

# Multidrug Resistance and the Lung Resistance-Related Protein in Human Colon Carcinoma SW-620 Cells

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**Background:** Lung resistance-related protein (LRP), the major vault protein in humans, is sometimes overexpressed in multidrug-resistant cells. Because cells transfected with the LRP gene did not express the multidrug-resistant phenotype, we investigated whether LRP is involved in multidrug resistance. **Methods:** SW-620 cells, a human colon carcinoma cell line, alone or transfected with an expression vector carrying a LRP-specific ribozyme or with an empty vector, were treated with sodium butyrate to induce differentiation. Expression of P-glycoprotein, multidrug resistance protein, and LRP in the cells was examined by northern and western blotting, and the efflux of doxorubicin in the cells or isolated nuclei was examined by fluorescence microscopy. **Results:** A 2-week treatment with sodium butyrate induced LRP and conferred resistance to doxorubicin, vincristine, etoposide, gramicidin D, and paclitaxel (Taxol) in SW-620 cells. Insertion of either of two LRP-specific ribozymes into SW-620 cells inhibited these activities. Levels of drugs accumulating in the cells were not decreased by sodium butyrate, suggesting that the adenosine triphosphate-binding cassette transporter is not involved in sodium butyrate-induced multidrug resistance. Doxorubicin was mainly located in the nuclei of untreated cells and in the cytoplasm of sodium butyrate-treated cells. Isolated nuclei from untreated cells or sodium butyrate-treated cells incubated with anti-LRP polyclonal antibodies contained more doxorubicin than the nuclei of sodium butyrate-treated cells alone. Efflux of doxorubicin was greater from the nuclei of sodium butyrate-treated cells than the nuclei of untreated cells or of sodium butyrate-treated cells transfected with a LRP-specific ribozyme and was inhibited by an anti-LRP polyclonal antibody. **Conclusions:** LRP is involved in resistance to doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D and has an important role in the transport of doxorubicin from the nucleus to the cytoplasm. [J Natl Cancer Inst 1999;91:1647-53]

Multidrug resistance is a major cause of the failure of chemotherapy (1). P-glycoprotein (P-gp), encoded by the gene MDR1, and multidrug-resistance protein (MRP) are known to be associated with multidrug resistance. P-gp, an integral membrane glycoprotein with a molecular mass of 170 kd, has been postulated to function as a pump to remove hydrophobic anticancer agents from drug-resistant cells (2). MRP, a 190-kd 1531-amino-acid membrane glycoprotein (3), is overexpressed in most non-P-gp-mediated multidrug resistant cell lines (4). MRP and P-gp are members of the adenosine triphosphate (ATP)-binding cassette superfamily of membrane transporter proteins (3). Findings suggest that MRP is an ATP-dependent organic anion transporter (5). The 110-kd lung resistance-related protein (LRP) frequently is overexpressed in multidrug resistant

cells, but it has not been proven that LRP is involved in multidrug resistance. Transfection of cells with the LRP gene was insufficient to confer the multidrug-resistant phenotype. LRP is the major vault protein in humans (6,7). Vaults are cytoplasmic organelles that were originally isolated in association with coated vesicles (8), but approximately 5% of the vaults reportedly are localized in nuclear pore complexes (9). The diameter (26–35 nm) and shape (two halves, each with eightfold radial symmetry) of the vaults are similar to those of the central plug (or transporter) of the nuclear pore complex (9). Vaults are thought to be involved in the vesicular and nucleocytoplasmic transport of drugs because both entrapment of drugs into vesicles and decreased nuclear/cytoplasmic ratios have been observed in some multidrug resistant cell lines that overexpress LRP (10,11). Because vaults are multisubunit particles containing LRP (or major vault protein), at least three other proteins, and an RNA moiety, hvg1, approaches other than transfection with the LRP gene are needed to elucidate the contribution of LRP to multidrug resistance.

Mickley et al. (12) reported modulation of the expression of a multidrug-resistance gene (*mdr-1/P-gp*) by agents that induce differentiation. They showed that some cell lines induced to differentiate by sodium butyrate express P-gp.

In this study, we show that LRP is also induced by sodium butyrate in human colorectal carcinoma SW-620 cells and that its expression confers the multidrug-resistance phenotype to the cells. We also show that the sodium butyrate-induced expression of LRP and the acquisition of the multidrug-resistance phenotype in SW-620 cells are inhibited by two LRP-specific ribozymes.

## MATERIALS AND METHODS

**Chemicals and antibodies.** The drugs sodium butyrate, paclitaxel (Taxol), doxorubicin (Adriamycin), vincristine, etoposide, and gramicidin D were obtained from Sigma Chemical Co. (St. Louis, MO). [<sup>3</sup>H]vincristine, [<sup>3</sup>H]paclitaxel, and [<sup>14</sup>C]doxorubicin were from Amersham International (Buckinghamshire, U.K.). C219 and MRK-16, monoclonal antibodies against P-gp, were from

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Centocor, Inc. (Malvern, PA), and Kamiya Biochemical (Thousand Oaks, CA), respectively. MR3PAB, a polyclonal antibody against MRP, was prepared as previously described (13). QCRL-3, a monoclonal antibody against MRP (14), was obtained from Chemicon International (Temecula, CA), and LRP-56, a monoclonal antibody against LRP, was obtained from Nichirei (Tokyo, Japan). A rabbit anti-human LRP polyclonal antibody was prepared by generating glutathione *S*-transferase-LRP fusion proteins as antigens. Two oligonucleotides (LRP2083F, 5'-ATGGATCCGACCAGTCAGAAGCCGAGAAAGC-3'; and LRP2383R, 5'-ATGAATTCACCTTCTTCACCTCCACCTCAGCC-3') were used as primers for amplification of LRP complementary DNA (cDNA) (residues 2083–2383) by the polymerase chain reaction (PCR) by use of the LRP cDNA (residues 1776–2689) as a template. The PCR product was digested with *Bam*HI and *Eco*RI and subcloned into pGEX-2T (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The antiserum was affinity purified with a glutathione *S*-transferase-LRP column.

