or with the lysosome inhibitors, monensin (20 μM) and NH_4Cl (20 mM). Cell lysates were immunoprecipitated with 1 μg anti-P-gp polyclonal antibody (mdr Ab-1), followed by immunoblotting with a monoclonal anti-ubiquitin antibody, as described in "Materials and Methods". Results are representative of three similar experiments.

Figure 8. Effect of exogenous ubiquitin (A) and tunicamycin (B) on doxorubicin accumulation in MCF-7/AdrR cells. MCF-7/AdrR cells grown in 100-mm culture dishes were transfected with the wild-type ubiquitin vector, PCW7 (A), or treated for 10 h with tunicamycin (5 μg/ml), or with vehicles. Cells were then incubated with 25 μM of doxorubicin for 2h, followed by washing three times with PBS. Doxorubicin accumulation was observed under a fluorescence microscope with 100X lens. Results are representative of two similar experiments.

Figure 9. Effect of exogenous ubiquitin on the sensitivity of MCF-7/AdrR cells to drugs. Transfected cells in 96-well plates in growth medium were incubated at 37^{o}C for 72 h in the presence of varying concentrations of vinblastine or hydroxyurea. The viability of cells was determined using Promega's CellTiter 96® Aqueous One Solution

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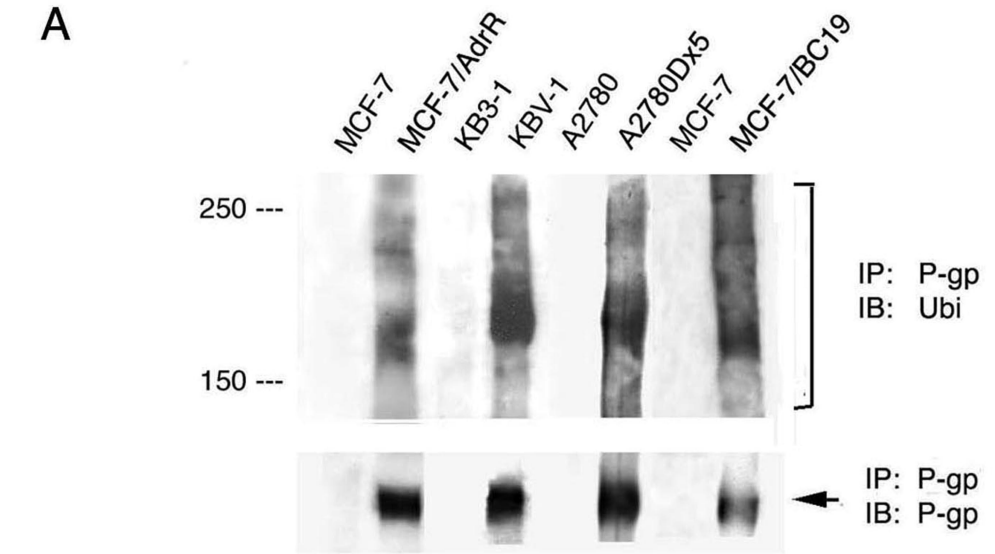
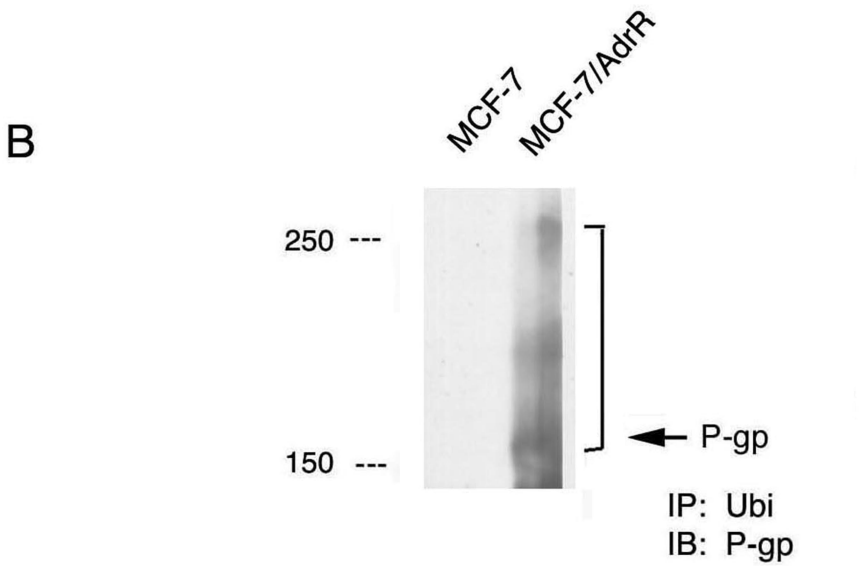
Cell Proliferation Assay. Each point represents the mean ± standard deviation of quadruplicate determinations. Results are representative of three similar experiments.

