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Original research article

Deoxycholic acid-formulated curcumin enhances caspase 3/7-dependent apoptotic induction in cholangiocarcinoma cell lines

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ABSTRACT

Cholangiocarcinoma (CCA), a bile duct cancer, is a significant health problem in Thailand. The major obstacle in CCA treatment is having no effective therapeutic regimen, particularly for late-stage disease. The potent effects of curcumin on CCA have previously been documented, but the application of curcumin has been limited by its solubility and stability. Therefore, this study used liposomes, a well-known drug delivery vehicle, to transport curcumin for enhancing its efficacy. Deoxycholic acid (DCA), a secondary bile acid that has its receptors located on bile duct cells, was selected for the preparation of liposome-mediated curcumin delivery by the thin-film hydration method. The efficacy of DCA-formulated curcumin-containing liposomes (dLip/Cur) on two CCA cells, KKU-213A and KKU-213B, were compared with curcumin-containing liposomes (Lip/Cur) and curcumin alone by determinations of cell proliferation and apoptosis. The results demonstrate that IC_{50}s of dLip/Cur in CCA cell lines were lower than those of other regimens. The growth inhibitory effect of dLip/Cur was partly due to the promotion of caspase-dependent apoptosis. Altogether, the usefulness of DCA-formulated liposomes as an anti-cancer delivery system for CCA treatment is suggested herein. It might be useful as a novel vehicle for CCA treatment in the future.

Keywords: cholangiocarcinoma, deoxycholic acid, liposomes, curcumin

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1. Introduction

Cholangiocarcinoma (CCA) is an epithelial cancer originating from bile duct lining cells. CCA is the second most common primary hepatic malignancy. The increasing incidence of newly diagnosed patients is considered a public health problem worldwide, which would contribute significant effects on both health and economic issues.[1] The major limitations of CCA diagnosis and treatment are lack of specific disease markers and effective treatment regimens. These would lead to disease progression and high mortality. The pathogenesis of CCA remains to be explored but the major contributing factors are exclusively related to chronic inflammation such as primary sclerosing cholangitis, hepatolithiasis, and hepatobiliary fluke infection (commonly referred to as *Opisthorchis viverrini*, *Ov*).[2-4] Hence, alleviation of chronic inflammation might provide benefits to CCA patients.

The standard treatment for CCA is surgical removal; however, this would be of benefit only for a resectable cancer. For those who are in the advanced stages of the disease, adjunct therapies including chemotherapy, radiotherapy, and targeted therapy are suggested; however, the serious side effects and high toxicity against normal cells are still a major concern. Curcumin has been well studied worldwide for its anticancer effects in many types of cancer such as B cell lymphoma,[5] colon cancer[6] and breast cancer.[7] In CCA, functional significance of curcumin has been established. Curcumin inhibits CCA cell growth and induces apoptosis through NF-κB-, STAT-3- and caspase-dependent pathways.[8,9] This suggests potent effects of curcumin on inflammation-related CCA. However, the major obstacles in curcumin usage are poor solubility and instability in a less acidic environment.[10] Thus, the development of a curcumin delivery system to enhance the efficacy of curcumin by improving target specificity might provide an opportunity for CCA treatment.

Liposomes are a drug delivery vehicle that is widely used to potentiate drug transport to a target site. Their amphipathic property provides a superior benefit to entrap medications with a wide range of physicochemical and biophysical properties. Phospholipid and cholesterol compositions are physiologically compatible. The lipid bilayer vesicle resembles the lipid bilayer and offers a large capacity for intravesicular drug loading.[11,12] Different types of liposomes have been developed, ranging from conventional liposomes, hydrophilic polymer coated liposomes to ligand-targeted liposomes.[13,14] Deoxycholic acid (DCA) is a secondary bile acid, which can bind to a G-coupled protein receptor, TGR5 (or GP-BAR1, or M-BAR) located on CCA cells.[15] The increased expression of TGR5 has been reported and the clinical significance in advanced CCA demonstrated.[15] The therapeutic advantages of bile acids have provided opportunities for treatment development against hepatobiliary diseases such as cholestatic and metabolic liver diseases, and cancers.[16] Moreover, DCA-modified liposomes are a novel carrier for an anticancer drug. DCA-modified epirubicin-containing liposomes have revealed their advantages for breast and melanoma treatment *in vitro* and *in vivo*,[17] adding further support that DCA can bind to the G-coupled protein receptor TGR5 (or GP-BAR1, or M-BAR) located on CCA. Hence, the development of DCA-modified liposomes for curcumin delivery to CCA might offer clinical benefits and is of interest in the current study.

