Bioorganic & Medicinal Chemistry 16 (2008) 8737–8744

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

New lithocholic and chenodeoxycholic piperazinylcarboxamides with antiproliferative and pro-apoptotic effects on human cancer cell lines

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ARTICLE INFO

Article history:
Received 3 April 2008
Revised 11 July 2008
Accepted 18 July 2008
Available online 23 July 2008

Keywords:
Steroid-heterocycle
Bile acid derivatives
Apoptosis
Anticancer
Multiple myeloma

ABSTRACT

Six new synthetic bile acid derivatives were synthesized and tested in vitro against various human cancer cells (glioblastoma multiforme (GBM), multiple myeloma (KMS-11), and colonic carcinoma (HCT-116) cell lines. The best activity was obtained with compound **IIIb** on multiple myeloma cells (LD_{50}: 8.5 ± 0.5 μM). This activity was associated with Mcl-1 and PARP-1 cleavage, inhibition of NFκB signaling, and DNA fragmentation, demonstrating an apoptotic cell death signaling pathway.

1. Introduction

Bile acids, the products of cholesterol catabolism, are synthesized in the liver and excreted into the bile canaliculus and the digestive tract. The primary bile acids (cholic acid, CA (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid, CDCA (3α,7α-dihydroxy-5β-cholan-24-oic acid) are conjugated with glycine or taurine in hepatocytes to form the corresponding *N*-acyl conjugates. As conjugates, they are secreted from the hepatocyte into the bile that is evacuated in the intestine. These conjugates are metabolized by enteric bacteria in the distal small intestine and in the colon to produce secondary bile acids by deconjugation followed by 7-dehydroxylation: deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid, DCA) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid, LCA) are obtained from cholic and chenodeoxycholic acids, respectively.[1–4] In addition, the bile contains 3β-, 7β- and 12β-epimers which mainly would come from the bacterial action in the distal ileum or colon. The urso family with 7β-epimer includes ursocholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, UCA) and ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid, UDCA).[5]

In a previous study, it has been reported that several unconjugated bile acids may play some role in the development of intestinal tumors.[6] Meanwhile, these acids possess pharmacological activities such as a potent and selective farnesoid X receptor (FXR) agonist,[7,8] and they also showed anticancer activities.[9,10] It is worth mentioning that the UDCA is used for the prevention of gastrointestinal disorders in patients having various cancers (stomach, colon, lung, breast, and liver).[11] It is also evaluated in clinical phase III trial therapy to prevent colorectal adenoma recurrence.[12] Another way for cancer treatment consists of coupling bile acids with anticancer molecules; cisplatin[13] or heparin[14] through covalent bonds. On the other hand, a series of bile acid–polyamine amides conjugated with 3′-azido-3′-deoxythymidine (AZT) were synthesized and their cytotoxic activities were screened against human cancer cells in vitro.[15]

Furthermore, the conjugation of bile acids with glycine and taurine is one of the mechanisms by which an organism could decrease its cytotoxicity at physiological concentrations.[16–18] For this reason, several synthetic bile acid derivatives have been developed. Among these synthesized ones, a conjugate of UDCA or CDCA with L-phenylalanine benzyl ester led HS-1183 or HS-1199, respectively. On the other hand, the conjugate of CDCA with β-alanine benzyl ester gave HS-1200, which were intensively studied.[19–21] These derivatives exerted antitumor effects against human cancer cells such as prostate cancer cells,[22] cervical carcinoma cells,[23]

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doi:10.1016/j.bmc.2008.07.046

colon cancer cells,[24] hepatocellular carcinoma cells,[20] stomach cancer cells,[25] leukemic T cells,[26] breast carcinoma cells,[27] and glioblastoma multiforme cells.[28] However, glycine or taurine conjugated bile acids are cleaved by intestinal and hepatic enzymes.[29] For this reason, another strategy was reported by introduction of a heterocycle amino acid (proline) in the bile acids side chain. The resulting compounds were found to be more resistant to bacterial deamidation than the corresponding natural conjugated molecules, but no anticancer activity was described.[30]

We wish to describe herein the synthesis of some new bile acids containing nitrogen heterocycles side chain. In vitro studies were then accomplished on glioblastoma multiforme (GBM, brain tumor cell line), multiple myeloma (KMS-11) and colonic carcinoma (HCT-116) cell lines.

Three piperazine derivatives, substituted in the 1-position by pyridine, pyrimidine or cinnamyl groups, were introduced in the bile acid side chain in place of the proline moiety. Therefore, a series of novel bile acid-substituted piperazine conjugates, derived from chenodeoxycholic and lithocholic acids, have been prepared (Scheme 1).

2. Results and discussion

2.1. Chemistry

Lithocholic and chenodeoxycholic acid derivatives were prepared as depicted in Scheme 2. Hydroxyl groups of cholic acids (3α-hydroxy-5β-cholan-24-oic acid (**1a**) and 3α,7α-dihydroxy-5β-cholan-24-oic acid (**1b**)) were acetylated with acetic anhydride in pyridine to give the acetates **2a** and **2b**, respectively. The latter acids **2a** and **2b** were converted into the corresponding mixed anhydride by treatment with triethylamine followed by ethyl chloroformate giving **3a** and **3b**, respectively. When the mixed anhydrides **3a** and **3b** were reacted with substituted piperazines (**a**, **b**, or **c**), the products were identified as the carboxamides (**4a**,**b**–**6a**,**b**). The latter amides were saponified by ethanolic potassium hydroxide to give the corresponding deacetylated amides **Ia**,**b**–**IIIa**,**b**. However, during purification of the resulting amides on silica gel, they were strongly adsorbed on the carrier material, thus they could be successfully purified on alumina deactivated with 6% water. The structures of the compounds prepared were confirmed by analytical and spectral analyses.

The hydrolytic stability of bile acids prepared was studied under physiological conditions (phosphate buffer, pH 7.4, at 37 °C). No degradation of these carboxamides was observed after 24 and 48 h.

2.2. Biology

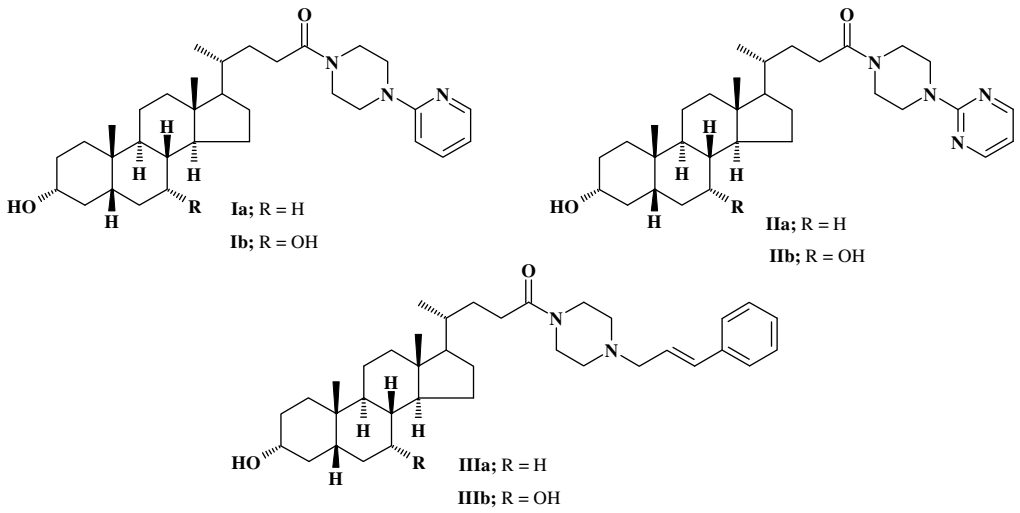
The anticancer activity of compounds (**Ia,b**–**IIIa,b**) was evaluated against three human cancer cells: glioblastoma multiforme (GBM),[31] colon (HCT-116),[32] and multiple myeloma (KMS-11)[33] using the neutral red uptake assay as described in Section 3. Multiple myeloma is a plasma-cell neoplasm characterized by the clonal expansion of malignant plasma cells within the hematopoietic bone marrow, glioblastoma multiforme is the most frequent tumor in the central nervous system; the prognosis of these cancers is extremely unfavorable. Both tumors are characterized by a potent evasion of apoptosis. Inhibition of this programmed cell death has been shown to be a major obstacle to chemo-resistance in many cancer cells.[34] Therefore, it would be important to identify new active agents that trigger apoptosis in these cells. HCT-116 cell line was chosen as bile acids may play an important role in intestinal tumorigenesis; the discovery and development of new products for both prevention and therapy remain an important field of research.

