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Original article

Synthesis of bile acid derivatives and in vitro cytotoxic activity with pro-apoptotic process on multiple myeloma (KMS-11), glioblastoma multiforme (GBM), and colonic carcinoma (HCT-116) human cell lines

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ABSTRACT

The novelty of this work derives from the use of nitrogenous heterocycles as building block in the synthesis of conjugate bile acid derivatives. New piperazinyl bile acid derivatives were synthesized and tested *in vitro* against various human cancer cells (GBM, KMS-11, HCT-116). The best pro-apoptotic activity was obtained with N-[4*N*-cinnamylpiperazin-1-yl)-3α,7β-dihydroxy-5β-cholan-24-amide (**7b**) and N-[4*N*-cinnamyllpiperazin-1-yl)- 3α,7α-dihydroxy-5β-cholan-24-amide (**7c**) on these human cancer cell lines (IC_{50}: 8.5–31.4 μM). This activity was associated with nuclear and DNA fragmentation, demonstrating that **7b** induces cell death by an apoptotic process as **7c.** This study shows the possibility of hydrid heterocycle-steroids as new anticancer agents with improved bioactivity and easy to synthesize.

1. Introduction

Bile acids, polar derivatives of cholesterol, are normal constituents of the colon and play key roles in the absorption of dietary lipids. A representative secondary bile acid, ursodeoxycholic acid (UDCA), is known to prevent gastrointestinal disorders of patients with various cancers (stomach, colon, lung, breast, and liver) [1–3]. It was also evaluated in clinical phase III trials to prevent colorectal adenoma recurrence [4]. Furthermore, recent clinical trials demonstrate that bile acid sequestrants can significantly reduce glucose levels in patients with type 2 diabetes mellitus [5,6]. 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. By analogy to these natural compounds, some authors have synthesized several bile acid derivatives, which are conjugates of bile acids with amino acids. These compounds have shown antitumor effects in human cancer cells such as stomach cancer cells [7], glioblastoma multiforme [8], colon cancer [9], breast carcinoma [10], leukemic T cells [11], prostate cancer cells [12], cervical carcinoma [13], hepatocellular carcinoma [14,15] and breast cancer cells [16]. Only a few conjugate bile acids with amino acids are synthesized but their biological activities have been widely studied. Due to their biological applicability, our approach consisted to introduce a nitrogenous heterocycle on the bile acids side chain. For this, we chose piperazine derivatives which are a pharmacologically interesting class of heterocycles [17–19]. This paper is the continuation of a previous study [20].

We report here the synthesis of new piperazinyl bile acid derivatives and discuss their cytotoxic activities on human cancer cell lines (Fig. 1).

2. Chemistry

Starting compounds were cholic acid (3α-,7α-,12α-trihydroxycholanic acid, **1a**), ursodeoxycholic acid (3α-,7β-dihydroxycholanic

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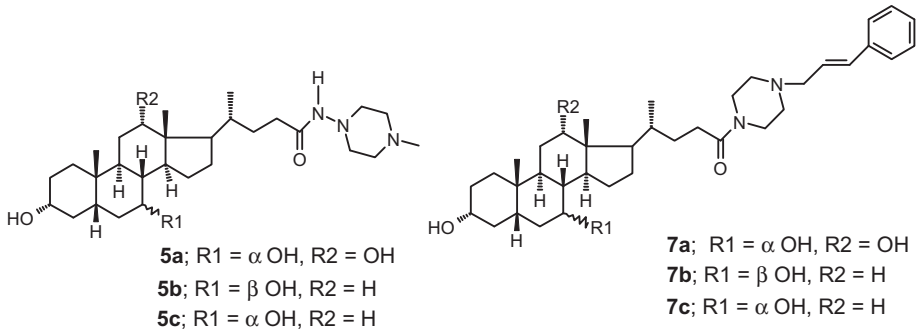


Fig. 1. Piperazinyl bile acid derivatives.

acid, **1b**) and chenodeoxycholic acid (3α-,7α-dihydroxycholanic acid, **1c**) which were acetylated with acetic anhydride in pyridine to give the acetates **2a**–**c** respectively. The latter acids **2a**–**c** were converted into the corresponding mixed anhydrides by treatment with ethyl chloroformate in the presence of triethylamine giving **3a**–**c** respectively. The mixed anhydrides **3a**–**c** were reacted with substituted piperazines (**a** or **b**), the products were identified as the carboxamides (**4a**–**c** and **6a**–**c**). Alcohol moieties were deprotected by ethanolic potassium hydroxide to give the corresponding deacetylated amides **5a**–**c** and **7a**–**c** which were purified on alumina deactivated with 6% water. The structures of the compounds prepared were confirmed by analytical and spectral analyses (Scheme 1).

The hydrolytic stability of compounds **5a**–**c** and **7a**–**c** was studied under physiological conditions (phosphate buffer, pH 7.4 at 37 °C). No degradation of these bile acid derivatives was observed.

The prediction of the lipophilicity of bile acid derivatives **5a**–**c** and **7a**–**c** has been determined by ALogP program [21].

3. Biology

3.1. Cytotoxicity

The compounds synthesized were tested for their initial *in vitro* cytotoxicity against a panel of tumor cells as described in the experimental section.

3.2. Pro-apoptotic activity

The pro-apoptotic activity of **7b** was explored using various approaches as described in the experimental section.

4. Results and discussion

Compounds **5a**–**c** and **7a**–**c** were synthesized to explore the relationship between structure and cytotoxic activity. We have investigated the role of the substituted lipid moiety in the cytotoxic activity. Cholic acid (**1a**), ursodeoxycholic acid (**1b**) and chenodeoxycholic acid (**1c**) were chosen because they allow controlled changes in the regiochemical and the stereochemical substitution of the hydroxyl groups on the cholane skeleton.

