BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 6–10 (1998)
ARTICLE NO. RC989029

Cellular Resistance to Adriamycin Conferred by Enhanced Rb Expression Is Associated with Increased *MDR1* Expression[1]

Young Do Yoo,[2] Seongman Kang,* and Yoon-Koo Kang
Laboratory of Experimental Therapeutics, Korea Cancer Center Hospital, Seoul 139-240, Korea; and
**Graduate School of Biotechnology, Korea University, Seoul, Korea*

Received June 22, 1998

In order to investigate if the enhanced expression of Rb confers increased cellular resistance to adriamycin, we made Rb stable transfectants from colon carcinoma cells, SW620. Rb stable transfectants exhibited 5- to 10-fold more resistance to adriamycin than the control cells. To study the correlation between enhanced Rb expression and *MDR1* expression, products of the Rb gene and the *MDR1* gene in Rb stable transfectants were measured by Western blot analysis. These Rb transfectants showed increased *MDR1* expression. Transient transfection of the *MDR1* promoter-CAT reporter gene and the Rb gene demonstrated that Rb up-regulated *MDR1* promoter activity in SW620 cells. Rb may, at least partly, contribute to a role in protecting cells from carcinogen exposure by up-regulating the *MDR1* gene. © 1998 Academic Press

Rb has been implicated in suppressing apoptosis. Rb protects radiation-induced apoptosis in the human osteosarcoma cells (1) and inhibit ceramide-induced apoptosis in human bladder tumor cells (2). Rb is also known to be important for inhibiting apoptosis in other cells. Cyclin D1-induced apoptosis is inhibited by Rb in neuronal cells (3). Overexpression of Rb inhibited cytokine-induced apoptosis and prevented p53-mediated apoptosis (4, 5, 6). These results are consistent with the previous reports that massive apoptosis was detected in the hematopoietic cells, central nervous systems, and the developing ocular lens of Rb-deficient mice (7, 8, 9, 10).

It has been suggested that Rb regulates genes negatively or positively through transcriptional controls. Hypophosphorylated Rb was shown to be a transcriptional repressor that is targeted to genes through interaction with E2F, the transcription factor regulating transcription of S-phase genes required for cell cycle progression, G1 to S, and for DNA synthesis (11, 12, 13, 14). It interacts with Elf-1 and inhibits Elf-1-dependent transcription in human T cells (15). The interaction of Rb with the transcription factors stimulates the transcription factors-mediated transcription (16, 17). Rb, also, stimulates Sp1- and ATF-2-mediated transcription (18, 19). It was recently reported that Rb stimulated expression of cyclin D1 at the transcriptional level, possibly, through Sp1 binding sites (20). These results suggested that Rb might be a transcriptional regulator that controls expression of several genes.

MDR1 expression is regulated in response to various stresses such as chemotherapeutic agents, cadminum, heat shock, arsenite, and differentiating agents (21). Several transcription factors have been suggested to be a mediator through which *MDR1* expression is induced by these stresses. The transcription factors such as Sp1 and Egr-1 are involved in inducible expression of the *MDR1* gene by v-*raf* kinase and TPA (22, 23). The Y-box-binding protein binds the *MDR1* promoter and induces *MDR1* expression, conferring on cells an increased resistance to some chemotherapeutic drugs (24, 25).

We observe that expression of the Rb gene was increased by exposure to adriamycin in colon carcinoma cells. We were interested in studying if Rb might have some role in protecting cells from toxic chemicals such as adriamycin. So we investigated the correlation between Rb levels and *MDR1* gene expression. In this study, we showed that enhanced expression of Rb caused by adriamycin treatment resulted in increased *MDR1* expression. We carried out co-transfection experiments with the CAT reporter genes under the control of the promoter of the *MDR1* gene. Our results, described in this study, demonstrated that Rb might

[1] This work was supported by the National Nuclear R & D Program of the Ministry of Science and Technology of Korea.

[2] To whom requests for reprints should be addressed at Laboratory of Experimental Therapeutics, Korea Cancer Center Hospital, Seoul 139-240. Fax: 82-2-977-0381. E-mail: ydy@kcchsun.kcch.re.kr.

