Induction of Human UDP-Glucuronosyltransferase 2B7 Gene Expression by Cytotoxic Anticancer Drugs in Liver Cancer HepG2 Cells

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ABSTRACT

We recently reported induction of UGT2B7 by its substrate epirubicin, a cytotoxic anthracine anticancer drug, via activation of p53 and subsequent recruitment of p53 to the UGT2B7 promoter in hepatocellular carcinoma HepG2 cells. Using the same HepG2 model cell line, the present study assessed the possibility of a similar induction of UGT2B7 by several other cytotoxic drugs. We first demonstrated by reverse transcriptase quantitative real-time polymerase chain reaction that, as observed with epirubicin, nine cytotoxic drugs including three anthracyclines (doxorubicin, daunorubicin, and idarubicin) and six nonanthracyclines (mitomycin C, 5-fluorouracil, camptothecin, 7-ethy1-10-hydroxycamptothecin, topotecan, and etoposide) significantly increased UGT2B7 mRNA levels. To investigate a potential involvement of p53 in this induction, we conducted further experiments with four of the nine drugs (doxorubicin, daunorubicin, idarubicin, and mitomycin C). The cytotoxic drugs studied increased p53 and UGT2B7 protein levels. Knockdown of p53 expression by small interfering RNA reduced cytotoxic drug-induced UGT2B7 expression. Luciferase reporter assays showed activation of the UGT2B7 promoter by cytotoxic drugs via a previously reported p53 site. Finally, chromatin immunoprecipitation assays demonstrated p53 recruitment to the UGT2B7 p53 site upon exposure to mitomycin C, the most potent UGT2B7 inducer among the nine tested drugs. Taken together, these results provide further evidence supporting UGT2B7 as a p53 target gene. The cytotoxic drug-induced UGT2B7 activity in target liver cancer cells or possibly in normal liver cells may affect the therapeutic efficacy of co-administered cytotoxic drugs (e.g., epirubicin) and nontoxic drugs (e.g., morphine), which are UGT2B7 substrates.

Introduction

UDP-glucuronosyltransferases (UGTs) are a superfamily of enzymes responsible for the glucuronidation of numerous endogenous and exogenous small lipophilic compounds, including bilirubin, bile acids, fatty acids, steroid hormones, and carcinogens, as well as therapeutic drugs; the resulting glucuronide products are generally inactive and water soluble, thus promoting their excretion from the body (Mackenzie et al., 1997, 2005). For example, UGT2B7 displays high activity toward a variety of endogenous compounds such as bile acids, sex hormones and their metabolites, mineralocorticoid and glucocorticoid hormones, fatty acids, and retinoic acids (Hu et al., 2014b). UGT2B7 has also been shown to conjugate many therapeutic drugs, such as morphine (Coffman et al., 1997), etoposide (Argikar and Remmel, 2009), mycophenolic acid (Picard et al., 2005), and nonsteroid anti-inflammatory drugs (Jin et al., 1993). UGT2B7 is widely expressed in human tissues, including liver, small intestine, kidney, and breast (Hu et al., 2014b). The broad substrate spectrum of UGT2B7 combined with its hepatic and extrahepatic expression highlights its physiological and pharmacological importance in metabolism of endogenous bioactive molecules and therapeutic drugs. Regulation of UGT2B7 expression and activity in human tissues, especially in the liver and gastrointestinal tract, is thus critical for determining substrate detoxification and clearance. As we have recently reviewed, studies to date have investigated the regulation of UGT2B7 using liver and colon cancer cell lines and transgenic mice and revealed regulation by a number of tissue-specific and ligand-dependent transcription factors (Hu et al., 2014b).

Chemotherapy using cytotoxic drugs continues to play an important role in treating most human cancers; however, both intrinsic resistance and acquired resistance limit their clinical value (Holohan et al., 2013). The cytotoxic drugs that have been frequently incorporated in chemotherapy protocols include DNA alkylating agents (e.g., mitomycin C), platinum coordination complexes, topoisomerases I (e.g., irinotecan and topotecan) and II (e.g., doxorubicin, etoposide, and etoposide) inhibitors, microtubule-damaging drugs, and anti-metabolites [e.g., 5-fluorouracil (5-FU)] (Pelvic, 2014). Multiple mechanisms have been shown to be involved in emergence of cancer resistance to cytotoxic drugs, including intratumoral overexpression of drug efflux transporters, drug-metabolizing enzymes, and drug-targeting molecules such as DNA topoisomerases (Szakács et al., 2006; Meijerman et al., 2008). Specifically, overexpression of cytotoxic drug-metabolizing enzymes in tumor cells enhances the inactivation of cytotoxic drugs, and thus reduces sensitivity to chemotherapy or even promotes resistance. For example, several cytotoxic drugs and/or their metabolites are primarily inactivated by

