Note

Identification of Carbonyl Reductase 1 as a Resveratrol-Binding Protein by Affinity Chromatography Using 4'-Amino-3,5-dihydroxy-*trans*-stilbene

Yuta ITO¹, Takakazu MITANI¹, Naoki HARADA¹, Atsushi ISAYAMA¹, Shinji TANIMORI¹,
Shigeo TAKENAKA², Yoshihisa NAKANO³, Hiroshi INUI¹ and Ryoichi YAMAJI^{1,*}

¹Division of Applied Life Sciences, and ²Division of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan

³Osaka Women's Junior College, Fujiidera, Osaka 583–8558, Japan

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Summary The mechanisms by which resveratrol (3,4',5-trihydroxy-*trans*-stilbene) elicits diverse health benefits remain unclear because the intracellular target molecules of resveratrol are poorly defined. We screened resveratrol-binding proteins from lysates of MCF-7 breast cancer cells using resveratrol-affinity resin, which was constructed by immobilizing 4'-amino-3,5-dihydroxy-*trans*-stilbene on activated CH-Sepharose. On SDS-PAGE, two bands were detected as proteins that specifically bound to the resveratrol-affinity resin. One of these, a 30-kDa protein, was identified as human carbonyl reductase 1 (CBR1) by hybrid linear ion trap/time-of-flight mass spectrometry. Similarly, recombinant CBR1 bound to the resveratrol-affinity resin in the absence of resveratrol, but not in the presence of resveratrol. Among its activities, CBR1 catalyzes a NADPH-dependent reduction of the anticancer drug doxorubicin to the cardiotoxin doxorubicinol. The effects of doxorubicin on viability of MCF-7 cells were enhanced by resveratrol, 3,5-dihydroxy-4'-methoxy-*trans*-stilbene, 3,4'-dihydroxy-5-methoxy-*trans*-stilbene, and 4'-amino-3,5-dihydroxy-*trans*-stilbene at concentrations of 1 and 10 μ M. Resveratrol and these derivatives inhibited CBR1 activities to a similar degree at concentrations of 100 and 200 μ M. However, 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene and *m*-hydroquinone had no influence on doxorubicin cytotoxicity or CBR1 activity. Resveratrol inhibited CBR1 activity through an apparent mix of competitive ($K_i = 55.8 \mu$ M) and noncompetitive ($\alpha K_i = 164 \mu$ M; $\alpha = 2.98$) inhibition kinetics. These results indicate that (i) resveratrol enhances the cytotoxic effects of doxorubicin on MCF-7 cells; (ii) the moiety that contains the 3,5-dihydroxyl groups of resveratrol, but not the *m*-hydroquinone structure alone, is required to bind CBR1; and (iii) resveratrol acts as a mixed-type inhibitor of CBR1 activity on doxorubicin.

Key Words resveratrol, carbonyl reductase 1, affinity chromatography, doxorubicin, breast cancer cells

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a phytopolyphenol that is present in a variety of edible plant products including berries and peanuts (1, 2). Because resveratrol is abundant in grape skin, its highest concentrations are found in grape products such as grape juice and wine, particularly red wine (3). For plants themselves, resveratrol is a natural phytoalexin produced in response to adverse conditions such as environmental stress or fungal infection (4). On the other hand, for humans, resveratrol exerts preventive effects against pathological processes such as inflammation (5), atherosclerosis (6), cardiovascular disease (7), and tumorigenesis in a variety of cancers including breast cancer

(8). Furthermore, resveratrol enhances the cytotoxic effect of doxorubicin on the growth of human breast cancer MCF-7 cells (9). However, the mechanisms by which resveratrol provides such diverse health benefits remain unclear because few of the intracellular target molecules of resveratrol have been identified.

