Curcumin Resistance Induced by Hypoxia in HepG2 Cells Is Mediated by Multidrug-resistance-associated Proteins

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Abstract. *Background: Tumor hypoxia, a common pathophysiological feature of solid tumors, contributes to drug resistance and treatment failure. Here, we demonstrate that hypoxia in HepG2 cells induces resistance towards cytotoxicity of curcumin, a promising anticancer agent. Materials and Methods: The number of surviving cells after exposure to chemotherapeutic drugs under normoxia (ambient O_2) and hypoxia (1% O_2) was determined by crystal violet staining. The expression levels of drug transporter genes were analyzed by quantitative real-time reverse transcription-polymerase chain reaction. Results: Increased resistance to curcumin, as well as to etoposide and doxorubicin, was observed in HepG2 cells under hypoxia. Gene expression analysis revealed that hypoxia increased the expression of ATP-binding cassette (ABC) drug transporter genes, sub-family C including ABCC1, ABCC2, and ABCC3, by more than two-fold. While expression of ABC drug transporter genes sub-family B member 1 and sub-family G member 2 (ABCB2/P-gp and ABCG2, respectively) did not change significantly. Both inhibitors of ABCC1/ABCC2 and depletion of intracellular glutathione levels were able to reverse hypoxia-induced curcumin resistance. Conclusion: ABCC1 and ABCC2 play an important role in hypoxia-induced curcumin resistance in human hepatocellular carcinoma.*

There has been much interest in improving the efficacy of cancer treatment by trying to find new anticancer agents or developing chemosensitizers or modulators to enhance the efficacy of existing chemotherapeutic drugs to combat drug-resistant cancers. This, however, has mostly been unsuccessful due to undesirable toxicological effects (1). Recently, curcumin, extracted from the spice turmeric (*Curcuma longa*), a well-known ancient herb, has been highlighted as a promising anticancer agent (2, 3). Curcumin has been shown to be well-tolerated in a phase II trial of patients with advanced pancreatic cancer (4) and is currently in clinical phase I and II, and recruiting for phase III trials (5). Curcumin inhibits cancer cell proliferation and/or induces apoptosis through modulating multiple apoptotic and survival signaling pathways, such as nuclear factor-κB (NF-κB) and signal transducers, and activators of transcription 3 (STAT3) (6-8). Additionally, curcumin has been shown to reverse multidrug resistance by inhibiting P-glycoprotein (P-gp, *ABCB1* gene product) expression in cervical carcinoma, gastric carcinoma, and multiple myeloma (9-11).

Drug resistance has become a major obstacle in cancer therapy, as cancer cells can acquire multidrug resistance following an initial round of chemotherapy. Multiple factors contribute to treatment failure including, but not limited to: (i) classical multidrug resistance involving increased ATP-binding cassette (ABC) drug transporters; (ii) environment-mediated drug resistance, where cell–cell interactions, extracellular matrix and growth factors are involved in reducing apoptosis; (iii) increased cell survivability due to up-regulated anti-apoptotic, pro-survival, and DNA repair proteins; and (iv) increased capacity for cellular de-toxification or inactivation of drug activity (12). Among these, the most common mechanism is increased drug efflux mediated by ABC drug transporter proteins (13). There are many variants of multidrug transporters that utilize ATP as an energy source. To date, 49 ABC transporters have been identified and classified into sub-family A to G, based on sequence similarities. For example, ABCB1 is member 1 of ABC transporter sub-family B (14).

Tumor hypoxia is a phenomenon in solid tumors where cells are exposed to uneven oxygen levels (15). The accelerated growth of tumor contributes to the disorganized arrangement of cells, resulting in constriction of existing blood vessels. The uneven distribution of oxygen to cells causes spatial and temporal hypoxia. Hypoxia-induced drug resistance has been observed in many cancer types, such as non-small cell lung cancer, gastric cancer, breast and ovarian cancer, and colon carcinoma (16-19).

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Although there is much interest in curcumin as an anticancer agent, to our knowledge, there have been no reports on the sensitivity to curcumin under hypoxia. In the present study, we have explored whether hypoxia can modulate sensitivity of a human hepatocellular carcinoma (HCC) cell line, HepG2, to curcumin and further investigated the underlying mechanism.