**Cell culture.** SW-620, a human colon carcinoma cell line, was obtained from A. T. Fojo (National Cancer Institute, Bethesda, MD). Cells were grown at 37 °C in 5% CO<sub>2</sub>-95% air in RPMI-1640 medium containing 10% newborn calf serum, 2 mM glutamine, and 100 U/mL penicillin. Sodium butyrate was added to a final concentration of 2 mM to induce differentiation. Culture medium alone or containing sodium butyrate was changed daily. KB-3-1 cells were cultured as described (15). Multidrug-resistant KB-C2 cells, overexpressing MDRI messenger RNA (mRNA) and P-gp, and C-A120 cells, a non-P-gp-mediated multidrug-resistant cell line overexpressing MRP, were isolated from KB-3-1 cell cultures and maintained as described (15,16). HT-1080 fibrosarcoma cells were used as positive controls for LRP.

**Northern blotting.** To prepare the RNA probe for LRP, the following synthetic oligonucleotides were used as PCR primers on the basis of the published LRP cDNA sequence (7): LRP1776F, 5'-TAAGAACTCAGCCCGCAT-CATTCG-3'; LRP2689R, 5'-AATCAGGAGTTAGCGCAGTACAGG-3'. This PCR product containing cDNA corresponding to LRP nucleotides 1776–2689 was subcloned into pBluescript II SK+ to give pBS-LRP. pBS-LRP was linearized with *Xho*I, and a digoxigenin-11-uridine 5'-triphosphate-labeled RNA probe was prepared by use of T3 RNA polymerase according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). A digoxigenin-labeled glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA probe was prepared in the pGEM-T vector with the PCR product of G3PDH corresponding to nucleotides 6–987 and was used as the control probe. For northern blotting, poly(A)+ RNA was resolved by electrophoresis on a formaldehyde-agarose-denaturing gel and was transferred onto a nylon membrane. After fixation with UV irradiation, the membrane was prehybridized for 2 hours at 68 °C in 50% formamide, 5× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 2% blocking reagent (Boehringer Mannheim GmbH), 1% sodium dodecyl sulfate (SDS), and 1 mg/mL yeast transfer RNA. The blot was hybridized for 18–24 hours at 68 °C with the digoxigenin-labeled RNA probe in prehybridization buffer. The membrane was washed twice in 2× SSC containing 0.1% SDS for 15 minutes at room temperature and twice in 0.1× SSC containing 0.1% SDS for 1 hour at 68 °C. The blot was then incubated with alkaline phosphatase-labeled anti-digoxigenin antibody and a chemiluminescent substrate as described by the manufacturer's instructions (Boehringer Mannheim GmbH).

**Western blotting.** Crude membranes were prepared as described (17). Protein concentrations were determined by the procedure of Bradford (18). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA) in transfer buffer (48 mM Tris and 39 mM glycine [pH 9.2] containing 20% [vol/vol] methanol) at 15 V for 36 minutes at room temperature (16). To block nonspecific binding, the blots were preincubated for 1 hour at room temperature in washing buffer (0.35 M NaCl, 10 mM Tris-HCl [pH 8.0], and 0.05% Tween 20) containing 3% skim milk. The blots were then incubated at 4 °C for 8 hours with C219, MR3PAB, or anti-LRP polyclonal antibody; washed with washing buffer; and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (for polyclonal antibody against MRP and LRP) or anti-mouse (for monoclonal antibody against P-gp) immunoglobulin G (IgG). Antibody binding was visualized with the ECL western blotting detection system (Amersham Pharmacia Biotech).

**Cell growth and survival.** The growth rate of SW-620 cells treated with sodium butyrate was measured in medium containing various concentrations of newborn calf serum (1%, 3%, 5%, and 10%) and 2 mM sodium butyrate. On days 2 and 4, cells were trypsinized and counted. Chemosensitivity *in vitro* was

measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay in 96-well plates as described (19).

[<sup>3</sup>H]Vincristine, [<sup>3</sup>H]paclitaxel, and [<sup>14</sup>C]doxorubicin accumulation in sodium butyrate-treated and untreated SW-620 cells. Cells (2 × 10<sup>5</sup> cells/well) were incubated for 1 hour at 37 °C in RPMI-1640 medium containing 1 μM [<sup>3</sup>H]vincristine (6.75 Ci/mmol), 0.1 μM [<sup>3</sup>H]paclitaxel (10 μCi/μmol), or 1 μM [<sup>14</sup>C]doxorubicin (2.7 μCi/mmol). Cells were then washed twice in phosphate-buffered saline and solubilized with 1% Triton X-100 and 0.2% SDS in phosphate-buffered saline (pH 7.3). Total [<sup>3</sup>H]paclitaxel, [<sup>14</sup>C]doxorubicin, or [<sup>3</sup>H]vincristine accumulation then was measured by liquid scintillation counting.

