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Effects of taurine-conjugated bile acids on colorectal cancer cell proliferation and tumor-associated cellular properties

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Abstract

Cholecystectomy has been associated with an increased risk of colorectal cancer (CRC), although the underlying mechanisms remain unclear. We investigated bile acid composition in

hepatic and gallbladder bile and examined the effects of bile samples and individual bile acids on non-malignant FHC colonic epithelial cells and Caco-2 and HCT-116 CRC cells. Cell proliferation, adhesion, migration, invasion, and colony formation were assessed, alongside potential signaling mechanisms involved in the response to taurocholic acid (TCA). Hepatic and gallbladder bile differed markedly in bile acid composition, and their effects on cell proliferation varied by cell line. TCA, taurochenodeoxycholic acid (TCDCA), and deoxycholic acid (DCA) modulated proliferation and other tumor-associated cellular properties in a bile acid-, cell line-, and endpoint-dependent manner. TCA increased sphingosine-1-phosphate receptor 2 (S1PR2) protein expression in Caco-2 and HCT-116 cells. Pharmacological inhibition of S1PR2, EGFR, AKT, MEK1/2, or COX-2 reduced proliferation in TCA-treated cells, although these inhibitors also affected basal proliferation. These findings demonstrate that bile acids can directly modulate colorectal cell behavior and suggest that S1PR2-associated signaling may contribute to cellular responses to TCA, warranting further mechanistic investigation.

Keywords: colorectal cancer; bile acids; taurocholic acid; taurine-conjugated bile acids; S1PR2; cell proliferation; cholecystectomy.

1. Introduction

Colorectal cancer (CRC) is the third most frequently diagnosed malignancy and remains one of the leading causes of cancer-related mortality worldwide [1,2]. Although genetic alterations are fundamental drivers of colorectal carcinogenesis, increasing evidence indicates that environmental and metabolic factors also contribute substantially to tumor initiation and progression [3,4]. Among these, bile acids (BAs) have emerged as important signaling molecules linking diet, intestinal microbiota, and CRC development [3–5].

Primary BAs are synthesized in the liver and subsequently conjugated with glycine or taurine before secretion into the biliary tract [6]. Following their release into the intestine, bacterial

metabolism converts a proportion of primary BAs into secondary BAs, thereby shaping the composition of the intestinal BA pool [7,8]. Besides their well-established role in lipid digestion, BAs function as signaling molecules that regulate cell proliferation, apoptosis, inflammation, metabolism, and epithelial homeostasis through the activation of multiple membrane and nuclear receptors [9]. Cholecystectomy has been associated with an increased risk of proximal or right-sided colon cancer, although its relationship with overall CRC incidence remains controversial [10,11]. Altered BA metabolism and continuous intestinal exposure to bile after gallbladder removal have been proposed to contribute to this association [12]. Previous mechanistic studies have primarily focused on secondary BAs, particularly deoxycholic acid (DCA), which can promote colorectal carcinogenesis through oxidative stress, DNA damage, EGFR/MAPK and Wnt/β-catenin signaling, and inflammatory pathways [13–18]. In contrast, considerably less is known about the role of taurine-conjugated BAs in CRC. Taurocholic acid (TCA) has mainly been linked to colorectal carcinogenesis through its microbial metabolism and the generation of tumor-promoting metabolites, including hydrogen sulfide and DCA [19]. However, the direct effects of TCA and taurochenodeoxycholic acid (TCDCA) on colorectal epithelial and cancer cells remain poorly understood. Emerging evidence indicates that conjugated BAs can also exert direct cellular effects through sphingosine-1-phosphate receptor 2 (S1PR2) [3,20,21]. In cholangiocarcinoma cells, , TCA has been reported to activate S1PR2-associated signaling involving epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase B (AKT), and cyclooxygenase-2 (COX-2), promoting tumor growth and invasiveness [21]. S1PR2-mediated signaling has also been implicated in the tumor-promoting effects of conjugated BAs in esophageal adenocarcinoma [20]. However, whether S1PR2 and these signaling pathways contribute to the cellular effects of taurine-conjugated BAs in CRC remains unclear.

Therefore, the present study investigated the effects of individual BAs, with particular emphasis on taurine-conjugated BAs, on non-malignant colonic epithelial cells and CRC cells. We compared the composition and biological effects of hepatic and gallbladder bile and evaluated the effects of individual BAs on cell viability, proliferation, adhesion, migration, invasion, and colony formation. Based on the observed proliferative effects of TCA, we further investigated its effect on S1PR2 protein expression and examined the potential involvement of S1PR2, EGFR, AKT, MEK/ERK, and COX-2 signaling in the proliferative response of colorectal cancer cells to TCA.

2. Results

2.1. Composition of hepatic and gallbladder bile

The composition of BAs in hepatic and gallbladder bile was analyzed using liquid chromatography–high-resolution mass spectrometry (LC–HRMS) (Fig. 1a, b). In gallbladder bile, conjugated BAs represented the predominant components, with glycine- and taurine-conjugated species present at substantially higher concentrations than unconjugated BAs. Hepatic bile was likewise dominated by conjugated BAs, particularly glycine-conjugated species, although their concentrations were considerably lower than those measured in gallbladder bile. A marked difference between the two bile types was observed in the abundance of taurine-conjugated BAs. While taurocholic acid (TCA), taurodeoxycholic acid (TDCA), and taurochenodeoxycholic acid (TCDCA) were detectable in gallbladder bile, only TCA was detected at measurable levels in hepatic bile, whereas TDCA and TCDCA remained below the detection limit. Unconjugated BAs, including cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA), were present at relatively low concentrations in gallbladder bile and at very low con-

centrations (<1 μg/mL) in hepatic bile. Ursodeoxycholic acid (UDCA) showed a distinct distribution, being below the detection limit in gallbladder bile but detectable at low concentrations in hepatic bile.

2.2. Effects of human bile on the proliferation of normal and tumor-derived colon cell lines

The effects of human gallbladder and hepatic bile on the proliferation of normal (FHC) and colorectal cancer (Caco-2 and HCT-116) cells were assessed (Fig. 2). Because the BA concentration and composition varied among individual samples (Fig. 1b), each sample was analyzed separately to account for interindividual differences. Gallbladder bile samples produced heterogeneous effects on cell proliferation. Among these, GB6 induced the most pronounced increase in proliferation, particularly in FHC and Caco-2 cells. Notably, GB6 contained relatively high concentrations of the taurine-conjugated BAs TCA, TDCA, and TCDCA compared with the other gallbladder bile samples. In HCT-116 cells, GB6 also increased proliferation, while several other gallbladder bile samples produced variable responses.

Hepatic bile samples likewise exerted heterogeneous and cell line-dependent effects. In FHC cells, most hepatic bile samples had little effect or reduced proliferation, whereas H4 and H8 increased proliferation at later time points. In Caco-2 cells, the responses varied considerably among samples and across treatment durations. In contrast, several hepatic bile samples increased proliferation in HCT-116 cells, particularly after prolonged exposure. When the responses to individual bile samples were averaged, neither gallbladder nor hepatic bile significantly altered proliferation in FHC or Caco-2 cells, whereas both bile types increased proliferation in HCT-116 cells.

Overall, these findings demonstrate substantial interindividual variability in the proliferative effects of human bile and indicate that the cellular response depends on both bile composition and cell type. The pronounced proliferative effects observed with selected samples containing relatively high levels of taurine-conjugated BAs prompted us to investigate the effects of individual BAs in subsequent experiments.

2.3. Effects of BAs on the viability, proliferation, and metastatic potential of normal and tumor-derived colon cell lines

To investigate whether the effects of human bile could be attributed to specific BA species, six conjugated BAs detected in human bile—GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA—were selected for further analysis. The presence of these compounds in the bile samples was confirmed by LC–HRMS. In addition, the unconjugated form of DCA was tested, given its well-documented carcinogenic effects in the colon [13,16,22]. Because BAs can exert cytotoxic effects at supraphysiological levels, dose–response assays were performed to determine concentrations compatible with cell viability (Fig. 3a–c). Treatments ranging from 100 to 1000 µM were tested for 24, 48, and 72 hours. Higher BA concentrations, particularly 500 and 1000 µM, generally reduced cell viability, although the magnitude of this effect varied depending on the BA, cell line, and exposure time. Interestingly, the toxic effects were more evident after 24–48 hours than at 72 hours, suggesting a partial cellular adaptation to BA exposure over time. Based on the dose-response experiments, 300 µM was selected for subsequent functional assays because it generally preserved cell viability while remaining within the range of intestinal BA concentrations reported previously [23].

