Up-Regulation of Carbonyl Reductase 1 Renders Development of Doxorubicin Resistance in Human Gastrointestinal Cancers

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Doxorubicin (DOX) is widely used for the treatment of a wide range of cancers such as breast and lung cancers, and malignant lymphomas, but is generally less efficacious in gastrointestinal cancers. The most accepted explanation for the DOX refractoriness is its resistance development. Here, we established DOX-resistant phenotypes of human gastric MKN45 and colon LoVo cells by continuous exposure to incremental concentrations of the drug. While the parental MKN45 and LoVo cells expressed carbonyl reductase 1 (CBR1) highly and moderately, respectively, the gain of DOX resistance further elevated the CBR1 expression. Additionally, the DOX-elicited cytotoxicity was lowered by overexpression of CBR1 and inversely strengthened by knockdown of the enzyme using small interfering RNA or pretreating with the specific inhibitor quercetin, which also reduced the DOX refractoriness of the two resistant cells. These suggest that CBR1 is a key enzyme responsible for the DOX resistance of gastrointestinal cancer cells and that its inhibitor is useful in the adjuvant therapy. Although CBR1 is known to metabolize DOX to a less toxic anticancer metabolite doxorubicinol, its overexpression in the parental cells hardly show significant reductase activity toward low concentration of DOX. In contrast, the overexpression of CBR1 increased the reductase activity toward an oxidative stress-derived cytotoxic aldehyde 4-oxo-2-nonenal. The sensitivity of the DOX-resistant cells to 4-oxo-2-nonenal was lower than that of the parental cells, and the resistance-elicited hyposensitivity was almost completely ameliorated by addition of the CBR1 inhibitor. Thus, CBR1 may promote development of DOX resistance through detoxification of cytotoxic aldehydes, rather than the drug’s metabolism.

Key words carbonyl reductase 1; doxorubicin; drug resistance; gastrointestinal cancer cell; quercetin

An anthracycline-based antibiotic doxorubicin (DOX) is widely utilized for the treatment of patients not only with solid tumors formed in many organs including breast, lung and stomach, but also with malignant lymphomas.1–3 One of the major anticancer actions of DOX is intercalation into the double-stranded DNA and the resultant inhibition of DNA/RNA polymerases.4 In addition, treatment with the drug is known to form the tripartite complex with DNA and topoisomerase-II, ultimately blocking the transcription and replication of DNA. Besides the events essential for protein biosynthesis and cell proliferation, the free radical formation is proposed as another cytopathological mechanism triggered by DOX1–3. Cellular reductases such as cytochrome P450 reductase4 and xanthine oxidase5 catalyze the one-electron reduction of the p-quinone structure in the anthraquinone ring of DOX into a semiquinone radical intermediate, which in turn reacts with molecular oxygen to yield superoxide anion radical. The radical is further converted into a more potent oxidizing agent hydroxyl radical, and thereby oxidatively modifies intracellular components such as nucleic acids, lipids and proteins, finally leading to the cancer cell death. Despite of its potent anticancer effect, continuation of the DOX therapy for gastrointestinal cancers provokes high recurrence rate and reduction in the drug efficacy, which are inferred to be due to acquisition of the DOX resistance in cancer cells. Literature showed that increases in transporters involved in the DOX excretion7 are responsible for development of the drug resistance. However, it remains unclear what other factors may progressively promote the chemoresistance.

