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Journal of Cellular Biochemistry 108:181–194 (2009)

Journal of Cellular Biochemistry

Celecoxib Enhanced the Sensitivity of Cancer Cells to Anticancer Drugs by Inhibition of the Expression of P-Glycoprotein Through a COX-2-Independent Manner

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ABSTRACT

The P-glycoprotein (p170, P-gp) encoded by human *MDR1* gene functions as a pump to extrude anticancer drugs from cancer cells. Over-expression of p170 is closely related to primary and induced drug resistance phenotype of tumor cells. Recent studies have demonstrated that expression of cyclooxygenase-2 (COX-2) is positively correlated with the p170 level, suggesting a potential of COX-2 specific inhibitors in regulation of cytotoxicity of anticancer agents. Celecoxib is one of the specific inhibitors of COX-2 and has been widely used in clinic. However, its function in the response of cancer cells to anticancer drugs and the related mechanism are still waiting to be investigated. To explore the correlation of celecoxib and the p170-mediated drug resistance, the role of celecoxib in drug response of cancer cells was analyzed with flow cytometry, high performance liquid chromatography (HPLC), and colony formation experiments. Celecoxib (50 μM) was found to significantly enhance the sensitivity of MCF-7 and JAR/VP16 cells to tamoxifen and etoposide, respectively, by inhibition of p170 expression and increase in intracellular accumulation of the drugs. However, celecoxib did not affect pump function of p170. Enzyme activity and methylation analyses demonstrated that the inhibitory effect of celecoxib on p170 was independent on COX-2 but closely related to hypermethylation of *MDR1* gene promoter. Our study suggested that celecoxib was a potential agent for enhancement of the sensitivity of cancer cells to anticancer drugs. It also provided a links between epigenetic change of *MDR1* and drug response of cancer cells. J. Cell. Biochem. 108: 181–194, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: P-GLYCOPROTEIN; CELECOXIB; CYCLOOXYGANES-2; DRUG RESISTANCE; DNA METHYLATION

Over-expression of ATP-binding cassette super-family of transporters, which function as pumps to extrude anticancer drugs from cancer cells, is closely related to drug resistance phenotype. Among the ATP-binding cassette transporters that frequently over-expressed in drug-resistant cancer cells are the multidrug resistance-associated protein 1 (MRP1) and P-glycoprotein (p170); the later is encoded by human *MDR1* (*ABCB1*) gene [Riordan et al., 1985]. A variety of anticancer drugs, including anthracene nucleus, vinblastine, daunorubicin, and epipodophyllotoxin, are the substrates of p170. Increased expression of p170 could be detected not only in post chemotherapy tumors, but also in tumor cells that did not treated by any anticancer drugs [Marie et al., 1991; Ludwig et al., 2006], suggesting over-expression of p170 is related to both induced and primary chemo-resistance phenotype of tumor cells.

Cyclooxygenase-2 (COX-2) is the key enzyme in the conversion of arachidonic acid to prostaglandins and is involved in tumor onset

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30772490, 30870978; Grant sponsor: Special Funds for Major State Basic Research Program of China (973 Program); Grant number: 2006CB503908; Grant sponsor: Basic Research Programme of College Natural Science Foundation from Jiang Su Province; Grant number: 06KJB310068.

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Received 31 October 2008; Accepted 11 May 2009 • DOI 10.1002/jcb.22239 • © 2009 Wiley-Liss, Inc.
Published online 26 June 2009 in Wiley InterScience (www.interscience.wiley.com).

and progression [Muller-Decker et al., 2002; Chang et al., 2004]. Additionally, COX-2 has been found to mediate drug resistance through up-regulation of *MDR1* transcription [Sorokin, 2004; Miller et al., 2006]. A close association between COX-2 and drug resistance phenotype provide a rationale to overcome or prevent drug resistance by COX-2 inhibitors [Ratnasinghe et al., 2001; Fantappie et al., 2002].

Selective inhibitors of COX-2 reduce the formation, growth, and metastases of tumor cells in vitro [Kawamori et al., 1998; Harris et al., 2000; Liu et al., 2000] and decrease the number of intestinal tumors in familial adenomatous polyposis (FAP) patients [Steinbach et al., 2000]. These anticancer effects can be either COX-2 dependent or independent [Grosch et al., 2006]. Celecoxib is one of the COX-2 specific inhibitors and has been widely used in clinic. It inhibits cancer cell proliferation by inhibition of COX-2 activity [Leahy et al., 2002; Sinicrope et al., 2004]. Additionally, it possesses the ability to inhibit NF-κB, AP-1, and Akt activation and causes cell cycle arrest, which are not common in other nonsteroidal anti-inflammatory drugs (NSAIDs) [Grosch et al., 2001; Tegeder et al., 2001; Arico et al., 2002; Lai et al., 2003]. Celecoxib has been approved to be used in chemopreventive treatment of FAP by FDA [Steinbach et al., 2000] because of its proved anticancer effects and the lowest level of adverse cardiovascular events among all COX-2 specific inhibitors [Kimmel et al., 2005; Ulrich et al., 2006]. Its application to chemotherapy of lung, prostate, and breast cancers has also been widely investigated in clinic [Dang et al., 2004; Csiki et al., 2005; Reckamp et al., 2006; Smith et al., 2006]. Therefore, celecoxib is a good candidate for investigation of the role of COX-2 inhibitors in drug resistance. Recently, several reports regarding correlation of celecoxib with the regulation of multidrug resistance (MDR) phenotype have been reported. In human lung cancer cells, celecoxib was reported to not only down-regulate MRP1 expression in a COX-independent manner but also functionally block p170 [Awara et al., 2004; Kang et al., 2005]. Other study, however, showed celecoxib could not substantially affect the p170-mediated drug efflux [de Vries et al., 2008]. Thus, the correlation of the effect of celecoxib on p170-mediated drug-resistance phenotype and related mechanism are still waiting to be elucidated.

In this study, we first investigated whether celecoxib suppressed p170-mediated resistance of cancer cells to anticancer drugs. The effects of celecoxib on expression level and pump function of p170 were then investigated using flow cytometry, MTT, plate colony formation, and HPLC in MCF-7 and JAR/VP16 cells, respectively. Our data indicated that celecoxib significantly enhanced the sensitivity of malignant cells to tamoxifen and etoposide (VP-16) by down-regulation of p170 expression. Further study revealed that the inhibitory effect of celecoxib on p170 was independent of COX-2 and mediated by hypermethylation of *MDR1* promoter.

MATERIALS AND METHODS

CELL LINES AND CULTURES

Human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC). Human choriocarcinoma cell line (JAR/VP16) that exhibited resistant to etoposide (VP-16) was established in our laboratory previously. MCF-7 cells were maintained in phenol red-free DMEM. VP-16 resistant JAR/VP16 cells were maintained in RPMI-1640 medium. The media were supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells were cultured under the condition of a humidified atmosphere with 5% CO_2 at 37°C.

REAGENTS AND ANTIBODIES

Celecoxib was purchased from Scenery Chemical Industry Co., Ltd (China). VP-16 was purchased from Jiangsu Hengrui Medicine Co., Ltd (China). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (for MTT assay) was purchased from Nanjing Keygen Biotech. Co., Ltd (China). Tamoxifen and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO). Phycoerythrin (PE)-labeled monoclonal antibody to human p170 and IgG1 for flow cytometry were obtained from Beckman Coulter, Inc. (USA) Rabbit anti-COX-2 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). HRP (horseradish peroxidase)-conjugated anti-rabbit IgG1 was obtained from Amersham Pharmacia Biotech. (Piscataway, NJ). Mouse monoclonal (C494) to p170 (ab3365) was from Abcam (Abcam plc. 332 Cambridge Science Park, Cambridge, UK). Arachidonic acid was purchased from Blood Research Institute of Suzhou University (China). Elite HPLC column (250 mm × 4.6 mm, Hypersil ODS2 5 μm) was purchased from Dalian Elite Analytical Instruments Co., Ltd (China). *MDR1* and negative control siRNA were purchased from Applied Biosystems (USA). PGE_2 enzyme linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc. (USA). 5-Aza-2′-deoxycytidine (5-aza-dC), a DNA demethylation agent, was purchased from Sigma (USA).