Materials and Methods

Chemical reagents. Curcumin (MW 368.39) was purchased from Fluka Chemika (Steinheim, Switzerland), etoposide (MW 588.56) and doxorubicin (MW 580.0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were dissolved in dimethylsulfoxide (DMSO) and kept as stock solution at −20˚C. MK-571 (sodium salt) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), while DL-Buthionine-[S,R]-sulfoximine (BSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and both compounds were dissolved in water and kept as stock solution at −20˚C. Experimental concentrations of all test compounds were obtained by dilutions with cell culture media, ensuring the final concentration of DMSO to be less than 0.2% (v/v). Cell culture medium, fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from Gibco (Grand Island, NY, USA). Crystal violet was obtained from Fluka Chemika (Steinheim, Switzerland).

Cell culture conditions. The human hepatocellular carcinoma cell line, HepG2, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM/high glucose) supplemented with 10% FBS, and 1% antibiotic-antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) in a humidified atmosphere of 5% CO_2 and 21% O_2 at 37˚C. For the hypoxic condition, cells were incubated at 37˚C in a hypoxic chamber (C-Chamber; Biospherix, NY, USA) with a constant humidified atmosphere of 5% CO_2 and 1% O_2 maintained by a regulated supply of CO_2 and N_2 gas.

Cytotoxicity assay. Crystal violet staining was used to assess the number of surviving cells after treatment with different chemotherapeutic drugs under either normoxic or hypoxic conditions. Cell suspensions were seeded into 96-well plates (100 μl/well) at a density of 4×10^4 cells/well (normoxia, 21% O_2) or 8×10^4 cells/well (hypoxia, 1% O_2) and incubated at 37˚C in a humidified atmosphere of 5% CO_2. After 20-24 h, cells were pre-exposed to the normoxic or hypoxic condition for 24 h, then treated with additional medium (100 μl) containing different concentrations of curcumin or other chemotherapeutic drugs, followed by further incubation for another 24 h under the normoxic or hypoxic condition. At the end of treatment, the number of surviving cells was determined by the crystal violet staining method, as previously described (20). Briefly, surviving cells in 96-well plates were washed twice with phosphate-buffered saline (PBS) and then fixed with 95% ethanol. Crystal violet solution (0.5% w/v in 25% methanol) was added to stain the cells, and after washing by tap water, 0.1 N HCl in methanol (100 μl/well) was added to lyse cells. The number of surviving cells was then determined by measuring absorbance at 550 nm with a microplate reader. Assays were performed in triplicate wells and data were computed as the percentage survival of drug-treated cells compared with that of the untreated control.

Quantitative real-time reverse transcription-polymerase chain reaction. Quantitative real-time RT-PCR (qRT-PCR) was used to determine differential expression of drug transporter genes induced by hypoxia. Total RNA from cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using Superscript III Reverse Transcriptase Kit (Invitrogen, San Diego, CA, USA).

Gene expression levels were detected by real-time PCR using LightCycler® 2.0 Instrument (Roche Applied Bioscience, Indianapolis, IN, USA). The real-time PCR protocol and primer sequences were described previously (21). Briefly, an aliquot of diluted cDNA and 10 pmol of primers were mixed with QuantiTect SYBR Green PCR master mix (Qiagen). PCR reactions were performed by initial activation at 95˚C for 15 min followed by 40 cycles of denaturing step at 95˚C for 30 s, annealing step at the appropriate temperature (56˚C for ABCC3, 62˚C for ABCB1, ABCC1, ABCC2, and ABCG2) for 30 s, and an extension step at 72˚C for 30 s. β-Actin was used as the reference gene and the relative amount of cDNA of each targeted gene was calculated from the crossing point (Cp) value of each sample using the LightCycler Software version 4.0.5. The differential fold expression of genes were calculated using the $2^{-\Delta\Delta Cp}$ equation (22).

Statistical analysis. All statistical analyses were carried out with Graphpad Prism5 software (GraphPad, La Jolla, CA, USA). Two-way analysis of variance (ANOVA) along with Bonferroni multiple comparisons were used to analyze the statistical significance of conditions between normoxia *vs*. hypoxia for the different treatment concentrations. One-way ANOVA along with Tukey's multiple comparison test was used to analyze the significance of results for different treatments under the hypoxic condition.

