Carbonyl Reductase 1 as a Novel Target of (−)-Epigallocatechin Gallate Against Hepatocellular Carcinoma

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Human carbonyl reductase 1 (CBR1) converts the antitumor drug and anthracycline daunorubicin (DNR) into the alcohol metabolite daunorubicinol (DNROL) with significantly reduced antitumor activity and cardiotoxicity, and this limits the clinical use of DNR. Inhibition of CBR1 can thus increase the efficacy and decrease the toxicity of DNR. Here we report that (−)-epigallocatechin gallate (EGCG) from green tea is a promising inhibitor of CBR1. EGCG directly interacts with CBR1 and acts as a noncompetitive inhibitor with respect to the cofactor reduced nicotinamide adenine dinucleotide phosphate and the substrate isatin. The inhibition is dependent on the pH, and the gallate moiety of EGCG is required for activity. Molecular modeling has revealed that EGCG occupies the active site of CBR1. Furthermore, EGCG specifically enhanced the antitumor activity of DNR against hepatocellular carcinoma SMMC7721 cells expressing high levels of CBR1 and corresponding xenografts. We also demonstrated that EGCG could overcome the resistance to DNR by Hep3B cells stably expressing CBR1 but not by RNA interference of CBR1-HepG2 cells. The level of the metabolite DNROL was negatively correlated with that of EGCG in the cell extracts. Finally, EGCG decreased the cardiotoxicity of DNR in a human carcinoma xenograft model with both SMMC7721 and Hep3B cells in mice. ***Conclusion:*** **These results strongly suggest that EGCG can inhibit CBR1 activity and enhance the effectiveness and decrease the cardiotoxicity of the anticancer drug DNR. These findings also indicate that a combination of EGCG and DNR might represent a novel approach for hepatocellular carcinoma therapy or chemoprevention.** (HEPATOLOGY **2010;52:703-714**)

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] Chemotherapy is a common treatment modality for inoperable HCC. However, resistance against anticancer drugs is a major problem in the chemotherapy of malignant tumors.

Anthracyclines such as daunorubicin (DNR) and doxorubicin (DOX) are among the most valuable cytostatic agents in chemotherapy.[2] However, their use is limited by the intrinsic or acquired resistance of tumor cells toward them and their toxicity in normal tissues (most notably chronic cardiomyopathy and congestive

Abbreviations: 5-FU, 5-fluorouracil; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CBR, carbonyl reductase; cTnT, cardiac troponin T; DNR, daunorubicin; DNROL, daunorubicinol; DOX, doxorubicin; DOXOL, doxorubicinol; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; IC_{50}, median inhibitory concentration; LDH, lactate dehydrogenase; MDA, malondialdehyde; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; RNAi, RNA interference; SDR, short-chain dehydrogenase/reductase; siRNA, small interfering RNA.

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heart failure).[3,4] Anthracycline resistance not only is a result of alterations in drug uptake and retention but also is caused by an enzymatic anthracycline detoxification pathway that is up-regulated upon the exposure of cancer cells to these drugs.[5,6] Interestingly, 13-hydroxy metabolites of anthracyclines, such as doxorubicinol (DOXOL) and daunorubicinol (DNROL), are significantly less potent than the parent drugs, and this suggests that the carbonyl reduction is an important biochemical mechanism in the detoxification of carbonyl group–bearing anthracyclines.[7,8] More importantly, DNROL and DOXOL have also been reported to be responsible for the cardiotoxicity of DNR and DOX, respectively.[9,10]

In humans, the conversion of DNR and DOX to DNROL and DOXOL is mainly catalyzed by carbonyl reductase 1 (CBR1).[11] CBR1 belongs to the short-chain dehydrogenase/reductase (SDR) family and is ubiquitously expressed in human tissues with particularly high levels in the liver.[12] CBR1 is believed to contribute significantly to the development of resistance toward DNR and DOX. This is supported by the finding that CBR1 overexpression results in DNR resistance in tumor cells.[13,14] DNR resistance in human stomach carcinoma cells has also been shown to result mainly from an induction of CBR1.[15] Furthermore, the role of CBR1 in the severe cardiotoxicity associated with anthracycline treatment has been documented. Mice heterozygous for the null allele of CBR1 have shown reduced sensitivity to anthracycline-induced cardiotoxicity because reduced CBR1 expression produces lower levels of DOXOL.[16]

Because of CBR1's role in the resistance to and toxicity of anthracyclines, it has been speculated that the inhibition of CBR1 to prevent carbonyl reduction may be an effective approach to enhancing the efficiency and reducing the toxicity of anthracyclines.[17] In the SDR family, several enzymes are sensitive to inhibition by flavonoids, a group of natural products of plant origin. Flavonoids were first identified as lens aldose CBR inhibitors in the 1970s.[18,19] More recently, hydroxy-PP has also been reported to inhibit CBR1 and increase the sensitivity of cancer cell lines to DNR treatment (Fig. 1A).[20]

Flavonoids with different chemical structures are widely distributed in plants, vegetables, fruits, and beverages, particularly in tea and red wine. The major flavonoids of green tea extracts are catechins. Among them, (−)-epigallocatechin gallate (EGCG) is most abundant. EGCG has been shown to possess a wide range of pharmacological properties, including chemopreventive, anticarcinogenic, and antioxidant activity.[21,22] We have noticed a structural similarity between catechins and known inhibitors of CBR1, such as quercetin and quercitrin (Fig. 1A). In this report, evidence is presented that EGCG has a previously unknown inhibitory effect on CBR1 and CBR1-mediated tumor resistance to DNR, and this makes EGCG a potential chemotherapeutic agent for HCC.

Materials and Methods

Additional experimental procedures are described in the Supporting Information.

CBR1 Assay and Kinetic Analysis. CBR1 activity was determined on a Jasco V-550 spectrophotometer (Jasco, Inc., Easton, MD) as follows: the decrease in reduced nicotinamide adenine dinucleotide phosphate (NADPH) absorbance at 340 nm at 25°C was monitored for 90 s. The standard assay mixture consisted of 0.1 M potassium phosphate (pH 7.0), 100 μM NADPH, and 200 μM isatin or other substrates as indicated.

DNR Carbonyl Reduction Assay. Cell lysates were prepared as previously described.[15] DNR carbonyl reduction was measured by the incubation of 150 μL of the cell lysate, 100 μM NADPH, and 100 μM DNR in a final volume of 200 μL at 37°C (a 0.1 M potassium phosphate buffer was used to bring up the volume). The reaction was stopped after 30 minutes by the addition of 100 μL of 0.4 M Na_2HPO_4 (pH 8.4). DOX (2 μg) was included as an internal standard. The samples were extracted with 900 μL of a 4:1 (vol/vol) chloroform/methanol mixture. After 15 minutes of vigorous shaking, samples were centrifuged for 10 minutes at 8000 rpm. The organic phase was transferred to a new tube, and the solvent was evaporated under a stream of nitrogen at 25°C. The residue was dissolved in the appropriate mobile phase and analyzed by high-performance liquid chromatography (HPLC). Control experiments were performed without biological material.