CELL PROLIFERATION ASSAY

Cells were seeded at a density of 5×10^3 per well in 96-well plates in growth media containing 10% FBS. On the following day, the media were changed and cells were treated with vehicle or anticancer agents and celecoxib. After 72 h of incubation, cell proliferation was evaluated by MTT assay. Cells were incubated with MTT reagent at the final concentration of 0.5 mg/ml. After 4 h of incubation, the medium was aspirated, and the cells were dissolved in DMSO. Optical density was read in an ELISA reader at 490 nm. The experiments were repeated three times, independently. Statistical analysis of the data was performed by one-way ANOVA using SPSS 11.5 software, and statistical significance accepted at $P < 0.05$.

PLATE COLONY-FORMING ASSAY

MCF-7 and JAR/VP16 cells were plated at 1×10^3 cells per well in 6-well plates, followed by treatment of anticancer agents (tamoxifen for MCF-7 and VP-16 for JAR/VP16) plus or minus celecoxib (50 μM) treatment for 72 h. The cells were allowed to grow for another 7 days. The culture medium was replaced with a fresh medium without treatment every other day. Colonies were stained with crystal violet staining solution (1% crystal violet, 5% formaldehyde, and 70% ethanol) on the 11th day of culture. Digital images of stained colonies were captured. The experiments were repeated three times, independently. Statistical analysis of the data was performed by one-way ANOVA using SPSS 11.5 software, and statistical significance accepted at $P < 0.05$.

DETECTION OF P170 EXPRESSION WITH FLOW CYTOMETRY

MCF-7 and JAR/VP16 cells were plated in duplicate at 2×10^5 per 35-mm dish. On the following day, MCF-7 cells were treated with tamoxifen plus or minus celecoxib and JAR/VP16 cells were treated with VP-16 plus or minus celecoxib for 72 h. After treatment, cells were washed gently for three times with cold phosphate buffered solution (PBS) and collected by trypsin digestion and centrifugation. The cells were then fixed with 75% ethanol overnight. The following morning, the cells were washed for another three times with cold PBS and labeled by p170 specific antibody for 30 min at room temperature. PE-labeled IgG1 was used as a negative control. Flow cytometer (FACSC alibur, BD Biosciences, USA) was used to detect p170 expression. Data acquisition and analysis were performed on 10,000 cells with the Cell Quest software (BD Biosciences).

DOWN-REGULATION OF p170 BY siRNA

MCF-7 and JAR/VP16 cells were plated in duplicate at 2×10^5 per 35-mm dish. The following day, cells were exposed to 50 nM of *MDR1* (*ABCB1*) siRNA or negative control siRNA for 5 h. After washed by PBS for three times, cells were maintained in phenol red-free medium supplemented with 1% FBS. The cells were then cultured under the condition of humidified atmosphere with 5% CO_2 at 37°C. After 48 h culture, cells were collected by trypsin digestion and centrifugation. The cells were then fixed with 75% ethanol overnight and p170 expression were investigated by flow cytometry.

MEASUREMENT OF TAMOXIFEN CONCENTRATION IN MCF-7 CELLS BY HPLC

MCF-7 cells were plated at the density of 2×10^6 cells per 10 cm dish. On the following day, the cells were treated with celecoxib (50 μM) or 0.1% DMSO for 72 h. After treatment, the supernatant was removed and cells were cultured with fresh medium containing tamoxifen (10 μM) for 2 h at 37°C. Then the cells were washed three times with cold PBS, and collected by trypsin digestion and centrifugation, then resuspended in 500 μl PBS. Cells were collected in 2 ml Eppendorf (EP) tubes and lysed by sonification. After centrifugation for 10 min at 10,000*g*, the supernatant (10 μl) was used to measure the protein concentration. Then, 20% perchloric acid (50 μl per 500 μl PBS) was added to denature the protein. Diethyl ether (1 ml) was added to each EP tube and mixed by vortex for 1 min. After centrifugation for 10 min at 10,000*g*, the organic phase (500 μl) was collected and evaporated to dryness. The dried residue was reconstituted with 500 μl of the mobile phase (93% methanol, 7% water, and 0.18% triethylamine) and used for HPLC.

An aliquot (about 20 μl) of the reconstituted residue was injected into a HPLC system. Samples were analyzed using a C-18 reverse-phase column and eluted isocratically with a mobile phase of water and triethylamine in methanol (HPLC grade). The flow rate was set at 0.5 ml/min and column temperature was 40°C. Tamoxifen was kept in the column for about 11 min and detected at 238 nm. Linearity was measured through a concentration range of 25–3,000 ng, including the sample concentration range, with a correlation coefficient of greater than 0.999. The tamoxifen concentration was calculated based on peak area and normalized by total cell protein amount.

DETECTION OF RHODAMINE123 CONTENT WITH FLOW CYTOMETRY

The p170-dependent transport activity was determined according to Huet et al. [1998]. Briefly, MCF-7 and JAR/VP16 cells were plated at 2×10^5 cells per 35-mm dish in duplicate. The following day, the cells were treated with celecoxib (10 or 50 μM) or vehicle for 2 or 72 h. After treatment, cells were washed gently for three times with cold PBS and collected by trypsin digestion and centrifugation. The cells were then resuspended in fresh medium with Rhodamine123 (5 μM) at 37°C for 0.5 h. Cells were then washed three times with PBS before data was acquired and analyzed by flow cytometry. Flow cytometer (FACSC alibur, BD Biosciences) was used to detect the intensity of Rhodamine123 fluorescence. Data acquisition and analysis were performed on 10,000 cells with the Cell Quest software (BD Biosciences).

DETECTION OF THE COX-2 AND p170 EXPRESSION WITH WESTERN BLOTS

MCF-7 or JAR/VP16 cells were lysed in ice-cold lysis buffer containing 50 mM Tris–HCl, pH 8.0, 1% NP-40, 150 mM NaCl, 0.1%SDS, 1 μg/ml aprotinin, 1 mM EDTA, 1 mM PMSF, and complete protease inhibitor cocktail tablets (one tablet per 10 ml, Roche Diagnostics GmbH, Mannheim, Germany). Protein aliquots were electrophoresed by 10% SDS–PAGE gel and transferred to PVDF (Bio-Rad, CA) membrane. The immunoblots were blocked in 5% dried nonfat milk in TST containing 0.1% Tween-20 at room temperature, and then washed with TST. The blots were incubated with rabbit polyclonal COX-2 antibodies diluted 1:5,000 (Rockland Immunochemicals, Inc., Pennsylvania) or mouse monoclonal p170 antibodies diluted 1:800 in TST at 4°C overnight. The blots were then washed and incubated for 1 h with HRP-conjugated secondary antibodies at 1:3,000 dilutions. The bands were visualized with ECL plus detection system (Amersham Pharmacia Biotech., Piscataway, NJ).

DETECTION OF COX ACTIVITY WITH ELISA

Cells (2×10^5 cells/well) were plated in 6-well tissue culture plates in triplicate for overnight culture. The following morning, cells were treated with celecoxib at the different concentrations (0.5, 5, 10, 25, 50 μM) or its vehicle (DMSO) for 2 h. The supernatant was then removed and cells were cultured with fresh medium containing 10 μM arachidonic acid at 37°C for 30 min. The supernate was collected, and acidified by 50 μl of 2 N HCl per 1 ml of sample. After sitting at 2–8°C for 15 min, the samples were centrifuged at 10,000*g* for 10 min and the supernate were collected. Then ethyl acetate was 1:1 (v/v) added into supernate and vortex for 1 min. After centrifugation for another 10 min at 10,000*g*, the organic phase (500 μl) was collected and evaporated to dryness. The dried residues were reconstituted with 500 μl of ELISA reagent (Calibrator Diluent RD5-39) and used for ELISA. PGE_2 was measured as described [Hull et al., 2004].

The cells were collected by trypsin digestion, and resuspended in 500 μl cold PBS. Cells were collected in 2 ml EP tubes and lysed by sonification. After centrifugation for 10 min at 10,000*g*, the supernatant (10 μl) was used to measure the protein concentration. PGE_2 concentrations in supernate were normalized by total cell

protein amount. Relative COX activity was calculated as the ratio of PGE_2 produced in the presence of celecoxib to the PGE_2 produced in vehicle alone.