Results

Hypoxia induces multidrug resistance in HepG2. In order to test the effect of hypoxia on drug sensitivity, HepG2 cells were pre-exposed to hypoxia for 24 h, followed by drug treatment for another 24 h. As shown in Figure 1, the hypoxic condition increased the number of surviving cells under all drug treatments. The percentage survival under normoxia *vs*. hypoxia for curcumin (50 μM) was 30% *vs*. 84%, for etoposide (51 μM) 37% *vs*. 86%, and for doxorubicin was 37% *vs*. 85% ($p<0.05$). Thus the results showed that hypoxia induced resistance to curcumin as well as to other anticancer drugs in HepG2 cells.

Determination of mechanisms involved in hypoxia-induced multidrug resistance. As increased drug efflux is the most common mechanism involved in the multidrug resistance phenotype, we evaluated whether up-regulation of ABC drug transporter genes are responsible for the observed hypoxia-induced curcumin resistance of HepG2 cells. The selected genes include: *ABCB1, ABCC1, ABCC2, ABCC3*, and *ABCG2*. Expression levels of selected genes under hypoxic and normoxic conditions were compared, and any gene expression with greater than two-fold difference was considered significant. Our results revealed that HepG2 cells under hypoxia had up-regulated expression levels of *ABCC1*, *ABCC2*,

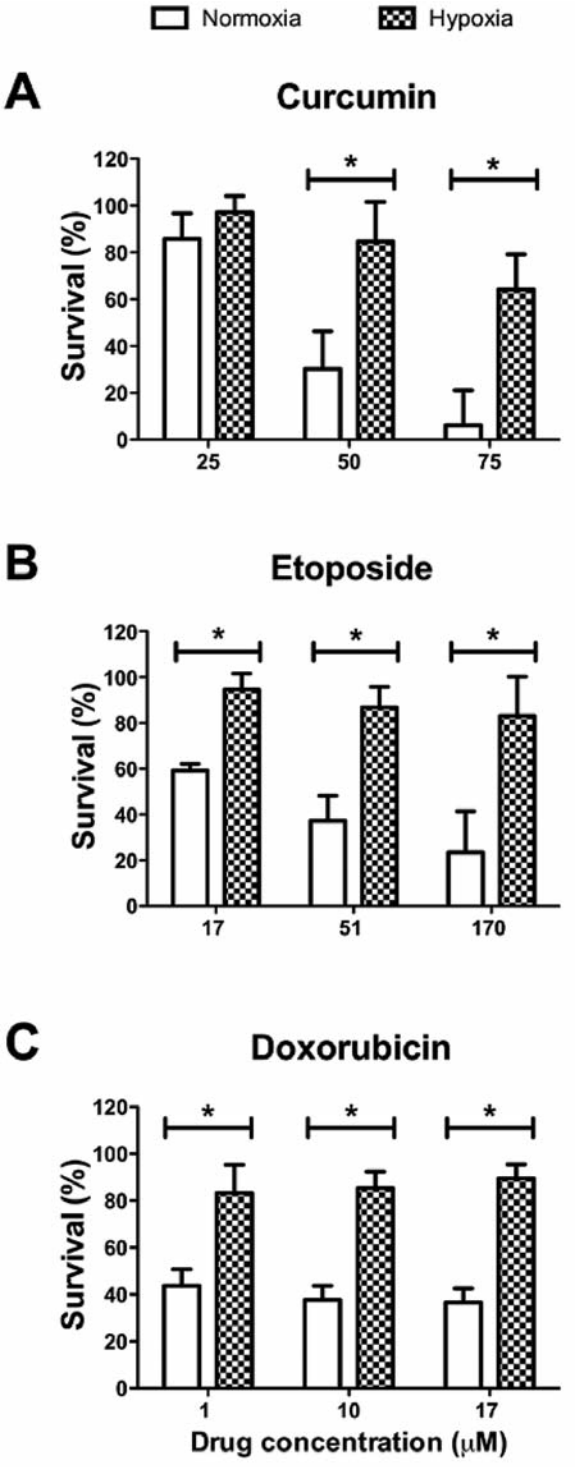

Figure 1. *Cytotoxicity of curcumin (A), etoposide (B) and doxorubicin (C) towards HepG2 cells under normoxic and hypoxic conditions. Cells were pre-incubated under normoxic or hypoxic condition for 24 h, and then treated with drugs under a normoxic or hypoxic condition for an additional 24 h. Cell survival was determined by crystal violet staining. Data are expressed as the percentage survival. Column and error bars indicate the mean±standard deviation obtained from three independent experiments. Statistical significance at p<0.05 is indicated by *.*

and *ABCC3* by greater than two-fold compared to their expression levels under normoxia (Figure 2). However, expression levels of *ABCB1* and *ABCG2* under hypoxia were not significantly increased. Our results suggest that the multidrug resistance phenotype of HepG2 cells induced by hypoxia was likely to result from up-regulation of the ABC transporter gene sub-family C members rather than *ABCB1* overexpression.

