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Synthesis, Structure-Activity Relationship, and Mechanistic Investigation of Lithocholic Acid Amphiphiles for Colon Cancer Therapy

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Abstract: We report a structure-activity relationship of lithocholic acid amphiphiles for their anticancer activities against colon cancer. We synthesized ten cationic amphiphiles differing in nature of cationic charged head groups using lithocholic acid. We observed that anticancer activities of these amphiphiles against colon cancer cell lines are contingent on nature of charged head group. Lithocholic acid based amphiphile possessing piperidine head group (**LCA-PIP$_1$**) is ~10 times more cytotoxic as compared to its precursor. Biochemical studies revealed that enhanced activity of **LCA-PIP$_1$** as compared to lithocholic acid is due to greater activation of apoptosis. **LCA-PIP$_1$** induces sub G$_0$ arrest and causes cleavage of caspases. A single dose of lithocholic acid-piperidine derivative is enough to reduce the tumor burden by 75% in tumor xenograft model.

Keywords: Bile acids, Cancer, Amphiphiles, Anticancer activities, Colon Cancer

Introduction: Colon cancer is third most malignant tumor type involving uncontrolled growth of cells in colon.[1] Many small molecule and antibody based therapeutics have been engineered and developed for cancer therapy to target this disease effectively.[2] Apart from genetic reasons, prolonged high consumption of western-type diet augments risk of colon cancer in majority of patients. Increase uptake of fatty diets need extensive recirculation of bile acids for its digestion. Therefore, colon epithelial cells gets enhanced exposure to these high concentration of bile acids. Clinical studies have revealed elevated levels of fecal secondary bile acids from patients diagnosed with colon cancer.[3] Therefore, bile acids are critical for pathogenesis of colon cancer progression and renewal of colon epithelium.

Bile acids are cholesterol-derived amphiphile molecules that help in absorption of fats and fat-soluble vitamins.[4] Primary bile acids like chenodeoxycholic acid and cholic acid are synthesized and secreted from liver and get re-circulated *via* portal system.[5] Gut microflora convert these primary bile acids to secondary bile acids, lithocholic acid and deoxycholic acid by de-hydroxylation.[6] High concentration of bile acids and its accumulation in the gut can induce apoptosis of colon epithelium.[7] Bile acid induced cytotoxicity causes colon cancer progression by disrupting the balance between cell growth and apoptosis, and helps in selection of bile acid resistant colon cancer cells.[8]

Bile acids exercise their cytotoxic effect *via* different cellular mechanisms that is contingent on structure, hydrophobicity, and stereochemistry of bile acids.[9] Glycine and taurine conjugated primary bile acids are least toxic due to their low hydrophobicity, whereas unconjugated hydrophobic secondary bile acids are more cytotoxic.[10] Diverse cellular mechanisms including membrane disruptions, role of PKC, MAPK pathways, and nuclear factor-kappa β activation have been proposed for toxicity of bile acids.[11] Stenson and coworkers suggested the enantiospecific bile-acid mediated apoptosis in colon cancer cells.[12] Natural bile acids induce apoptosis, escalated cellular detachment and enhanced caspases-3 and -9 cleavage as compared to synthetic enantiomers of bile acids in HT-29 and HCT-116 cells.[13]

Cationic lipid amphiphiles and their drug conjugates have been explored for potential biological activities as anticancer and antibacterial candidates as they have ability to interact with cellular membranes effectively due to their charged hydrophobic character.[14] Chikkara *et al.* synthesized lipophilic derivatives of anticancer drug doxorubicin for anticancer therapy[15] and Emtricitabine based prodrugs for enhanced anti-HIV activity.[16] Banerjee and co-workers synthesized haloperidol conjugated cationic lipids[17] and lipids targeting estrogen receptors for target delivery to cancer cells.[18] Sreekanth *et al* synthesized charged bile acid tamoxifen conjugates for breast cancer therapy and revealed that conjugation of multiple tamoxifen molecules to cationic cholic acid induces membrane perturbations and enhanced activity.[19] We have recently shown that conjugation of single-charged head groups on bile acids induces favorable interactions with model membranes and cancer cells for their cytotoxicity, whereas multi-headed amphiphiles does not interact with mammalian cells due to enhanced hydration barrier between amphiphiles and cellular membranes.[20]

In this paper, we hypothesize that anticancer activity of amphiphiles is contingent on nature of the single-charged head group on lithocholic acid amphiphile. Therefore, we synthesized ten lithocholic acid based amphiphiles differing in nature of the charged head group attached to hydroxyl group of lithocholic acid. Anticancer activities of these amphiphiles were investigated against three human colon cancer cell lines. Annexin-FITC and cell cycle analysis were performed to study the mechanism of cellular death by these amphiphiles. We then evaluated *in vivo* anticancer potential of amphiphiles in tumor xenograft models, and analyzed the mechanism of activity in tumors.

Results and discussion:

Synthesis of amphiphiles: We synthesized and characterized ten amphiphiles (**Fig. 1**) differing of charged head group using lithcholic acid. All the lithocholic acid amphiphiles were synthesized and characterized (except **LCA-PPZ$_1$**, **LCA-MOR$_1$**, **LCA-TMOR$_1$**) as described previously.[20] Amphiphiles **LCA-PPZ$_1$**, **LCA-MOR$_1$**, **LCA-TMOR$_1$** were synthesized by reaction of chloroacetyl derivative of

lithocholic acid methyl ester with corresponding amine, and purified by column chromatography as described in experimental section. All the synthesized amphiphiles were purified and characterized by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry, and HPLC (Waters, RI-410).

Anticancer activities in colon cancer cell lines (MTT Assay): We examined the cytotoxic effect of these amphiphiles against three human colon cancer cell lines (HCT-8, HCT-116 and DLD-1) at 48h. We discovered that introduction of soft charge ammonium head group (**LCA-AMM$_1$**) on lithocholic acid enhances its cytotoxicity by 3-6 fold (**Fig. 2, Table 1**). Other soft charge modifications on lithocholic acid like piperazine (**LCA-PPZ$_1$),** morpholine **(LCA-MOR$_1$),** thiomorpholine **(LCA-TMOR$_1$)** are not effective in enhancing the cytotoxic potential of lithocholic acid (**Fig. 2, Table 1**). Further quaternization of lithocholic acid with trimethyl ammonium head group (**LCA-TMA$_1$**) lowers its cytotoxicity as compared to ammonium head group amphiphiles. Amphiphile **LCA-PIP$_1$** bearing piperidine head group is most active with IC$_{50}$ value of ~12-15 µM in HCT-116, HCT-8 cells and DLD-1 cells (**Fig. 2**). Amphiphile **LCA-PIP$_1$** is nearly 6-10 fold more cytotoxic as compared to lithocholic acid **LCA**, and 3-fold more effective than amphiphile **LCA-AMM$_1$** bearing ammonium head group (**Table 1**). Structure-activity studies revealed that aliphatic 6-membered head group based amphiphile **LCA-PIP$_1$** is 2-3 fold more cytotoxic as compared to aromatic head group based amphiphile **LCA-PYR$_1$** and 5-membered aliphatic derived amphiphile **LCA-PYROL$_1$** as well. Introduction of a hydrophobic 6-membered piperidine head group allows its efficient interactions with intra-cellular targets making it more effective. Therefore, structure-activity studies conclude that anticancer activities of amphiphiles are strongly contingent on nature of the head group. As bile acids are known to bind with cell membrane TGR5 receptors, or nuclear FXR receptors; nature of the charged head group may help in preferential interactions of these amphiphiles with cell surface membrane receptors or intracellular receptors. We believe that nature of the head group may also help in preferential activation of death

receptors, or intracellular proteins responsible for apoptosis; as these amphiphiles induce apoptosis by intrinsic and extrinsic pathways as described later.