ABBREVIATIONS: 5-FU, 5-fluorouracil; MT, mutant; PCR, polymerase chain reaction; si, small interfering; SN-38, 7-ethyl-10-hydroxycamptothecin; UGT, UDP-glucuronosyltransferase.
UGTs, including etoposide and 7-ethyl-10-hydroxycamptothecin (SN-38) (the active metabolite of irinotecan) by UGT1A1 (O’Dwyer and Catalano, 2006; Wen et al., 2007) and epirubicin by UGT2B7 (Innocenti et al., 2001). Basseville et al. (2011) have recently shown that SN-38 stimulates UGT1A1 expression via the PXR in colorectal cancer LS180 cells and HepG2 cells, suggesting a potential role in conferring irinotecan resistance. In support of this view, Takahata et al. (2008) have demonstrated that overexpression of UGT1A1 reduced the sensitivity of hepatocellular cancer cells to irinotecan. We recently reported the induction of UGT2B7 by epirubicin via activation of p53 and the subsequent recruitment of p53 to a p53 response element (5’-TGCCATGTCCATACAGATC-3’, core sequence of p53 motif underlined) at the UGT2B7 proximal promoter in liver cancer HepG2 cells (Hu et al., 2014c). Epirubicin has been administered alone (monotherapy), in combination with cisplatin or mitomycin C (double therapy), or together with cisplatin and mitomycin C (triple therapy) for treating patients with unresectable advanced hepatocellular carcinoma (Marelli et al., 2007). Therefore, it is believed that the epirubicin-induced UGT2B7 glucuronidation activity promotes epirubicin metabolism and thus reduces therapeutic efficacy or may even contribute to the development of cancer resistance to epirubicin-containing hepatocellular carcinoma chemotherapy (Hu et al., 2014c).

That UGT2B7 is a p53 target gene is also supported by a recent finding that silencing of p53 expression by stably expressing an anti-p53 short-hairpin RNA reduces basal UGT2B7 mRNA levels in HepG2 cells (Goldstein et al., 2013). In addition to epirubicin, many other drugs that belong to different classes of cytotoxic drugs have also been shown to activate p53 in cancer cells, such as doxorubicin, camptothecin, etoposide, 5-FU, and mitomycin C (Fritsche et al., 1993; Tishler et al., 1993, 1995; Müller et al., 1997). The present study hypothesized that, similar to epirubicin, these p53-activating cytotoxic drugs would also be able to stimulate the expression of UGT2B7 in HepG2 cells, and presents several lines of evidence to support this hypothesis.

**Materials and Methods**

Chemical compounds of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO), including doxorubicin hydrochloride, epirubicin hydrochloride, daunorubicin hydrochloride, idarubicin hydrochloride, nutlin-3a, mitomycin C, dimethyl sulfoxide, 5-FU, (S)-(+)-camptothecin, SN-38, topotecan

**Fig. 1.** Anthracyclines elevate both UGT2B7 mRNA and protein levels in the liver cancer HepG2 cell line. HepG2 cells were treated in triplicate (A and B) or duplicate (C) for 24 hours with vehicle (0.1% ethanol) or one of four anthracyclines at 1 μM as indicated. (A) Data shown are the fold induction (i.e., mean ± S.D. of three independent experiments) in target gene mRNA levels in drug-treated cells over those in vehicle-treated cells (set to a value of 1). (B) Shown are immunosignals (top) and calculated fold induction in UGT2B7 protein levels in drug-treated cells over those in vehicle-treated cells (set to a value of 1) following background subtraction and normalizing to the control calnexin protein levels (bottom) from a representative experiment of two independent experiments as described in Materials and Methods. (C) Whole cell lysates were prepared from treated HepG2 cells as indicated and subjected to Western blotting assays (~30 μg each sample). Blots were sequentially probed with an anti-p53 antibody and an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody as described in Materials and Methods. Shown are immunosignals from one experiment performed in duplicate. This experiment was repeated and similar results were obtained. Eth, ethanol; EPI, epirubicin; Dox, doxorubicin; Dau, daunorubicin; Ida, idarubicin. Statistical analyses of data presented in (A) and (B) used one-way analysis of variance followed by Tukey’s post hoc multiple comparison test. * < 0.05, ** < 0.01, *** < 0.001.
hydrochloride hydrate, and etoposide. Primers were synthesized by Sigma-Genosys (Castle Hill, NSW, Australia).