The effects of DCA-formulated curcumin-containing liposomes (dLip/Cur) on CCA proliferation and apoptosis were investigated in CCA cell lines. Curcumin solution (Cur) and curcumin-containing liposomes (Lip/Cur) were used for comparison purposes. The underlying mechanism related to dLip/Cur-induced apoptosis was revealed. Altogether, the information from the current study might provide the opportunity for anti-CCA targeted treatment.

2. Materials and Methods

2.1 Cell culture

Two human CCA cell lines, KKU-213A and KKU-213B, were previously established from *Ov*-associated CCA tissues from Thai patients.[18] Cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan. Cells were cultured with DMEM (Wako Pure Chemical Industries, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C under a 5% CO_2 atmosphere.

2.2 DCA-formulated curcumin

Liposomes were formulated by the thin-film hydration method.[19] Twenty-five mg DCA was added to generate DCA-modified liposome. Each preparation was evaporated and rehydrated by adding 10 ml of 0.1 M sodium chloride solution. The liposomes obtained were further sonicated on ice to reduce the particle size. To generate curcumin-containing liposome, 5 mg of curcumin (Merck, Darmstadt, Germany) was added to each formulation. After that Lip/Cur and dLip/Cur were filtrated through a 0.45 µm cellulose membrane to remove non-encapsulated curcumin particles. The ratios of phosphatidylcholine : DCA : curcumin were 0 mg : 0 mg : 5 mg (Cur; standard solution), 100 mg : 0 mg : 5 mg (Lip/Cur; formulation control), and 100 mg : 25 mg : 5 mg (dLip/Cur; tested formula). The concentrations indicated in this study refer to the curcumin concentration in each formulation analyzed by entrapment efficiency (%EE) using a direct method. Briefly, curcumin-entrapped liposomes were solubilized in methanol then filtrated to be analyzed by HPLC. The area under the curve (AUC) of each curcumin formulation was calculated compared to the curcumin standard solution.

2.3 MTT assay

The effect of Cur, Lip/Cur, and dLip/Cur on cell number was determined in KKU-213A and KKU-213B cell lines by measuring mitochondrial dehydrogenase activity using 3-[4,5,-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) as the substrate.[20] Briefly, 10^4 cells/well of 96-well plates were incubated with 10, 25 and 50 µM Cur (as control) and 10, 25, 50, 75 and 100 µM Cur for Lip/Cur, or dLip/Cur, for 24 h. All curcumin formulations were dissolved in complete DMEM for the required concentrations. Then MTT solution was added onto cells to reach a final concentration of 0.5 mg/mL and the cells were incubated for an additional 2 hours. Afterwards, formazan crystals were dissolved with 0.04 N HCl in isopropanol. The absorbance was measured at 570 nm using a microplate reader (Multiskan; Thermo Fisher Scientific, Inc., MA, USA). The OD570 of untreated samples was adjusted to 100%.

2.4 Flow cytometry analysis

KKU-213A were treated with 50 µM Cur and 100 µM Lip/Cur, or dLip/Cur and incubated for 6-8 hours. Cells were washed twice with phosphate buffered saline (PBS). Apoptotic induction was assessed by Muse™ Annexin V & Dead Cell Kit (Merck, Darmstadt, Germany) following the manufacturer's instructions. In brief, 10^5 cells were stained with a mixture of annexin V-phosphatidyl serine and 7-AAD-dead cell marker. Stained cells were analyzed by Muse™ Cell Analyzer (Merck, Darmstadt, Germany). $5x10^3$ acquired cells were gated to detect apoptotic cells as annexin V-positive (+).