0006-291X/98 $25.00

have another role, protecting cells from toxic chemicals, besides growth control and the regulation of gene expression.

MATERIALS AND METHODS

Cell culture. SW620 cell lines and the adriamycin-resistant cell lines for SW620 cells were grown in RPMI1640 (Life Technologies, Inc., Grand Island, NY) containing 7% fetal bovine serum (Life Technologies, Inc), 2 mg/ml sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C and 5% CO_2.

Construction of Rb-overexpressed cell lines. Exponentially growing SW620 cells (5×10^5) were transferred to 60 mm tissue culture plate containing 4 ml of RPMI1640 medium. After washing with serum-free medium, cells were incubated with a mixture of 20 μl of Lipofectin (Life Technologies, Inc.) and 4 μg of Rb DNA in 2 ml of serum-free medium for 12 hours. The cells were then cultured in RPMI1640 medium containing 500 μg/ml G418 (Life Technologies, Inc.) for 2-3 weeks.

Plasmids. All the *MDR1* promoters in the *MDR1* promoter-reporter CAT gene were generated by polymerase chain reamplification. Amplified DNA fragments, −1572 to +130, −500 to +130, and −70 to +130, were cloned into promoterless CAT expression plasmid (pGEM4-SVOCAT) using *Hin*dIII and *Kpn*I restriction sites built into the oligonucleotides used for amplification.

CAT assay. The *MDR1* promoter-reporter CAT gene with or without the Rb gene were transfected into the SW620 cells by the Lipofectin method. After 48 hours, the cells were harvested and the protein concentration of the cell lysates was determined by using the Bio-Rad (Hercules, CA) protein assay. Equal amounts of protein were used to assay for the CAT enzyme.

Cytotoxicity assay. Drug sensitivity to adriamycin was measured using a sulforhodamine B assay as previously described (26).

Cell lysates and Western blotting. 2×10^6 cells were prepared by lysis with 50 μl of RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris [pH 8.0]) containing phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM NaF, and 30 mM NaPPi). Samples (50 μg/land) were resolved by SDS-PAGE and blotted on nitrocellulose membranes. Filters were incubated for 1 hr at room temp. with anti-Rb, #PC13 (Oncogene Science, Uniondale, NY) or with anti-p-glycoprotein, C-19 (Santa Cruz Biotechnology, Santa Cruz, CA). The antigen-antibody complexes were detected by enhanced chemiluminescence, according to the manufacturers instructions (Amersham, Little Chalfont, England).

RESULTS

Enhanced Expression of Rb and Adriamycin Resistance

In order to investigate the correlation between enhanced expression of Rb and adriamycin resistance, we established SW620 cell lines overexpressing the Rb protein. Three G418-resistant clones, SW620-Rb1, -Rb2, and -Rb3, were selected from G418-resistant clones. Cellular Rb concentrations were determined by Western blot analysis. An increased Rb expression was observed in three G418-resistant cell lines as compared with SW620 cells and neo-transfected SW620 cells (Fig. 1A). The Rb in SW620-Rb3 cells was expressed higher than in other cell lines. Adriamycin sensitivity was measured for SW620-neo, Rb1, Rb2, and Rb3 cell lines. Cellular response to adriamycin was evaluated using sulforhodamine B assay at various doses. The greatest viability was in SW620-Rb3, which expresses more Rb protein than other cell lines. The result demonstrates that enhanced Rb expression results in increased resistance of SW620 cells to adriamycin treatment. We also examined cell cycle distributions of transfectants (SW620-neo, SW620-Rb1, and SW620-Rb3). Fractions of cell population in the G0-G1 phase (in %) were 43, 54, and 64, respectively.

