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Bile acids, carriers of hepatoma-targeted drugs?

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Previous studies display that bile acids (Bas) could be used as carriers and pharmaceutical excipients. In this study, the selective cytotoxicity of 6 bile acids (BAs) was evaluated against hepatoma cell line HepG2, human colon carcinoma cell line HT-29, gastric cancer cell line BGC823, cervical cancer cell line Hela and hepatocyte line L02. Our study suggested that most of the BAs showed cytotoxicity against a broader spectrum of tumor cells and display high cell selectivity toward HepG2. In particular, chenodeoxycholic acid (CDCA) exerted the most potent selective cytotoxicity against HepG2 ($IC_{50} = 54.62 \pm 3.5$ μM) and low toxicity on L02 cells ($IC_{50} > 200$ μM). According to the structure-activity relationship, the position, configuration and number of OH groups in BAs could affect cell proliferation and selectivity. Moreover, the pre-mechanism of CDCA on HepG2 cells was studied by Giemsa staining, DAPI staining, AO/EB staining, apoptosis analysis and mitochondrial membrane potential assay. Results showed that CDCA could induce apoptosis and loss of mitochondrial transmembrane potential in HepG2 cells. The study inferred that CDCA might be a carrier and parent pharmaceutical excipient for hepatic carcinoma targeting drug.

1. Introduction

Bile acids (BAs) are cholesterol metabolites and their main function is to ensure fat solubilization, emulsification and thus promote digestion (Baptissart et al. 2013). Numerous studies showed that BAs had essential regulatory functions, as they interact with a large number of intracellular and extracellular cytokines such as tumor necrosis factor-alpha (TNF-α), interlukin-1 beta (IL-1β) (Hua et al. 2013), murine double minute 2 (MDM2) and murine double minute 4 (MDM4) (Vogel et al. 2012). It has also been reported that BAs play key homeostatic roles in hepatic metabolism, glucose homeostasis, energy expenditure acterial growth and inflammation (Neuschwander-Tetri et al. 2014; Hirschfield et al. 2014; Sipka et al. 2014). Moreover, BAs have been found to have anti-neoplastic properties in a multitude of digestive system cancer cell models, such as liver cancer, colon cancer, and gastric cancer (Ao et al. 2013; Chien et al. 2014; Röhrl et al. 2014; Komori et al. 2014). More recently, BAs could be used as carriers and pharmaceutical excipients of drugs, in combination with BAs, the uptake of drugs on liver diseases were improved and the activities of the complexes were reserved and even enhanced (Li et al. 2014; Li et al. 2013; Liang et al. 2012; Cirri et al. 2015).

To date, the role of BAs as pharmaceutical excipients, their liver-targeting effects and dose-related cytotoxicity have not been evaluated systematically. In this study, we investigated the cytotoxicity and organ specificity of six BAs (Table 1) against both tumor and normal cells. The antiproliferative activities of BAs were evaluated against HepG2, HT-29, BGC823, Hela and L02 cell lines by MTT assay. Subsequent fluorescence staining and flow cytometry analysis on treated HepG2 cells were studied. Structure-activity relationships (SARs) of these BAs were also discussed.

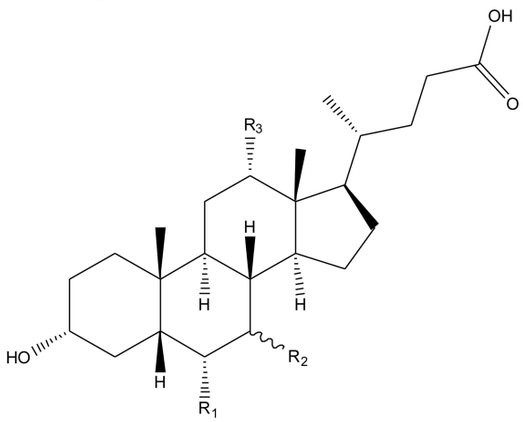
2. Investigations, results and discussion

2.1. In vitro cytotoxicity

The cytotoxicity of six BAs against L02, HepG2, HT-29, BGC823 and Hela cell lines was evaluated by MTT assay. Each experiment was repeated at least three times. The results are presented in Table 2 .