Activation of caspase-3/7 was measured by Muse™ Caspase-3/7 Kit as recommended by the manufacturer (Merck, Darmstadt, Germany). In brief, $5x10^4$ cells were incubated with caspase-3/7-FITC for 30 minutes, then stained with 7-AAD. The stained cells were measured by the Muse™ Cell Analyzer (Merck, Darmstadt, Germany). $3x10^3$ acquired cells were gated to caspase 3/7-activated apoptotic cells which were caspase-3/7 positive (+).

3. Results

3.1 Cell viability

The effects of Cur, Lip/Cur, and dLip/Cur on KKU-213A and KKU-213B viabilities were determined by MTT assay. Curcumin concentration of 10, 25 and 50 µM (for Cur, Lip/Cur and dLip/Cur) and 75 and 100 µM

(for Lip/Cur and dLip/Cur) were added onto the CCA cells. Cell viability was observed at 24 h after treatment. The viability of untreated cells was adjusted to 100%. Percent viabilities of KKU-213A and KKU-213B are shown in Fig. 1. The half maximal inhibitory concentration (IC_{50}) was calculated by an equation obtained from a linear graph, shown in Table 1. IC_{50}s of Cur, Lip/Cur, and dLip/Cur in KKU-213A were 40.7, >100 and 32.8 μM, and were >50, >100 and 41.8 μM in KKU-213B. The results show that dLip/Cur suppressed KKU-213A and KKU-213B with higher cytotoxicity than Cur and Lip/Cur.

Table 1. Half maximal inhibitory concentration (IC_{50}) of different curcumin formulations on cell viability of KKU-213A and KKU-213B cells at 24 h**.**

Curcumin formulation	IC_{50} (μM)	
	KKU-213A	KKU-213B
Cur	40.7	>50
Lip/Cur	>100	>100
dLip/Cur	32.8	41.8

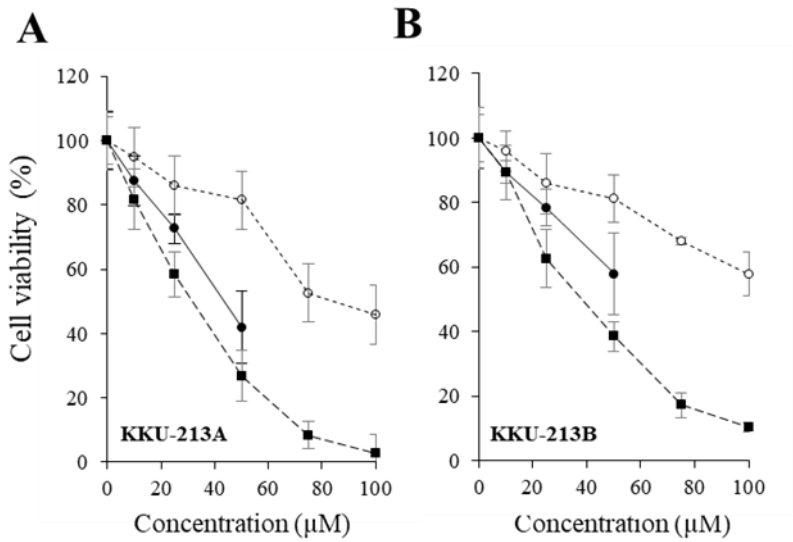

Fig. 1. The anti-proliferative effects of 3 curcumin formulations on two CCA cell lines, KKU-213A (A) and KKU-213B (B). Cells were treated with various concentrations of curcumin (●, Cur), curcumin-containing liposomes (○, Lip/Cur) and DCA-modified curcumin-containing liposomes (■, dLip/Cur) for 24 h. The relative cell numbers were determined by MTT assay. The number of untreated cells was set as 100%. Data are presented as means ± standard deviations (SD) from three independent experiments.

3.2 Apoptosis induction

The growth inhibitory effect of dLip/Cur was further studied with a focus on apoptotic induction. KKU-213A was selected as a representative cell line for investigation. Cells were treated with 50 µM of Cur and 100 µM Lip/Cur or dLip/Cur for 6 hours, then apoptotic cells were measured by Muse™ Annexin V & Dead Cell Kit and Muse™ Cell Analyzer. The percentage of apoptotic cells was calculated fromAnnexin V positive cells as shown in Fig. 2A. Dot plots of dLip/Cur induced KKU-213A apoptosis are presented in Fig. 2B. Apoptotic cells observed in untreated control, Cur-, Lip/Cur- and dLip/Cur-treated cells were 0.28±0.03%, 6.91±2.12%, 6.8±1.23% and 37.14±2.65%, respectively.