Carbonyl reductase 1 (CBR1), a member of the short-chain dehydrogenase/reductase superfamily, is a 30-kDa cytosolic enzyme that catalyzes reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of several endogenous carbonyl compounds such as prostaglandins, 3-ketosteroids, isatin and a lipid peroxidation-derived cytotoxic aldehyde, 4-oxo-2-nonenal (ONE).8–13 In particular, the enzyme effectively catalyzes the reduction of the ketone, aldehyde and C=C bond of ONE into 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenol and 4-oxononal, respectively.13 Because the aldehyde reduction of ONE is the most efficient among the three reactions catalyzed by CBR1, the enzyme is suggested to function as an antioxidant enzyme that protects cells from the ONE-induced damage through its conversion to the less toxic 4-oxo-2-nonenol. CBR1 also reduces a variety of exogenous carbonyl compounds, which include ketone-containing drugs, such as haloperidol, metyrapone, loxopro-
fen, and anthracycline drugs. In the reduction of DOX and daunorubicin, the enzyme reduces their C13-keto groups into the C13-alcohol metabolites [doxorubicinol (DOXol) and daunorubicinol, respectively], which are considered to elicit the serious adverse effect, cardiotoxicity. The CBR1-mediated DOX reduction is suggested to develop the chemoresistance in cancer cells, based on the findings that the alcohol metabolite DOXol exerts less anticancer effect compared to DOX. To our knowledge, there are only three reports that propose the contribution of CBR1 to the mechanism underlying DOX resistance of breast cancer cells and hepatocellular carcinomas.

In this study, we monitored the expression of CBR1 in seven gastrointestinal cells, of which MKN45, LoVo and DLD1 cells with intrinsically different levels of CBR1 expression were chosen in order to establish the DOX-resistant variants. Since reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses revealed the up-regulation of CBR1 in the established DOX-resistant phenotypes of MKN45 and LoVo cells, the pathophysiological role of the enzyme in the DOX resistance was investigated by analyzing effects of its transient overexpression and specific inhibition on cytotoxicity of DOX. In addition, we propose that high expression of CBR1 in the cells facilitates the DOX resistance through its catalytic reduction of ONE rather than the drug.

**MATERIALS AND METHODS**

**Materials**  DOX and DOXol were obtained from Kyowa Hakko Kogyo (Tokyo, Japan) and Pharmacia & Upjohn (Milan, Italy), respectively. ONE was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.); quercetin (QUE) was from Sigma-Aldrich (St. Louis, MO, U.S.A.); sulforaphane (SFN) was from LKT Laboratories (St. Paul, MN, U.S.A.); and Taq DNA polymerase was from TaKaRa (Kusatsu, Japan). The cDNA for the reagent, Superscript III reverse transcriptase, oligo(dT)12-18 primer and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, U.S.A.). All other chemicals were of the highest grade that could be obtained commercially.

**Cell Culture and Transfection**  Six human colon cancer (RKO, LoVo, DLD1, HCT15, HT29, and SW480) and gastric cancer MKN45 cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and Health Science Research Resources Bank (Osaka, Japan), respectively. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified incubator containing 5% CO2. For establishment of the subpopulation resistant to DOX, DLD1, LoVo and MKN45 cells were continuously treated in the growth medium supplemented with DOX, whose concentration was increased in a stepwise manner. At each DOX concentration of 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 µM, the cells were passaged three times. The sensitivity to toxic compounds including DOX and growth potential of the cells were estimated by monitoring the viability, which was evaluated by a tetrazolium dye-based cytotoxicity assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt.

In order to overexpress CBR1 in cells, the cDNA for the enzyme was amplified by PCR using the following primers from the bacterial expression vector. The forward primer, 5'-CCC CGA ATT GCC cacc ATG TCG TCC GGC ATC C-3', contains an EcoRI site, a Kozak sequence, and a start codon, which are shown in italic, small, and underlined letters, respectively. The reverse primer, 5'-CCC CGT CGA ACT TCA CC ACT GTG TTC ACT T-3', is complementary to the bacterial expression vector containing a SalI site, which is shown in italic letters. The amplified cDNA was subcloned at the EcoRI and SalI sites into the mammalian pGW1 expression vector. The sequence of the insert was verified by DNA sequencing using a CEQ2000XL DNA sequencer (Beckman Coulter, Fullerton, CA, U.S.A.). The pGW1 expression vector harboring the cDNA for CBR1 was transfected into DLD1 and LoVo cells using Lipofectamine 2000 when the cells were grown to 90% confluence in microplates or dishes. The empty vector was similarly transfected into the cells, which were used as control cells. After 48-h culture, the cells were washed twice with serum-free growth medium containing the antibiotics alone, and then treated with the agents. To prepare MKN45 cells silenced for CBR1, 10 pmol of the duplex small interfering RNAs (siRNAs), (obtained from Nippon EGT (Toyama, Japan), was transfected using Lipofectamine 2000 into the 50%-confluent cells. The siRNA sequences were as follows: CBR1 siRNA, 5'-GCC CAG AGCUG CAG ACA TTT-3' (sense), 5'-UUCUG UG CGA GC CUG GCTT-3' (antisense). The overexpression and knockdown of the target gene were verified by Western blot analysis described below.