The bile acids (**Ia**,**b**–**IIIa**,**b**), **LCA**, **CDCA**, and amine building blocks (**a**, **b**, and **c**) were first tested at 50 μM. The percentage of viability cell was determined after 24 and 48 h of incubation. A representative experiment out of three is given in Figure 1(1 and 2). No cytotoxicity was observed for **LCA**, **CDCA**, amines (**a**, **b**, and **c**), **Ib** and **IIIa**. Compound **IIIb** induced a complete death as soon as 24 h, while compounds **Ia** and **IIb** showed various cytotoxicities at this concentration. No significant difference of activity was observed between 24 and 48 h of treatment as determined by the student test.

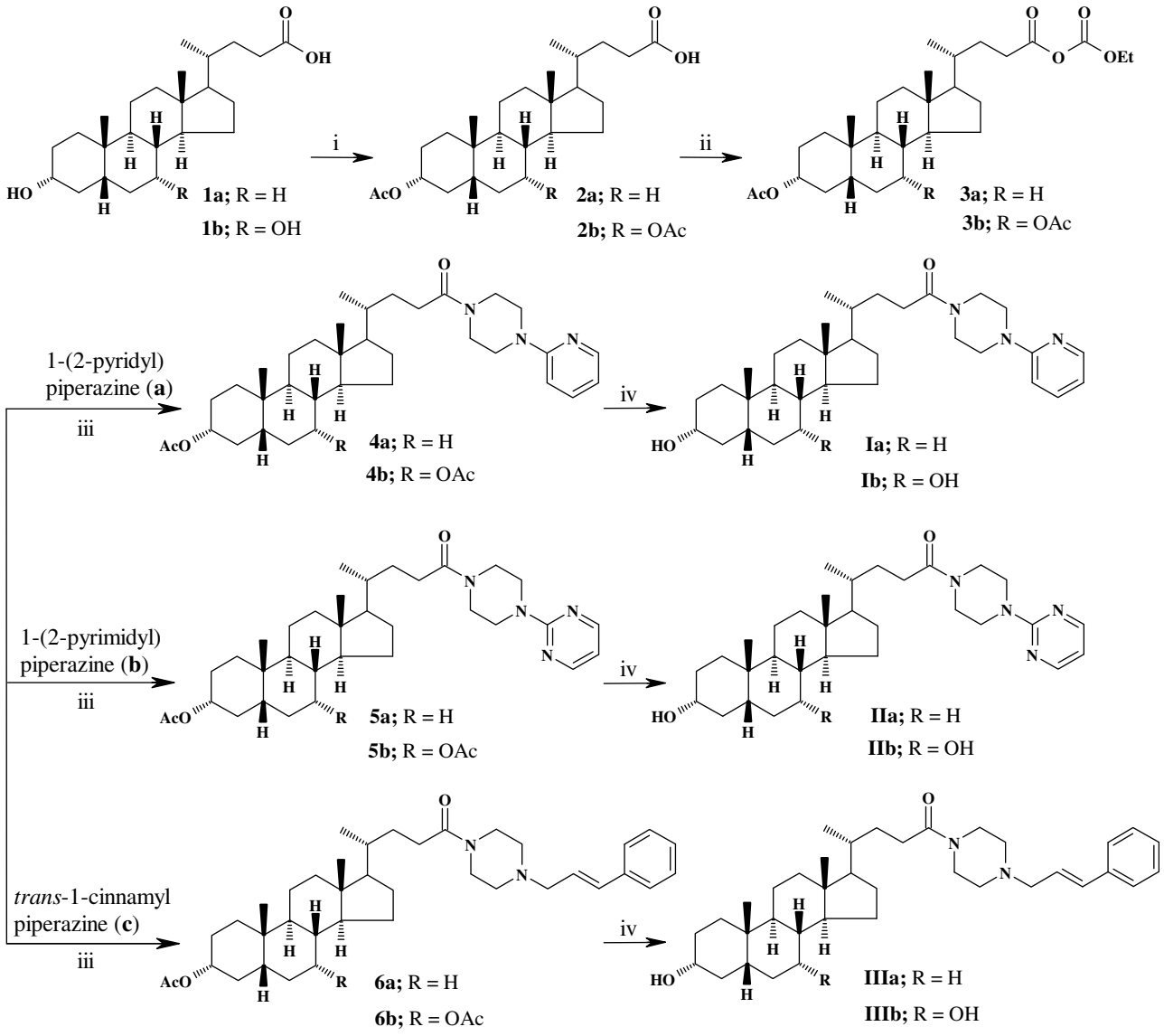
In order to calculate the LD_{50} after a 24 h treatment, dose–response experiments were carried out. An example is given in Figure 2A showing the dose–response curve of **IIIb** effect toward the three cell lines. The bile acid derivative **IIIb** caused a concentration dependent increase in cytotoxicity toward all the cells with KMS-11 cell line being the more sensitive. Morphological modifications induced by **IIIb** (12 μM–24 h) are illustrated in Figure 2B.

The mean LD_{50} (lethal dose 50% ± standard error, calculated from the results of at least three independent dose responsiveness experiments, after 24 h treatment) for all products is summarized in Table 1.

Taken together, these results showed that **LCA**, **CDCA**, **Ib**, and **IIIa** had no cytotoxic activity. Compound **IIIb** was the most active



Scheme 1. Amide derivatives of lithocholic and chenodeoxycholic acids.



Scheme 2. Synthesis of lithocholic and chenodeoxycholic acid derivatives. Reagents and conditions: (i) Ac_2O, pyridine, rt, 15 h; (ii) EtOCOCl, NEt_3, $CHCl_3$, rt, 50 min; (iii) piperazine (a, b or c), NEt_3, $CHCl_3$, rt, 16 h; (iv) KOH, EtOH, reflux, 24 h.

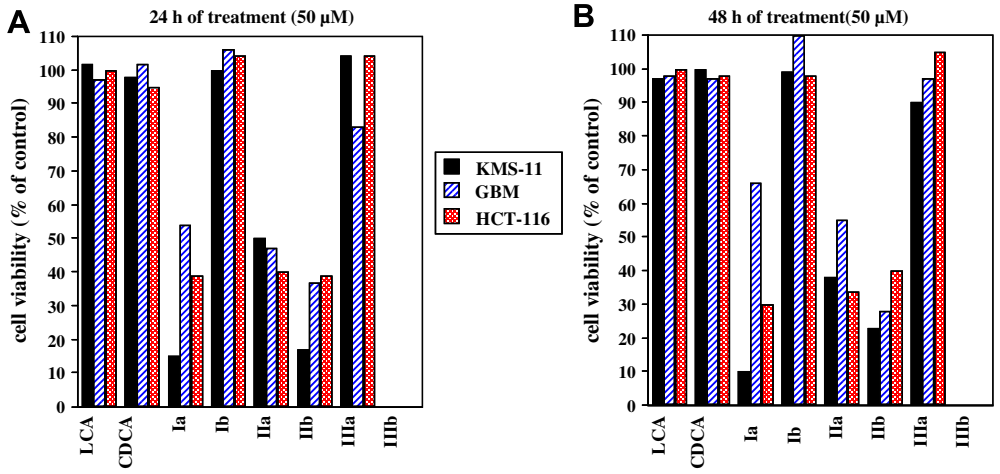


Figure 1. Cell viability after 24 h (A) and 48 h (B) exposure with bile acid derivatives (50 μM) evaluated using the neutral red assay. Results are expressed as the percentage of untreated cells (one representative experiment).

