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A Leukotriene Receptor Antagonist, ONO-1078, Modulates Drug Sensitivity and Leukotriene C_4 Efflux in Lung Cancer Cells Expressing Multidrug Resistance Protein

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ONO-1078 is a new class of peptide leukotriene receptor antagonist, and multidrug resistance protein (MRP) is a membrane tranporter of multiple anticancer drugs and endogenous leukotriene C_4 (LTC_4). We investigated the effects of ONO-1078 on drug sensitivity and LTC_4-efflux in MRP-expressing lung cancer cells. Drug sensitivity, intracellular vincristine accumulation, and intracellular and extracellular LTC_4 concentrations were measured with or without ONO-1078. The effect of ONO-1078 on MRP-mediated calcein-efflux was determined by flow cytometry. ONO-1078 (1 to 10 μM) dose-dependently enhanced the sensitivity of NCI-H520 cells to vincristine with the reduced accumulation, and also enhanced the sensitivity to doxorubicin and etoposide. ONO-1078 inhibited both LTC_4- and calcein-efflux from the cells with increased intracellular accumulations. Our findings indicate that ONO-1078 modulates multidrug resistance and inhibits LTC_4-efflux in lung cancer cells, by inhibition of MRP function. © 1998 Academic Press

Peptide leukotrienes (LTs) including LTC_4, LTD_4 and LTE_4 are metabolites of arachidonic acid, and constitute potent mediators of bronchoconstriction, inflammation, and slow reacting substances of anaphylaxis (1). Recently, LTs have attracted interest as targets in the treatment of bronchial asthma, and several LT receptor antagonists (LTRAs) with different structures have been synthesized (2–4). LTRAs bind to multiple LT receptors *in vitro*, resulting in competitive inhibition of LT activities in target cells expressing LT receptors (3, 5). This leads to remarkable inhibition of LTC_4- and LTD_4-induced contractions in airway smooth muscle (3). In fact, certain LTRAs have proven clinically effective in the treatment of bronchial asthma (4, 6–8). In addition, LTRAs have multiple biological effects reducing inflammatory cell recruitment, vascular leakage, mucus production, neuronal dysfunction, and airways remodeling, however, the exact mechanisms remain undetermined (2, 3).

Multidrug resistance protein (MRP) is a 190 kDa transmembrane protein belonging to the ATP-binding cassette (ABC) superfamily which also includes the cystic fibrosis transmembrane conductance regulator and human P-glycoprotein (Pgp) encoded by the MDR1 gene (9). MRP and Pgp function as a drug-efflux pump confering multidrug resistance (MDR) of cancer cells, and they are broadly distributed in normal lung, heart, kidney, skeletal muscle, testis, or peripheral mononuclear cells (9–12). In addition, *in vitro* studies have shown that MRP is an ATP-energy-dependent glutathione-conjugates transporter, by using membrane vesicles of cells (13–15). Regarding LT transport, MRP binds to endogenous LTC_4 of a cytoplasmic glutathione conjugate, and sequentially pump out LTC_4 into the extracellular space (16). This probably leads to LT-inducing inflammation *in vivo* (16).

A number of agents such as verapamil and cyclosporin A are known to reverse Pgp-mediated MDR *in vitro* (17). For MRP-mediated MDR, reversal agents such as genistein, buthionine sulphoximine, and probenecid have been reported (18–20). Recently, MK-571 of an anionic quinoline LTD_4 receptor antagonist was also found to specifically modulate MRP-mediated MDR in MRP-overexpressing human cancer cells, and inihibit LTC_4 transport *in vitro* (21, 22). Thus, the interaction between LTRAs and MRP as a LTC_4 transporter is of great interest. ONO-1078, a new class of peptide leukotriene receptor antagonist with quite different structure from MK-571, is an orally active and

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selective LTRA (4). In this study, we investigated the effects of ONO-1078 on anticancer drug sensitivity and LTC_4-efflux in MRP-expressing human lung cancer cells.