**Localization of doxorubicin.** Sodium butyrate-treated and untreated cells were incubated for 2 minutes at 37 °C in RPMI-1640 medium with 10% newborn calf serum and 20 μM doxorubicin. The cells were then washed with phosphate-buffered saline and examined by fluorescence microscopy (Olympus, Tokyo, Japan). We then measured uptake of doxorubicin by isolated nuclei. Nuclei were isolated as described (20) from SW-620 cells alone and SW-620 cells treated with sodium butyrate. The isolated nuclei were suspended in solution A (250 mM sucrose, 1 mM dithiothreitol, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 15 mM PIPES-NaOH [pH 7.4], 0.5 mM spermidine, 0.2 mM spermine, and 1 mM phenylmethylsulfonyl fluoride) and incubated with 1 μM doxorubicin for 2 minutes at 32 °C in the presence or absence of anti-LRP polyclonal antibody (0.11 mg/mL), rabbit IgG (21.1 mg/mL), a monoclonal antibody against MRP (QCRL3 at 0.98 mg/mL), or a monoclonal antibody against P-gp (MRK-16 at 1.01 mg/mL). The nuclei were then washed with solution A and examined by fluorescence microscopy (Olympus).

**Accumulation and efflux of [<sup>14</sup>C]doxorubicin in isolated nuclei.** To measure [<sup>14</sup>C]doxorubicin accumulation in isolated nuclei, we incubated 1 × 10<sup>6</sup> nuclei from control cells, 1 × 10<sup>6</sup> nuclei from sodium butyrate-treated cells, and 1 × 10<sup>6</sup> sodium butyrate-treated cells transfected with Rz789 at 37 °C as indicated with 1 μM [<sup>14</sup>C]doxorubicin in the presence or absence of anti-LRP polyclonal antibody and washed them three times with solution A. Radioactivity in the nuclei was then determined. To study [<sup>14</sup>C]doxorubicin efflux, we incubated nuclei at 37 °C for 10 minutes with 1 μM [<sup>14</sup>C]doxorubicin in the presence or absence of anti-LRP polyclonal antibody. The nuclei were then washed three times with ice-cold solution A, and fresh solution A with or without the antibody was added. Nuclei were then incubated at 37 °C as indicated and washed once, after which radioactivity in the nuclei was determined.

**Design and expression of LRP-specific ribozymes in SW-620 cells.** Two single-stranded polynucleotides that contained ribozyme-flanking *Hind*III and *Apa*I restriction sites on both ends were synthesized and 5'-phosphorylated by T4 polynucleotide kinase (Toyobo, Osaka, Japan). The polynucleotide sequences were 5'-AGCTTACTGTGGTCTGATGAGTCCGTGAGGACGAAACCAGCATGGGCC-3' and 5'-CATGGCTGGTTTCGTCCTCAGGACTCATCAGACCAGTA-3' for Rz594 and 5'-AGCTTCTCGTGTCTGATGAGTCCGTGAGGACGAAACATCTGGGCC-3' and 5'-CAGATGTTTCGTCCTCAGGACTCATCAGCAGGAGGA-3' for Rz789. Each ribozyme was cloned into pRc/CMV (Invitrogen, NV Leek, The Netherlands). The sequences and orientations of the ribozymes in the vector were confirmed by DNA sequencing. The plasmid carrying the ribozyme was digested with *Bgl*III and transfected into logarithmically growing SW-620 cells by electroporation.

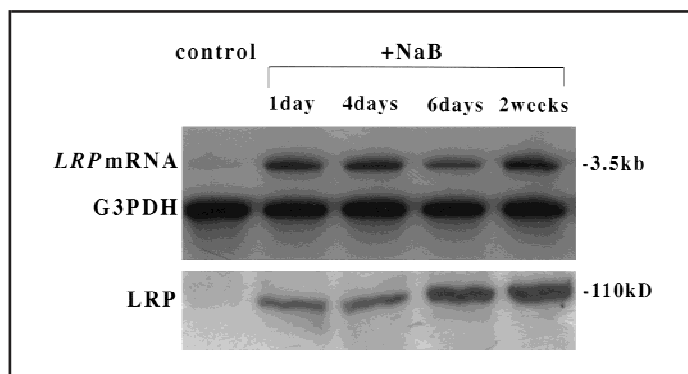
**Statistical analyses.** Differences between groups were tested by the analysis of variance or Mann-Whitney *U* test. All *P* values are two-sided. A two-sided *P* value of less than .05 was considered to be statistically significant.

## RESULTS

### Expression of LRP mRNA and Protein in Sodium Butyrate-Treated Cells

SW-620 cells were incubated with sodium butyrate for 1, 4, 6, or 14 days, and sodium butyrate-mediated modulation of LRP mRNA expression was investigated by northern blot analysis (Fig. 1). In the absence of sodium butyrate, the cells expressed a very low level of LRP mRNA. After the addition of sodium butyrate, an increased level of LRP mRNA was observed within 24 hours. LRP mRNA reached a maximum on day 1 and remained at this level as long as sodium butyrate was present.