At 300 µM, the effects of individual BAs on cell proliferation and adhesion were further investigated (Fig. 4). In normal FHC cells, short-term (24–48 h) treatment with most BAs either reduced or did not alter proliferation. However, after 72 hours, TCA and TCDCA significantly

increased cell proliferation (Fig. 4aIn Caco-2 cells, TCA, TCDCA, and DCA significantly increased proliferation, particularly after 48 and 72 h of exposure. HCT-116 cells showed a broader response, with several glycine- and tau-rine-conjugated BAs significantly increasing proliferation, particularly after 48 and 72 h. To examine whether enhanced proliferation was accompanied by changes in adhesive behavior, cell adhesion assays were performed (Fig. 4b). In FHC cells, TCA and TCDCA treatment led to a significant increase in adhesion, mirroring their proliferative effects. In Caco-2 cells, only TCA and DCA significantly increased adhesion, while in HCT-116 cells, several BAs significantly increased cell adhesion, with the most pronounced effects observed following GCA, TCA, and DCA treatment. The potential impact of individual BAs on metastatic properties was then evaluated by measuring invasion, migration, and colony-forming ability (Fig. 5a-e).

Several BAs enhanced these tumor-associated cellular properties, although the responses differed between the two cancer cell lines. TCA, TCDCA, and DCA markedly increased invasion, migration, and colony size in both cancer cell lines, indicating an overall enhancement of metastatic potential. By contrast, TDCA had little or no stimulatory effect and in some cases slightly reduced invasiveness. Among the glycine-conjugated BAs, GCDCA increased invasion in both cell lines and promoted migration specifically in HCT-116 cells. Collectively, these findings demonstrate that taurine-conjugated BAs—particularly TCA and TCDCA—exert strong, time-dependent proliferative and pro-metastatic effects on colon cancer cells, comparable in magnitude to those induced by DCA. This highlights their potential contribution to CRC progression in conditions characterized by increased taurine-conjugated BA exposure, such as after cholecystectomy.

2.4. TCA increases S1PR2 expression and S1PR2 inhibition reduces proliferation in TCA-treated CRC cells

As S1PR2 is a G protein-coupled receptor responsive to conjugated BAs, including TCA, next we investigated whether TCA treatment affects S1PR2 protein expression in CRC cells. Immunocytochemical analysis was performed in HCT-116 and Caco-2 cells following treatment with TCA (300 μM) for 72 h. In both cell lines, TCA treatment resulted in increased S1PR2 immunoreactivity compared with untreated controls (Figure 6a). Quantitative analysis of fluorescence intensity confirmed a significant increase in S1PR2 immunoreactivity following TCA treatment (Figure 6b). Negative controls performed in the absence of the primary antibody showed minimal fluorescence, supporting the specificity of the immunostaining. To confirm these findings, S1PR2 protein expression was further assessed by Western blot analysis. TCA treatment increased S1PR2 protein expression in both HCT-116 and Caco-2 cells compared with untreated controls (Figure 6c). Densitometric analysis normalized to GAPDH confirmed a statistically significant increase in both cell lines (Figure 6d). In HCT-116 cells, normalized S1PR2 expression increased from 0.651 ± 0.099 in control cells to 0.981± 0.044 following TCA treatment (unpaired two-tailed Student's t-test, $t(4) = -3.021$, $p = 0.0391$). Similarly, in Caco-2 cells, S1PR2 expression increased from 0.779 ± 0.022 to 1.043 ± 0.021following TCA treatment ($t(4) = -8.50$, $p = 0.0011$). Together, the immunocytochemical and Western blot analyses demonstrate that pro-longed TCA exposure is associated with increased S1PR2 protein expression in both CRC cell lines.

Based on these findings, we next investigated whether pharmacological inhibition of S1PR2 affects the proliferative response to TCA. HCT-116 and Caco-2 cells were pretreated with the S1PR2 antagonist JTE-013 before exposure to TCA (300 μM) for 72 h. JTE-013 was initially tested at 1, 5, and 10 μM. As increasing concentrations did not produce a consistent dose-dependent inhibitory effect across both cell lines, 5 μM JTE-013, which significantly reduced proliferation in both HCT-116 and Caco-2 cells, was selected for further analysis. As shown in (Figure 6e), JTE-013 alone significantly reduced basal proliferation compared with

untreated control cells in both cell lines. Combined treatment with JTE-013 and TCA resulted in significantly lower proliferation than TCA treatment alone. Thus, pharmacological inhibition of S1PR2 reduced proliferation under TCA-treated conditions in both HCT-116 and Caco-2 cells. However, because JTE-013 also reduced basal proliferation in the absence of TCA, the reduction observed during combined treatment cannot be attributed exclusively to inhibition of the TCA-induced proliferative response. Taken together with the TCA-induced increase in S1PR2 protein expression, these findings are consistent with the potential involvement of S1PR2 in the cellular response to TCA.

2.5. Pharmacological inhibition of EGFR, AKT, MEK/ERK, and COX-2 reduces proliferation in TCA-treated CRC cells

To further investigate signaling pathways potentially involved in the proliferative response to TCA, the effects of pharmacological inhibition of EGFR, AKT, MEK/ERK, and COX-2 were examined. All inhibitors were initially tested at three different concentrations: gefitinib (1, 5, and 10 μM), MK-2206 (1, 2.5, and 5 μM), U0126 (5, 10, and 20 μM), and celecoxib (10, 30, and 50 μM). As increasing inhibitor concentrations did not produce a consistent dose-dependent effect across both cell lines, one concentration showing a statistically significant inhibitory effect in both HCT-116 and Caco-2 cells was selected for further analysis for each inhibitor. At the concentrations selected for further analysis—gefitinib (5 μM), MK-2206 (2.5 μM), U0126 (10 μM), and celecoxib (10 μM)—all four inhibitors significantly reduced basal proliferation compared with untreated control cells in both HCT-116 and Caco-2 cells (Figure 7a-d). In TCA-treated cells, inhibition of EGFR with gefitinib, AKT with MK-2206, Mitogen-Activated Protein Kinase 1 and 2 (MEK1/2) with U0126, or COX-2 with celecoxib also resulted in significantly lower proliferation compared with TCA treatment alone. Thus, pharmacological

inhibition of each of these signaling components reduced proliferation under TCA-treated conditions in both CRC cell lines. However, because all four inhibitors also reduced basal proliferation in the absence of TCA, the reduction observed during combined inhibitor and TCA treatment cannot be attributed exclusively to inhibition of the TCA-induced proliferative response. These findings are consistent with the potential involvement of EGFR, AKT, MEK/ERK, and COX-2 signaling in the cellular response to TCA, although the present pharmacological experiments alone do not establish that the inhibitory effects are specific to TCA-induced signaling.

3. Discussion

Colon diseases, especially CRC, are responsible for increased morbidity and mortality worldwide. CRC is the second leading cause of cancer death globally, and its incidence is steadily rising [1,2]. The relationship between CRC and BAs, especially DCA and lithocholic acid (LCA), has long been known [13,16,22,24,25]. DCA and LCA are secondary BAs produced from primary BAs under the influence of bacterial flora. During enterohepatic circulation, most of the primary and secondary BAs return to the liver, where they are conjugated with glycine or taurine and excreted in the bile. However, this process occurs differently in cholecystectomized patients as the composition of bile and its secretion into the duodenum change. Several studies have indicated that cholecystectomy promotes CRC development. However, the mechanism is still not well understood.

This study used different cell assays and showed that the different composition of hepatic and gallbladder bile alone is not responsible for the development or progression of CRC. However, a difference in the effects of individual BAs was observed. Previous studies highlighted the role of secondary BAs, especially DCA and LCA, in the development and progression of CRC. These studies have identified several intracellular pathways through which these BAs

exert their effects. DCA has been shown to enhance the proliferation and metastatic potential of colon cells by the production of reactive oxygen species[15,17], induction of DNA damage and mutation [14,26], which causes genetic instability, or activation of a range of signal transduction pathways [13,18,27–30]. However, the study of other BAs was far behind that of DCA. This comprehensive study showed for the first time that TCA and TCDCA strongly increase the proliferation and metastatic potential of colon cells, indicating that the concentration of these BAs in the BA pool is likely a key factor in the development and progression of CRC.

The composition of hepatic and gallbladder bile was first examined using the LC–HRMS technique. Glycine-conjugated BAs (GCA, GDCA, and GCDCA) and TCA were present in both hepatic and gallbladder bile. However, the concentrations of these BAs were considerably lower in the liver. In the case of TDCA and TCDCA, detectable amounts were found only in the gallbladder. Unconjugated BAs were present at relatively low concentrations in gallbladder bile and at very low concentrations in hepatic bile. These differences should, however, be interpreted cautiously because hepatic and gallbladder bile were obtained from different donor populations, and the present study was not designed as a paired comparison before and after cholecystectomy. The glycine:taurine ratio is greatly influenced by the protein and fat content of the food. High meat consumption increases taurine uptake and the amount of taurine-conjugated BAs in bile [31,32]. Therefore, the low taurine-conjugated BA ratio observed in cholecystectomized patients can presumably be explained by the special diet of these patients.