MATERIALS AND METHODS

Chemical agents and cell lines. RPMI 1640 cell culture medium and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, NY). Etoposide (VP-16) and cisplatin (CDDP) were gifts from Nippon Kayaku Co. (Tokyo, Japan), and doxorubicin (DOX) from Kyowa Hakko Kogyo Co. (Tokyo). Vincristine (VCR) was purchased from Sigma Chemical Co. (St. Louis, MO), [^{3}H]-VCR from Amersham Co. (Tokyo), and calcein acetoxy-methyl ester (calcein-AM) from Molecular Probes (Eugene, OR). ONO-1078, 4-oxo-8-[4-(4-phenylbutoxy)-benzoylamino]-2-(tetrazol-5-yl)-4*H*-1-benzopyran hemihydrate, was a gift from Ono Pharmaceutical Co. (Osaka, Japan).

The multidrug-resistant HL60R human promyelocytic leukemia cell line was selected by continuous exposure to DOX, as reported previously (23). The adriamycin-resistant MCF7/ADR human breast cancer cell line was a kind gift from Dr. Kenneth H. Cowan (National Cancer Institute, Bethesda, MD) (24). Unselected human lung cancer cell lines, NCI-H520 (H520) squamous cell carcinoma and NCI-H23 (H23) adenocarcinoma cells, were purchased from the American Type Culture Collection (ATCC; Rockville, MD). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 4% L-glutamine, and 80 mg/L kanamycin sulfate.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA from cultured cells was obtained using the guanidinium isothiocyanate method (25). RT-PCR for the MRP, MDR1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes was performed as previously described (23, 26). HL60R and MCF7/ADR cells were used as positive controls for the MRP and MDR1 genes, respectively.

Drug sensitivity assay. The sensitivity to anticancer drugs was determined using the tetrazolium dye assay (27). Prior to the execution of these assays, we checked the linear relationship between cell number and absorbance at 570 nm, followed by cell growth studies as described previously (28). Cells (2,500 cells/well) were seeded in 96-well plates, at least in triplicate, with varying concentrations of the anticancer drug, with or without ONO-1078. Following incubation for 4 days at 37°C, we added 15 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to each well. The cells were lysed after incubation for 4 hr at 37°C by adding 100 μl of 2-propanol-0.1 N HCl to each well. Absorbance was read on a microplate reader (MR 600; Dynatech Co., Alexandria, VA) with test and reference wavelengths of 570 and 630 nm, respectively. The IC_{50} was defined as the concentration of the anticancer drug that reduced the absorbance in each test by 50%. The modulation effect was expressed as the ratio of the IC_{50} value with and without ONO-1078. Each study was repeated three times.

Intracellular accumulation of [^{3}H]-VCR. Cells (1×10^4 cells/well for H520 cells; 6×10^3 cells/well for H23 cells) were seeded onto 12-well plates and cultured for 72 hr prior to the assay. The cells were incubated at 37°C with 30 nM [^{3}H]-VCR with or without ONO-1078. Aliquots (0.5 ml) of cells were removed at the appropriate times and ice-cold PBS was added to stop accumulation. After two washes with ice-cold PBS, cells were lysed with 1% sodium dodecyl sulfate, and the cell-associated radioactivity was determined using a liquid scintillation system. Each study was performed in triplicate.

Measurement of LTC_4. The intracellular and extracellular LTC_4 concentrations were measured using a LTC_4 specific [^{3}H] assay system (Amersham Co., Bucks, UK) (29). Briefly, 1.5 to 2.5×10^3 cells were incubated without or with ONO-1078 for 4 days. Then, the cell pellet and the supernatant of culture medium were obtained by centrifugation at 4°C, and they were used for the measurement. Each experiment was performed in triplicate.