The expression of LRP was examined by immunoblot analy-



**Fig. 1.** Expression of lung resistance-related protein (LRP) messenger RNA (mRNA) and LRP in SW-620 cells. mRNA (0.5  $\mu$ g) was applied to each well and hybridized with LRP RNA probe. Positions of 3.5-kilobase (kb) LRP mRNA are indicated. A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA probe was used as the control probe. Cytosolic fractions (75  $\mu$ g of protein) from untreated and sodium butyrate (NaB)-treated SW-620 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) membranes. LRP levels in the cytosol were determined with the polyclonal antibody against LRP. kD = kilodaltons.

sis (Fig. 1). LRP was not detected in untreated cells but was induced in cells treated with sodium butyrate for 24 hours. Maximum LRP expression was observed after 14 days of treatment. The maximum level of LRP protein lagged behind the maximum expression of LRP mRNA.

#### Effect of LRP-Specific Ribozymes on LRP Expression

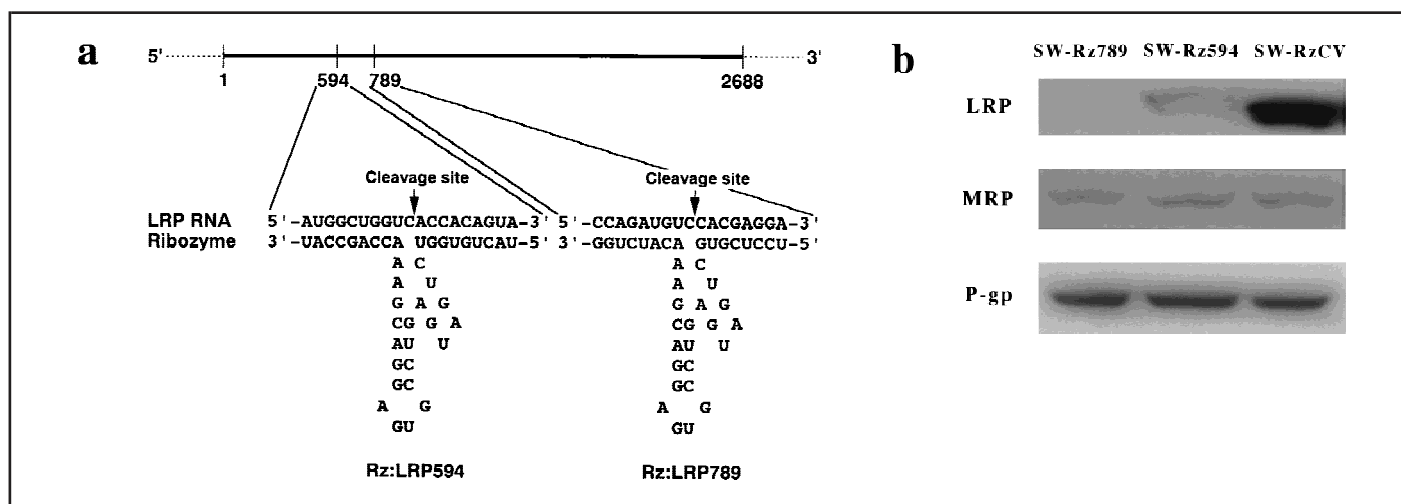
SW-620 cells were transfected with the expression vectors carrying Rz594 to make SW-Rz594 cells or Rz789 to make SW-Rz789 cells (Fig. 2, a). LRP was not detected in SW-Rz789 cells when the cells were treated with sodium butyrate. LRP was only minimally detected in SW-Rz594 cells after treatment with sodium butyrate. LRP was induced by sodium butyrate in SW-RzCV cells; SW-620 cells transfected with empty vector. MRP and P-gp were also induced by sodium butyrate in all transfected cell lines studied, and their expression was not affected by the two ribozymes (Fig. 2, b).

#### Sensitivity of Sodium Butyrate-Treated SW-620 Cells to Anticancer Agents

Sodium butyrate slows the growth rate of SW-620 cells, and the growth rate may affect the sensitivity of the cells to anticancer agents. Consequently, we first compared the growth rates of SW-620 cells in medium containing various concentrations of newborn calf serum (1%, 3%, and 5%) with the growth rates of SW-620 cells treated with sodium butyrate for 14 days. The growth rates of SW-620 cells cultured in medium containing 5% newborn calf serum and of cells treated with sodium butyrate were similar. We next compared the anticancer agent sensitivity of untreated SW-620 cells cultured in medium containing 5% or 10% newborn calf serum with that of sodium butyrate-treated SW-620 cells cultured in medium containing 10% newborn calf serum. The cytotoxic effects of the anticancer agents doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D on SW-620 cells were determined by the MTT assay. We found that the levels of toxicity (measured as the concentration that inhibits the response by 50%) induced by doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D in sodium butyrate-treated cells were 6.2-, 2.0-, 8.9-, 7.4-, and 12.8-fold higher, respectively, than the toxicity of cells cultured in medium containing 5% newborn calf serum (Table 1). Except for the sensitivity to vincristine, the differences in sensitivity to these agents were statistically significant. Sodium butyrate-treated SW-Rz789 and SW-Rz594 cells and SW-620 cells not treated with sodium butyrate had similar sensitivities to these anticancer agents and to gramicidin D. On the other hand, SW-RzCV cells treated with sodium butyrate were resistant to these anticancer agents, and the levels of resistance were similar to those of SW-620 cells treated with sodium butyrate.