The cytotoxicity experiments with the synthesized compounds were performed *in vitro* in various human cancer cells: glioblastoma multiforme (GBM-12, brain tumor cell line), multiple myeloma (KMS-11) and colonic carcinoma (HCT-116) cell lines. The IC_{50} cytotoxicity values obtained are summarized in Table 1. These compounds displayed micromolar cytotoxic concentrations. The highest activity was obtained with bile acid derivatives **7b** and **7c**. The best activity was observed for **7c** on multiple myeloma with IC_{50} 8.5 μM, but **7b** is quite similar in these three human cancer cell lines with IC_{50} 24.7–31.4 μM.

The marked difference in activity among the piperazinyl derivatives shows the importance of the cinnamyl system. The cinnamylpiperazine derivatives **7b** and **7c** appear to be the most active compounds on these human cancer lines. It can be noticed that no activity was observed with **7a** which is a cinnamylpiperazinyl building block with cholic acid.

On the other hand, the lipophilicity (LogP) of bile acid derivatives **5a**–**c** and **7a**–**c** was predicted from structures using ALogP program (Table 1). These data show that compounds **7b** and **7c** have the highest lipophilicity suggesting that the lipophilicity plays a role in the activity of these molecules.

Following the results obtained with the **7b**, and because the apoptotic effect of **7c** was already described [20], we decided to investigate how **7b** induces cell death.

As shown by annexin V staining which reveals the early stages of apoptosis [22], the percentage of dead cells strongly increased after a 24 h treatment with 20 μM of **7b** (Fig. 2). Percentage of specific apoptosis was of 96% (KMS-11), 78% (GBM-12, data not shown), 45% (HCT-116). These data are in accordance with IC_{50} values and suggest that induction of apoptosis is delayed in HCT-116 compared to the two other cell lines.

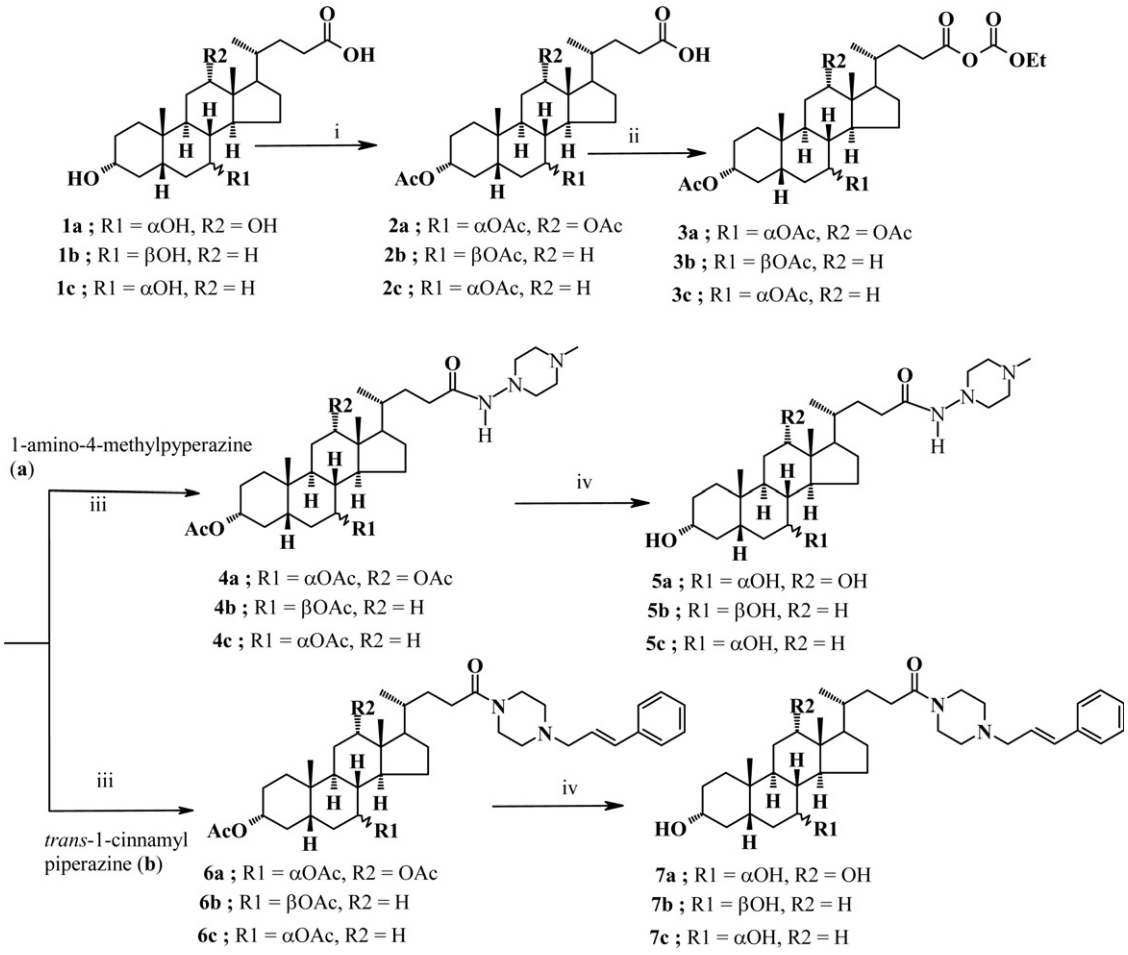
Characteristic images of apoptotic nuclei are shown in Fig. 3. Cell density of KMS-11, evaluated after 24 h of treatment by counting the number of nuclei using metamorph software, decreased with increased **7b** concentrations (84% for 10 μM, 59% for 20 μM, 35% for 40 μM) relative to the control. Final DNA fragmentation which is a consequence of apoptotic death [23], was observed after 48 hours of treatment (Fig. 4).

Taken together, these results (exposure of phosphatidylserines on the external surface of cells, nuclear and DNA fragmentation) demonstrate that **7b** induces cell death by an apoptotic process as **7c** [20].