**Western Blot Analysis**  For the analysis of nuclear factor erythroid 2-related factor 2 (Nrf2) translocation into nucleus, the cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS) and the nuclear fraction was prepared according to the method reported by Mohan et al. In the analysis of CBR1, the cells were washed with DPBS, suspended in ice-cold DPBS containing 0.5% Triton X-100 and 0.3 mM phenylmethylsulfonyl fluoride, and homogenized by sonication. The cell extract was prepared by centrifugation (12000 g for 15 min) of the homogenate, and the protein concentration was determined with a Pierce bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, U.S.A.). For Western blotting, the cell extracts (40 µg) were electrophoretically separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and then the proteins were transferred to a Millipore polyvinylidene difluoride membrane by electroblotting. After blocking with 50 mM Tris-buffered saline (TBS) containing 0.5% bovine serum albumin, the membrane was allowed to be incubated for 2 h in TBS supplemented with 0.5% Tween 20 and 1 µg/mL primary antibodies against Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), CBR1 and β-actin (Santa Cruz Biotechnology), and then for 1.5 h with the saline supplemented with 0.5% Tween 20 and 1 µg/mL secondary antibody conjugated to horseradish peroxidase. The immunoreactive proteins were visualized using an enhanced chemiluminescence substrate system (GE-Healthcare, Buckinghamshire, U.K.). The band densities were estimated using a Bio-Rad GelDoc 2000 (Bio-Rad Laboratories, Segrate, Italy) and the attached program, Quantity One.

**PCR Analyses**  Total RNA was isolated from treated cells using the TRIzol reagent, and single-stranded cDNA was prepared from the total RNA sample by incubation for 50 min at 42°C with Superscript III reverse transcriptase and oligo(dT)12-18 primer. The cDNA for CBR1 was amplified from the
single-stranded cDNA sample (5 µg) by semiquantitative PCR using the specific primers as reported previously. 23) The PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. The cDNA for human β-actin constitutively expressed was also amplified as an internal control using specific primers (Toyobo, Osaka, Japan) to ensure that equal amounts of RNA were added.

To compare expression level of CBR1 between the DOX-resistant and parental cells, real-time PCR analysis was performed using a TaKaRa Thermal Cycler Dice Real-Time PCR System with the SYBR Green Supermix. PCR amplification of CBR1 cDNA (5 µg) was carried out under the thermal conditions consisting of an initial denaturation step (95°C/3 min) and 40 cycles of denaturation (95°C/15 s) and annealing and extension (60°C/1 min). The primer pairs and product size were 5′-ATA CGG GGT GAC GAA GAT TG-3′ (forward), 5′-CAC TTT CTT CTT GGC GCT TTT G-3′ (reverse), and 164 bp. Specific amplification of the target product was verified by the subsequent analysis of melt curve profiles and DNA sequencing, and PCR efficiency for the primers was determined by amplifying serial dilutions of the template cDNA. The expression ratio of transcripts for the enzymes was calculated by normalizing to that for β-actin as the internal standard.