Inhibitor of ABCC1 and ABCC2 reverses hypoxia-induced curcumin resistance. Up-regulation of ABCC1 and ABCC2 might be the main cause of curcumin resistance under the hypoxic condition. Therefore, a potent inhibitor of ABCC1 and ABCC2 was employed as co-treatment with curcumin under hypoxia to suppress ABCC1- and ABCC2-mediated drug efflux. MK-571 is a leukotriene LTD4 receptor antagonist that is capable of inhibiting transport by ABCC1 and ABCC2. Treatment with MK-571-alone of HepG2 cells under hypoxia did not affect cell survivability (Figure 3). Combinatorial treatment of curcumin (50 μM) and MK-571 (10-100 μM) under the hypoxic condition was able to significantly reverse hypoxia-induced curcumin resistance in a dose-dependent manner ($p<0.05$) (Figure 3), implying that the function of ABCC1 and ABCC2 contribute to this resistance.

Reduction of glutathione synthesis sensitizes hypoxic HepG2 cells to curcumin. Hypoxia-induced curcumin resistance observed in HepG2 cells was postulated to be due to the ability of ABCC1 and ABCC2 to efflux curcumin from the cells. ABCC1 and ABCC2 recognize substrates that are anionic or conjugates of glutathione, glucuronide, and sulfate (23). Curcumin may conjugate to glutathione (24) and can be recognized by ABCC1 and ABCC2 (25). A glutathione synthesis inhibitor (BSO) was used to reduce conjugation of curcumin–glutathione conjugates, thereby reducing the efflux of curcumin by ABCC1 and ABCC2. BSO reduces intracellular glutathione by inhibiting gamma-glutamylcysteine synthetase, an enzyme crucial for glutathione synthesis. Addition of BSO-alone to HepG2 cells under hypoxia did not reduce cell survivability at concentrations of 1 and 5 mM (Figure 4). However, the results clearly show that treatment with BSO for 24 h before the addition of curcumin significantly sensitized hypoxia-exposed HepG2 cells to curcumin, when compared to cells treated with curcumin in the absence of BSO, confirming the crucial role of ABCC1 and ABCC2 in curcumin resistance under hypoxic conditions.

Discussion

To our knowledge, this is the first report showing that hypoxia can induce resistance of human HCC cells to curcumin, through up-regulation of ABC drug transporter genes. Previous studies have reported the ability of curcumin to suppress the

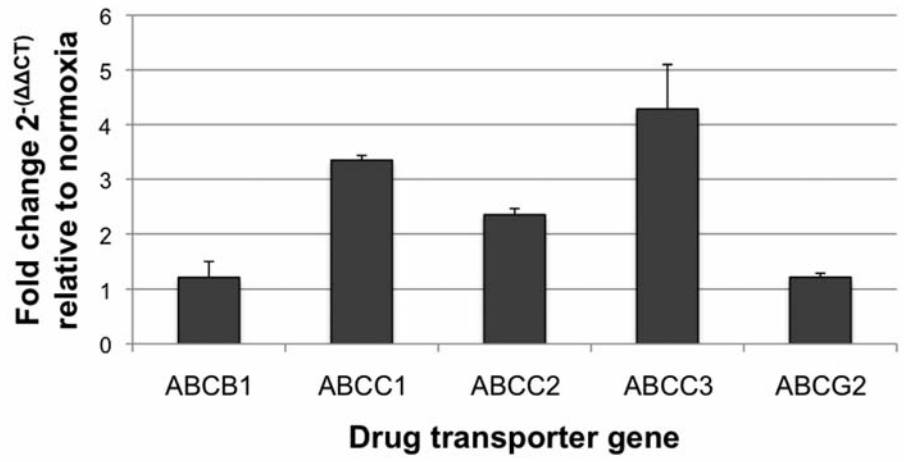

Figure 2. *Expression level of ATP-binding cassette (ABC) drug transporter genes in HepG2 cells under the hypoxic condition compared to the normoxic condition. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of selected genes in HepG2 cells after 24 h exposure to normoxia or hypoxia. Fold increase in expression was calculated using $2^{-\Delta\Delta CT}$ value for each gene. Column and error bars represents the mean±standard deviation obtained from two independent experiments.*