3.3 Caspase-3/7-dependent apoptosis

To demonstrate the involvement of caspase 3/7 in apoptosis induction, its activation was determined in drug-treated KKU-213A by Muse™ Caspase-3/7 Kit and Muse™ Cell Analyzer. As shown in Fig. 3, caspase-3/7 activation was clearly observed in dLip/Cur-treated KKU-213A when compared to Cur treatment. The proportions of caspase 3/7-activated cells were 5.08±2.43% and 35.12±6.29% in Cur- and dLip/Cur-treated cells. The percentage of caspase 3/7 activation in dLip/Cur-treated cells was 6.9-fold higher than in Cur-treated cells.

4. Discussion

The higher potency of dLip/Cur over Cur is similar to that of DCA-modified liposomes containing paclitaxel,[21] sorafeni[22] orepirubicin[17] compared to chemotherapeutic agent alone. Previous studies of DCA-modified anticancer drug-loaded liposomes have suggested that the higher toxicity of DCA-modified liposomes is due to the enhanced cellular uptake. However, the advantages of DCA-modified liposome and dLip/Cur in CCA were first reported in the current study.

The percentage of apoptotic cells in dLip/Cur treatment was approximately 5 times higher than for curcumin alone. Curcumin-induced apoptosis in this study was similar to that of a previous report.[9] DCA induced apoptosis has been reported elsewhere,[23,24] but the effects of DCA on TGR5 and transcriptional factor activation might hint at growth promoting effects rather than apoptosis induction.[25,26] The strong apoptosis inducing effect of dLip/Cur was similar to that of other DCA-modified liposomes,[21,27,28] but greater potency is reported herein when compared to another study in breast cancer cells.[21] Paclitaxel loaded DCA micelles induced 2-fold higher apoptotic cell death than those treated with free paclitaxel in MCF-7 cells.[21] The stronger apoptosis induction might be promoted by the higher of TGR5 in CCA cells,[15] and the requirement of TGR5 for cholangiocyte proliferation.[29]

The curcumin-induced caspase-3-dependent apoptotic pathway has been reported in several cancer cells, including CCA.[9,30,31] Moreover, activation of caspase-3, -8, -9 and PARP cleavage were reported in curcumin-treated CCA cells.[9] In the current study, the percentage of caspase-3/7-activated cells was less than the percentage of annexin V positive cells. This might imply the involvement of a caspase-independent apoptosis pathway in dLip/Cur-treated CCA cells.[32,33] However, strong caspase 3/7 activation in dLip/Cur suggests caspase-dependent apoptosis as a major pathway.