Resveratrol has two kinds of phenols. Some structural features of resveratrol such as the number and the position of hydroxyl groups play an important role in its inhibitory effects on various enzymes (10, 11). The hydroxyl group in the 4' position of resveratrol with stereoisomery in the *trans*-conformation (4'-hydroxystyryl moiety) is required for binding to estrogen receptor (ER) α and anti-proliferative activity against MCF-7 cells (12). Furthermore, 4-hydroxy-*trans*-stilbene, or perhaps just its 4-hydroxystyryl moiety, up-regulates the expression of cyclins A, E, and B₁ (13). On the other hand, the *m*-hydroquinone moiety of resveratrol (resorcinol) is the

*To whom correspondence should be addressed.

E-mail: yamaji@biochem.osakafu-u.ac.jp

Abbreviations: CBR1, carbonyl reductase; ER, estrogen receptor.

minimum structure necessary to inactivate cyclooxygenase-1 (14).

Quinone reductase 2 (15), glutathione sulfotransferase- π , and ER β (16) have been identified as resveratrol-binding proteins by resveratrol-affinity chromatography. Resveratrol is immobilized on epoxy-activated agarose through any one of three hydroxyl groups. Thus, the resveratrol-specific bio-affinity strategy provides no information about which hydroxyl groups of resveratrol are required for the binding of resveratrol-binding proteins to the affinity resin. The affinity resin is useful as a tool to search for resveratrol-binding proteins, which are required for at least the 4'-hydroxyl group of resveratrol, because ER β , which is in the same protein family as ER α , binds to the affinity resin. However, it remains unclear whether the affinity resin is useful as a tool to search for resveratrol-binding proteins, which are required for the hydroxyl group of 3 or 5 position of resveratrol. In the present study, we prepared a resveratrol-affinity Sepharose resin by immobilizing 4'-amino-3,5-dihydroxy-*trans*-stilbene on activated CH-Sepharose through its 4'-amino group and identified carbonyl reductase 1 (CBR1) as a resveratrol-binding protein.

CBR1 metabolizes physiological (endogenous) substrates such as prostaglandin E2 (17) and *S*-nitroglutathione (18) or xenobiotic (exogenous) substrates such as anthracycline anticancer drugs (doxorubicin and daunorubicin) (19). Doxorubicin is used to treat many cancers including breast cancer because it results in DNA damage by intercalating the DNA base pairs of the double helix. However, CBR1 catalyzes the reduction of doxorubicin to doxorubicinol, which is less potent in killing cancer cells and induces chronic cardiotoxicity. Some tumor cells acquire resistance to doxorubicin through the increased expression of CBR1 (20), indicating that resistance and toxicity limit the effectiveness of doxorubicin. Therefore, we further investigated the effects of resveratrol and its derivatives on the cytotoxicity of doxorubicin in MCF-7 cells and CBR1 activity using doxorubicin as a substrate and characterized the kinetics of resveratrol as a CBR1 inhibitor.

Materials and Methods

Reagents. Resveratrol, 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene, and 3,4'-dihydroxy-5-methoxy-*trans*-stilbene were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 4'-Amino-3,5-dihydroxy-*trans*-stilbene was synthesized according to the method of Sun et al. (21), and 3,5-dihydroxy-4'-methoxy-*trans*-stilbene was synthesized according to the method of Paul et al. (22).

Cell culture. Human breast cancer MCF-7 cells were obtained from the RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured in growth medium (Dulbecco's modified Eagle's medium-high glucose (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin) at 37°C in a 5% CO₂/95% air atmosphere at 100% humidity.

Preparation of resveratrol-affinity resin. Activated

CH-Sepharose (1 g freeze-dried powder; GE Healthcare, Uppsala, Sweden) was activated by 1 mM HCl and incubated with 4'-amino-3,5-dihydroxy-*trans*-stilbene (25 mg) in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, and 50% ethanol) at 4°C for 4 h. The Sepharose resin which was washed several times with coupling buffer and incubated with 1 M monoethanolamine at 4°C for 1 h, followed by washing with coupling buffer, was termed resveratrol-affinity resin.