Comparing **7a** with **7b** and **7c**, it can be concluded that the hydroxyl functionality at C-7 position of steroid nucleus in cinnamylpiperazinylsteroid is crucial for the cytotoxic activity. In addition, this activity was enhanced with 7-hydroxyl group in the stereochemistry alpha. Indeed, no activity was observed with N-[4*N*-cinnamylpiperazin-1-yl)-3α-hydroxy-5β-cholan-24-amide without hydroxyl group at the C-7position [20]. Moreover, the activity was suppressed when the steroid possess the hydroxyl group on the C-12 position (**7a**) even in the presence of the hydroxyl at C-7 position.

5. Conclusion

In conclusion, some new piperazinyl bile acid derivatives were synthesized and their cytotoxic activities on human cancer cell lines were evaluated. Some of them exhibited good activity in all



Scheme 1. Synthesis of piperazinyl bile acid derivatives. *Reagents and conditions:* (i) Ac_2O, pyridine, r.t., 15 h; (ii) EtOCOCl, NEt_3, $CHCl_3$, r.t., 50 min; (iii) piperazine (a or b), NEt_3, $CHCl_3$, r.t., 16 h; (iv) KOH, EtOH, reflux, 24 h.

cancer cells tested. The best activity shown by **7b** and **7c** may be due to the presence of the cinnamylpiperazinyl group in the side chain of the ursodeoxycholic and chenodeoxycholic acids bearing a hydroxyl group at C-7 position. These derivatives constitute a starting point towards a new platform development of this type of steroid-heterocyclic hybrid, which could easily be prepared in large amounts for biological evaluation. Further research in this area is in progress in our laboratory.

Table 1
Cytotoxic activity of piperazinyl bile acid derivatives on three human cancerous cell lines.[a]

Compounds	Log P[b]	KMS-11 IC_{50} (μM)	GBM IC_{50} (μM)	HCT-116 IC_{50} (μM)
5a	2.162	>50	>50	>50
5b	3.264	>50	>50	>50
5c	3.264	≥50	≥50	>50
7a	4.705	>50	>50	>50
7b	5.807	28.1 ± 5.2	31.4 ± 6.3	24.7 ± 1.9
7c	5.807	8.5 ± 0.5	18.5 ± 1	16.5 ± 2
CA	2.912	>50	>50	>50
CDCA	4.014	>50	>50	>50
UDCA	4.014	>50	>50	>50

[a] IC_{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.
[b] LogP was determined by ALogP program.

6. Experimental

6.1. Chemistry

6.1.1. 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 ionisation energy from 30 to 40 eV. ESI mass spectra were recorded on a LC/MS Waters alliance. ^1H NMR and ^{13}C NMR spectra were recorded using $CDCl_3$ and methanol-d_4 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 ^1H-decoupled. Elemental analyses were performed at the "Institut de Recherche en Chimie Organique Fine" (Rouen, France).

6.1.2. Chemical synthesis

*6.1.2.1. General procedure for **2a**, **2b** and **2c**.* Acetic anhydride (78.8 mmol, 6.7 mL) was added dropwise to a solution of **1a**, **1b** or

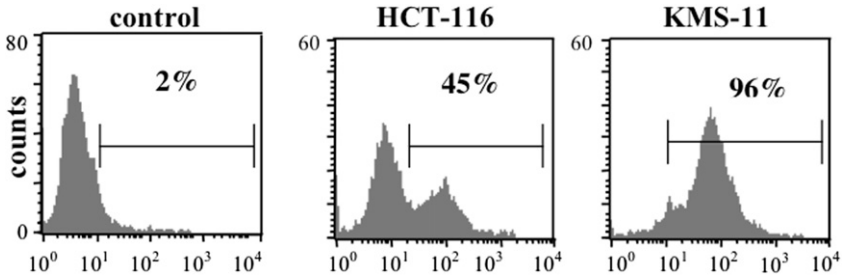


Fig. 2. Percentage of apoptotic KMS-11 and HCT-116 cells in the absence or presence of **7b** (20 μM) determined after 24 h, by annexin V staining.

1c (8.8 mmol) in pyridine (25 mL). The reaction mixture was stirred at room temperature under argon atmosphere for 15 h and then ice water was added. The white precipitate formed was dissolved in CH_2Cl_2 (100 mL), washed with 1 M HCl (2 × 20 mL), 5% $NaHCO_3$ (2 × 20 mL), brine (2 × 20 mL) and then with water (20 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate, 8:2) to afford **2a**, **2b** or **2c**.

*6.1.2.2. 3α,7α-,12α-triacetyloxy-5β-cholan-24-oic acid (**2a**).* The crude product was recrystallized from acetone to give **2a** (4.15 g, 88 %) as a white powder. IR (KBr): *ν* (cm^{-1}): 3404–3010 (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): $\delta = 0.73$ (s, 3H, Me-18), 0.82 (d, $^3J = 6.8$ Hz, 3H, Me-21), 0.91 (s, 3H, Me-19), 2.05 (s, 3H, $-OCOCH_3$), 2.09 (s, 3H, $-OCOCH_3$), 2.14 (s, 3H, $-OCOCH_3$), 4.52–4.62 (m, 1H, H-3), 4.86–4.95 (m, 1H, H-7), 5.08–5.12 (m, 1H, H-12) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.2$ (C-18), 17.4 (C-21), 21.4 ($-OCOCH_3$), 21.5($-OCOCH_3$), 21.6($-OCOCH_3$), 22.5(C-19), 22.7, 25.5, 26.8, 27.1, 28.8, 30.5, 30.8, 31.2, 34.3, 34.5 (2C), 34.6, 37.7, 40.8, 43.3, 45.0, 47.3, 70.7, 74.1, 75.3, 170.4($-OCOCH_3$), 170.5($-OCOCH_3$), 170.6($-OCOCH_3$), 179.6(C-24) ppm. MS (30 eV, EI): *m/z* (%) = 418.5 (3) [$M^{+\cdot}$], 358.4 (100) [$M^{+\cdot} - CH_3CO_2H$], 343.4 (12), 304.3 (6), 257.3 (9), 230.3 (21), 215.3 (33).