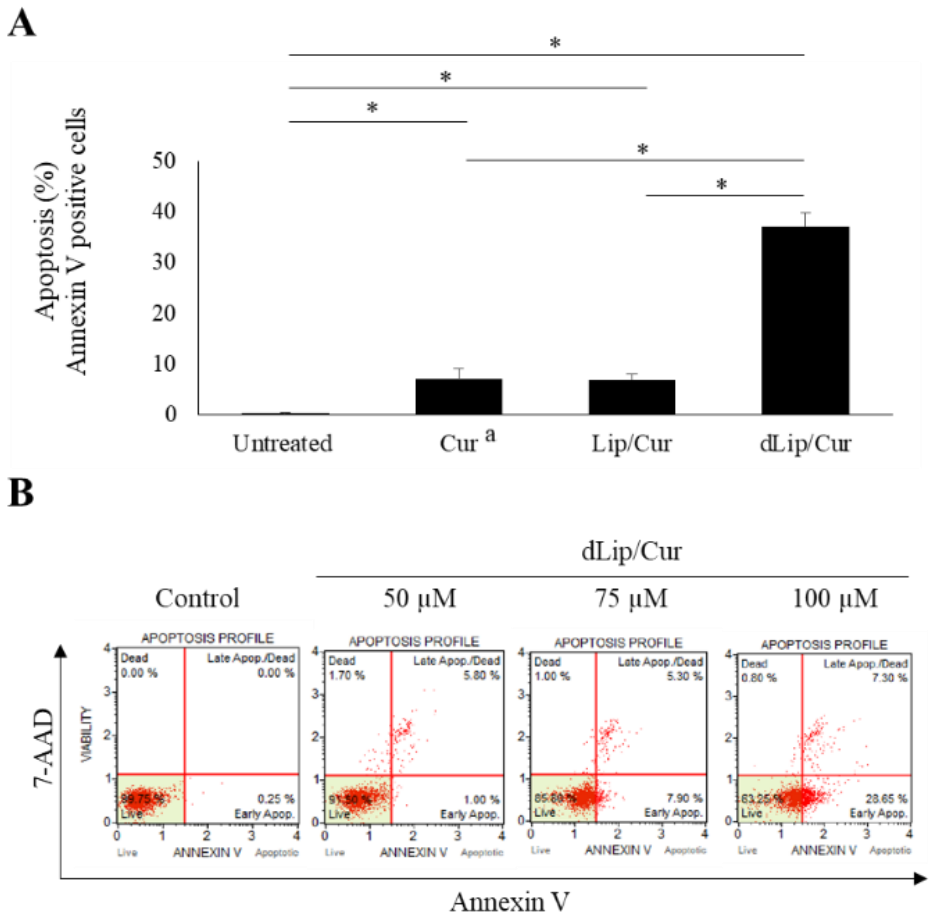

Fig. 2. dLip/Cur induces apoptosis in KKU-213A cells. Cells were treated with Cur (0 and 50 µM), Lip/Cur, and dLip/Cur (0, 50, 75 and 100 µM) for 6 h, and apoptotic cells were stained by Muse™ Annexin V & Dead Cell Kit and analyzed by Muse™ Cell Analyzer. **(A)** Apoptotic cells are Annexin V-positive. Percentages of apoptotic cells are presented as mean ± SD from three independent experiments for [a] Cur (0 µM), Lip/Cur, and dLip/Cur (100 µM). (**B**) Percentages of early apoptotic, late apoptotic and dead cells are demonstrated. Early apoptotic cells are Annexin V-positive (+)/7-AAD-negative (-); late apoptotic cells are Annexin V-(+)/7-AAD-(+); dead cells are Annexin V-(-)/7-AAD-(+). The statistical significance is shown as follows: *p<0.05.

5. Conclusion

The growth inhibition and caspase-dependent apoptosis induction of DCA-formulated curcumin-containing liposome (dLip/Cur) on CCA cells has been reported in the current study. The effects were stronger than those of curcumin alone (Cur) and curcumin-containing liposome (Lip/Cur). dLip/Cur effectively inhibits CCA cell viability and induces caspase-dependent apoptosis. Altogether, the results suggest that DCA-modified liposomes are a candidate delivery system for poorly soluble agents and that dLip/Cur is a potential regimen for CCA treatment. However, additional studies *in vivo* and on the effects of DCA in CCA cell apoptosis are required.