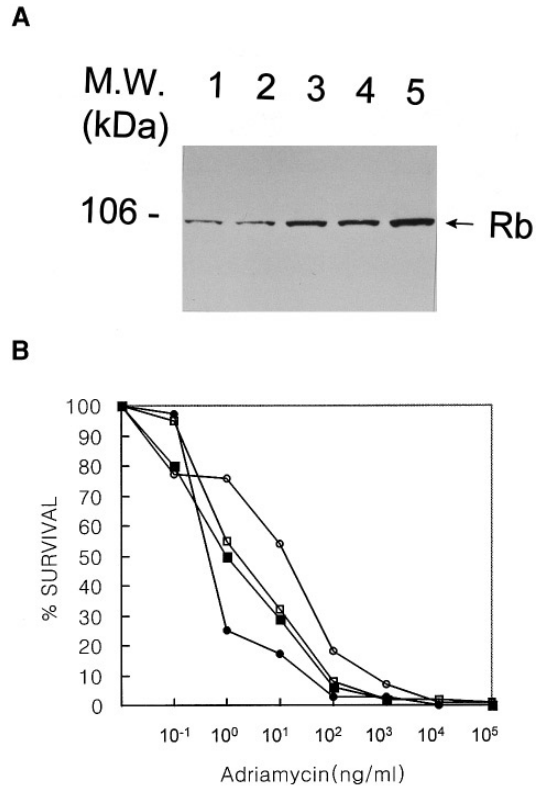

FIG. 1. (A) Rb expression in Rb stable transfectants. Rb stable transfectants were constructed as described under Materials and Methods. Protein lysates were separated by SDS-PAGE and Rb expression was detected by Western blotting. Equal loading of protein was confirmed by ponceau S staining after Western blotting. *Lane* 1, SW620; *Lane* 2, SW620-neo; *Lanes* 3-5, Rb stable transfectants SW620-Rb1, SW620-Rb2, and SW620-Rb3, respectively. (B) Viability analysis of Rb stable transfectants SW620-Rb1, SW620-Rb2, and SW620-Rb3 after treatment with various doses of adriamycin. Drug sensitivity to adriamycin was measured using sulforhodamine B assay after 72-h drug exposure. ●, SW620-neo; ■, SW620-Rb1; □, SW620-Rb2; ○, SW620-Rb3.

Rb Expression Is Associated with MDR1 Expression

Multidrug resistance to anticancer drugs is associated with overexpression of the *MDR1* gene. To exam-

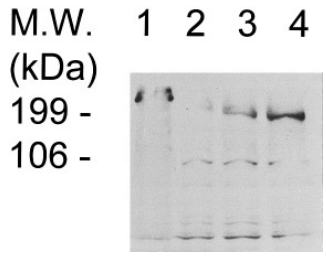

FIG. 2. *MDR1* expression in Rb stable transfectants. Protein lysates from Rb stable transfectants were separated by SDS-PAGE and Western blotting for p-glycoprotein was done. Equal loading of protein was confirmed by ponceau S staining after Western blotting. *Lane* 1, SW620-neo; *Lanes* 2-4, SW620-Rb1, SW620-Rb2, and SW620-Rb3, respectively.

ine which factor is involved in adriamycin resistance of Rb transfectants by increased Rb expression, we studied *MDR1* expression in three Rb transfectant cell lines. Cells were cultured in RPMI1640 medium without G418 for 3 days to eliminate the effect of G418 in *MDR1* expression and then Western blotting for p-glycoprotein was done. The highest level of *MDR1* expression was observed in SW620-Rb3 cell line, which had also shown the highest Rb expression among the tested cell lines (Fig. 2). In contrast, Rb expression in SW620-Rb1 cell line was much lower than that in SW620-Rb3 cell lines. *MDR1* expression in SW620-Rb1 cell line was slightly increased as compared with SW620 cells.

Figure 3 shows that enhanced *MDR1* expression was caused by increased Rb expression. Rb has been known to induce transcription of some genes. So, we tested the ability of Rb to induce transcription of *MDR1* by co-