Identification of resveratrol-binding proteins. MCF-7 cells were sonicated in lysis buffer (20 mM Hepes-NaOH, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/mL of leupeptin, and 1 μ g/mL of aprotinin) and centrifuged at 20,000 $\times g$ at 4°C for 10 min. The supernatant was used as a crude extract throughout the experiments. The crude extract (2 mg protein) was incubated with resveratrol-affinity resin (100 μ L; 50% slurry) in the presence or absence of resveratrol (1 mM), and proteins bound to the resin were subjected to SDS-PAGE, followed by silver staining. The selected gel bands, which were not detected in the presence of resveratrol, were subjected to in-gel trypsin digestion as described previously (23) and analyzed with an ion trap/time-of-flight mass spectrometer (LCMS IT-TOF, Shimadzu Corp., Kyoto, Japan). Data were further analyzed by the Mascot MS/MS Ions Search algorithm to identify proteins corresponding to the bands.

Recombinant CBR1 protein. Human CBR1 cDNA was amplified by nested PCR and cloned into pCR2.1-TOPO vector, followed by sub-cloning into pET30 vector. The resultant pET30-CBR1 was transformed into *Escherichia coli* BL21(DE3), and the C-terminal His-tagged recombinant CBR1 was purified using Ni-Sepharose FF (GE Healthcare). The purified protein, which was detected as a single band by SDS-PAGE, was used throughout the experiments.

CBR1 activity. Purified recombinant CBR1 was prepared as described above. For the inhibitory effects of resveratrol and its derivatives on CBR1 activity, the enzyme activities were measured at 37°C for 10 min in 100 μ L of reaction mixture containing 100 mM sodium phosphate buffer, pH 7.0, 100 μ M NADPH, 500 μ M doxorubicin, enzyme (4 μ g protein), and resveratrol or its derivatives (100 and 200 μ M). For the kinetics studies, the assay reaction was performed in 100 μ L of reaction mixture containing 100 mM sodium phosphate buffer, pH 7.0, 100 μ M NADPH, doxorubicin (0–500 μ M), enzyme (4 μ g protein), and resveratrol (0–200 μ M). The measured rates for 10 min were in the linear range of the enzyme kinetic curve. The reaction was stopped by the addition of 100 μ L of ice-cold ethanol on ice, followed by the further addition of 10 μ L of 250 μ M daunorubicin as an internal standard and vortex mixing. The mixture was centrifuged for 20,000 $\times g$ at 4°C for 5 min, and the supernatant was injected into a HPLC system (Nihonbunkoh, Tokyo, Japan) with a fluorescence detector (excitation at 480 nm and emission 560 nm). Separation was performed on a reverse phase

column (C18 AR II, 4.6 mm I.D.-150 mm; Nakalai Tesque, Inc., Kyoto, Japan), with a mobile phase (50 mM monosodium phosphate buffer/acetonitrile [(v/v) 3 : 1] (pH 4.0)) at a flow rate of 0.5 mL/min. A calibration curve with doxorubicinol standard (10–400 pmol) was prepared.

Western blot. Crude extract (2 mg protein) or recombinant CBR1 (10 μ g protein) was incubated with resveratrol-affinity resin (100 μ L; 50% slurry) in the presence or absence of resveratrol or quercetin, and proteins bound to the resin were subjected to SDS-PAGE, followed by Western blot analysis with anti-CBR1 antibody (Aviva Systems Biology, San Diego, CA). Immunoreactive proteins were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody and reacted with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), followed by detection with an LAS4000 imaging system (GE Healthcare).