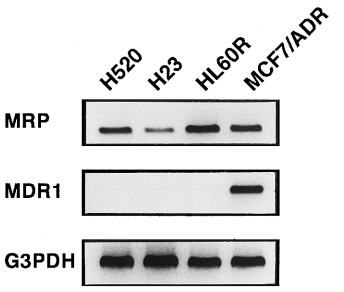

FIG. 1. RT-PCR for the MRP and MDR1 genes. Note the 430 bp MRP gene PCR product present in H520 and H23 cells, while the 308 bp MDR1 gene PCR product is absent. HL60R and MCF7/ADR cells are positive controls for the MRP and MDR1 genes, respectively.

Calcein-AM efflux assay. Calcein-AM is a highly lipophilic and non-fluorescent compound, which rapidly penetrates the plasma cell membrane, and it is transformed into calcein of the intensively fluorescent organic anion by intracellular esterases (30). MRP actively pumps out calcein-AM and fluorescent calcein from the cytoplasm and sequentially reduces an intracellular calcein accumulation after loading MRP-expressing cells with calcein-AM (31, 32). Briefly, the efflux function of MRP in the cells is evaluated by measurement of intracellular fluorescence of calcein using a flow cytometer.

As a functional assay, fluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson Medical Systems, Sharon, MA), equipped with an argon laser. A suspension of log phase cells was incubated in 0.25 μM calcein-AM for 30 min with or without ONO-1078. Cells were washed in ice-cold PBS and kept in the dark until flow cytometric analysis. The fluorescence of 10,000 cells was logarithmically measured at a laser excitation wavelength of 488 nm. The fluorescence of calcein and propidium iodide was collected through a 530 nm band-pass filter. Samples were gated on forward scatter versus side scatter to exclude cell debris and aggregates. The logarithmically amplified signals were converted into absolute values on a linear scale and expressed as relative fluorescence units (FU), from which the mean fluorescence was calculated.

Statistical analysis. Data were expressed as means $\pm$ SD, and differences between groups were tested for statistical significance by the unpaired Student's *t*-test. A $p < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

MRP Expression in Lung Cancer Cells

The H520 and H23 cells expressed MRP mRNA but not MDR1 mRNA, and the density of the MRP gene band in H520 cells was relatively higher than that in H23 cells (Fig. 1). Also in immunostaining with anti-human MRP antibody, the plasma membrane of these cells was positively stained, and the staining intensity was consistent with the result in PCR analysis (data not shown).

Effect of ONO-1078 on Drug Sensitivity

In preliminary experiments, we examined the cellular toxicity of ONO-1078 in the two cell lines used here,

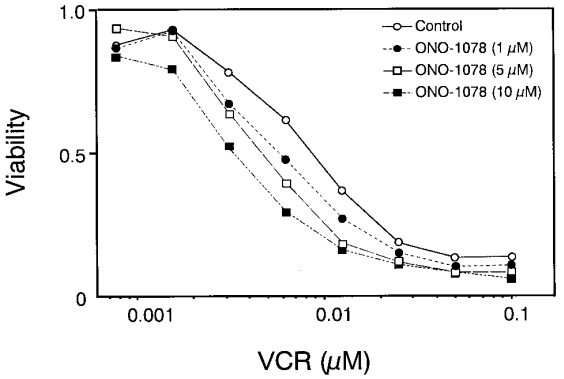

FIG. 2. Modulatory effect of ONO-1078 on the cytotoxicity of VCR. The sensitivity of H520 cells to VCR was determined without (control) and with 1, 5, and 10 μM ONO-1078. Data represent the mean value of triplicate MTT assays in a single experiment, and the SD values were within the dots. *VCR*, vincristine.

and no toxicity was observed within a range of 0.1 to 10 μM ONO-1078 (data not shown). The effect of ONO-1078 on VCR sensitivity of H520 cells was subsequently examined (Fig. 2). ONO-1078 enhanced the sensitivity of H520 cells to VCR, and there were significant differences among the IC_{50} values at 1, 5 and 10 μM ONO-1078 ($p < 0.001$).

