Cancer Chemother Pharmacol (2006) 58: 348–353
DOI 10.1007/s00280-005-0162-9

ORIGINAL ARTICLE

Seita Matsunaga · Takeshi Asano
Arisa Tsutsuda-Asano · Yoshitaka Fukunaga

# Indomethacin overcomes doxorubicin resistance with inhibiting multi-drug resistance protein 1 (MRP1)

Received: 19 August 2005 / Accepted: 15 November 2005 / Published online: 6 December 2005


**Abstract** Drug resistance continues to be a serious problem in cancer therapy. We investigated whether indomethacin, which inhibited cyclooxygenases, would overcome doxorubicin resistance in K562/ADR leukemia cells. Indomethacin at 10 μM increased the cytotoxicity of doxorubicin, as well as vincristine in K562/ADR. Both multi-drug resistant protein1 (MRP1) and P-glycoprotein were overexpressed in K562/ADR cells when compared with K562 parent cells (K562/P). Expression of MRP1 mRNA and protein, but not P-glycoprotein, was significantly decreased in K562/ADR cells after indomethacin treatment. Indomethacin treatment increased 5(6)-carboxyfluorescein diacetate (CFDA) efflux, as well as decreased accumulation in K562/ADR cells. The activity of the MRP1 promoter decreased after indomethacin treatment in Hela cells. These data strongly suggest that the cyclooxygenase inhibitor, indomethacin, increased the cytotoxicity of doxorubicin with decreasing expression of MRP1 through inhibition of MRP1 promoter activity.

**Keywords** K562 · Doxorubicin-resistant · Multi-drug resistance protein 1 · Indomethacin · Promoter

## Introduction

Drug resistance remains a serious problem in cancer therapy and has been attributed to mechanisms such as overexpression of P-glycoprotein genes in doxorubicin-resistant leukemia cell lines and in leukemia patients [5]. Although several therapeutic approaches, including the use of reversal agents against P-glycoprotein export have attempted to overcome doxorubicin resistance in leukemia patients, the results have been unsatisfactory to date [6]. To analyze the mechanism of doxorubicin drug resistance, we recently investigated the gene expression profile of a doxorubicin-resistant myeloid leukemia cell line by microarray and confirmed cyclooxygenase-1 (Cox-1) overexpression in the doxorubicin-resistant cells [2]. These results prompt us to investigate whether indomethacin, which is an inhibitor of cyclooxygenases, is able to increase the cytotoxicity of doxorubicin in doxorubicin-resistant leukemia cells. In the present study, we found that indomethacin sensitized the doxorubicin-resistant leukemia cells to doxorubicin and vincristine with decreasing expression of multi-drug resistance protein 1 (MRP1), as a result of inhibition of MRP1 promoter activity.

S. Matsunaga · T. Asano (✉) · A. Tsutsuda-Asano · Y. Fukunaga
Department of Pediatrics, Nippon Medical School,
Chiba Hokusoh Hospital, 1715 Kamagari, Inba-mura,
270-1894 Inba-gun, Chiba, Japan
E-mail: VFF13540@nifty.ne.jp
Tel.: +81-476-991111
Fax: +81-476-991925

## Materials and methods

### Reagents and drugs

RPMI 1640 medium, phosphate buffer saline (PBS), foetal calf serum (FCS), and Hanks Balanced Salt Solution (HBSS) were purchased from Invitrogen Inc. (Tokyo, Japan). Adriamycin (doxorubicin), etoposide, vincristine, indomethacin, verapamil, Rhodamine 123, 5(6)-carboxyfluorescein diacetate (CFDA), and proteinase inhibitors were purchased from Wako Pure Chemicals Co Ltd. (Osaka, Japan).

### Cell lines

K562 cells (human erythroleukemia cell line) and Hela cells were obtained from American Type Culture Collection (Manassas, VA. USA). Parent cells (K562/P) were incubated in RPMI 1640 containing 10% FCS (R10 media) under 5% $CO_2$ in a humidified incubator. A doxorubicin-resistant cell line (K562/ADR) was established by stepwise and continuous exposure to doxorubicin using the limiting dilution method [2]. Cell lines

were confirmed free from *mycoplasma* organisms using the MycoFluor$^{TM}$ Mycoplasma detection kit (Molecular Probes, Eugene, OR, USA).