Next, the effects of hepatic and gallbladder bile on colon cells were compared. In the case of normal cells, one gallbladder sample (GB5) decreased the proliferation of the cells, whereas another sample (GB6) significantly increased it. Comparing the composition of the two samples, GB5 did not contain non-conjugated BAs, and the concentrations of TCA and TCDCA were relatively low. The concentration of taurine-conjugated BAs was extremely high in sample GB6. Sample GB6 also increased the proliferation of adenocarcinoma cells, whereas sample

GB5 caused a decrease only in Caco-2 cells. Since the TCA concentration was still higher in GB5 than in the hepatic bile samples, this assumes that other factors play a role in the effect of this sample. These results emphasize the key role of taurine-conjugated BAs, especially TCA, in cell proliferation. This is also supported by the fact that, in the case of hepatic bile, where the samples did not contain TDCA and TCDCA, the increase was significant in those cases only where the concentration of TCA was relatively high (sample H4 and H8).

Furthermore, the effects of individual BAs were examined. TCA, TCDCA, and DCA treatments increased the rate of proliferation and adhesion in all three cell lines. Additionally, these BAs promoted colony formation and increased the migration and invasion capacity of cells. These results suggest that these BAs accelerate colon tumor progression by enhancing the proliferation and metastasis potentials of the cells. Among the glycine-conjugated BAs, GDCA and GCDCA exerted a similar effect on the HCT-116 cell line, but not on the Caco-2 cells. This discrepancy may be because the two cell lines do not express the same BA transporters or the BAs do not activate the same signaling pathways within the cells.

Although the role of DCA in CRC is well recognized, fewer results are reported regarding the role of taurine-conjugated BAs. Taurine is an essential amino acid. The human body cannot produce taurine only to a limited extent, so most of the taurine enters the body with food. Red meat, mollusks, and energy drinks contain large amounts of taurine, which is either excreted in the urine or bound to free BAs in the liver [32]. Diets rich in dark meat have been known to increase the chance of CRC development [33,34]. Since red meats are rich in taurine, high consumption of these foods increases the proportion of taurine-conjugated BAs in bile, which may contribute to the development of CRC, among other mechanisms. A recent review by Ridlon et al. described a novel mechanism for the role of TCA in CRC [19]. According to their hypothesis, during the bacterial metabolism of TCA, two potentially toxic substances are formed, namely, hydrogen sulfide and DCA, which are known to be involved in the biological

processes of colon cancer. This hypothesis assumes that substances formed during the decomposition of TCA lead to CRC. However, our results showed that TCA also has a direct effect on colon cells, as shown in esophageal cancer or cholangiocarcinoma [20,21].

To further investigate the mechanism underlying the direct effect of TCA, we examined the involvement of S1PR2, a receptor previously implicated in the cellular response to conjugated BAs. TCA exposure increased S1PR2 protein expression in both HCT-116 and Caco-2 cells, as demonstrated by immunocytochemistry and Western blotting. Moreover, pharmacological inhibition of S1PR2 with JTE-013 attenuated the TCA-induced increase in proliferation in both cell lines. However, JTE-013 also reduced basal proliferation in both cell lines, indicating that the observed effect cannot be attributed specifically to inhibition of the TCA-induced proliferative response. Taken together, the TCA-induced increase in S1PR2 expression and the anti-proliferative effect of S1PR2 inhibition are consistent with a potential involvement of S1PR2 in the cellular response to TCA, although further mechanistic studies are required to establish a direct causal relationship. These findings are also consistent with previous studies linking conjugated BAs to S1PR2-associated signaling in gastrointestinal malignancies [20,21].

Because S1PR2 signaling can interact with pathways involved in cellular proliferation, we next examined the contribution of EGFR, AKT, MEK/ERK, and COX-2 signaling. Inhibition of EGFR with gefitinib, AKT with MK-2206, MEK1/2 with U0126, or COX-2 with celecoxib attenuated the proliferative effect of TCA. However, all four inhibitors also reduced basal proliferation in the absence of TCA, indicating that their effects cannot be attributed specifically to inhibition of TCA-induced signaling. Taken together, these pharmacological findings are consistent with the potential involvement of EGFR, AKT, MEK/ERK, and COX-2 signaling in the cellular response to TCA. However, they do not establish a linear signaling cascade downstream of S1PR2 or define the hierarchical relationship between these signaling components.

Further studies using genetic approaches and direct assessment of pathway activation will be required to determine their specific roles and their relationship to S1PR2 signaling.

To the best of our knowledge, this study is among the first to directly compare the effects of human hepatic and gallbladder bile on colorectal cells and to systematically examine the effects of individual bile acids using one non-malignant and two colorectal adenocarcinoma cell lines. Although hepatic and gallbladder bile differed markedly in BA composition, their effects on cell proliferation were heterogeneous and cell line dependent, and no consistent relationship between bile type and proliferative response was observed. Importantly, because hepatic and gallbladder bile samples were obtained from different donor populations, the observed differences cannot be attributed specifically to cholecystectomy. Altered intestinal BA exposure following gallbladder removal may nevertheless represent a potential mechanism linking cholecystectomy to CRC risk and warrants further investigation in appropriate clinical and experimental models. Our findings further demonstrate that, in addition to DCA, the taurine-conjugated BAs TCA and TCDCA can enhance proliferation and several tumor-associated cellular properties, including adhesion, migration, invasion, and colony formation, although these effects varied according to cell type and experimental endpoint.

Taken together, the present findings extend the potential role of taurine-conjugated BAs in CRC beyond their proposed microbial conversion to secondary metabolites by demonstrating direct effects of TCA and TCDCA on colorectal cells *in vitro*. TCA treatment was associated with increased S1PR2 protein expression, while pharmacological inhibition of S1PR2, EGFR, AKT, MEK1/2, or COX-2 reduced proliferation in TCA-treated cells. These findings are consistent with the potential involvement of these signaling components in the cellular response to TCA, although the present pharmacological experiments do not establish their hierarchical relationship or distinguish TCA-specific effects from general antiproliferative effects of the in-

hibitors. Further studies incorporating genetic approaches, direct assessment of pathway activation, appropriate inhibitor-only controls, and *in vivo* models will be required to define the underlying mechanisms and establish the relevance of taurine-conjugated BAs to CRC development and progression.

4. Materials and Methods

4.1. Chemicals and solutions

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and type I collagen were obtained from Thermo Fisher Scientific (Darmstadt, Germany). Technical Manual Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, MD, USA). GDCA was obtained from Cayman Chemical Company (Michigan, MI, USA). Methanol (liquid chromatography–mass spectrometry (LC–MS) grade), acetonitrile (LC–MS grade), and water (LC–MS grade) were obtained from VWR (Budapest, Hungary). Matrigel matrix was ordered from Corning (Glendale, AZ, US). All other laboratory chemicals were purchased from Sigma-Aldrich Kft. (Budapest, Hungary).

4.2. Cell lines and culture conditions

HCT-116, a human colon adenocarcinoma cell line, was obtained from Sigma-Aldrich Kft. (Budapest, Hungary). HCT-116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 Ham media supplemented with 15% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, and 2% (v/v) antimycotics/antibiotics (AA). Caco-2, a human colon adenocarcinoma cell line, was a kind gift from Dr. Maria A. Deli (Biological Research Center, Szeged, Hungary). Caco-2 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 15% (v/v) FBS, 1% (v/v) L-glutamine, and 2% (v/v) AA. FHC, a normal colon epithelial cell line, was obtained from American Type Culture Collection (Manassas, VA,

United States). FHC cells were cultured in DMEM/F-12 Ham media supplemented with 15% (v/v) FBS, 10 mM HEPES (v/v) (for a final concentration of 25 mM), 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, and 20 ng/mL human recombinant epidermal growth factor. The cells were incubated at 37°C and gassed with a mixture of 5% CO2 and 95% air. The media were replaced every second day. Passage numbers were between 3 and 15 in each experiment.