**Assay for Enzyme Activity** The reductase activity for isatin was assayed by measuring oxidation rate of NADPH at 340 nm, in 2.0-mL reaction mixture containing 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, 0.1 mM isatin, and the cell extract (100 µg). The reductase activities for DOX and ONE were determined with 10 µM DOX and 10 µM ONE, respectively, as the substrates. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 µmol NADPH per minute at 37°C. The recombinant CBR1, 21) aldo-keto reductase (AKR) 1B10, 24) AKR1C1, 25) AKR1C2 26) and AKR1C3 27) were expressed from expression plasmids harboring their cDNAs and purified to homogeneity as described previously. The IC₅₀ (concentration required for 50% inhibition) value for QUE was determined in the NADPH-linked reduction using 0.1 mM isatin (for CBR1) 22) and 0.2 mM pyridine-3-aldehyde (for AKR1B10) 23) as the substrates, and the values with AKR1C1, AKR1C2 and AKR1C3 were estimated in nicotinamide adenine dinucleotide phosphate-linked oxidation of (S)-(−)-1,2,3,4-tetrahydro-1-naphthol as described. 28)

**Quantitation of DOXol** Enzymatic reduction of DOX was conducted in a 10 mL system composed of 0.1 M potassium phosphate, pH 7.4, 0.4 mM NADPH, 10 µM DOX, and CBR1. The reaction products were extracted into 20 mL ethyl acetate 8 h after the reaction was started at 37°C. In the analysis of DOX metabolism in MKN45 cells, 10 µM DOX was added into the culture medium and incubated for 24 h. DOX and its metabolites in the medium were extracted into ethyl acetate. The organic phase was evaporated to dryness, and then dissolved in 50 µL of acetonitrile. The metabolite were analyzed by LC/MS (Hewlett Packard, Waldbronn, Germany) using a Mightysil 5-µm C₁₈ column (Kanto Chemical, Tokyo, Japan), which was eluted isocratically with a mobile phase consisting of 0.1% formic acid–acetonitrile (3:1) at a flow rate of 0.4 mL/min. DOX and DOXol were detected by monitoring their molecular ions (m/z 544.5 and 546.5, respectively), and eluted at the retention times of 24 and 14 min, respectively, which were identical to those of the two authentic compounds.

**Statistical Analysis** Data are expressed as means± standard deviation (S.D.) of at least three independent experiments, unless otherwise noted. Statistical evaluation of the data was performed by using the unpaired Student’s t-test and ANOVA followed by Fisher’s test. A p value <0.05 was considered statistically significant.

**RESULTS**

**Relationship between CBR1 Expression in Gastrointestinal Cancer Cells and DOX Resistance** Western blot analysis of six colon cancer (RKO, LoVo, DLD1, HCT15, HT29 and SW480) and gastric cancer MKN45 cells revealed that basal expression level of CBR1 is high in MKN45, HCT15 and RKO cells, moderate in LoVo cells and low in HT29, SW480 and DLD1 cells (Fig. 1). When sensitivity to DOX was calculated from the change in the cell viability after 24-h treatment with the drug, the LD₅₀ values of MKN45, LoVo, and DLD1 cells were 47.8±6.8, 34.7±6.1, and 22.6±5.1 µM (n=4), respectively. Considering the close relatedness between the CBR1 expression levels of the cells and their sensitivities to DOX, it is possible that CBR1 is a predominant enzyme engaged in acquisition of the DOX resistance. To test this possibility, we established DOX-resistant variants of MKN45, LoVo and DLD1 cells by continuous exposure to stepwise increasing concentrations (from 0.01 to 0.5 µM) of the drug, and monitored alteration in CBR1 expression associated with development of the drug resistance. Although DLD1 cells with low CBR1-expression level were unable to survive against toxicity by treatment with DOX concentrations more than 0.2 µM, 0.5-µM DOX-resistant variants of LoVo and MKN45 cells were easily generated during the drug exposure. The two established variants (LoVo-R and MKN-R) exhibited at least 3-fold higher LD₅₀ values for DOX compared to the parental cells. Semiquantitative PCR- (Fig. 2A) and Western blotting-based
expression analyses (Fig. 2B) showed remarkable higher expression of CBR1 in LoVo-R and MKN-R, compared to their respective parental cells. Being consistent with the results of the both analyses, quantitative PCR assay also resulted in 2.4-fold higher expression of the CBR1 gene in the LoVo-R cells than that in the unresistant LoVo cells (data not shown). The up-regulation of CBR1 with the DOX resistance was further more evident by assaying the reductase activity toward isatin, the preferred substrate for CBR112) (Fig. 2C). The activities in the extracts of LoVo-R and MKN45-R cells (4.5 and 3.6 mU/mg, respectively) were approximately 2-fold higher than those of parental LoVo and MKN45-R cells (2.5 and 2.2 mU/mg, respectively).

