Causes of hOCT1-Dependent Cholangiocarcinoma Resistance to Sorafenib and Sensitization by Tumor-Selective Gene Therapy

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Although the multi-tyrosine kinase inhibitor sorafenib is useful in the treatment of several cancers, cholangiocarcinoma (CCA) is refractory to this drug. Among other mechanisms of chemoresistance, impaired uptake through human organic cation transporter type 1 (hOCT1) (gene SLC22A1) has been suggested. Here we have investigated the events accounting for this phenotypic characteristic and have evaluated the interest of selective gene therapy strategies to overcome this limitation. Gene expression and DNA methylation of SLC22A1 were analyzed using intrahepatic (iCCA) and extrahepatic (eCCA) biopsies (Copenhagen and Salamanca cohorts; n = 132) and The Cancer Genome Atlas (TCGA)-CHOL (n = 36). Decreased hOCT1 mRNA correlated with hypermethylation status of the SLC22A1 promoter. Treatment of CCA cells with decitabine (demethylating agent) or butyrate (histone deacetylase inhibitor) restored hOCT1 expression and increased sorafenib uptake. MicroRNAs able to induce hOCT1 mRNA decay were analyzed in paired samples of TCGA-CHOL (n = 9) and Copenhagen (n = 57) cohorts. Consistent up-regulation in tumor tissue was found for miR-141 and miR-330. High proportion of aberrant hOCT1 mRNA splicing in CCA was also seen. Lentiviral-mediated transduction of eCCA (EGI-1 and TFK-1) and iCCA (HuCCT1) cells with hOCT1 enhanced sorafenib uptake and cytotoxic effects. In chemically induced CCA in rats, reduced rOct1 expression was accompanied by impaired sorafenib uptake. In xenograft models of eCCA cells implanted in mouse liver, poor response to sorafenib was observed. However, tumor growth was markedly reduced by cotreatment with sorafenib and adenoviral vectors encoding hOCT1 under the control of the BIRC5 promoter, a gene highly up-regulated in CCA. Conclusion: The reason for impaired hOCT1-mediated sorafenib uptake by CCA is multifactorial. Gene therapy capable of selectively inducing hOCT1 in tumor cells can be considered a potentially useful chemosensitization strategy to improve the response of CCA to sorafenib. (Hepatology 2019;70:1246-1261).