## Cytotoxicity assay and cell treatment with indomethacin

Cytotoxicity was measured by trypan blue dye exclusion assay, as described previously [2]. Briefly, $1\times10^6$ cells were incubated with various concentrations of doxorubicin for 72 h and viable cells were counted after trypan blue staining. Indomethacin treatment was carried out at 10 μM for 72 h [7], and verapamil treatment was carried out at 5 μg/ml for 72 h [10].

## P-glycoprotein expression in leukemia cells by flow cytometric analysis

Cultured under various conditions, $5\times10^5$ cells were incubated with PE-labeled anti-human P-glycoprotein antibody (Immunotech, Marseille, France) for 30 min at room temperature, washed with PBS three times and analyzed by flow cytometry (Coulter, CA, USA).

## RNA extraction and northern blot analysis

RNA extraction was conducted as described previously [1, 2]. Fragment of complimentary DNA (cDNA) synthesis and RT-PCR were performed according to manufacturer's instructions (Takara, Ohtsu). Briefly, total RNA (1 μg) of samples were added to RT-PCR buffer (Takara, Tokyo), 5 mM of $MgCl_2$, 1 mM dNTPs, oligo dT-adaptor primer (final concentration 0.125 μM), RNase inhibitor (0.8 U/μl), AMV reverse transcriptase (0.1 U/μl), AMV-optimized Taq polymerase (0.1 U/μl) to provide a final total volume of 50 μl. RT-PCR conditions were: 30 min 50°C, 2 min 94°C, following 25×30 s/94°C; 30 s/55°C; 60 s/72°C; and 7 min 72°C for MRP1, Cox-1, and *β*-actin and 30 min 50°C, 2 min 94°C, following 25×30 s/94°C; 30 s/55°C; 60 s/72°C; and 7 min 72°C for Cox-2. Primers used were: for MRP1, 5′-CCGTGTACTCCAACGCTGC-3′, 5′-CTGGACCGCTGACGCCGTGAC-3′, yielding a PCR product of 326 bp [13]; Cox-1, 5′-TGTTCGGTGTCCAGTTCCAAT-3′, 5′-CGCAACCGCATTGCCATGGAGT-3′, yielding a PCR product of 80 bp [14]; for Cox-2, 5′-GTTTGCATTCTTTGCCCAGC-3, 5′-CAGGCACCAGACCAAAAGACC-3′, yielding a PCR product of 300 bp [16]; and for *β*-actin, 5′-GTGGGGCGCCCCAGGCACA-3′, 5′-CTCCTTAATGTCACGCACGATTTC-3′, yielding a PCR product of 548 bp [3]. The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining. Northern blot was performed as described previously [1]. cDNAs were labeled with digoxigenin using digoxigenin labeling kit (Roche Molecular Biochemicals, Tokyo), and hybridized with the membranes according to manufacture's instruction. For densitometric analysis, band intensity was assessed using NIH image software (http://www.rsb.info.nih.gov/nih-image/). After band intensity was adjusted by *β*-actin intensity, data were calculated the mean ± standard deviation of at least three experiments. Results were presented as percentage in intensity compared with K562/P cells without indomethacin treatment.

## Protein extraction and western blotting

Protein blots were performed as described before [15]. Briefly, total cell lysates were made by lysing harvested cells in 10 mM KCl/1.5 mM $MgCl_2$/10 mM Tris–HCl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). DNA was sheared by sonication and samples containing 25 μg of protein were fractionated by 7.5% polyacrylamide gel. The proteins were transferred onto nitrocellulose filter (Bio-Rad, Hercules, CA, USA) by electroblotting. Non-specific binding sites were blocked for 2 h with Tris-buffered saline containing 0.2% Tween 20 and 10% non-fat skim milk. Mouse monoclonal anti-MRP1 antibody (1:50 dilution, MRPm6, Kamiya Biochemcal, Seattle, WA, USA) was incubated to the membrane for 1 h at room temperature. Following a second series of washes, a secondary horseradish peroxidase mouse Ig antibody in Tris-buffered saline with 0.1% Tween 20 was added (1:15,000 dilution) and incubated for 60 min at room temperature. After washing with Tris-buffered saline containing 0.2% Tween 20, the blot was visualized by chemiluminescence (ECL) detection using the enhanced ECL system (Amersham Bioscience, Buckinghamshire, UK) according to manufacture's instruction.