METHYLATION SPECIFIC PCR (MSP)

Genomic DNA was extracted from MCF-7 cells after 72 h treatment of different drugs. DNA (1 μg) was subjected to sodium bisulfite modification using CpGenome DNA Modification kit (Chemicon Company, USA). On the basis of the functional promoter sequence [Ueda et al., 1987], methylation-specific and unmethylation-specific primers were used as reported previously [Enokida et al., 2004]. The sense and antisense methylation primers sequence were CGAGGAATTAGTATTTAGTTAATTCGGGTCGG, and ACTCAACC-CACGCCCCGACG. The sense and antisense unmethylation primers sequence were TGAGGAATTAGTATTTAGTTAATTTGGGTTGG and ACTCAACCCACACCCCAACA. The PCR conditions were: annealing temperature/time/cycle (67°C/30 s/5 then 65°C/30 s/5 then 62°C/30 s/5 and 59°C/30 s/11). The PCR products (95 bp) were analyzed by electrophoresis on 2% agarose gel. The area under the curve corresponding to each band was analyzed using the ImageJ software.

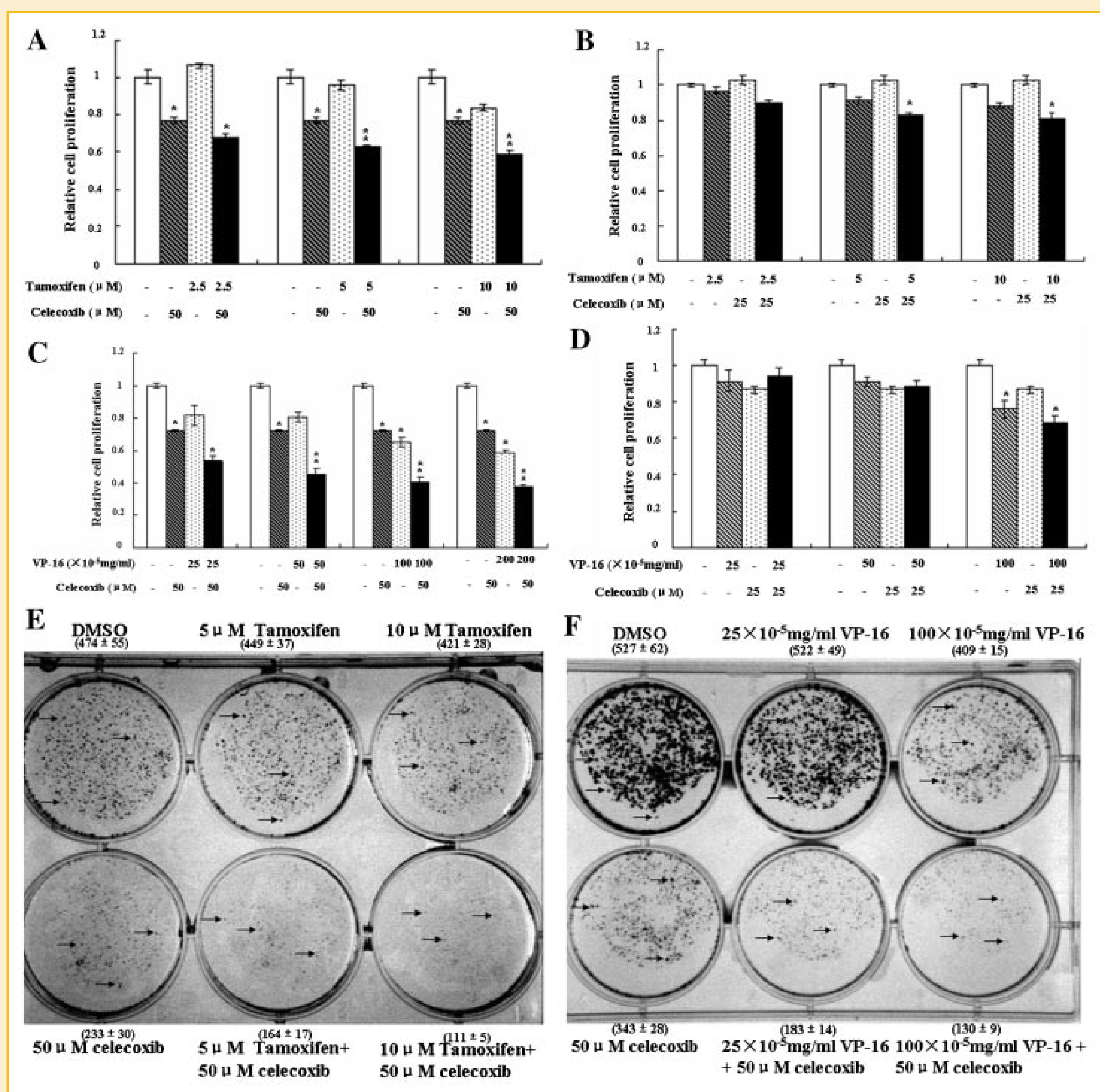

Fig. 1. Celecoxib enhanced the sensitivities of MCF-7 and JAR/VP16 cells to anticancer drugs. A: MCF-7 cells were treated with celecoxib (50 μM) combined with tamoxifen at various concentrations for 72 h. B: MCF-7 cells were treated with celecoxib (25 μM) combined with tamoxifen at various concentrations for 72 h. C: JAR/VP16 cells were treated with celecoxib (50 μM) combined with VP-16 at various concentrations for 72 h. D: JAR/VP16 cells were treated with celecoxib (25 μM) was combined with various concentrations of VP-16 to treat JAR/VP16 cells for 72 h. Cell proliferation of MCF-7 and JAR/VP16 cells were determined by MTT. *Indicated a significance of $P < 0.05$ compared to the vehicle, **indicated a significance of $P < 0.05$ compared to the drug used alone. E,F: The colony-forming assay was performed as described in Materials and Methods Section; the arrow indicated a typical colony, the numbers indicated the average colonies of different treatments.