Next, we examined the modulation effects of 10 μM ONO-1078 on the sensitivity of H520 to VCR, DOX, VP-16 and CDDP (Fig. 3). ONO-1078 significantly enhanced the sensitivity of H520 cells by approximately 3-fold in VCR, 4-fold in VP-16, and 2-fold in DOX, but had no effect on CDDP which is not a substrate for MRP. The modulation effects on H23 cells were between 1.5- and 2-fold in VCR, VP-16 and DOX, and no effect on CDDP was observed (data not shown).

Effect of ONO-1078 on Intracellular Accumulation of [^{3}H]-VCR

To explore the modulation mechanism of ONO-1078, intracellular accumulation of [^{3}H]-VCR in H520 and H23 cells was examined. The accumulation in H520 cells time-dependently increased in both without and with 10 μM ONO-1078 (Fig. 4); the accumulation at each time interval was significantly higher in the presence, than in the absence, of ONO-1078 ($p < 0.0001$). At 180 min, 10 μM ONO-1078 increased the accumulation by 1.6- and 1.3-fold in H520 and H23 cells, respectively (Fig. 5), compared with each control ($p < 0.003$).

Effect of ONO-1078 on LTC_4 Efflux and Accumulation

In our preliminary experiments, LTC_4 was not detected in the medium only, and LTC_4 concentrations time-dependently increased in the culture medium of both cell lines for 4 days. The mean LTC_4 concentration

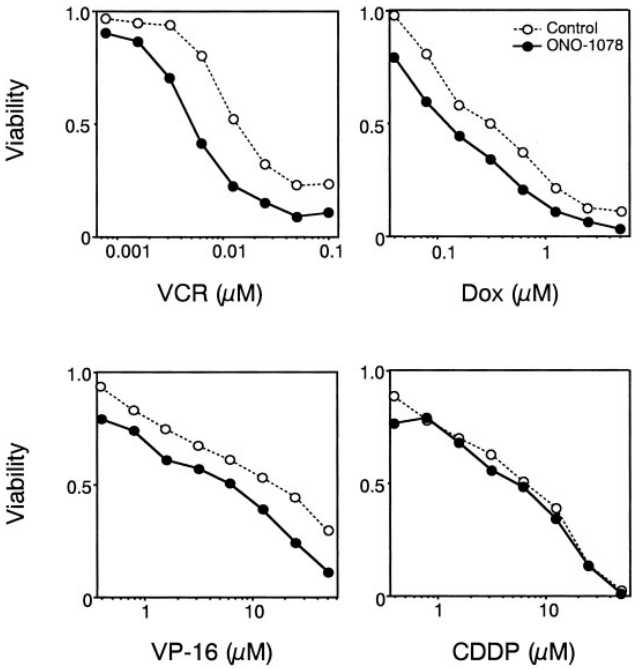

FIG. 3. Modulation effect of ONO-1078 on the cytotoxicity of VCR, DOX, VP-16 and CDDP. The sensitivity of H520 cells to drugs was determined without (control) and with 10 μM ONO-1078. Data represent the mean value of triplicate MTT assays in a single experiment, and the SD values were within the dots. *VCR*, vincristine; *DOX*, doxorubicin; *VP-16*, etoposide; *CDDP*, cisplatin.

in the medium of H520 cells was 31.3 and 22.1 pg/ml in the absence (control) and presence of 10 μM ONO-1078 (Fig. 6), respectively, showing that ONO-1078 produced a 29.4% decrease relative to control ($p < 0.001$). Conversely, the mean concentration of intracellular LTC_4 in H520 cells was 59.3 and 111 pg/protein in the absence and presence of 10 μM ONO-1078 (Fig. 6),