**Cell Culture, Drug Treatment, RNA Extraction, and Reverse Transcriptase Quantitative Real-Time Polymerase Chain Reaction (PCR).** The HepG2 cell line was purchased from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C in a 5% CO2 atmosphere. For drug treatment, cells were plated in 6-well plates and cultured for 3 to 4 days to reach 100% confluence. Cells were then treated with drugs in triplicate at intervals of time as indicated in the figures 1–7. The concentrations of drugs used (i.e., anthracyclines at 1 μM, mitomycin

**Fig. 2.** siRNAs against p53 reduce anthracycline-induced UGT2B7 mRNA levels in HepG2 cells. Briefly, cells were plated and transfected in 6-well plates in four wells at 100 nM with either p53 siRNA or nontargeting siRNA (neg-siRNA). Forty-eight hours after transfection, cells were harvested from one well of each transfection to prepare whole cell lysates for Western blotting assays using an antibody recognizing p53 (top) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bottom) (A), and the remaining three wells of each transfection were treated for 24 hours with one of the four anthracyclines at 1 μM as indicated, followed by quantitative real-time PCR to quantify the mRNA levels of three genes (UGT2B7, UGT2B10, and p21) (B), as described in Materials and Methods. Shown are the immunosignals from a representative experiment (A) and the calculated (i.e., mean ± S.D. of three independent experiments) target gene mRNA levels in anthracycline-treated and p53 siRNA-transfected HepG2 cells compared with those in anthracycline-treated and neg-siRNA-transfected cells (set to a value of 100%) (B). Statistical analyses used two-way analysis of variance followed by independent t tests with Bonferroni correction. **P < 0.01, ***P < 0.001.

**Fig. 3.** Anthracylines activate the UGT2B7 promoter in HepG2 cells via a reported p53 site in its proximal promoter. The UGT2B7-575/-1 plasmid contains the proximal 575 base pair (bp) UGT2B7 promoter fragment cloned in the promoterless pGL3-basic vector; UGT2B7-575/-1/MT3 plasmid contains the 575 bp promoter with a mutated p53 site (A); UGT2B7-575/-1/MT5 contains the 575 bp promoter with mutation of a sequence located 7 bp downstream from the p53 site (A). HepG2 cells were transfected with these constructs and subsequently treated for 24 hours with vehicle [0.1% ethanol (ETH) for anthracyclines or 0.1% dimethylsulfoxide (DMSO) for nutlin-3a], the p53 activator nutlin-3a (10 μM), or one of the anthracylines [i.e., epirubicin (EPI), doxorubicin (DOX), daunorubicin (DAU), and idarubicin (IDA), all at 1 μM]. After treatment, cells were harvested and assayed for luciferase activity as described in Materials and Methods. (A) Shown is the UGT2B7 promoter sequence between nucleotides -278 and -238 containing the p53 site (underlined and core sequence in bold) and both the positions and sequences of the two introduced mutations (MT3 and MT5). (B) Data are presented as fold induction of the activities of promoter constructs relative to those of the promoterless pGL3-basic vector (set at a value of 1). Data shown are mean ± S.D. of three independent experiments performed in triplicate. Statistical analyses used one-way analysis of variance followed by Tukey’s post hoc multiple comparison test. **P < 0.01, ***P < 0.001.
C at 10 μM, 5-FU at 300 ng/ml, camptothecin at 0.5 μM, SN-38 at 10 ng/ml, topotecan at 0.1 μg/ml, and etoposide at 50 μM) are clinically relevant and/or approximate those that have been frequently used in similar cell culture studies (Italia et al., 1983; Pieret et al., 2006; Roudier et al., 2007; L’Espérance et al., 2008; Malmlof et al., 2008; Cosse et al., 2010; Saunders et al., 2010; Basseville et al., 2011; Hu et al., 2014c). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA) and converted to cDNA using Invitrogen reverse transcription reagents (Mulgrew, VIC, Australia) as previously reported (Hu and Mackenzie, 2009). Real-time PCR was performed using a RotorGene 3000 instrument (Corbett Research, NSW, Australia) and either QuantiTect SYBR Green PCR master mix (QIAGEN) or GoTaqqPCR master Mix (Promega, Madison, WI) in a 20 μl reaction containing ~60 ng of cDNA sample and previously published primers (Hu and Mackenzie, 2009; Hu et al., 2014c). The mRNA levels of target genes (i.e., UGT2B7, UGT2B10, and p21) relative to those of 18S rRNA were quantified using the 2^-ΔΔCt method (Livak and Schmittgen, 2001).