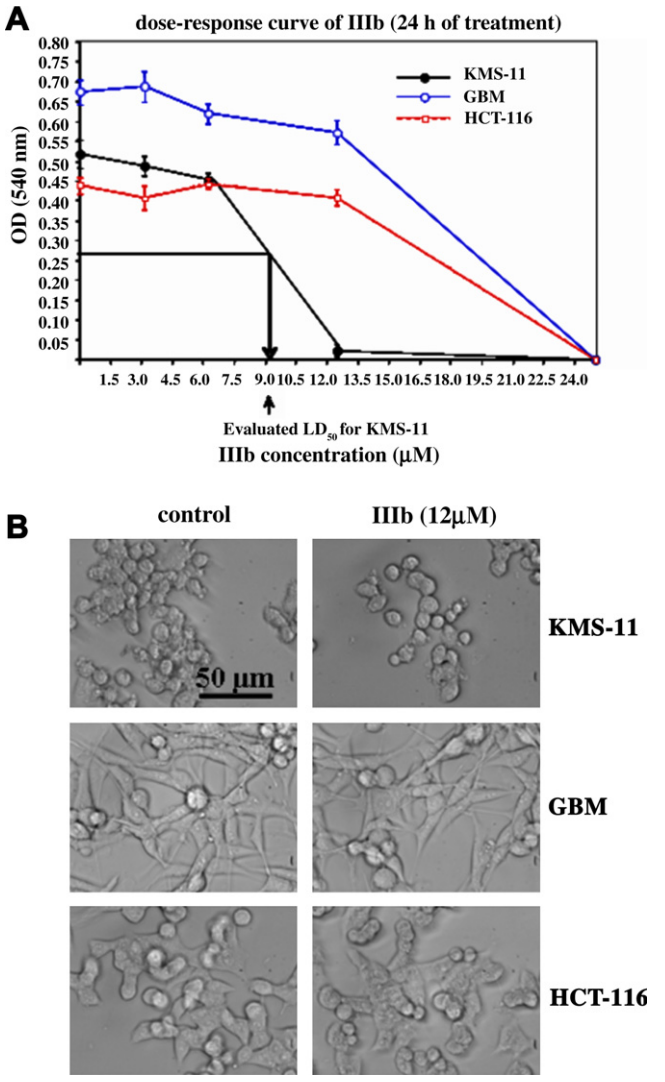


Figure 2. Dose–response effect of **IIIb** on KMS-11, GBM, and HCT-116 cell lines. (A) Cell survival was determined using the neutral red uptake assay (one representative experiment). Each concentration was done in triplicate, results are expressed in the mean OD, error bars represent the standard deviation. (B) Morphological aspect of three cell lines treated with **IIIb** at 12 μM for 24 h.

Table 1
Conjugated bile acids cytotoxicity towards three human cancerous cell lines

Compound	KMS-11 LD_{50} (μM)	GBM LD_{50} (μM)	HCT-116 LD_{50} (μM)
Ia	9 ± 3	50 ± 10	40 ± 10
Ib	>50	>50	>50
IIa	50 ± 15	50 ± 15	40 ± 10
IIb	17 ± 8	30 ± 10	35 ± 10
IIIa	>50	>50	>50
IIIb	8.5 ± 0.5	18.5 ± 1	16.5 ± 2
LCA	⩾50	>50	>50
CDCA	⩾50	>50	>50

LD_{50} values were determined from the dose–response curves after 24 h of incubation using the neutral red uptake assay. Results are expressed as the mean of three experiments ± standard error.

toward the three cell lines, complete toxicity being obtained at 25 μM (data not shown); therefore, we focused our study on this compound. The effect of **IIIb** on KMS-11 cell proliferation was evaluated by using the [^{3}H]thymidine incorporation assay. In contrast to **LCA** and **CDCA,** an exposure with **IIIb** at 20 μM for 24 h completely inhibits cell proliferation (Fig. 3 illustrates one representative experiment; results are given as the percentage of untreated cells).

Next, we analyzed the mechanisms of KMS-11 cell death with emphasis to apoptotic processes. For this purpose, we investigated whether 7A6 antigen (detected with the specific antibody APO2.7, specifically expressed on the mitochondrial membrane during the early stages of apoptosis)[35] could be detected after cells exposure to **IIIb**: a 24 h treatment leads to a significant dose dependent increase in APO2.7-positive cells ($p < 0.001$) (Fig. 4), therefore suggesting an apoptotic signal.

To confirm this KMS-11 cells apoptotic death, some intracellular signaling pathways were analyzed by immunoblotting. Western blots revealed the cleavage of Mcl-1 (Myeloid Cell Leukemia-1) and PARP-1 (Poly(ADP-Ribose)Polymerase-1) as well as the hyperphosphorylation of I*k*B*a* (Fig. 5A), an inhibitor of NFkB (nuclear factor-κB). Activated NFκB is involved in the transcriptional activation of some critical apoptotic genes, while inactivated NFκB is maintained in the cytoplasm compartment because of its binding to non-phosphorylated IκB.[36] Modifications of these key signaling proteins have clearly been associated with induction of apoptosis in multiple myeloma cell lines.[37–39] Moreover, nuclear staining clearly proved DNA fragmentation, which is the final stage of apoptosis (Fig. 5B).

These preliminary results showed that **IIIb** induced cell death in three cancer cell lines. Further experiments are needed to better understand the balance between cell cycle inhibition and apoptotic process in **IIIb** cytotoxic activity. Difference of sensitivity between cell lines could result from the implication of various survival factors and signaling pathways.

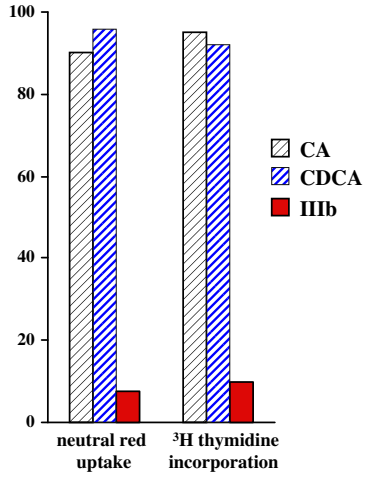


Figure 3. Effects of **IIIb**, **LCA**, and **CDCA** (20 μM for 24 h) on cell viability (evaluated by neutral red uptake) and cell proliferation (evaluated by tritiated thymidine uptake) on KMS-11 multiple myeloma cell line. Results are expressed as the percentage of untreated cells (one representative experiment).

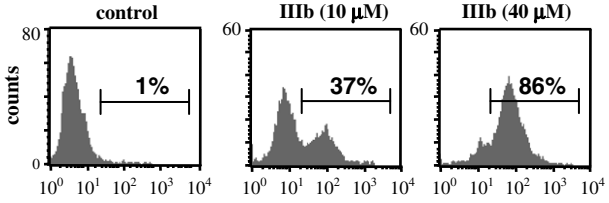


Figure 4. Apoptotic index with Apo2.7 staining analyzed by flow cytometry of KMS-11 cells after a 24 h treatment with **IIIb** at the indicated concentrations. Results are presented as the percentage of APO2.7-positive cells.