## Rhodamine 123, CFDA accumulation and efflux assays

Rhodamine 123 and CFDA were used as transport dyes for P-glycoprotein and MRP1, respectively [4, 11, 12]. Prior to the study, the cells were incubated with or without 10 μM of indomethacin for 72 h. To study uptake, $1\times10^6$ of the treated cells each of K562/P and K562/ADR were incubated with 1 mg/ml of Rhodamine 123 or 2 μM CFDA for 30 min. For accumulation assay, the cells were harvested at 0, 10, and 30 min. The cells were washed with ice-cold PBS(−) twice and then suspended with 1 ml of PBS and the fluorescence intensity was measured. For efflux assay, the tumor cells were further washed with ice-cold PBS(−) twice, then incubated in drug-free medium for 120 min. At each time point, $1\times10^6$ cells were removed and washed twice with 5 ml of ice-cold PBS(−) and then suspended with 1 ml of PBS and the fluorescence intensity was measured. Fluorescence intensity of the samples was determined with a fluorescence spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Tokyo, Japan) at

excitation and emission wavelengths of 490 and 550 nm. For accumulation assay, the fluorescence arbitrary units were calculated with subtraction of the value at time 0. For efflux assay, % retain was calculated the fluorescence arbitrary units at 30, 60, 90, and 120 min after removal of fluorescence dye subtracted from the fluorescence arbitrary units from the cells incubated without fluorescence dye (auto-fluorescence) divided by the fluorescence arbitrary units at 0 min after removal of fluorescence dye subtracted from the auto-fluorescence.

### β-galactosidase assay for MRP1 promoter activity

The promoter region of the MRP1 gene [8] was amplified using primer set 5′-ATCAGGCTGCTCACGGGTTTG-3′ (−612 to −591), and 5′-CCGCAACGCCGCCTGGT-3′ (+42 to +22; yielding a 655-bp product) from K562/ADR cells. Samples were added to PCR buffer (Takara, Tokyo), 2 mM of $MgCl_2$, 0.25 mM dNTPs, 1 units Taq polymerase, and provided a final total volume of 50 μl. PCR conditions were: 45×45 s/94°C; 45 s/60°C; 60 s/72°C; and 7 min 72°C. The PCR product was cloned into a pBlue-TOPO vector (Invitrogen). Correctly oriented cloned genes in the pBlue-TOPO vector were confirmed by *Sma* I digestion (correct orientation showed 4,462 and 3,991 bp bands) and PCR using primer sets of the T7 promoter *LacZ* gene (PCR conditions were: 45×45 s/94°C; 45 s/58°C; 60 s/72°C, and 7 min 72°C) and the inserted gene. Reporter genes (20 μg) were transfected into $2\times10^6$ of Hela cells cultured under various conditions using Lipofect AMINE (Invitrogen) for 48 h. The cells were lysed using freeze and thaw cycle and the extracted protein were assayed using a β-gal assay kit (Invitrogen) according to the manufacturer's instructions. Protein concentration was measured using a Bradford protein assay kit (BioRad, Tokyo, Japan) and β-gal strength was adjusted with protein concentration.

## Results

### Cytotoxicity in doxorubicin resistant leukemia cells

As shown in Table 1, the doxorubicin-resistant cell line (K562/ADR) was strongly resistant to doxorubicin and vincristine, but not to etoposide, when compared with the parent cell line (K562/P). Indomethacin treatment significantly increased the cytotoxicity of doxorubicin and vincristine in K562/ADR cells ($p < 0.05$, Table 1).