*6.1.2.3. 3α,7β-diacetyloxy-5β-cholan-24-oic acid (**2b**).* The crude product was recrystallized from acetonitrile/diethyl ether (2/8) to give **2b** (3.86 g, 92 %) as a white powder. IR (KBr): (ν cm^{-1}): 3493-3050 (O–H acid), 2939–2870 (C–H alkane), 1729 (C=O ester and acid). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.90 (s, 3H, Me-19), 0.95 (d, $^3J = 8.0$ Hz, 3H, Me-21), 2.02 (s, 6H, 2 × $-OCOCH_3$), 4.65–4.67 (m, 1H, H-3), 4.73–4.79 (m, 1H, H-7) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.0$ (C-18), 18.3 (C-21), 21.2, 21.4 (2 × $-OCOCH_3$), 21.8 (C-19), 23.2, 25.6, 26.4, 28.4, 29.7, 30.8, 30.9, 32.9, 34.0, 34.5, 35.2, 39.4, 39.9, 40.0, 42.0, 43.6, 55.0, 55.2, 73.6 (C-3), 170.6 ($-OCOCH_3$), 170.7 ($-OCOCH_3$), 178.7 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 476.3 (1) [$M^{+\cdot}$], 434.3 (3), 416.3 [$M^{+\cdot} - CH_3CO_2H$], 374.3 (7), 356.3 (100) [$M^{+\cdot} - 2 \times CH_3CO_2H$], 341.2 (33), 302.2 (10), 255.2 (22), 228.2 (38), 213.2 (28).

*6.1.2.4. 3α,7α-diacetyloxy-5β-cholan-24-oic acid (**2c**).* Compound **2c** have been prepared according to a reported procedure [20].

*6.1.3. General procedure for compounds **4a**, **4b**, **4c**, **6a**, **6b** and **6c***

Ethyl chloroformate (1.13 mL, 11.8 mmol) was added to a stirred solution of acid **2a**, **2b** or **2c** (8.9 mmol) in chloroform (30 mL) under argon atmosphere at room temperature. Triethylamine (2.50 mL, 17.7 mmol) was added dropwise and the solution was kept under stirring for 45 min. The reaction mixture was diluted with methylene chloride (40 mL), washed with water (2 × 20 mL), dried over anhydrous magnesium sulfate and evaporated. The crude product of anhydride (**3a** or **3b**) was used to next step without purification.

Under nitrogen atmosphere, piperazine derivative (**a** or **b**) (17.8 mmol) and triethylamine (1.25 mL, 8.9 mmol) were added to a solution of anhydride **3a, 3b** or **3c** (8.9 mmol) in chloroform

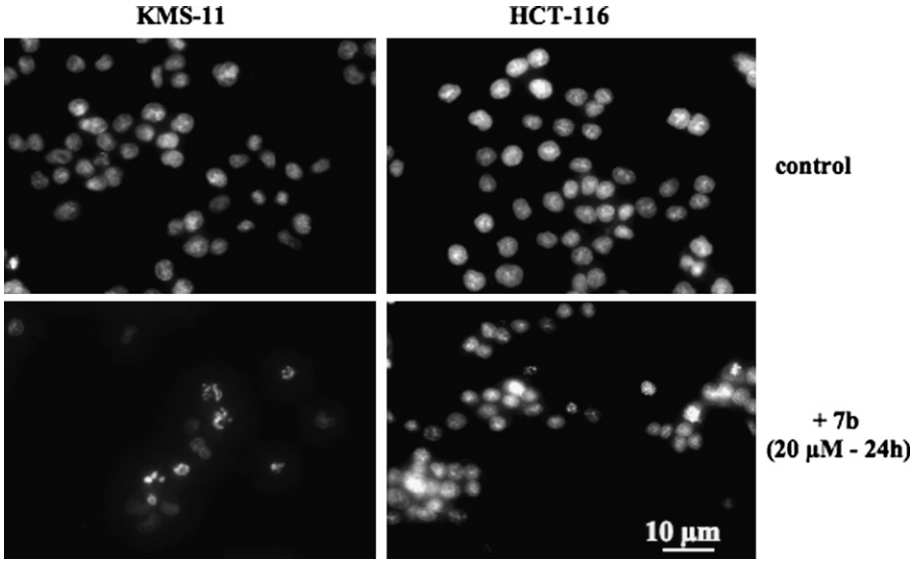


Fig. 3. Nuclear fragmentation of KMS-11 and HCT-116 cells induced by 7b treatment (20 μM–24 h) visualized by DNA staining with Hoëchst.

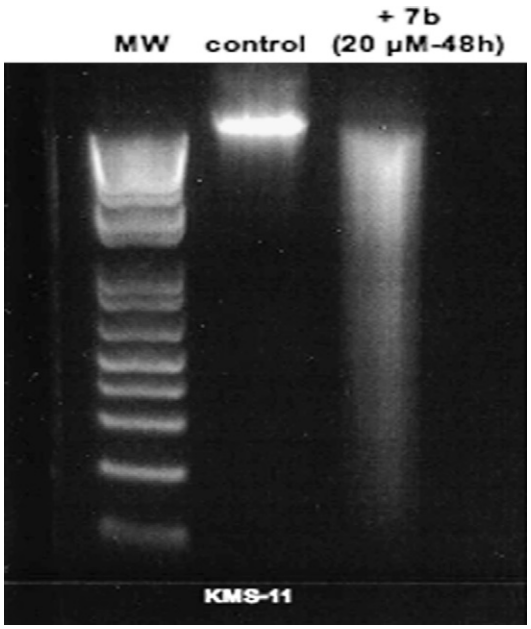


Fig. 4. Supernatants of control and **7b** treated (20 μM–48 h) KMS-11 cultures analyzed by agarose gel electrophoresis (MW, 100 bp DNA ladder).

(30 mL). The reaction mixture was stirred at room temperature for 12 h. The solution was diluted with methylene chloride (40 mL), washed with water (2 × 20 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.