Viability. MCF-7 cells (2.5×10^3 cells/well) were plated in a 48-well plate and cultured in growth medium for 24 h, followed by incubation in the presence of vehicle (DMSO), doxorubicin (0–10 nM), or resveratrol or its derivatives (0–10 μ M) for 72 h. Cells were further incubated in growth medium without phenol red for 1 h, followed by treatment with Alamar Blue reagent at a final concentration of 5% (Alamar Bioscience, Chicago, IL) for an additional 4 h according to the manufacturer's instructions. The fluorometric assay (excitation at 544 nm and emission 590 nm) was performed using Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis. The effects of resveratrol and its derivatives on cell viability and CBR1 activity were evaluated by two-way analysis of variance with Tukey's post hoc testing. Statistical analysis was performed using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC). Data are expressed as mean $\pm$ SD, and differences were considered statistically significant at a *p* value of <0.05.

Results

To screen proteins that interact with one or both of the hydroxyl groups in the *m*-hydroquinone moiety of resveratrol, 4'-amino-3,5-dihydroxy-*trans*-stilbene was synthesized according to the method of Sun et al. (21) and was immobilized on Sepharose through its amino group (Fig. 1A). Proteins in MCF-7 cell lysates were incubated with the resin in the presence or absence of resveratrol, and proteins bound to the resin were analyzed by SDS-PAGE, followed by silver staining (Fig. 1B). Two bands (arrows in Fig. 1B) were detected as proteins that specifically bound to resveratrol. Peptide sequences predicted by the mass spectrometric analysis were blasted against the protein databases, and nine peptides from one (lower arrow in Fig. 1B) of the two bands matched CBR1 (Fig. 1C). The sequence coverage was 36%. Western blot analysis with anti-CBR1 antibody showed that CBR1 bound to the resveratrol-affinity resin in the absence of resveratrol, but not in the presence of resveratrol (Fig. 1D). Furthermore, to determine

Fig. 1. Identification of CBR1 as a resveratrol-affinity protein. (A) Schematic representation of the resveratrol-immobilized resin (resveratrol-affinity Sepharose) constructed in this study. (B) MCF-7 cell lysates were incubated with the resveratrol-affinity resin in the presence or absence of resveratrol. Proteins bound to the resin were subjected to SDS-PAGE, followed by silver staining. Arrows indicate protein bands that were not detected in the presence of resveratrol. (C) Amino acid sequences of peptide fragments were obtained with an ion trap/time-of-flight mass spectrometer and Mascot MS/MS ion Search algorithm. (D) MCF-7 cell lysates were incubated with the resveratrol-affinity resin in the presence or absence of resveratrol, followed by Western blot analysis with anti-CBR1 antibody. (E) Recombinant CBR1 was incubated with the resveratrol-affinity resin in the presence or absence of resveratrol or quercetin, followed by Western blot analysis with anti-CBR1 antibody.

whether CBR1 directly binds to resveratrol, a C-terminal His-tagged recombinant CBR1 protein was purified and incubated with the resin in the presence or absence of resveratrol. The binding of recombinant CBR1 protein to the resin was inhibited in the presence of resveratrol (Fig. 1E). When the recombinant CBR1 protein was incubated with the resin in the presence of quercetin, the binding of CBR1 to the resin was inhibited. These results indicate that resveratrol physically interacts with CBR1 and suggest that the interaction of quercetin with CBR1 occurs at the same site as the resveratrol-binding site on CBR1. On the other hand, although another resveratrol-affinity resin was reported to bind ER β (16), a Western blot of the proteins bound to our resin using anti-ER β antibody detected no bands (data not shown).

Fig. 2. Enhancing effects of resveratrol and its derivatives on cytotoxicity of doxorubicin in MCF-7 cells. (A) Chemical structures. (B) MCF-7 cells were cultured in the presence or absence of doxorubicin (DOX) (5 or 10 nM), and cell viabilities were determined. (C) MCF-7 cells were cultured in the presence of vehicle (Veh), 3,4',5-trihydroxy-*trans*-stilbene (Res), 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene (4'-OH), 3,5-dihydroxy-4'-methoxy-*trans*-stilbene (3,5-OH), 3,4'-dihydroxy-5-methoxy-*trans*-stilbene (3,4'-OH), 4'-amino-3,5-dihydroxy-*trans*-stilbene (4'-amino), and *m*-hydroquinone (*m*-OH) (1 or 10 μM), and cell viabilities were determined. (D) Cells were cultured with resveratrol or its derivatives (1 or 10 μM) in the presence of doxorubicin (5 nM), and cell viabilities were determined. Data are expressed as mean ± SD, and differences were considered statistically significant at a *p* value of <0.05. In all experiments, the result is representative of three independent experiments.