**Table 1** $IC_{50}$ in K562 parent cells and resistant cells

| | Doxorubicin (nM) | Vincristine (nM) | Etoposide (nM) |
|---|---|---|---|
| K562/P | 20 ± 3 | 2.0 ± 2.1 | 10 ± 4 |
| K562/P with indomethacin | 18 ± 3 | 1.8 ± 2.0 | 9 ± 5 |
| K562/ADR | 250 ± 20* | 20.2 ± 8.1* | 15 ± 10 |
| K562/ADR with indomethacin | 50 ± 50** | 5 ± 3** | n.d. |

Leukemia cells were incubated with various doses of anti-cancer drugs for 72 h, and cytotoxicity was evaluated by trypan blue dye exclusion. Data are average ± SD from three experiments
*$p < 0.05$; resistant cells versus parent cells
**$p < 0.05$; Cytotoxicity with indomethacin treatment versus cytotoxicity without indomethacin treatment

### RT-PCR analysis of cyclooxygenase RNA expression in K562/ADR cells after indomethacin treatment

We then examined whether indomethacin treatment decreased expression of cyclooxygenases in K562/ADR cells. Cox-1 expression was increased in K562/ADR cells when compared with K562/P cells. Indomethacin treatment decreased Cox-1 and decreased Cox-2 expressions in K562/ADR cells, but had little effect in K562/P cells (data not shown).

### P-glycoprotein expression in K562/P and ADR cells after indomethacin treatment

K562/ADR cells showed an increase in P-glycoprotein expression on the cell surface when compared with K562/P cells. Indomethacin treatment did not affect expression of P-glycoprotein in K562/ADR cells or in K562/P cells when compared with untreated cells (data not shown).

### MRP1 RNA and protein expression in K562/ADR cells was decreased after indomethacin treatment

We next investigated MRP1 expression in K562/P and K562/ADR cells. As shown in Fig. 1, MRP1 expression was significantly increased in K562/ADR cells ($p < 0.05$). Indomethacin treatment significantly decreased expression of MRP1 in K562/ADR cells ($p < 0.05$).

### Accumulation and efflux of Rhodamine 123 in K562 cells after indomethacin treatment

We examined the transport activity of P-glycoprotein in K562/P and K562/ADR cells. Decreased accumulation and increased efflux of Rhodamine 123, which is transported by P-glycoprotein efflux pump, were observed in K562/ADR cells when compared with K562/P cells ($p < 0.05$). Indomethacin treatment showed slight increase Rhodamine 123 concentration in K562/ADR cells, but not significant. Indomethacine did not affect accumulation and efflux of Rhodamine 123 in K562/P cells when compared with non-treated cells (data not shown). These results were similar to murine and human cell lines expressing MRP1 [4]. However, verapamil treatment significantly increased accumulation and decreased efflux of Rhodamine 123 in K562/ADR cells (data not shown).