Biliary tract cancers are a type of heterogeneous tumors that include cholangiocarcinoma (CCA), both intrahepatic (iCCA) and extrahepatic (eCCA), and gallbladder cancer, with diverse phenotypic characteristics.1 CCA, whose incidence is increasing worldwide, is currently the...
second most frequent primary hepatic malignancy after hepatocellular carcinoma (HCC). Surgical resection constitutes the best option for complete cure. However, owing to the lack of accurate noninvasive CCA markers and to the fact that these tumors grow up asymptptomatically, they are often detected at an unresectable advanced stage.\(^{(2,3)}\) This partly accounts for the very poor prognosis of this cancer, from which most patients die within 12 months after diagnosis. Classical chemotherapy offers a 5-year survival rate lower than 10\%, which is due to the negligible degree of response of all types of CCA to available chemotherapeutic regimens.\(^{(4,5)}\) Gemcitabine plus cisplatin has become the reference regimen for systemic chemotherapy in patients with biliary tract cancers;\(^{(6)}\) nonetheless, this chemotherapy is poorly effective. Therefore, it is essential to understand the molecular bases of the strong chemoresistance of CCA and to seek new therapeutic approaches. Despite their heterogeneity regarding several clinical and biological aspects, biliary cancers share their marked chemoresistance. Among the so-called targeted therapies, sorafenib, a multitargeted tyrosine kinase inhibitor (TKI) that blocks the activity of Raf serine/threonine kinase isoforms, as well as vascular endothelial growth factor receptor-2/3, platelet-derived growth factor receptor, c-KIT, FLT-3, and RET, to inhibit tumor angiogenesis and tumor cell proliferation,\(^{(7,8)}\) has been approved for the treatment of HCC.\(^{(9)}\) Sorafenib is one of the few TKIs that is active even against mutated BRAF, which appears with a high frequency in CCA, especially in iCCA. Moreover, sorafenib has been reported to have some anticancer activity against CCA in experimental models both \textit{in vitro} and \textit{in vivo}, which has not been consistently confirmed in clinical studies.\(^{(10-14)}\) The lack of response of patients with CCA to sorafenib may be the result of the combined action of several mechanisms of chemoresistance (MOCs).\(^{(5)}\) We have demonstrated that the organic cation transporter 1 (hOCT1, \textit{SLC22A1} gene) can play a key role in sorafenib effectiveness because the mechanism of action of this drug depends on its access to the intracellular domains of the tyrosine kinases that are inhibited by sorafenib.\(^{(15)}\) Moreover, hOCT1 mediates the uptake of this drug by target cells.\(^{(15)}\) Of note, it has been demonstrated that decreased expression of hOCT1 constitutes a shared characteristic of liver tumors (HCC, CCA, and hepatoblastoma).\(^{(16)}\) In addition, not all synthesized \textit{hOCT1} mRNA is translated into functional protein because of the high proportion of inactive variants that are generated as a result of alternative splicing mechanisms or single-nucleotide polymorphisms (SNPs).\(^{(15)}\)
In the present study, we have evaluated the hypothesis that mechanisms that cause impaired hOCT1 expression/function in CCA may be involved in the lack of response of these tumors to sorafenib, and we have investigated the usefulness of epigenetic manipulation and gene therapy in order to sensitize CCA to this drug by selectively enhancing hOCT1 expression in cancer cells under the control of a tumor-specific promoter.

**Materials and Methods**

**HUMAN SAMPLES AND DATA**

Whole transcriptome profiling was performed using human Ref-8v2 BeadChips (Illumina) on 68 iCCA and 36 eCCA surgical specimens (T) and 60 samples from adjacent nontumor tissue (NT), as described (GEO: GSE26566; “Copenhagen cohort” of patients). For a subset of these patients (T = 48, NT = 41), DNA methylation profiling was performed using Infinium HumanMethylation27 BeadChip (Illumina). The methylation status of the hOCT1 promoter was measured using beta (β)-value metric (range: 0-1; 0%-100% methylation) (see detailed description in Supporting Information). To further investigate the degree of DNA hypermethylation in hOCT1, level 1 Infinium HumanMethylation450 BeadChip (Illumina Inc.) data (T = 36 and NT = 9) obtained from The Cancer Genome Atlas (TCGA)-CHOL consortium were analyzed. For an extended subset of the “Copenhagen cohort,” miRNA-sequencing (miR-seq) data were also available for 57 paired T and NT samples and 22 normal livers. For some analyses, we have used specimens of iCCA (n = 16) and eCCA (n = 12) and paired NT samples (n = 17) obtained after tumor resection at University Hospital of Salamanca (“Salamanca cohort,” see Supporting Table S1). Research protocols were approved by the Ethical Committees for Clinical Research of supporting institutions, and all patients signed written consents for the use of their samples for biomedical research.