Detection and Quantitation of DNROL. After enzymatic conversion, DNR and DNROL were detected on a Shimadzu LG-4A reverse-phase HPLC system with Intertsil ODS-3 (250 × 4.6 mm; GL Science, Inc.) by a published method with some modifications.[23] The mobile phase consisted of a 2:1 (vol/vol) mixture of 50 mM monobasic sodium phosphate and acetonitrile adjusted to pH 4.0 with orthophosphoric acid and filtered through a 0.22-μm membrane (Millipore). The mobile phase was freshly prepared

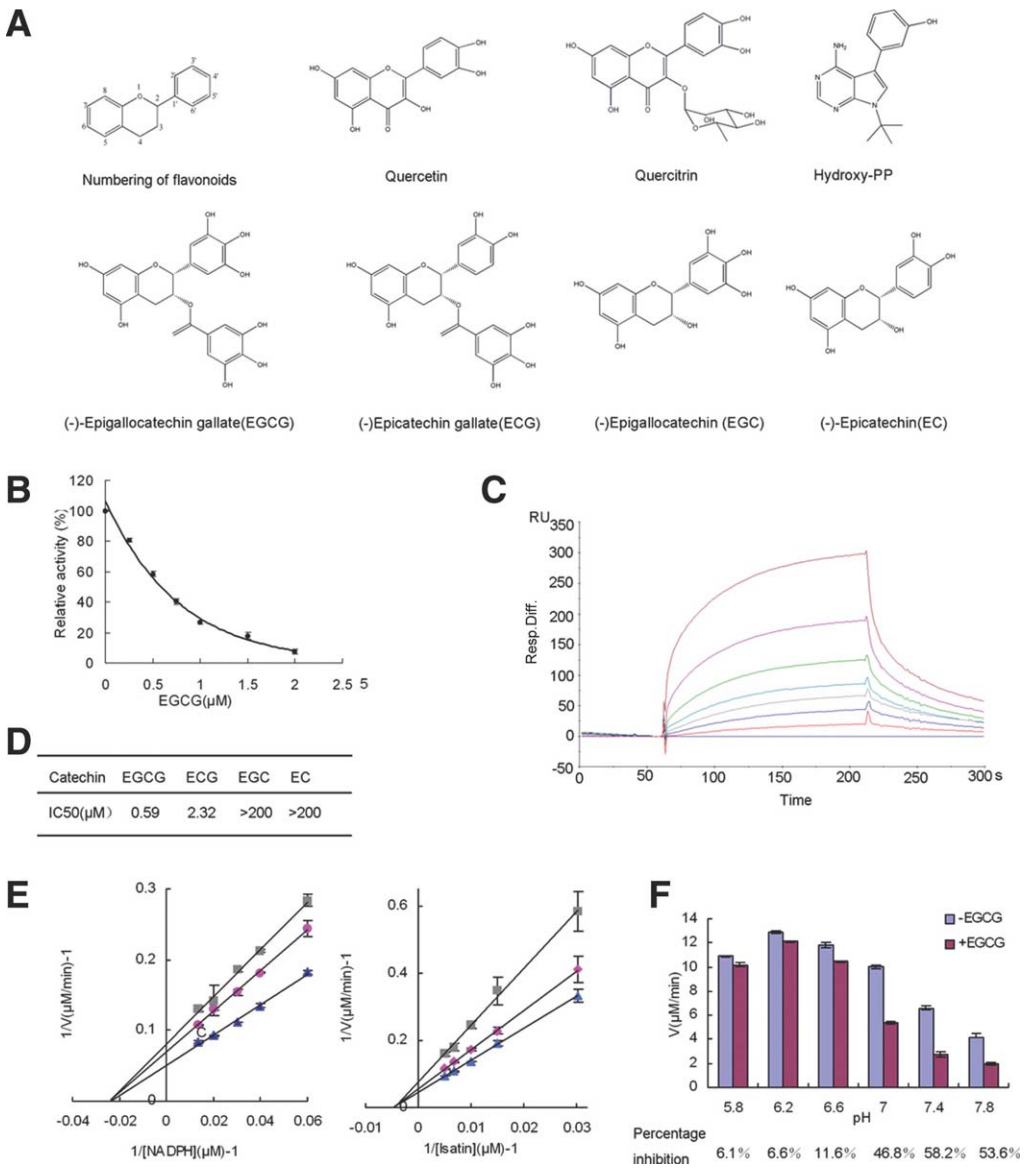

Fig. 1. Effects of EGCG on CBR1 activity. (A) Structures of the flavonoids mentioned in this study. (B) Inhibition of CBR1 activity by different concentrations of EGCG. The reaction of CBR1 was measured in the presence of various concentrations of EGCG with isatin as the substrate. (C) Interaction between CBR1 and EGCG as determined by the Biacore assay. Various concentrations of EGCG were applied to a CBR1-immobilized sensor chip. The resonance unit reflects the number of attached molecules. Red, blue, gray, sky blue, green, purple, and brown lines indicate the binding curves of 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, and 5μM EGCG with CBR1, respectively. The data show the resonance units after the subtraction of the resonance units of CBR1 (−) as background from the experimental unit. (D) Structure-activity relationship of catechins versus CBR1: inhibition of CBR1 activity by catechins. (E) Determination of the inhibitory mechanism of EGCG on CBR1. The concentrations of EGCG were (▲) 0, (●) 0.5, and (■) 0.75 μM. The concentration of isatin was fixed at 200 μM, and NADPH was the variable substrate (left), or the concentration of NADPH was fixed at 100 μM, and isatin was the variable substrate (right). (F) pH dependence of the inhibition of CBR1 activity by EGCG. The activities of the reaction with or without 0.5 μM EGCG were measured at different pHs: 5.8, 6.2, 6.6, 7.0, 7.4, and 7.8. The data represent means and standard deviations (n = 3). V = reaction velocity of the enzyme activity of CBR1; Resp. Diff. = response difference from baseline.

each day and was degassed before use. The flow rate was 1 mL/minute, and the injection volume was 10 μL. Substances were monitored with a Shimadzu SPD-10A ultraviolet-visible detector at an excitation wavelength of 470 nm. Metabolite quantification was performed with the aid of calibration curves generated with known concentrations of authentic DNR.

Results

EGCG Inhibits CBR1 Activity. We overexpressed CBR1 in *Escherichia coli*, purified recombinant CBR1 nearly to homogeneity, and verified its purity and authenticity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and mass spectrometry (Supporting

Fig. 1 and Supporting Information Table 1). The NADPH-dependent CBR activity with isatin and DNR as substrates was determined with Michaelis constants of 0.021 and 0.10 mM, respectively, which were comparable to those reported in the literature.[24] Next, we determined the effect of EGCG on purified recombinant CBR1 with isatin as a substrate. EGCG had a dose-dependent inhibitory effect on CBR1 activity with a median inhibitory concentration (IC_{50}) of 0.59 μM (Fig. 1B). The direct interaction between EGCG and CBR1 was assessed with Biacore. The resonance units of EGCG to CBR1 increased in a dose-dependent manner, and the affinity constant was estimated to be 2.73 μM (Fig. 1C).