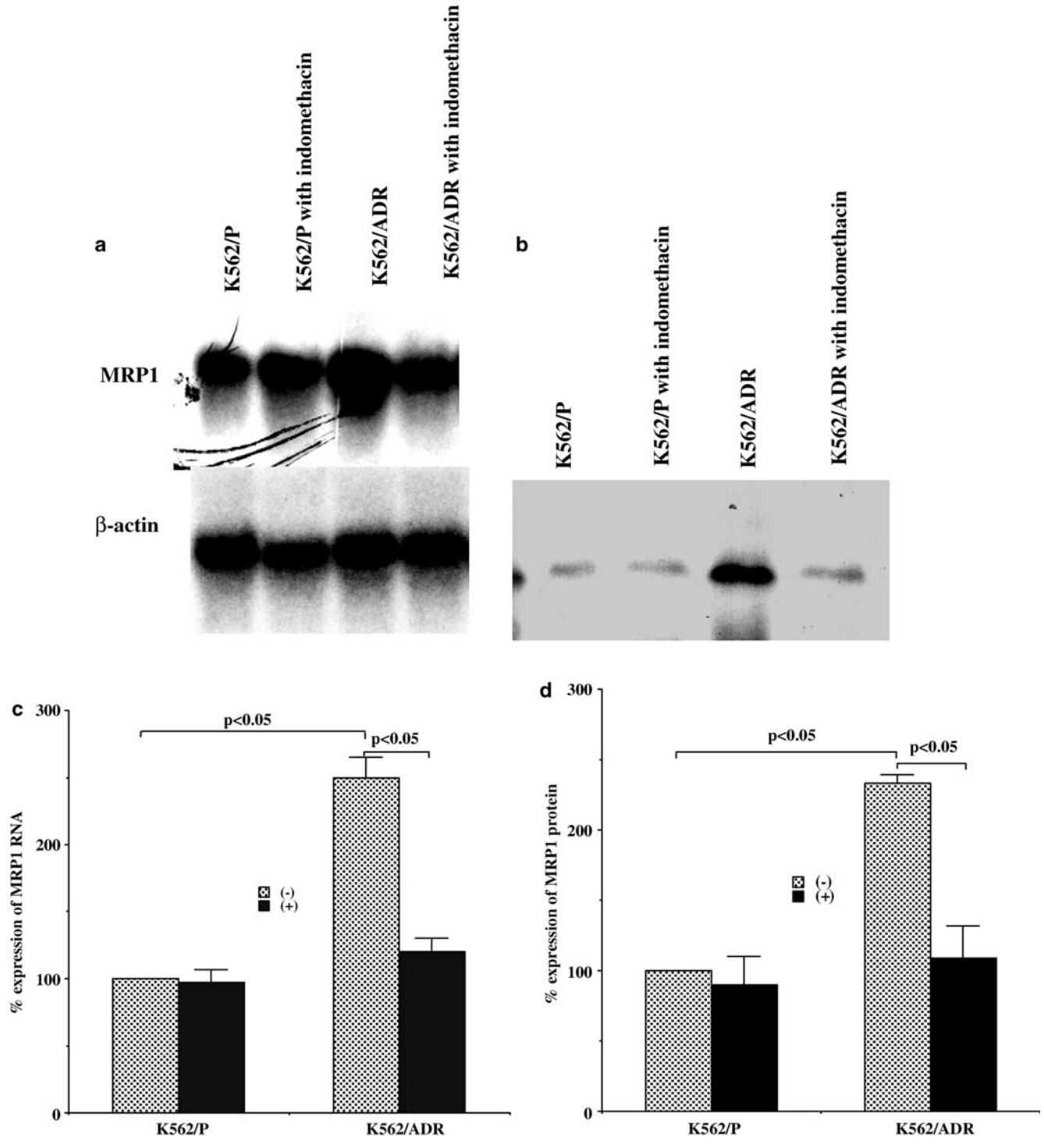


**Fig. 1** MRP1 mRNA (**a**), and protein (**b**) expression in K562/P, and K562/ADR with or without indomethacin treatment Increased expression of MRP1 was observed in K562/ADR cells when compared with K562/P cells. Indomethacin treatment decreased expression of MRP1 in K562/ADR cells. Representative data from five independent experiments. Densitometric analysis of MRP1 mRNA (**c**), and protein (**d**) expression in K562/P, and K562/ADR with or without indomethacin treatment. Significant increased expression of MRP1 was observed in K562/ADR cells when compared with K562/P cells ($p < 0.05$). Indomethacin treatment significantly decreased expression of MRP1 in K562/ADR cells ($p < 0.05$). For densitometyric analysis, band intensity was assessed using NIH image software. After band intensity was adjusted by $\beta$-actin intensity, data were calculated the mean ± SD of five independent experiments. Results were presented as percentage in intensity compared with K562/P cells without indomethacin treatment. (−) represents without indomethacin treatment and (+) with indomethacin treatment

Accumulation and efflux of CFDA in K562 cells after indomethacin treatment

Accumulation of CFDA, which is a representative chemical transported by MRP1, was significantly decreased in K562/ADR cells when compared with K562/P cells (Fig. 2, $p < 0.05$), and efflux was also significantly increased in K562/ADR cells (Fig. 2, $p < 0.05$). Indomethacin treatment significantly decreased efflux of CFDA in K562/ADR cells ($p < 0.05$) and intracellular concentration of CFDA was increased in indomethacin-treated K562/ADR cells when compared with non-treated K562/ADR cells (Fig. 2, $p < 0.05$).