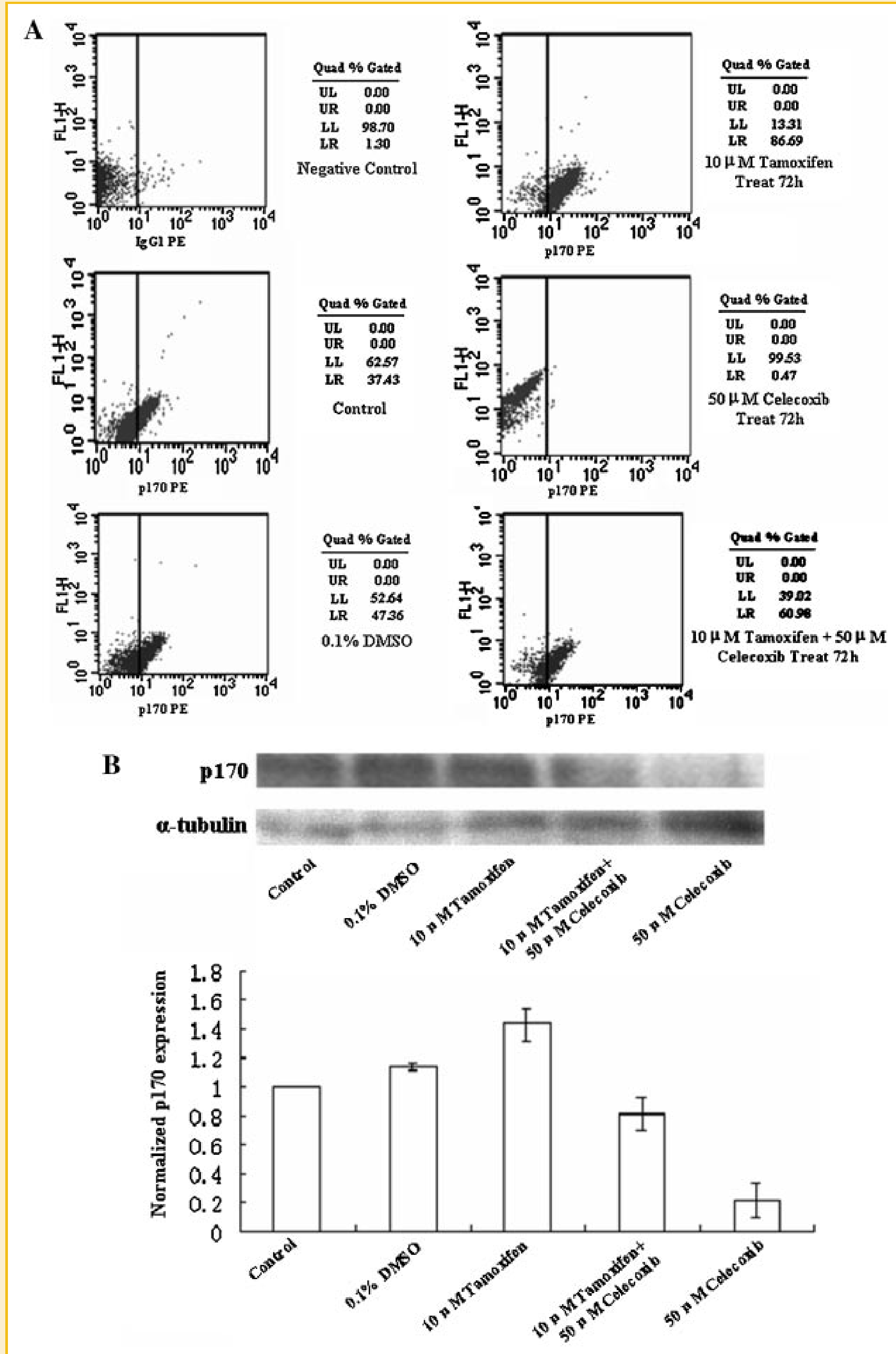

Fig. 2. Celecoxib inhibited p170 expression but not its pump function in MCF-7 and JAR/VP16 cell lines. As described in Materials and Methods Section, p170 expression and the content of Rhodamine123 within cells was detected by flow cytometry (A,C,D) and Western blot (B). The right part of the panels indicated the p170 positive cells in flow cytometry assay. A,B: MCF-7 cells were treated with celecoxib (50 μM) and tamoxifen (10 μM) either alone or in combination, or with the drug vehicle only (0.1% DMSO) for 72 h. C: JAR/VP16 cells were treated by celecoxib (50 μM) and VP-16 (100×10^{-5} mg/ml) either alone or in combination, or with the drug vehicle only (0.1% DMSO), for 72 h. D: The left part indicated the p170 positive cells after treated by celecoxib (50 μM) for 2 h. The right part indicated the content of Rhodamine123 within cells after treatment of celecoxib (50 μM) or CS-A (10 μM) for 2 h.

RESULTS

CELECOXIB ENHANCED SENSITIVITY OF MCF-7 AND JAR/VP16 CELLS TO ANTICANCER DRUGS

To evaluate the role of celecoxib in the response of cancer cells to anticancer drugs, MCF-7 cells that expressed p170 were treated with celecoxib (25, 50 μM) alone or combined with tamoxifen (2.5–10 μM). The response of cells to drugs was evaluated by cell proliferation-inhibiting-rate determined with MTT assay. As indicated in Figure 1A, at the concentrations of 2.5–10 μM, tamoxifen had no significant effect on cell proliferation ($P > 0.05$). Celecoxib alone could inhibit the proliferation of cells

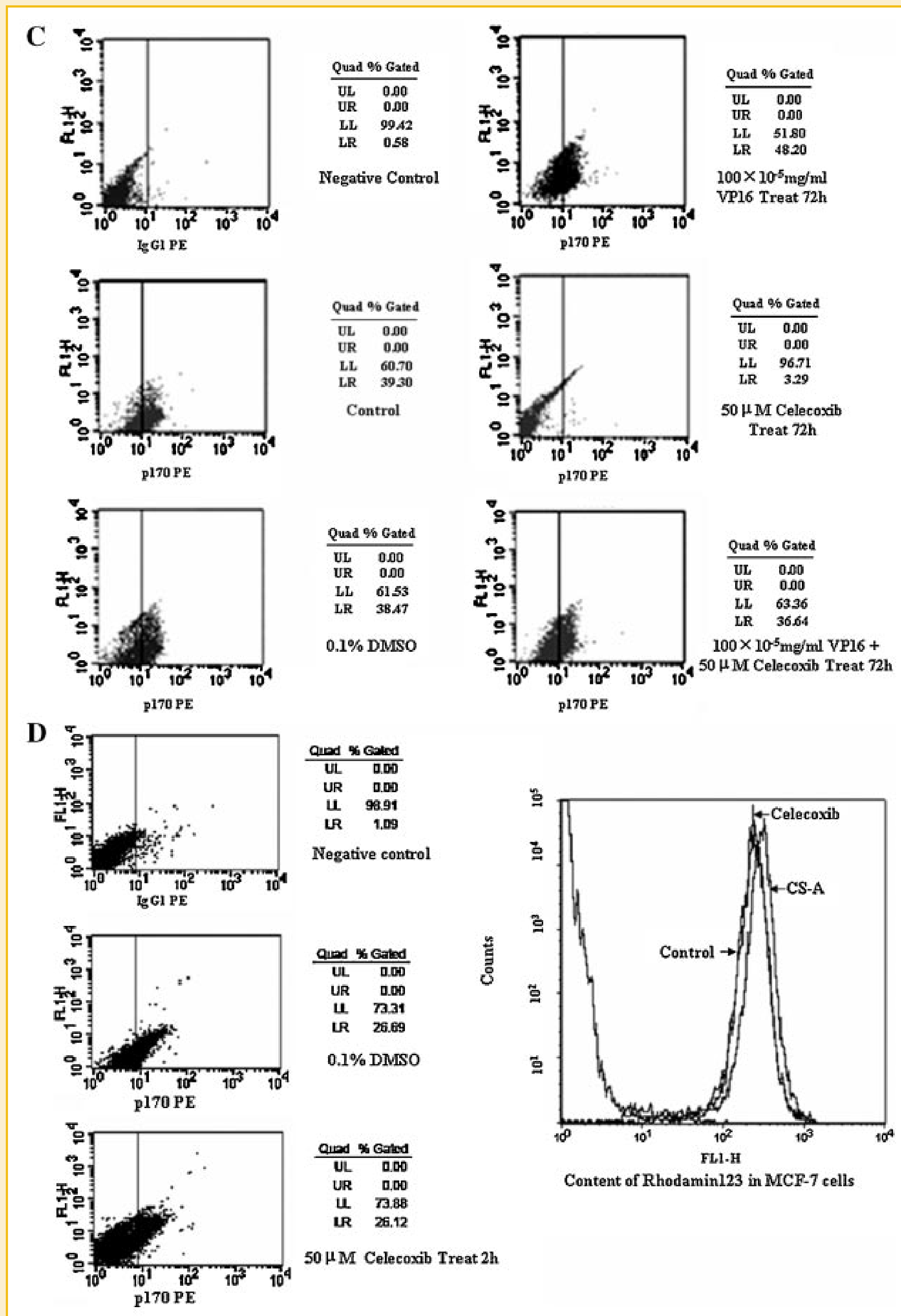

Fig. 2. (*Continued*)

in a dose-dependent manner (data not shown). However, when combined with celecoxib, tamoxifen at the same concentrations significantly inhibited the cell proliferation. The inhibitory effect was greater than that when celecoxib was applied alone ($P < 0.05$). Notably, 5 μM tamoxifen that did not inhibit MCF-7 cell proliferation increased cell proliferation-inhibiting-rate significantly when combined with celecoxib. These observations showed that celecoxib could enhance the sensitivity of MCF-7 cells to tamoxifen. In our experiments the effective concentration of celecoxib was 50 μM. Celecoxib (25 μM) had no significant effect on the response of cells to tamoxifen (Fig. 1B). The effect of celecoxib was subsequently examined in another p170 expressing cell line, JAR/VP16. Similar results were obtained in JAR/VP16 cells treated with VP-16 at various concentrations ($25–200 \times 10^{-5}$ mg/ml) combined with celecoxib (25 and 50 μM) (Fig. 1C,D). These results suggested that celecoxib could enhanced the sensitivity of cancer cells to anticancer drugs in cell type independent way.

To confirm our observations, plate colony-forming assays were performed. As indicated in Figure 1E, tamoxifen alone did not inhibit colony formation ($P < 0.05$), while celecoxib inhibited colony-formation significantly ($P < 0.05$), compared with the control, respectively. When combined with celecoxib, tamoxifen significantly inhibit MCF-7 cells colony formation. The colonies were less than celecoxib used alone ($P < 0.05$). Similar results were obtained in JAR/VP16 cells (Fig. 1F). So, we concluded that celecoxib could enhance the tamoxifen- and VP-16-induced inhibition of colony-formation significantly, supporting the experimental observations described above.