Fig. 1





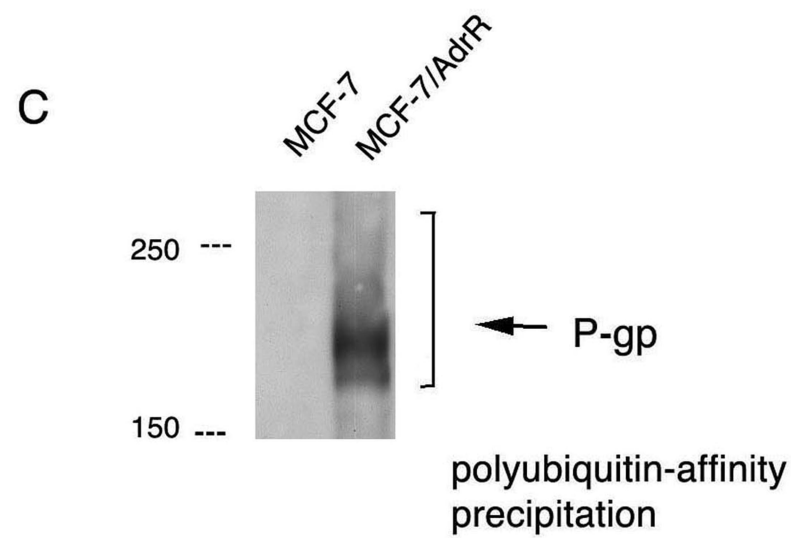
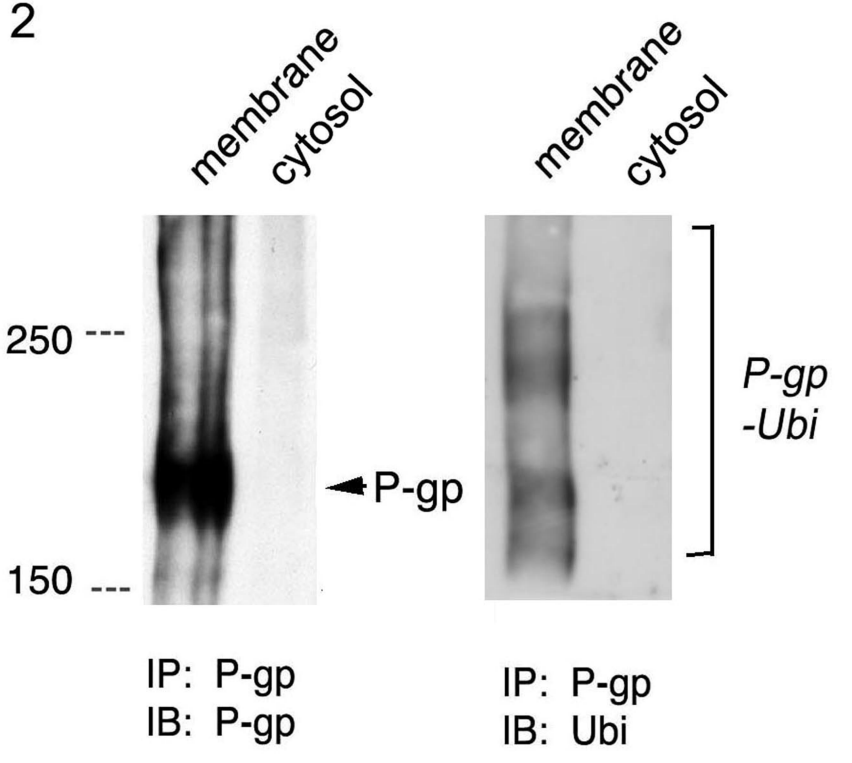