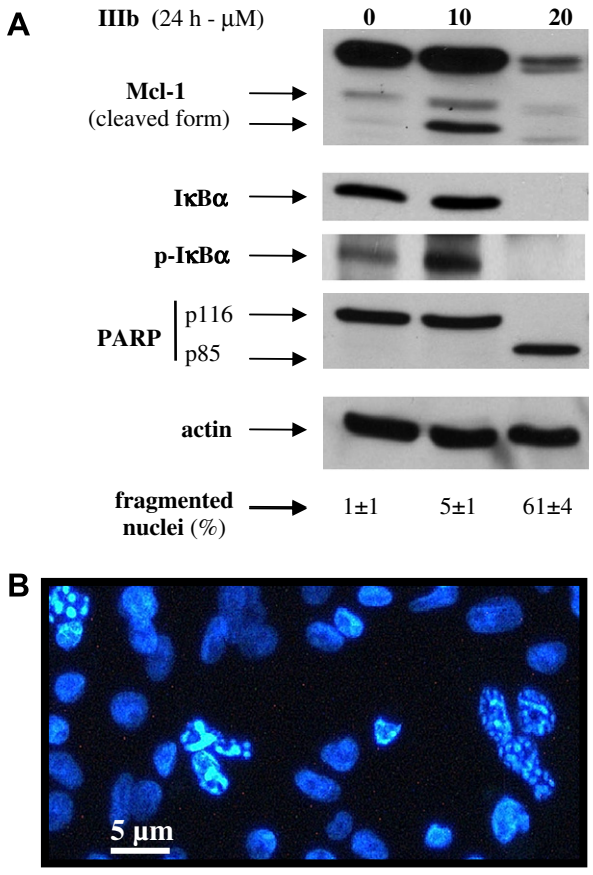


Figure 5. Pro-apoptotic effects of **IIIb** (24 h of treatment) on KMS-11 cells. (A) Western blotting showing the cleavage of Mcl-1 and PARP-1 as well as the hyperphosphorylation of IκB. (B) DNA fragmentation is illustrated after nuclear staining with Hoëchst.

In conclusion, we have developed a new series of cholic acids combined with nitrogen heterocycles, and demonstrated that some of these synthetic bile acid derivatives possess cytotoxic functions against glioblastoma multiforme (GBM), colorectal adenocarcinoma (HCT-116), and multiple myeloma (KMS-11) cell lines, whereas **LCA** and **CDCA** have no effect. Concerning the structure–activity relationship, the best activity was obtained with compound **IIIb** having cinnamylpiperazinyl group in the side chain and hydroxyl group at C-7 of steroid skeleton (compound **IIIb** in the range of 8–20 μM). These results prompt us in a future study to incorporate other amino substituted cinnamylpiperazinyl groups in the side chain of chenodeoxycholic acid.

3. Experimental

3.1. Chemistry

3.1.1. General remarks

All solvents were distilled and dried prior to use. Reagents and materials were obtained from commercial suppliers and were used without further purification. All the reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The spots were detected using a UV lamp (254 nm) and by spraying sulfuric acid/ethanol (2:8) on TLC and heating. Column chromatography was carried out using deactived neutral alumina (6% water) and silica gel 60 (0.063–0.2 mm) (Merck). Melting points were determined on a Kofler block. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. EI mass spectra were recorded on a Jeol-GCmate (GC–MS system) spectrometer with ionization energy from 30 to 40 eV. ESI mass spectra were recorded on a LC/MS Waters alliance. ^{1}H NMR and ^{13}C NMR spectra were recorded using $CDCl_3$ and DMSO-d_6, respectively, at 400 MHz (Jeol Lambda 400 spectrometer) and at 100 MHz. Chemical shifts are reported relative to TMS; *J* values are given in Hz. ^{13}C NMR spectra are ^{1}H-decoupled.

3.1.2. General procedure for 2a and 2b

Acetic anhydride (106.2 mmol, 10 mL) was added dropwise to a solution of **1a** or **1b** (13.3 mmol) in pyridine (40 mL). The reaction mixture was stirred at room temperature (rt) under argon atmosphere for 15 h and then ice water was added. The white precipitate formed was dissolved in CH_2Cl_2 (150 mL), washed with 1 M HCl (2× 20 mL), 5% $NaHCO_3$ (2× 25 mL), brine (2× 25 mL), and then with water (25 mL). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate, 8:2) to afford **2a** or **2b**.

3.1.2.1. 3α-Acetyloxy-5β-cholan-24-oic acid (2a). The crude product was recrystallized from acetone to give **2a** (4.73 g, 83%) as a white powder. Mp 167 °C (lit.[40] 168–169 °C). IR (KBr): *ν* (cm^{-1}): 3404 (br, O–H acid), 2950–2869 (C–H alkane), 1736 (C=O ester), 1709 (C=O carboxylic acid). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.92 (d, ^3J = 5.4 Hz, 3H, Me-21), 0.93 (s, 3H, Me-19), 2.03 (s, 3H, –OCOCH$_3$), 4.68–4.76 (m, 1H, H-3), 10.86 (br s, 1H, OH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 12.0 (C-18), 18.2 (C-21), 20.8, 21.4 (–OCOCH$_3$), 23.3 (C-19), 24.1, 26.3, 26.5, 26.9, 28.1, 30.8, 31.2, 32.2, 34.5, 35.0, 35.3, 35.7, 40.1, 40.3, 41.8, 42.7, 55.9, 56.4, 74.4 (C-3), 170.7 (–OCOCH$_3$), 180.2 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 418.5 (3) [M$^{+\cdot}$], 358.4 (100) [M$^{+\cdot}$–CH$_3$CO$_2$H], 343.4 (12), 304.3 (6), 257.3 (9), 230.3 (21), 215.3 (33).

3.1.2.2. 3α,7α-Bis(acetyloxy)-5β-cholan-24-oic acid (2b). The crude product was recrystallized from acetonitrile/diethyl ether to give **2b** (6.25 g, 95%) as a white powder. Mp 99 °C (dec). IR (KBr): (*ν* cm^{-1}): 3493 (br, O–H acid), 2939–2870 (C–H alkane), 1729 (C=O ester and acid). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.64 (s, 3H, Me-18), 0.89 (s, 3H, Me-19), 0.91 (d, ^3J = 8.0 Hz, 3H, Me-21), 1.98 (s, 6H, 2× –OCOCH$_3$), 3.81–3.84 (m, 1H, H-7), 4.50–4.59 (m, 1H, H-3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.7 (C-18), 18.2 (C-21), 20.5, 21.5 (2× –OCOCH$_3$), 22.7 (C-19), 23.6, 26.6, 28.1, 30.7, 31.0, 32.7, 34.3, 34.9, 35.0, 35.2, 35.3, 39.3, 39.5, 41.1, 42.7, 50.3, 55.7, 68.5 (C-7), 74.4 (C-3), 170.9 (2× –OCOCH$_3$), 179.9 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 476.3 (1) [M$^{+\cdot}$], 434.3 (3), 416.3 [M$^{+\cdot}$– CH$_3$CO$_2$H], 374.3 (7), 356.3 (100) [M$^{+\cdot}$–2× CH$_3$CO$_2$H], 341.2 (33), 302.2 (10), 255.2 (22), 228.2 (38), 213.2 (28).