**ANIMALS AND IN VIVO EXPERIMENTS**

Male Wistar rats (University of Salamanca Animal House) and female nude mice (Swiss nu/nu; Charles River Laboratories, Barcelona) were used. Nude mice were maintained under pathogen-free environment and handled under stringent sterile conditions. The animals were fed on standard rat or mouse chow (Panlab, Madrid) and water *ad libitum*. Temperature (20°C) and the light/dark cycle (12 hours:12 hours) were controlled and the protocols were approved by the Ethical Committee of the University of Salamanca. To study sorafenib uptake by CCA, this tumor was induced in rats by including 0.05% thioacetamide (TAA) in the drinking water for 30 weeks (Supporting Information). Xenografts of CCA in nude mice were generated by subcutaneous injection of ≈1 × 10^6 human EGI-1 eCCA cells to donor nude mice under isoflurane anesthesia. EGI-1 cells were selected because of their acceptable tumorigenesis ability *in vivo* as compared with TFK-1 and HuCCT1 cells. Seven weeks later, generated CCA tumors were resected and dissected into ~1 mm^3 pieces that were implanted under anesthesia in the livers of several host nude mice. The following day, the animals were randomly divided into four groups for the coadministration of control adenoviruses (Ad-Mock: Ad-BIRC5pr-enhanced green fluorescent protein [EGFP]) or adenoviruses containing Ad-BIRC5pr-hOCT1-EGFP plus sorafenib or the vehicle alone (saline). Additional control mice were used to measure serum levels of routine biochemical parameters for comparative purposes. Adenoviral particles (VP) were injected every 5 days (4 × 10^7 VP/mice in each administration, intravenously through the tail vein). Sorafenib (10 mg/kg body weight) or saline were administered (intraperitoneally) twice per week. At the end of the experiment (after 2 months), the animals were anesthetized with sodium pentobarbital to measure the tumor volume and to collect tissues and blood samples. Serum levels of routine biochemical parameters were determined in a dry chemistry automated analyzer Spotchem EZ SP-4430 (Arkray Factory, A. Menarini Diagnostics, Badalona, Spain).

**LENTIVIRAL AND ADENOVIRAL VECTORS**

The human OCT1 open reading frame (ORF) was amplified from total RNA isolated from healthy liver by reverse transcription followed by high-fidelity PCR using AccuPrime Pfx DNA polymerase (Life Technologies) and specific primers (Supporting Table S2). OCT1 ORF
was cloned into the Pad site of the pWPI lentiviral vector (that contains the constitutive EF1α promoter). Recombinant lentiviruses production and lentiviral transduction are described in the Supporting Information.

The promoter region of the BIRC5 gene (BIRC5pr, 1467-bp zone of the 5′-flanking region) was cloned from human hepatoma PLC/PRF/5 (Alexander) cells using AccuPrime Pfx DNA polymerase, and specific oligonucleotide primers (Supporting Table S2). The cloning procedure is described in detail in the Supporting Information.

**IN VITRO EXPERIMENTS**

Cells transduced with lentiviral vectors (Lent MOCK or Lent-OCT1) were seeded onto 96-well plates at subconfluence (5,000 to 7,500 cells/dish). After 24 hours, the cells were exposed to 5 µM sorafenib for 6 hours. The formazan test using thiazolyl blue tetrazolium bromide (Sigma-Aldrich) was used to determine cell viability 66 hours later.

The transport function of hOCT1 was measured by the uptake of organic cations, such as dihydroethidium (DHE, 5 µM) by flow cytometry and [14C]-tetraethylammonium (TEA; 150 µM) by radioactivity determination. Specific hOCT1 inhibition was determined using quinine (250 µM). Sorafenib (5 µM) uptake was also determined using an adaptation of a published method using high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). In uptake experiments, the results were corrected by protein content.

The role of miRNAs potentially involved in regulating hOCT1 expression was studied in EGI-1 and TFK-1 cells, using lentivirus designed to mimic the pre-miRNA (hsa-mir-141/330/1468) sequence including the loop (Supporting Information).

**QUANTITATIVE RT-PCR (RT-QPCR)**

Total RNA extraction from cells and tissues and retro-transcription were performed as described (Supporting Information). The primer oligonucleotide sequences to carry out QPCR are described in Supporting Table S3. The results of mRNA abundance of target genes in each sample were normalized on the basis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT1), or rat β-actin (ACTB) mRNA abundance.