Selectivity over normal cells: We then studied the selectivity of most potent amphiphile **LCA-PIP$_1$** over normal cells, and investigated the toxicity of amphiphile against macrophages (THP-1), CHO and A549, and Red blood cells. As shown in **Fig. 3**, amphiphile **LCA-PIP$_1$** is five times less toxic in normal macrophages as compared to colon cancer cells; and ~3-fold more selective for colon cancer cells over CHO cells. **LCA-PIP$_1$** is also ~2-fold more selective over lung cancer A549 cells (**Fig. 3**). As these are charged amphiphiles, we investigated the membrane disruptive character of amphiphile **LCA-PIP$_1$** and performed the hemolytic assay. Amphiphile **LCA-PIP$_1$** was found to be non-hemolytic against RBCs with MHC$_{50}$ > 1mM concentration.[20b] These results suggest that lithocholic acid derived amphiphile **LCA-PIP$_1$** is inducing the apoptotic machinery in colon cancer cells for its activity, and does not disrupt the cellular membranes in a detergent manner for its activity. We therefore investigated the mechanism of cell death induced by amphiphile **LCA-PIP$_1$** against colon cancer cells.

Effect of Ester and Amide linkages: In **LCA-PIP$_1$** amphiphile, piperidine head group is conjugated to lithocholic acid through ester linkage. To explore the effect of linkage between piperidine head group and lithocholic acid, we synthesized an amide derivative of **LCA-PIP$_1$** and compared the cytotoxicity of ester and amide derivatives of **LCA-PIP$_1$** against three colon cancer cell lines (**Fig. S1**, ESI). MTT studies suggested that ester derivative of **LCA-PIP$_1$** is more effective than amide derivative of **LCA-PIP$_1$ (Fig. S1**, ESI**)** that might be due to easy cleavage of piperidine head group for enhanced activity.

Colony forming assay: We then studied colony-forming ability of colon cancer cells on treatment with **LCA** and **LCA-PIP$_1$** at different concentrations. Treatment of **LCA** on these cells does not inhibit colony formation at 15, 25 and 50 μM conc. whereas **LCA-PIP$_1$** on treatment at 15μM induces ~50% inhibition in HCT-116 cells (**Fig. 4a**). We have not observed any colony on treatment with 50μM of **LCA-PIP$_1$**.

Similarly treatment of **LCA-PIP$_1$** inhibits colony formation in DLD-1 and HCT-8 colon cancer cells (**Fig. 4b, c**). These studies conclude that introduction of single piperidine head group on **LCA** makes it highly active and inhibit colony-forming properties of cancer cells.

Cell cycle and Annexin-FITC analysis: We then performed cell cycle analysis to know the fate of cells in different phases of cell cycle on treatment with **LCA** and **LCA-PIP$_1$** at different concentrations for 48h. Cell cycle analysis revealed that **LCA** alone does not induce any concentration dependent change in cell cycle in HCT-116, DLD-1 and HCT-8 cells, whereas treatment of **LCA-PIP$_1$** arrests cells in sub-G$_0$ phase and does not allow them to enter in G$_0$-G$_1$ phase (**Fig. 5**). **LCA-PIP$_1$** at 50μM induces ~80% arrest in sub-G$_0$ phase in HCT-116 and DLD-1 cells, and ~40% arrest in HCT-8 cells. To ensure whether arrest in cell cycle phase by **LCA-PIP$_1$** induces apoptosis, we probed **LCA** and **LCA-PIP$_1$** treated HCT-116 cells with Annexin-V-FITC apoptosis assay. As shown in **Fig. 6**, treatment of **LCA-PIP$_1$** induces 1.3 and 2.0-fold increase in apoptotic cells at 25μM and 50μM conc. as compared to **LCA**. **LCA-PIP$_1$** at 50μM induces ~40% cells to early apoptosis and ~26% cells to late apoptosis. Therefore, cell cycle and Annexin-FITC studies conclude that introduction of a piperidine head group on **LCA** in case of **LCA-PIP$_1$** enhances sub-G$_0$ arrest of cells and induces apoptosis.

***In vivo* anticancer activities:**

HCT-116 Nude mice colon tumor model: To explore *in vivo* potential of **LCA-PIP$_1$** in xenograft tumor model, we studied anticancer activity of **LCA-PIP$_1$** in xenograft tumor model in NUDE mice. We used HCT-116 cancer cells in NUDE mice to develop tumors on flank. To test the potential of **LCA-PIP$_1$** in larger tumor volumes, we developed tumors of ~750 mm^3 as compared to ~100-200 mm^3 tumors usually reported in the studies. Single injection of 20 mg/kg body weight is able to reduce tumor volume by 75% as shown in **Fig. 7**. We observed more than 50% decrease in tumor weight after 6-days of

treatment. These studies conclude that introduction of a single piperidine head group on **LCA** make it highly potent anticancer agent.

***In vitro* and *in vivo* mechanism**: To unravel the mechanism of cell death, we performed western studies in HCT-116 cells on treatment with **LCA** and **LCA-PIP$_1$** to see the expression of caspases required for apoptosis. Western studies showed increased expression of pro-caspase 8 on **LCA-PIP$_1$** treatment at low concentrations, which gets decreased at high concentration (**Fig. 8a**). We observed increased levels of cleaved casapase-7 and caspase-3 on **LCA-PIP$_1$** treatment. To determine the mechanism involved in tumor regression upon treatment with **LCA-PIP$_1$**, we harvested tumors from mice and subjected them to western blot analysis for expression of caspases (**Fig. 8b**). We observed the elevated expression of activated (cleaved) caspases 3, 7 and 8 in **LCA-PIP$_1$** treated tumors as compared to vehicle control group (**Fig. 8b**). Induction of apoptotic pathway leads to cascade of events, releasing various apoptotic mediators from mitochondria, and activation of caspase-3 and caspase-7 required for apoptosis.[21,22] Caspase 3 that is critical for apoptosis by intrinsic apoptotic pathway and activation of caspase 8 critical for extrinsic apoptotic pathway.[23] Therefore these studies conclude that amphiphile **LCA-PIP$_1$** induces tumor regression through activation of intrinsic and extrinsic pathways.