#### [<sup>3</sup>H]Vincristine, [<sup>3</sup>H]Paclitaxel, and [<sup>14</sup>C]Doxorubicin Accumulation in Sodium Butyrate-Treated and Untreated SW-620 Cells and Multidrug-Resistant Cells

The accumulation of vincristine and doxorubicin in sodium butyrate-treated SW-620 cells was 1.43-fold and 1.85-fold higher, respectively, than in untreated cells. There was no dif-



**Fig. 2.** Effect of lung resistance-related protein (LRP)-specific ribozymes on LRP expression. **a)** Targeted sequences and cleavage sites of LRP-specific ribozymes. **b)** SW-620 cells transfected with LRP-specific ribozymes, Rz:LRP594 (SW-Rz594 cells) and Rz:LRP789 (SW-Rz789 cells), or empty vector RzCV (SW-RzCV cells) were treated with sodium butyrate for 14 days, and the expression of LRP, multidrug-resistance protein (MRP), and P-glycoprotein (P-gp) was examined by immunoblot analysis.



**Table 1.** Effect of 2 mM sodium butyrate (NaB) on drug sensitivity of SW-620 cells

Drugs	IC <sub>50</sub> *						
	SW-620 (10%)	SW-620 (5%)	SW-620 + NaB	<i>P</i> †	SW-Rz789 + NaB	SW-Rz594 + NaB	SW-RzCV + NaB
ADM‡	0.011 (0.002–0.019)	0.020 (0.017–0.023)	0.124 (0.117–0.131)	.049	0.008 (0.006–0.009)	0.020 (0.018–0.025)	0.10 (0.064–0.164)
VCR‡	2.052 (2.045–2.057)	2.971 (2.969–2.973)	6.030 (6.029–6.032)	.513	3.363 (3.342–3.384)	4.561 (4.514–4.608)	6.011 (5.978–6.045)
Etoposide	0.149 (0.116–0.181)	0.433 (0.423–0.443)	3.851 (3.836–3.865)	.040	0.192 (0.190–0.195)	0.393 (0.387–0.398)	3.991 (3.986–3.997)
Paclitaxel	0.032 (0.025–0.039)	0.011 (0.009–0.013)	0.081 (0.079–0.083)	.041	0.011 (0.008–0.015)	0.032 (0.019–0.046)	0.074 (0.065–0.083)
Gramicidin D	0.090 (0.083–0.097)	0.063 (0.060–0.067)	0.809 (0.785–0.833)	.050	0.052 (0.047–0.058)	0.102 (0.098–0.105)	0.329 (0.318–0.340)

\*Cell survival was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and reported as the concentration that inhibits the response by 50% (IC<sub>50</sub>). Data are the mean (95% confidence interval).

†SW-620 (5%) versus SW-620 + NaB, two-sided *P* values were obtained from Mann–Whitney *U* test.

‡ADM = doxorubicin; VCR = vincristine.

ference in the accumulation of paclitaxel between untreated and treated cells (Fig. 3). These findings suggested that active efflux pumps were not involved in the drug resistance of cells treated with sodium butyrate. The intracellular concentrations of vincristine, paclitaxel, and doxorubicin in KB-C2 cells, which overexpress P-gp, were lower than in the parental KB-3-1 cells. The intracellular concentrations of vincristine and doxorubicin in C-A120 cells, which overexpress MRP, were also lower than those in the parental KB-3-1 cells, and the accumulation of paclitaxel in C-A120 cells was only slightly lower than that in KB-3-1 cells.

#### Localization of Doxorubicin in Sodium Butyrate-Treated SW-620 Cells and Untreated Control SW-620 Cells

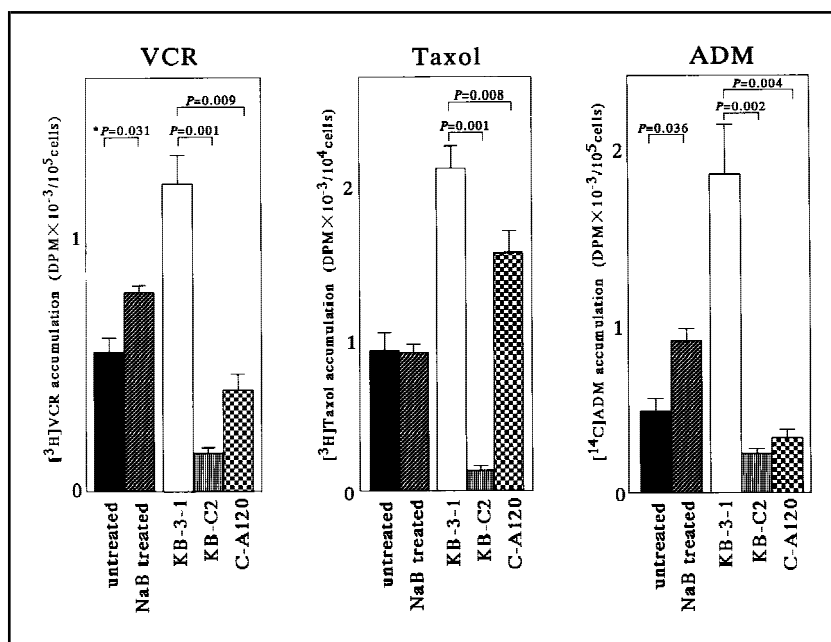
Sodium butyrate-treated and untreated viable SW-620 cells were incubated with doxorubicin at 37 °C and examined by fluorescence microscopy without fixation. There was strong fluorescence in the nuclei of untreated SW-620 cells (Fig. 4, a) and of sodium butyrate-treated cells transfected with the LRP-specific ribozyme SW-Rz789 (Fig. 4, c). In contrast, the treated SW-620 cells showed statistically significantly reduced doxorubicin fluorescence in their nuclei and strong fluorescence in their cytoplasm (Fig. 4, b).