CELECOXIB INHIBITED THE EXPRESSION OF p170 BUT NOT AFFECT THE PUMP FUNCTION IN MCF-7 AND JAR/VP16 CELLS

In order to investigate whether p170 is involved in the sensitizing effect of celecoxib on cancer cells, we first determined the expression level of p170 in MCF-7 cells treated with celecoxib in the presence or absence of tamoxifen by flow cytometry. As indicated in Figure 2A, in the control, 47.4% of cells were p170 positive. The p170 positive cells were increased to 88.6% after treatment of cells with tamoxifen (10 μM) alone for 72 h, showing tamoxifen induced expression of p170. However, when cells were treated with tamoxifen and celecoxib together, p170 positive cells were only increased to 60.1%, suggesting celecoxib could significantly inhibit tamoxifen-induced p170 expression. The inhibitory effect of celecoxib on p170 expression was further confirmed with Western blot analysis (Fig. 2B). Similarly, celecoxib inhibited the drug-induced expression of p170 in JAR/VP16 cells treated with VP-16 (Fig. 2C).

We further asked whether celecoxib affected the pump function of p170. For this purpose, the cells were treated with 50 μM celecoxib for 2 h, the intracellular content of Rhodamin123, a well-known p170 substrate, was then tested using flow cytometry. Cells treated with 10 μM Cyclosporin-A (CS-A), a well-known p170 pump inhibitor, for 2 h were used as positive control. As shown in Figure 2D, treatment of celecoxib did not significantly change the content of Rhodamin123 within cells, indicating celecoxib was not able to affect pump function of p170. All together, our observations

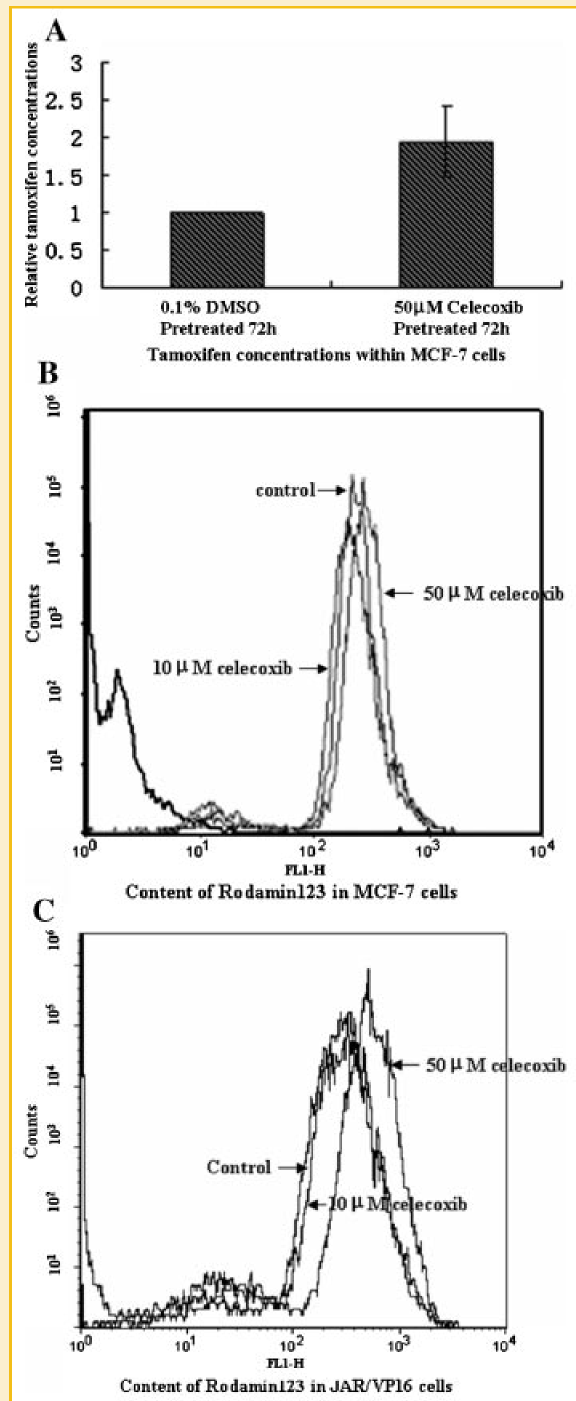

Fig. 3. Celecoxib increased the concentrations of anticancer drug and p170 substrate within MCF-7 and JAR/VP16 cells. A: HPLC results showed the change of tamoxifen within MCF-7 cells, between control and celecoxib (50 μM) pretreated cells. B,C: The contents of Rhodamin123 in MCF-7 and JAR/VP16 cells, respectively, pretreated with celecoxib (50 μM) or with the vehicle only (DMSO), for 72 h, were measured by flow cytometry. The peak area indicated the content of Rhodamin123.

demonstrated that celecoxib was able to inhibit the expression of p170, but not affect the pump function of p170.

THE CELECOXIB-INDUCED ENHANCEMENT OF SENSITIVITY OF CANCER CELLS TO ANTICANCER DRUGS WAS MEDIATED BY INHIBITION OF p170 EXPRESSION

To investigate whether the sensitization effect of celecoxib was mediated by inhibiting of p170 expression which ultimately results in accumulation of p170 substrates within cells, we determined the intracellular contents of tamoxifen within MCF-7 cells pretreated by celecoxib or not using HPLC. As expected, tamoxifen concentration within cells pretreated with 50 μM celecoxib for 72 h increased twice as much as that in cells without pretreatment (Fig. 3A). Similarly, pretreatment of cells with celecoxib enhanced the intracellular accumulation of Rhodamin123 in both MCF-7 and JAR/VP16 cells (Fig. 3B,C). These observations demonstrated that celecoxib-induced enhancement of sensitivity of cancer cells to anticancer drugs was dependent on inhibition of p170 expression. This notion was further supported by our observation that down-regulation of p170 by siRNA increased the intracellular concentration of tamoxifen (Fig. 4A,B) and correlation between methylation of *MDR1* promoter and celecoxib treatment (see Celecoxib Down-Regulated p170 Expression by Induction of Hypermethylation of *MDR1* Gene Promoter Section).

THE INHIBITORY EFFECT OF CELECOXIB ON THE EXPRESSION OF p170 WAS INDEPENDENT ON COX-2

Considering COX-2 could up-regulate *MDR1* transcription, we examined whether the inhibitory effect of celecoxib on p170 expression was COX-2 dependent or not. The correlation between COX-2 expression and celecoxib-induced down-regulation of p170 was first evaluated in MCF-7 and JAR/VP16 cell lines treated with celecoxib. Unexpectedly, the expression of COX-2 was not significantly changed when cells were treated with 50 μM celecoxib (Fig. 5A), although celecoxib at this concentration significantly down-regulated the expression of p170 (Fig. 5B). In JAR/VP16 cells, celecoxib treatment even increased expression of COX-2 slightly (Fig. 5C). Thus, the inhibitory effect of celecoxib on p170 was not related to the expression level of COX-2.