Conclusions: In summary, we have shown that conjugation of charged head groups on lithocholic acid modulates their interactions with cancer cells. Anticancer potential of these amphiphiles is contingent on nature of the charged head groups. Piperidine head group based amphiphile **LCA-PIP$_1$** is most active in as compared to **LCA** suggesting the effective intracellular interactions with targets responsible for inducing apoptosis. Annexin-FITC studies and cell cycle studies suggest induction of apoptosis and sub-G$_0$ cell cycle arrest by **LCA-PIP$_1$**. Single dose of **LCA-PIP$_1$** could induce ~75% reduction in tumor volume and ~50% reduction in tumor weight for high aggressive HCT-116 tumor models. Western studies on tumors indicated the activation of caspases for inducing apoptosis. As these molecules are

based on based on bile acids, further modification of these molecules would have future applications for treatment of different gastrointestinal cancers. Amphiphilic character of these molecules can further be explored to form nanoparticles and its delivery. Therefore, engineering of these lithocholic acid amphiphiles open up a new class of molecules as therapy for colon cancer.

Experimental Section:

Materials and methods: All the chemicals and solvents used are of ACS grade. Lithocholic acid (LCA), Propidium iodide, Annexin-FITC kit was purchased from Sigma-Aldrich. All antibodies were purchased from Cell Signaling, except β-actin that was purchased from Sigma. MTT and ECL kit was purchased from Amersham. Cell culture media, trypsin, and antibiotics were purchased from Hyclone, USA or Sigma-Aldrich. Plasmocin prophylactic and plasmocin treatment was purchased from invivogen. All synthesized compounds were purified using Combi-flash or glass chromatography using 230-400 mesh size silica gel. ^{1}H and ^{13}C NMR spectra were recorded using Bruker 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm with tetramethyl silane as internal standard. High-resolution mass spectra were measured on LC-MS/MS (AB-SCIEX TRIPLE TOF-5600) and MALDI (AB SCIEX TOF/TOF 5800) mass spectrometer.

General procedure for the synthesis of cationic amphiphiles: Lithocholic acid amphiphiles were synthesized and characterized (except **LCA-PPZ₁**, **LCA-MOR₁**, **LCA-TMOR₁**) from chloroacetyl derivative of lithocholic acid methyl ester as described previously.[20] Amphiphiles **LCA-PPZ₁**, **LCA-MOR₁**, **LCA-TMOR₁** were synthesized from chloroacetyl derivative of lithocholic acid methyl ester[20] as described below.

Amphiphile LCA-PPZ₁,: Piperazine (86 mg, 1 mmol) was added to a solution of chloroacetyl derivative of lithocholic acid methyl ester (200 mg, 0.42 mmol) in ethyl acetate (5 mL) and K_2CO_3 in a sealed tube and reaction mixture was refluxed for 36 h. After completion of reaction, reaction mixture

was filtered to remove K_2CO_3. Solvent was removed by evaporation, and the product was purified by column chromatography using EtOAc : Pet Ether (20:50) as a colorless solid (185 mg, 85%; R_f = 0.40, EtOAc : Pet Ether, 50: 50); ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 0.63 (s, 3H, -CH$_3$), 0.91 (s, 6H, 2 x CH$_3$), 0.99-2.23 (steroid), 2.67(s, 4H, -N-(CH$_2$)$_2$), 3.19 (m, 2H, -CO-CH$_2$-N-), 3.66 (s, 3H, -CO-OCH$_3$), 4.78 (s, 1H, -O-CH); ^{13}C-NMR (CDCl$_3$, 100 MHz) δ: 12.04, 18.28, 20.84, 23.30, 24.19, 26.32, 26.66, 27.01, 28.19, 29.70, 31.23, 34.60, 35.01, 35.79, 40.13, 40.41, 42.74, 51.49, 52.68, 55.99, 56.48, 74.92, 169.59, 174.78; MALDI Mass: m/z ($C_{31}H_{52}N_2O_4$) calculated 516.39; found (M)$^+$ 517.402.

Amphiphile LCA-MOR$_1$: To a solution of chloroacetyl derivative of lithocholic acid methyl ester (250 mg, 0.53 mmol) in ethyl acetate (5 mL) was added K_2CO_3 and Morpholine (95 mg , 1.1 mmol) in a sealed tube and the mixture was refluxed for 48h. After completion of reaction, K_2CO_3 was removed by filtration and solvent was removed by evaporation. Final product was purified by column chromatography using EtOAc : Pet Ether (20:50) as a colorless solid (246 mg, 90%; R_f = 0.35, EtOAc : Pet Ether, 50: 50); ^{1}H-NMR (CDCl$_3$, 400 MHz) δ: 0.63 (s, 3H, -CH$_3$), 0.92 (s, 6H, 2 x CH$_3$), 0.99-2.38 (steroid), 2.59 (s, 4H, -N-(CH$_2$)$_2$), 3.18 (m, 2H, -CO-CH$_2$-N-), 3.66 (s, 3H, -CO-OCH$_3$), 3.76 (t, J = 4.4, 4H, -N-(CH$_2$)$_2$), 4.78 (s, 1H, -O-CH); ^{13}C-NMR (CDCl$_3$, 100 MHz) δ: 12.04, 18.28, 20.84, 23.31, 24.19, 26.32, 26.67, 27.01, 28.19, 29.70, 31.01, 31.07, 32.25, 34.60, 35.00, 35.38, 35.79, 40.14, 40.43, 41.91, 42.74 51.50, 53.25, 56.00, 56.50, 59.97, 66.75, 74.97, 74.92, 169.50, 174.78; MALDI Mass: m/z ($C_{31}H_{51}NO_5$) calculated 517.38; found (M)$^+$ 518.377.