We then determined the effects of EGCG analogues, including (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin (EGC), on CBR1 activity (Fig. 1A). ECG, which contains the gallate moiety, also inhibited CBR1 activity in a manner similar to that of EGCG (IC_{50} = 2.32 μM). The other analogues, EC and EGC, showed much weaker inhibition of CBR1 activity with only partial inhibition of CBR1 at 200 μM (Fig. 1D). These results indicate that the gallate moiety of EGCG is crucial for inhibition of CBR1. We examined the kinetic mechanism of CBR1 inhibition by EGCG by holding the concentration of EGCG constant and measuring the effect of increasing cofactor NADPH concentrations on the initial reaction rate. Lineweaver-Burk plots indicated that EGCG inhibits CBR1 noncompetitively with respect to NADPH (Fig. 1E, left panel). Similarly, EGCG is also a noncompetitive inhibitor of CBR1 against isatin (Fig. 1E, right panel).

The activity of CBR1 is known to be sensitive to the pH. Therefore, we examined whether the inhibition of CBR1 activity by EGCG is also dependent on the pH. The pH of the assay buffer was varied from 5.8 to 7.8 with other conditions fixed. The pH for optimal CBR1 activity was 6.2, and this was consistent with the literature.[24] The inhibition by EGCG was strong under neutral and weakly alkali conditions (the percentage inhibition was about 50%) but was significantly weaker under weakly acidic conditions (the percentage inhibition was about 10%; Fig. 1F). The absorption spectrum of EGCG was also strongly affected by the pH (Supporting Information Fig. 2A). The absorbance maximum underwent a bathochromic displacement from alkali conditions to acidic conditions. The absorption spectrum of the nongalloylated counterpart EGC showed no obvious change in the pH range of 5.8 to 7.8 (Supporting Information Fig. 2B).

Molecular Modeling of the CBR1-EGCG Complex. To further dissect the interaction of EGCG with CBR1, we established a binding model for the CBR1-EGCG complex with molecular modeling techniques. The model was tested by some active site mutants of CBR. The CBR1 mutants were generated and purified nearly to homogeneity (Supporting Information Fig. 3). The carbonyl reduction activities of the mutants were not significantly different from those of the wild-type enzyme, and this made it possible to determine the inhibition of those mutants by EGCG (Supporting Information Table 2).

The docking analysis selected the conformation of EGCG in the active site of CBR1 with the lowest free energy. EGCG was bound to CBR1 by geometric placement in the active site of CBR1. As shown in Fig. 2A, all residues in the active site were selected to generate a cavity, and EGCG was docked into the cavity. Six hydrogen bonds were established between hydroxyl groups of EGCG and hydrogen-bond acceptors (nitrogen or oxygen) in CBR1. The polyphenol structure of EGCG appeared to be crucial for its binding to CBR1. Importantly, the phenolic hydroxyl group in the gallate moiety of EGCG reached deeply into the active site and interacted with Ser139 and Tyr193 of the catalytic triad. The phenolic oxygen was positioned 3.43 Å from Oγ of Ser139 and 3.48 Å from Oη of Try193, and this suggested the existence of strong hydrogen-bond interactions (Fig. 2B).

EGCG is positioned differently from hydroxy-PP, which binds to the substrate-binding site of CBR1.[21] The structure of the substrate isatin is similar to that of hydroxy-PP and has the same pyrazolopyrimidine core, and it is thus not surprising that they compete against each other for the same site of CBR1. This suggests that EGCG does not bind to the substrate-binding site as hydroxy-PP does. EGCG is also positioned differently from NADPH. This model is in agreement with the results of an enzyme assay, which showed that EGCG is a noncompetitive inhibitor against both isatin and NADPH.

The model was further verified by an examination of the inhibitory activity of EGCG on CBR1 mutants. The R95A and K231A mutants, which were as active as the wild-type enzyme, were significantly less sensitive to EGCG with IC_{50} values 8.3-fold and 9.2-fold higher than that of the wild-type enzyme, respectively (Supporting Information Table 2).

EGCG Sensitizes Human Hepatoma Cancer Cells to DNR. As the metabolism of DNR by CBR1 in tumor cells has been shown to contribute to drug resistance, it was expected that EGCG would enhance the antitumor

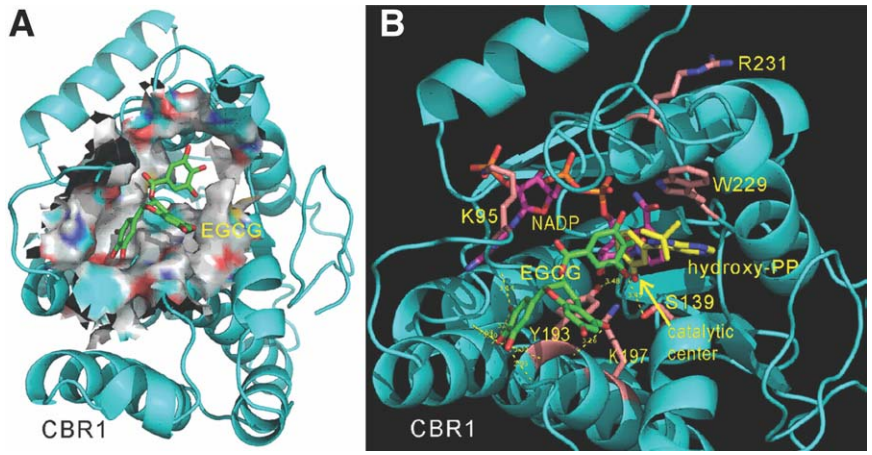

Fig. 2. Stereo images of EGCG docked to the active site of CBR1. (A) CBR1 is shown in ribbon form with the active site layered with a transparent surface [it is represented with a water-accessible surface and is colored by the atom type (red for oxygen, blue for nitrogen, gray for carbon and hydrogen)]. EGCG is represented in stick form and is colored green with oxygen in red. (B) An overview of EGCG's binding mode to CBR1 is also shown. CBR1 is shown in ribbon form, and binding site amino acids, EGCG, hydroxy-PP, and NADPH are shown in stick form. The hydrogen bonds (broken yellow lines) between EGCG and CBR1 are presented along with the distances from the residue to EGCG. Hydroxy-PP (yellow) is positioned behind EGCG. The catalytic residues (Ser139, Lys197, and Tyr193) and alkaline residues (Arg95 and Lys231) are shown in pink, and nicotinamide adenine dinucleotide phosphate (NADP) is shown in purple.

effect of DNR by inhibition of the CBR1-mediated metabolism. To test this possibility, we measured the ability of EGCG to block CBR1-mediated metabolism of DNR in hepatoma cells with a cell viability assay. We carried out a protein western blot analysis to determine endogenous protein levels of CBR1 in different hepatoma cells (Fig. 3A). The expression levels of CBR1 in most of the HCC cells were comparable to those in human hepatocytes (L02). Only in Hep3B was the CBR1 expression significantly reduced for some reason. We selected HepG2 and SMMC7721 as CBR1 high-expression cells and Hep3B as CBR1 low-expression cells in the ensuing studies. The concentration of EGCG that exhibited minimal cytotoxicity in hepatoma cell lines when used alone was selected for the treatment in combination with DNR (Supporting Information Fig. 4). In HepG2 cells, EGCG induced a 16.2% enhancement of DNR-mediated growth inhibition (Fig. 3B, left panel), and the enhancement was 20.5% in SMMC7721 cells (Fig. 3B, middle panel). The enhancement effect of EGCG was dose-dependent. In contrast, EGCG did not affect the sensitivity of DNR in Hep3B cells (Fig. 3B, right panel), and this further supports the idea that the enhancement effect of EGCG is CBR1-dependent. The protein levels of CBR1 in HCC cells (HepG2, SMMC7721, and Hep3B) did not undergo obvious changes when they were treated with EGCG, and this suggests that the synergic effect of EGCG and DNR is not due to changes in protein levels of CBR1 (Supporting Information Fig. 5).