4.3. Bile acid and human bile treatments

For BA treatments, the cells were treated with seven different types of BAs (GCA, GDCA, GCDCA, TCA, TDCA, TCDCA, and DCA) in four different concentrations (100, 300, 500, and 1000 μM) for 24, 48, and 72 h. For human bile treatments, the cells were treated with gallbladder or hepatic bile. Gallbladder bile samples were obtained from the gallbladder of 6 cadavers (average age: 78.42 ± 4.7; male-to-female ratio: 3:3) in the Department of Pathology, University of Szeged. The cadavers had no known history of hepatic or biliary disease. Hepatic bile samples were obtained during routine endoscopic retrograde cholangiopancreatography procedure from patients (average age: 66 ± 5.6; male/female ratio: 2/6) who previously underwent cholecystectomy at the Department of Medicine, University of Szeged. Patients' data are shown in Table 1. Bile samples were kept at −20°C until use. A small part of the samples was used for HPLC measurements, while the remaining bile samples were used for cell treatments for 24, 48, and 72 h in a 100× dilution. The cells were seeded in 25 cm^2 tissue culture flasks or 96-well plates and grown to 70%–80% of confluence two days before BA or human bile treatments.

4.4. Ethical approval

The clinical part of the study was carried out with the approval of the Ethics Committee of the University of Szeged (No. 5016), followed by the EU Member States' Directive 2004/23/EC

on presumed consent practice for tissue collection, the guidelines of the Helsinki Declaration, and the General Data Protection Regulation (EU) 2016/679. Written informed consent was obtained from all patients for sample and data collection.

4.5. LC–HRMS measurements

The frozen samples were allowed to thaw at 4°C. Gallbladder and hepatic bile samples were diluted 1:500 and 1:200, respectively and samples were prepared in cold methanol:water 1:1. 100 µL of each sample was spiked with 10 µL methanol, containing 100 µg/mL internal standards (GCDA-D4 used for TCA, GCA, GCDA, and GDCA and DCA-D4 used for DCA, UDCA, CDCA, TDCA, and TCDCA, respectively). Samples were vortex-mixed (LSE W, Corning, NY, USA) for 1 min and then incubated for 30 min at −20°C. The incubated samples were centrifuged (Heraeus Fresco 17, Thermo Scientific, Waltham, MA, USA) at 13000 rpm for 15 min at 10°C. 5 µL of the supernatants was subjected to liquid chromatography–high-resolution mass spectrometry (LC–HRMS) analysis.

LC–HRMS measurements were performed using a Thermo Scientific Dionex Ultimate 3000 UHPLC System (Dionex) coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer operating with a heated electrospray interface. BAs were separated using a Purospher STAR RP-18 Hibar HR 100 × 2.1 mm, 2 µm reversed-phase columns (Merck KGaA, Darmstadt, Germany) tempered at 40°C. Solvent A was water, and solvent B was methanol:acetonitrile 1:1, and both were supplemented with 0.1% formic acid. The flow rate was 300 µL/min, and the following gradient elution was applied: 0–1 min, 5% B; 12 min, 95% B; 15 min, 95% B; 15.5 min, 5% B; 20 min, 5% B.

The ion source had the following settings: the temperature of the probe heater and ion transfer capillary, spray voltage, sheath gas flow rate, auxiliary gas flow rate, and S-lens radio frequency level were set to 350°C, 275°C, 3.5 kV, 30 arbitrary units, 20 arbitrary units, and 50

arbitrary units, respectively. The mass spectrometer acquired data using parallel reaction monitoring in negative polarity mode at 17,500 (at m/z = 200) resolution and a minimum automatic gain control target of 2.00 × 105 with a maximum injection time of 100 ms. The isolation window was 0.4 m/z. Fragmentation was performed with a normalized collision energy of 60.

4.6. Cell adhesion

96-well tissue plates were coated with 40 µg/ml type I collagen (diluted in phosphate-buffered saline (PBS)) at 4°C. After 12 h of coating, the collagen solution was removed, and the wells were air-dried and then washed with PBS. The cells were serum-starved for 8 h. Then, 100 µl cell suspension (105 cells/ml) was added to each coated well and incubated for 20 min at 37°C. The non-adherent cells were removed by washing the wells with a culture medium four times. After washing, the cells were incubated with different BAs for 24, 48, and 72 h. After treatments, 10 µl of MTT substrate was added to each well, and the cells were incubated for an additional 3 h. The MTT-treated cells were then lysed in dimethyl sulfoxide, and absorbance was measured at 560 nm using a FLUOstar OPTIMA Spectrophotometer (BMG LabTech, Ortenberg, Germany). All treatments were carried out in triplicate.

4.7. Proliferation

Cell proliferation was determined using the CCK-8 solution. Briefly, the cells (5 × 103) were seeded in a sterile 96-well plate and incubated with BAs for 24, 48, and 72 h. After treatments, the cells were incubated with 10 µl of the CCK-8 solution for 3 h. Then, absorbance was measured at 450 nm using a FLUOstar OPTIMA Spectrophotometer (BMG LabTech, Ortenberg, Germany). All treatments were carried out in triplicate.

4.8. Viability

Cell viability was measured using Lactate Dehydrogenase (LDH) Cytotoxicity Colorimetric Assay Kit according to the manufacturer's instructions. Briefly, 100 µl cell suspension (2 ×

104 cells) was seeded in a sterile 96-well tissue culture plate and incubated overnight to allow the cells to adhere. The following day, the cells were treated with 0.1% Triton X-100 or BAs. The LDH release induced by Triton X-100 was assigned to 100%. After incubation, 100 µl supernatant was carefully removed from each well and transferred into a new sterile 96-well plate. Then, 100 µl reaction mixture was added to each well and incubated for 30 min at room temperature. Absorbance was measured at 490 nm using a FLUOstar OPTIMA Spectrophotometer (BMG LabTech, Ortenberg, Germany) to determine LDH release. All treatments were carried out in triplicate. The average absorbance values of each triplicate were calculated, and the average value of the background control (200 µl assay medium) was subtracted from each sample to reduce background noise. Then, the percentage of cytotoxicity was calculated using the following formula: Cytotoxicity (%) = (exp. value − low control/high control − low control) × 100. For low control, 100 µl cell suspension plus 100 µl assay medium was added, and for high control, 100 µl cell suspension plus 100 µl Triton X-100 was added.

4.9. Migration

The cells (250,000 cells/well) were seeded in 24-well plates and allowed to adhere overnight. The following day, the confluent monolayer was gently scratched using P2 tips. Only wells containing even-sided and sharp-edged wounds were used for experiments. After washing, reagents were added to the wells. Pictures were taken before and after the 72h BA treatment using an Olympus IX71 inverted microscope (Olympus, Budapest, Hungary). The digital images were analyzed using Image J to quantify the distance between the two edges of the wound, and the migration rate was expressed as the percentage of migration using the following equation: Wound closure (%) = $[(W_0 - W_{72})/W_0] \times 100$, where W_0 represents the initial wound width and W_{72} represents the wound width measured after 72 h.

4.10. Clonogenic assay

The cells (1,000 cells/ well) were seeded in 6-well plates and allowed to adhere overnight. The following day, BAs were added to the wells, and the cells were incubated for 24, 48, and 72 h. After treatments, the normal culture medium was returned, and the cells were allowed to grow until day 9. Then, the media were removed, and the cells were washed with PBS, fixed with methanol–ethanol solutions (3:1 dilution), and stained with Giemsa. Pictures were taken before and after the 72 h BA treatment using an Olympus IX71 inverted microscope (Olympus, Budapest, Hungary). Colony formation was quantified using ImageJ as the percentage of the total well area covered by colonies.

4.11. Invasion assay

Matrigel pre-coated transwell inserts were used for invasion assays. 200 µl cell suspension (~ 2.5 × 105/ml in serum-free medium) was loaded into the upper chamber of the transwell. In contrast, the lower chamber contained 750 µl complete medium with or without BAs. Then, the cells were incubated at 37°C for 72 h in a humidified incubator. The cells that migrated to the bottom surface of the transwell were fixed in formaldehyde (3.7% in PBS), permeabilized with 100% methanol, and stained with Giemsa. The non-invading cells on the upper surface of the membrane were gently scraped off using a cotton swab. Cell invasion was quantified by counting the average number of invaded cells in five randomly selected microscopic fields. All treatments were carried out in triplicate.