Amphiphile LCA-TMOR$_1$: Reaction mixture of chloroacetyl derivative of lithocholic acid methyl ester (300 mg, 0.64 mmol) in ethyl acetate (6 mL), K_2CO_3 (300 mg) and thiomorpholine (124 mg , 1.2 mmol) was refluxed in a sealed tube for 40 h. Solid K_2CO_3 was removed from reaction mixture and solvent was removed by evaporation. The product was purified by column chromatography using EtOAc : Pet Ether (20:50) as a colorless solid (310 mg, 91%; R_f = 0.62, EtOAc : Pet Ether, 50: 50); ^{1}H-NMR (CDCl$_3$, 400

MHz) δ: 0.63 (s, 3H, -CH$_3$), 0.91 (s, 6H, 2 x CH$_3$), 0.99-2.33 (steroid), 2.72 (s, 4H, -S-(CH$_2$)$_2$), 2.84 (d, J = 4.4, 4H, -N-(CH$_2$)$_2$) 3.20 (s, 2H, -CO-CH$_2$-N-), 3.66 (s, 3H, -CO-OCH$_3$), 4.77 (s, 1H, -O-CH); ^{13}C-NMR (CDCl$_3$, 100 MHz) δ: 12.04, 18.27, 20.84, 23.31, 24.18, 26.32, 26.71, 27.00, 27.81, 28.18, 29.69, 31.01, 31.07,3 32.28, 34.59, 35.00, 35.37, 35.79, 40.13, 40.44, 41.92, 42.74, 51.48, 54.53, 56.00, 56.49, 60.51, 74.94, 169.75, 174.74; MALDI Mass: m/z (C$_{31}$H$_{51}$NO$_4$S) calculated 533.35; found (M)$^+$ 534.284.

Amide derivative of LCA-PIP$_1$: Amino derivative of lithocholic acid was synthesized as described previously.[24] Amide derivative of **LCA-PIP$_1$** was synthesized using amino derivative of lithocholic acid (LCA-NH$_2$) with similar procedures.[20] Yield 80% (solid). **^{1}H-NMR** (CDCl$_3$, 400 MHz) δ: 0.62 (d, J=5.6, 3H, -CH$_3$), 0.90 (s, 6H, -2 x CH$_3$), 0.99-2.37 (steroid), 3.39 (s, 3H, -N-CH$_3$); 2H, -N-CH$_2$), 3.65 (s, 3H, -CO-OCH$_3$), 3.91 (m, 2H, -N-CH$_2$), 4.69 (m, 2H, -CO-CH$_2$-N-); ^{13}**C-NMR** (CDCl$_3$, 100 MHz) δ: 12.03, 18.27, 20.16, 20.87 23.38, 23.64, 24.18, 26.27, 27.07, 28.16, 30.56, 32.75, 34.58, 35.37, 35.64, 40.00, 40.17, 42.73, 50.92, 51.46, 55.97, 56.62, 62.69, 71.84, 162.86, 174.86; **MALDI Mass**: m/z (C$_{33}$H$_{57}$N$_2$O$_3$)$^+$ calculated 529.44; found (M)$^+$ 529.431.

Cell cultures: HCT-116, DLD-1, HCT-8, A549 and CHO cell lines were maintained as monolayers. HCT-116 cells in McCoy`s medium, DLD-1 and HCT-8 cells in RPMI-1640 medium, and A549 and CHO cells in DMEM media with 10% (v/v) fetal bovine serum, and antibiotics were maintained at 37°C in a humidified atmosphere with 5% CO$_2$. THP-1 cells were grown in DMEM media and differentiated to adherent macrophages by adding Phorbol myristic acid (PMA).

Cytotoxicity Assay:[25] MTT studies were performed for anticancer activities of all amphiphiles in three different colon cancer cell lines. Cells were plated in 96 well plates at a density of 3-4 x 10^3 cells per well for 24h for their adherence. Cells were treated at different concentrations of 25, 50, 100, 150 and 200μM of synthetic lithocholic acid amphiphiles for 48h. MTT solution (25μL of 5mg/mL) was added to cells for incubation to get formazan crystals. After 3h of incubation, media was replaced with 150μL of DMSO to

lyse the cells. Absorbance of formazen crystals was recorded at 555nm using spectramax M5 (Molecular devices). Cell viability was then calculated using equation [{A_{555} (treated cells) - background]/[A_{555} (untreated cells) - background}] 100.

Colony formation assay:[26] Colony formation studies were performed with colon cancer cells on treatment with **LCA** and **LCA-PIP$_1$** at different concentrations. 200 cancer cells per well were plated in a 6-well plate. After adherence (24h), cells were treated with different conc. of **LCA** and **LCA-PIP$_1$**. After 2-weeks, cells were stained with crystal violet and colonies were counted. From the number of colonies, we first calculated the plating efficacy (PE) from untreated samples as

$$PE = \frac{number\ of\ colonies\ formed}{number\ of\ cells\ seeded}\ X\ 100$$

The number of colonies after treatment of cells, called survival fraction, is calculated using following equation:

$$SE = \frac{number\ of\ colonies\ formed\ after\ treatment}{number\ of\ cells\ seeded\ X\ PE}$$

Cell cycle and Annexin-FITC analysis: In a 6-well plate, colon cancer cells at a density of ~2 x 10^5 cells/well were seeded. After 24h, cells were treated with amphiphiles for 48h. Cells were trypsinized and collected by centrifugation after treatment. For cell cycle analysis, cells were washed twice with cold PBS and fixed in 70% ice-cold ethanol. Ethanol was removed by washing the cells again with PBS and cells were treated with RNase (10μL of 20 mg/ml) at 37°C for 1h. Cells were stained with propidium iodide (50 μg/ml) at room temperature for 20 min and counted on a FACS (Becton Dickinson,

Mountain View, CA). Annexin-FITC studies were performed using kit from Sigma-Aldrich according to manufacturer's instructions. Cells were stained simultaneously with FITC labeled Annexin V (50µg/mL) and propidium iodide (100µg/mL) after their re-suspension in binding buffer and analyzed using a flow cytometer (Becton Dickinson).

***In vivo* experiments:** 6 weeks old female NUDE mice were received from Institutional Animal Facility and were allowed to acclimatize to facility conditions before any experimental procedure. All the protocols for animal experiments were approved from Institutional Animal Ethical Committee of National Institute of Immunology. Mycoplasma testing was performed in the cells to make sure that cells were free of any sort of contamination. We injected 1.5×10^6 HCT-116 cells/mice in right flank of each mice to generate tumor models. Tumors became palpable after 7 days and we started measuring tumor sizes after 10th day. Digital calipers were used for measuring tumor sizes throughout the study. Tumor volume was calculated using the formula: Volume = 0.5 L x W^2, where W = width of tumor, L = length of tumor. Once the tumors attained an average volume of ~750 mm^3, we divided mice into two groups of 5 mice each. On the 19th day, we started treatment of mice with either vehicle control or amphiphile **LCA-PIP$_1$** at dose of 20 mg/kg near tumor sites. Tumors measurements were performed regularly after giving treatment and eventually mice were sacrificed on 25th day.

Western blot studies in cell lysates and tumor samples: Tumors were harvested from control and treated NUDE mice. These tumors were then subjected to homogenization using RIPA lysis buffer. Tumor lysates were loaded in equal concentration and then electro-transferred onto polyvinylidene fluoride membranes. Blots were then blocked with 5% non-fat dry milk (NFDM) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2h at room temperature. Blots were washed three times with TBST and incubated overnight at 4°C, with specific primary antibodies in TBST containing 2% NFDM. Next day membranes were washed three times with TBST and incubated for 2h at room

temperature with secondary horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG in TBST containing 2% NFDM. Detection was performed using enhanced chemiluminescence reagent (ECL) and a bioluminescent image analyzer LAS-4000. Western blots were performed from lysates originating from at least two different tumors. Similarly, western blot studies were performed using cell lysates.