Transient Transfection and Luciferase Reporter Assay. Three pGL3 luciferase constructs containing the proximal 575 base pair UGT2B7 promoter fragment used in this study have been reported previously (Hu et al., 2014c). As shown in Fig. 3A, the UGT2B7-575/-1 construct contained a wild-type p53 site, the UGT2B7-575/-1/MT3 (where MT denotes mutant) carried a mutated p53 site, and the UGT2B7-575/-1/MT5 construct had a mutation in the sequence that is 7 base pairs downstream from the p53 site. Transient transfection of these constructs together with the internal control pRL-null vector into HepG2 cells and the subsequent drug treatment and luciferase assays using the Dual-Luciferase Reporter Assay System (Promega) were conducted essentially as reported previously (Hu et al., 2014c).

Small Interfering RNA (siRNA) Knockdown Experiments. On-TARGETplusSMARTpool siRNA against p53 and On-TARGETplus non-targeting pool siRNA (neg-siRNA) were purchased from Dharmacon RNAi Technologies (Lafayette, CO). HepG2 cells were transfected in either 6- or 24-well plates at a concentration of 100 nM using Lipofectamine2000 (Invitrogen) as recently reported (Hu et al., 2014c). 48 hour post transfection, cells were treated with drugs at the concentrations and time indicated in the figures 2 and 6. After treatment, cells were then harvested either for total RNA, followed by quantitative real-time PCR to quantify target mRNA levels as described previously or for whole cell lysates for Western blotting analysis as described subsequently.

Western Blotting. Whole cell lysates were prepared from drug- or vehicle-treated HepG2 cells or p53 siRNA (or neg-siRNA)–transfected HepG2 cells in radioimmunoprecipitation assay buffer [50 mM Tris-HCl, pH 8.0, 1% nonidet p40 substitute (Astral Scientific, Gymea, NSW, Australia), 150 mM sodium chloride, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate]. Protein concentrations were determined using the Bradford Protein Assay (BioRad) (Gladeville, NSW, Australia), and 30 μg protein was separated on SDS-polyacrylamide gels (10%) and transferred to nitrocellulose membranes for immunodetection. The development and specificity of the rabbit anti-UGT2B7 antibody has been reported previously (Kerdpin et al., 2009; Hu et al., 2014a). Other primary antibodies used were anti-calnexin antibodies obtained from Sigma-Aldrich, anti-p53 antibodies (FL-393 or DO-1) obtained from Santa Cruz Biotechnology (Dallas, Texas), and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies (G9545 or G8795) obtained from Sigma-Aldrich. Membranes were incubated first with the primary antibodies at supplier-recommended concentrations and then with a horseradish peroxidase-conjugated donkey anti-rabbit (or anti-mouse) secondary antibody (NeoMarkers) (Fremont, CA). Immunosignals were detected with the SuperSignalWest Pico Chemiluminescent kit (Thermo-Fisher Scientific, Waltham, MA) and an ImageQuant LAS 4000 luminescence image analyzer (GE Healthcare, Chalfont St. Giles, United Kingdom). Quantitation of band intensity and background subtraction was performed using NIH ImageJ analysis (Qin et al., 2011; Schneider et al., 2012).

Chromatin Immunoprecipitation Assay and Quantitative Real-Time PCR. The chromatin immunoprecipitation assay and quantitative PCR were performed essentially as reported previously (Hu et al., 2014c). In brief, HepG2 cells were treated with vehicle, 1 μM epirubicin, 10 μM mitomycin C, or 10 μM mitulxin-3α for 24 hours and then crosslinked using 1% formaldehyde for 30 minutes at 37°C, followed by quenching using 125 mM glycine solution. Cells were lysed, sonicated, and then subjected to immunoprecipitation with 8 μg of the rabbit preimmune IgG control (sc-2027, Santa Cruz Biotechnology) or equivalent amounts of the anti-p53 antibody (FL-393). Precipitated chromatin