#### Accumulation of Doxorubicin in Isolated Nuclei

Nuclei were isolated from untreated SW-620 cells or SW-620 cells that were treated with sodium butyrate. LRP, but not P-gp and MRP, was detected by immunoblotting in the nuclei from the cells treated with sodium butyrate (Fig. 5). The accumulation of doxorubicin in the nuclei was examined by fluorescence microscopy (Fig. 6). Doxorubicin accumulated in the nuclei of the control untreated SW-620 cells but was not detected in the nuclei of the sodium butyrate-treated cells. However, doxorubicin accumulated in the nuclei of the sodium butyrate-treated cells that had been incubated with an anti-LRP polyclonal antibody but not with QCRL3, a monoclonal antibody against MRP, or MRK-16, a monoclonal antibody against P-gp. The LRP level in the nuclei of the sodium butyrate-treated cells was not affected by the treatment with anti-LRP antibody (data not shown).

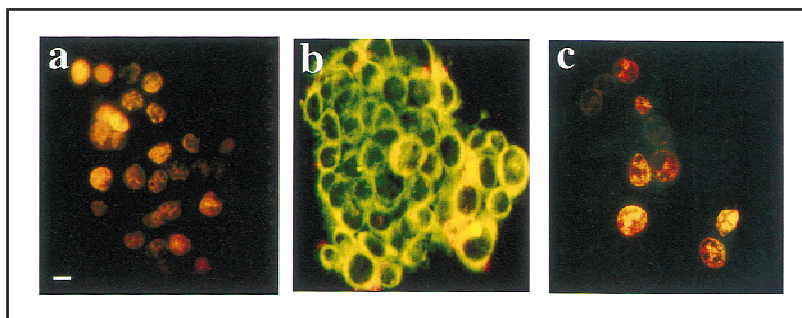
#### Accumulation and Efflux of [<sup>14</sup>C]Doxorubicin in Isolated Nuclei

We next examined the effect of the polyclonal antibody against LRP on the accumulation and efflux of [<sup>14</sup>C]doxorubicin from the nuclei isolated from the sodium butyrate-treated cells. The accumulation of [<sup>14</sup>C]doxorubicin as a function of time in

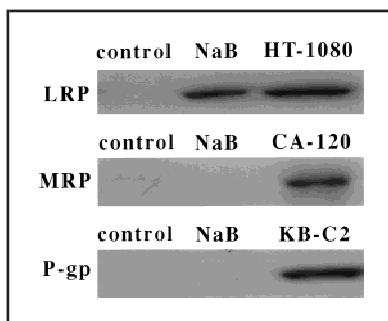


**Fig. 3.** Accumulation of vincristine (VCR), paclitaxel (Taxol), and doxorubicin (ADM). The intracellular levels of vincristine, paclitaxel, and doxorubicin in control untreated SW-620 cells, sodium butyrate (NaB)-treated SW-620 cells, parental KB-3-1 cells, P-glycoprotein-expressing KB-C2 cells, and multidrug-resistance protein-expressing C-A120 cells were determined. Data are the mean of three determinations. Error bars are 95% confidence intervals. \* = two-sided *P* values (Mann–Whitney *U* test).

**Fig. 4.** Fluorescence visualization of doxorubicin accumulation in living SW-620 cells. Untreated (a), sodium butyrate-treated (b), or sodium butyrate-treated cells transfected with lung resistance-related protein (LRP)-specific ribozyme (c) were incubated for 2 minutes at 37 °C with 20  $\mu$ M doxorubicin and then visualized. Scale bar = 10  $\mu$ m.



**Fig. 5.** Immunoblots for lung resistance-related protein (LRP), P-glycoprotein (P-gp), and multidrug-resistance protein (MRP) in isolated nuclei. Nuclear protein (50  $\mu$ g) from control untreated SW-620 cells or from SW-620 cells treated with sodium butyrate (NaB) and membrane vesicles (50  $\mu$ g) from KB-C2 and C-A120 (CA-120) cells, and cytosolic protein (50  $\mu$ g) from HT-1080 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a poly(vinylidene difluoride) membrane. The transferred proteins were immunoblotted with a polyclonal antibody against LRP, a monoclonal antibody against P-gp (C-219), or a monoclonal antibody against MRP (QCRL3).



the nuclei isolated from the control and sodium butyrate-treated cells is shown in Fig. 7, a.

Rapid uptake of [ $^{14}$ C]doxorubicin was observed within 1 minute, and the uptake reached a plateau at 10 minutes. The [ $^{14}$ C]doxorubicin accumulation in the nuclei of the sodium butyrate-treated cells was about one half that of the control cells and the sodium butyrate-treated cells transfected with Rz789. In the presence of the polyclonal antibody against LRP, the accumulation of [ $^{14}$ C]doxorubicin in the nuclei of the sodium butyrate-treated cells was similar to that of the control cells.