4.12. Immunocytochemical analysis of S1PR2 expression

To determine the effect of TCA on S1PR2 protein expression, HCT-116 and Caco-2 cells were seeded at a density of 4×10^4 cells per 35-mm cell culture dish (CellView, PS, 35/10 mm) and allowed to adhere overnight. Cells were treated with TCA (300 µM) for 72 h; untreated cells served as controls. Following treatment, cells were fixed with acetone for 5 min at room

temperature, permeabilized with 0.1% Triton X-100, and rehydrated in PBS. Non-specific binding was blocked with 10% normal goat serum in phosphate-buffered saline (PBS, pH 7.4; containing 0.1% sodium azide). Cells were incubated overnight at 4°C with rabbit anti-S1PR2 primary antibody (Abcam, Cat. No. ab235919; 1:100). Negative controls were processed in parallel without primary antibody. Cells were then incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Thermo Fisher Scientific, Cat. No. A-11034; 1:400 in 0.5% BSA-TBS) and counterstained with DAPI. Samples were imaged using a Zeiss LSM 880 laser-scanning confocal microscope equipped with a 40× oil-immersion objective. S1PR2 fluorescence was quantified as integrated density using ImageJ2 (FIJI) software (v.1.54p). The same image acquisition settings were maintained for control and TCA-treated samples.

4.13. Western blotting

HCT-116 and Caco-2 cells (250,000 cells per 75-cm² culture flask) were seeded in culture flasks and allowed to attach for 24 h. Following the attachment period, cells were treated with 300 μM TCA for 72 h. Control cells were treated with suitable culture medium only and maintained under the same experimental conditions. After 72 h of treatment, cells were harvested for protein extraction. Total cellular proteins were extracted using RIPA lysis buffer solution supplemented with protease inhibitors. The lysis solution was prepared by mixing 5 mL RIPA buffer with 22.5 mL distilled water, followed by the addition of protease inhibitors at a ratio of 5 μL per 1 mL of the prepared lysis solution. Protein concentrations were determined using the Bradford assay, and 25 μg of protein per sample was loaded onto 10% acrylamide gels for SDS-PAGE and then transferred onto PVDF membranes. After blocking the membranes with 5% nonfat dry milk for 1 h at room temperature, the membranes were probed overnight at 4°C with the primary antibodies, anti-S1PR2 (1:750, Abcam, Cat. No. ab235919). Following this, the

membranes were incubated for 1 h at room temperature with the secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:8000, Southern Biotech, 4030-05). Chemiluminescent signals were detected using the ECL Prime Western Blotting Detection Reagent (cytiva, Amersham) and visualized with the ChemiDoc Imaging System (Bio-Rad). GAPDH (1:10000, Thermo Fisher Scientific, PA1-988) served as a loading control. Band intensities were quantified using ImageJ (FIJI) software (v.1.54p).

4.14. Pharmacological inhibition of TCA-induced proliferation

To investigate the signaling pathways involved in TCA-induced proliferation, HCT-116 and Caco-2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured overnight. Cells were pretreated for 1 h with the S1PR2 antagonist JTE-013 (1, 5, or 10 μM), the EGFR inhibitor gefitinib (1, 5, or 10 μM), the AKT inhibitor MK-2206 (1, 2.5, or 5 μM), the MEK1/2 inhibitor U0126 (5, 10, or 20 μM), or the COX-2 inhibitor celecoxib (10, 30, or 50 μM). Following pretreatment, the medium was replaced with fresh medium containing the corresponding inhibitor together with TCA (300 μM), and cells were incubated for an additional 72 h. The final solvent concentrations did not exceed 0.1% (v/v) DMSO for JTE-013, gefitinib, MK-2206, and celecoxib or 0.2% (v/v) ethanol for U0126. Corresponding vehicle controls were included. For each inhibitor, experiments included untreated control, vehicle control, TCA alone, inhibitor alone, and inhibitor plus TCA groups. Cell proliferation was determined using the CCK-8 assay as described above. Each condition was measured in technical triplicate, and experiments were independently repeated three times.

4.15. Statistical analysis

Quantitative data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Microsoft Excel (Microsoft Office Professional Plus 2016) and

IBM SPSS Statistics, version 25 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was performed using Microsoft Excel for comparisons involving multiple groups, whereas unpaired two-tailed Student's t-tests were performed using IBM SPSS Statistics for comparisons between two independent groups. Data were considered statistically significant when $P \leq 0.05$.

4.16. GenAI disclosure

During the preparation of this manuscript, the authors used (ChatGPT OpenAI) for language editing and improvement of readability. The authors reviewed and edited the output and take full responsibility for the content of the publication.

5. References

1. Morgan, E. *et al.* Global burden of colorectal cancer in 2020 and 2040: incidence and mortality estimates from GLOBOCAN. *Gut* **72**, 338–344 (2023).
2. Xi, Y. & Xu, P. Global colorectal cancer burden in 2020 and projections to 2040. *Transl. Oncol.* **14**, 101174 (2021).
3. Caliceti, C. *et al.* New Insights into Bile Acids Related Signaling Pathways in the Onset of Colorectal Cancer. *Nutrients* **14**, 2964 (2022).
4. Ocvirk, S. & O'Keefe, S. J. Influence of Bile Acids on Colorectal Cancer Risk: Potential Mechanisms Mediated by Diet - Gut Microbiota Interactions. *Curr. Nutr. Rep.* **6**, 315–322 (2017).
5. Ridlon, J. M., Harris, S. C., Bhowmik, S., Kang, D.-J. & Hylemon, P. B. Consequences of bile salt biotransformations by intestinal bacteria. *Gut Microbes* **7**, 22–39 (2016).
6. Di Ciaula, A. *et al.* Bile Acid Physiology. *Ann. Hepatol.* **16**, s4–s14 (2017).
7. Bustamante, J.-M. *et al.* Impact of Fecal Microbiota Transplantation on Gut Bacterial Bile Acid Metabolism in Humans. *Nutrients* **14**, 5200 (2022).
8. Winston, J. A. & Theriot, C. M. Diversification of host bile acids by members of the gut microbiota. *Gut Microbes* **11**, 158–171 (2020).

9. Keitel, V., Kubitz, R. & Häussinger, D. Endocrine and paracrine role of bile acids. *World J. Gastroenterol.* **14**, 5620–5629 (2008).
10. Reid, F. D., Mercer, P. M., harrison, M. & Bates, T. Cholecystectomy as a risk factor for colorectal cancer: a meta-analysis. *Scand. J. Gastroenterol.* **31**, 160–169 (1996).
11. Zhang, Y. *et al.* Cholecystectomy can increase the risk of colorectal cancer: A meta-analysis of 10 cohort studies. *PloS One* **12**, e0181852 (2017).
12. Jiang, X. *et al.* Cholecystectomy promotes the development of colorectal cancer by the alternation of bile acid metabolism and the gut microbiota. *Front. Med.* **9**, 1000563 (2022).
13. Centuori, S. M. *et al.* Deoxycholic acid mediates non-canonical EGFR-MAPK activation through the induction of calcium signaling in colon cancer cells. *Biochim. Biophys. Acta* **1861**, 663–670 (2016).
14. Glinghammar, B., Inoue, H. & Rafter, J. J. Deoxycholic acid causes DNA damage in colonic cells with subsequent induction of caspases, COX-2 promoter activity and the transcription factors NF-kB and AP-1. *Carcinogenesis* **23**, 839–845 (2002).
15. Bile acids mimic oxidative stress induced upregulation of thioredoxin reductase in colon cancer cell lines | Carcinogenesis | Oxford Academic. https://academic.oup.com/carcin/article-abstract/23/8/1281/2390239?redirectedFrom=fulltext.
16. Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness - PubMed. https://pubmed.ncbi.nlm.nih.gov/15004225/.
17. Payne, C. M. *et al.* Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. *Carcinogenesis* **28**, 215–222 (2007).
18. Zhu, Y., Zhu, M. & Lance, P. Stromal COX-2 signaling activated by deoxycholic acid mediates proliferation and invasiveness of colorectal epithelial cancer cells. *Biochem. Biophys. Res. Commun.* **425**, 607–612 (2012).
19. Ridlon, J. M., Wolf, P. G. & Gaskins, H. R. Taurocholic acid metabolism by gut microbes and colon cancer. *Gut Microbes* **7**, 201–215 (2016).
20. Liu, R., Li, X., Hylemon, P. B. & Zhou, H. Conjugated Bile Acids Promote Invasive Growth of Esophageal Adenocarcinoma Cells and Cancer Stem Cell Expansion via Sphingosine 1-Phosphate Receptor 2-Mediated Yes-Associated Protein Activation. *Am. J. Pathol.* **188**, 2042–2058 (2018).