**IMMUNOFLUORESCENCE AND IMMUNOBLOTTING ASSAYS**

Immunofluorescent staining was performed on tissue cryosections air-dried and fixed in cold methanol using appropriate antibodies against hOCT1, platelet/endothelial cell adhesion molecule 1, and Na+/K+-ATPase. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal laser-scanning microscopy was performed using a Leica TCS SP2 confocal microscope. Immunoblotting analyses of cell lysates were carried out in 10-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, loading 30 µg of protein per lane. Appropriate primary antibodies for survivin and GAPDH were diluted in phosphate-buffered saline-Tween. Immunoreactive protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) after incubation with appropriate secondary antibodies (immunoglobulin G–horseradish peroxidase linked). See Supporting Information for antibodies characteristics and conditions.

**DETERMINATION OF ALTERNATIVE SPLICING**

Based on previous reports of alternative spliced hOCT1 variants, we designed primers annealing in exon 6 (Forward) and exon 11 (Reverse) that are shared by all hOCT1 isoforms (Supporting Table S3). PCR was carried out with Platinum-Taq DNA polymerase (Life Technologies) using 30 cycles of amplification. The presence and size of the PCR products were determined by gel electrophoresis, and a semi-quantitative determination of the amount of each splicing variant was carried out by densitometry of the bands using a LAS-4000 luminescent image analyzer.

**STATISTICAL ANALYSES**

Results were statistically analyzed using GraphPad program. For comparisons between two groups, parametric paired t test or Student t test and nonparametric Mann-Whitney U test were used. After analysis of variance, Bonferroni method of multiple-range testing was used to calculate the statistical significance of differences among groups.
Results

ROLE OF DNA METHYLATION IN HOCT1 DOWN-REGULATION

The relationship between hOCT1 expression and the methylation status of the \textit{SLC22A1} promoter was analyzed in resected CCA specimens from two different cohorts of patients: (1) TCGA-CHOL, including TCGA Infinium 450k data, and (2) the “Copenhagen cohort.” We have confirmed hOCT1 down-regulation in CCA versus peritumor tissue in both cohorts (Fig. 1A,C). hOCT1 promoter was significantly hypermethylated in CCA (using the probe cg27292431, corresponding to exon 1 of \textit{SLC22A1}) compared with peritumor tissue (Fig. 1B,D). Owing

![Figure 1](https://example.com/figure1.png)

**FIG. 1.** \textit{hOCT1} expression and promoter methylation in CCA. Levels of \textit{hOCT1} mRNA (A,C) determined by microarray and methylation status (B,D) in tumor (T) compared with paired non-tumor (NT) tissue analyzed in (A,B) TCGA-CHOL (NT, \(n = 9\); T, \(n = 36\)) and (C,D) Copenhagen cohort (NT, \(n = 60\); T, \(n = 104\)). Methylation was determined using cg27292431 probe at exon 1 of \textit{SLC22A1}. Values are individual measurements of T (open triangles) and NT (open circles) samples or mean ± SD (solid lines). Comparison between T and NT was carried out using Mann-Whitney U test. Separate analysis of hOCT1 expression in iCCA and eCCA tumors and adjacent NT liver tissue in (E) the Copenhagen cohort (iCCA, \(n = 68\); eCCA, \(n = 36\); NT, \(n = 60\)) and (F) the Salamanca cohort (iCCA, \(n = 16\); eCCA, \(n = 12\); NT, \(n = 17\)). Values are represented as mean ± SD. Abbreviations: A.U., arbitrary units; RPKM, reads per kilobase mapped. N.S., no significant difference (\(P > 0.05\)).
to the importance of the location of CpG dinucleotide hypermethylation in relationship to gene expression, we analyzed 450k data from TCGA-CHOL, which provides increased hOCT1 promoter coverage. Accordingly, we uncovered three additional hypermethylated probes mapping to the transcriptional start site and 5′-untranslated region (UTR) of hOCT1 (Supporting Fig. S1). When, both in the “Copenhagen cohort” (Fig. 1E) and the “Salamanca cohort” (Fig. 1F), hOCT1 expression was analyzed separately in iCCA and eCCA, similar marked down-regulation in both types of tumors was found.