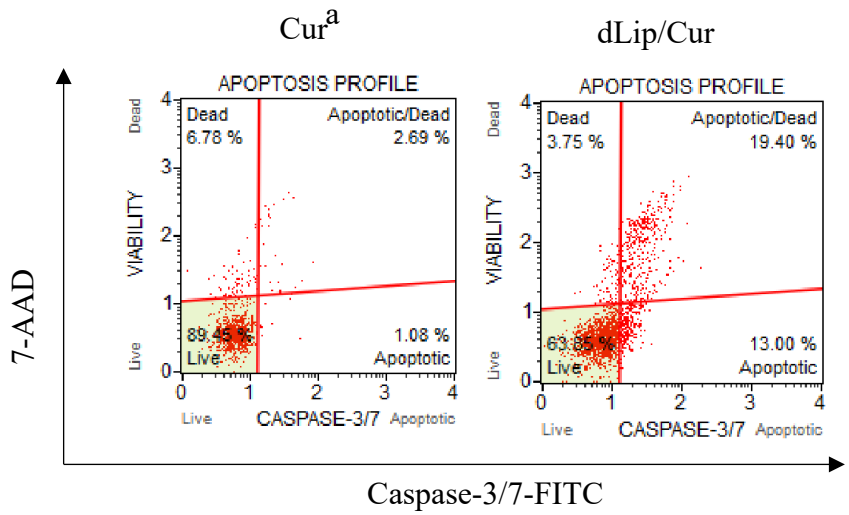

Fig. 3. dLip/Cur induced caspase 3/7 activation in KKU-213A cells. Cells were treated with 50 µM Cur, or 100 µM dLip/Cur for 6 h and caspase 3/7-activated cells were quantified by Muse™ Caspase-3/7 staining and Muse™ Cell Analyzer. Percentages of caspase 3/7-activated cells equal the percentages of caspase 3/7-FITC-positive cells.

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References

[1] Khuntikeo N, Thinkhamrop B, Bundham-charoen K, Andrews RH, Grundy-Warr C, Yongvanit P, et al. The socioeconomic burden of cholangiocarcinoma associated with *Opisthorchis viverrini* sensu lato infection in Northeast Thailand: a preliminary analysis. Adv Parasitol. 2018; 102:141-163.

[2] Sripa B, Brindley PJ, Mulvenna J, Laha T, Smout MJ, Mairiang E, et al. The tumorigenic liver fluke *Opisthorchis viverrini*--multiple pathways to cancer. Trends Parasitol. 2012; 28(10):395-407.

[3] Kamsa-Ard S, Luvira V, Pugkhem A, Luvira V, Thinkhamrop B, Suwanrungruang K, et al. Association between praziquantel treatment and cholangiocarcinoma: a hospital-based matched case-control study. BMC Cancer. 2015;15:776.

[4] Saengsawang P, Promthet S, Bradshaw P. Reinfection by *Opisthorchis viverrini* after treatment with praziquantel. Asian Pac J Cancer Prev. 2016;17(2):857-862.

[5] Han SS, Chung ST, Robertson DA, Ranjan D, Bondada S. Curcumin causes the growth arrest and apoptosis of B cell lymphoma by downregulation of egr-1, c-myc, bcl-XL, NF-kappa B, and p53. Clin Immunol. 1999; 93(2):152-161.

[6] Chauhan DP. Chemotherapeutic potential of curcumin for colorectal cancer. Curr Pharm Des. 2002;8(19):1695-1706.

[7] Liu D, Chen Z. The effect of curcumin on breast cancer cells. J Breast Cancer. 2013; 16(2):133-137.

[8] Plengsuriyakarn T, Viyanant V, Eursitthichai V, Picha P, Kupradinun P, Itharat A, et al. Anticancer activities against cholangio-carcinoma, toxicity and pharmacological

activities of Thai medicinal plants in animal models. BMC Complement Altern Med. 2012;12:23.

[9] Prakobwong S, Khoontawad J, Yongvanit P, Pairojkul C, Hiraku Y, Sithithaworn P, et al. Curcumin decreases cholangiocarcinogenesis in hamsters by suppressing inflammation-mediated molecular events related to multistep carcinogenesis. Int J Cancer. 2011;129(1): 88-100.

[10] Kharat M, Du Z, Zhang G, McClements DJ. Physical and chemical stability of curcumin in aqueous solutions and emulsions: impact of pH, temperature, and molecular environment. J Agric Food Chem. 2017; 65(8):1525-1532.

[11] Samad A, Sultana Y, Aqil M. Liposomal drug delivery systems: an update review. Curr Drug Deliv. 2007;4(4):297-305.

[12] Zylberberg C, Matosevic S. Pharmaceutical liposomal drug delivery: a review of new delivery systems and a look at the regulatory landscape. Drug Deliv. 2016; 23(9):3319-3329.

[13] Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev. 2013; 65(1):36-48.

[14] Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. Advances and challenges of liposome assisted drug delivery. Front Pharmacol. 2015;6:286.

[15] Li AD, Xie XL, Qi W, Wang WB, Ma JJ, Zhao DQ, et al. TGR5 promotes cholangiocarcinoma by interacting with mortalin. Exp Cell Res. 2020;389(2):111855.