*6.1.3.1. N-[4N-methylpiperazin-1-yl)-3α,7α,12α-tri-acetoxy-5β-cholan-24-amide (**4a**).* 3.24 g (58 % yields) as a yellow oil. IR (KBr): (ν cm^{-1}): 3448–3150 (N–H amide), 2944–2872 (C–H alkane), 1735 (C=O ester), 1666 (C=O amide). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.74$ (s, 3H, Me-18), 0.85 (d, $^3J = 6.8$ Hz, 3H, Me-21), 0.92 (s, 3H, Me-19), 2.05 (s, 3H, –C$_3$–OCOCH$_3$), 2.09 (s, 3H, –C$_7$–OCOCH$_3$), 2.15 (s, 3H, –C$_{12}$–OCOCH$_3$), 2.30 (s, 3H, N–CH$_3$), 2.51–2.60 (m, 4H, 2 × –CH$_2$- piperazinyl), 2.75–2.95 (m, 4H, 2 × –CH$_2$– piperazinyl), 4.52–4.62 (m, 1H, H-3), 4.86–4.95 (m, 1H, H-7), 5.08–5.12 (m, 1H, H-12), 6.65 (br s, 1H, NH, D_2O exchange) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.0$ (Me-18), 17.4 (Me-21), 21.2 (3 × –OCO*C*H$_3$), 22.3 (Me-19), 22.5, 25.3, 26.6, 26.9, 28.6, 29.0, 30.8, 31.3, 34.0, 34.3, 34.5, 34.7, 37.4, 40.6, 43.1, 44.8, 45.4, 47.3, 54.0, 54.3, 55.0, 56.1, 70.4 (C-7), 73.8 (C-3), 75.1 (C-12), 170.1(–O*C*OCH$_3$), 170.3(–O*C*OCH$_3$), 170.5(–O*C*OCH$_3$), 176.6 (O=C–N) ppm. MS (30 eV, EI): *m/z* (%) = 631.4 (82) [M$^{+\cdot}$], 616.5 [M$^{+\cdot}$ – CH$_3$], 572.4 (100) [M$^{+\cdot}$ – CH$_3$CO$_2$], 556.4 (13) [M$^{+\cdot}$ – CH$_3$CO$_2$H – CH$_3$], 512.4 (16) [M$^{+\cdot}$ – CH$_3$CO$_2$H – CH$_3$CO$_2$], 413.3 (18), 354.3 (27), 253.2 (37.9), 170.1 (74). HRMS (EI) *m/z* [M$^+$] calcd. for $C_{35}H_{57}N_3O_7$: 631.41963, found: 631.41943.

*6.1.3.2. N-[4N-methylpiperazin-1-yl)-3α,7β-di-acetoxy-5β-cholan-24-amide (**4b**).* 3.14 g (62 % yield) as a yellow oil. IR (KBr): (ν cm^{-1}): 3445-3140 (N–H amide), 2948–2873 (C–H alkane), 1736 (C=O ester), 1667 (C=O amide). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.90 (d, $^3J = 6.8$ Hz, 3H, Me-21), 0.97 (s, 3H, Me-19), 2.00 (s, 3H, –C$_3$–OCOCH$_3$), 2.03 (s, 3H, –C$_7$–OCOCH$_3$), 2.31 (s, 3H, N–CH$_3$), 2.52–2.61 (m, 4H, 2 × –CH$_2$- piperazinyl), 2.70–3.70 (m, 4H, 2 × –CH$_2$- piperazinyl), 4.62–4.70 (m, 1H, H-3), 4.72–4.80 (m, 1H, H-7), 6.27 (br s, 1H, NH, D_2O exchange) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.0$, 18.5, 21.1(1 × –OCO*C*H$_3$), 21.3(1 × –OCO*C*H$_3$), 23.1, 25.5, 26.3, 28.4, 29.0, 31.2, 31.6, 32.7, 33.9, 34.4, 35.2, 35.3, 39.3, 39.8, 41.9, 43.5, 45.6, 54.1, 54.5, 54.9, 55.0, 55.1, 55.4, 56.4, 66.7 (C-7), 73.4 (C-3), 170.4 (O–C=O), 170.5(O–C=O), 176.9 (O=C–N) ppm. MS (30 eV, EI): *m/z* (%) = 573.4 (25) [M$^{+\cdot}$], 558.4 (19) [M$^{+\cdot}$ – CH$_3$], 198.1 (4) [M$^{+\cdot}$ – StC23], 170.1 (100) 198.1 (4) [M$^{+\cdot}$ – StC21]. HRMS (EI) *m/z* [M$^+$] calcd. for $C_{33}H_{55}N_3O_5$: 573.41413, found: 573.41556.

*6.1.3.3. N-(4N-methylpiperazin-1-yl)-3α,7α-di-acetoxy-5β-cholan-24-amide (**4c**).* 3.49 g (69 % yield) as a yellow oil. IR (KBr): (ν cm^{-1}): 3450–3220 (N–H amide), 2942–2871 (C–H alkane), 1735 (C=O ester), 1665 (C=O amide), 1247 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.65$ (s, 3H, Me-18), 0.93 (s, 3H, Me-19), 0.96 (d, $^3J = 5.8$ Hz, 3H, Me-21), 2.00 (s, 3 H, C$_3$ –OCOCH$_3$), 2.05 (s, 3 H, C$_7$ –OCOCH$_3$), 2.30 (s, 3H, N–CH$_3$), 2.52–2.66 (m, 4H, 2 × –CH$_2$- piperazinyl), 2.72–2.96 (m, 4H, 2 × –CH$_2$- piperazinyl), 4.50–4.62 (m, 1H, H-3), 4.82–4.92 (m, 1H, H-7), 6.66 (br s, 1H, NH, D_2O exchange) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 11.5$, 18.1, 20.4, 21.2(1 × –OCO*C*H$_3$), 21.3(1 × –OCO*C*H$_3$), 22.4, 23.3, 26.5, 27.8, 29.1, 31.0, 33.8, 34.3, 34.5, 34.6, 35.2, 35.4, 37.6, 39.2, 40.6, 42.4, 45.4, 50.1, 53.9, 54.3, 54.9, 55.6, 56.1, 70.1 (C-7), 73.9 (C-3), 170.3 (O–C=O), 170.7 (O–C=O), 176.9 (O=C–N) ppm. MS (ESI): [M + H]$^+$ 574.10. HRMS (EI) *m/z* [M$^+$] calcd. for $C_{33}H_{35}N_3O_5$: 573.41413, found: 573.41588.