Supporting Information: Comparison of cytotoxicity of ester and amide derivatives of **LCA-PIP$_1$**. Characterization of final compounds **LCA-PPZ$_1$**, **LCA-MOR$_1$**, **LCA-TMOR$_1$**. HPLC profiles of all amphiphiles.

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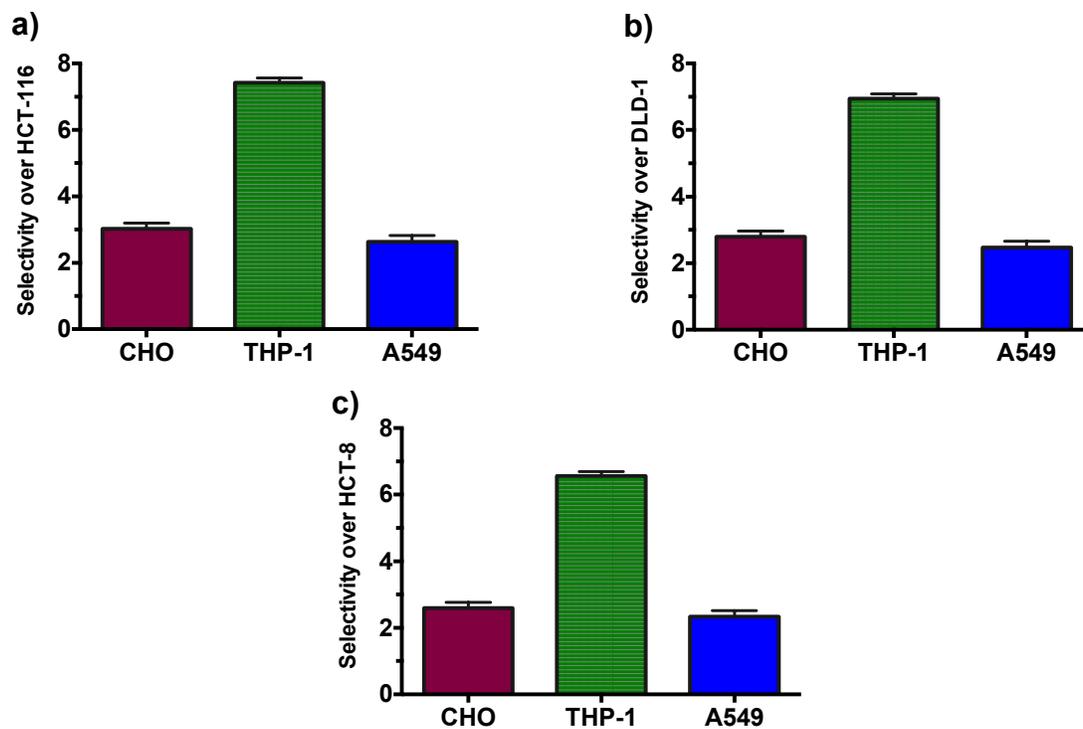
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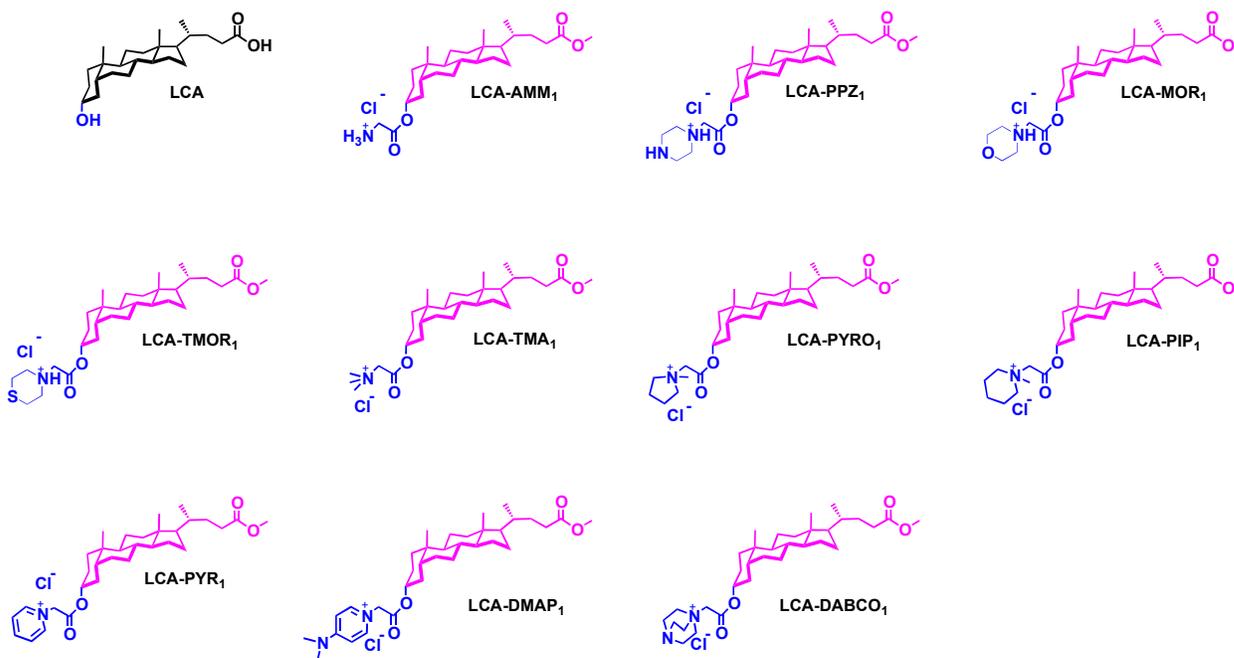
**Fig. 1.** Molecular structures of lithocholic acid amphiphiles synthesized and studied.
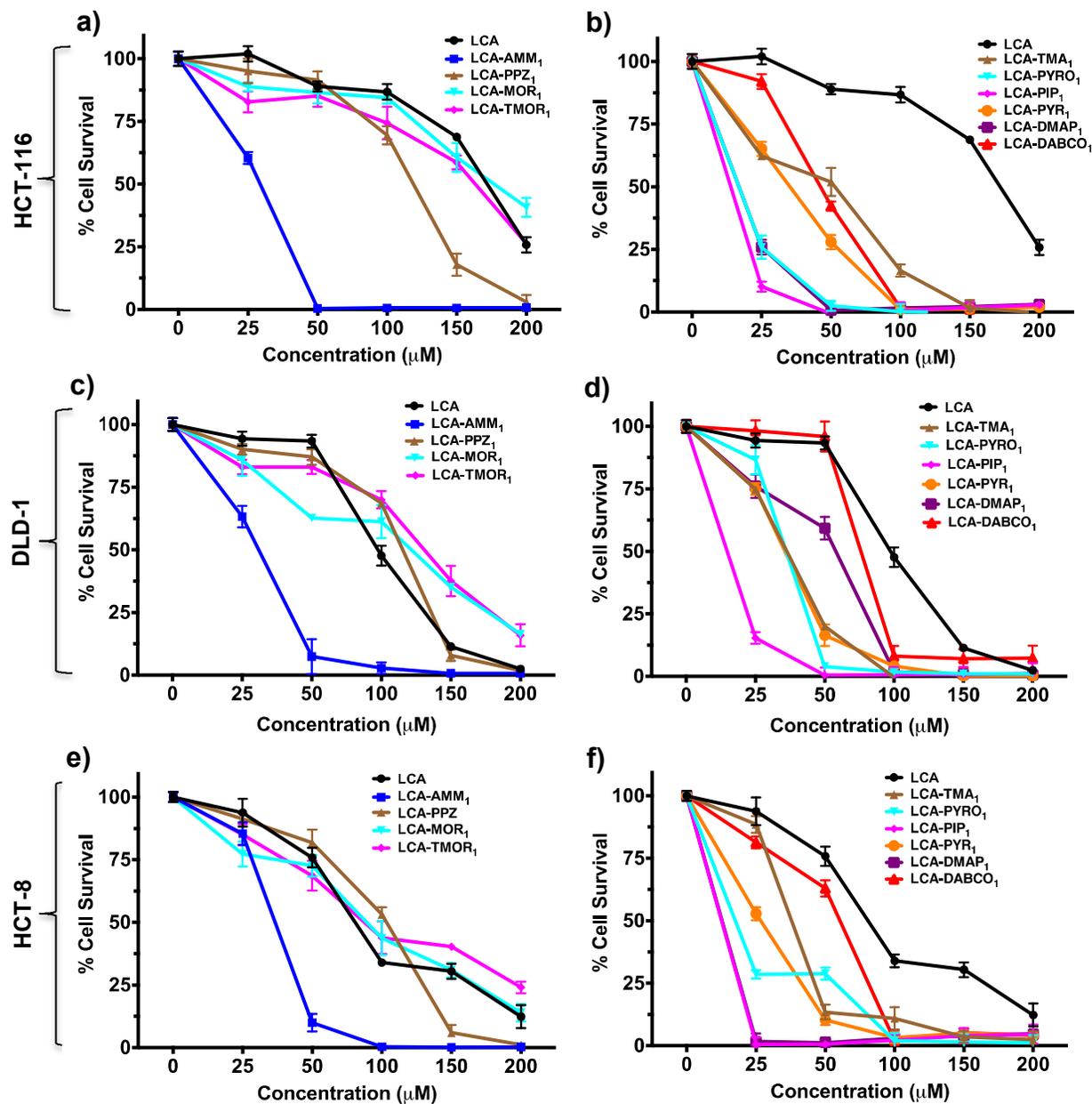
Fig. 2. a-f) Structure-activity investigation showing IC_{50} values of different amphiphiles in three colon cancer cell lines **a-b**) HCT-116, **c-d**) DLD-1, and **e-f**) HCT-8. All experiments have been performed at least two times in four replicates and IC_{50} values reported are mean $\pm$ SD.