To further inquire into the relationship between the up-regulation of CBR1 and DOX resistance in gastrointestinal cancer cells, we prepared the overexpressing phenotypes (1-Ex and 2-Ex) by transient transfection of the expression vector harboring the cDNA for CBR1 in the parental cells. The overexpression of CBR1 was confirmed by Western blotting, in which the band densities were increased by 4.3- (1-Ex) and 5.6-fold (2-Ex) in DLD1 cells and by 2.9- (1-Ex) and 3.8-fold (2-Ex) in LoVo cells, compared to their vector-transfected cells (Figs. 3A, B, insets). The transfectants of DLD1 and LoVo cells significantly mitigated the lethal damage provoked by DOX in a manner dependent on the expression level of CBR1 (Figs. 3A, B). In addition, the silencing for CBR1 strengthened the MKN45 cell toxicity elicited by 40 µM DOX (Fig. 3C), although the siRNA-mediated depletion of the enzyme was incomplete (46% of the basal level). These results clearly indicate that the CBR1 up-regulation plays a central role in acquisition of the DOX resistance.

It is well accepted that transcriptional induction of CBR1 is triggered by ligand binding to an arylhydrocarbon receptor.29) By contrast, Nrf2 has recently been suggested to be a major transcriptional regulator of CBR1 gene.30) We therefore assessed the contribution of Nrf2 to gain of DOX tolerance in gastrointestinal cancer cells. The 24-h incubation of LoVo cells with DOX up-regulated the CBR1 expression, which was declined to the untreated control level by pretreatment with an antioxidant N-acetyl-L-cysteine (NAC) (Fig. 4A). In addition, the expression level of CBR1 was highly elevated by treating not only with HNE, a reactive aldehyde that is produced by peroxidation of membrane lipids and reported to activate Nrf2,31) but also with a potent Nrf2 activator SFN.32) Furthermore, the SFN pretreatment significantly reduced the LoVo cell sensitivity to DOX toxicity (Fig. 4B), suggesting that the DOX treatment up-regulates CBR1 through reactive oxygen species (ROS) generation and the resultant Nrf2 activation, finally leading to the hyposensitivity to the drug. Western blot analysis revealed that levels of nuclear Nrf2 (i.e., its activated form) in their DOX-resistant cells were much higher than those in the parental cells (Fig. 4C). The data may imply that continuous exposure of the cells to DOX causes the aberrant activation of Nrf2, driving to CBR1 up-regulation.
Effects of CBR1 Inhibitor QUE on DOX Resistance  
A survey of previous literature revealed several CBR1 inhibitors, such as such as QUE,12,33) hydroxy-PP,34) epigallocatechin gallate,35) chrysin and zearalenone analogues.36) The IC$_{50}$ values of the inhibitors range from 0.2 to 10 $\mu$M, although the assay conditions are different among the reports. Our evaluation of inhibition of isatin reductase activity in recombinant CBR1 revealed that QUE is a potent inhibitor with an IC$_{50}$ value of 0.15±0.1 $\mu$M. In contrast, QUE showed less potent inhibition against AKR1B10, AKR1C1, AKR1C2 and AKR1C3 (IC$_{50}$ value: 1.9±1, 1.4±0.1, 4.2±0.3, and 8.0±3.0 $\mu$M, respectively), which are suggested to be involved in colon cancer chemoresistance.37) Since QUE was found to be a potent and selective inhibitor of CBR1, its effect on sensitivity of MKN45 cells to DOX was examined (Fig. 5A). Treatment with DOX elicited MKN45 cell damage, which was significantly augmented by the pretreatment with sublethal concentrations (20 and 40 $\mu$M) of QUE. Intriguingly, addition of QUE also made LoVo-R and MKN-R cells more sensitive to DOX (Fig. 5B). In the case of MKN-R cells, the DOX sensitivity increased by 20 $\mu$M QUE was the same as that of MKN45 cells, implying that the inhibitor almost completely overcame the DOX resistance of MKN45 cells (Fig. 5Bb). The proliferative rates of the two resistant cells in the presence of DOX were also decreased by the addition of QUE, and this effect was sustained for at least 4 d (Fig. 5C). QUE did not affect the expression of CBR1 in LoVo and MKN45 cells as illustrated by representative results of its long-term (15 d) exposure (Fig. 5D). Together with the results that QUE significantly potentiates DOX sensitivity of two lymphoma cells (Supplementary Figure S1), these suggest that QUE is an effective adjuvant chemotherapeutic agent for hyperresponsiveness of cancer cells toward DOX.