IN VITRO hOCT1 EXPRESSION

To investigate the functional impact of SLC22A1 hypermethylation, CCA cell lines also with markedly reduced hOCT1 mRNA levels\(^{15,16}\) were treated with decitabine (5-aza-2′-deoxycytidine) (DAC). This DNA demethylating agent restored hOCT1 expression in EGI-1 (Fig. 2A) and TFK-1 (Fig. 2B) cells. Because acetylation of histones facilitates gene expression and histone deacetylases (HDACs) are overexpressed in CCA cells, which leads to a reduced expression of genes involved in differentiation,\(^{22}\) we evaluated whether HDAC inhibitors (HDACI) could affect hOCT1 mRNA expression in CCA cells. Sodium butyrate induced hOCT1 up-regulation in both EGI-1 (Fig. 2C) and TFK-1 (Fig. 2D) cells. The magnitude of the effect after 5 days of treatment was DAC>butyrate in EGI-1 cells but butyrate>DAC in TFK-1 cells. Other HDACIs, such as phenyl butyrate, increased hOCT1 mRNA levels, but only in TFK-1 cells and to a lesser extent than butyrate, whereas trichostatin A had no significant effect in any of these cell lines (Fig. 2C,D). Using EGI-1, in which DAC effect was stronger, it was demonstrated that the recovery of hOCT1 expression was accompanied by enhanced sorafenib uptake (Fig. 2E).

ROLE OF microRNA IN hOCT1 DOWN-REGULATION

In a separate study, we have carried out in silico analysis of microRNA-induced hOCT1 mRNA decay. Among six selected microRNAs, only three showed activity in HepG2 hepatoma cells.\(^{23}\) Here, we have shown that the same microRNAs, i.e., 141, 1468, and 330, reduced hOCT1 mRNA in EGI-1 (Fig. 2F) and TFK-1 (Fig. 2G) cells. The expression of these three microRNAs in paired T and NT specimens from both TCGA-CHOL and the extended “Copenhagen cohort” was analyzed and compared with that of hOCT1 (Fig. 3). Both miR-141 and miR-330 were consistently up-regulated in both groups of CCA. In the same paired specimens, hOCT1 mRNA was consistently decreased (Fig. 3F,L). In contrast, there was a discrepancy between both series regarding changes in miR-1468 expression (Fig. 3B,H).

ABERRANT SPLICING

To quantify the importance of aberrant splicing in overall hOCT1 expression/function, we evaluated the presence of splicing variants by PCR using specific primers to amplify the hOCT1 amplicon between the exons 6 and 11 (Fig. 4A). The length of the amplified fragment was used to distinguish wild-type from shorter variants (Fig. 4B,C). Measurement by densitometry of the abundance of splicing variants revealed a higher proportion of aberrant forms in CCA (both in biopsies and in cell lines) than in healthy liver. Interestingly, aberrant splicing was also present in peritumor tissue (Fig. 4D).

ROLE OF mRNA STABILITY/DECAY PROTEINS

To investigate whether changes in the balance of RNA binding proteins (RBPs) involved in mRNA stability/decay could affect the levels of hOCT1 mRNA in CCA, the expression of genes involved in mRNA decay (AUF1, BRF1, BRF2, CUGBP, FBP2, and TTP) or mRNA stability (HuR) was measured in paired samples of tumor and peritumor tissue (“Salamanca cohort”). We found no significant difference between T and NT tissue for any of these genes (Supporting Fig. S2A-F), except for that encoding tristetraprolin (TTP) (Supporting Fig. S2G), that was down-regulated in CCA samples. Similar results were found when the expression levels of RBP genes were determined in CCA cell lines (data not shown).

hOCT1 DOWN-REGULATION CORRELATES WITH DECREASED SORAFENIB UPTAKE BY CCA TUMORS

A rat model of chemically induced CCA\(^ {19}\) was used to study whether down-regulation of this
transporter results in impaired uptake of sorafenib by CCA tumors in vivo (Fig. 5). Sorafenib was intravenously administered to CCA-bearing rats. Samples from both T and NT tissue were collected 1 hour later, and their sorafenib content was determined using HPLC-MS/MS. The results indicated that peritumor tissue, which maintained higher Oct1 levels in comparison with CCA (Fig. 5D), was able to efficiently take up sorafenib (Fig. 5E). In contrast, in the tumors, the reduced expression of Oct1 (Fig. 5D) was accompanied with a consistent and significant decrease in sorafenib content (Fig. 5E).