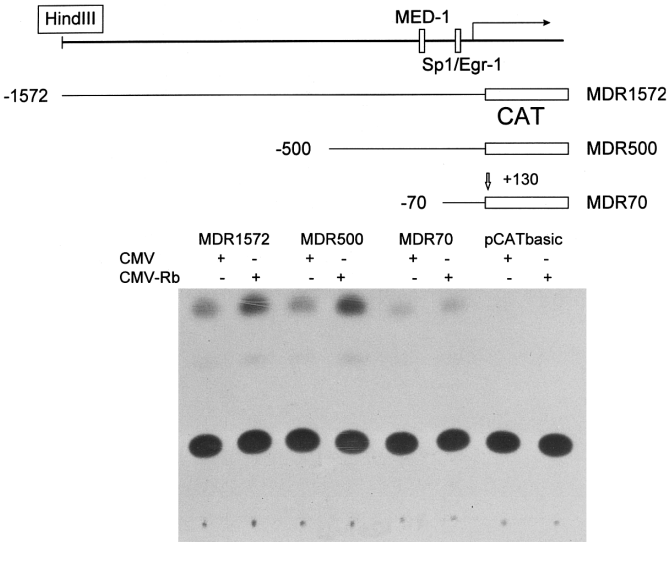

FIG. 3. Stimulation of MDRCAT expression by Rb. MDRCAT plasmids with deletions at their 5′-ends were co-transfected with either CMV expression vector control or Rb.

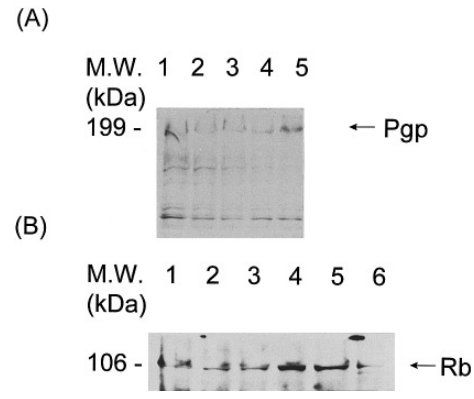

FIG. 4. (A) *MDR1* expression after adriamycin treatment. SW620 cells were treated with adriamycin and maintained in culture for 1 day and then Western blotting for p-glycoprotein was performed. *Lane* 1, untreated; *Lanes* 2-5, treated with 0.64, 3.2, 16, and 80 ng/ml adriamycin, respectively; Left, location of the Mr 199,000 marker; arrow, p-glycoprotein. (B) Increase in Rb expression after adriamycin treatment. SW620 cells were treated with adriamycin and maintained in culture for 1 day. Rb expression after adriamycin treatment was determined by Western blotting. *Lane* 1, untreated; *Lanes* 2-6, treated with 0.64, 3.2, 16, 80, or 400 ng/ml adriamycin, respectively; Left, location of the Mr 106,000 marker; arrow, Rb.

transfection with the *MDR1* promoter-reporter and the Rb gene. The *MDR1* promoter-CAT construct with CMV expression vector or Rb was transiently transfected into SW620 cells and CAT activity was measured. The minimal promoter construct pSVOCAT was used as a control for Rb-induced nonspecific increases in transcription. There was little induction in minimal *MDR1*-promoter driven CAT activity. However, an increase in CAT expression of −1572 *MDR1* promoter-CAT and −500 *MDR1* promoter-CAT constructs was observed in the presence of Rb. The induction was abolished when the deletion reached nucleotide position −70 (Fig. 3). These data suggest that Rb stimulates *MDR1* expression at the transcriptional level in SW620 cells.

Induction of Rb by Adriamycin

MDR1 expression could be modulated by adriamycin treatment (Fig. 4A) and we showed that Rb stimulated *MDR1* expression at the transcriptional level in Fig. 3. We examined whether adriamycin treatment could cause increases in expression of Rb. Figure 4 shows that the expression levels of *MDR1* and Rb were simultaneously increased after exposure to adriamycin in SW620 cells. These results demonstrate that enhanced Rb expression resulting from exposure to adriamcyin may induce an increase in *MDR1* expression.