Fig. 2


membrane
cytosol
membrane
cytosol
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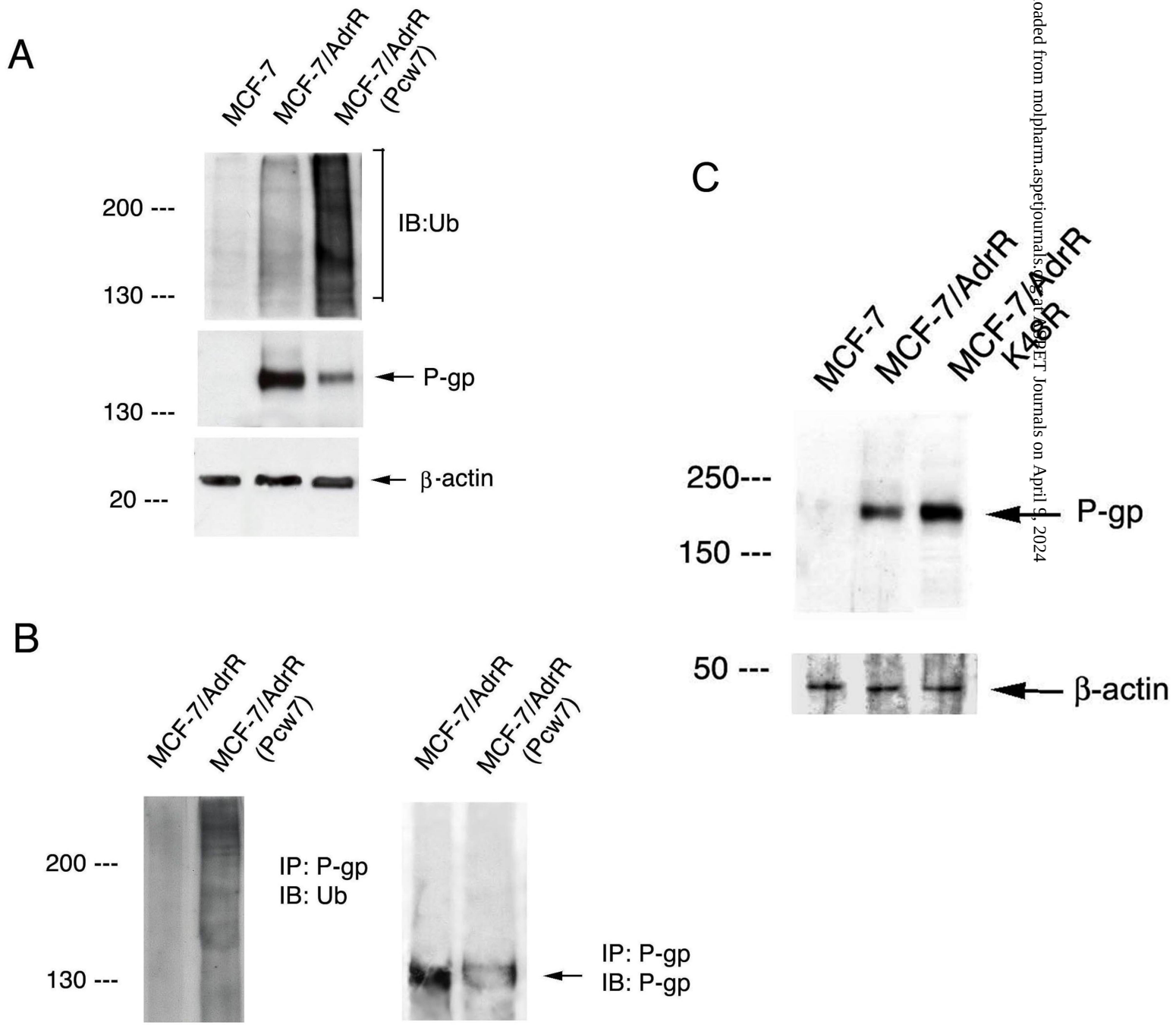
150
P-gp
P-gp
-Ubi
IP: P-gp
IB: P-gp
IP: P-gp
IB: Ubi