RELATIONSHIP BETWEEN hOCT1 EXPRESSION AND SORAFENIB UPTAKE/ACTIVITY

To evaluate whether the experimental overexpression of hOCT1 could be used to increase the cytotoxic effect of sorafenib, eCCA (TFK-1 and EGI-1) and iCCA (HuCCT1) cells were transduced using lentivirus, either empty (MOCK) or containing hOCT1 coding sequence (Lent-OCT1). The high efficacy of transduction, as analyzed by counting EGFP-positive cells (=95%, =60%, and =80% in TFK-1, EGI-1, and HuCCT1 cells, respectively), resulted in a marked increase in hOCT1 mRNA expression (Fig. 6A-C). Consequently, hOCT1-transduced cells showed enhanced ability to take up, in a quinine-sensitive manner, organic cations, such as DHE (Fig. 6D-F) and TEA (Supporting Fig. S3). Moreover, in comparison with MOCK-transduced cells, hOCT1-expressing CCA cells showed higher ability to take up (Fig. 6G-I) and respond (Fig. 6J-L) to sorafenib. Enhanced cytotoxic effect of sorafenib in CCA cells overexpressing hOCT1 was consistent with the inhibition of the phosphorylation of the sorafenib-known target signal transducer and activator of transcription 3 (STAT3) as revealed by immunoblot analysis (Supporting Fig. S4).

TUMOR-SELECTIVE CHEMOSENSITIZATION BY GENE THERAPY

In order to restrict transgene expression to tumor cells, we designed an adeno viral vector in which hOCT1 expression was driven by BIRC5pr, due to the high activity of this promoter found in clinical CCA specimens. As it has been reported and we have confirmed here, the levels of both BIRC5 mRNA and survivin protein were greatly increased in CCA cells compared with healthy liver and normal human cholangiocytes (Fig. 7A,B). Promoter activity was evaluated in transfection experiments with Alexander cells using vectors encoding firefly luciferase (Luc2) under the control of either CMVpr or BIRC5pr. Both promoters were similarly potent in stimulating Luc2 expression (Fig. 7C,D). Based on these results, adenoviruses bearing BIRC5pr-EGFP (Ad-MOCK) (Fig. 7E) or BIRC5pr-hOCT1-EGFP (Ad-hOCT1) (Fig. 7F) were used to treat nude mice with intrahepatic CCA xenograft (Fig. 8). Gene therapy resulted in selective overexpression of hOCT1 at the plasma membrane of tumor cells, whereas no detectable expression in adjacent peritumor tissue (Fig. 8C-F) or endothelial cells (Supporting Fig. S5) was found. In mice treated with Ad-MOCK, sorafenib treatment failed to affect tumor growth (Fig. 8G,H). In contrast, coadministration of sorafenib plus Ad-hOCT1 resulted in a marked antitumor effect (Fig. 8G,H), which was not accompanied by decreased vascularization (Supporting Fig. S6). Interestingly, in the absence of treatment with sorafenib, the administration of these vectors did not affect tumor growth. Moreover,
signs of renal or hepatic toxicity were not found (Supporting Table S4).