DISCUSSION

Rb has an important role in apoptosis, senescence, differentiation, and suppressing tumorigenesis. Func-

tional Rb gene loss is seen in retinoblastoma and many kinds of tumors (27, 28) and is associated with tumor progression in bladder carcinoma (29). A low level of Rb is also implicated in shortened survival in acute mylogenous leukemia (28). Deletions or mutations in tumor suppressor genes is associated with multidrug resistance. There have been several reports that Rb inhibits apoptosis. Massive apoptosis was observed in Rb-deficient mice (7, 8, 9, 10). Rb prevents apoptosis and increases viability following treatment of radiation in Saos-2 cells expressing Rb which originally lacked Rb expression (1). The mechanism by which Rb protect cells from apoptosis have been explained by its functions in the regulation of the transition from G1 phase to the S phase of cell cycle where it binds to E2F, inactivating it. Overexpressing of endogenous E2F-1 is associated with premature S-phase entry and subsequent apoptosis (30) and deregulated E2F-1 expression leading to S-phase entry induces p53-mediated apoptosis (31). Furthermore, MEFs lacking the Rb gene enters inappropriate S-phase entry and results in elevated mRNA and protein levels of E2F target genes (32). In this study, we showed that excess Rb expression conferred on cells increased viability to adriamycin. This result is consistent with previous reports that Rb inhibits apoptosis (4, 5, 6). However, the role of Rb in SW620 cells reported in this paper may be somehow different from previous reports. Rb may be involved in a pathway that protects cells from carcinogen exposure.

P-glycoprotein (Pgp), encoded by the *MDR1* gene, is a 170 kDa protein which functions as an energy-dependent drug efflux pump. The expression of Pgp is associated with a resistance to chemotherapy in tumor cells. The cells transfected with the *MDR1* gene showed cross-resistance to natural product drugs including adriamycin (33). High levels of Pgp is expressed in the kidney, colon, liver, and adrenal gland (34). Tumors occurring in these organs are usually classified as nonresponsive to chemotherapy. *MDR1* is also expressed in normal lymphocytes. Pgp is known to be involved in protecting cells from natural product, anticancer drugs, as well as many other cytotoxic compounds (21). *MDR1* expression may play a role in the protection of epithelial tissues from toxic chemicals exposure and low *MDR1* expression may be associated with an increased risk of developing bladder cancer (35). We show that enhanced Rb expression is associated with increased *MDR1* expression and Rb stimulates *MDR1* expression at the transcriptional level in colon carcinoma cells. We also demonstrate this increase in Rb expression can be caused by the treatment of adriamycin. On the basis of these observations, we suggest that cellular resistance to adriamycin conferred by enhanced Rb expression is associated with increased *MDR1* expression. As shown in Fig. 4, *MDR1* expression is increased by the treatment of adriamycin. This increase in *MDR1* expression might have been from enhanced expression of the Rb gene. From this study we hypothesize that Rb may have a role in protecting cells from carcinogens such as adriamycin by reducing the intracellular concentration of toxic compounds. Also, we cannot rule out that enhanced Rb expression may cause the growth arrest associated with cell damage, which provides time for DNA repair, thereby preventing adriamycin-induced apoptosis. Decreased drug sensitivity in Rb-overexpressing SW620 cells may be associated with both increased *MDR1* expression and enhanced repair function associated with G1 arrest.