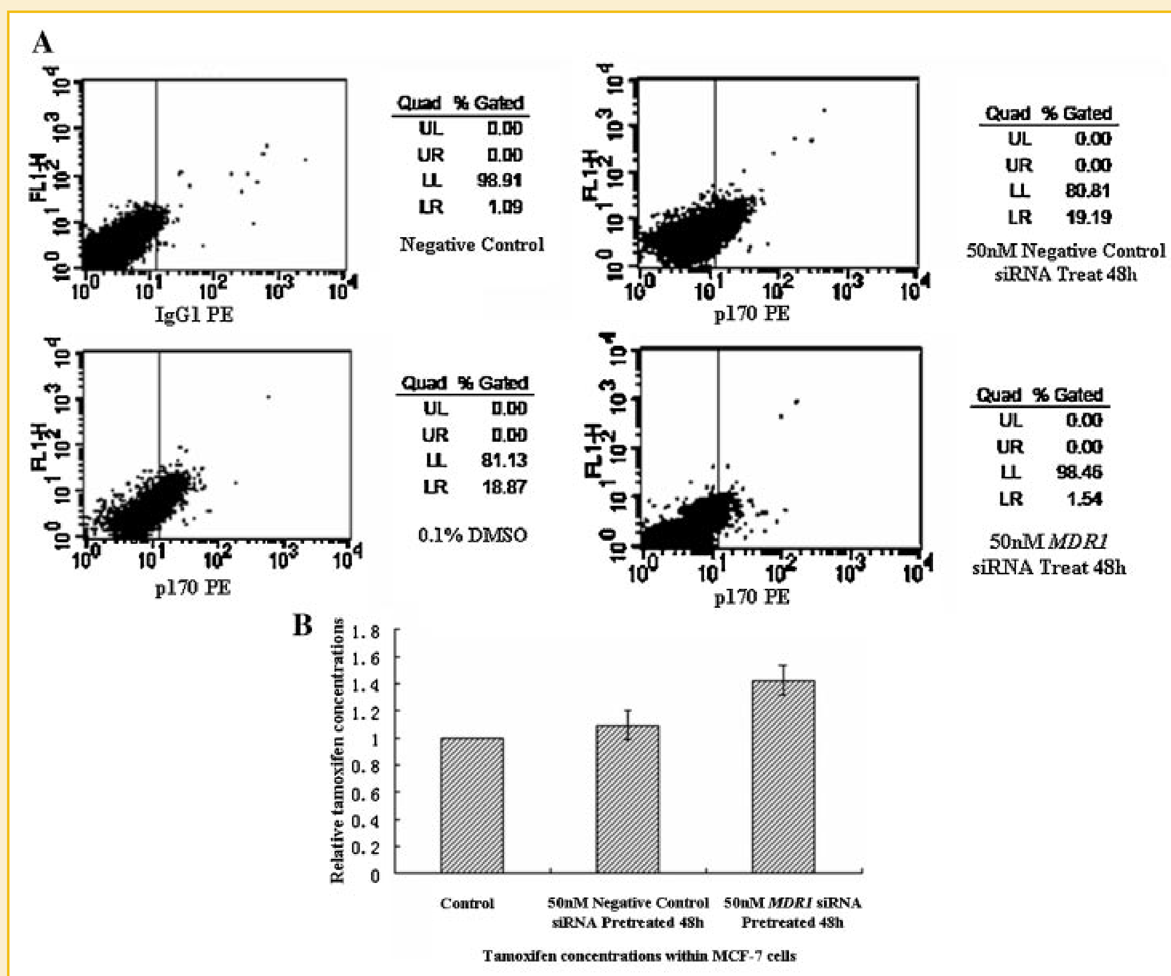

Fig. 4. The p170 specific siRNA increased the concentrations of tamoxifen in MCF-7 cells. A: Flow cytometry showed that the MDR1 specific siRNA (50 nM) down-regulated p170 expression while negative control siRNA had no effect on p170 expression. B: HPLC showed that the concentration of tamoxifen within specific siRNA pretreated and control cells.

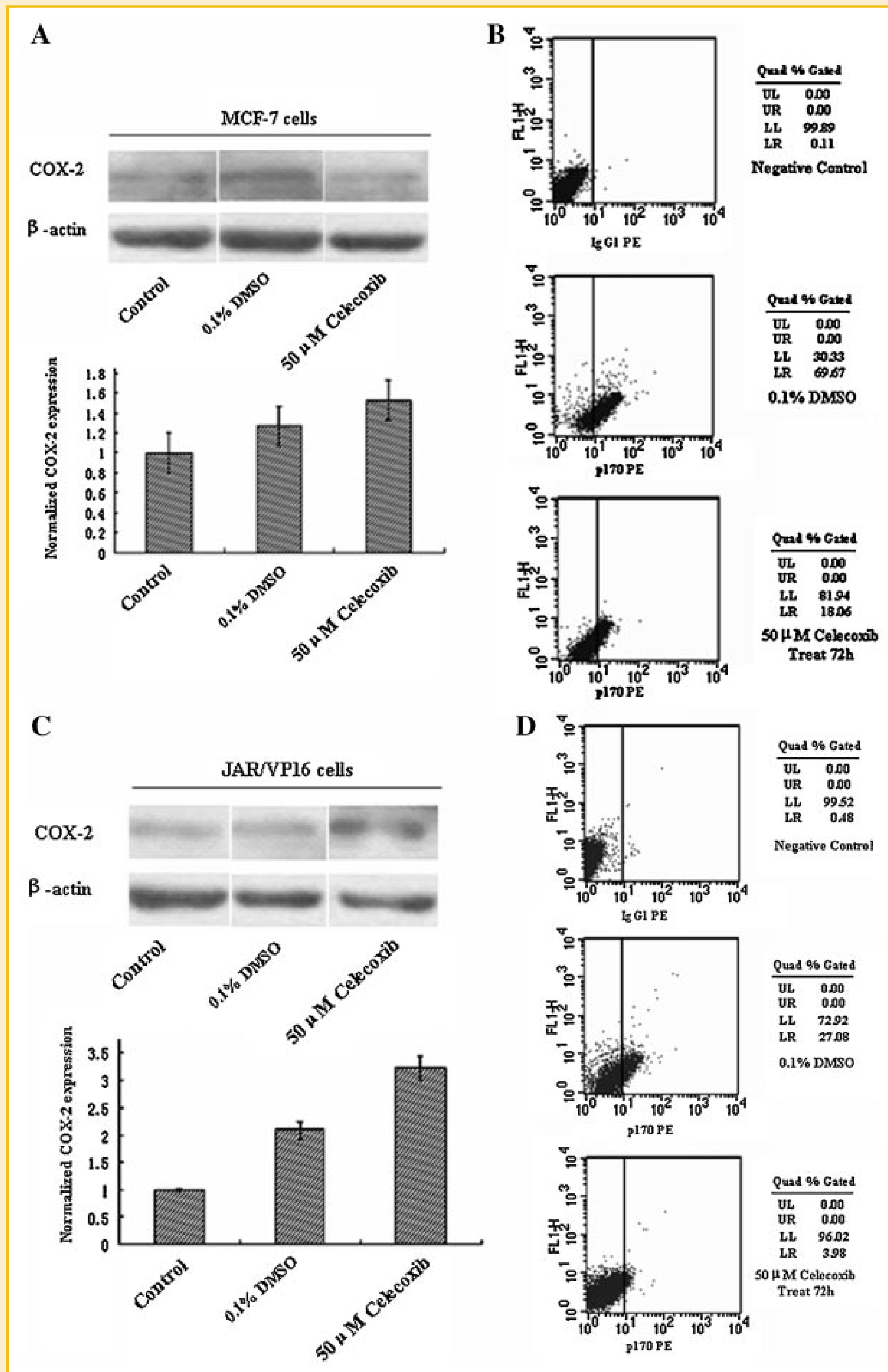

Fig. 5. Celecoxib-induced down-regulation of p170 expression was independent of COX-2 expression and activity. A,C: COX-2 expression in MCF-7 and JAR/VP16 cells were detected by Western blots. B,D: p170 expression in MCF-7 and JAR/VP16 cells were investigated by flow cytometry. E,F: MCF-7 and JAR/VP16 cells were exposed to different concentrations of celecoxib. The supernatant was collected and PGE_2 was measured by ELISA to determine the activity of COX-2. Relative COX activities were measured as the ratio of PGE_2 produced in the presence of celecoxib to that of the control. G,H: The expression of p170 in MCF-7 and JAR/VP16 cells exposed to celecoxib (10 and 50 μM) or vehicle for 72 h were determined by flow cytometry.

The correlation between COX-2 activity and celecoxib-induced down-regulation of p170 was then investigated by examining release of PGE_2 into the culture supernatant with ELISA (Fig. 5E,F). We found that celecoxib could inhibit COX-2 activity at the concentration as low as 0.5 μM and this inhibitory effect reached the maximum at the concentration of 10 μM in both cell lines. However, the effective concentration of celecoxib for inhibition of p170 was 50 μM in our experiment. Lower than this concentration, celecoxib could not down-regulate the expression of p170 (Fig. 5G,H). Obviously, the inhibitory effect of celecoxib on p170 expression was not mediated by change of COX-2 activity. Altogether, our data indicated the inhibitory effect of celecoxib on p170 expression was independent on COX-2.