Molecular Mechanisms by Which Up-Regulation of CBR1 Decreases the DOX Sensitivity  
In vitro measurement using purified CBR1 showed that the enzyme is one of the effective reductases for catalyzing the two-electron reduction of the C-13 carbonyl group of DOX into its alcohol DOXol, but its $V_{\text{max}}/K_{\text{m}}$ value is lower than those for the two other reductases, AKR1C3 and AKR7A2.38) To elucidate whether CBR1 up-regulated during acquisition of the DOX
resistance decreases chemosensitivity of cells via its catalytic conversion of DOX into DOXol, we first compared the DOX reductase activities in the resistant (LoVo-R and MKN-R) and parental (LoVo and MKN45) cells. The activities in the resistant cells were more than 2-fold higher than those in the parental cells (Fig. 6A), but were much lower than the reductase activities toward isatin (Fig. 2C). When measurement of DOXol formed in 8-h culture of Lovo or MKN45 cells with 10 \( \mu \text{M} \) DOX followed, its amount was only 3% of DOX. In addition, the transient overexpression of CBR1 in the cells did not alter the DOXol formation (Fig. 6B) and DOX-reductase activity (0.23±0.05 mU/mg in LoVo 1-Ex cells versus 0.21±0.03 mU/mg in vector-transfected LoVo cells). Even in the reaction that was incubated for 8 h with the low concentration (10 \( \mu \text{M} \)) of DOX, 0.4 mM NADPH and 200 \( \mu \text{g} \) of the recombinant CBR1, only 6% of DOX was converted into DOXol. In the NADPH-linked reduction containing less than 100 \( \mu \text{g} \) of the recombinant enzyme, DOXol was hardly yielded (data not shown). The data suggest that the elevation of DOX reductase activity by developing the drug resistance is due to enzymes other than CBR1.

CBR1 is known to efficiently reduce ONE that is more cytotoxic than HNE.13) The reductase activities toward ONE in the resistant (LoVo-R and MKN-R) cells were significantly higher compared to the parental cells (Fig. 7A). The CBR1 overexpression also resulted in a significant increase in the ONE-reductase activity in LoVo cells (0.62±0.07 mU/mg in 1-Ex cells versus 0.36±0.05 mU/mg in vector-transfected control cells). When the ONE-elicited damage was compared between MKN45 and MKN-R cells, it was lower in MKN-R cells than in the parental cells (Fig. 7B). In addition, the ONE-elicited damage in the resistant cells was restored to the level of the parental cells by pretreating with the CBR1 inhibitor QUE. The results clearly suggest that CBR1 facilitates the DOX resistance of gastrointestinal cells probably through the detoxification of reactive lipid-derived aldehydes such as ONE.
DISCUSSION