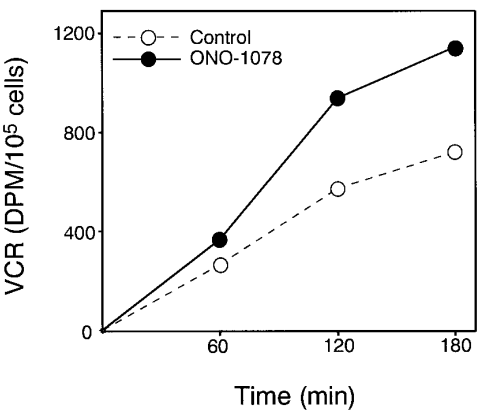

FIG. 4. Effect of ONO-1078 on intracellular accumulation of VCR in H520 cells. Accumulation of [^{3}H]-VCR in H520 cells was measured without (control) and with 10 μM ONO-1078 at each time point. Data represent the mean value of triplicate determinations in a single experiment, and the SD values were within the dots. *VCR*, vincristine.

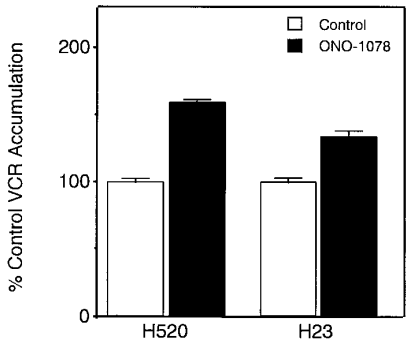

FIG. 5. Effect of ONO-1078 on intracellular accumulation of VCR. Accumulation of [^{3}H]-VCR in H520 and H23 cells was measured 180 min after exposure to [^{3}H]-VCR alone (control) and to [^{3}H]-VCR with 10 μM ONO-1078. Data represent the mean ± SD of triplicate determinations in a single experiment. *VCR*, vincristine.

respectively, indicating a significant difference ($p < 0.001$).

Effect of ONO-1078 on Calcein-AM Efflux

To investigate whether ONO-1078 inhibits MRP function as an efflux pump, a calcein-AM efflux assay was performed. The assay was performed by loading H520 and H23 cells with calcein-AM without or with 10 μM ONO-1078. The mean fluorescence of H520 cells was 8.84 and 17.8 in the absence and presence of ONO-1078, respectively, and that of H23 cells was 5.06 and 8.30, respectively (Fig. 7).

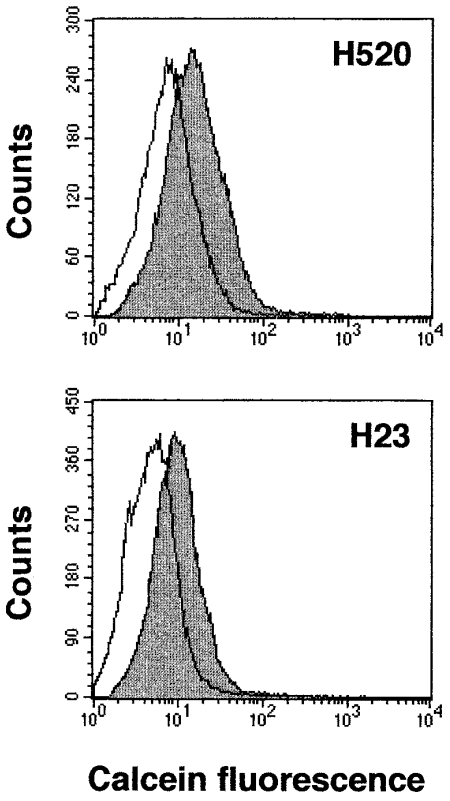

FIG. 7. Effect of ONO-1078 on calcein-AM efflux. Fluorescence of intracellular calcein was determined by flow cytometry without (*open*) and with (*shaded*) 10 μM ONO-1078.

DISCUSSION

This study demonstrated that ONO-1078 of a new class LTRA enhanced the sensitivity of MRP-expressing human lung cancer cells to VCR, DOX, and VP-16. The modulatory effect in VCR was dose-dependent, and it was due to increased intracellular VCR accumulation. Furthermore, we demonstrated that ONO-1078 inhibited MRP-mediated calcein-efflux in these cells. Accordingly, ONO-1078 is thought to specifically inhibit the drug-efflux function of MRP and then enhance the cytotoxicity of VCR, DOX and VP-16 in the cells. These findings indicate that ONO-1078 is an inhibitor of MRP.