CELECOXIB DOWN-REGULATED p170 EXPRESSION BY INDUCTION OF HYPERMETHYLATION OF *MDR1* GENE PROMOTER

To elucidate the mechanism by which celecoxib suppresses p170 expression, we examined the methylation status of CpG islands within the *MDR1* gene promoter in celecoxib treated and untreated MCF-7 cells. As shown in Figure 6A, the treatment of cells with 50 μM celecoxib induced hypermethylation of the CpG islands and this effect could be reversed by treatment of 5 μM 5-aza-dC. The celecoxib-induced hypermethylation was closely accompanied by the suppression of p170 expression, while treatment of 5 μM 5-aza-dC induced demethylation of CpG islands within the *MDR1* promoter and resulted in increase in p170 expression. Obviously, the celecoxib-induced down-regulation of p170 could be reversed

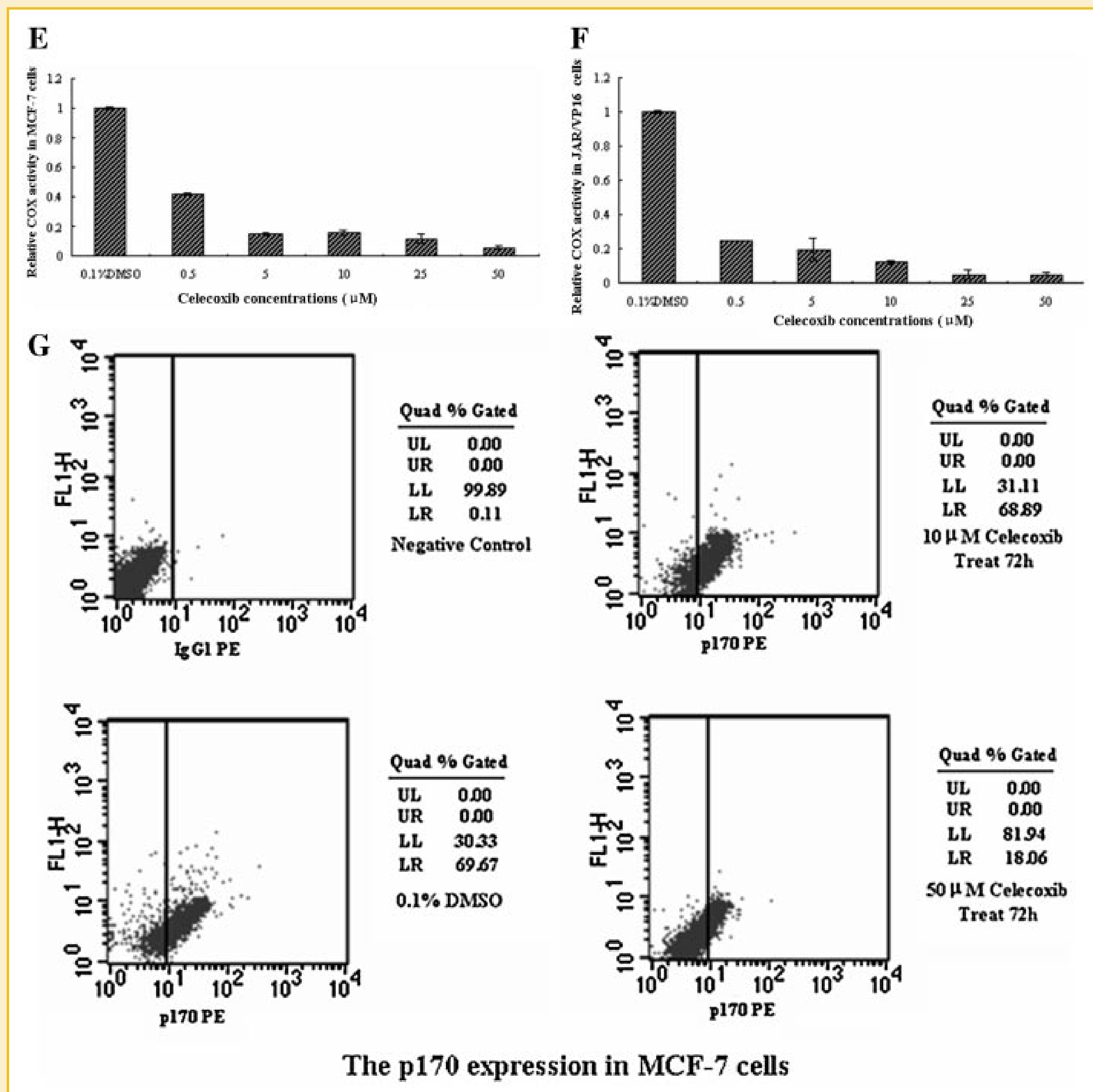

Fig. 5. (*Continued*)

by 5-aza-dC (Fig. 6B). These findings suggested that celecoxib inhibited p170 expression by induction of hypermethylation of *MDR1* gene promoter.

DISCUSSION

P-Glycoprotein (p170) encoded by the *MDR1* gene is an important member of the ATP-binding cassette transporters that function as pumps to extrude anticancer drugs from cancer cells, thereby closely related to MDR phenotype in cancer patients. The purpose of this study was to determine whether celecoxib, a COX-2 specific inhibitor, suppressed the expression and pump function of p170 as well as to explore the correlation of this effect to COX-2. Our data demonstrated that celecoxib could enhance the sensitivity of cancer cells with drug-induced or primary expression of p170 to anticancer drugs by down-regulation of the expression of p170. The pump function of p170 was not significantly affected by celecoxib. This observation established a clear link between the sensitization effect of celecoxib on cancer cells and p170 expression level. It provided strong evidence that celecoxib might be a good candidate for preventing or reversing drug resistance phenotype. Further investigation of the effect of celecoxib in experiment-induced tumor animal model will be of interest.

An increasing number of reports are challenging the notion that the anti-tumor potential of the selective COX-2 inhibitor celecoxib is mediated primarily via the inhibition of COX-2 [Chuang et al., 2008]. In addition to inhibiting COX-2 activity, celecoxib has been known to trigger endoplasmic reticulum stress (ERS) [Chuang et al., 2008], modulate expression or function of apoptosis-related proteins, such as death receptor-5 [He et al., 2008] and protein kinase Akt [Lai et al., 2003] in COX-2 dependent or independent way. In our present study down-regulation of p170 by celecoxib occurred at the concentration much higher than that required for inhibiting PGE_2 release, suggesting that the inhibitory effect of celecoxib on p170 was likely mediated through a novel mechanism independent of the inhibition of COX-2 and the resulting production of PGE_2. Inhibition of COX-2 is not necessary for this anticancer ability of celecoxib. Similar result was also obtained in earlier study of another drug efflux pump, MRP1, from human lung cancer [Kang et al., 2005].

Celecoxib was reported to modulate DNA methylation [Pereira et al., 2004] and the expression of *MDR1* gene is known to be regulated by the methylation of CpG islands within the *MDR1* promoter [Enokida et al., 2004; Qiu et al., 2007; Ellinger et al., 2008]. We supposed that celecoxib inhibited the expression of p170 by increasing the methylation of *MDR1* promoter. Our experimental data proved this to be the case. Treatment of 50 μM celecoxib significantly increased the methylation of CpG islands within *MDR1* promotor, which was accompanied by suppression of p170, while reverse of the methylation of *MDR1* promoter by 5-aza-dC resulted in increase in p170 expression. This finding not only provided a direct evidence that sensitization effect of celecoxib on cancer cells was mediated through p170, but also established a link between