![Fig. 4. Nonanthracycline cytotoxic drugs enhance UGT2B7 mRNA levels in the liver cancer HepG2 cell line. Nearly 100% confluent HepG2 cells in 6-well plates were treated for 24 hours with vehicle [water for mitomycin C or 0.1% dimethylsulfoxide (DMSO) for the remaining five drugs] or one of the six drugs, namely, mitomycin C (10 μM), 5-FU (300 ng/ml), camptothecin (0.5 μM), SN-38 (10 ng/ml), topotecan (0.1 μg/ml), and etoposide (50 μM). Total RNA was extracted and reverse transcribed to generate cDNA, followed by quantitative real-time PCR to quantify the mRNA levels of three genes (UGT2B7, UGT2B10, and p21) as described in Materials and Methods. Data are presented as the fold induction in target gene mRNA levels in drug-treated cells over those in vehicle-treated cells (set as a value of 1). Data shown are mean ± S.D. from a representative experiment of two independent experiments performed in triplicate. Statistical analysis used one-way analysis of variance followed by Tukey’s post hoc multiple comparison test. ** p < 0.01, *** p < 0.001.](https://dmd.aspetjournals.org/doi/abs/10.1124/dmd.117.072750)
was captured by Protein A Sepharose CL-4B beads (GE Healthcare), and subsequently eluted from the beads as described previously (Hu and Mackenzie, 2009). Crosslinking was reversed by heating the eluates at 65°C overnight. The resulting DNA/protein precipitates were digested with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellets were resuspended in 100 μl of Tris-EDTA buffer. Using 3 μl of each of the resultant DNA samples as the template, real-time quantitative PCR was conducted to quantify three UGT2B7 promoter regions, namely, the UGT2B7 p53 site (nucleotides -355/-209 relative to the translation start site) spanning the recently reported p53 recognition element (Hu et al., 2014c), and two negative control regions located at -3026/-2907 (negative control 1 region) and -7906/-7747 (negative control 2 region) relative to the UGT2B7 translation start site. In addition, the CYP3A4 promoter region covering the known p53 site (termed the CYP3A4 p53 site) was quantified as a positive control (Goldstein et al., 2013). Data from negative control region 2 were used to normalize the starting amounts of immunoprecipitated DNA added to each PCR. Enrichment of p53 binding at the UGT2B7 and CYP3A4 p53 sites and negative control region 1 were quantified relative to negative control region 2 using the 2^−ΔΔCt method (Livak and Schmittgen, 2001).

**Morphine Glucuronidation Assay.** HepG2 cells were plated in T75 flasks and cultured until they reached 90% confluence. Cells were then treated in triplicate with vehicle (water) or mitomycin C at 5, 10, or 20 μM for 24 hours. Cells were harvested, washed twice with 1× phosphate-buffered saline, and then lysed in 250 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), followed by protein concentration determination using the Bradford Protein Assay (Bio-Rad). Morphine glucuronidation assays using these samples were conducted essentially the same as previously reported (Hu et al., 2014c).

**Statistical Analysis.** Log transformed data met the assumption of equal variance at a significance level of less than 0.01 (Levene’s test). Statistical analyses of the transformed data were conducted by one- or two-way analysis of variance with the Tukey’s post hoc test or independent t test using the IBM SPSS program (version 22) (Chicago, IL) as detailed in the legends of Figures 1–7. A P value of less than 0.05 was considered statistically significant.

**Results**

**Induction of UGT2B7 by Anthracyclines.** Our recent demonstration of induction of UGT2B7 by epirubicin in HepG2 cells (Hu et al., 2014c) prompted us to test whether other anthracyclines could also stimulate UGT2B7 expression. As shown in Fig. 1A, the anthracyclines, doxorubicin, epirubicin, daunorubicin, and idarubicin, at 1 μM significantly elevated UGT2B7 mRNA levels in HepG2 cells following a 24 hour treatment, with epirubicin showing a significantly greater induction than the three other anthracylines. In contrast, UGT2B10 mRNA levels remained unchanged. Western blot analysis demonstrated that all four anthracyclines significantly increased UGT2B7 protein levels in HepG2 cells following a 24 hour exposure to these drugs (Fig. 1B). Epirubicin and doxorubicin showed a similar
induction in UGT2B7 protein levels despite the fact that epirubicin elicited a greater induction in UGT2B7 mRNA levels compared with doxorubicin (Fig. 1, A and B).

Induction of UGT2B7 by epirubicin was previously found to be mediated by p53 activation and subsequent binding of p53 to a recognition element in the UGT2B7 proximal promoter (Hu et al., 2014c). As shown in Fig. 1C, Western blot analyses using whole cell lysates of HepG2 cells treated with four anthracyclines for 24 hours demonstrated that, similar to epirubicin, all three other anthracyclines significantly increased p53 protein levels. Daunorubicin gave the lowest level of induction while the three other anthracyclines exhibited a similar level of induction. This activation of p53 was functionally relevant because all four anthracyclines increased the mRNA levels of p21, a p53 target gene (el-Deiry et al., 1993) (Fig. 1A). Knockdown of p53 by transfection of p53 siRNA into HepG2 cells was conducted to confirm a role for p53 in anthracyline-induced UGT2B7 expression. As shown in Fig. 2A, p53 siRNA significantly reduced the levels of endogenously expressed p53 protein; this reduction significantly decreased the anthracyline-elevated mRNA levels of both UGT2B7 and p21 but did not affect UGT2B10 mRNA levels (Fig. 2B). Furthermore, transient luciferase reporter assays demonstrated that, similar to epirubicin and the p53-activator nutlin-3a, doxorubicin and idarubicin activated the UGT2B7 promoter and this activation was abolished by mutating the p53 site (MT3) but not by a mutation (MT5) that changed the sequence immediately downstream from the p53 site (Fig. 3, A and B). Daunorubicin was not able to stimulate the UGT2B7 promoter, suggesting that factors additional to p53 are required to regulate its effect on UGT2B7 gene expression and that other UGT2B7 cis regulatory elements may be involved. The reduction of basal UGT2B7 promoter activity resulting from mutation of the p53 site (MT3) is indicative of a role for p53 in controlling constitutive expression of UGT2B7 as previously reported (Goldstein et al., 2013; Hu et al., 2014c). Taken collectively, our results demonstrate that classic anthracyclines stimulate the expression of UGT2B7 in HepG2 cells via a p53-signaling pathway.