We determined the effects of resveratrol or its derivatives (Fig. 2A) on cell viability in the presence or absence of doxorubicin. Doxorubicin decreased cell viability at a concentration of 10 nM, but had no influence on cell viability at a concentration of 5 nM (Fig. 2B). Resveratrol and its derivatives at concentrations of 1 and 10 μM had no influence on cell viability (Fig. 2C). However, in the presence of 5 nM doxorubicin, cell viability was repressed to a similar degree by resveratrol, 3,5-dihydroxy-4'-methoxy-*trans*-stilbene, 3,4'-dihydroxy-5-methoxy-*trans*-stilbene, and 4'-amino-3,5-dihydroxy-*trans*-stilbene individually at concentrations of 1 and 10 μM (Fig. 2D). However, 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene and the *m*-hydroquinone moiety of resveratrol (resorcinol) had no influence on cell viability. These results indicate that resveratrol enhances the potential of the anticancer drug doxorubicin in MCF-7 cells.

We assessed the effects of resveratrol on the activity of CBR1 using doxorubicin as a substrate. The IC₅₀ of resveratrol on CBR1 activity was estimated to be

approximately 140 μM. When the effects of resveratrol derivatives on CBR1 activity were determined, CBR1 activity was inhibited to a similar degree by resveratrol, 3,5-dihydroxy-4'-methoxy-*trans*-stilbene, 3,4'-dihydroxy-5-methoxy-*trans*-stilbene, and 4'-amino-3,5-dihydroxy-*trans*-stilbene, each at concentrations of 100 and 200 μM (Fig. 3). In contrast, 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene and the *m*-hydroquinone moiety of resveratrol (resorcinol) had no influence on CBR1 activity. These results indicate that the moiety with 3,5-dihydroxyl groups of resveratrol is required as the binding site or binding spatial structure to bind CBR1, perhaps through either one of the 3,5-dihydroxyl groups.

To characterize the mechanism by which resveratrol inhibits CBR1, we examined the enzyme kinetics parameters. Resveratrol inhibited the activity of CBR1 in a concentration-dependent manner, and the lines for each inhibitor concentration crossed on the left of the Y-axis on the Lineweaver-Burk plots (Fig. 4A), indicating that

Fig. 3. Inhibition of CBR1 activities by resveratrol and its derivatives with doxorubicin. CBR1 activities were determined in the presence of vehicle (Veh), 3,4',5-trihydroxy-*trans*-stilbene (Res), 3,5-dimethoxy-4'-hydroxy-*trans*-stilbene (4'-OH), 3,5-dihydroxy-4'-methoxy-*trans*-stilbene (3,5-OH), 3,4'-dihydroxy-5-methoxy-*trans*-stilbene (3,4'-OH), 4'-amino-3,5-dihydroxy-*trans*-stilbene (4'-amino), and *m*-hydroquinone (*m*-OH). Data are expressed as mean $\pm$ SD, and differences were considered statistically significant at a *p* value of <0.05 . The result is representative of three independent experiments.

resveratrol acts on CBR1 activity as a mixed-type inhibitor when using doxorubicin as a substrate (Fig. 4B). To calculate K_i and α , the slope and the intercept of the Lineweaver-Burk lines for concentrations of resveratrol as an inhibitor were plotted as a function of inhibitor concentration (Fig. 4C). The intersections of the lines from slope and intercept with the X-axis show K_i ($55.8 \pm 12.0 \mu\text{M}$) and αK_i ($164 \pm 51.5 \mu\text{M}$; $\alpha = 2.98 \pm 0.98$), respectively. Furthermore, the Lineweaver-Burk plots showed a K_m value of $158 \pm 40.1 \mu\text{M}$, indicating an αK_m value of $448 \pm 44.0 \mu\text{M}$. These results indicate that the affinity of the CBR1-doxorubicin complex for resveratrol is approximately 3-fold lower than that of CBR1 for doxorubicin.