Fig. 3



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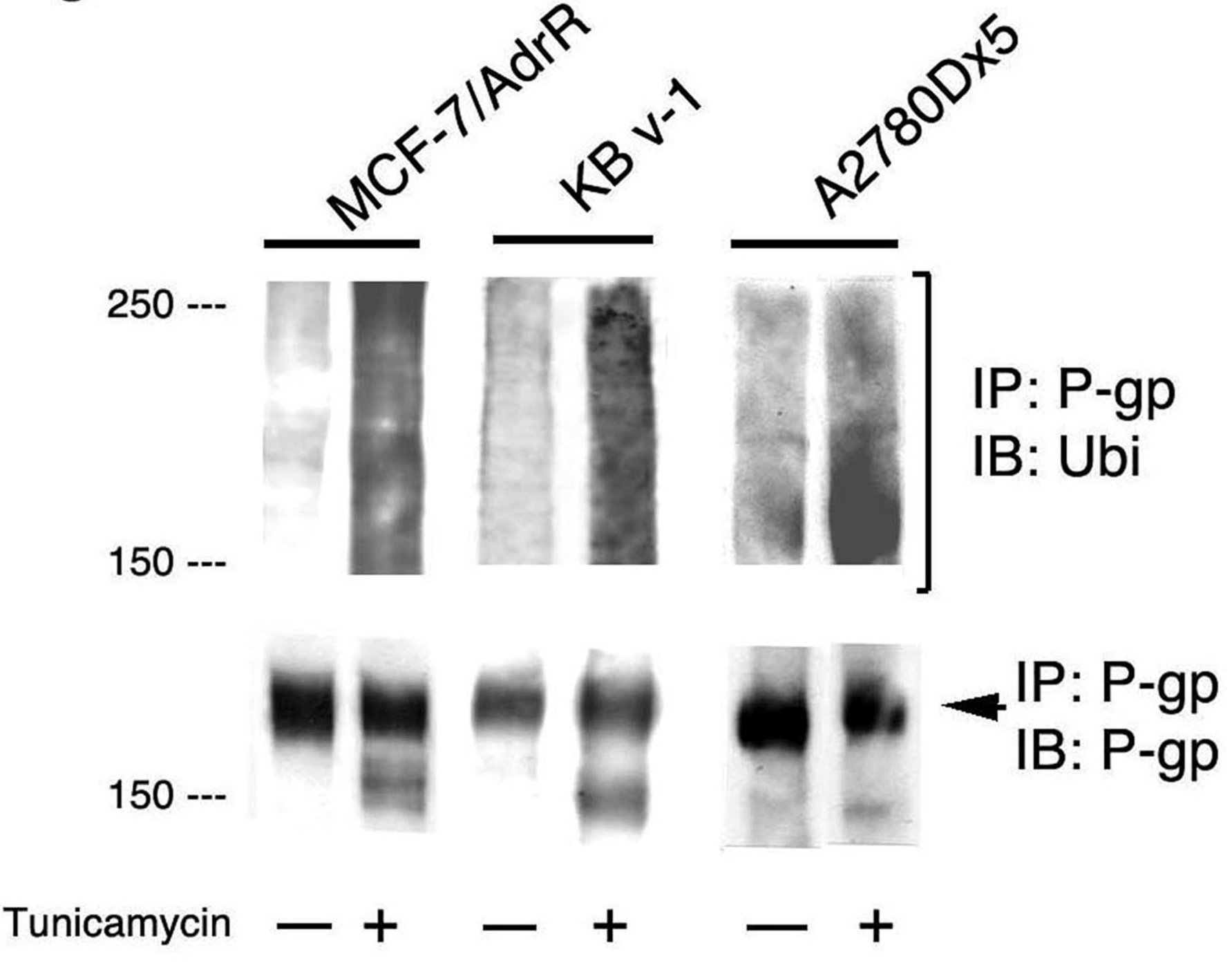
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KB v-1
A2780Dx5