hypoxic response of cancer cells through hypoxia inducible factor 1 down-regulation, when curcumin is applied simultaneously with hypoxic exposure (26-28). In our study, resistance to curcumin and other anticancer drugs was observed when HepG2 cells were pre-exposed to hypoxia for 24 h before drug treatment. Therefore, we believe that under this circumstance, cells pre-exposed to hypoxia have elevated expression of ABC transporters, as shown in Figure 2, leading to an increased capacity to efflux curcumin from the cells before it can exert its cytotoxic effect. Bachmeier *et al.* observed that the M14 melanoma cell line is resistant to curcumin by intrinsic overexpression of ABCA1, another ABC transporter protein (29). Our study reveals that curcumin resistance in the HepG2 cell line could be acquired by increased expression of *ABCC1* and *ABCC2* as a result of exposure to hypoxia before drug treatment.

Hypoxia-induced drug resistance has been shown to be associated with increased expression of various multidrug resistant proteins such as: ABCB1 in human colon carcinoma (16, 30) and non-small cell lung cancer (19), and ABCC1 and lung resistant protein in gastric cancer (18, 31). Under the normoxic condition, overexpression of ABCB1, ABCC1, and ABCC2 is associated with doxorubicin (32) and etoposide resistance (33) by increasing cellular drug efflux, indicating overlapping substrate specificity between ABC transporter proteins. ABCG2 has similar substrate specificity to ABCB1, as reviewed by Kuo (14). While ABCB1 has broad substrate specificity, ABCC1 and ABCC2 appear to have varying substrate specificity between the members that mostly include unconjugated anionic compounds and conjugates of glutathione, glucuronide, and sulfate (23). In our study, expressions of *ABCC1* and *ABCC2*, rather than *ABCB1*, were significantly increased in HepG2 cells by exposure to hypoxia. Up-regulation of both *ABCB1* and *ABCC1* in hypoxic HepG2 cells has been previously reported (34), but we did not observe a significant increase in *ABCB1* in this study. We believe that this difference in gene expression might be due to differences in the levels of O_2 and in the culture medium under the hypoxic condition (2% O_2 in RPMI-1640 medium), compared to the ones used here (1% O_2 in DMEM). Nevertheless, the previous study does support our findings that hypoxia up-regulates *ABCC1* expression in HepG2 cells.

The reversal of hypoxia-induced curcumin resistance by the inhibitor MK-571 indicated that ABCC1 and ABCC2 play a significant role in curcumin resistance. Clinical study of curcumin pharmacokinetics in healthy humans showed curcumin–glucuronide and curcumin–sulfate in plasma after 1 h of administration (35), while in mice, curcumin–glucuronide was the major metabolite *in vivo* (36). Intracellular curcumin– glutathione, curcumin–glucuronide, and curcumin–sulfate conjugates serve as possible substrates of ABCC1 and ABCC2. As demonstrated by Wortelboer *et al.*, high-performance liquid chromatography (HPLC) analysis showed that curcumin– glutathione conjugates are substrates of ABCC1 and ABCC2 in Madin-Darby canine kidney cells, while curcumin itself was not a substrate for these molecules (25). We, therefore, postulate that the hypoxia-induced curcumin resistance observed here is most likely due to increased ABCC1- and ABCC2-mediated efflux of curcumin–glutathione conjugates, resulting in a decrease of overall curcumin levels inside the cell and to increased cell survival under this condition. MK-571 thus serves as a stop cork to prevent efflux of curcumin from hypoxic cells, allowing curcumin to exert its cytotoxic effects. This is