We determined whether the decreased accumulation of doxorubicin in the nuclei isolated from the sodium butyrate-treated cells was due to enhanced efflux. Release of [ $^{14}$ C]doxorubicin as a function of time after 10 minutes of accumulation is shown in

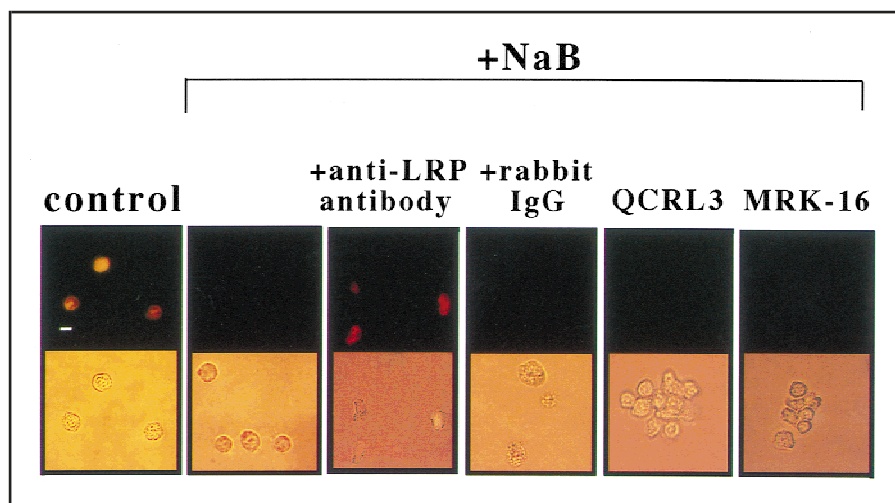
Fig. 7, b. Efflux of doxorubicin from the nuclei isolated from the sodium butyrate-treated cells was enhanced compared with that of the control cells and the sodium butyrate-treated cells expressing Rz789. The polyclonal antibody against LRP almost completely inhibited the enhanced efflux from the nuclei isolated from the sodium butyrate-treated cells.

## DISCUSSION

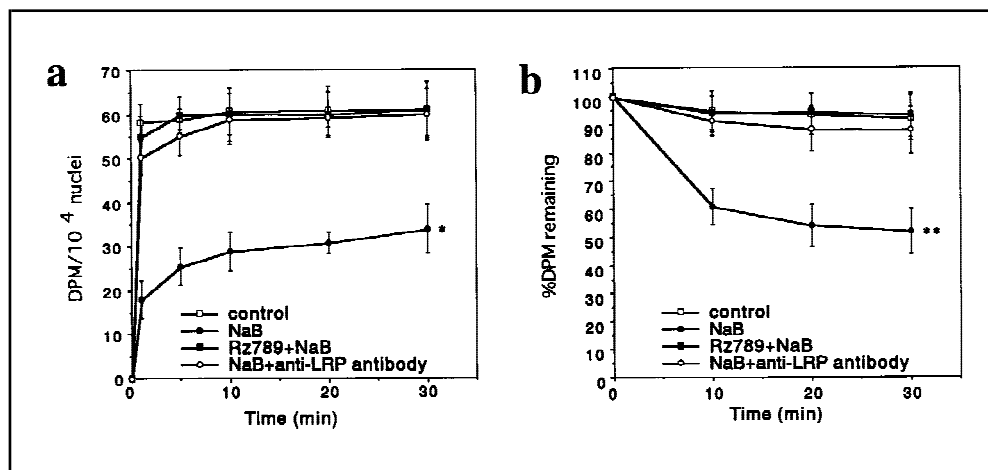
LRP is overexpressed in several multidrug-resistant cell lines that have different histogenetic origins and is thought to mediate multidrug resistance (6). The human LRP gene was cloned and the deduced LRP amino acid sequence showed 87.7% identity with the 104-kd rat major vault protein (7). These findings indicate that LRP is the major vault protein in humans. Vaults are cytoplasmic ribonucleoprotein organelles specifically associated with the nuclear envelope and the nuclear pore complex (9) and are similar to the central plug of nuclear pore complex in shape and size (9). These findings suggest that vaults are involved in nucleocytoplasmic transport. Although transfection with full-length LRP cDNA was not sufficient to confer the multidrug-resistant phenotype (7), vault synthesis was linked directly to multidrug resistance (21). Vaults, but not LRP alone, seem to be involved in multidrug resistance.

We found that sodium butyrate induced LRP, as well as MRP and P-gp, in SW-620 cells. The two LRP-specific ribozymes inhibited the sodium butyrate-induced expression of LRP, but not of MRP and P-gp in SW-620 cells, and almost completely abolished the acquisition of the multidrug-resistant phenotype in sodium butyrate-treated SW-620 cells. These findings indicate that LRP, but not MRP and P-gp, is involved in multidrug resistance in sodium butyrate-treated cells. In accordance with this

**Fig. 6.** Doxorubicin accumulation in isolated nuclei from SW-620 cells. Nuclei were isolated from sodium butyrate-treated or from control untreated cells and were incubated for 2 minutes at 32 °C with doxorubicin in the absence or presence of anti-lung resistance-related protein (LRP) antibody, rabbit immunoglobulin G (IgG), anti-multidrug-resistance protein (MRP) antibody (QCRL3), or anti-P-glycoprotein antibody (MRK-16). The accumulation of doxorubicin in nuclei was assessed by fluorescence microscopy.