3.1.3. General procedure for 4a, 4b, 5a, 5b, 6a, and 6b

Ethyl chloroformate (1.13 mL, 11.8 mmol) was added to a stirred solution of acid **2a** or **2b** (5.9 mmol) in chloroform (20 mL) under argon atmosphere at room temperature. Triethylamine (1.66 mL, 11.8 mmol) was added dropwise and the solution was kept under stirring for 50 min. The reaction mixture was diluted with methylene chloride (30 mL), washed with water (2× 15 mL), dried over anhydrous magnesium sulfate, and evaporated. The crude product of anhydride (**3a** or **3b**) was used in next step without purification.

Under nitrogen atmosphere, piperazine derivative (**a**, **b**, or **c**) (59.0 mmol) and triethylamine (0.83 mL, 5.9 mmol) were added to a solution of anhydride **3a** or **3b** (5.9 mmol) in chloroform (20 mL). The reaction mixture was stirred at room temperature for 16 h. The solution was diluted with methylene chloride (20 mL), washed with water (2× 15 mL), dried over anhydrous magnesium sulfate and evaporated. The crude product was purified by column chromatography on deactivated neutral alumina (6% water) eluting by cyclohexane/ethyl acetate, 7:3.

3.1.3.1. 3α-Acetoxy-17β-[1-methyl-3-(1*N*-(4*N*-(pyridin-2-yl))piperazin-1-yl)carboxy-propyl]etiocholane (4a). 1.41 g, 43%. Yellow oil. IR (KBr): (ν cm^{-1}): 2927–2860 (C–H alkane), 1733 (C=O ester), 1657 (C=O amide), 1241 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.95 (d, J = 6.4 Hz, 3H, Me-21), 2.02 (s, 3H, –OCOCH$_3$), 3.47–3.62 (m, 8H, 4 –CH$_2$– piperazinyl), 4.68–4.74 (m, 1H, H-3), 6.64–6.68 (m, 2H, H-3$_{py}$ and H-5$_{py}$), 7.49–7.53 (m, 1H, H-4$_{py}$), 8.20 (d, J = 4.7 Hz, 1H, H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 12.1 (C-18), 18.5 (C-21), 20.8, 21.5 (–OCO*C*H$_3$), 23.3 (C-19), 24.2, 26.3, 26.6, 27.0, 28.3, 30.4, 31.5, 32.2, 34.6, 35.0, 35.7, 35.8, 40.2, 40.4, 41.1, 41.9, 42.8, 45.2, 45.4 (2C), 56.1, 56.5, 74.4 (C-3), 107.3 (C-3$_{py}$), 113.9 (C-5$_{py}$), 137.7 (C-4$_{py}$), 147.9 (C-6$_{py}$), 158.9 (C-2$_{py}$), 170.7 (–O*C*OCH$_3$), 172.4 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 563.4 (56) [M$^{+\cdot}$], 548.4 (19) [M$^{+\cdot}$–CH$_3^{\cdot}$], 218.1 (80), 205.1 (55), 120.0 (100).

3.1.3.2. 3α,7α-Diacetoxy-17β-[1-methyl-3-(1*N*-(4*N*-(pyridin-2-yl))piperazin-1-yl) carboxypropyl]etiocholane (4b). 0.88 g, 24%. White powder. Mp 200 °C. IR (KBr): (ν cm^{-1}): 2973–2849 (C–H alkane), 1732 (C=O ester), 1619 (C=O amide), 1248 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.67 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.96 (d, J = 6.4 Hz, 3H, Me-21), 2.01 (s, 6H, 2× –OCOCH$_3$), 3.47–3.76 (m, 8H, 4 –CH$_2$– piperazinyl), 3.83–3.87 (m, 1H, H-7), 4.54–4.62 (m, 1H, H-3), 6.65–6.70 (m, 2H, H-3$_{py}$ and H-5$_{py}$), 7.47–7.53 (m, 1H, H-4$_{py}$), 8.20 (d, J = 4.9 Hz, 1H, H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.8 (C-18), 18.5 (C-21), 20.6, 21.5 (2× –OCO*C*H$_3$), 22.7 (C-19), 23.8, 26.8, 28.3, 30.3, 31.4, 32.8, 34.4, 35.1 (2C), 35.3, 35.6, 39.4, 39.6, 41.2 (2C), 42.7, 45.3, 45.4 (2C), 50.4, 55.8, 68.5 (C-7), 74.4 (C-3), 107.2 (C-3$_{py}$), 113.9 (C-5$_{py}$), 137.7 (C-4$_{py}$), 148.0 (C-6$_{py}$), 159.0 (C-2$_{py}$), 170.7 (2× –O*C*OCH$_3$), 172.2 (C-24) ppm. MS (ESI): [M+H]$^+$ 622.

3.1.3.3. 3α-Acetoxy-17β-[1-methyl-3-(1*N*-(4*N*-(pyrimidyl-2-yl))piperazin-1-yl)carboxypropyl]etiocholane (5a). 1.33 g, 40%. White powder. Mp 190 °C. IR (KBr): (ν cm^{-1}): 2928–2869 (C–H alkane), 1734 (C=O ester), 1652 (C=O amide), 1244 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.95 (d, J = 6.6 Hz, 3H, Me-21), 2.03 (s, 3H, –OCOCH$_3$), 3.52–3.87 (m, 8H, 4 –CH$_2$– piperazinyl), 4.68–4.74 (m, 1H, H-3), 6.54 (t, J = 4.7 Hz, 1H, H-5$_{py}$), 8.33 (d, J = 4.7 Hz, 2H, H-4$_{py}$ and H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 12.2 (C-18), 18.7 (C-21), 21.0, 21.7 (–OCO*C*H$_3$), 23.5 (C-19), 24.4, 26.5, 26.8, 27.2, 28.5, 30.7, 31.6, 32.4, 34.8, 35.2, 35.9, 36.0, 40.3, 40.6, 41.5, 42.1, 42.9, 43.8, 44.0, 45.6, 56.3, 56.7, 74.6 (C-3), 110.6 (C-5$_{py}$), 158.0 (C-4$_{py}$ and C-6$_{py}$), 161.7 (C-2$_{py}$), 170.9 (–O*C*OCH$_3$), 172.6 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 564.2 (9) [M$^{+\cdot}$], 549.2 (4) [M$^{+\cdot}$–CH$_3^{\cdot}$], 489.2 (2) [M$^{+\cdot}$–(CH$_3^{\cdot}$ + CH$_3$CO$_2$H)], 219.0 (30), 206.0 (100), 121.0 (74).

3.1.3.4. 3α,7α-Diacetoxy-17β-[1-methyl-3-(1*N*-(4*N*-(pyrimidyl-2-yl))piperazin-1-yl)carboxy-propyl]etiocholane (5b). 2.50 g, 68%. White powder. Mp 80 °C. IR (KBr): (ν cm^{-1}): 2937–2866 (C–H alkane), 1732 (C=O ester), 1644 (C=O amide), 1248 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.67 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.97 (d, J = 6.4 Hz, 3H, Me-21), 2.01 (s, 6H, 2× –OCOCH$_3$), 3.52–3.82 (m, 8H, 4 –CH$_2$– piperazinyl), 3.83–3.87 (m, 1H, H-7), 4.53–4.63 (m, 1H, H-3), 6.54 (t, J = 4.7 Hz, 1H, H-5$_{py}$), 8.33 (d, J = 4.7 Hz, 2H, H-4$_{py}$ and H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.8 (C-18), 18.5 (C-21), 20.6, 21.5 (2× –OCO*C*H$_3$), 22.7 (C-19), 23.8, 26.7, 28.3, 30.3, 31.4, 32.8, 34.4, 35.0, 35.1, 35.3, 35.6, 39.4, 39.6, 41.2, 41.3, 42.7, 43.6, 43.8, 45.5, 50.5, 55.9, 68.5 (C-7), 74.4 (C-3), 110.5 (C-5$_{py}$), 157.8 (C-4$_{py}$ and C-6$_{py}$), 161.8 (C-2$_{py}$), 170.6 (2× –O*C*OCH$_3$), 172.2 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 622.6 (6) [M$^{+\cdot}$], 580.5 (10), 563.4 (7), 269.0 (20), 206.1 (72), 121.0 (100).