These results showed that BAs had hepatoma-targeted and dose-related cytotoxicity towards the four cell lines. Most of BAs displayed stronger toxicity against a broader spectrum of tumors at high concentrations while friendly to L02 cells, and none of BAs were toxic at low concentrations on L02 cells. Among them, CDCA, HDCA, LCA and UDCA were more toxic to HepG2 cells than to L02, HT-29, Hela and BGC823 cell lines, suggesting that R_3 without a substituent may be favorable for the selective cytotoxicity. For example, the effect of CDCA ($IC_{50} > 200$ μM) on L02 showed nontoxicity at the concentration of 100 μM, while the inhibition rate against HepG2 ($IC_{50} = 54.62 \pm 3.5$ μM) was up to 76% at the same concentration (Fig. 1), which was consistent with our structure-activity relationship analysis. Moreover, the α-OH group in R_2 was considered to be a critical pharmacophore in CDCA, which resulted in an increased proliferation on L02 cells (< 100 μM). Interestingly, UDCA, the epimer of CDCA with β-OH in R_2, showed a strong cytotoxicity on L02 cells. Besides, it was found that the

Table 1: Chemical structures of six bile acids investigated



Full name	Trivial name	R_1	R_2	R_3
Hyodeoxycholic acid	HDCA	OH	H	H
Deoxycholic acid	DCA	H	H	OH
Lithocholic acid	LCA	H	H	H
Ursodeoxycholic acid	UDCA	H	β-OH	H
Chenodeoxycholic acid	CDCA	H	α-OH	H
Cholic acid	CA	H	α-OH	OH

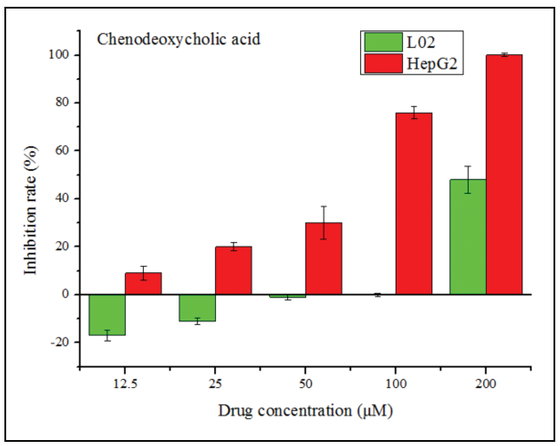


Fig. 1: Effect of CDCA against the human liver cell line L02 and the hepatocellular carcinoma cell line HepG2.

cytotoxicity against BGC823 cells was significantly enhanced as the number of OH groups decreased, the activity order was LCA > CDCA, DCA, UDCA, HDCA > CA.

In addition, we found that most BAs elicited a great inhibitory effect on human colon carcinoma cell line HT-29. Previous studies have suggested that OATP1B3, a member of the organic anion transporting polypeptides(OATPs) family, was markedly increased in colon tumor tissues (Pressler et al. 2011). Besides, the uptake of bile acids into HepG2 cells was mediated only

Table 2: Inhibitory activity of 6 BAs on different cell lines proliferation

Compounds	Cell lines	inhibition rate (%)[a]					IC_{50}
		12.5 μM	25 μM	50 μM	100 μM	200 μM	
HDCA	L02	13 ± 5.2	36 ± 7.5	38 ± 5.7	45 ± 5.1	46 ± 2.0	> 200
	HepG2	11 ± 6.1	12 ± 2.5	23 ± 2.1	44 ± 2.6	92 ± 1.2	109.5 ± 3.5
	HT-29	18 ± 1.2	25 ± 2.2	36 ± 2.6	46 ± 0.4	66 ± 2.9	77.38 ± 2.3
	BGC823	-6 ± 3.3	-5 ± 3.9	16 ± 2.0	49 ± 3.3	68 ± 2.3	115.67 ± 3.6
	Hela	8 ± 4.3	17 ± 3.6	26 ± 2.3	30 ± 1.4	50 ± 5.4	> 200
DCA	L02	8 ± 3.5	32 ± 5.8	49 ± 1.7	57 ± 3.7	73 ± 1.4	33.25 ± 3.9
	HepG2	13 ± 1.9	14 ± 2.2	31 ± 4.9	67 ± 1.7	100 ± 0.33	58.14 ± 2.6
	HT-29	8 ± 2.5	25 ± 1.7	35 ± 3.6	52 ± 2.1	68 ± 3.4	80.11 ± 3.2
	BGC823	-33 ± 0.70	-18 ± 1.2	8 ± 1.7	43 ± 8.1	85 ± 3.3	111.53 ± 6.2
	Hela	7 ± 5.0	19 ± 1.6	26 ± 1.1	27 ± 3.8	58 ± 2.0	116.27 ± 3.2
LCA	L02	9 ± 5.4	31 ± 1.4	36 ± 2.9	59 ± 1.5	62 ± 1.9	75.92 ± 2.4
	HepG2	-1 ± 1.3	1 ± 2.4	27 ± 2.4	98 ± 0.07	100 ± 0.42	58.95 ± 1.7
	HT-29	21 ± 0.5	30 ± 3.9	45 ± 2.1	52 ± 4.3	61 ± 0.2	68.54 ± 2.8
	BGC823	11 ± 7.0	14 ± 4.2	16 ± 1.6	23 ± 8.3	99 ± 1.2	89.81 ± 5.4
	Hela	22 ± 1.3	32 ± 1.1	34 ± 8.0	37 ± 6.5	66 ± 2.4	76.85 ± 4.3
UDCA	L02	41 ± 2.7	44 ± 1.4	47 ± 4.4	63 ± 2.4	63 ± 0.29	46.98 ± 2.7
	HepG2	11 ± 4.1	12 ± 9.2	39 ± 2.1	60 ± 1.9	91 ± 0.87	64.39 ± 4.6
	HT-29	23 ± 2.3	35 ± 4.6	33 ± 3.1	42 ± 3.7	55 ± 1.4	79.83 ± 3.8
	BGC823	19 ± 10	26 ± 2.9	33 ± 7.9	50 ± 1.9	77 ± 2.0	68.78 ± 5.9
	Hela	20 ± 1.6	30 ± 8.9	47 ± 1.2	48 ± 4.4	67 ± 1.4	66.55 ± 4.2
CDCA	L02	-17 ± 2.3	-11 ± 1.4	-1 ± 0.97	0 ± 0.64	48 ± 5.7	> 200
	HepG2	9 ± 2.8	20 ± 1.7	30 ± 6.8	76 ± 2.7	100 ± 0.68	54.62 ± 3.5
	HT-29	19 ± 2.0	28 ± 1.8	41 ± 1.9	48 ± 2.2	70 ± 1.3	69.14 ± 2.3
	BGC823	6 ± 4.9	8 ± 4.7	9 ± 3.7	28 ± 4.1	89 ± 1.6	108.11 ± 4.6
	Hela	16 ± 1.7	18 ± 2.8	31 ± 3.5	43 ± 3.1	56 ± 1.7	95.26 ± 3.1
CA	L02	-21 ± 6.6	17 ± 4.3	18 ± 10	28 ± 9.1	54 ± 7.8	132.18 ± 9.1
	HepG2	18 ± 1.0	19 ± 0.46	25 ± 3.1	36 ± 0.86	51 ± 2.3	106.25 ± 1.9
	HT-29	14 ± 1.6	33 ± 4.8	47 ± 0.8	57 ± 0.7	74 ± 0.9	60.71 ± 2.1
	BGC823	-16 ± 2.8	-2 ± 2.2	5 ± 1.1	31 ± 5.8	37 ± 5.9	> 200
	Hela	14 ± 4.5	24 ± 3.8	30 ± 3.6	38 ± 1.1	56 ± 0.42	96.93 ± 3.2