**Induction of UGT2B7 by Nonanthracycline Cytotoxic Drugs.**

Nonanthracycline cytotoxic drugs have also been shown to be able to activate p53, including DNA topoisomerase II inhibitors (e.g., etoposide), DNA topoisomerase I inhibitors (e.g., camptothecin, SN-38, and topotecan), DNA alkylating agents (e.g., mitomycin C), and antimetabolite anticancer drugs (e.g., 5-FU) (Fritsche et al., 1993; Tishler et al., 1993, 1995; Müller et al., 1997; Inoue et al., 2001; Daoud et al., 2003). To test the possibility of induction of UGT2B7 by nonanthracyline p53-activating cytotoxic drugs, we treated HepG2 cells for 24 hours with six nonanthracycline cytotoxic drugs (i.e., mitomycin C, 5-FU, camptothecin, SN-38, topotecan, and etoposide) at concentrations as detailed in the Fig. 4 legend. Results from these experiments demonstrated that all six cytotoxic drugs significantly elevated UGT2B7 mRNA levels with mitomycin C showing the highest induction. In control experiments, all six drugs enhanced the mRNA levels of p21 (a p53-target gene) but only SN-38 had any effect on the mRNA levels of UGT2B10 (a small but significant reduction). These results demonstrated the capacity of nonanthracycline p53-activating cytotoxic drugs to induce UGT2B7 expression.

To investigate the involvement of p53 in the above-described induction of UGT2B7 by nonanthracycline cytotoxic drugs, we carried out further experiments in HepG2 cells using mitomycin C, the most potent inducer of UGT2B7 among the tested six nonanthracycline cytotoxic drugs (Fig. 4). First, Western blot assays demonstrated that mitomycin C elevated the protein levels of both p53 and UGT2B7 in a dose-dependent manner but did not alter the control glyceraldehyde-3-phosphate dehydrogenase protein levels (Fig. 5, A and B); UGT2B7 activity, using morphine as the substrate was also increased by mitomycin C in a dose-dependent manner (Fig. 5B, middle panel). Second, knockdown of p53 protein levels by anti-p53 siRNA (Fig. 6A) significantly reduced the mitomycin C–induced expression of UGT2B7 mRNA levels in HepG2 cells. Briefly, cells were transfected at 100 nM with either p53 siRNA or nontarget siRNA (neg-siRNA). Forty-eight hours after transfection, cells were treated for 24 hours with mitomycin C (10 μM) or the p53 activator nutlin-3a (10 μM), followed by quantitative real-time PCR to quantify the mRNA levels of UGT2B7 and p21 as described in Materials and Methods. Shown are the immunosignals (A) and the fold induction (i.e., mean ± S.D.) (B) in target gene mRNA levels in drug-treated and p53 siRNA-transfected HepG2 cells compared with those in drug-treated and nontarget siRNA-transfected cells (set as a value of 100%) from a representative experiment of two independent experiments performed in triplicate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Statistical analyses used two-way analysis of variance followed by independent *t* tests with Bonferroni correction, *P* < 0.05, **P** < 0.01.