21. Liu, R. *et al.* Taurocholate Induces Cyclooxygenase-2 Expression via the Sphingosine 1-phosphate Receptor 2 in a Human Cholangiocarcinoma Cell Line. *J. Biol. Chem.* **290**, 30988–31002 (2015).
22. Milovic, V., Teller, I. C., Murphy, G. M., Caspary, W. F. & Stein, J. Deoxycholic acid stimulates migration in colon cancer cells. *Eur. J. Gastroenterol. Hepatol.* **13**, 945–949 (2001).
23. Zeng, H., Umar, S., Rust, B., Lazarova, D. & Bordonaro, M. Secondary Bile Acids and Short Chain Fatty Acids in the Colon: A Focus on Colonic Microbiome, Cell Proliferation, Inflammation, and Cancer. *Int. J. Mol. Sci.* **20**, 1214 (2019).
24. Baek, M. K. *et al.* Lithocholic acid upregulates uPAR and cell invasiveness via MAPK and AP-1 signaling in colon cancer cells. *Cancer Lett.* **290**, 123–128 (2010).
25. Halvorsen, B., Staff, A. C., Ligaarden, S., Prydz, K. & Kolset, S. O. Lithocholic acid and sulphated lithocholic acid differ in the ability to promote matrix metalloproteinase secretion in the human colon cancer cell line CaCo-2. *Biochem. J.* **349**, 189–193 (2000).
26. Powolny, A., Xu, J. & Loo, G. Deoxycholate induces DNA damage and apoptosis in human colon epithelial cells expressing either mutant or wild-type p53. *Int. J. Biochem. Cell Biol.* **33**, 193–203 (2001).
27. Centuori, S. M. & Martinez, J. D. Differential regulation of EGFR-MAPK signaling by deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) in colon cancer. *Dig. Dis. Sci.* **59**, 2367–2380 (2014).
28. Lee, D. K. *et al.* [Deoxycholic acid-induced signal transduction in HT-29 cells: role of NF-kappa B and interleukin-8]. *Korean J. Gastroenterol. Taehan Sohwagi Hakhoe Chi* **43**, 176–185 (2004).
29. Lee, H. Y., Crawley, S., Hokari, R., Kwon, S. & Kim, Y. S. Bile acid regulates MUC2 transcription in colon cancer cells via positive EGFR/PKC/Ras/ERK/CREB, PI3K/Akt/IkappaB/NF-kappaB and p38/MSK1/CREB pathways and negative JNK/c-Jun/AP-1 pathway. *Int. J. Oncol.* **36**, 941–953 (2010).
30. Li, Z. *et al.* EphA2 Up-regulation induced by deoxycholic acid in human colon carcinoma cells, an involvement of extracellular signal-regulated kinase and p53-independence. *J. Cancer Res. Clin. Oncol.* **129**, 703–708 (2003).
31. Stapleton, P. P., Charles, R. P., Redmond, H. P. & Bouchier-Hayes, D. J. Taurine and human nutrition. *Clin. Nutr.* **16**, 103–108 (1997).
32. Trefflich, I. *et al.* Associations between Dietary Patterns and Bile Acids-Results from a Cross-Sectional Study in Vegans and Omnivores. *Nutrients* **12**, 47 (2019).

33. English, D. R. *et al.* Red meat, chicken, and fish consumption and risk of colorectal cancer. *Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol.* **13**, 1509–1514 (2004).
34. Norat, T. *et al.* Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J. Natl. Cancer Inst.* **97**, 906–916 (2005).

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Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Szeged (protocol No. 5016).

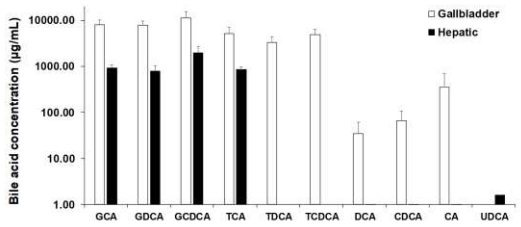
Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflicts of interest.

Figure 1

a



b

	Gallbladder bile (µg/ml)									
Patient No.	*GCA*	*GDCA*	*GCDCA*	*TCA*	*TDCA*	*TCDCA*	*DCA*	*CDCA*	*CA*	*UDCA*
GB1	16340	12370	30250	5590	2875	8725	162.18	237.84	2007.05	BDL
GB2	9060	9350	12080	3665	2610	4205	32.65	141.9	112.39	BDL
GB3	6260	10350	7285	4345	4210	4290	5.01	0	0	BDL
GB4	1400	2570	1224	413	353.5	127.5	4.09	12.89	7.05	BDL
GB5	6600	1900	6530	2935	1425	2445	0	0	0	BDL
GB6	8630	9860	9460	13915	8345	9475	3.27	0	0	BDL
Average	8048.3	7733.3	11138.2	5143.8	3303.1	4877.9	34.5	65.4	354.4	—

	Hepatic bile (µg/ml)									
Patient No.	*GCA*	*GDCA*	*GCDCA*	*TCA*	*TDCA*	*TCDCA*	*DCA*	*CDCA*	*CA*	*UDCA*
H1	880	425	576	981	BDL	BDL	0	0	0.59	BDL
H2	505	155	882	497	BDL	BDL	0	0	0.62	1.61
H3	67	16.5	35.7	61.5	BDL	BDL	0	0	0.363	BDL
H4	1449	736	1556	1118	BDL	BDL	0.24	0.87	0.74	BDL
H5	916	1312	1474	949	BDL	BDL	0.64	0	0	BDL
H6	1292	785	6550	971	BDL	BDL	0.21	2.58	0	BDL
H7	1249	2069	3321	1023	BDL	BDL	0.47	1.01	0	BDL
H8	1073	890	1458	1181	BDL	BDL	0	0.79	0.23	BDL
Average	928.9	798.6	1981.6	847.7	—	—	0.20	0.66	0.52	1.61

Figure 1. (a) Concentrations of individual bile acids (BAs) in gallbladder (n = 6) and hepatic (n = 8) bile samples, measured using liquid chromatography–high-resolution mass spectrometry. Data are presented as mean ± SEM. (**b**) Concentrations of individual BAs in gallbladder (GB1–GB6) and hepatic (H1–H8) bile samples. GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; TCA: taurocholic acid; TDCA: taurodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; DCA: deoxycholic acid; CDCA: chenodeoxycholic acid; CA: cholic acid; UDCA: ursodeoxycholic acid; BDL: below the detectable level.

Figure 2

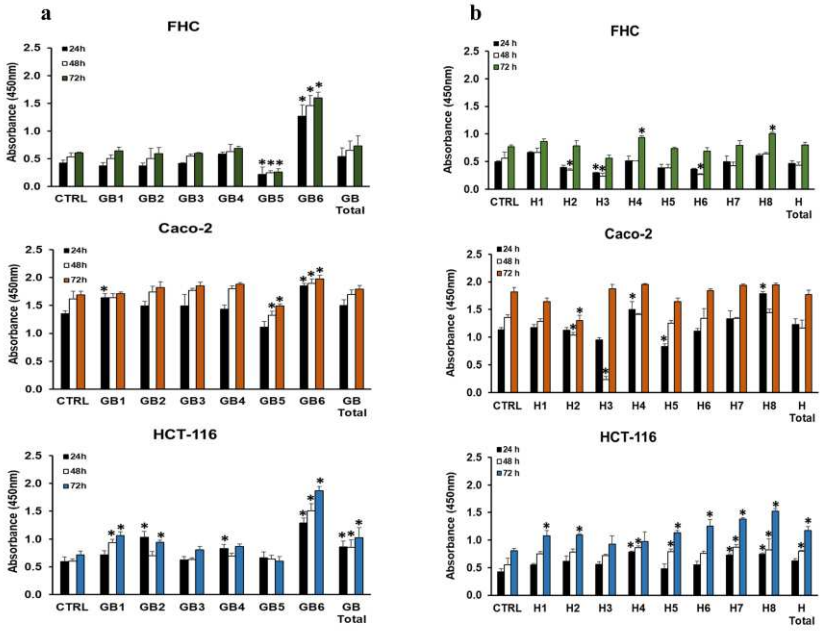


Figure 2. Effects of human bile treatment on cell proliferation. FHC, Caco-2, and HCT-116 cells were treated with individual gallbladder (a, GB1–GB6) or hepatic (b, H1–H8) bile samples for 24, 48, and 72 h, and cell proliferation was assessed using the CCK-8 assay. GB Total and H Total represent the mean responses to all individual gallbladder and hepatic bile samples, respectively. Data were presented as mean ± SEM of three independent experiments. Statistical significance was determined using one-way ANOVA. * =P ≤ 0.05 vs. the corresponding un-treated control. GB, gallbladder bile; H, hepatic bile.