Discussion

There is increasing evidence that resveratrol has health benefits. Although resveratrol is speculated to exert diverse biological functions through specific cellular mediators such as proteins, few proteins have been shown to bind directly to resveratrol. In the present study, we developed a resveratrol-affinity resin as a tool to search for and analyze specific cellular mediators of resveratrol. The resveratrol-affinity resin was prepared by binding the 4'-amino group of 4'-amino-3,5-dihydroxy-*trans*-stilbene to Sepharose. CBR1 must interact with 3,5-dihydroxyl groups of resveratrol to physically interact with resveratrol. Another resveratrol-affinity resin was prepared by immobilizing any one of three hydroxyl groups of resveratrol on agarose (15, 16). That resin bound quinone reductase 2 (15), glutathione sulfotransferase- π , and ER β (16). The interaction between resveratrol and ER α , which is in the same protein fam-

Fig. 4. Kinetics analysis of CBR1 inhibition by resveratrol. (A) CBR1 activities using the substrate doxorubicin were determined in the presence of various concentrations of resveratrol. For each concentration of resveratrol, the inverse of the velocity is plotted as a function of the inverse of substrate concentration. (B) Schematic representation of the inhibitory mechanism is shown. (C) The slope (closed circle) and the intercept (open square) of the Lineweaver-Burk representations are plotted as a function of the inhibitor concentration. The result is representative of three independent experiments.

ily as ER β , requires the 4'-hydroxyl group of resveratrol (12). This, together with result that ER β did not bind to our resin, indicates that the present resveratrol-affinity resin provides the structural orientation needed to bind resveratrol-binding proteins because the proteins that bind to resveratrol on the resin are variable depending on how the resveratrol is bound to the resin.

CBR1 catalyzes the conversion of doxorubicin to its cardiotoxic metabolite doxorubicinol. Mice with a null allele of CBR1 are protected from doxorubicinol-induced cardiotoxicity (24), indicating that CBR1 is the main enzyme responsible for the doxorubicinol formation. Therefore, it is expected that the inhibition of CBR1 activity will improve the cardiotoxic side effect of doxorubicinol and increase the therapeutic efficacy of

doxorubicin against tumors in patients during chemotherapy. The present study demonstrates that resveratrol at concentrations of 1 and 10 μM inhibits the growth of MCF-7 cells in the presence of 5 nM doxorubicin although 10 μM resveratrol alone or 5 nM doxorubicin alone has no influence on cell viability. Likewise, Osman et al. (9) reported that cytotoxicity of doxorubicin is enhanced by resveratrol at a concentration of 66 μM although the viability of MCF-7 cells is determined under experimental conditions different from the present study. Thus, combined treatment with resveratrol and doxorubicin is effective in inhibiting the growth of MCF-7 cells, compared to treatment with doxorubicin alone. These results suggest that resveratrol increases the chemosensitivity of MCF-7 cells to doxorubicin by inhibiting CBR1 activity.