We also determined the effects of EGCG analogues, including EC, ECG, and EGC, on DNR-mediated growth inhibition (Fig. 3C). ECG, which like EGCG also inhibits CBR1 *in vitro*, showed significant enhancement of DNR-mediated cell growth inhibition in both HepG2 ($P < 0.01$) and SMMC7721 ($P < 0.05$), whereas EGC and EC, which weakly inhibited CBR1 *in vitro*, did not show an obvious synergic effect with DNR. Thus, there is a correlation between the inhibition of CBR1 and the enhancement of DNR-mediated tumor cell growth by EGCG and its analogues.

We next examined the effect of EGCG on DNR-induced G2/M cell cycle arrest by fluorescence-activated cell sorting analysis. As shown in Fig. 3D and Supporting Information Fig. 6A, DNR treatment of cells induced a reduction of the cell number in the G1 phase and a corresponding increase in the G2/M phase population. In contrast, 10 μM EGCG alone had no effect on the cell cycle progression. However, a combination of 10 μM EGCG and 0.04 μM DNR resulted in an increase in the percentage of G2/M cells from 52.8% (DNR alone) to 62.4% (EGCG and DNR) in HepG2 cells. For SMMC7721 cells, EGCG and 0.03 μM DNR induced a 10.4% increase in cells in the G2/M phase versus DNR alone. EGCG was thus capable of enhancing the DNR-induced G2/M cell cycle arrest, and this reflected the ability of EGCG to enhance the inhibition of cell proliferation by DNR. We also examined the effect of EGCG on DNR-induced apoptosis with flow cytometry (Fig. 3E and Supporting Information Fig. 6B). EGCG alone at 20 μM did not induce apoptosis. However, EGCG at the same concentration increased DNR-induced apoptosis from 36.4% to 45.2% in

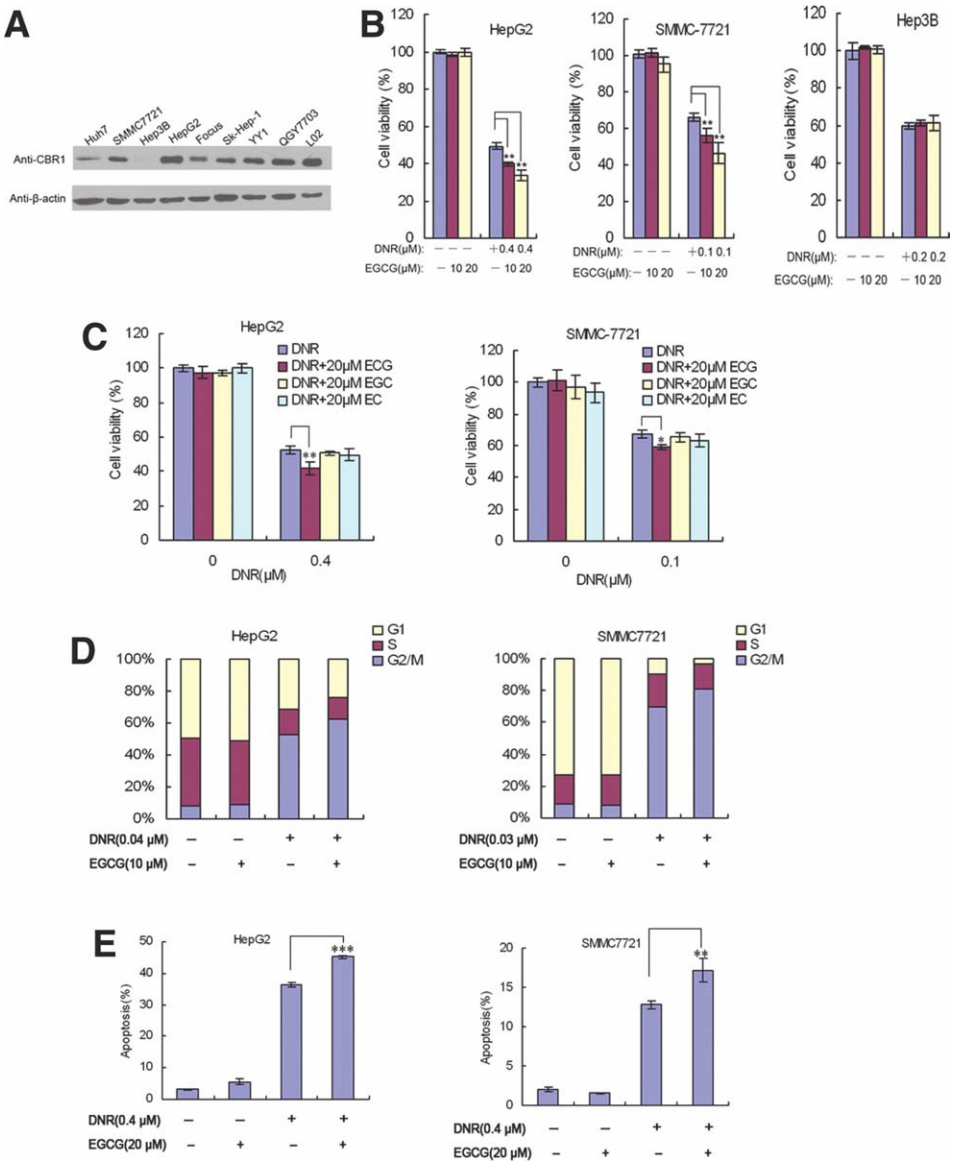

Fig. 3. Effects of catechins on DNR-induced cytotoxicity and G2/M arrest in hepatoma cells. (A) CBR1 expression in HCC cells. Expression of CBR1 was determined in nine HCC cells by western blotting with an anti-CBR1 polyclonal antibody. β-Actin was used as the loading control. The lysate from human hepatocyte cells (L02) was used as a positive control. (B) Effect of EGCG on DNR-induced cytotoxicity with respect to HepG2 (left), SMMC7721 (middle), and Hep3B (right) cell viability. (C) Effects of ECG, EGC, and EC on DNR-induced cytotoxicity with respect to HepG2 (left) and SMMC7721 (right). Cells were cultured for 48 hours after the addition of DNR in the presence or absence of EGCG or an EGCG analogue. Viable cells were identified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The numbers of viable cells are expressed as percentages of the untreated control (absorbance). The data represent means and standard deviations (n = 5). $*P < 0.05$ and $**P < 0.01$. (D) Flow cytometry analysis of the cell cycle of HepG2 (left) and SMMC7721 (right) after the addition of DNR in the presence or absence of EGCG. The fractions of viable cells in the G1, S, and G2/M phases of the cell cycle were quantified by flow cytometry analysis of propidium iodide–stained cells. Results of ModFit analysis are shown with the percentage attributed to the respective cell cycle stage. (E) Flow cytometry analysis of the apoptosis of HepG2 (left) and SMMC7721 (right) cells upon treatment with DNR in the presence or absence of EGCG. At 48 hours after drug treatment, cells were labeled with annexin V–fluorescein isothiocyanate and propidium iodide, and this was followed by fluorescence-activated cell sorting analysis. Early-stage apoptotic cells were identified by high annexin staining and low propidium iodide staining. The data represent means and standard deviations (n = 3). $**P < 0.01$ and $***P < 0.001$ versus the DNR-alone group.