Discussion

At present, sorafenib is the reference drug used in the pharmacological treatment of HCC. The high proportion of genetic alterations in critical signaling pathways involved in cell proliferation in CCA has led to an interest in the development of clinical trials investigating targeted therapies including sorafenib. However, data on the effectiveness of sorafenib, and other TKIs, in patients with CCA are controversial, reporting both beneficial effect\(^{(12,25,26)}\) and poor activity against this type of liver cancer.\(^{(27,28)}\) The latter is not surprising considering the strong multidrug resistance (MDR) phenotype of CCA, in which several MOCs involved in the lack of response to sorafenib have been identified.\(^{(5)}\) These include the overexpression of ABC proteins, such as MDR1 and BCRP, which reduce intracellular drug content (MOC-1b); enhanced drug inactivation by uridine glucuronosyl transferase 1A (MOC-2); or the appearance of genetic variants in the intracellular targets of sorafenib (MOC-3).\(^{(29)}\) In addition, we have reported that because cell uptake, which is mediated mainly by hOCT1, is an essential requirement for sorafenib...
to reach its intracellular targets and carry out its pharmacological effect, changes in the expression/activity of this carrier can lead to poorer response to sorafenib. As a natural continuation of this line of research, in the present study, we have analyzed the causes of hOCT1 down-regulation in CCA, and we have explored strategies to restore its expression and hence increase its sensitivity to sorafenib.

We have reported that hOCT1 expression is reduced in HCC and CCA. Epigenetic abnormalities, including DNA hypermethylation, have been described. Moreover, hypermethylation of SLC22A1 promoter in HCC has been reported and, as we have demonstrated here, these changes also occur in CCA. An inverse behavior regarding hOCT1 mRNA expression and hypermethylation status of three critical CpG regions (5′-UTR, transcriptional start site, and exon 1) has been found. This is consistent with the concept that methylation of the first exon correlates with transcriptional silencing of SLC22A1 gene. Although further studies are needed, our data suggest that methylation of SLC22A1 could be a prognostic biomarker in CCA, as has been proposed in HCC.

As suggested by our results, DAC treatment is an efficient strategy to restore OCT1 expression in CCA cell lines, which enhances sorafenib uptake. These findings support that demethylating agents could be useful in restoring hOCT1 expression in CCA tumors and hence improving sorafenib uptake/response. Moreover, clinical data indicate that DAC treatment retards CCA tumor growth. Thus, combination therapy of DAC plus sorafenib should be explored in patients with CCA. In support of this view, treatment of acute myeloid leukemia with DAC plus sorafenib has shown synergistic antitumor effect.
FIG. 6. Role of hOCT1 in sorafenib uptake and response in vitro. Extrahepatic (TFK-1 and EGI-1) and intrahepatic (HuCCT1) CCA cells were transduced with control lentivirus (MOCK) or hOCT1 lentivirus (Lent-OCT1). (A–C) Relative hOCT1 mRNA levels were evaluated in WT-, MOCK-, or OCT1-transduced cells. Measurements were carried out 4 days after transduction. (D–F) MOCK or Lent-OCT1 CCA cells were incubated with 5 μM DHE in the absence (Control) or the presence of 250 μM quinine for 15 minutes to determine hOCT1 function. (G–I) MOCK or Lent-OCT1 CCA cells were incubated with 5 μM sorafenib for 1 hour, and its levels in the cells were measured by HPLC-MS/MS. (J–L) Cell viability was evaluated in MOCK or Lent-OCT1 CCA after incubation with 5 μM sorafenib for 6 hours, and cell viability was measured 66 hours later to evaluate sorafenib response. Values are mean ± SD from nine wells of three different cultures in each cell line. *, P < 0.05, on comparing with MOCK cells. †, P < 0.05, on comparing cells incubated with (D–F) quinine or (J–L) sorafenib with their corresponding Control/DMSO groups. AUF, arbitrary units of fluorescence.
Although the up-regulation induced by DAC in EGI-1 cells was strong (=15-fold) (Fig. 2A), the impact of this change in the functional experiments was modest (Fig. 2E). This could be partly due to the fact that a fraction of the synthesized hOCT1 pre-mRNA was processed to inactive variants by an aberrant splicing that was quantitatively important in both CCA tumors and cell lines (Fig. 4C). In a previous study on a low number of specimens of CCA, several variants of aberrant splicing of hOCT1 pre-mRNA were identified. This is pharmacologically relevant because these variants encode truncated non-functional proteins. Interestingly, here we have found aberrant variants in both tumor and peritumor tissues, whereas this was negligible in healthy liver. The presence of enhanced alternative splicing in the mRNA of some genes has been described to occur during early stages of several liver diseases. An ongoing study on liver samples from patients with different liver diseases, without cancer, suggests a heterogeneous degree of aberrant splicing of hOCT1 pre-mRNA, which is lower than in CCA (data not shown).
Other epigenetic mechanisms, such as histone deacetylation, seem to also play a role in the modulation of hOCT1 expression, although to a lesser extent. RBPs, which bind AU-rich elements in the 3′ UTR of many mRNAs and target them for stabilization or rapid decay, might also be involved in hOCT1 mRNA decay. As the only change observed was a decreased expression of TTP, which participates in mRNA decay, our results do not support a role of RBPs in hOCT1 mRNA down-regulation. However, this cannot be ruled out because the complex regulation of the function of RBPs has not been explored in depth here. In contrast, in vitro activity measurements together with analysis of microRNA expression...
in CCA samples suggest a possible role of miR-141 and miR-330 in hOCT1 mRNA modulation in CCA.