**Fig. 6.** siRNAs against p53 reduce mitomycin C–induced UGT2B7 mRNA levels in HepG2 cells. Briefly, cells were transfected at 100 nM with either p53 siRNA or nontarget siRNA (neg-siRNA). Forty-eight hours after transfection, cells were treated for 24 hours with mitomycin C (10 μM) or the p53 activator nutlin-3a (10 μM), followed by quantitative real-time PCR to quantify the mRNA levels of UGT2B7 and p21 as described in Materials and Methods. Shown are the immunosignals (A) and the fold induction (i.e., mean ± S.D.) (B) in target gene mRNA levels in drug-treated and p53 siRNA-transfected HepG2 cells compared with those in drug-treated and nontarget siRNA-transfected cells (set as a value of 100%) from a representative experiment of two independent experiments performed in triplicate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Statistical analyses used two-way analysis of variance followed by independent *t* tests with Bonferroni correction, *P* < 0.05, **P** < 0.01.
both UGT2B7 and p21 (Fig. 6B). As expected, this strategy reduced the induction of both genes by the p53-activator nutlin-3a. Third, transient luciferase reporter assays showed that mitomycin C significantly increased the activity of the wild-type UGT2B7 promoter (UGT2B7-575/-1) but not the p53 site-mutated UGT2B7 promoter (UGT2B7-575/-1/MT3) (Fig. 7A). Fourth, similar to epirubicin and the p53 activator nutlin-3a, chromatin immunoprecipitation analysis showed that mitomycin C recruited p53 to the region covering the recently reported UGT2B7 p53 site (Hu et al., 2014c) but not to the region that is approximately 3 kb upstream of the UGT2B7 p53 site (Fig. 7B). Taken together, these results demonstrated that, similar to epirubicin, mitomycin C stimulates UGT2B7 expression via p53 activation and subsequent binding of p53 to the reported p53 recognition element in the UGT2B7 promoter.

**Discussion**

Recent studies have shown that a novel group of genes involved in drug metabolism are induced by the cytotoxic drug-activated p53-signaling pathway, including CYP enzymes (Goldstein et al., 2013) and UGTs (Dellinger et al., 2012; Hu et al., 2014c). This enhanced drug metabolism activity promotes drug clearance and may be relevant to reduced chemotherapeutic efficacy or the development of drug resistance. A recent example is the induction of UGT2B7 expression by epirubicin in the liver cancer HepG2 cell line via a p53 site in the proximal UGT2B7 promoter (Hu et al., 2014c). Because epirubicin is a substrate of UGT2B7 (Innocenti et al., 2001), this induction enhances epirubicin inactivation via glucuronidation. The present study demonstrates that, similar to epirubicin, several other cytotoxic drugs, including three anthracyclines (i.e., doxorubicin, daunorubicin, and idarubicin) and six nonanthracyclines (i.e., mitomycin C, 5-FU, camptothecin, SN-38, topotecan, and etoposide), stimulated UGT2B7 expression via the p53 pathway. UGT2B7 is not involved in the metabolism of these nine cytotoxic drugs, and thus this enhanced UGT2B7 activity would not affect the therapeutic efficacy of these cytotoxic drugs themselves; however, cytotoxic drugs with different modes of action are frequently used in combination (termed combinatorial chemotherapy) to enhance chemotherapeutic efficacy and/or to prevent/delay development of chemotherapy resistance. Frequently, two of these nine cytotoxic drugs are used in combinatorial chemotherapy for treating various cancers; for example, the double (epirubicin/mitomycin C) and triple (epirubicin/mitomycin C/cisplatin) chemotherapeutic regimens for treating advanced hepatocellular carcinoma (Marelli et al., 2007). Thus, if induction of UGT2B7 by mitomycin C occurs in vivo the enhanced glucuronidation of epirubicin may reduce the overall efficacy of regimens that contain both of these drugs. In addition to the cytotoxic drugs tested in the present study, other cytotoxic and noncytotoxic drugs have also been shown to be able to activate the p53-signaling pathway, such as methotrexate, cisplatin, cyclophosphamide, bleomycin, taxol, vinblastine, nocardazole, arsenite, and deferoxamine mesylate (Fritsche et al., 1993; Tishler et al., 1993, 1995; Müller et al., 1997; Appella and Anderson, 2001; Pluquet and Hainaut, 2001). The capacity of these

![Figure 7](https://example.com/figure7.png)