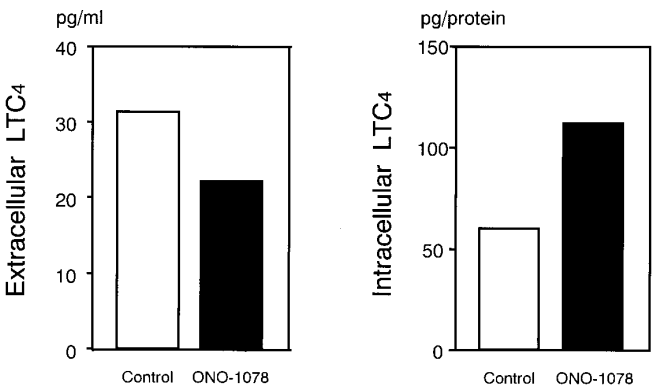

FIG. 6. Effect of ONO-1078 on LTC_4 efflux and intracellular accumulation of H520 cells. The concentration of LTC_4 was measured in the cell culture media (*left*) and whole cell pellet (*right*), after 4-day culture without (control) or with 10 μM ONO-1078. Data represent the mean of triplicate determinations in a single experiment, and the SD values were very small.

In this study, ONO-1078 inhibited the LTC_4 excretion from the MRP-expressing H520 lung cancer cells with the increased intracellular LTC_4 accumulation, though the ONO-1078 concentration used here was higher than those obtained at clinical dosage. Briefly, this means that ONO-1078 inhibits endogenous LTC_4-efflux from the cells rather than LTC_4 synthesis in the cell, also suggesting that ONO-1078 is probably an inhibitor of MRP, as described above. This close association between LTRAs and endogenous LTC_4-efflux is of great interest in LT-inducing inflammation. Recent studies identified human canalicular multispecific organic anion transporter (cMOAT), a novel member of the ABC superfamily, which transports endogenous LTC_4 and confers resistance against VCR, DOX and CDDP (33–35). We also reported that the cMOAT gene was expressed in various human cancer cells including H520 and H23 cells (36). Thus, there is a possibility

that ONO-1078 might inhibit cMOAT-mediated LTC_4 transport in this study. However, ONO-1078 did not enhance the sensitivity of H520 cells to CDDP (Figure 3). Accordingly, we think that ONO-1078 inhibits MRP-mediated LTC_4 transport but probably not cMOAT-mediated transport. On the other hand, considered together with the prior report that MK-571 modulated drug sensitivity of MRP-overexpressing cancer cells (21), it is likely that other types of LTRAs may also inhibit MRP-mediated LTC_4-efflux. If our hypothesis is correct, in the airways of patients with bronchial asthma LTRAs block not only LT receptors on smooth muscle cells but may also inhibit LTC_4-efflux from inflammatory cells such as eosinophils, mast cells and basophils. Namely, certain LTRAs as well as ONO-1078 are thought to have dual activities towards both LT-producing effector cells and LT-receptor-expressing target cells in LT-inducing inflammation.

MRP is distributed in most human tissues including peripheral blood cells (10, 12), where its exact physiological functions remain undetermined (10). In the lung, MRP is mainly localized to the cytoplasm of bronchial epithelium and alveolar macrophages (12), where MRP is speculated to play an important role in inflammation and detoxification, consistent with its function as a glutathione conjugate transporter. Wijnholds and co-workers recently established MRP-deficient mice ($mrp^{-/-}$ mice), and examined LT production, LT-mediated inflammatory response to arachidonic acid, and VP-16 sensitivity in these mice (16). The $mrp^{-/-}$ mice had decreased LTC_4 excretion with increased intracellular LTC_4 accumulation in the bone marrow-derivatived mast cells, a poor response to arachidonic acid, and hypersensitivity to VP-16 (16). These results strongly indicate that physiologically MRP plays an important role in LT-inducing inflammation and drug sensitivity *in vivo*. Thus, as shown in this study of ONO-1078, MRP inhibitors including LTRAs are not only MDR-modulating drugs but also may be drugs against LT-related inflammatory diseases such as bronchial asthma.