[16] Schaap FG, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. Nat Rev Gastroenterol Hepatol. 2014;11(1): 55-67.

[17] Gao F, Li L, Zhang H, Yang W, Chen H, Zhou J, et al. Deoxycholic acid modified-carboxymethyl curdlan conjugate as a novel carrier of epirubicin: in vitro and in vivo studies. Int J Pharm. 2010;392(1-2): 254-260.

[18] Sripa B, Seubwai W, Vaeteewoottacharn K, Sawanyawisuth K, Silsirivanit A, Kaewkong W, et al. Functional and genetic characterization of three cell lines derived from a single tumor of an *Opisthorchis viverrini*-associated cholangio-carcinoma patient. Hum Cell. 2020;33(3): 695-708.

[19] Zhang H. Thin-Film Hydration Followed by Extrusion Method for Liposome Preparation. Methods Mol Biol. 2017;1522:17-22.

[20] Bharti AC, Takada Y, Shishodia S, Aggarwal BB. Evidence that receptor activator of nuclear factor (NF)-kappaB ligand can suppress cell proliferation and induce apoptosis through activation of a NF-kappaB-independent and TRAF6-dependent mechanism. J Biol Chem. 2004; 279(7):6065-6076.

[21] Wang F, Chen Y, Zhang D, Zhang Q, Zheng D, Hao L, et al. Folate-mediated targeted and intracellular delivery of paclitaxel using a novel deoxycholic acid-O-carboxymethylated chitosan-folic acid micelles. Int J Nanomedicine. 2012;7:325-337.

[22] Lei M, Ma G, Sha S, Wang X, Feng H, Zhu Y, et al. Dual-functionalized liposome by co-delivery of paclitaxel with sorafenib for synergistic antitumor efficacy and reversion of multidrug resistance. Drug Deliv. 2019; 26(1):262-272.

[23] Redlak MJ, Dennis MS, Miller TA. Apoptosis is a major mechanism of deoxycholate-induced gastric mucosal cell death. Am J Physiol Gastrointest Liver Physiol. 2003; 285(5):G870-G879.

[24] Sousa T, Castro RE, Pinto SN, Coutinho A, Lucas SD, Moreira R, et al. Deoxycholic acid modulates cell death signaling through changes in mitochondrial membrane properties. J Lipid Res. 2015;56(11):2158-2171.

[25] Duboc H, Tache Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. Dig Liver Dis. 2014;46(4):302-312.

[26] Guo C, Chen WD, Wang YD. TGR5, not only a metabolic regulator. Front Physiol. 2016;7:646.

[27] Faustino C, Serafim C, Rijo P, Reis CP. Bile acids and bile acid derivatives: use in drug delivery systems and as therapeutic agents. Expert Opin Drug Deliv. 2016; 13(8):1133-1148.

[28] Piktel E, Niemirowicz K, Watek M, Wollny T, Deptula P, Bucki R. Recent insights in nanotechnology-based drugs and formulations designed for effective anticancer therapy. J Nanobiotechnology. 2016;14(1): 39.

[29] Reich M, Deutschmann K, Sommerfeld A, Klindt C, Kluge S, Kubitz R, et al. TGR5 is essential for bile acid-dependent cholangiocyte proliferation in vivo and in vitro. Gut. 2016; 65(3):487-501.

[30] Kuttikrishnan S, Siveen KS, Prabhu KS, Khan AQ, Ahmed EI, Akhtar S, et al. Curcumin induces apoptotic cell death via inhibition of PI3-Kinase/AKT pathway in B-precursor acute lymphoblastic leukemia. Front Oncol. 2019;9:484.

[31] O'Sullivan-Coyne G, O'Sullivan GC, O'Donovan TR, Piwocka K, McKenna SL. Curcumin induces apoptosis-independent death in oesophageal cancer cells. Br J Cancer. 2009;101(9):1585-1595.

[32] Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol. 2013;14:32.

[33] Broker LE, Kruyt FA, Giaccone G. Cell death independent of caspases: a review. Clin Cancer Res. 2005;11(9):3155-3162.