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150 ---
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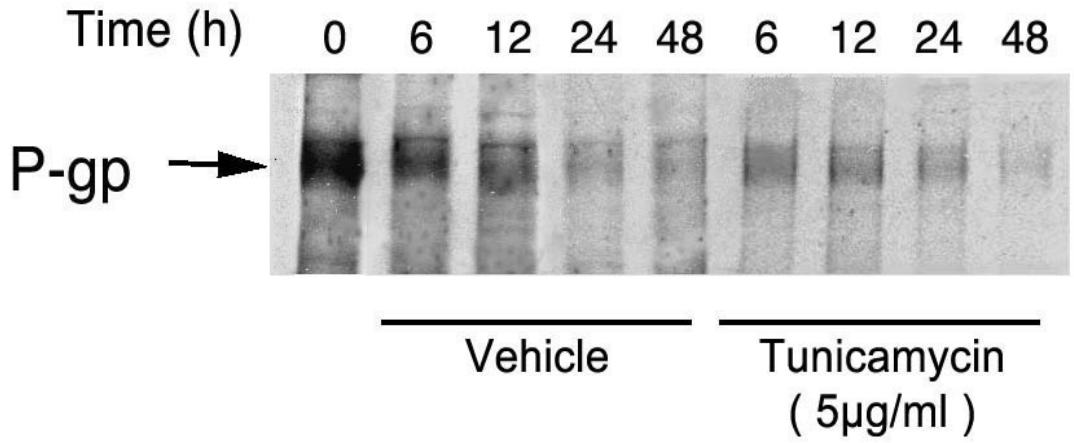
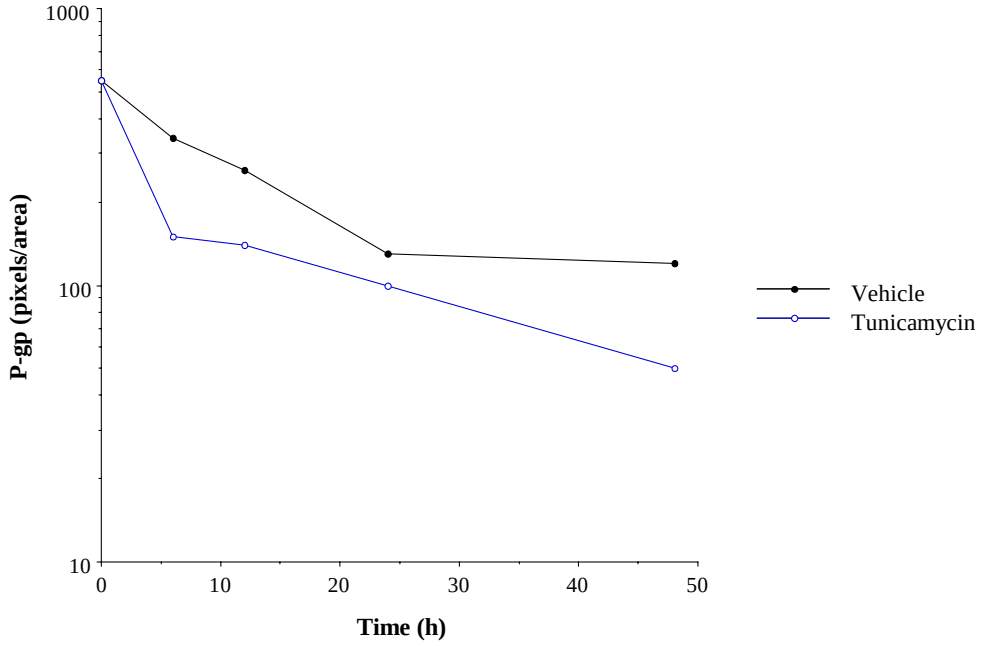
Tunicamycin — + — + — +