Fig. 3. Selectivity of **LCA-PIP$_1$** for different colon cancer cell lines **a**) HCT-116, **b**) DLD-1, **c**) HCT-8) over CHO, THP-1 and A549 cell lines.
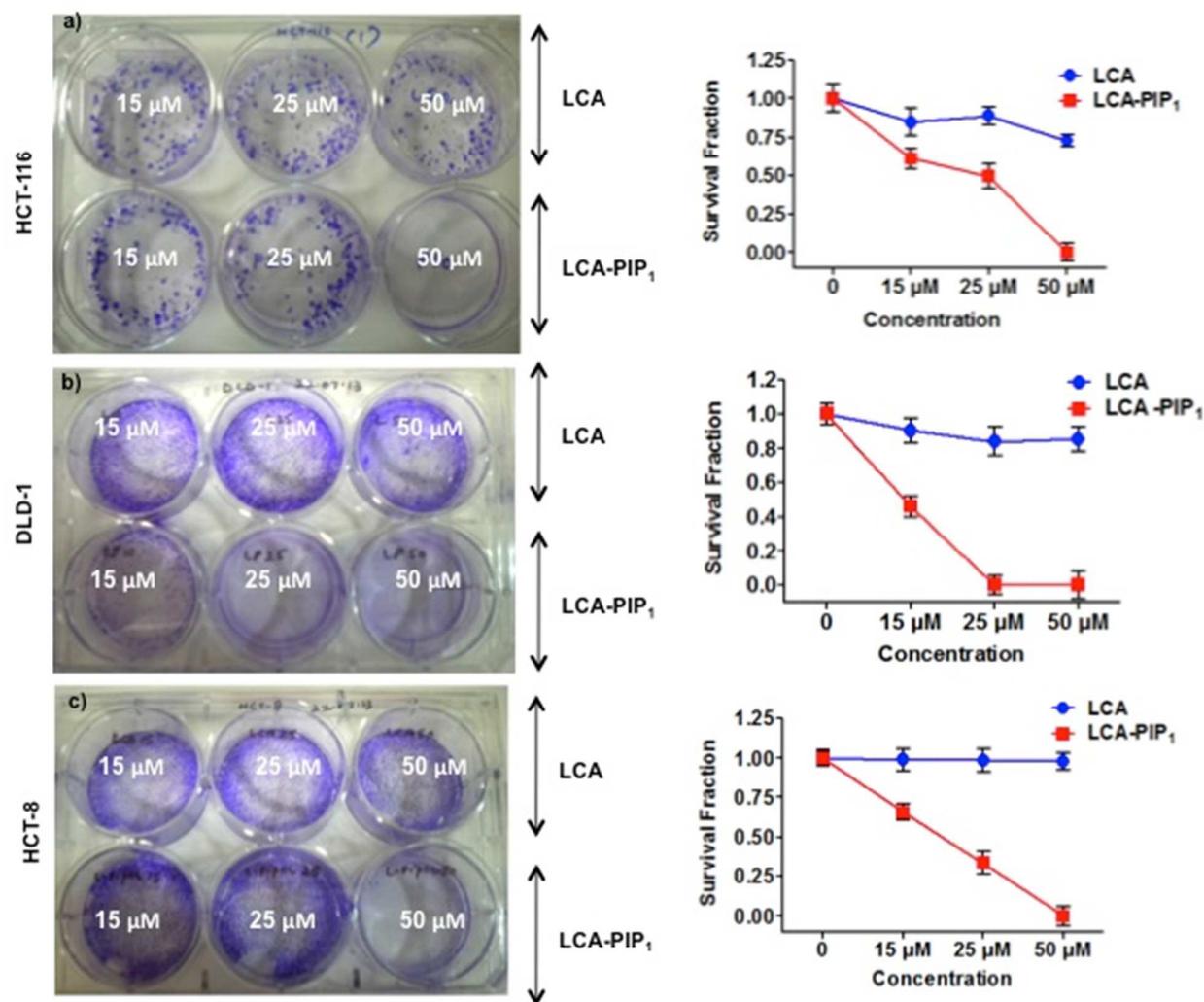
Fig. 4. Colony forming assay showing effect of **LCA** and **LCA-PIP$_1$** at different concentrations on colony forming abilities and survival fraction of **a**) HCT-116, **b**) DLD-1, and **c**) HCT-8 cells. All experiments were done two times in duplicates and survival fraction values are reported as mean $\pm$ SD.
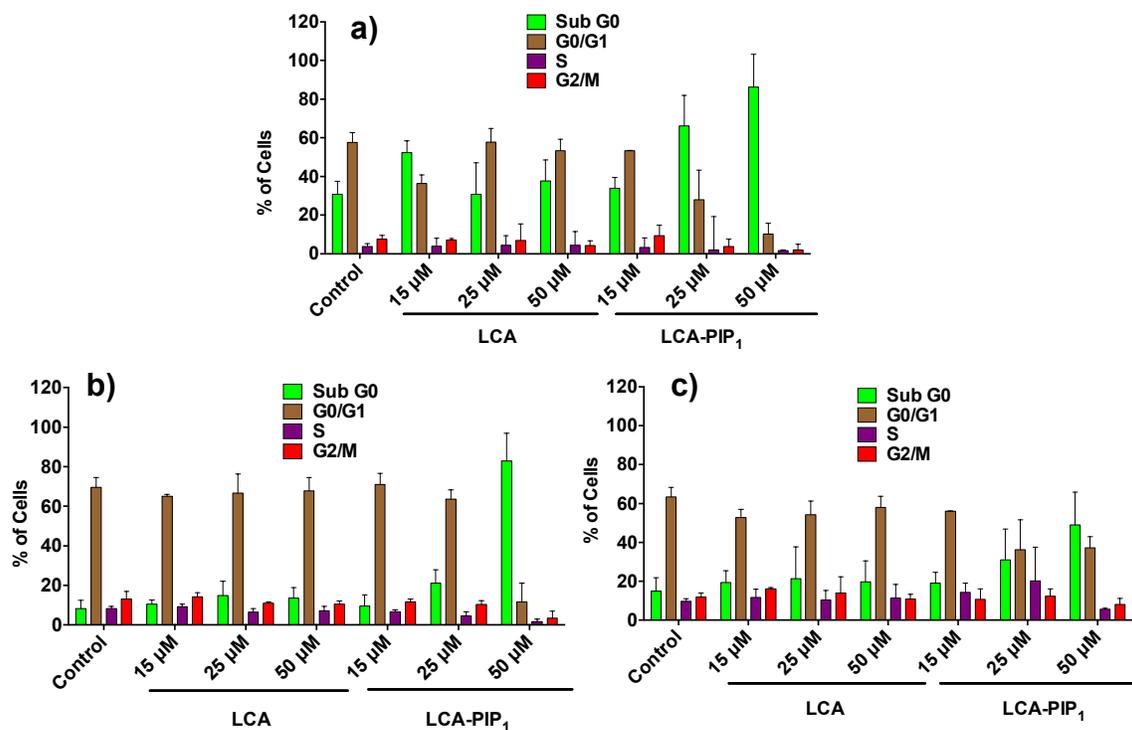
Fig. 5. Cell cycle analysis of **a)** HCT-116, **b)** DLD-1, and **c)** HCT-8 cells treated with **LCA** and **LCA-PIP$_1$** at different concentrations of 15, 25 and 50μM for 48h showing enhanced sub-G$_0$ arrest on treatment with **LCA-PIP$_1$**. All experiments were performed in triplicates, and values are reported as mean $\pm$ SD.