REFERENCES

1. Haas-Kogan, D. A,. Kogan, S. C., Levi, D., Dazin, P., T'Ang, A., Fung, Y.-K. T., and Israel, M. A. (1995) *EMBO J.* **14,** 461–472.
2. McConkey, D. J., Goodrich, D., Bucana, C., and Klostergaard, J. (1996) *Oncogene* **13,** 1693–1700.
3. Kranenburg, O., van der Eb, A. J., and Zantema, A. (1996) *EMBO J.* **15,** 46–54.
4. Berry, D. E., Lu, Y., Schmidt, B., Fallon, P. G., O'Connell, C., Hu, S.-X., Xu, H.-H., and Blanck, G. (1996) *Oncogene* **12,** 1809–1819.
5. Fan, G., Ma, X., Kren, B. T., and Steer, C. J. (1996) *Oncogene* **12,** 1909–1919.
6. Haupt, Y., Rowan, S., and Oren, M. (1995) *Oncogene* **10,** 1563–1571.
7. Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. (1992) *Nature* **359,** 328–330.
8. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992) *Nature* **359,** 295–300.
9. Lee, E. Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C.J., Lai, C.-C., Herrup, K., Lee W.-H., and Bradley, A. (1992) *Nature* **359,** 288–294.
10. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994) *Nature* **371,** 72–74.
11. Bagchi, S., Weinmann, R., and Raychaudhuri, P. (1991) *Cell* **65,** 1063–1072.
12. Bandara, L. R., Adamczewski, J. P., Hunt, T., and La Thangue, N. B. (1991) *Nature* **352,** 249–251.
13. Chellappan, S. P., Heibert, S., Mudryl, M., Horowitz, J. M., and Nevins, J. R. (1991) *Cell* **65,** 1053–1061.
14. Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. (1991) *Cell* **65,** 103–1082.
15. Wang, C.-Y., Petryniak, B., Thompson, C. B., Kaelin, W. G., and Leiden, J. M. (1993) *Science* **260,** 1330–1335.
16. Rustgi, A. K., Dyson, N., and Bernards, R. (1991) *Nature* **352,** 541–544.
17. Hagemeier, C., Brannister, A. J., Cook, A., and Kouzarides, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1580–1584.
18. Kim, S.-J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R., and Robbins, P. D. (1992) *Mol. Cell. Biol.* **12,** 2455–2463.
19. Kim, S.-J., Wagner, S., Liu, F., O'Reilly, M. A., Robbins, P. D., and Green, M. R. (1992) *Nature* **358,** 331–334.
20. Muller, H., Lukas, J., Schneider, A., Warthoe, P., Bartek, J., Eilers, M., and Strauss, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2945–2949.
21. Chin, K. V., Pastan, I., and Gottesman, M. M. (1993) *Adv. Cancer Res.* **60,** 157–180.

22. Cornwell, M. M., and Smith, D. E. (1993) *J. Biol. Chem*. **268,** 15347–15350.

23. McCoy, C., Smith, D. E., and Cornwell, M. M. (1995) *Mol. Cell. Biol*. **15,** 6100–6108.

24. Ohga, T., Koike, K., Ono, M., Makino, Y., Itagaki, Y., Tanimoto, M., Kuwano, M., and Kohno, K. (1996) *Cancer Res.* **56,** 4224–4228.

25. Ohga, T., Uchiumi, T., Makino, Y., Koike, K., Wada, M., Kuwano, M., and Kohno, K. (1998) *J. Biol. Chem*. **273,** 5997–6000.

26. Wu, L., Smythe, A. M., Stinson, S. F., Mullendore, L. A., Monks, A., Scudiero, D. A., Paull, K. D., Koutsoukos, A. D., Rubinstein, L. V., Boyd, M. R., and Shoemaker, R. H. (1992) *Cancer Res.* **52,** 3029–3034.

27. Horowitz, J. M., Park, S.-H., Bogenmann, E., Cheng, J.-C., Yandell, D. M., Kaye, F. J., Minna, J. D., Dryja, T. P., and Weinberg, R. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2775–2779.

28. Kornblau, S. M., Xu, H.-J., Zhang, W., Hu, S.-X., Beran, M., Smith, T. L., Hester, J., Estey, E., Benedict, W. F., and Deisseroth, A. B. (1994) *Blood* **84,** 256–261.

29. Logothetis, C. J., Xu, H. J., Ro, J. Y., Hu, S. X., Sahin, A., Ordonez, N., and Benedict, W. F. (1992) *J. Natl. Cancer Inst.* **84,** 1256–1261.

30. Shan, B., and Lee, W.-H. (1994) *Mol. Cell. Biol.* **14,** 8166–8173.

31. Qin, X.-Q., Livingston, D. M., Kaelin, Jr., W. G., and Adams, P. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10918–10922.

32. Almasan, A., Yin, Y., Kelly, R. E., Lee, E. Y.-H. P., Bradley, A., Li, W., Bertino, J. R., and Wahl, G. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5436–5440.

33. Ueda, K., Cardarelli, C., Gottesman, M. M., and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3004–3008.

34. Dalton, W. S. (1994) *Curr. Opin Oncol.* **6,** 595–600.

35. Clifford, S. C., Neal, D. E., and Lunec, J. (1996) *Carcinogenesis* **17,** 601–604.