**Fig. 7.** Mitomycin C activates the UGT2B7 promoter and recruits p53 to the UGT2B7 promoter encompassing the previously reported p53 site in HepG2 cells. (A) HepG2 cells were transfected with the internal control pRL-null vector and one of the three luciferase constructs (pGL3-basic vector, UGT2B7-575/-1, and UGT2B7-575/-1/MT3); 24–48 hours after transfection, cells were treated for 24 hours with vehicle (water) or mitomycin C (5 μM). After treatment, cells were harvested and assayed for luciferase activity as described in Materials and Methods. Data are presented as the fold induction of the activities of promoter constructs relative to those of the promotorless pGL3-basic vector (set as a value of 1). Data shown are mean ± S.D. from a representative experiment of two independent experiments performed in triplicate. (B) Cells were treated for 24 hours with vehicle, 1 μM epirubicin, 10 μM nutlin-3a, or 10 μM mitomycin C, and then subjected to chromatin immunoprecipitation assays, followed by real-time PCR to quantify the amount of precipitated DNA corresponding to (1) the previously defined UGT2B7 p53-binding site; (2) a region located approximately 3 kb upstream of the UGT2B7 p53 site (negative control 1); and (3) the reported CYP3A4 p53 site (positive control). Data were normalized as described in Materials and Methods using amplification values for the negative control 2 locus, and were then expressed as the fold enrichment in DNA samples precipitated with the anti-p53 antibody (FL393) compared with that in the control DNA samples precipitated with equivalent amounts of preimmune IgG serum (IgG) (set as a value of 1). Data shown are mean ± S.D. from one representative experiment of two independent experiments performed in triplicate. Statistical analyses used two-way analysis of variance followed by Tukey’s post hoc multiple comparison test. **P < 0.001.**
drugs to stimulate UGT2B7 expression and activity remains to be investigated. In addition to cytotoxic drugs, the p53-signaling pathway has also been shown to be activated by other genotoxic stress, including hypoxia, UV light, radiation, and nucleotide depletion (Appella and Anderson, 2001; Pluquet and Hainaut, 2001; Resnick-Silverman and Manfredi, 2006; Riley et al., 2008). It remains to be determined whether UGT2B7 is induced upon exposure to these diverse genotoxic stimuli.

Because the liver is the major glucuronidation site in the human body, hepatic UGT expression and activity governs systemic metabolism and clearance of UGT substrates. As reviewed recently (Hu et al., 2014b), UGT2B7 displays high activity toward a variety of noncytotoxic drugs. This represents a novel mechanism that may clear cytotoxic and noncytotoxic drugs that are primarily nonhepatic cancers may induce the expression and activity of UGT2B7, which represents a novel mechanism that may clear cytotoxic and noncytotoxic drugs that are primarily nonhepatic.

Table 1: Cytotoxic drugs studied in this study and their indications

<table>
<thead>
<tr>
<th>Drug</th>
<th>Major Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>Lymphomas, Breast cancer, Sarcomas,</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular cancer, Leukaemias</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>Breast cancer, Hepatocellular</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Acute myelogenous leukemia, Acute</td>
</tr>
<tr>
<td></td>
<td>lymphocytic leukemia, Acute myelogenous</td>
</tr>
<tr>
<td>Irinotecan (SN-38)</td>
<td>Testicular cancer, Small cell lung</td>
</tr>
<tr>
<td></td>
<td>cancer, Ovarian cancer, Pancreatic</td>
</tr>
<tr>
<td></td>
<td>cancer, Ovarian cancer, Lung cancer</td>
</tr>
<tr>
<td>Topotecan</td>
<td>Small cell lung cancer, Ovarian cancer</td>
</tr>
<tr>
<td></td>
<td>Lung cancer</td>
</tr>
<tr>
<td>5-FU</td>
<td>Colorectal cancer, Breast cancer,</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer, Hepatocellular cancer,</td>
</tr>
<tr>
<td></td>
<td>Stomach cancer, Pancreatic cancer,</td>
</tr>
<tr>
<td></td>
<td>Breast cancer, Colon cancer, Anal</td>
</tr>
<tr>
<td></td>
<td>cancer, Lung cancer</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Not approved for clinical use</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Hepatocellular cancer, Stomach cancer,</td>
</tr>
<tr>
<td></td>
<td>Pancreatic cancer, Colon cancer,</td>
</tr>
<tr>
<td></td>
<td>Breast cancer, Anal cancer,</td>
</tr>
<tr>
<td></td>
<td>Lung cancer</td>
</tr>
</tbody>
</table>

In conclusion, the present study demonstrates that similar to epirubicin, another nine cytotoxic drugs stimulate UGT2B7 expression in HepG2 cells through the p53-signaling pathway, most likely via the previously reported UGT2B7 p53 site in the proximal promoter. These observations further support our previous finding that UGT2B7 is a p53 target gene in a hepatic cellular context and shed more insight into the molecular mechanisms controlling the hepatic expression and activity of UGT2B7. This cytotoxic drug-induced UGT2B7 activity in target liver cancer cells within the tumor or possibly in normal liver cells could promote intratumoral or systemic metabolism and clearance of cytotoxic drugs and other co-administered drugs that are inactivated by UGT2B7, and thus it may relate to reduced efficacy of cancer therapy or even lead to development of drug resistance.

Authorship Contributions

Participated in research design: Hu, Mackenzie, McKinnon. Conducted experiments: Hu, Lu. Performed data analysis: Hu, Mackenzie. Wrote or contributed to the writing of the manuscript: Hu, Mackenzie, Meec, McKinnon.

References