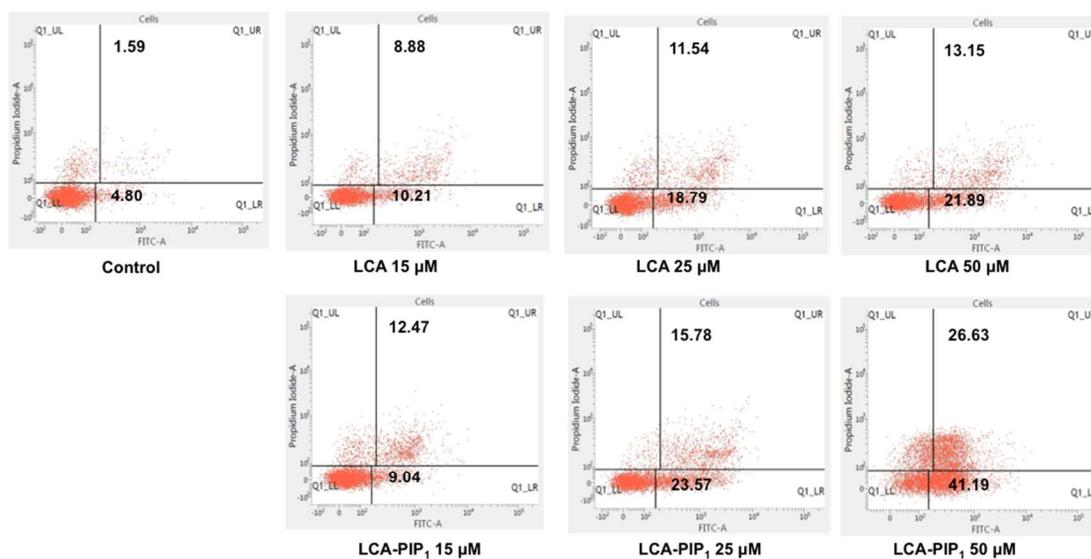
Fig. 6. Annexin-V/FITC apoptosis assay graphs in HCT-116 cells treated with **LCA** and **LCA-PIP$_1$** at different concentrations of 15μM, 25μM, 50μM for 48h showing enhanced apoptosis by **LCA-PIP$_1$**.
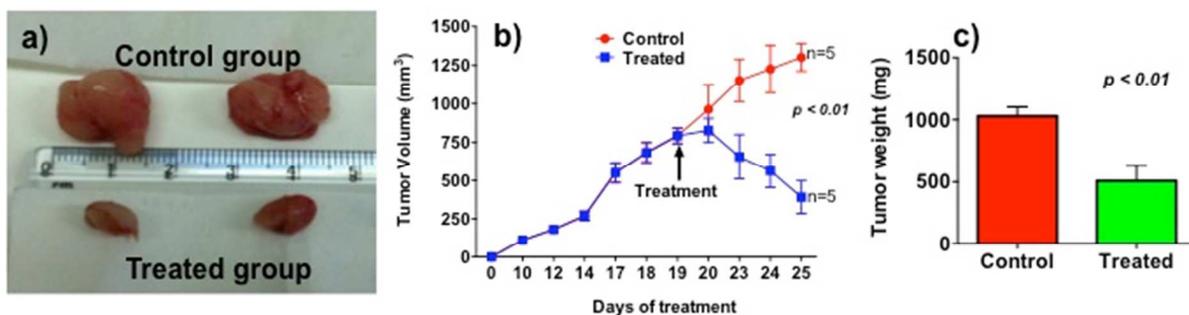
Fig. 7. *In vivo* anticancer effect of **LCA-PIP$_1$** in HCT-116 tumor models in NUDE mice, **a**) representative excised tumors from control and treated group; **b**) change in tumor volume on treatment with **LCA-PIP$_1$**; **c**) change in tumor weight after treatment with **LCA-PIP$_1$**.
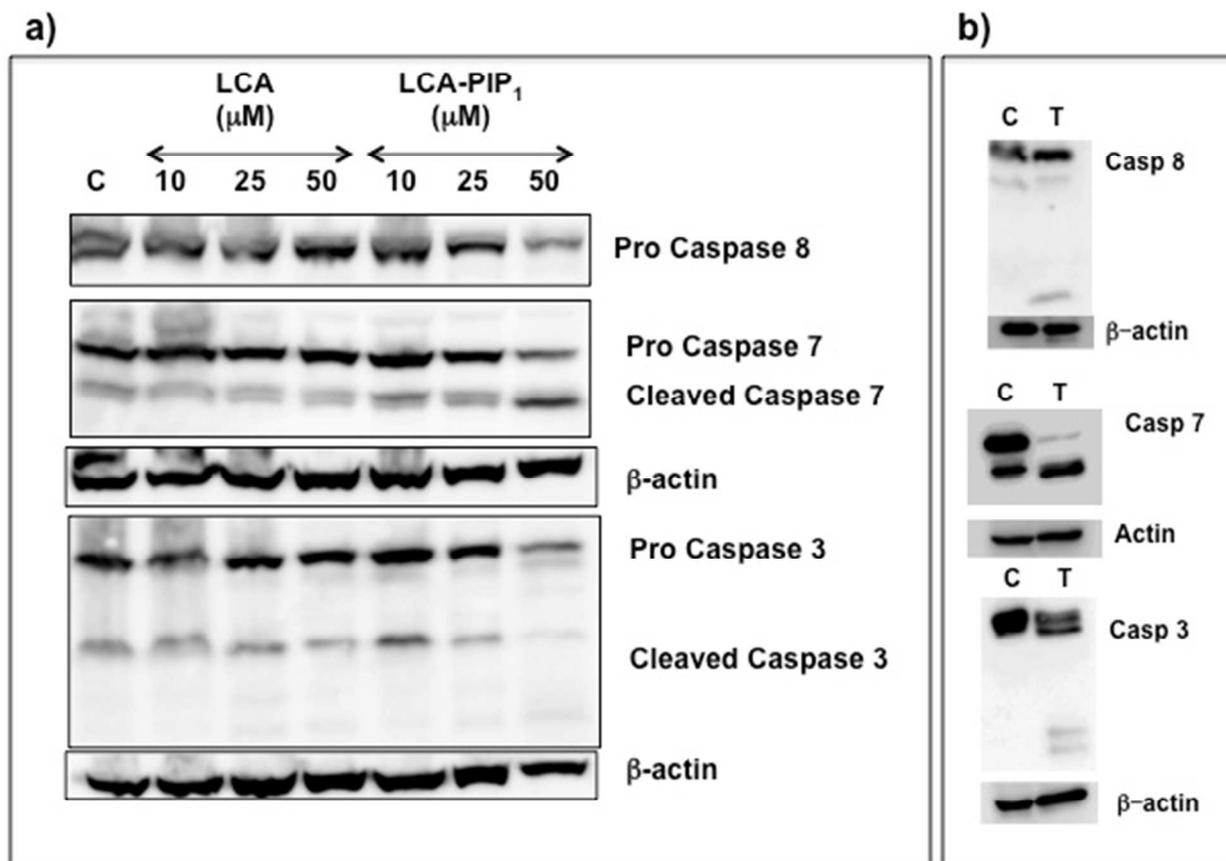
Fig. 8. a) Representative western blots of lysates of **LCA** and **LCA-PIP₁** treated HCT-116 cells, **b)** Representative western blots of lysates of control and **LCA-PIP₁** treated tumors showing the activation of caspases.