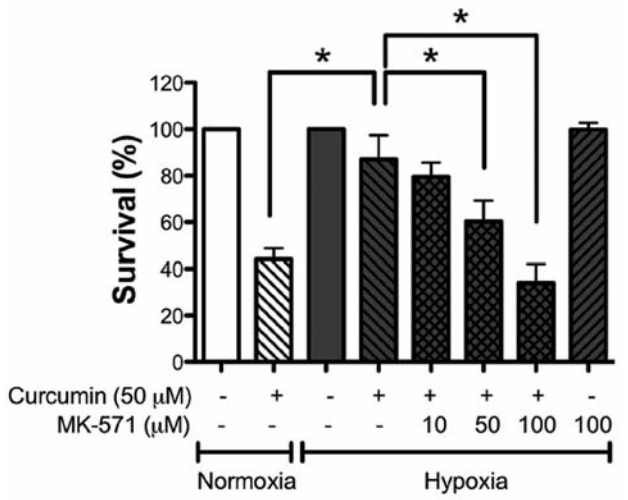

Figure 3. *Reversal of curcumin resistance by inhibitor of ABCC1 and ABCC2, MK-571, under the hypoxic condition. Cells were pre-incubated under the normoxic or hypoxic condition for 24 h then treated with curcumin, MK-571, or their combination and exposed to hypoxia for an additional 24 h. Data are expressed as the percentage survival. Column and error bars indicate the mean±standard deviation obtained from three independent experiments. Statistical significance at p<0.05 is indicated by *.*

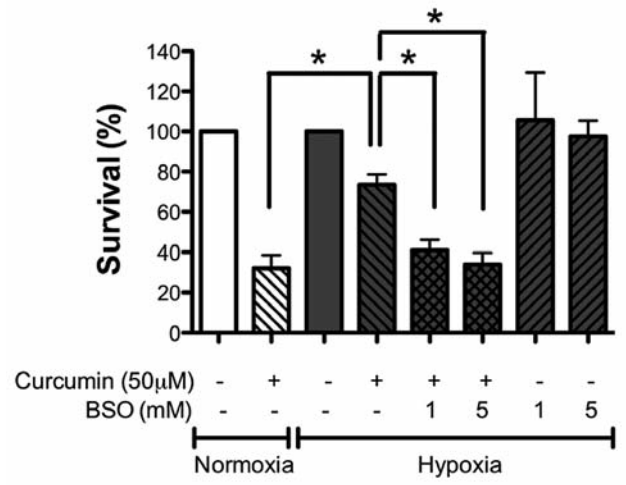

Figure 4. *Sensitization of hypoxic HepG2 cells to curcumin by inhibiting glutathione synthesis. Cells were pre-incubated under the normoxic or hypoxic condition for 24 h with 1 mM or 5 mM DL-Buthionine-[S,R]-sulfoximine (BSO), and then treated with 50 μM curcumin and exposed to the normoxic or hypoxic condition for another 24 h. Data are expressed as the percentage survival. Column and error bars indicate the mean±standard deviation obtained from three independent experiments. Statistical significance at p<0.05 is indicated by *.*

confirmed by the dose-dependent decrease in cell survival after the addition of MK-571.

As curcumin and its derivatives (demethoxycurcumin, *bis*demethoxycurcumin, tetrahydrocurcumin, and turmerones) are metabolized in the liver, they can form various conjugates with glutathione (monoglutathionyl curcumin, diglutathionyl curcumin, monoglutathionyl demethoxycurcumin, diglutathionyldemethoxycurcumin, monoglutathionyl *bis*demethoxy curcumin, glutathionyl feruloyl methyl ketone, glutathionyl ferulic acid, and glutathionyl feruloyl aldehyde) (24). Addition of BSO, a compound used to reduce the level of cellular glutathione by inhibiting glutathione synthesis, 24 h prior to curcumin treatment sensitized hypoxia-exposed cells to curcumin. This probably results from a decrease in curcumin–glutathione conjugation, and further confirms the essential role of ABCC1 and ABCC2 in hypoxia-induced resistance to curcumin in HepG2 cells.

In conclusion, our finding that ABCC1 and ABCC2 contribute to curcumin resistance in hypoxia-exposed cells has impact on the use of curcumin in cancer treatment for patients with non-resectable tumors, such as liver cancer with tumor mass near the portal vein. As hypoxia is a common pathophysiological feature of solid tumors (15), the use of curcumin-alone for treatment might fail to eradicate all cancerous cells. Thus, treatment of curcumin together with an inhibitor of ABCC1 and ABCC2 might be a better alternative to combat hypoxia-exposed HCC.

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