[a] Inhibition rate (%) are presented as the mean ± SD (standard deviation) from 3 separated experiments.

by OATPs (Kullak-Ublick et al. 1996). Meanwhile, OATP1B1 and OATP1B3 are highly expressed in liver and are regarded as "liver-specific" OATPs. These associations suggesting that OATPs may play an important role in the organ specificity effects of BAs, which will be investigate in our continuing research.

Based on the above mentioned evidence, we reasoned that CDCA may be a desirable carrier and parent pharmaceutical

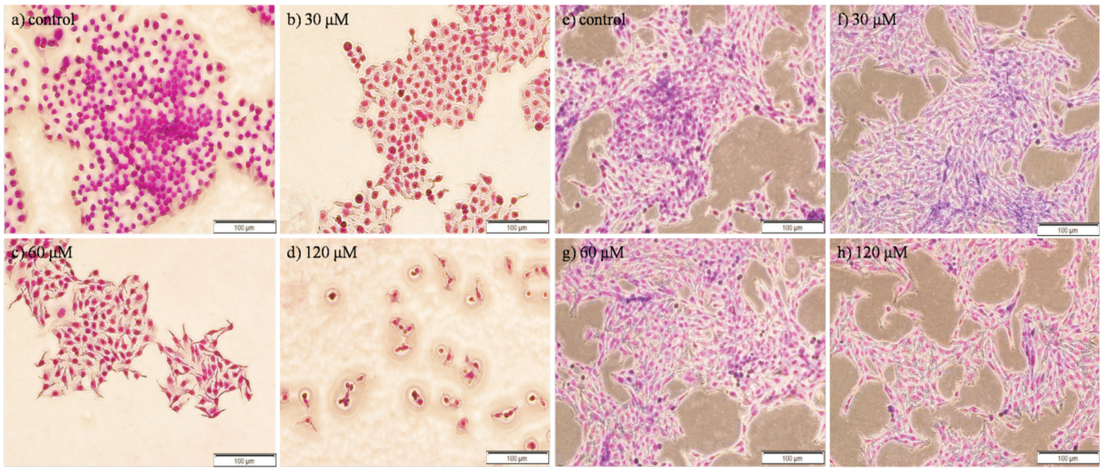


Fig. 2: The Giemsa staining of CDCA in L02 and HepG2 cell lines. For HepG2 group (a-d); For L02 group (e-h) (×200): HepG2 and L02 cells were either untreated or treated with 30, 60, or 120 μM CDCA for 48 h and stained with Giemsa.