Table 1: IC_{50} of lithocholic acid amphiphiles againt three colon cancer cell lines, HCT-116, DLD-1 and HCT-8.[a]

Amphiphile	HCT-116 (µM)	DLD-1 (µM)	HCT-8 (µM)	HPLC, RT[b] (min)
LCA	81.1 ± 3.8	173.1 ± 3.0	97.4 ± 2.5	-[c]
LCA-AMM$_1$	36.7 ± 3.4	29.4 ± 2.3	30.7 ± 6.9	-[d]
LCA-PPZ$_1$	102.0 ± 4.3	118.4 ± 4.5	114.6 ± 4.1	-[d]
LCA-MOR$_1$	89.0 ± 2.7	176.2 ± 3.8	120.9 ± 3.4	24.17
LCA-TMOR$_1$	87.3 ± 3.1	163.2 ± 2.7	130.5 ± 4.1	28.63
LCA-TMA$_1$	38.1 ± 2.9	53.0 ± 1.0	37.0 ± 2.3	7.94
LCA-PYRO$_1$	17.8 ± 2.4	17.0 ± 4.6	36.4 ± 2.9	8.23
LCA-PIP$_1$	11.7 ± 3.0	13.7 ± 1.0	14.7 ± 3.0	8.12
LCA-PYR$_1$	26.5 ± 2.0	35.4 ± 2.8	35.4 ± 4.3	7.84
LCA-DMAP$_1$	13.2 ± 3.0	17.0 ± 2.9	59.1 ± 4.5	7.90
LCA-DABCO$_1$	60.7 ± 1.2	46.4 ± 2.9	76.4 ± 5.9	7.98

a = IC_{50} values were calculated from MTT experiments, b = Mobile phase for all amphiphiles was AcCN:MeOH (95:5) except for **LCA-MOR$_1$** and **LCA-TMOR$_1$**, where mobile phase was AcCN. **LCA-MOR$_1$** and **LCA-TMOR$_1$** stock solutions were made in 10% EtOAc in AcCN. c = not determined, d = could not be determined as this amphiphile is soluble in DMSO only.

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