To date, many agents with different structures and activities, such as calcium channel blockers, immunosuppressants and protein kinase inhibitors, have been shown to modulate MRP-mediated MDR in cancer cells (10). This suggests that these agents as well as ONO-1078 may inhibit LTC_4-efflux from cells by their functional inhibition of MRP, as described above. Nguyen and co-workers recently reported that probenecid of an MRP inhibitor inhibited LTC_4 efflux from normal murine mast cells lacking MRP (37). It has been speculated that other probenecid-sensitive LTC_4 transporters exist, in addition to MRP, in murine mast cells, though they did not examine murine cMOAT expression (37). At present, ONO-1078 appears not to inhibit cMOAT-mediated LTC_4 transport as the above-mentioned, but it is unknown whether other LTRAs also interact with probenecid-sensitive LTC_4 transporters. However, to develop more effective LTRAs that can inhibit LTC_4-efflux from inflammatory cells should be extremely useful in the treatment of LT-related inflammatory diseases. These issues remain to be investigated in future.

In conclusion, we showed that ONO-1078 of a new class LTRA modulated the sensitivity to anticancer drugs and inhibited LTC_4-efflux in MRP-expressing lung cancer cells by functional inhibition of MRP. Our findings indicate that ONO-1078 not only blocks LT receptors on cells but also inhibits MRP-mediated endogenous LTC_4 efflux from cells.

REFERENCES

1. Samuelsson, B. (1983) *Science* **220,** 568–575.
2. O'Byrne, P. M. (1997) *Chest* **111,** 27S–34S.
3. Hay, D. W. (1997) *Chest* **111,** 35S–45S.
4. Barnes, N. C., de Jong, B., and Miyamoto, T. (1997) *Chest* **111,** 52S–60S.
5. Nakagawa, N., Obata, T., Kobayashi, T., Okada, Y., Nambu, F., Terawaki, T., and Aishita, H. (1992) *Jpn. J. Pharmacol.* **60,** 217–225.
6. Dahlen, B., Margolskee, D. J., Zetterstrom, O., and Dahlen, S. E. (1993) *Thorax* **48,** 1205–1210.
7. Smith, L. J., Geller, S., Ebright, L., Glass, M., and Thyrum, P. T. (1990) *Am. Rev. Respir. Dis.* **141,** 988–992.
8. Taniguchi, Y., Tamura, G., Honma, M., Aizawa, T., Maruyama, N., Shirato, K., and Takishima, T. (1993) *J. Allergy Clin. Immunol.* **92,** 507–512.
9. Cole, S. P. C., Bhardwaj, B., Gerlach, J., Mackie, J., Grant, C., Almquist, K., Stewart, A., Kurz, E., Duncan, A., and Deeley, R. G. (1992) *Science* **258,** 1650–1654.
10. Loe, D. W., Deeley, R. G., and Cole, S. P. C. (1996) *Eur. J. Cancer* **32A,** 945–957.
11. Gottesman, M., and Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427.
12. Flens, M. J., Zaman, G. J., Valk, P., Izquierdo, M. A., Schroeijers, A. B., Scheffer, G. L., Groep, P., Hass, M., Meijer, C. J., and Scheper, R. J. (1996) *Am. J. Pathol.* **148,** 1237–1247.
13. Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) *Cancer Res.* **54,** 4833–4836.
14. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeley, R. G., and Keppler, D. (1994) *J. Biol. Chem.* **269,** 27807–27810.
15. Müller, M., Meijer, C., Zaman, G. J., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G., and Jansen, P. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 13033–13037.
16. Wijnholds, J., Evars, R., van Leusden, M. R., Mol, C. A. A. M., Zaman, G. J. R., Mayer, U., Beijnen, J. H., van der Valk, M., Krimpenfort, P., and Borst, P. (1997) *Nat. Med.* **3,** 1275–1279.
17. Ford, J. M., and Hait, W. N. (1993) *Cytotechnology* **12,** 171–212.
18. Versantvoort, C. H. M., Schuurhuis, G. J., Pinedo, H. M., Eekman, C. A., Kuiper, C. M., Lankelma, J., and Broxterman, H. J. (1993) *Br. J. Cancer* **68,** 939–946.
19. Zaman, G. J. R., Lankelma, J., Van Tellingen, O., Beijnen, J., Dekker, H., Paulusma, C., Oude Elferink, R. P. J., Baas, F., and Borst, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7690–7694.
20. Berger, W., Elbling, L., Hauptmann, E., and Micksche, M. (1997) *Int. J. Cancer* **73,** 84–93.