*6.1.3.4. N-(4N-cinnamylpiperazin-1-yl)-3α,7α,12α-tri-acetoxy-5β-cholan-24-amide (**6a**).* 3.78 g (59 % yield) as a yellow oil. IR (KBr): (ν cm^{-1}): 2942–2808 (C–H alkane), 1732 (C=O ester), 1645 (C=O amide), 1248 (C–N amine). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.72$ (s, 3H, Me-18), 0.82 (s, 3H, Me-19), 0.91 (d, 3H, $J = 6.6$ Hz, Me-21), 2.07 (s, 3H, –C$_3$–OCOCH$_3$), 2.08 (s, 3H, –C$_7$–OCOCH$_3$), 2.13 (s, 3H, –C$_{12}$–OCOCH$_3$), 2.46–2.50 (m, 4H, 2 × –CH$_2$- piperazinyl), 3.17 (d, $J = 6.8$ Hz, 2H, –CH$_2$–CH=CH-Ph), 3.46–3.48 (m, 2H, 2 × –C H$_{AX}$H$_{EQ}$- piperazinyl), 3.60–3.65 (m, 2H, 2 × –C H$_{AX}$H$_{EQ}$- piperazinyl), 4.53–4.65 (m, 1H, H-3), 4.87–4.95 (m, 1H, H-7), 5.05–5.12 (m, 1H, H-12), 6.25 (dt, $^dJ = 15.6$ Hz and $^tJ = 6.8$ Hz, 1H, –CH$_2$–CH= CH-Ph), 6.52 (d, $J = 15.6$ Hz, 1H, –CH$_2$–CH=CH-Ph), 7.22–7.39 (m, 5H, H$_{ar}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.1$(C-18), 17.6 (C-21), 21.3 (2 × –OCO*C*H$_3$), 21.5 (1 × –OCO*C*H$_3$), 22.4, 22.7, 25.4, 26.7, 27.1, 28.7, 30.2, 31.1, 34.1, 34.4, 34.5, 34.8, 37.5, 40.7, 41.3, 43.2, 44.9, 45.4, 47.5, 52.7, 53.1 (2C), 60.7 (–CH$_2$–CH=CH-Ph), 70.5 (C-7), 73.9 (C-3), 75.2 (C-12), 125.7, 126.2 (2C), 127.5, 128.4 (2C), 133.4, 136.5, 170.2(C=O), 170.4 (2 C=O), 171.6 (O=C–N) ppm. MS (30 eV, EI): *m/z* (%) = 718.3 (100) [M$^{+\cdot}$], 658.3 (27) [M$^{+\cdot}$ – CH$_3$CO$_2$H], 600.3 (36) [M$^{+\cdot}$ – Ph-CH=CH–CH$_2$], 542.3 (34), 358.2 (84), 313.1 (43), 257.1 (48).

*6.1.3.5. N-(4N-cinnamylpiperazin-1-yl)-3α,7β-di-acetoxy-5β-cholan-24-amide (**6b**).* 4.16 g (71 % yield) as a yellow oil. IR (KBr): (ν cm^{-1}): 2948–2871 (C–H alkane), 1735 (C=O ester), 1647 (C=O amide). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.94 (d, $J = 6.8$ Hz, 3H, Me-21), 0.97 (s, 3H, Me-19), 2.00 (s, 3H, 1 × –OCOCH$_3$), 2.02 (s, 3H, 1 × –OCOCH$_3$), 2.44–2.52 (m, 4H, 2 × –CH$_2$- piperazinyl), 3.17 (d, $J = 5.8$ Hz, 2H, –CH$_2$–CH=CH-Ph), 3.46–3.48 (m, 2H, 2 ×–C H$_{AX}$H$_{EQ}$- piperazinyl), 3.60–3.64 (m, 2H, 2 × -C H$_{AX}$H$_{EQ}$- piperazinyl), 4.62–4.67 (m, 1H, H-3), 4.72–4.79 (m, 1H, H-7), 6.25 (dt, $^dJ = 15.6$ Hz and $^tJ = 6.8$ Hz, 1H, –CH$_2$–CH= CH-Ph), 6.53 (d, $J = 15.6$ Hz, 1H, –CH$_2$–CH=CH-Ph), 7.22–7.39 (m, 5H, H$_{ar}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 11.9$, 18.5, 21.2 (–OCO*C*H$_3$), 21.7(–OCO*C*H$_3$), 23.0, 25.4, 26.1, 28.3, 30.0, 31.2, 32.7, 33.8, 34.3, 35.3, 39.2, 39.7, 41.3, 41.8, 43.4, 45.4, 52.7, 54.8, 55.0, 60.7 (–CH$_2$–CH=CH-Ph), 73.3 (C-3 and C-7), 125.7, 126.1 (2C), 127.5, 128.4 (2C), 133.3, 136.5, 170.3 (C=O), 170.4 (C=O), 171.8 (O=C–N) ppm. MS (30 eV, EI): *m/z* (%) = 660.3 (30) [M$^{+\cdot}$], 600.3 (9) [M$^{+\cdot}$ – Ph-CH=CH-CH$_2$], 569.1 (10), 449.3 (9), 257.0 (43) [M$^{+\cdot}$ – StC21], 172.0 (100).

*6.1.3.6. N-(4N-cinnamylpiperazin-1-yl)-3α,7α-di-acetoxy-5β-cholan-24-amide (**6c**).* Compound **6c** have been prepared according to a reported procedure [20].

*6.1.4. Typical procedure of compounds **5a**, **5b**, **5c**, **7a** and **7b***

An aqueous solution (1 mL) of potassium hydroxide (0.84 g, 15.04 mmol) was added to a stirred solution of **4a** (1.18 g, 1.88 mmol) in ethanol (15 mL). 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 **5a** (0.53 g, 56 %) as a white powder.