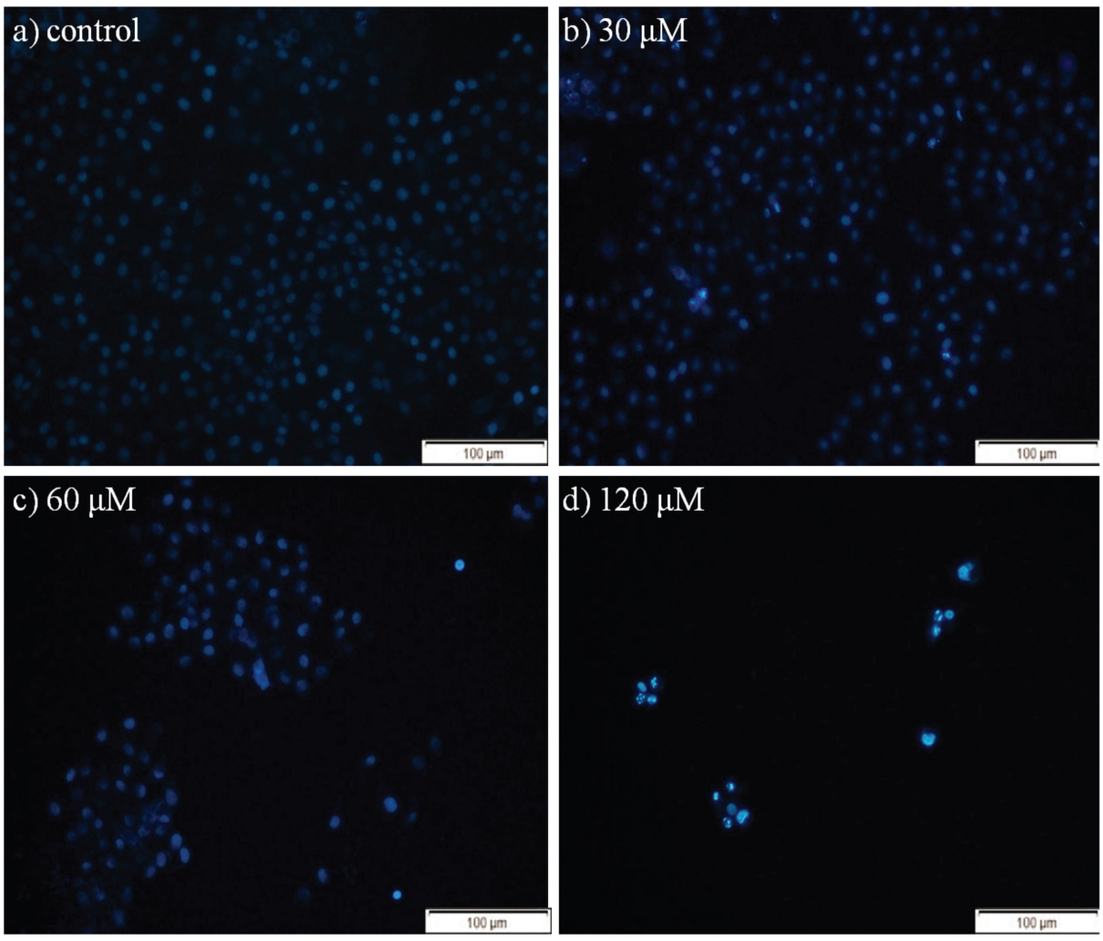


Fig. 3: The DAPI staining of CDCA in HepG2 cells (× 200): HepG2 cells were either untreated or treated with 30, 60, or 120 μM CDCA for 48 h and stained with DAPI.

excipient for hepatoma-targeted drugs. The current analysis was in agreement with the previous study that berberine-CDCA analog mediated apoptosis in hepatocarcinoma SMMC-7721 cells (Li et al. 2013). In the continuing research, CDCA was selected to analyze the mechanism of growth inhibition on HepG2 cell line at high concentration by fluorescence staining, apoptosis analysis and mitochondrial membrane potential detection.

2.2. Fluorescence staining

2.2.1. Giemsa Staining

To further confirm that cell apoptosis was induced by CDCA in HepG2 cells and the selective cytotoxicity between L02 and HepG2, a morphological examination was performed by Giemsa staining. As shown in Fig. 2, both L02 and HepG2 cells in control group demonstrated round cell bodies with clean and intact cell edges. Treated with CDCA, L02 cells retained regular morphology and no marked changes were detected with increasing concentration of CDCA (Fig. 2e-h), while HepG2 cells showed obvious apoptotic characteristics, such as condensation of nuclear, increased cell debris, loss of intercellular contacts and apoptotic bodies (Fig. 2a-d). Meanwhile, we could clearly observe that the morphological changes of HepG2 cells significantly deteriorated with increasing concentration of CDCA.