The finding that resveratrol inhibited CBR1 activity on doxorubicin with a mixed-type inhibition (Fig. 3) indicates that the inhibition involves binding not only to the CBR1 but also to the CBR1-doxorubicin complex. The flavonoid rutin (25) and the semi-synthetic flavonoid 7-monohydroxyethylrutoside (26) act as a mixed-type inhibitor and a competitive inhibitor of CBR1 activity for daunorubicin, respectively, indicating that the inhibitory mechanisms by which flavonoids inhibit CBR1 activity for anthracyclines depend on the structure of the flavonoids. The *m*-hydroquinone moiety of resveratrol and the flavonoid A-ring of rutin are structurally similar. Taken together, these results suggest that the *m*-hydroquinone moiety of resveratrol contributes to the mixed-type inhibition of resveratrol for CBR1 activity. Furthermore, the effective resveratrol derivatives for cell viability exerted inhibitory effects on CBR1 activity, indicating that the hydroxyl group in the 3 or 5 position of resveratrol plays important roles in inhibiting CBR1 activity and enhancing the cytotoxicity of doxorubicin.

CBR1 catalyzes endogenous substrates such as prostaglandin E2 (17) and *S*-nitrosoglutathione (18) in addition to exogenous substrates. CBR1 activity is inhibited by 7-monohydroxyethylrutoside as a competitive inhibitor for daunorubicin and as an uncompetitive inhibitor for menadione (26). Thus, the mechanisms by which CBR1 is inhibited by certain inhibitors depend on substrates. It is of interest to determine whether resveratrol inhibits CBR1 activity for endogenous substrates.

Some flavonoids inhibit reduction of anthracyclines such as doxorubicin and daunorubicin (25–27). The IC_{50} values of 7-monohydroxyethylrutoside and quercetin, which is the aglycone moiety of rutin, on CBR1 activity for doxorubicin are 59 μM and 43 μM , respectively (26), and these flavonoids seem to be more effective in inhibiting CBR1 activity than resveratrol (IC_{50} = 140 μM). 7-Monohydroxyethylrutoside protects against doxorubicin-induced cardiotoxicity in rats (28), and quercetin enhances therapeutic efficacy of doxorubicin in mice bearing 4T1 breast cancer (29). However, these IC_{50} values are not physiologically achievable concentrations. On the other hand, although the IC_{50} values of doxorubicin for the growth of MCF-7 cells are approximately 690 to 770 nM (9, 30), the K_m value

of CBR1 for doxorubicin was 158 μM , almost identical to previous reports that the K_m values are 287 and 226 μM when recombinant CBR1 and MCF-7 cell lysate are used to determine the kinetics of reduction of doxorubicin, respectively (19, 30). At this time, we have no obvious explanation for the discrepancy between the IC_{50} values (or the K_m values) and the effective concentrations of resveratrol and doxorubicin against cell viability. Recombinant CBR1 might require certain factors to exert the same catalytic function as the native form of CBR1.

Other dietary components besides resveratrol have been shown to have medicinal effects. Quercetin inhibits the activity of CBR1 for doxorubicin and daunorubicin (25). Furthermore, quercetin attenuates hypoxia-induced resistance to doxorubicin in breast cancer 4T1 cells by suppressing the expression of hypoxia-inducible factor-1 α , which is a key regulator of the transcriptional activation of hypoxia-inducible genes (29). Resveratrol, which enhances the cytotoxic effect of doxorubicin on the growth of MCF-7 cells (9), also inhibits the expression of hypoxia-inducible factor-1 α in human ovarian cancer cells (31) and human tongue squamous cell carcinoma (32). However, there is no evidence that quercetin or resveratrol directly interacts with hypoxia-inducible factor-1 α . The present findings suggest that resveratrol attenuates resistance to doxorubicin treatment (for example, hypoxia-induced resistance to doxorubicin) and toxicity of doxorubicin by inhibition of CBR1 activity and by suppression of hypoxia-inducible factor-1 α expression. Identification of the target molecules of resveratrol should contribute to elucidating the function of resveratrol.

Acknowledgments

Both Y. I. and T. M. carried out the experiments and equally contributed to this study. A. I. and S. T. synthesized resveratrol derivatives. S. T. analyzed data of mass spectrometry. N. H., Y. N., and H. I. provided valuable advice. R. Y. contributed to the concept and design of this study. T. M. and R. Y. drafted and revised the manuscript. All authors discussed all results and commented on the manuscript.

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