HepG2 cells. For SMMC7721 cells, the percentage of apoptosis increased from 12.8% (DNR alone) to 17.2% (DNR and EGCG). These results strongly suggest that EGCG is capable of enhancing the antitumor activity of DNR.

EGCG Can Reverse CBR1-Mediated Resistance to DNR. To further verify that the synergic effect of EGCG with DNR is mediated by CBR1, we generated Hep3B-CBR1 cells stably expressing CBR1 and control Hep3B cells stably transfected with empty

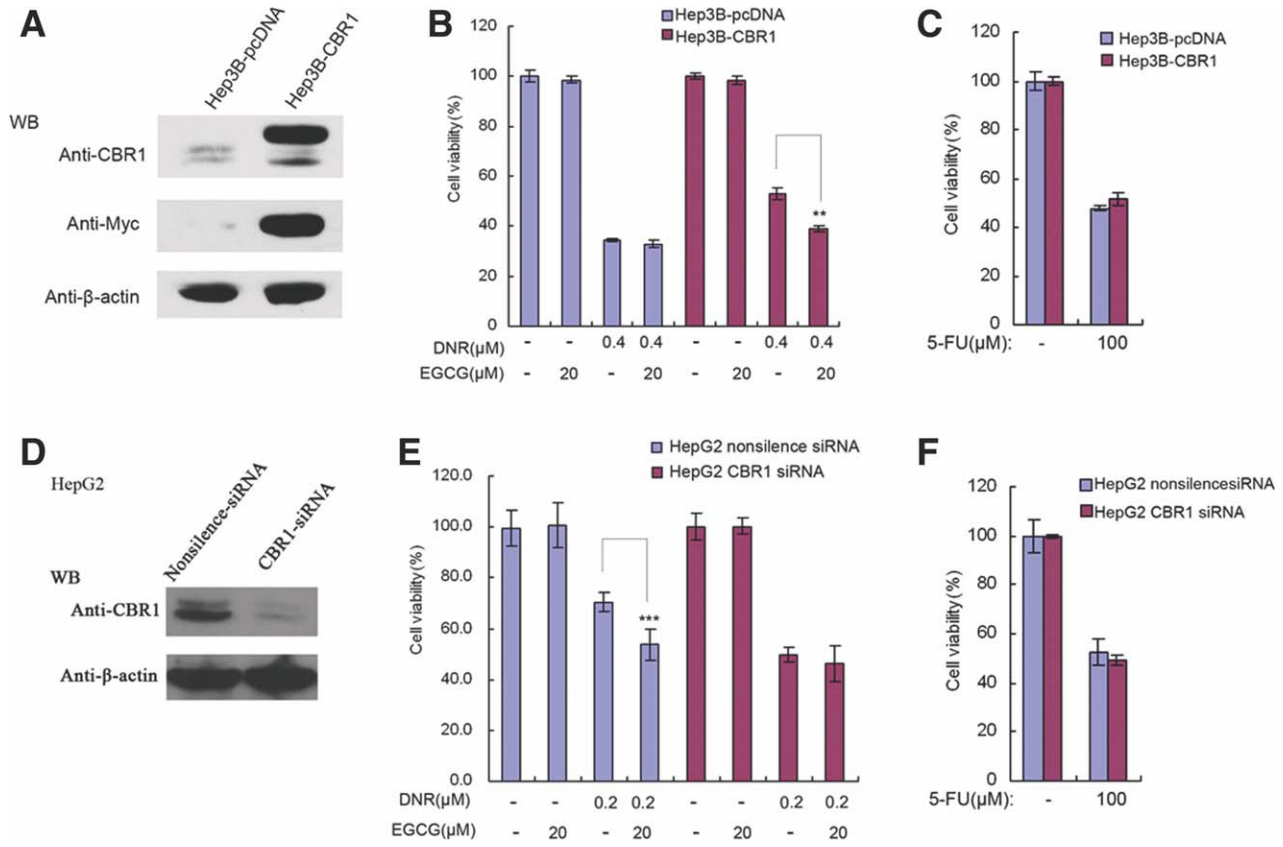

Fig. 4. Effect of EGCG on CBR1-mediated resistance to DNR. (A) Protein levels of CBR1 in Hep3B-pcDNA and Hep3B-CBR1 cells were analyzed by western blotting with antibodies against CBR1 and Myc. β-Actin was used for normalization. (B) Effect of EGCG on DNR-induced cytotoxicity with respect to Hep3B-pcDNA and Hep3B-CBR1 cell viability. Cells were cultured for 48 hours after the addition of DNR in the presence or absence of EGCG. (C) Effect of 5-FU on Hep3B-pcDNA and Hep3B-CBR1 cell viability. (D) Knockdown of the expression of endogenous CBR1 in HepG2 cells was analyzed by western blotting with antibodies against CBR1. β-Actin was used for normalization 24 hours after transfection. (E) Effect of EGCG on DNR-induced cytotoxicity with respect to nonsilence siRNA and CBR1 siRNA knockdown HepG2 cell viability. Drugs were added 24 hours after transfection. Cells were cultured for 48 hours with DNR in the presence or absence of EGCG. (F) Effect of 5-FU on nonsilence siRNA and CBR1 siRNA knockdown HepG2 cell viability. Throughout the study, viable cells were identified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; the data are expressed as percentages of the untreated control (absorbance). The data represent means and standard deviations (n = 5). $^{**}P < 0.01$ and $^{***}P < 0.001$.

pcDNA3.1(-)/myc-HIS vector (pcDNA). The ectopic expression of CBR1 was confirmed by western blotting in Hep3B-CBR1 cells (Fig. 4A). Hep3B-pcDNA cells and Hep3B-CBR1 cells were treated with DNR, EGCG, or EGCG and DNR. As shown in Fig. 4B, the treatment of Hep3B-pcDNA cells with 0.4 μM DNR led to 34.4% cell viability in comparison with the untreated cells, whereas the cell viability of Hep3B-CBR1 was 52.9%. Hep3B-CBR1 cells were more resistant to DNR than Hep3B-pcDNA, whereas no differences were observed for these two lines in their resistance to 5-fluorouracil (5-FU; $P > 0.05$; Fig. 4C). The treatment of Hep3B-CBR1 cells with EGCG and 0.4 μM DNR decreased the cell viability from 52.9% to 39.0% ($P < 0.01$; Fig. 4B). These results indicated that the specific increased resistance to DNR of Hep3B-CBR1 cells was due to the elevated level of CBR1 expression. Also, treatment with EGCG in combination with DNR seemed at least partially to overcome the acquired resistance to DNR in Hep3B-CBR1 cells.