Fig. 5





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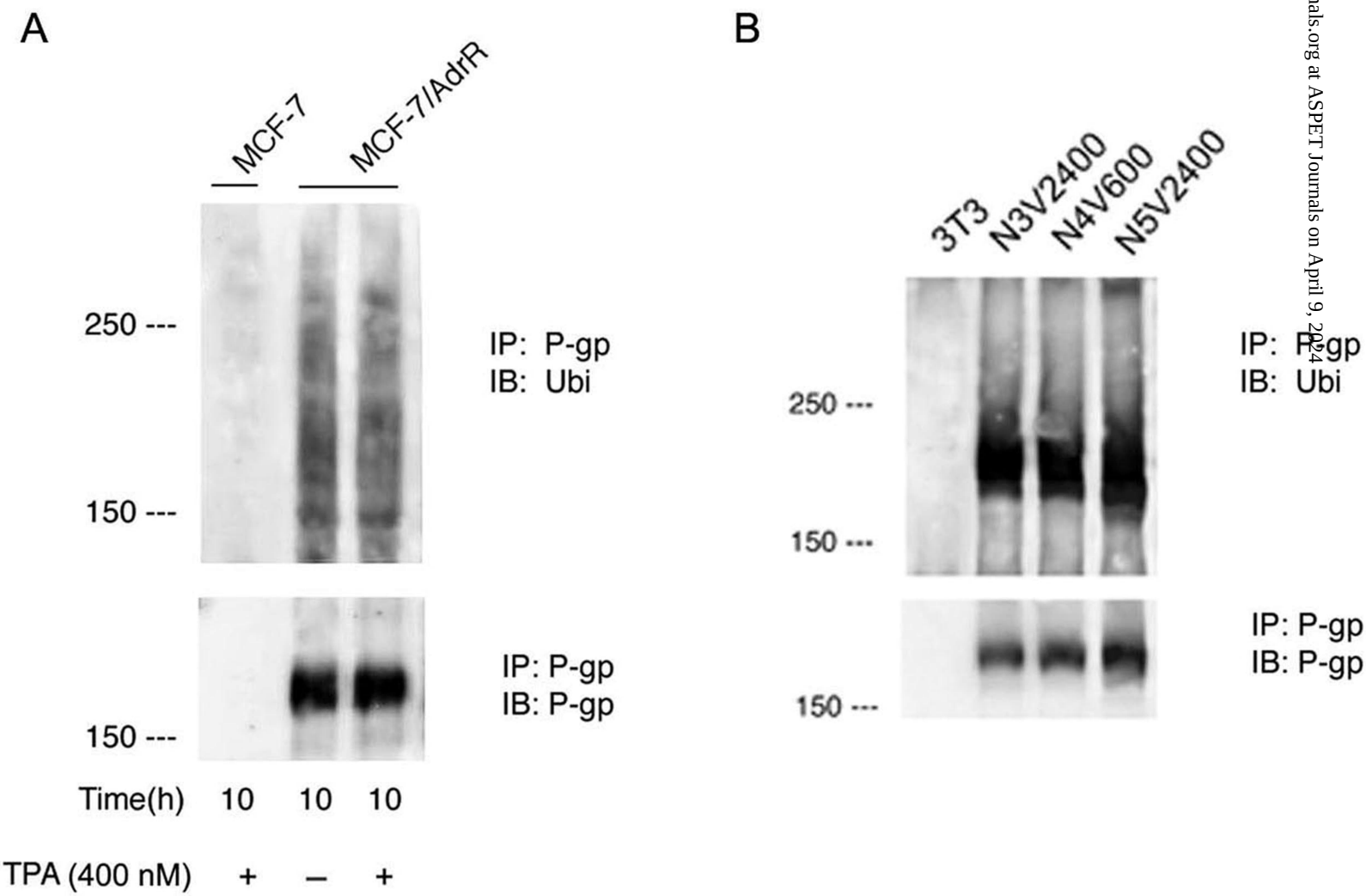