21. Gekeler, V., Ise, W., Sanders, K. H., Ulrich, W. R., and Beck, J. (1995) *Biochem. Biophys. Res. Commun.* **208,** 345–352.
22. Pulaski, L., Jedlitschky, G., Leier, I., Buchholz, U., and Keppler, D. (1996) *Eur. J. Biochem.* **241,** 644–648.
23. Narasaki, F., Matsuo, I., Ikuno, N., Fukuda, M., Soda, H., and Oka, M. (1996) *Anticancer Res.* **16,** 2079–2082.
24. Fairchild, C., Ivy, S., Kao-Shan, C.-S., Whang-Peng, J., Rose, N., Israel, M., Melara, P., Cowan, K. H., and Goldsmith, M. (1987) *Cancer Res.* **47,** 5141–5148.
25. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
26. Tomonaga, M., Oka, M., Narasaki, F., Fukuda, M., Nakano, R., Takatani, H., Ikeda, K., Terashi, K., Matsuo, I., Soda, H., Cowan, K. H., and Kohno, S. (1996) *Jpn. J. Cancer Res.* **87,** 1263–1270.
27. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
28. Graaf, W. T. A., de Vries, H. E. G. E., Timmer-Bosscha, H., Meersma, G., Mesander, G., Vellanga, E., and Mulder, N. (1994) *Cancer Res.* **54,** 5368–5373.
29. Levine, L., Morgan, R. A., Lewis, R. A., Austen, K. F., Clark, D. A., Marfat, A., and Corey, E. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7692–7696.
30. Hollo, Z., Homolya, L., Davis, C., and Sarkadi, B. (1994) *Biochim. Biophys. Acta* **1191,** 384–388.
31. Feller, N., Kuiper, C. M., Lankelma, J., Ruhdal, J. K., Scheper, R. J., Pinedo, H. M., and Broxterman, H. J. (1995) *Br. J. Cancer* **72,** 543–549.
32. Homolya, L., Hollo, Z., Germann, U. A., Pastan, I., Gottesman, M. M., and Sarkadi, B. (1993) *J. Biol. Chem.* **268,** 21493–21496.
33. Keppler, D., and Kartenbeck, J. (1996) *Prog. Liver Dis.* **14,** 55–67.
34. Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S., and Kuwano, M. (1996) *Cancer Res.* **56,** 4124–4129.
35. Koike, K., Kawabe, T., Tanaka, T., Toh, S., Uchiumi, T., Wada, M., Akiyama, S., Ono, M., and Kuwano, M. (1997) *Cancer Res.* **57,** 5475–5479.
36. Narasaki, F., Oka, M., Nakano, R., Ikeda, K., Fukuda, M., Nakamura, T., Soda, H., Nakagawa, M., Kuwano, M., and Kohno, S. (1997) *Biochem. Biophys. Res. Commun.* **240,** 606–611.
37. Nguyen, T., and Gupta, S. (1997) *J. Immunol.* **158,** 4916–4920.