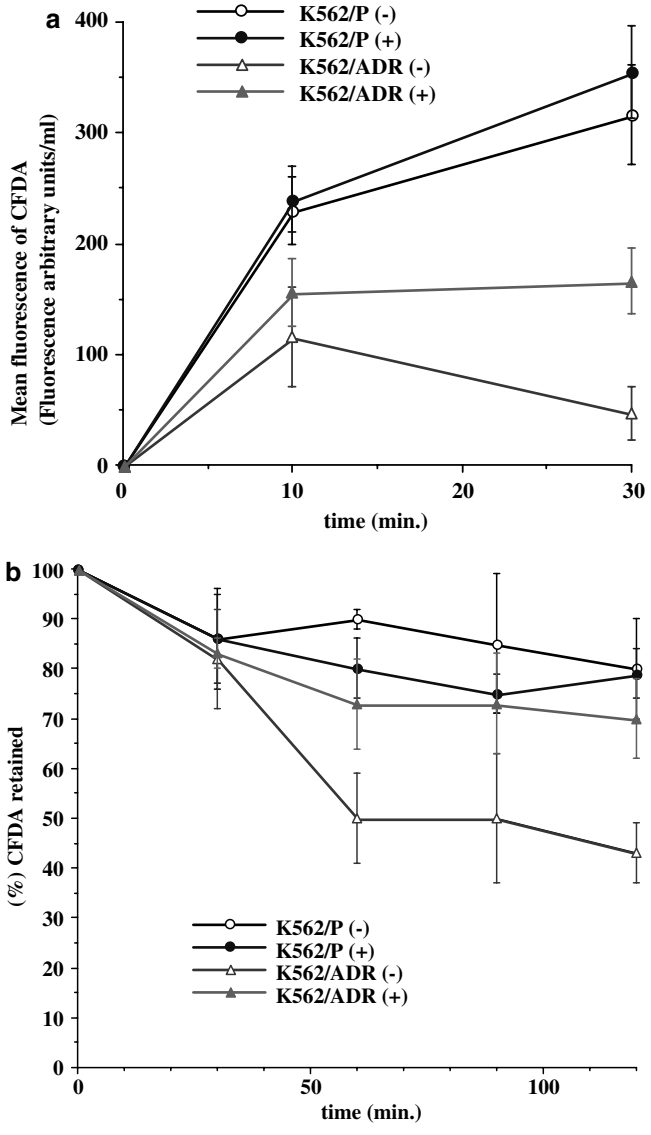


**Fig. 2** **a** CFDA accumulation in K562/Parent and K562/ADR cells after indomethacin treatment. CFDA accumulation was significantly decreased in K562/ADR cells when compared with K562/P cells ($p < 0.05$). Accumulation of CFDA was significantly increased in indomethacin-treated K562/ADR cells when compared with untreated K562/ADR cells. Data are average ± SD from six independent experiments. (−) represents without indomethacin treatment and (+) with indomethacin treatment. **b** CFDA efflux in K562/Parent and K562/ADR cells after indomethacin treatment. CFDA efflux was significantly increased in K562/ADR cells compared to K562/P cells ($p < 0.05$). Indomethacin treatment significantly decreased efflux of CFDA in K562/ADR cells ($p < 0.05$) and consequently inctracellular concentration of CFDA was increased in indomethacin-treated K562/ADR cells when compared with untreated K562/ADR cells. Data are average ± SD from six independent experiments. (−) represents without indomethacin treatment and (+) with indomethacin treatment

MRP1 promoter activity after indomethacin treatment

To investigate the mechanism of MRP1 RNA alternation by indomethacin, we performed promoter assay for MRP1 gene, which encompass from −612 to +42, from K562/ADR cells. Indomethacin treatment significantly decreased the activity of the MRP1 promoter in Hela cells ($p < 0.05$, Fig. 3).