*6.1.4.1. N-(4N-methylpiperazin-1-yl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (**5a**).* IR (KBr): (ν cm^{-1}): 3417–3231 (O–H alcohol and N-H amide), 2936–2862 (C–H alkane), 1629 (C=O amide). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.67$ (s, 3H, Me-18), 0.88 (s, 3H, Me-19), 0.98 (d, $J = 6.8$ Hz, 3H, Me-21), 2.06–2.26 (m, 4H, 2 × $-CH_2$- piperazinyl), 2.29 (s, 3H, N-CH_3), 2.51–2.68 (m, 2H, 2 × –C $H_{\underline{AX}}H_{\underline{EQ}}$- piperazinyl), 2.75–2.98 (m, 2H, 2 × –C $H_{AX}H_{\underline{EQ}}$- piperazinyl), 3.35–3.46 (m, 1H, H-3), 3.80–3.85 (m, 1H, H-7), 3.92–3.96 (m, 1H, H-12), 5.30 (s, 3H, 3 x OH, D_2O exchange), 6.61 (br s, 1H, –NH, D_2O exchange) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.37$, 17.5, 22.4, 23.3, 27.6, 27.7, 28.0, 30.3, 30.4, 31.2, 31.3, 34.8, 35.3, 35.8, 39.5, 41.5, 41.6, 45.5, 46.3, 47.0 (2C), 54.2 (2C piperazinyl), 55.9 (2C piperazinyl), 68.4 (C-7), 71.7 (C-3), 73.0 (C-12), 177.4 (C=O) ppm. MS (30 eV, EI): *m/z* (%) = 505.4 (28) [$M^{+\cdot}$], 490.4 (25) [$M^{+\cdot} - CH_3$], 472.3 (8) [$M^{+\cdot} - (H_20 + CH_3)$], 271.2 (18), 170.1 (100) [$M^{+\cdot}$ – StC21]. HRMS (EI) m/z [M^+] calcd. for $C_{29}H_{51}N_3O_4$: 505.38792, found: 505.38805. Anal. calcd. for $C_{29}H_{51}N_3O_4$: C, 68.87; H, 10.16; N, 8.31; O, 12.65 Found: C, 68.90; H, 10.20; N, 8.31; O, 12.66.

*6.1.4.2. N-(4N-methylpiperazin-1-yl)-3α,7β-dihydroxy-5β-cholan-24-amide (**5b**).* 0.61 g (66 % yield) as a yellow amorphous solid. IR (KBr): (ν cm^{-1}): 3424–3000 (O–H alcohol and NH amide), 2926–2852 (C–H alkane), 1654 (C=O amide). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.92 (s, 3H, Me-19), 0.97 (d, $J = 5.9$ Hz, 3H, Me-21), 2.31 (s, 3H, N-CH_3), 2.58–2.80 (m, 2H, 2 × –C $H_{\underline{AX}}H_{\underline{EQ}}$- piperazinyl), 2.85-2.96 (m, 2H, 2 × –C $H_{AX}H_{\underline{EQ}}$- piperazinyl), 3.55–3.79 (m, 2H, H-3 and H-7), 5.30 (s, 2H, 2 × OH, D_2O exchange), 6.18 (br s, 1H, -NH, D_2O exchange) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): $\delta = 12.7$, 19.1, 22.4, 24.0, 24.3, 28.0, 29.7, 31.0, 34.1, 35.2, 36.1, 36.3, 37.3, 38.0, 38.7, 40.8, 41.6, 44.1, 44.5, 44.8, 56.8, 57.6, 72.0 (C-7), 72.2 (C-3), 180.4 (C=O) ppm. (Carbon signals were probably included in Methanol-d4 signal). MS (30 eV, EI): *m/z* (%) = 489.4 (83) [$M^{+\cdot}$], 474.3 (48) [$M^{+\cdot} - CH_3$], 447 (18), 170 (100) [$M^{+\cdot}$ – StC21]. HRMS (EI) m/z [M^+] calcd. for $C_{29}H_{51}N_3O_3$: 489.39301, found: 489.39199. Anal. calcd. for $C_{29}H_{51}N_3O_3$: C, 71.12; H, 10.50; N, 8.58; O, 9.80 Found: C, 71.34; H, 10.69; N, 8.58; O, 9.84.

*6.1.4.3. N-(4N-methylpiperazin-1-yl)-3α,7α-dihydroxy-5β-cholan-24-amide (**5c**).* 0.59 g (64 % yield) as a yellow amorphous solid. IR (KBr): (ν cm^{-1}): 3419 (br, O–H alcohol and NH amide), 2935–2865 (C–H alkane), 1662 (C=O amide). ^1H NMR (400 MHz, CD_3OD, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.97 (s, 3H, Me-19), 1.01 (d, $J = 5.84$ Hz, 3H, Me-21), 2.30 (s, 3H, N–CH_3), 2.15–2.48 (m, 4H, 2 × $-CH_2$- piperazinyl), 2.15–2.48 (m, 4H, 2 × -CH_2- piperazinyl), 3.21–3.40 (m, 2H, H-3 and H-7), 3.78 (br s, 1H, –NH) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): $\delta = 12.2$ (Me-18), 18.9 (Me-21), 21.8, 23.4 (Me-19), 24.6, 29.3, 31.4, 32.3, 33.2, 34.0, 35.3, 35.9, 36.2, 36.6, 36.9, 40.5, 40.8, 41.1, 43.2, 43.7, 45.6, 51.5, 55.1, 55.3, 55.8, 56.3, 69.0 (C-7), 72.8 (C-3), 174.3 (C=O) ppm. MS (30 eV, EI): *m/z* (%) = 489.4 (76) [$M^{+\cdot}$], 474.3 (56), 447 (12), 170 (100) [$M^{+\cdot}$ – StC21]. HRMS (EI) *m/z* [M^+] calcd. for $C_{29}H_{51}N_3O_3$: 489.39301, found: 489.39303. Anal. calcd. for $C_{29}H_{51}N_3O_3$: C, 71.12; H, 10.50; N, 8.58; O, 9.80 Found: C, 71.31; H, 10.72; N, 8.60; O, 9.83.