2.2.2. DAPI Staining

HepG2 cells were treated with CDCA at 0, 30, 60 and 120 μM for 48 h, then stained with DAPI staining. As shown in control cells (Fig. 3a), the nuclear staining was slightly blue and homogeneous, cells with smaller nuclei were rarely seen. After exposure to CDCA under the concentration gradient, the contours of some cells became irregular, the nuclei condensed (as brightly blue fluorescence indicated) and the apoptotic bodies appeared (Fig. 3b-d). Taken all together, the results indicated that CDCA could induce apoptosis in HepG2 cells.

2.2.3. AO/EB Staining

Acridine orange (AO) and ethidium bromide (EB) are fluorescent intercalating DNA dyes. AO can stain nuclear DNA across an intact cell membrane, while EB is only taken by cells that had lost their membrane integrity. Therefore, after stained with AO and EB, live cells will be stained green and regular-sized, early apoptotic cells will be stained orange with condensed or fragmented chromatin, while late apoptotic and necrotic cells will be stained as condensed and fragmented red chromatin.
HepG2 cells were treated with CDCA (0, 30, 60 and 120 μM) for 48 h, then stained with AO/EB. As shown in Fig. 4a, the control cells excluded EB and stained by AO showing green, after exposure to CDCA under the concentration gradient, the changes on the cell morphology could be obviously observed.

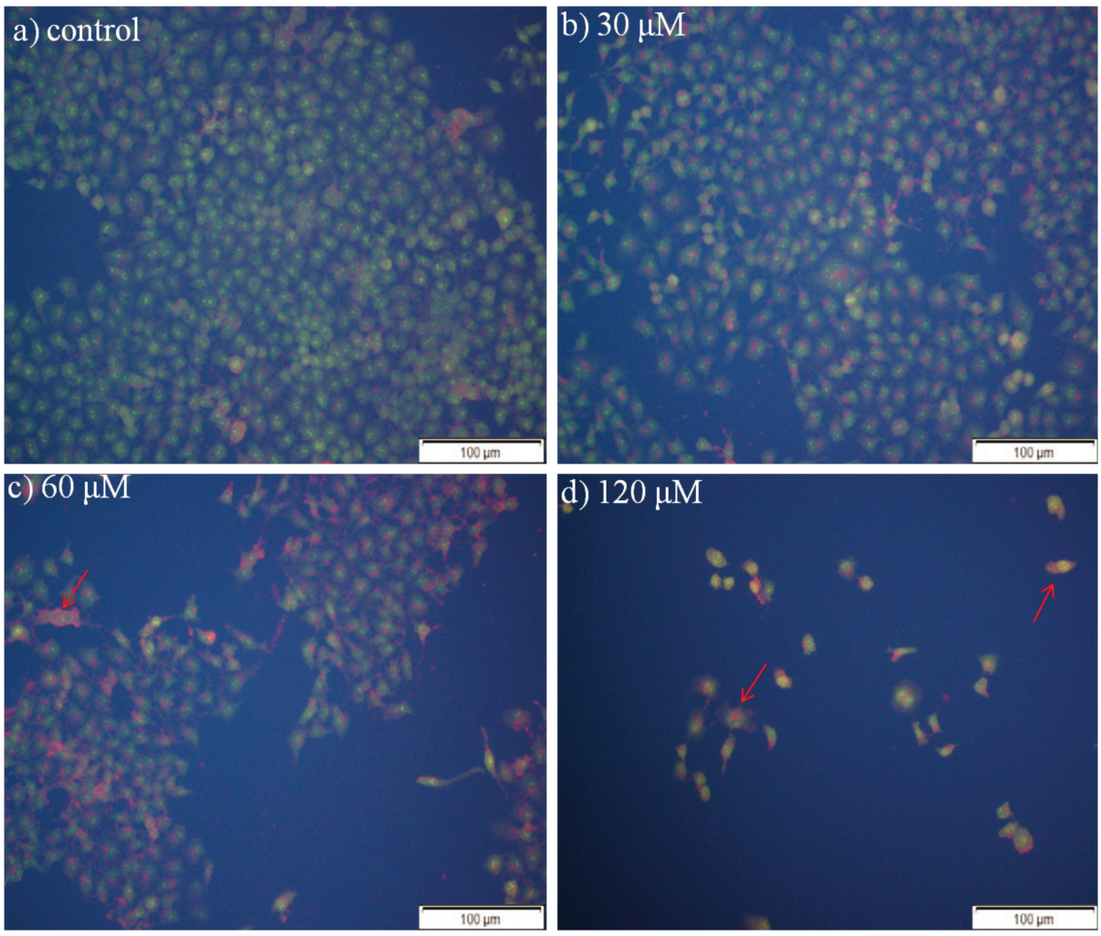


Fig. 4: The AO/EB staining of CDCA in HepG2 cells (× 200): HepG2 cells were either untreated or treated with 30, 60, or 120 μM CDCA for 48 h and stained with AO/EB, the red arrow indicates apoptotic cell.