In a complementary experiment, we decreased the expression of CBR1 in HepG2 cells by RNA interference (RNAi). The efficiency of small interfering RNA (siRNA) in knocking down the expression of CBR1 in HepG2 cells was verified (Fig. 4D). Upon CBR1 knockdown, HepG2-CBR1 siRNA cells became more sensitive to DNR. With 0.2 μM DNR, the cells showed 49.7% viability in comparison with 70.4% for the control cells (HepG2 nonsilence RNAi; Fig. 4E). Again, no differences were observed in their sensitivity to 5-FU ($P > 0.05$; Fig. 4F). In control HepG2 cells, EGCG significantly enhanced the DNR-induced inhibition of proliferation, which was similar to that of wild-type HepG2 cells, whereas EGCG did not show a marked enhancing effect on DNR activity in HepG2-CBR1

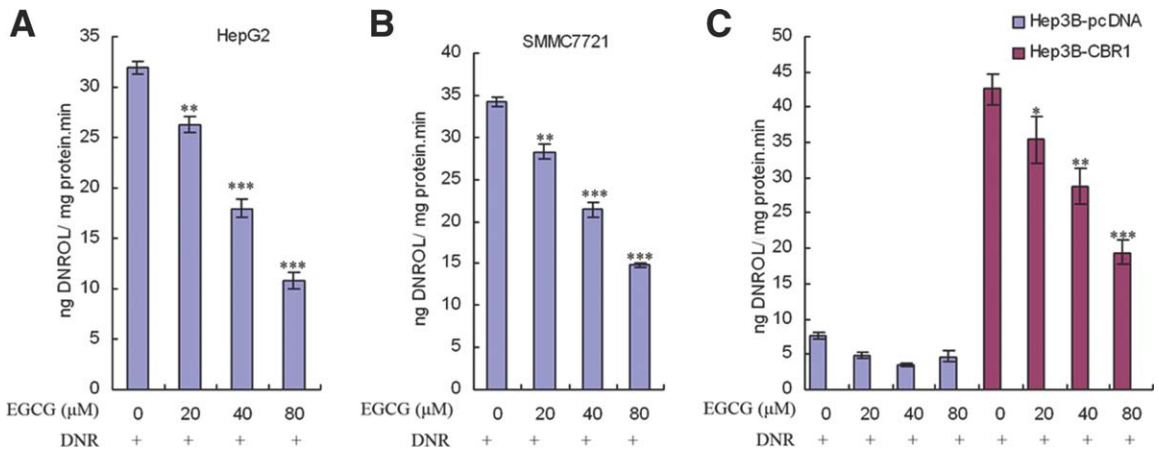

Fig. 5. Dose-dependent effect of EGCG on the DNR carbonyl reduction to DNROL in (A) HepG2, (B) SMMC7721, and (C) Hep3B-pcDNA and Hep3B-CBR1 cells. Experiments were performed through the incubation of the cell lysate in a 0.1 M potassium phosphate buffer (pH 7.0) with 100 μM DNR, 200 μM NADPH, and different concentrations of EGCG (0-80 μM) for 30 minutes. Enzyme activity is presented as nanograms of DNROL per milligram of protein per minute. The data represent means and standard deviations (n = 3). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the control group (without EGCG).

RNAi cells (Fig. 4E). Taken together, these results clearly demonstrate that CBR1 specifically affects the sensitivity of cancer cells to DNR and that EGCG can reverse CBR1-mediated resistance to DNR.

EGCG Inhibits DNR Carbonyl Reduction in Cells. To obtain direct evidence that EGCG enhances the activity of DNR by inhibiting DNR reduction by CBR1, cellular concentrations of DNR and DNROL were measured with HPLC. HepG2 cell lysates contained a DNROL level of 32.0 ng/mg of protein/minute, and levels of DNROL were reduced by 17.7%, 43.8%, and 66.2% in the presence of 20, 40, and 80μM EGCG, respectively (Fig. 5A). SMMC7721 cell lysates showed a DNROL level of 34.1 ng/mg of protein/minute, and the lowest dose of EGCG (20 μM) could significantly affect DNR carbonyl reduction ($P < 0.01$; Fig. 5B). The dose-dependent effect of EGCG on DNR reduction further supports the notion that EGCG specifically inhibits DNR reduction.

The control Hep3B-pcDNA cell lysates showed DNR-reducing activity of 7.7 ng/mg of protein/minute, whereas Hep3B-CBR1 cells stably expressing CBR1 had higher DNR-reducing activity (42.6 ng/mg of protein/minute, i.e., an increase of 5.4-fold). The DNR-reducing activity of the Hep3B-CBR1 cell lysate was decreased to 35.4, 28.8, and 19.4 ng/mg of protein/minute when 20, 40, and 80 μM EGCG was added, respectively (Fig. 5C). These results are consistent with Fig. 4B, which shows that CBR1 contributes to the acquired resistance toward DNR and that EGCG can reverse the resistance by inhibiting CBR1 activity.

Effect of EGCG on DNR in an HCC Xenograft Model. In order to evaluate the potential benefit of a combination therapy using EGCG and DNR for HCC, we determined the effects of EGCG and DNR (alone or in combination) in a xenograft model using HCC cells with high (SMMC7721) or low (Hep3B) CBR1 expression levels.

For SMMC7721 xenografts, the EGCG and DNR group showed a higher level of inhibition in comparison with the EGCG-alone group or the DNR-alone group (Fig. 6A). As shown in Fig. 6B, the average tumor weight in the control group was 0.50 $\pm$ 0.12 g. The tumor weight of the EGCG-alone group and the DNR-alone group was decreased by 15.7% and 16.8% in comparison with the control group, respectively. The combination of EGCG with DNR reduced the tumor weight by 45.6% in comparison with the control group, and this was significantly lower than that of the EGCG-alone group and the DNR-alone group ($P < 0.01$). The antitumor activity of the EGCG and DNR group was higher than the sum of the EGCG-alone and DNR-alone groups (32.5% inhibition), and this suggested synergy between EGCG and DNR. For the Hep3B xenograft, however, the antitumor effect was not obviously different in the EGCG and DNR group and the DNR-alone group (Fig. 6C,D).

Safety of the Drug Combination in Mice with an HCC Xenograft. To assess the general toxicity of the combination of EGCG and DNR in animals, we determined and compared the body weights and several biochemical parameters for the same animals receiving xenografts. For those receiving xenografts of SMMC7721, the EGCG treatment group did not experience substantial decreases in body weight in comparison with the control group (Fig. 7A). The average