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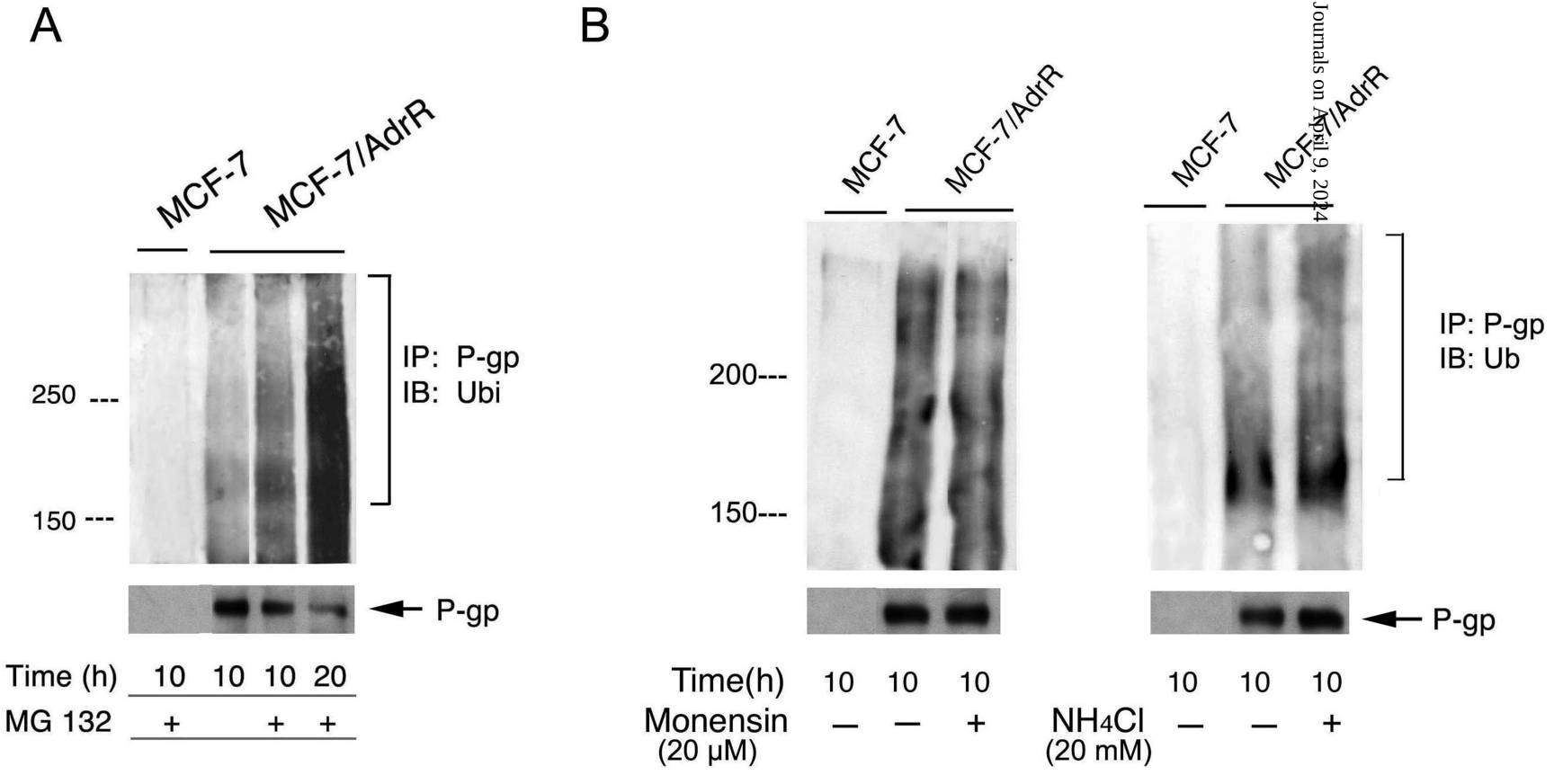