## Discussion

Drug resistance is a serious problem in cancer therapy. Using indomethacin, a cyclooxygenase inhibitor, we were able to overcome doxorubicin resistance in K562/ADM cells with decreasing expression of MRP1, as well as decreasing the export functions of MRP1, but not p-glycoprotein. There have been numerous clinical

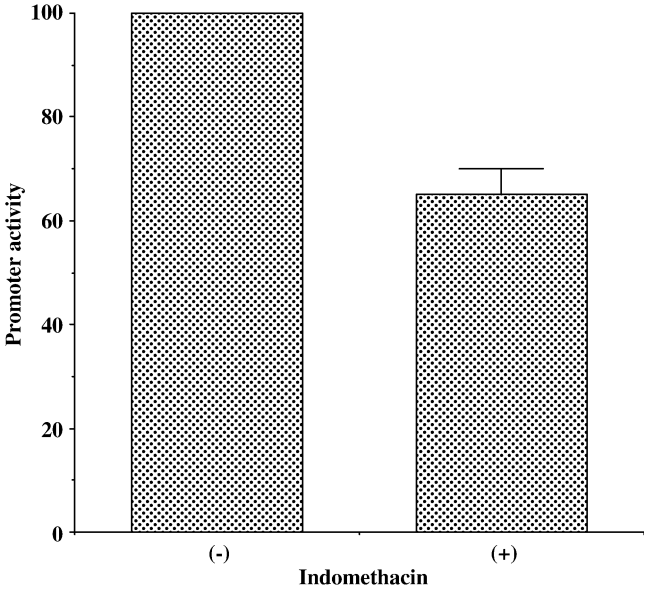


**Fig. 3** MRP1 promoter activity in Hela cells with or without indomethacin treatment. The MPR1 promoter isolated from K562/ADR cells was cloned into a pBlue-TOPO vector. Expression vectors were then transfected into Hela cells with or without indomethacin treatment, and β-galactosidase activity was measured and normalized for protein concentration described in Materials and methods. Indomethacin treatment significantly decreased promoter activities ($p < 0.05$). Results were presented as percentage in intensity compared with Hela cells without indomethacin treatment. Data are average ± SD from three independent experiments

attempts to overcome resistance related to P-glycoprotein [6], but the results have been unsatisfactory to date. In refractory cases, drug-resistant leukemia often exhibits dual expression of P-glycoprotein and MRP1 [9]. Combination treatment of verapamil and indomethacin in K562/ADR cells, which overexpressed in P-glycoprotein and MRP1, significantly sensitized the cell to doxorubicin and vincristine when compared with treatment of verapamil or indomethacin alone [Asano T, unpublished data]. We believe that simultaneous blocking of both P-glycoprotein and MRP1 activity is necessary to overcome drug resistance to doxorubicin.

The major promoter activity of MRP1 resides in a highly GC-rich region of −91 to +103. It was also observed that the activity of the MRP1 promoter could be modulated both by positive and negative regulatory elements in −92 to −411 [8]. It is of interest that the region contains the sequence AACCTCTCT, which is characteristic of the NE-1-negative element found in a number of different genes [17]. The possibility, therefore, exists in a coordinated control of MRP1 gene expression through the use of NE-1 (−380 to −370) and PRE (−454 to −449). Thus, these two elements residing in a tandem array could function as molecular switches turning MRP1 on or off during certain developmental processes. Decreased MRP1 promoter activity by indomethacin treatment in our experiments indicated that those elements might contribute decreased activity.

In conclusion, we found that indomethacin was a novel sensitizer in doxorubicin resistant leukemia cells, with decreased export pump function. As indomethacin is available in human as an anti-inflammatory drug and 10 μM of indomethacin, which we used, is tolerate dose in human, and it might be worthy for clinical use in refractory cases of leukemia.