Figure 3

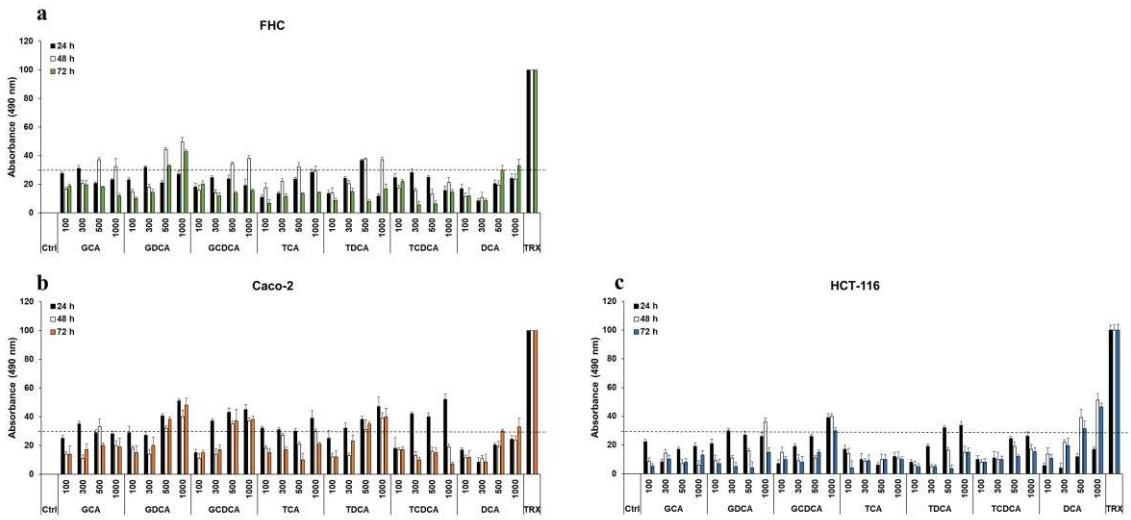


Figure 3. Cytotoxic effects of BAs. FHC, Caco-2, and HCT-116 cells were treated with different concentrations of BAs (100, 300, 500, and 1000 μM) for 24, 48, and 72 h, and cell death was assessed using an LDH release assay. 0.1% Triton X-100 (TRX) was used as a positive control. The dashed line indicates 30% cell death. Data are presented as mean ± SEM of three independent experiments. GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; TCA: taurocholic acid; TDCA: taurodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; DCA: deoxycholic acid.

Figure 4

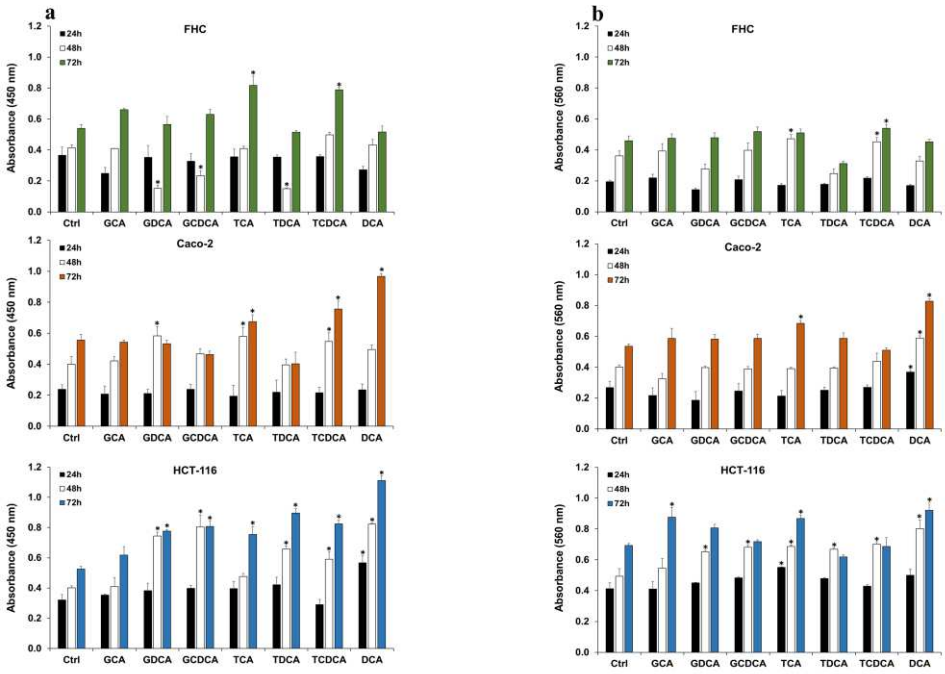


Figure 4. Effects of BAs on cell proliferation and adhesion. FHC, Caco-2, and HCT-116 cells were treated with BAs (300 μM) for 24, 48, and 72 h, and the effects on proliferation (a) and adhesion (b) were investigated. Data are presented as mean ± SEM of three independent experiments. *P ≤ 0.05 vs. control. GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; TCA: taurocholic acid; TDCA: taurodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; DCA: deoxycholic acid.

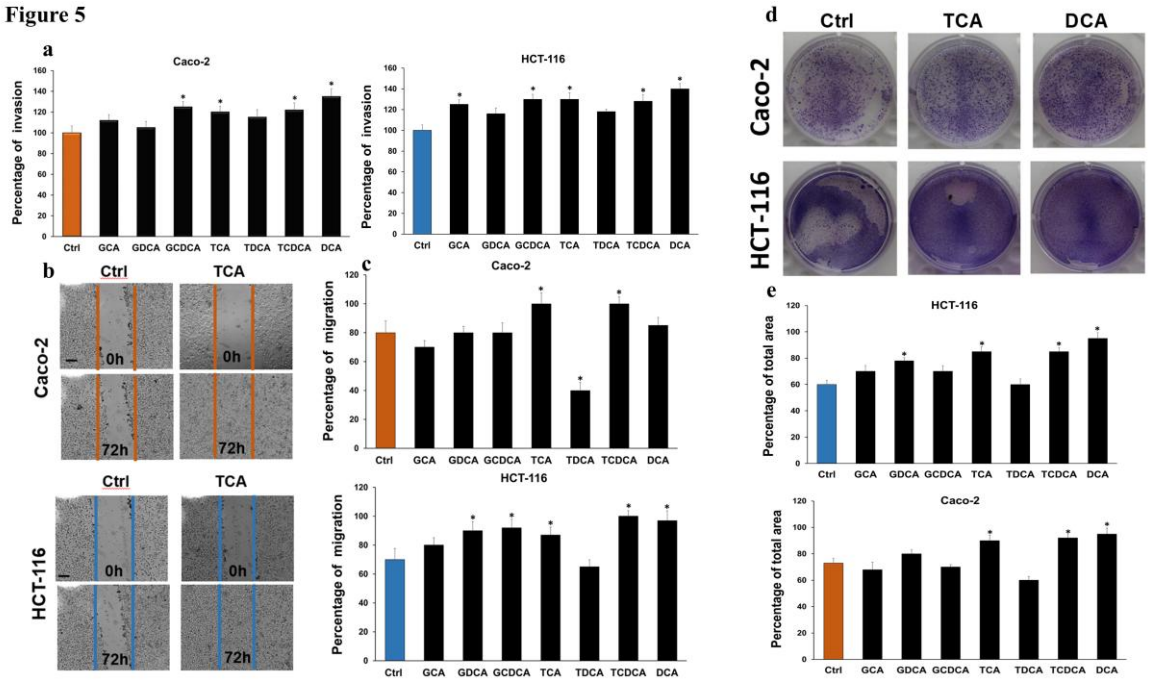


Figure 5. Effects of bile acids (BAs) on invasion, migration, and colony formation of colon adenocarcinoma cells. Caco-2 and HCT-116 cells were treated with BAs (300 µM) for 72 h, and the effects on cell invasion (**a**), migration (**b** and **c**), and colony formation (**d** and **e**) were investigated using different assays. (**a**) Cell invasion was assessed using an invasion assay and expressed as the percentage of the un-treated control. (**b**) Cell migration was investigated using the wound-healing assay. Representative pictures were taken before and 72 h after TCA (300 µM) treatment using Olympus IX71 inverted microscope and wound closure was quantified after 72 h (**c**). Representative pictures show the effects of TCA and DCA (300 µM) on the colony formation of the cells (**d**). The colony formation rate after 72 h of BA treatment was expressed as the percentage of the total area covered by colonies (**e**). Data are presented as mean ± SEM of three independent experiments. *P ≤ 0.05 vs. control. GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; TCA: taurocholic acid; TDCA: taurodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; DCA: deoxycholic acid. Scale bar: 200 µm.