Although epigenetic therapies such as DAC administration may be useful for recovering hOCT1 mRNA levels, the generation of aberrant isoforms and the presence of inactivating SNPs that appear in CCA(15) would limit the gaining of hOCT1 function. In contrast, in vitro studies clearly demonstrate that enhanced expression of exogenous hOCT1 results in higher sorafenib uptake and sensitivity. One of the mechanisms accounting for sorafenib antitumor activity involves inhibition of survival Janus kinase/STAT3 signaling pathway that includes reduced STAT3 phosphorylation.(10,38) Immunoblot analysis revealed that treatment of TFK-1 cells with sorafenib induced a marked reduction in the proportion of phosphorylated STAT3 only if the cells overexpressed hOCT1 (Supporting Fig. S4).

Prompted by these findings, we have evaluated the alternative of inducing hOCT1 expression by viral transduction in CCA cells. Thus, as a proof of concept, we have assayed an in vivo strategy of gene therapy using adenoviruses, which can infect a broad range of human cells with high gene transduction efficiency (39,40) and have been proposed for the treatment of neoplastic diseases, including CCA.(41) We have used adenoviruses serotype 5 (Ad5), which has become the most popular system in virotherapy. The fact that Ad5 has hepatotropic properties is an advantage for targeting this organ, but it could be a partial obstacle for its successful application in liver tumors such as CCA. To overcome this problem, we have used a transcriptional targeting strategy that exploits the activity of tumor-specific promoters that are preferentially active in tumor cells in comparison with normal cells.(42) Survivin (BIRC5 gene), a member of the inhibitor of apoptosis family that is involved in controlling mitotic progression and preventing cell death, is overexpressed in many cancers, including CCA, but not in normal adult tissues. (43-45) Previous studies have suggested the interest of BIRC5pr for tumor-targeted therapy,(46) because of its high tumor activity, as high as CMVpr (Fig. 7), and low activity in healthy cells.(47) This accounts for the specific transduction reached in the experimental model of orthotopic CCA xenograft. Accordingly, the expression of hOCT1 at the plasma membrane of tumor cells dramatically improved the antitumor effect of sorafenib.

Taken together, our study demonstrates that events such as promoter hypermethylation, microRNA-mediated degradation, and aberrant splicing lead to decreased hOCT1 mRNA and sorafenib uptake/response in CCA. Gene therapy able to selectively induce hOCT1 expression in tumor cells, but not in adjacent healthy liver tissue, is a useful chemosensitization strategy to improve the response of CCA to sorafenib.

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Supporting Information

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