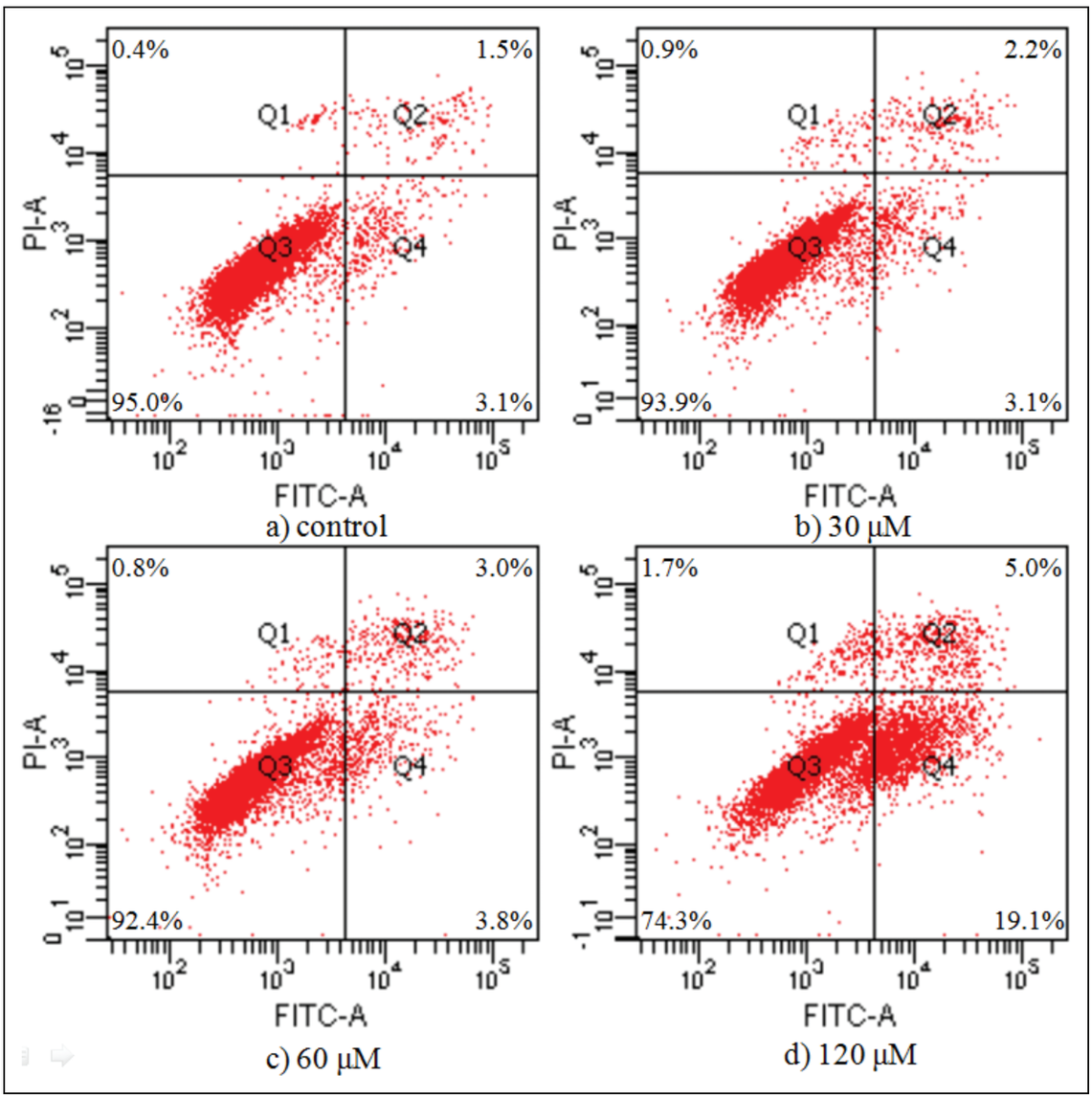


Fig. 5: Effect of CDCA on apoptotic rate in HepG2 cells: HepG2 cells were either untreated or treated with 30, 60, or 120 μM CDCA for 48 h and measured by flow cytometric using annexin V-FITC/PI staining. Q1, necrotic cells; Q2, late apoptotic cells; Q3, viable cells; Q4, early apoptotic cells.

The nuclei clearly stained as yellow green, orange or red, displayed pycnosis, nuclear fragmentation and cell budding (Fig. 4b-d). It suggested the significant cell apoptosis induced by CDCA on HepG2 cells in a drug dose-dependent manner, which was consistent with the previous results for Giemsa and DAPI staining.