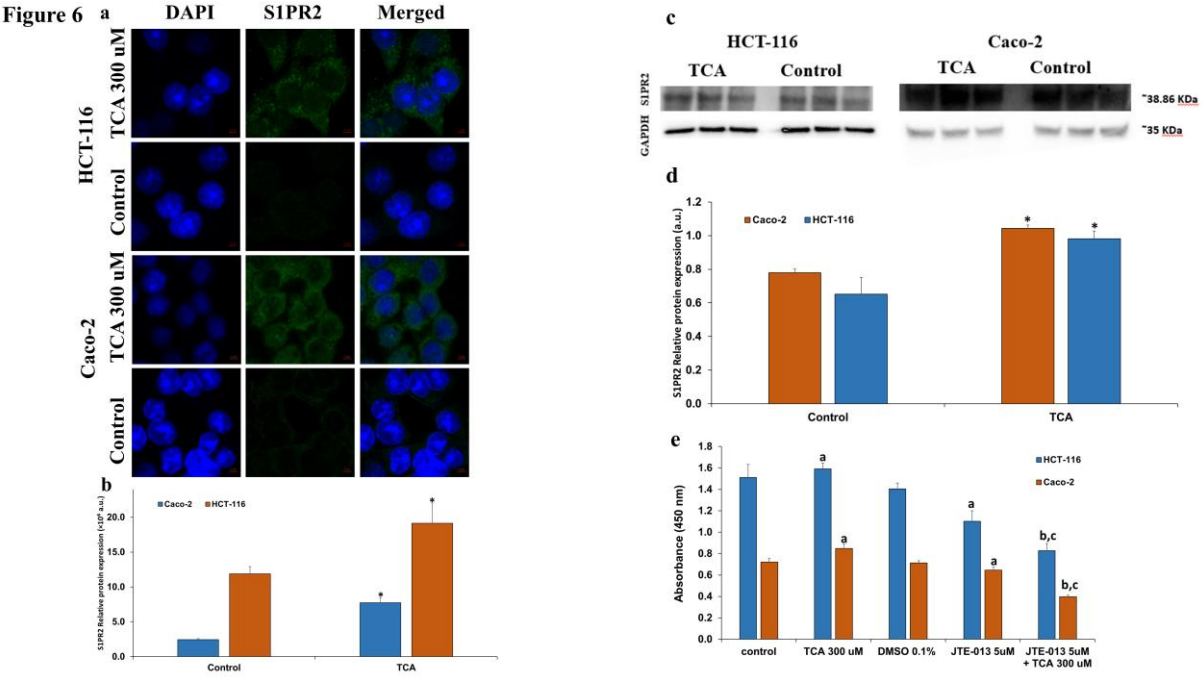


Figure 6. Taurocholic acid (TCA) increases sphingosine-1-phosphate receptor 2 (S1PR2) reduces proliferation under TCA-treated conditions in HCT-116 and Caco-2 cells. (**a**) Representative immuno-cytochemical images showing S1PR2 immunoreactivity in HCT-116 and Caco-2 cells following treatment with TCA (300 µM) for 72 h. (**b**) Quantitative analysis of S1PR2 fluorescence intensity in control and TCA-treated cells. (**c**) Representative Western blot images showing S1PR2 protein ex-pression, with GAPDH used as the loading control, in HCT-116 and Caco-2 cells following TCA treatment. (**d**) Densitometric quantification of S1PR2 protein expression normalized to GAPDH. (**e**) Effect of the S1PR2 antagonist JTE-013 (5 µM) on cell proliferation in the presence or absence of TCA (300 µM) for 72 h. DMSO (0.1%) was included as the vehicle control. Cell proliferation was assessed using the CCK-8 assay. Data are presented as mean ± SEM from three independent experiments. For S1PR2 protein expression (**b** and **d**), * = $P \leq 0.05$ vs. the corresponding untreated control. For proliferation experiments (**e**), a = $P \leq 0.05$ vs. untreated control; b = $P \leq 0.05$ vs. TCA (300 µM); and c = $P \leq 0.05$

vs. JTE-013 alone. Statistical significance was determined using an unpaired two-tailed Student's t-test for S1PR2 expression analyses and one-way ANOVA for proliferation experiments.

Figure 7

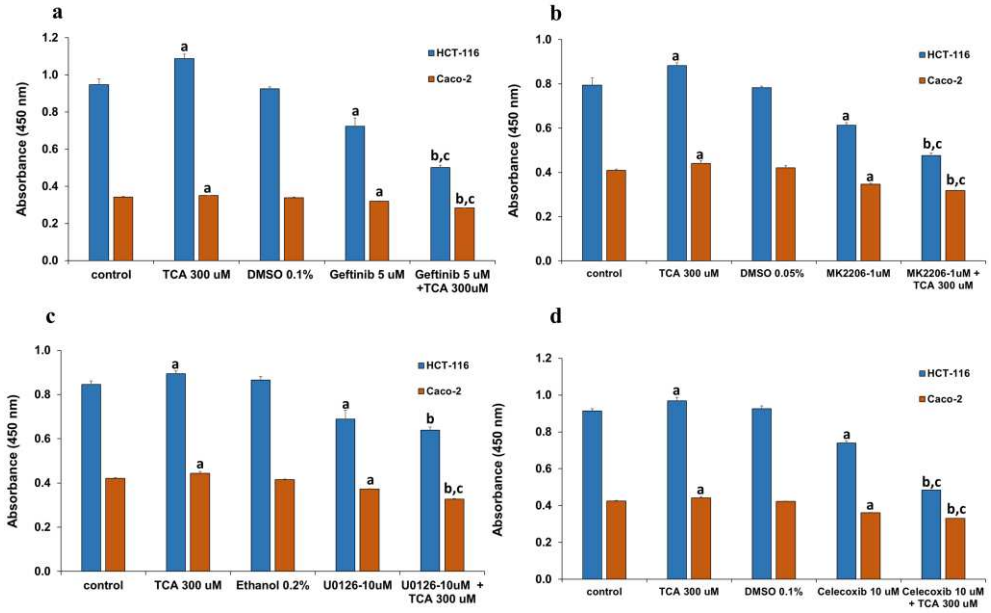


Figure 7. Pharmacological inhibition of epidermal growth factor receptor (EGFR), protein kinase B (AKT), Mitogen-activated protein kinase 1 and 2 (MEK1/2), and and cyclooxygenase-2 (COX-2) reduces proliferation in TCA-treated CRC cells. HCT-116 and Caco-2 cells were pretreated with pharmacological inhibitors targeting (**a**) EGFR (gefitinib, 5 μM), (**b**) AKT (MK-2206, 2.5 μM), (**c**) MEK1/2 (U0126, 10 μM), or (**d**) COX-2 (celecoxib, 10 μM), followed by treatment with TCA (300 μM) for 72 h. For each inhibitor, untreated control, vehicle control, inhibitor-alone, TCA-alone, and combined inhibitor-plus-TCA conditions were compared. DMSO was used as the vehicle for gefitinib, MK-2206, and celecoxib, whereas ethanol was used for U0126. Cell proliferation was assessed using the CCK-8 assay. Data are presented as mean ± SEM from three independent experiments. a = $P \leq 0.05$ vs. untreated control; b = $P \leq 0.05$ vs. TCA (300 μM); and c = $P \leq 0.05$ vs. the corresponding inhibitor-alone group.

Table 1. Clinicopathological characteristics and the level of bile acids (BAs) in the gallbladder and liver.

Clinicopathological characteristics and the level of bile acids in gallbadder and liver				
Variable	Gallbladder (n=6)		Liver (n=8)	
	n	(%)	N	(%)
Gender:				
Male	3	(50.50)	2	(25.00)
Female	3	(50.00)	6	(75.00)
Age:				
<65	1	(16.66)	2	(25.00)
≥65	5	(83.33)	6	(75.00)
Cause of death:				
Sepsis	1	(16.66)	0	(0.0)
Acute left ventricular failure	3	(50.02)	0	(0.0)
Stroke	1	(16.66)	0	(0.0)
Tumour	1	(16.66)	3	(37.5)
Bile acids in human serum (μg/mL)				
GCA	8 048 ± 1996	(19.77)	929 ± 160	(20.37)
GDCA	7 733 ± 1790	(19.00)	799 ± 233	(17.51)
GCDCA	11 138 ± 4096	(27.37)	1 982 ± 736	(43.46)
TCA	5 144 ± 1890	(12.64)	848 ± 133	(18.59)
TDCA	3 303 ± 1142	(8.11)	0±0.0	(0.00)
TCDCA	4 877 ± 1474	(11.98)	0±0.0	(0.00)
DCA	34.5 ± 25.9	(0.09)	0.20 ± 0.08	(0.004)
CDCA	65.4 ± 41.3	(0.16)	0.65 ± 0.31	(0.014)
CA	354 ± 331	(0.88)	0.32 ± 0.1	(0.007)
UDCA	0±0.0	(0.00)	1.61	(0.035)
Total Bile Acids:	40 699.05		4 559.49	

Supplementary Files

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- SupplementaryInformation.pdf