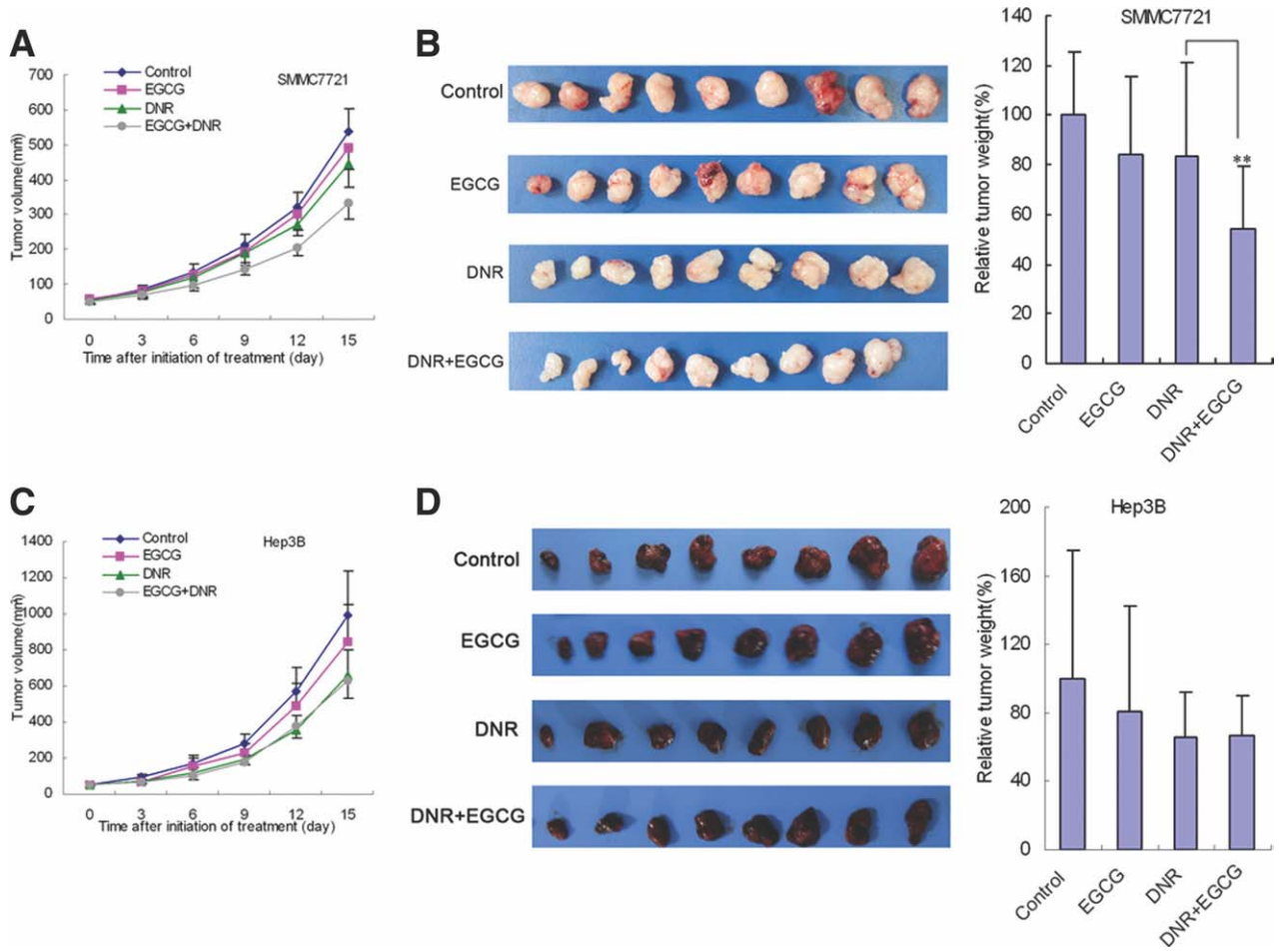

Fig. 6. Effect of EGCG in the xenograft HCC model. The effect of EGCG on the change in the average tumor volume induced by DNR in the xenograft HCC model with (A) SMMC7721 and (C) Hep3B is shown. The data represent means and standard errors of the mean (n = 9 or 8). The tumor size was measured every 3 days. The effect of EGCG on the change in the tumor weight induced by DNR in the xenograft HCC model with (B) SMMC7721 and (D) Hep3B is shown. The tumors were photographed (left), and the average tumor weights for the various treatment groups are shown (right). The data represent means and standard deviations (n = 9 or 8). $*P < 0.05$ and $**P < 0.01$.

body weight of the DNR group was 2.31 g lighter than that of the control group, and average body weight of the EGCG and DNR group was 1.34 g lighter than that of the control group (Fig. 7B). Thus, EGCG significantly reversed the weight loss caused by DNR ($P < 0.05$). The administration of DNR did not affect the alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels in serum in comparison with the control group, and these are good indicators of liver disease or damage. Heart injury was tested by markers such as lactate dehydrogenase (LDH), serum creatine kinase MB isoenzyme (CK-MB), malondialdehyde (MDA), and cardiac troponin T (cTnT) in serum. The administration of DNR led to a significant elevation of MDA and cTnT levels but did not affect the levels of the other two markers. The levels of MDA and cTnT were restored to those seen in the control group by a combination with EGCG (Table 1). As shown in Fig. 7C, EGCG also significantly reversed the weight loss caused by DNR in the Hep3B xenograft model ($P < 0.05$). Similar results were obtained for biochemistry parameters in serum for EGCG in the Hep3B xenograft model (Table 1). These results suggest that EGCG could increase the safety of DNR therapy in both CBR1-overexpressing and CBR1-underexpressing HCC xenografts *in vivo* and the coadministration of DNR. Also, EGCG is a promising strategy for overcoming resistance and decreasing toxicity for the anthracycline family of anticancer drugs.

Discussion

Drug resistance is a major challenge in the treatment of malignant tumors. The resistance to the anthracyclines DNR and DOX is mediated in large part by one enzyme, CBR1, which reduces the C13 carbonyl group into alcohols, DNROL and DOXOL,

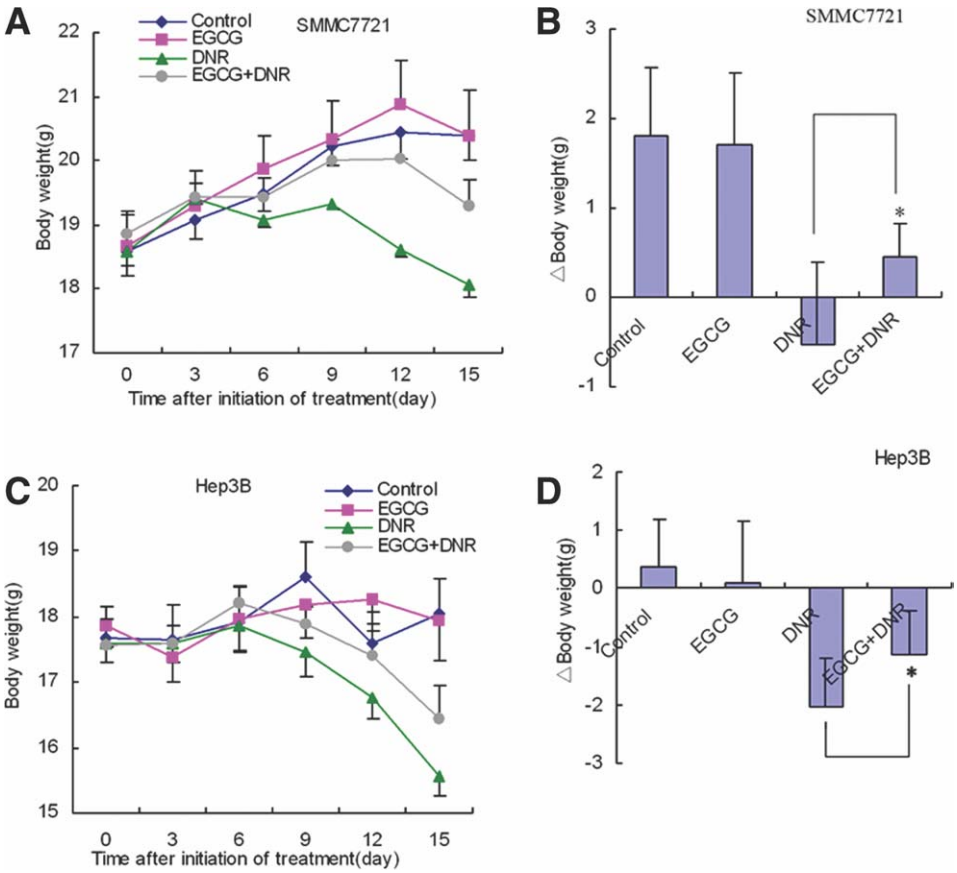

Fig. 7. Effects of DNR, EGCG, and their combination on body weight. The body weights of (A) the SMMC7721 group and (C) the Hep3B group are shown with the various treatments from the beginning of treatment. The average weights for the different treatment groups were measured every 3 days. The data represent means and standard errors of the mean (n = 9 or 8). The average changes in body weight for (B) the SMMC7721 group and (D) the Hep3B group are shown from the beginning to the end of the drug treatment. A positive number represents an increase, whereas a negative number indicates a decrease in body weight. The data represent means and standard deviations (n = 9 or 8). *$P < 0.05$ and **$P < 0.01$.

that are not only less active against tumor cells but also cardiotoxic. High levels of CBR1 in HCC cells thus contribute to drug resistance to both DNR and DOX. The pharmacological inhibition of CBR1 activity has been proposed as a strategy to reverse the resistance in tumor cells and minimize the clinical incidence of anthracycline-related cardiotoxicity.