2.3. Apoptosis analysis

In an attempt to explicate whether the loss in cell viability induced by CDCA was associated with apoptosis, the interactions of HepG2 cells with CDCA were further performed by annexin V-FITC/PI double staining. The apoptosis ratios induced by CDCA in HepG2 cells were quantitatively determined using flow cytometry. HepG2 cells not treated with CDCA were used as control. Apoptosis results were obtained after 48 h of treatment at three concentrations of CDCA. As shown in Fig. 5, apoptosis ratios (including the early and late apoptosis ratios) were found to increasing from 5.3% (30 μM) to 6.8% (60 μM) and 24.1% (120 μM), respectively, while that of control was 4.6%. The results indicated that CDCA could induce apoptosis in HepG2 cells.

2.4. Mitochondrial membrane potential assay

The depletion of $\Delta\psi m$ is a critical step that occurs in all cell types undergoing apoptosis. To determine whether an early loss of $\Delta\psi m$ occurred during treatment with CDCA in HepG2 cells, we performed $\Delta\psi m$ measurement using JC-1 staining. In non-apoptotic cells, JC-1 accumulates as aggregates in the mitochondria which emit red fluorescence. Whereas in apoptotic cells, JC-1 exists in a monomeric form and emits green fluorescence. As shown in Fig. 6, the green fluorescent ratio increasing from 6.3% in non-CDCA treated cells to 7.6%, 8.1% and 22.5%, respectively by 30, 60 and 120 μM concentration of CDCA treatment. This indicated that CDCA was able to induce mitochondrial membrane potential disruption in HepG2 cells.

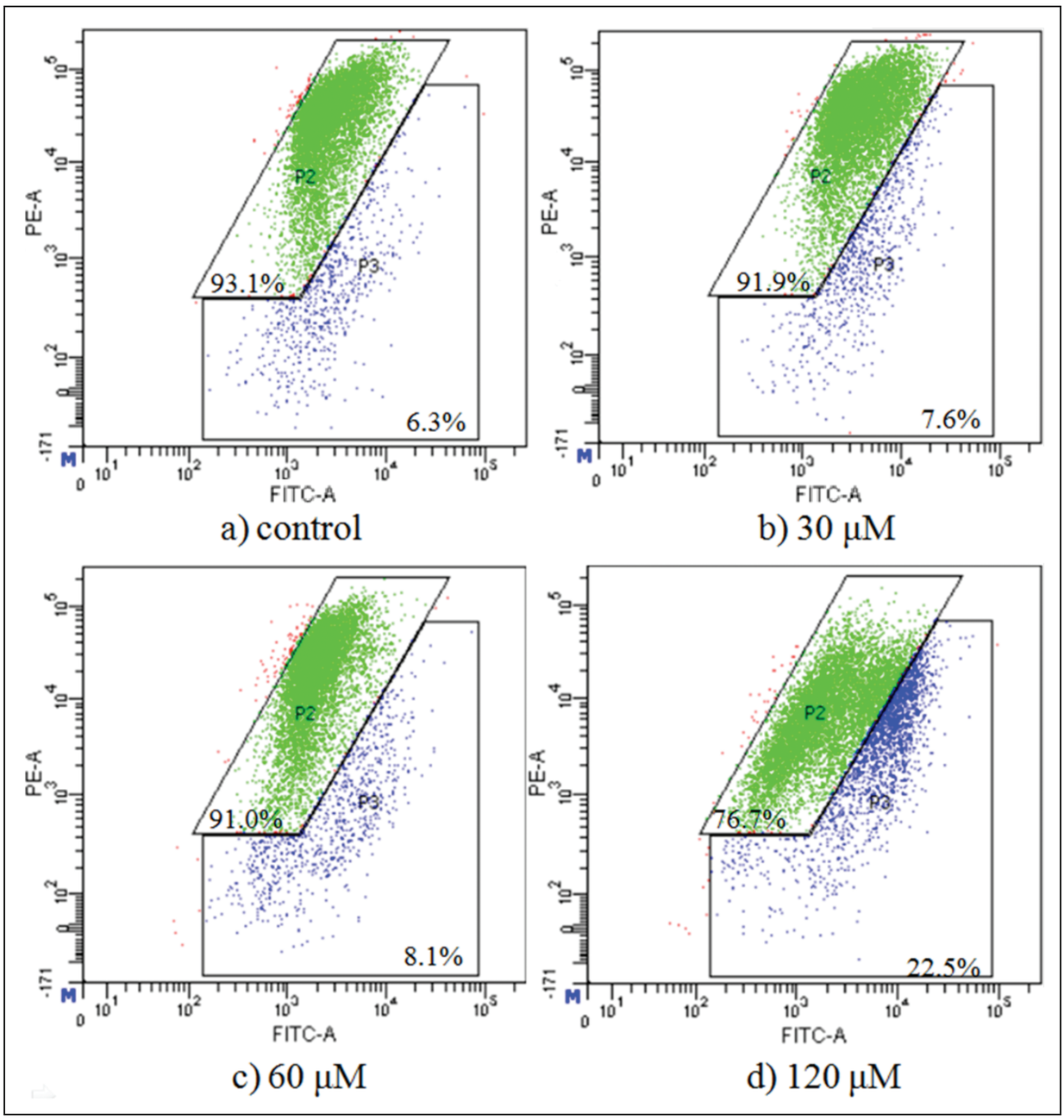


Fig. 6: Effect of CDCA on mitochondrial transmembrane potential ($\Delta\psi m$) in HepG2 cells: HepG2 cells were either untreated or treated with 30, 60, or 120 μM CDCA for 48h and measured by flow cytometry with JC-1.