*6.1.4.4. N-[4N-cinnamylpiperazin-1-yl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (**7a**).* 0.54 g (48 % yield) as a white solid. IR (KBr): (ν cm^{-1}): 3419 (br, O–H alcohol), 2934–2813 (C–H alkane), 1627 (C=O amide). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.64$ (s, 3H, Me-18), 0.84 (s, 3H, Me-19), 1.00 (d, $J = 5.8$ Hz, 3H, Me-21), 2.98–3.08 (m, 4H, 2 × $-CH_2$- piperazinyl), 3.15 (d, $J = 5.8$ Hz, 2H, $-C\underline{H}_2-CH{=}CH$-Ph), 3.38-3.43 (m, 1H, 3-H), 3.39 (m, 2H, 2 × –C $H_{\underline{AX}}H_{\underline{EQ}}$- piperazinyl), 3.61 (m, 2H, 2 × $-CH_{AX}H_{\underline{EQ}}$- piperazinyl), 3.80 (m, 1H, 7-H), 3.94 (m, 1H, 12-H), 4.55 (s, 3H, 3 × OH, D_2O exchange), 6.18–6.30 (m, 1H, $-CH_2-C\underline{H}{=}CH$-Ph), 6.52 (d, $J = 15.6$ Hz, 1H, $-CH_2-CH{=}C\underline{H}$-Ph), 7.21–7.38 (m, 5H, H_{ar}) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.3$, 17.3, 22.3, 23.1, 26.1, 27.5, 27.9, 29.8, 30.0, 31.2, 34.5, 34.6, 35.2, 35.4, 39.3, 41.3, 41.4, 44.6 (2C), 45.4, 46.2, 46.5, 51.2, 51.9, 60.9, 68.1 (C-7), 71.4 (C-3), 72.8 (C-12), 125.5, 126.2 (2C), 127.5, 128.4 (2C), 133.5, 136.5, 172.3 (C-24) ppm. MS (ESI): $[M + H]^+$ 593.2. Anal. calcd. for $C_{37}H_{56}N_2O_4$: C, 74.96; H, 9.52; N, 4.73; O, 10.79 Found: C, 74.91; H, 9.57; N, 4.73; O, 10.80.

*6.1.4.5. N-[4N-cinnamylpiperazin-1-yl)-3α,7α-dihydroxy-5β-cholan-24-amide (**7b**).* 0.68 g (63 % yield) as a white solid. IR (KBr): (ν cm^{-1}): 3410 (br, O–H alcohol), 2931–2813 (C–H alkane), 1628 (C=O amide). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.68$ (s, 3H, Me-18), 0.94 (s, 3H, Me-19), 0.95 (d, $J = 5.8$ Hz, 3H, Me-21), 2.43–2.52 (m, 4H, 2 × $-CH_2$- piperazinyl), 3.17 (d, $J = 6.8$ Hz, 2H, $-C\underline{H}_2-CH{=}CH$-Ph), 3.42–3.51 (m, 4H, 2 × $-CH_2$- piperazinyl), 3.52-3.61 (m, 1H, 3-H), 3.62–3.68 (m, 1H, 7-C), 5.30 (s, 2H, 2 × OH, D_2O exchange), 6.27 (dt, $^dJ = 15.6$ Hz and $^tJ = 6.7$ Hz, 1H, $-CH_2-C\underline{H}{=}CH$-Ph), 6.52 (d, $J = 15.6$ Hz, 1H, $-CH_2-CH{=}C\underline{H}$-Ph), 7.23–7.39 (m, 5H, H_{ar}) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 12.1$ (C-18), 18.5 (C-21), 21.1 (C-19), 23.3, 26.8, 28.6, 30.2, 31.4, 33.0, 34.0, 34.9, 35.5, 36.8, 37.2, 39.1, 40.0, 41.4, 42.4, 43.6, 43.7, 45.5, 52.8, 53.2, 54.9, 55.6, 60.8, 71.2 (C-3 and C-7), 125.7, 126.3 (2C), 127.6, 128.5 (2C), 133.5, 136.6, 172.1 (C-24) ppm. MS (30 eV, EI): *m/z* (%) = 576.3 (27) [$M^{+\cdot}$], 561.3 (3) [$M^+ - CH_3$], 485.3 (9), 257.1 (19), 201.1 (24), 172.1 (100). HRMS (EI) *m/z* [M^+] calcd. for $C_{37}H_{56}N_2O_3$: 576.42907, found: 576.42697. Anal. calcd. for $C_{37}H_{56}N_2O_3$: C, 77.04; H, 9.78; N, 4.86; O, 8.82 Found: C, 77.08; H, 9.75; N, 4.85; O, 8.84.

*6.1.4.6. N-[4N-cinnamyllpiperazin-1-yl)-3α,7α-dihydroxy-5β-cholan-24-amide (**7c**).* Compound **7c** have been prepared according to a reported procedure [20].

6.1.5. Hydrolytic stability of the bile acid derivatives

Bile acid derivative (**5a–c and 7a–c**, 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).

6.2. Bioassays

Evaluation of cytotoxic activities against glioblastoma multiforme (GBM), multiple myeloma (KMS-11) and colonic carcinoma (HCT-116) cell lines, were realized according to the method detailed before in our previous work [20].

For DNA fragmentation analysis, KMS-11 controls and **7b** treated (20 μM – 48 h) cultures were treated according to Gentra-Puregen Kit of Qiagen recommandations.

Percentage of apoptotic cells was determined after FITC-conjugated annexin V labeling (Beckman Coulter) on a FACSCalibur flow cytometer (BD Biosciences).

Nuclei were visualized after Hoëchst (Sigma–Aldrich, St. Louis, MO) staining using a LEICA DMI 6000B microscope and data were quantified using Metamorph software of ROPER (PICell, Nantes).

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