In the present study, we identified EGCG as a novel inhibitor of human CBR1 with an IC_{50} value of 0.59 μM. Its potency against CBR1 compares favorably with the potency of the known natural flavonoid inhibitors of the same enzyme, including quercetin, kaempferol, quercitrin, and genistein with IC_{50} values between 1 and 10 μM, and the synthetic inhibitor

Table 1. Effects of Drug Administration on Changes in Biochemical Markers in the Sera of Animals

	Parameter					
	Liver Function		Heart Function			
Group	ALT (U/L)	AST (pg/mL)	CK-MB (U/L)	LDH1 (U/L)	MDA (nmol/L)	cTnT (ng/L)
Hep3B						
Control	20.18 ± 2.86	743.81 ± 82.00	45.09 ± 8.08	47.10 ± 4.13	12.70 ± 1.38	249.69 ± 13.71
EGCG	18.56 ± 0.81	722.38 ± 44.92	41.55 ± 5.79	45.95 ± 4.11	12.54 ± 0.94	243.08 ± 11.80
DNR	17.88 ± 1.32	699.83 ± 32.90	41.34 ± 4.26	43.10 ± 4.00	15.63 ± 0.99*,§	290.55 ± 7.96*,§
DNR and EGCG	19.74 ± 1.67	766.83 ± 46.91	41.85 ± 7.09	45.44 ± 5.32	12.87 ± 1.70†,‡	260.64 ± 20.78†,§
SMMC7721						
Control	18.63 ± 6.43	608.05 ± 90.51	41.83 ± 6.02	41.36 ± 8.04	11.11 ± 2.73	122.59 ± 9.10
EGCG	21.40 ± 6.53	675 ± 62.02	42.99 ± 5.02	41 ± 9.22	11.25 ± 2.24	122.49 ± 10.29
DNR	20.46 ± 4.15	652.5 ± 116.1	41.44 ± 8.21	42.4 ± 4.25	14.86 ± 2.06*,‡	157.34 ± 14.64*,§
DNR and EGCG	20.56 ± 8.5	711 ± 99.38	42.79 ± 5.72	38.31 ± 7.65	11.21 ± 2.39†,‡	121.82 ± 14.91†,§

CK-MB, creatine kinase MB isoenzyme.
The data are means and standard deviations from eight or nine mice.
*Significantly different for the DNR group versus the control group.
†Significantly different for the EGCG and DNR group versus the DNR-alone group.
‡$P < 0.01$
§$P < 0.001$.

hydroxy-PP with an IC_{50} value of 0.79 μM.[20,24] Unlike most known inhibitors of CBR1, however, EGCG is already taken by humans through tea and other beverages, and purified EGCG and its analogues have been entered into different clinical trials for cancer chemoprevention and treatment; this is paving the way for EGCG to be evaluated for HCC in light of this study.

EGCG is the most abundant and active compound with anticancer activity in tea. The mechanism for the cancer-preventive effect of EGCG is still under active investigation. Several putative binding proteins, including salivary proline-rich proteins, fibronectin, fibrinogen, and histidine-rich glycoproteins, have been identified; more recently, proteins such as the 67-kDa laminin receptor,[25] B cell lymphoma 2,[26] vimentin,[27] insulin-like growth factor 1 receptor,[28] FYn,[29] glucose-regulated protein 78,[30] and zeta chain associated protein kinase 70,[31] among others, have been identified. None of these putative EGCG-binding proteins, however, can account for the inhibition of reduction of DNR by EGCG, except for CBR1, which is identified in this study.

It has been reported that EGCG alone can inhibit the growth of human HCC cell lines *in vitro* and induce apoptosis in HCC cells,[32] and this is consistent with our observations (Supporting Information Figs. 4 and 7). The inhibitory effect of EGCG on HCC xenografts has also been shown to be associated with inhibition of the vascular endothelial growth factor/vascular endothelial growth factor receptor axis.[33] The aim of our research was to test the synergic effect of EGCG on DNR by inhibiting CBR1. We therefore avoided using toxic EGCG concentrations and deliberately selected lower doses of EGCG that showed minimal toxicity in HCC cells. Several lines of evidence suggest that inhibition of CBR1 by EGCG is responsible for its ability to block DNR resistance and its synergy with DNR for the inhibition of HCC both *in vitro* and *in vivo*. EGCG specifically enhanced DNR-induced G2/M phase cell cycle arrest and cell apoptosis in HCC cells with higher CBR1 expression such as HepG2 cells. In contrast, knockdown of CBR1 expression in HepG2 cells by RNAi recapitulated EGCG's effect. On the other hand, Hep3B with spontaneously lower expression of CBR1 exhibited little synergistic response to EGCG and DNR. Overexpression of CBR1 in Hep3B conferred resistance to DNR, which was overcome by EGCG. In addition, the levels of the metabolite DNROL in the cell extract correlated with the reduction activity of CBR1, and EGCG was found to significantly decrease the concentration of DNROL. These results also support the idea that CBR1 is a key contributor to drug resistance in human HCC toward DNR.

The protein levels of CBR1 vary in different human HCC cells. Although CBR1 has been shown to be decreased in HCC by immunohistochemical analysis,[34] other biochemical analyses have revealed the opposite change.[35] In this study, we analyzed a total of 59 cases of human HCC. CBR1 was down-regulated in 30 (50.8%) and up-regulated in 8 (13.6%) and was similar to corresponding nontumor tissues in 21 cases (35.6%; Supporting Information Fig. 8); this calls into question whether the combination of EGCG with DNR can be of general use to HCC patients. EGCG enhanced the antitumor effects of DNR in cell toxicity assays and in animal xenograft models using cells with high expression of CBR1 (SMMC7721). However, we found that although EGCG did not bring additional benefits to DNR with respect to the inhibition of tumor growth, it clearly decreased the cardiotoxicity of DNR in Hep3B and SMMC7721 xenografts independently of CBR1 expression levels. We speculate that the reduction of DNR occurs not only in tumor cells but also in normal liver cells that contribute to the reduction of DNR to DNROL and hence the cardiotoxicity of DNR. The ability of EGCG to enhance the anticancer activity of DNR, together with its known safety and pharmacological properties, renders EGCG a generally applicable component of a combination therapy using DNR and EGCG for HCC.

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