3.1.3.5. 3α-Acetoxy-17β-[1-methyl-3-(1*N*-(4*N*-cinnamyl)piperazin-1-yl)carboxy-propyl]etiocholane (6a). 2.22 g, 61%. Yellow oil. IR (KBr): (ν cm^{-1}): 2938–2866 (C–H alkane), 1734 (C=O ester), 1646 (C=O amide), 1243 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.63 (s, 3H, Me-18), 0.91 (s, 3H, Me-19), 0.95 (d, 3H, J = 6.6 Hz, Me-21), 2.00 (s, 3H, –OCOCH$_3$), 2.40–2.62 (m, 4H, 2 –CH$_2$– piperazinyl), 2.97–3.06 (m, 2H, –CH$_2$– piperazinyl), 3.15 (d, J = 5.9 Hz, 2H, –C*H*$_2$–CH=CH–Ph), 3.44–3.66 (m, 2H, –CH$_2$– piperazinyl), 4.65–4.75 (m, 1H, H-3), 6.17–6.27 (m, 1H, –CH$_2$–C*H*=CH–Ph), 6.50 (d, J = 15.9 Hz, 1H, –CH$_2$–CH=C*H*–Ph), 7.19–7.36 (m, 5H, H$_{ar}$), 7.66 (br s, 1H, NH) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 12.0 (C-18), 18.5 (C-21), 20.8, 21.5 (–OCO*C*H$_3$), 23.3 (C-19), 24.2, 26.3, 26.6, 27.0, 28.2, 30.3, 31.4, 32.2, 34.5, 35.0, 35.6, 35.7, 40.1, 40.4, 41.8, 42.7, 44.3, 45.5, 51.8, 52.8, 56.0, 56.5, 61.0, 74.4 (C-3), 125.7, 126.3 (2C), 127.7, 128.6 (2C), 133.6, 136.6, 170.7 (–O*C*OCH$_3$), 172.2 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 602.4 (71) [M$^+$], 542.3 (5) [M$^{+\cdot}$–CH$_3$CO$_2$H], 511.3 (12), 451.3 (14), 257.1 (34), 201.1 (41), 172.1 (100).

3.1.3.6. 3α,7α-Diacetoxy-17β-[1-methyl-3-(1*N*-(4*N*-cinnamyl)-piperazin-1-yl)carboxy-propyl]etiocholane (6b). 2.07 g, 53%. Yellow oil. IR (KBr): (ν cm^{-1}): 2939–2868 (C–H alkane), 1732 (C=O ester), 1632 (C=O amide), 1247 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.66 (s, 3H, Me-18), 0.91 (s, 3H, Me-19), 0.95 (d, J = 6.6 Hz, 3H, Me-21), 2.00 (s, 6H, 2× –OCOCH$_3$), 2.46–2.50 (m, 4H, 2 –CH$_2$– piperazinyl), 3.17 (d, J = 6.6 Hz, 2H, –C*H*$_2$–CH=CH–Ph), 3.47–3.67 (m, 4H, 2 –CH$_2$– piperazinyl), 3.83–3.87 (m, 1H, H-7), 4.53–4.61 (m, 1H, H-3), 6.25 (dt, ^dJ = 15.8 Hz and ^tJ = 6.6 Hz, 1H, –CH$_2$–C*H*=CH–Ph), 6.53 (d, J = 15.8 Hz, 1H, –CH$_2$–CH=C*H*–Ph), 7.22–7.39 (m, 5H, H$_{ar}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.9 (C-18), 18.7 (C-21), 20.7, 21.7 (2× –OCO*C*H$_3$), 22.9 (C-19), 23.9, 26.9, 28.4, 30.3, 31.6, 32.9, 34.6, 35.1, 35.2, 35.4, 35.8, 39.5, 39.7, 41.4, 41.6, 42.8, 45.8, 50.6, 53.1, 53.4, 56.0, 61.1, 68.6 (C-7), 74.5 (C-3), 126.0, 126.5 (2C), 127.8, 128.8 (2C), 133.7, 136.8, 170.9 (2× –O*C*OCH$_3$), 172.3 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 660.3 (18) [M$^{+\cdot}$], 618.1 (96), 600.3 (17), 257.1 (41), 201.1 (39), 172.1 (100).

3.1.4. Typical procedure of compounds Ia, Ib, IIa, IIb, IIIa, and IIIb

Compound **4a** (1.06 g, 1.88 mmol) was added to a solution of potassium hydroxide (0.84 g, 15.04 mmol) in ethanol. The mixture was refluxed for 24 h. Aqueous 0.1 N HCl was added to neutral pH and the solution was evaporated. The residue was dissolved in chloroform, washed with 5% $NaHCO_3$, water and dried over anhydrous sodium sulfate. The crude product was purified by column chromatography on deactived neutral alumina (6% water) (eluent/cyclohexane/ethyl acetate, 7:3) to afford **Ia** (0.29 g, 30%) as a white powder.

3.1.4.1. 17β-[1-Methyl-3-(1*N*-(4*N*-(pyridin-2-yl))piperazin-1-yl)-carboxypropyl] etiocholan-3α-ol (Ia). 0.29 g, 30%. White powder. Mp 168 °C. IR (KBr): (ν cm^{-1}): 3414 (br, O–H alcohol), 2928–2861 (C–H alkane), 1638 (C=O amide), 1241 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.96 (d, J = 6.6 Hz, 3H, Me-21), 3.48–3.76 (m, 9H, 4 –CH$_2$– piperazinyl, and H-3), 6.66–6.69 (m, 2H, H-3$_{py}$, and H-5$_{py}$), 7.49–7.54 (m, 1H, H-4$_{py}$), 8.20 (d, J = 4.9 Hz, 1H, H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 12.2 (C-18), 18.6 (C-19), 20.9 (C-21), 23.5, 24.4, 26.5, 27.3, 28.4, 30.5, 30.7, 31.6, 34.7, 35.5, 35.8, 36.0, 36.6, 40.3, 40.5, 41.2, 42.2, 42.9, 45.3, 45.5 (2C), 56.1, 56.6, 71.9 (C-3), 107.4 (C-3$_{py}$), 114.0 (C-5$_{py}$), 137.8 (C-4$_{py}$), 148.1 (C-6$_{py}$), 159.2 (C-2$_{py}$), 172.5 (C-24) ppm. MS (ESI): [M+H]$^+$ 522.1, [M+H+MeCN]$^+$ 564.1. HRMS (EI) *m/z* [M$^+$] calcd. for $C_{33}H_{51}N_3O_2$: 521.3981, found: 521.4005.