**Acknowledgements** This work was supported by Grant-in-Aid for Scientific Research C (2) from the Ministry of Education, Culture, Sports, Science, and Technology (13670848).

## References

1. Asano T, An T, Mayes J, Zwelling LA, Kleinerman ES (1996) Transfection of human topoisomerase IIα gene increase in sensitivity followed by down-regulation of the endogenous gene. Biochem J 319:307–313
2. Asano T, Zhi C-L, Hayakawa J, Fukunaga Y (2003) Analysis of resistance-related gene expression in doxorubicin resistant leukemia cell line by DNA microarray. Jpn J Pediatr Hematol 17:348–353
3. Brenner CA, Tam AW, Nelson PA, Engleman EG, Suzuki N, Fry KE, Larrick JW (1989) Message amplification phenotyping (MAPPing): a technique to simultaneously measure multiple mRNAs from small numbers of cells. Bio Tech 7:1096–1103
4. Draper MP, Martell RL, Levy SB (1997) Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell line overexpressing MRP, but not P-glycoprotein. Br J Cancer 75:810–815
5. Kartner N, Riordan JR, Ling V (1985) Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science (Washington DC) 316:820–823
6. Kolk DM van der, Vries EGE de, Muller M, Vellenga E (2002) The role of drug efflux pumps in acute myeloid leukemia. Leuk Lymphoma 43:685–701
7. Kundu N, Fulton AM (2002) Selective cyclooxygenase (Cox)-1 or Cox-2 inhibitors control metastatic disease in a murine model of breast cancer. Cancer Res 62:2343–2346
8. Kurz EU, Cole SP, Deeley RG (2001) Identification of DNA-protein interactions in 5′ flanking and 5′ untranslated regions of the human multidrug resistance protein (MRP1) gene evaluation of a putative antioxidant response element/AP-1 binding site. Biochem Biophys Res Commun 285:981–990
9. Legrand O, Simonin G, Beauchamp-Nicoud A, Zittoun R, Marie J-P (1999) Simultaneous activity of MRP1 and Pgp is correlated with in vitro resistance to daunorubicin and with in vivo resistance in adult acute myeloid leukemia. Blood 94:1045–1056
10. Mahon F-X, Belloc F, Legarde V, Chollet C, Moreau-Gaudy F, Reiffers J, Goldman JM, Melo JV (2003) MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 101:2368–2373
11. Meaden ER, Hoggard PG, Khoo SH, Back DJ (2002) Determination of P-gp and MRP1 expression and function in peripheral blood mononuclear cells in vivo. J Immunol Method 262:159–165
12. Payen L, Courtois A, Campion J-P, Guillouzo A, Fardel O (2000) Characterization and inhibition by a wide range of xenobiotics of organic anion excretion by primary human hepatocytes. Biochem Pharmacol 60:1967–1975
13. Roller A, Baehr O, Streffer J, Winter S, Heneka M, Deininger M, Meyermann R, Naumann U, Gulbins E, Weller M (1999) Selective potentiation of drug cytotoxicity by NSAID in human glioma cells: the role of Cox-1 and MRP. Biochem Biophys Res Commun 259:600–605
14. Sales KJ, Katz AA, Howard B, Soeter RP, Millar RP, Jabbour HN, Soeters RP (2002) Cyclooxygenase-1 is up-regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin E receptors, and angiogenic factors by cyclooxygenase-1. Cancer Res 62:424–432
15. Zaman GJR, Flens MJ, van Leusden MR, de Haas M, Muller HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ, Borst P (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proc Natl Acad Sci USA 91:8822–8826
16. Zhi H, Zhang J, Hu G, Lu J, Wang X, Zhou C, Wu M, Liu Z (2003) The deregulation of arachidonic acid metabolism-related genes in human esophageal squamous cell carcinoma. Int J Cancer 106:327–333
17. Zhu Q, Center MS (1994) Cloning and sequence analysis of the promoter region of the MRP gene of HL60 cells isolated for resistance to adriamycin. Cancer Res 54:4488–4492