2.5. Conclusions

Bile acids are important products of cholesterol catabolism which have been extensively studiedIn our study, we evaluated the selective cytotoxicity, dose-response effect and structure-activity relationships of six BAs against the L02, HepG2, BGC823 and Hela cell lines. Our research suggested that most of BAs displayed high selectivity related to different cell lines and drug concentrations, high concentration of BAs cell resulted in hepatoma cell apoptosis. Among 6 BAs, CDCA was identified to be the most significant candidate targeting the HepG2 cells. In addition, the apoptosis-inducing activity of CDCA at high concentration in HepG2 cell line was investigated by following assays: Giemsa staining, DAPI staining, AO/EB staining, apoptosis analysis and mitochondrial membrane potential assay. The preliminary mechanistic studies indicated that the CDCA might inhibit HepG2 cell proliferation by apoptosis. Based on the above evidence, we reasoned that CDCA may be a carrier and parent pharmaceutical excipient for liver tumor-targeted drugs, which may also synergy with endogenous BAs to reach a higher concentration and inducing apoptosis in hepatoma cell. According to the structure-activity relationships, the position, configuration and number of OH group in BAs could affect cell proliferation and selectivity. These findings provided a powerful incentive for further research on the pharmaceutical excipient and chemical modification of bile acids.

3. Experimental

3.1. Materials

All commercially available solvents and reagents used were of analytical grade and without further purification. All compounds were purchased from Aladdin (Shanghai) Industrial Corporation. The cell lines were provided by the Chinese Academy of Medical Sciences & Peking Union Medical College. The DAPI and AO/EB staining dyes, Annexin V-FITC Apoptosis Kit were purchased from Solarbio science & technology CO., LTD. The JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Beyotime Institute of Biotechnology, China.

3.2. Cytotoxicity evaluation

According to our previous studies (Wang et al. 2012; Wang et al. 2013; Xu et al. 2014), cytotoxicity of BAs was evaluated in three human cancer cell lines (HepG2, HT-29, BGC823, Hela) and the human liver cell line L02. The growing cells were plated at a density of 2×10^4 cells/mL and incubated in a 96-well plate for 24 h (37 °C, 5% CO_2). Then the cells were exposed to various concentrations of the tested compounds (12.5, 25, 50, 100 and 200 μM) for 48 h. After the incubation, MTT solution (20 μL, 5 mg/mL) was added to each well, and the plate was incubated for a further 4 h before removing the media. Formazan crystals were dissolved with DMSO (150 μL). After mixing well, the absorbance was read at 490 nm with a BIORAD 550 spectrophotometer. Wells containing no drugs were used as negative controls. Tumor cell growth inhibitory rate was calculated in the following Eq. (1):

$$\text{inhibition}\,\% = (1 - \text{Sample group OD}/\text{Control OD}) \times 100\% \quad (1)$$

3.3. Fluorescence staining

3.3.1. Giemsa/DAPI staining

Giemsa/DAPI staining was performed according to our previous study (Chu et al. 2014) with minor modifications. In brief, HepG2 cells were seeded at 5×10^3 cells/well in a volume of 800 μL on a 12-well plate. After incubation, CDCA of various concentrations was added to the cultures, and the plate was incubated for further 48 h. Then the culture medium containing compounds was removed. PBS wished twice, cells were fixed in ethanol for 10 min and stained with Giemsa/DAPI. After washing with PBS for twice, cells were photographed under fluorescence microscope.

3.3.2. AO/EB staining

The pre-processing method was mentioned above. The cultured cells were stained with 20 μL of AO/EB stain (100 μg/mL) for 10 min and then the fluorescence was read using fluorescence microscope.

3.4. Apoptosis analysis

The HepG2 cells (3×10^3/mL) were incubated with or without CDCA (120 μL) for 48 h, washed twice with cold PBS and re-suspended gently in 200 μL binding buffer. Then the cells were stained in 10 μL Annexin V-FITC and shaked well. After incubated for 20 min in a dark place, 200 μL binding buffer and 5 μL PI was added to these cells, and then analyzed by flow cytometry analysis.

3.5. Mitochondrial membrane potential assay

Prepared HepG2 cells were washed with PBS and incubated in RPMI-1640 medium containing JC-1 dye for 20 min at 37 °C in the dark. The mitochondrial depolarization patterns of the cells were observed by flow cytometry analysis according to the manufacturer's instructions (Beyotime, China).

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Declaration of interest statement: The authors declare no conflict of interest.

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