3.1.4.2. 17β-[1-Methyl-3-(1*N*-(4*N*-(pyridin-2-yl))piperazin-1-yl)-carboxypropyl]etiocholan-3α,7α-diol (Ib). Yield: 86%. White powder. Mp 218 °C. IR (KBr): (ν cm^{-1}): 3402 (br, O–H alcohol), 2931–2845 (C–H alkane), 1606 (C=O amide), 1238 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.66 (s, 3H, Me-18), 0.90 (s, 3H, Me-19), 0.96 (d, J = 6.6 Hz, 3H, Me-21), 3.45–3.50 (m, 3H, 1 –CH_2– piperazinyl, and H-3), 3.56–3.63 (m, 4H, 2 –CH_2– piperazinyl), 3.72–3.75 (m, 2H, 1 –CH_2– piperazinyl), 3.82–3.86 (m, 1H, H-7), 6.64–6.68 (m, 2H, H-3$_{py}$ and H-5$_{py}$), 7.50 (ddd, ^3J = 8.4 Hz, ^3J = 7.7 Hz and ^4J = 2.2 Hz, 1H, H-4$_{py}$), 8.19 (d, J = 4.9 Hz, 1H, H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.9 (C-18), 18.7 (C-19), 20.7 (C-21), 22.9, 23.9, 28.4, 30.3, 30.8, 31.5, 33.0, 34.7, 35.2, 35.4, 35.7, 39.5, 39.8, 40.0, 41.3, 41.6, 42.8, 45.3, 45.5 (2C), 50.6, 55.9, 68.6 (C-7), 72.1 (C-3), 107.4 (C-3$_{py}$), 114.0 (C-5$_{py}$), 137.8 (C-4$_{py}$), 148.1 (C-6$_{py}$), 159.2 (C-2$_{py}$), 172.5 (C-24) ppm. MS (30 eV, EI): m/z (%) = 537.2 (3) [M$^{+\cdot}$], 519.3 (2) [M$^{+\cdot}$–H_2O], 501.3 (1) [M$^{+\cdot}$–2 H_2O], 218.1 (12), 206.1 (10), 120.1 (100), 107.0 (19). HRMS (EI) m/z [M$^+$] calcd. for $C_{33}H_{51}N_3O_3$: 537.3930, found: 537.3967.

3.1.4.3. 17β-[1-Methyl-3-(1*N*-(4*N*-(pyrimidyl-2-yl))piperazin-1-yl)carboxypropyl] etiocholan-3α-ol (IIa). Yield: 75%. White powder. Mp 144 °C. IR (KBr): (ν cm^{-1}): 3422 (br, O–H alcohol), 2934–2862 (C–H alkane), 1652 (C=O amide), 1244 (C–N amine). ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C): δ = 0.61 (s, 3H, Me-18), 0.87 (s, 3H, Me-19), 0.90 (d, J = 6.4 Hz, 3H, Me-21), 3.49–3.53 (m, 4H, 2 –CH_2– piperazinyl), 3.49–3.53 (m, 4H, 2 –CH_2– piperazinyl), 3.67–3.76 (m, 4H, 2 –CH_2– piperazinyl), 6.66 (t, J = 4.6 Hz, 1H, H-5$_{py}$), 8.38 (d, J = 4.6 Hz, 2H, H-4$_{py}$ and H-6$_{py}$) ppm (H-3 signal was probably included in H_2O signal). ^{13}C NMR (100 MHz, DMSO-d_6, 25 °C): δ = 12.1 (C-18), 18.6 (C-19), 20.6 (C-21), 23.5, 24.1, 26.4, 27.1, 28.0, 29.6, 30.6, 30.9, 31.1, 34.4, 35.2, 35.3, 35.6, 36.5, 40.9, 41.7, 42.5, 43.3, 43.7, 44.8, 55.7, 56.3, 70.1 (C-3), 110.6 (C-5$_{py}$), 158.2 (C-4$_{py}$ and C-6$_{py}$), 161.2 (C-2$_{py}$), 171.4 (C-24) ppm (one carbon signal was probably included in DMSO-d_6 signal). MS (30 eV, EI): m/z (%) = 522.3 (8) [M$^{+\cdot}$], 507.4 (4) [M$^{+\cdot}$–Me$^\cdot$], 219.1 (35), 206.1 (100), 164.1 (9), 121.1 (45). HRMS (EI) m/z [M$^+$] calcd. for $C_{32}H_{50}N_4O_2$: 522.3934; found: 522.3953.

3.1.4.4. 17β-[1-Methyl-3-(1*N*-(4*N*-(pyrimidyl-2-yl))piperazin-1-yl)carboxypropyl] etiocholan-3α,7α-diol (IIb). Yield: 79%. White powder. Mp 198 °C. IR (KBr): (ν cm^{-1}): 3400 (br, O–H alcohol), 2962–2845 (C–H alkane), 1615 (C=O amide), 1250 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.91 (s, 3H, Me-19), 0.95 (d, J = 6.6 Hz, 3H, Me-21), 3.53–3.56 (m, 2H, 1 –CH_2– piperazinyl), 3.57–3.67 (m, 1H, H-3), 3.68–3.71 (m, 2H, 1 –CH_2– piperazinyl), 3.81–3.88 (m, 5H, 2 –CH_2– piperazinyl, and H-7), 6.55 (t, J = 4.7 Hz, 1H, H-5$_{py}$), 8.34 (d, J = 4.7 Hz, 2H, H-4$_{py}$ and H-6$_{py}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.9 (C-18), 18.7 (C-19), 20.7 (C-21), 22.9, 23.9, 28.4, 30.4, 30.8, 31.5, 33.0, 34.7, 35.2, 35.5, 35.7, 39.6, 39.8, 40.0, 41.5, 41.6, 42.9, 43.7, 44.0, 45.6, 50.6, 55.9, 68.7 (C-7), 72.2 (C-3), 110.6 (C-5$_{py}$), 157.9 (C-4$_{py}$ and C-6$_{py}$), 161.7 (C-2$_{py}$), 172.6 (C-24) ppm. MS (30 eV, EI): m/z (%) = 538.3 (11) [M$^{+\cdot}$], 523.3 (5) [M$^{+\cdot}$–Me$^\cdot$], 487.4 (3) [M$^{+\cdot}$–(2 H_2O+Me$^\cdot$)], 335.1 (4), 267.1 (7), 219.2 (37), 206.1 (100), 164.1 (16), 121.1 (83). HRMS (EI) m/z [M$^+$] calcd. for $C_{32}H_{50}N_4O_3$: 538.3883; found: 538.3866.

3.1.4.5. 17β-[1-Methyl-3-(*1N*-(4*N*-cinnamyl)piperazin-1-yl)carboxypropyl]etiocholan-3α-ol (IIIa). Yield: 73%. Beige powder. Mp 159 °C (dec). IR (KBr): (ν cm^{-1}): 3401 (br, O–H alcohol), 2960–2863 (C–H alkane), 1638 (C=O amide). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.64 (s, 3H, Me-18), 0.91 (s, 6H, Me-19 and Me-21), 2.45–2.57 (m, 4H, 2 –CH_2– piperazinyl), 2.94–2.97 (m, 4H, 2 –CH_2– piperazinyl), 3.17 (d, J = 6.8 Hz, 2H, –CH_2–CH=CH–Ph), 3.57–3.68 (m, 1H, H-3), 6.27 (dt, ^dJ = 15.6 Hz and ^tJ = 6.8 Hz, 1H, –CH_2–C*H*=CH–Ph), 6.53 (d, J = 15.6 Hz, 1H, –CH_2–CH=C*H*–Ph), 7.17–7.39 (m, 5H, H$_{ar}$) ppm. MS (ESI): [M+H]$^+$ 561.5. MS (30 eV, EI): m/z (%) = 560.4 (15) [M$^{+\cdot}$], 469.3 (3), 358.2 (90), 343.2 (18), 230.2 (26), 215.1 (100), 202.1 (38). HRMS (EI) m/z [M$^+$] calcd. for $C_{37}H_{56}N_2O_2$: 560.4342; found: 560.4354.