In the present study, we show that there is a close relationship between CBR1 expression and DOX sensitivity in the gastrointestinal cancer cells. In addition, it was found that CBR1 is highly induced during the development of DOX resistance through formation of ROS, as well as previously reported hypoxia. Based on results of our previous promoter gene analysis and the marked induction during the treatment of LoVo cells with the Nrf2 activator SFN (Fig. 4A), we assume that up-regulation of CBR1 is mainly mediated due to activation of Nrf2/Keap1 signaling pathway. The assumption may be also supported by other reports indicating up-regulation of CBR1 by three known Nrf2 activators (butylated hydroxyanisole39) and phenetyl isothiocyanate 41) other than SFN. Under the basal conditions, intracellular levels of Nrf2 are maintained at the low levels through the ubiquitin-dependent proteosomal degradation. 42,43) In response to oxidative stress including ROS and electrophiles, Nrf2 is released from the Nrf2–Keap1 heterodimer by modifying reactive cysteines within Keap1. The released Nrf2 translocates into the nucleus to bind to antioxidant response elements in the promoters of many phase-II drug metabolizing enzyme genes [NAD(P)H quinone oxidoreductase-1, heme oxygenase-1, 3-glutamylcysteine synthetase and glutathione S-transferases] that play important roles in the defense against oxidative stress. A 24-h treatment of MKN45 cells with DOX increased the protein adducts with HNE, that activates Nrf2, by approximately 3-fold of the untreated control cells (data not shown). Thus, it is suggested that the CBR1 overexpression in the DOX-resistant cells results from Nrf2 activation through continuous formation of ROS and HNE. Previous studies have speculated that the aberrant expression of Nrf2 genes confers advantage potentials to cancer cells due to up-regulation of the above phase-II drug metabolizing enzymes for protection against various cell stresses. Additionally, the hyper-activation of Nrf2 is in many cases considered to result from various mutations in Keap1, contributing to the development of chemotherapy-resistant cells. Indeed, knockdown of Nrf2 using the siRNA appears to elevate chemosensitivity of colon cancer cells. On the other hand, an investigation using clinical specimens by Yoo et al. showed that among 499 cancer tissues from lung, breast, colon, stomach, liver, larynx and prostate, somatic mutations of Keap1 are the highest in gastrointestinal cancers (18.9%), followed in turn by hepatocellular carcinomas (8.9%) and lung cancers (4.6%). These may imply that the constitutive Nrf2 activation observed in the DOX-resistant cells is due to the Keap1 mutation during the continuous exposure of DOX. As
Colorectal and liver cancers exhibit high refractory to single drug and combination therapies. Therefore, there is an urgent need for the development of effective therapeutic strategies and novel adjuvant chemotherapeutics that do not cause the refractoriness. Although the detailed mechanisms underlying the low responsiveness of the anticancer drugs in the cancers are ambiguous, one of the most convincing reasons might be high expressions of metabolic enzymes that conjugate and detoxify the drugs. Although CBR1 is ubiquitously distributed in normal human organs and its high expression is detected in the liver, kidney and gastrointestinal tract, clinical investigation using tumor specimens indeed revealed overexpression of CBR1 in the hepatocellular carcinoma cells and colon cancer tissues. In this study, we demonstrate that CBR1 is a diagnostic and prognostic biomarker for developing DOX resistance of gastrointestinal cancers. In addition, the CBR1 up-regulation is considered to be a key mechanism supporting gain of the resistance to the chemotherapy, as obvious from the CBR1-dependent reduction of DOX damage in Fig. 3. This study also provides evidence that addition of QUE, a potent and specific CBR1 inhibitor, is capable of significantly suppressing the DOX resistance of the two gastrointestinal cells (Figs. 5A to C). The results may indicate that CBR1 is a predominant factor involved in the DOX resistance of the cancer cells. Although treatment with QUE potentiates colorectal cancer cell apoptosis elicited by 5-fluorouracil and hypoxia, CBR1 may be in part involved in the apoptotic potentiation. Due to its anti-proliferative activity of gastrointestinal cancer cells and long-term efficacy on the chemoresistance (Figs. 5C, D), QUE is useful not only as an anti-cancer drug, but also as an adjuvant therapeutic agent that prevents the DOX resistance. CBR1 exhibits reductase activity toward DOX and is considered to be the predominant DOX reductase in human liver, whereas its \( K_m \) value for DOX (167 or 540 \( \mu M \)) is substantially higher than the blood concentration (0.1 to 1 \( \mu M \)) detected in patients who received the chemotherapy. Under our assay conditions using DOX at the low concentration (10 \( \mu M \)), recombinant CBR1 (less than 100 \( \mu g \)) and the overexpression into LoVo cells hardly reduced DOX into its C-13 alcohol metabolite DOXol. These results strongly indicate that CBR1 scarcely reduces the pharmacokinetic concentrations of DOX in the gastrointestinal cancer cells. Here, we propose that the potentiation of antioxidant properties, rather than the DOX reduction, is the key process for obtaining the drug resistance. The possible target molecule of CBR1 is the lipid-derived reactive aldehyde ONE that exerts potent cytotoxicity, as apparent from the clear improvement of ONE resistance in the MKN-R cells by treating with QUE (Fig. 7B). In addition, the \( k_{cat}/K_m \) value for ONE is 110-fold higher than that for DOX. The positive relationship between detoxification activity towards the reactive aldehydes (ONE and HNE) and chemoresistance development is consistent with that of colon cancer cells resistant to mitomycin-C and cisplatin, both of which generate ROS as their anticancer mechanism. It is reasonable to assume that elevation of cytoprotective activities against ROS and the resultant reactive aldehydes such as ONE is one of the most crucial steps in the development of DOX resistance in gastrointestinal cancer mentioned above, some researches argued that transcriptional activation of CBR1 is regulated through a transcription factor hypoxia-inducible factor-1 \( \alpha \). Further accumulation of precise information about the factors responsible for the transcriptional regulation of CBR1 and sequence of the promotor region is therefore required to explain the clear discrepancy.