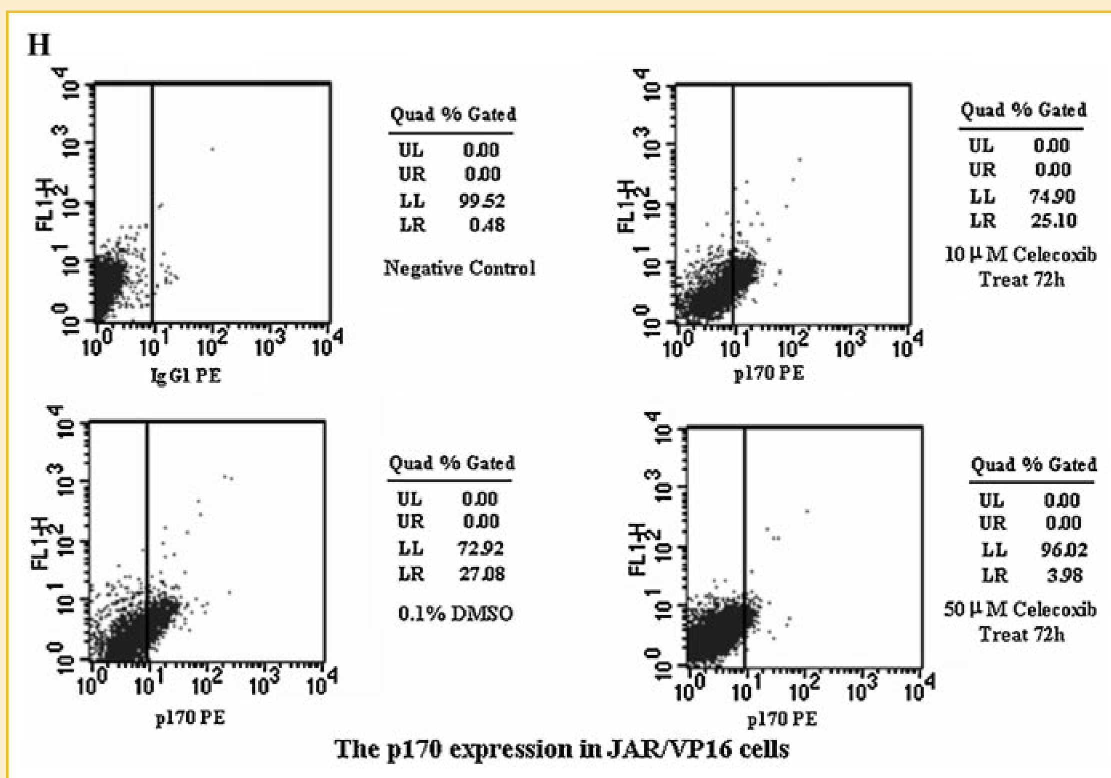

Fig. 5. (*Continued*)

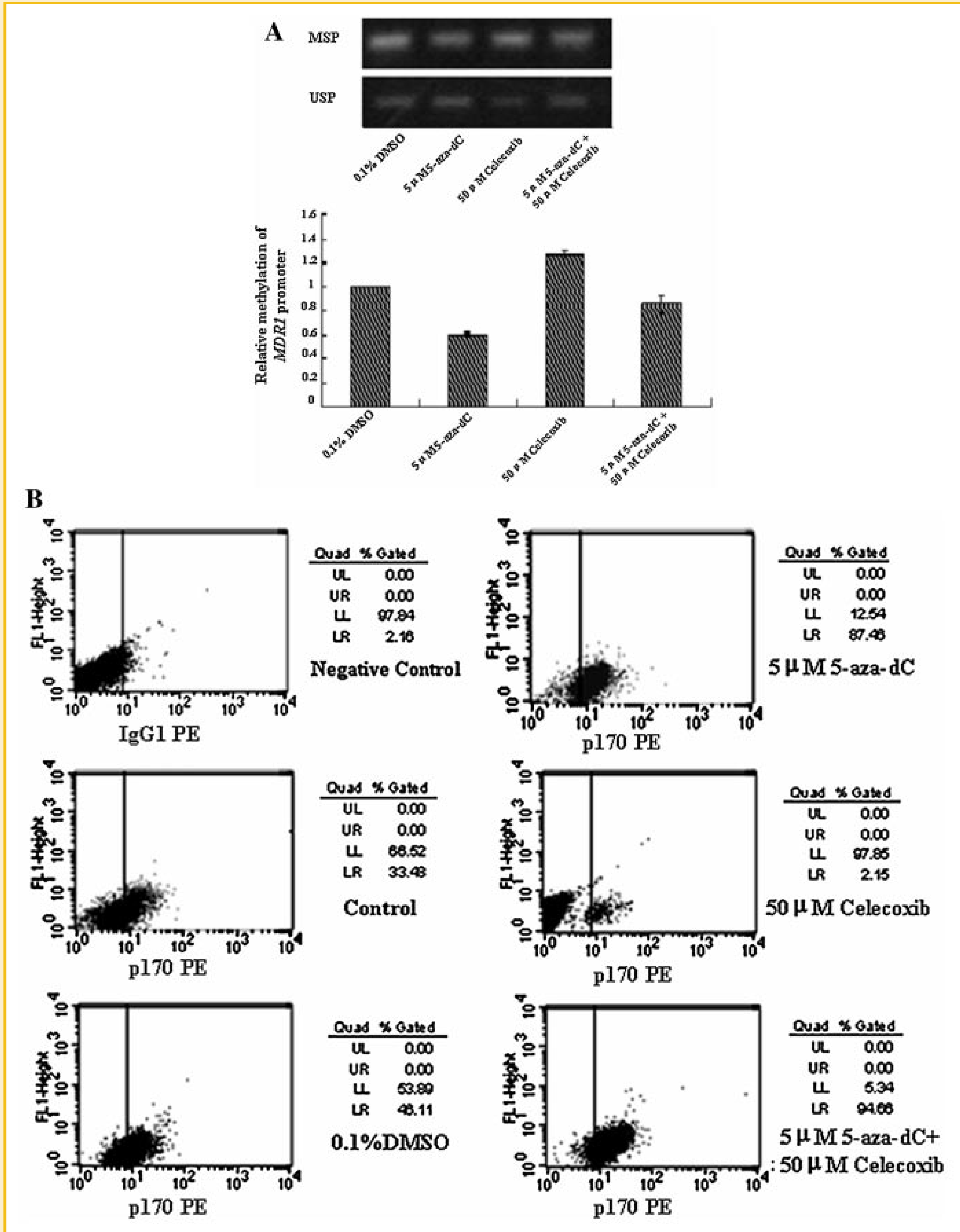

Fig. 6. Celecoxib down-regulate p170 expression by induction of hypermethylation of MDR1 gene promoter. MCF-7 cells were treated by celecoxib and/or 5-aza-dC for 72 h. A: Methylation-specific PCR was performed to investigate MDR1 gene promotor CpG islands methylation level. B: Flow cytometry was used to investigate p170 expression after treatment of celecoxib and/or 5-aza-dC for 72 h.

epigenetic change of *MDR1* gene and the effect of celecoxib, suggesting that celecoxib may be used as a methylation agent to reverse p170-related drug resistance of cancer cells.

Several studies have demonstrated that celecoxib suppressed the activity of Sp1 transcriptional factor [Wei et al., 2004]. The promoter of *MDR1* gene possesses Sp1 binding sites [Cornwell and Smith, 1993; Rohlff and Glazer, 1998]. As each forward and reverse M/USP primer used for methylation analysis in our experiments was designed to cover the Sp1 sites in their 3′-region [Enokida et al., 2004], the detected hypermethylation may affected the binding of

Sp1. It is possible that the hypermethylation-induced by celecoxib could suppress the binding of Sp1 transcription factor to its binding sites within the *MDR1* promoter and ultimately resulted in inhibition of expression of *MDR1* gene. Therefore, it is of interest to determine links between the transcriptional activity of Sp1 and methylation status of *MDR1* promoter.

The concentration of celecoxib used in our experiments was higher than that observed in plasma samples from patients or animals after treatment of celecoxib [Davies et al., 2001; Niederberger et al., 2001]. However, there is reason to treat cells with higher concentration of celecoxib in vitro. First, the distribution of celecoxib in tissues was quite different. The concentrations of celecoxib in the plasma samples of patients could not represent that of the drug concentration in the target tissues that may accumulate high concentration of the celecoxib [Paulson et al., 2000]. Second, celecoxib has a high protein binding capacity [Maier et al., 2004]. This property limited the free and therefore effective celecoxib in our cell culture systems, which included 10% of serum and thus higher concentration of the drug was necessary. Furthermore, the concentration of celecoxib used in this study was comparable with that in previous studies in vitro [Williams et al., 2000; Pyrko et al., 2006; van Wijngaarden et al., 2007], although lower concentration was reported for the experiments in vitro during the preparation of this manuscript [Fantappie et al., 2007].

In summary, our data demonstrated that celecoxib enhanced the sensitivity of cancer cells to anticancer drugs by inhibition of the expression of p170 through a COX-2-independent manner. Celecoxib may inhibit p170 expression by induction of hypermethylation of CpG islands within the *MDR1* promoter. This study suggests that celecoxib could be a potential agent for improving chemotherapy. It also provided a links between epigenetic change of *MDR1* and drug response of cancer cells.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science Foundation of China (30772490 and 30870978), the Special Funds for Major State Basic Research Program of China (973 Program, 2006CB503908) and the Basic Research Programme of College Natural Science Foundation from Jiang Su Province (06KJB310068). We thank Shukui Wang, Yan Li et al. at Department of Radioimmunity in Nanjing First Hospital for their help in flow cytometry analysis and we thank Qunwei Xu, Peng Wang, Dongfei Liu for their help in HPLC. We also thank Dr. Linda Durrin for careful reading of this manuscript.

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