3.1.4.6. 17β-[1-Methyl-3-(1*N*-(4*N*-cinnamyl)piperazin-1-yl)carboxypropyl]etiocholan-3α,7α-diol (IIIb). Yield: 60%. Yellow powder. Mp 126 °C. IR (KBr): (ν cm^{-1}): 3412 (br, O–H alcohol), 2929–2864 (C–H alkane), 1629 (C=O amide). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.65 (s, 3H, Me-18), 0.90 (s, 3H, Me-19), 0.94 (d, J = 6.6 Hz, 3H, Me-21), 2.45–2.50 (m, 4H, 2 –CH_2– piperazinyl), 3.17 (d, J = 6.9 Hz, 2H, –CH_2–CH=CH–Ph), 3.47–3.50 (m, 3H, 1 –CH_2– piperazinyl, and H-3), 3.62–3.67 (m, 2H, 1 –CH_2– piperazinyl), 3.83–3.86 (m, 1H, H-7), 6.25 (dt, ^dJ = 15.8 Hz and ^tJ = 6.9 Hz, 1H, –CH_2–C*H*=CH–Ph), 6.53 (d, J = 15.8 Hz, 1H, –CH_2–CH=C*H*-Ph), 7.23–7.39 (m, 5H, H$_{ar}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 11.9 (C-18), 18.6 (C-21), 20.7, 22.9 (C-19), 23.9, 28.3, 30.2, 30.8, 31.5, 33.0, 34.7, 35.2, 35.4, 35.7, 39.5, 39.7, 40.0, 41.6 (2C), 42.8, 45.7, 50.6, 53.0, 53.4, 55.9, 61.1, 68.7 (C-7), 72.1 (C-3), 126.0, 126.5 (2C), 127.7, 128.7 (2C), 133.7, 136.8, 172.1 (C-24) ppm. MS (ESI): [M+H]$^+$ 577.5. HRMS (EI) m/z [M$^+$] calcd. for $C_{37}H_{56}N_2O_3$: 576.4291; found: 576.4313.

3.1.5. Hydrolytic stability of the bile acid derivatives

Bile acid derivative (**Ia**, **Ib**, **IIa**, **IIb**, **IIIa**, or **IIIb**, 50 mg) was dissolved in ethanol (0.5 mL) and added to aqueous solution of phosphate buffer at pH 7.4 (0.1 M, 10 mL). The mixture solution was stirred at 37 °C for 24 and 48 h. The solution was extracted with methylene chloride, dried over anhydrous magnesium sulfate, and evaporated. The crude product was found unchanged as monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm).

3.2. Biology

3.2.1. Cell lines and culture

Three tumor cell lines were tested in this study: KMS-11 (an adherent plasma-cell line obtained from a patient with multiple myeloma),[31] GBM (astrocytoma cells obtained after tumor resection of patients with glioblastoma multiforme (GBM primary culture),[32] HCT-116 (colorectal adenocarcinoma cells derived from a patient with Lynch's syndrome).[33]

Cells were cultured in RPMI 1640 medium supplemented with 5% (KMS-1l) or 10% (GBM and HCT-116) fetal calf serum, 2 mM glutamine, antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) (Life Technologies). Cells were subcultured at confluency after dispersal with 0.025% trypsin in 0.02% EDTA. For experiments cells were used at 70–80% confluency.

3.2.2. Evaluation of cytotoxicity (neutral red uptake assay)

Twenty thousand cells (GBM and HCT-116) and 50,000 cells (KMS-11) (200 μL) were plated in 96-well culture microtiter plates (Falcon) and incubated at 37 °C in 5% CO_2. After 24 h, drugs were added in 50 μL fresh medium and after 45 h cells were loaded for 3 h with neutral red (3-amino-7,7-dimethylamino-2-methylphenazine hydrochloride) (Sigma–Aldrich, St Quentin, France) at a final concentration of 50 μg/mL in culture medium. This weakly cationic dye penetrates cell membranes by non-ionic diffusion, and binds intracellularly to anionic carboxylic and/or phosphate groups of the lysosomal matrix. Thereafter, the medium was removed, cells were fixed for 5 min with a mixture of 1% formaldehyde–1% $CaCl_2$ and the dye extracted with 0.2 mL of 1% acetic acid in 50% ethanol. Plates were left overnight at 4 °C and absorbance (OD, optical density) was recorded at 570 nm (Multiskan EX—Thermo-Electron Corporation). Control experiments were carried out using appro-

priate DMSO dilutions. Experiments were performed at least in triplicate, four wells per cell line being used. LD_{50} (lethal dose 50%) values were calculated from the dose–response curves as illustrated in Figure 2A. Values are expressed as the mean of at least three independent experiments ± standard error. Statistical analyses were performed by Student's *T* test.

3.2.3. Evaluation of cell proliferation ([^{3}H]thymidine incorporation assay)

Twenty thousand (GBM and HCT-116) and 500,000 (KMS-11) cells were plated in triplicates in 96-well culture microtiter plates (Falcon) and incubated at 37 °C in 5% CO_2. After 24 h drugs or appropriate vehicle were added in fresh medium. After 24 h cells were pulsed with 1 μCi/well of [^{3}H]thymidine. After the incubation period, medium was removed, the cell monolayer detached by incubation with 100 μL of 0.025% trypsin–0.02% EDTA for 5 min. Cell suspensions were pooled with their culture medium then cells were harvested onto glass filters with an automatic cell harvester (Perkin-Elmer). [^{3}H]Thymidine uptake was monitored using a 1450 Microbeta Jet counter (Perkin-Elmer). Results are representative of three independent experiments.

3.2.4. Nuclear staining

KMS-11 cells, seeded on glass lamellae, were fixed with 2% formaldehyde in culture medium for 10 min, washed with saline buffer (PBS), incubated with Hoechst 33342 which binds to DNA (5 min–1 μg/mL in PBS—Sigma, St. Louis, MO, USA), and then rinced in PBS. Slides were observed under ultraviolet (350 nm) illumination (maximal emission at 461 nm) using a Zeiss epifluorescence microscope.

3.2.5. APO2.7 staining

Cell death was assessed using the APO2.7 kit (Beckman Coulter, France) according to manufacturer's instruction (phycoerythrin-labeled APO2.7 antibody binds the 7A6 antigen exposed on mitochondria of cells undergoing apoptosis). Flow cytometry analyses were performed on a FACSCalibur cytometer and quantified using the Cell Quest software (Becton Dickinson, San Jose, CA, USA).

3.2.6. Western blot analysis

Cells (5×10^6) were resuspended in 150 μL lysis buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM ethylene-diamine-tetra-acetic acid (EDTA) and 1% Triton X-100) containing 2 mL phenyl-methylsulfonyl fluoride (PMSF) and 2 g/mL aprotinin. After 40 min on ice, lysates were cleared by centrifugation (10,000 g, 30 min, 4 °C). Protein concentrations were determined using bicinchoninic acid (BCA protein assay reagent kit, Pierce, Rockford, IL USA). Equal amounts of total protein were separated by SDS–PAGE (fractionated in polyacrylamide gel under reducing conditions) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, St. Quentin en Yvelines, France). Membranes were blocked with Western blocking reagent (Roche Diagnostics, Meylan, France) for 1 h at room temperature and incubated with primary antibody over night at 4 °C. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody for 30 min. Protein bands were vizualized by chemiluminescence detection (Roche Diagnostics). Antibody against PARP-1 was from Calbiochem (Germany). Antibody against Mcl-1 (S-19 clone) was from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France), antibodies against IκBα and phosphorylated- IκBα were from Cell Signaling (Beverly, MA, USA). Anti-actin, used as protein loading control, was purchased from Chemicon International (Temecula, CA, USA).

Acknowledgments

The authors thank A. Roger and H. Tauvel for their technical assistance. Dr. Rémi Legay is gratefully acknowledged for his assistance in measurement of mass spectra. We are grateful to L. Oliver (CHU, Nantes, France) for providing GBM cells.

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