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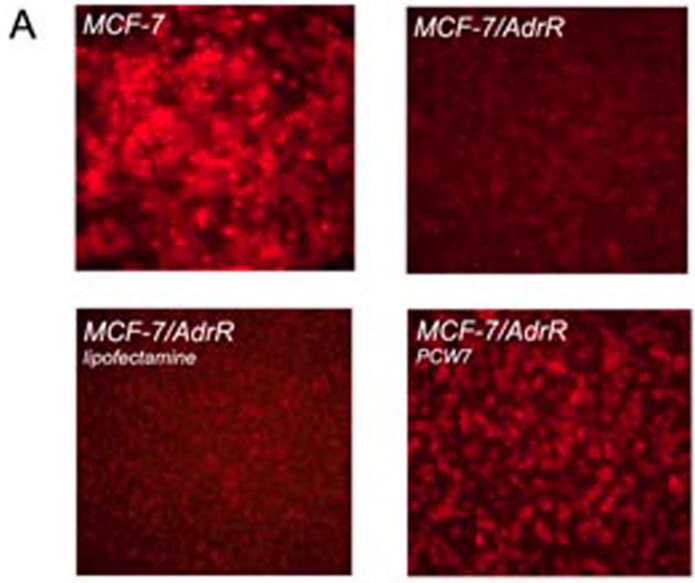
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MCF-7/AdrR
MCF-7/AdrR
lipofectamine
MCF-7/AdrR
PCW7

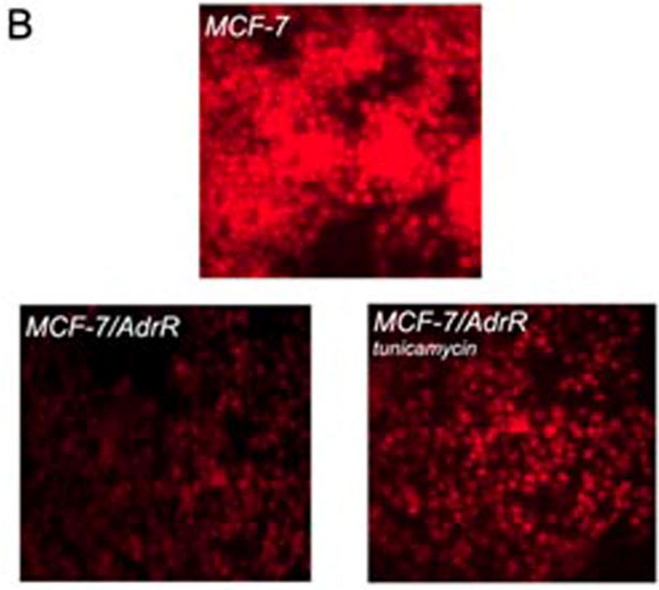
B
MCF-7
MCF-7/AdrR
MCF-7/AdrR
tunicamycin

Fig. 9