**Fig. 6. Relationship between DOX-Reductase Activity and CBR1 Expression in the Drug-Resistant Cells**

(A) DOX-reductase activity in extracts of parental (LoVo and MKN45) and resistant (LoVo-R and MKN-R) cells. *Significant difference from the parental cells (open bar), \( p<0.05 \). (B) Effect of CBR1 overexpression on DOXol formation in LoVo and MKN45 cells. (a) The CBR1-overexpressing cells (1-Ex) and control cells (Vector) were prepared as described in Fig. 3. The cells were treated for 8h with 10 \( \mu M \) DOX, and the DOX metabolites extracted from the medium were analyzed by LC/MS. The LC/MS chromatograms (m/z=545.5) of the DOX metabolites in the medium of vector- and CBR1-transfected MKN45 cells are shown in (b). n.s.: no significance. 1: DOXol, 2: DOX.
cells. Although DOX reductase activities in the resistant cells are significantly higher than those in the control cells (Fig. 6A), the major metabolic enzyme is not identified yet. Since three AKRs (1C1, 1C3 and 1B10) are reported to be also Nrf2-driven genes\(^{36,37}\) and to reduce the anthracycline drugs into their corresponding alcohols,\(^{38,49}\) further studies in our laboratory are underway to define pathophysiological roles of the four AKRs in development of the chemoresistant gastrointestinal cancer cells.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**REFERENCES**


**Fig. 7. Resistance to ONE Toxicity by CBR1 Up-Regulation**

(A) ONE-reductase activity. The activity was analyzed using the cell extracts prepared in Fig. 6A. * Significant difference from the parental cells (open bar), p<0.05. (B) Effect of DOX resistance on cellular damage elicited by ONE. MKN45 and MKN-R cells were treated for 24h with 10µM ONE. A group of MKN-R cells was pretreated for 2h with 40µM QUE prior to the 24h treatment with 10µM ONE. The cellular damage was assessed by measuring viability, in which the value of MKN45 cells treated with DMSO alone was taken as 100%. * Significant difference from the MKN45 cells treated with ONE, p<0.05. * Significant difference from the MKN-R cells treated with ONE alone, p<0.05.


Kensler TW, Wakabayashi N, Nrf2, friend or foe for chemoprevention? *Carcinogenesis*, 31, 90–99 (2010).


Matsunaga T, Yamane Y, Iida K, Endo S, Banno Y, El-Kabbani O, Hara A. Involvement of the aldo-keto reductase, AKR1B10, in