**Fig. 7.** Accumulation and efflux of [ $^{14}$ C]doxorubicin in isolated nuclei. **a)** [ $^{14}$ C]Doxorubicin accumulation in nuclei isolated from control untreated cells, from sodium butyrate (NaB)-treated cells, and from NaB-treated cells transfected with Rz789 was measured for 30 minutes in the absence or presence of polyclonal antibody against lung resistance-related protein (LRP). **b)** Efflux of [ $^{14}$ C]doxorubicin. [ $^{14}$ C]Doxorubicin retained in nuclei isolated from control cells, from NaB-treated cells, from NaB-treated cells transfected with Rz789, and from NaB-treated cells also treated with polyclonal antibody against LRP are shown after 10 minutes of [ $^{14}$ C]doxorubicin accumulation. DPM = disintegrations per minute. Data are the mean of three determinations. Error bars = 95% confidence intervals. \* = two-sided  $P = .009$ ; \*\* = two-sided  $P = .008$  (analysis of variance).



observation, the accumulation of doxorubicin, vincristine, and paclitaxel was not decreased in sodium butyrate-treated cells, suggesting that the efflux pumps are not involved in the resistance to doxorubicin, vincristine, and paclitaxel in these cells. Bates et al. (22) reported that the increased levels of P-gp expression in the sodium butyrate-treated cells were not necessarily associated with decreased accumulation of cytotoxic drugs.

Sodium butyrate was reported to inhibit the phosphorylation of P-gp and to diminish the transport of doxorubicin, vinblastine, and dactinomycin by P-gp (22). Although recent mutation analysis suggests that phosphorylation and/or dephosphorylation mechanisms do not play an essential role in the establishment of multidrug resistance mediated by P-gp, phosphorylation may modulate P-gp-mediated multidrug resistance (23). Moreover, P-gp and MRP may have been induced in sodium butyrate-treated SW-620 cells but at a level that was insufficient to confer drug resistance because the expression levels of P-gp and MRP in SW-620 cells treated with sodium butyrate for 2 weeks were less than 2.0% of levels in KB-C2 and C-A120 cells, respectively (data not shown). KB-C2 and C-A120 cells were 57- and 61-fold more resistant to doxorubicin, respectively, than the parental KB-3-1 cells (24,25).

Sodium butyrate-treated cells were reported to be resistant to colchicine but not to doxorubicin or vinblastine (12,22). However, we found that sodium butyrate-treated cells were resistant to doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D. Differences in the duration of sodium butyrate treatment may explain this discrepancy. We treated SW-620 cells with sodium butyrate for 2 weeks before the MTT assay, whereas the cells were treated with sodium butyrate for only 3 days in the previous study (22). In sodium butyrate-treated SW-620 cells, we found that the level of LRP expression at day 14 was considerably higher than the level at day 4.

Most of the doxorubicin was found in the nuclei in control untreated SW-620 cells and in the nuclei of sodium butyrate-treated cells transfected with a LRP-specific ribozyme. However, doxorubicin was mainly located in the cytoplasm and was only slightly detected in the nucleus in sodium butyrate-treated SW-620 cells, which express LRP. In accordance with this observation, decreased nuclear to cytoplasmic drug ratios were reported in multidrug-resistant cells overexpressing LRP

(10,26). The most likely explanation for these findings is that doxorubicin in the nucleus was transported into the cytoplasm by vaults.

Our findings also suggest that vaults have functions other than transport between nucleus and cytoplasm because sodium butyrate-treated SW-620 cells were resistant to vincristine, paclitaxel, and gramicidin D, all of which have non-nuclear targets. Entrapment of drugs in vesicles has been observed in some multidrug-resistant cell lines that overexpress LRP (7,11). These findings suggest that vaults are also involved in the vesicular transport of drugs. The accumulation of vincristine and doxorubicin in sodium butyrate-treated cells was statistically significantly increased compared with untreated cells. This increased accumulation may be attributed to the compartmentalization of the agents in vesicles.

It seems possible that vaults transported doxorubicin in vesicles and inhibited the accumulation of doxorubicin in the nuclei. Therefore, we examined the accumulation of doxorubicin in isolated nuclei. We detected LRP in nuclei isolated from sodium butyrate-treated cells. Other investigators (27) have reported evidence that vaults dwell inside the nucleus. LRP seemed to inhibit the accumulation of doxorubicin in the nuclei because the accumulation defect of doxorubicin was observed in the nuclei from sodium butyrate-treated cells but was reversed by a polyclonal antibody against LRP. Efflux of doxorubicin from LRP-expressing nuclei was enhanced and the efflux was inhibited by anti-LRP polyclonal antibody. These findings suggest that the vaults are involved in the efflux of doxorubicin from nuclei.

The functional vault structure is a multisubunit particle containing LRP, at least three other proteins, and an RNA moiety. Vault formation may be limited by expression of the other vault proteins, the small-vault RNA, and LRP. Because overexpression of LRP alone was not sufficient to confer a multidrug-resistant phenotype (7), sodium butyrate may have induced the other vault proteins and the small-vault RNA and LRP in SW-620 cells. Alternatively, excess levels of components of vaults other than LRP may have been present in the untreated SW-620 cells. In fact, a small-vault RNA was reported to be in considerable excess with respect to major vault protein in small-cell lung cancer and myeloma cells (21).



Expression of LRP, but not P-gp or MRP, was an independent prognostic factor for predicting tumor response to standard chemotherapy and survival in patients with advanced ovarian carcinoma (28). In patients with LRP-positive acute myelogenous leukemia, LRP expression was also associated with an inferior response to induction chemotherapy (29). We are now studying the association of LRP expression with refractoriness to chemotherapy in other kinds of tumors and studying how vaults contribute to the vesicular transport of drugs and to the transport of drugs between the nucleus and cytoplasm.

In conclusion, LRP was induced in sodium butyrate-treated SW-620 cells, and the expression of LRP was involved in the resistance of the cells to doxorubicin, vincristine, etoposide, paclitaxel, and gramicidin D. Vaults have an important role in the transport of doxorubicin between the nucleus and the cytoplasm.

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## NOTES

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