Bioorganic & Medicinal Chemistry 18 (2010) 6886–6895

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Bile acid toxicity structure–activity relationships: Correlations between cell viability and lipophilicity in a panel of new and known bile acids using an oesophageal cell line (HET-1A)

Ruchika Sharma [a,b,*], Ferenc Majer [a], Vijaya Kumar Peta [a], Jun Wang [a], Ray Keaveney [a], Dermot Kelleher [b], Aideen Long [b], John F. Gilmer [a,*]

[a] School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland
[b] Cell Signalling, Institute of Molecular Medicine, Trinity Centre for Health Science, St. James's Hospital, Dublin 8, Ireland

ARTICLE INFO

Article history:
Received 25 March 2010
Revised 12 July 2010
Accepted 13 July 2010
Available online 19 July 2010

Keywords:
Bile acids
Lipophilicity
Physicochemical characteristics
Cell viability
Cytotoxicity
Azido steroid

ABSTRACT

The molecular mechanisms and interactions underlying bile acid cytotoxicity are important to understand for intestinal and hepatic disease treatment and prevention and the design of bile acid-based therapeutics.

Bile acid lipophilicity is believed to be an important cytotoxicity determinant but the relationship is not well characterized. In this study we prepared new azido and other lipophilic BAs and altogether assembled a panel of 37 BAs with good dispersion in lipophilicity as reflected in RPTLC R_{Mw}. The MTT cell viability assay was used to assess cytotoxicity over 24 h in the HET-1A cell line (oesophageal). R_{Mw} values inversely correlated with cell viability for the whole set ($r^2 = 0.6$) but this became more significant when non-acid compounds were excluded ($r^2 = 0.82$, $n = 29$). The association in more homologous subgroups was stronger still (r^2 >0.96). None of the polar compounds were cytotoxic at 500 μM, however, not all lipophilic BAs were cytotoxic. Notably, apart from the UDCA primary amide, lipophilic neutral derivatives of UDCA were not cytotoxic. Finally, CDCA, DCA and LagoDCA were prominent outliers being more toxic than predicted by R_{Mw}. In a hepatic carcinoma line, lipophilicity did not correlate with toxicity except for the common naturally occurring bile acids and their conjugates. There were other significant differences in toxicity between the two cell lines that suggest a possible basis for selective cytotoxicity. The study shows: (i) azido substitution in BAs imparts lipophilicity and toxicity depending on orientation and ionizability; (ii) there is an inverse correlation between R_{Mw} and toxicity that has good predictive value in homologous sets; (iii) lipophilicity is a necessary but apparently not sufficient characteristic for BA cytocidal activity to which it appears to be indirectly related.

1. Introduction

Bile acids (BAs) exert multiple biological effects, non-specifically on organic solutes and membranes and specifically as signalling agents by binding to membrane and nuclear receptors.[1–3] Their peculiar effects on cell proliferation (negative and positive) attract attention because of the relevance of these phenomena to the pathogenesis of cholestatic liver diseases and intestinal carcinogenesis.[4–7] An understanding of the structural basis for the apoptotic effects of BAs is important to ongoing efforts to harness this property pharmacologically in the design of selective cytocidal and cytoprotective agents.[8–12]

A specific binding target(s) for BAs in triggering apoptotic processes remains elusive but there is substantial evidence for the involvement of membrane interactions.[13] The ability to induce necrosis through detergent effects is one manifestation of this.[14] At lower concentration, BAs can induce apoptosis through extrinsic and intrinsic pathways, and through ER stress.[7] Membrane effects are important in this context too. BAs can modify membrane fluidity and composition[15–17] as well as protein mobilization and activation[18] without directly affecting barrier function.[19] Some of these effects resemble those observed with non-BA hydrophobic solutes such as cholesterol myristate and detergents *n*-octylglycoside and Triton-X, at sub-lytic concentrations.[20]

Abbreviations: GCA, glcyocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; HSA, hydrophobic surface area; HCA, hyocholic acid; NCA, nutriacholic acid; UCA, ursocholic acid; PSA, polar surface area; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

* Corresponding authors. Tel.: +353 18963350/8962844; fax: +353 18962810 (R.S.); tel.: +353 18962795 (J.F.G.).
E-mail addresses: rsharma@tcd.ie (R. Sharma), gilmerjf@tcd.ie (J.F. Gilmer).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.07.030

An influential early observation about the SAR of BA toxicity was that deoxycholate (DCA) causes more membrane damage than cholic acid (CA) and its conjugates.[21] Numerous studies have since shown that DCA, chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) have greater effects on cell viability[7] and mitochondrial function than other BAs.[22] They are also more toxic than their own glyco- and tauro-conjugates.[7,23] In partitioning experiments these BAs are lipophilic relative to other less toxic physiological BAs suggesting that lipophilicity and toxicity are associated or even correlated. The activation of PKC in vitro in constituted micelles correlated with chromatographic measures of hydrophobicity for DCA, ursodoexycholic acid (UDCA), CA and CDCA but especially with their taurine conjugates.[24] In a HCT116 cell line, growth arrest and apoptosis roughly paralleled a RPHPLC hydrophobicity ranking for a range of 16 BAs, though interestingly the behaviour of DCA and CDCA was not contiguous with the rest of the series.[25] LCA, DCA and CDCA occupy the toxic/hydrophobic end of the spectrum of common BAs but their toxicity may not be attributable to their hydrophobicity alone. In a recent study, natural bile acids LCA, CDCA and DCA were reported to initiate more apoptosis than their enantiomers *ent*-LCA, *ent*-CDCA and *ent*-DCA in HT-29 and HCT-116 cell lines. However, hydrophobic interactions are predicted to be identical in enantiomeric pairs unless the environment is itself chiral.[26,27]

In order to further explore the requirements for BA toxicity we constructed a panel of 37 naturally occurring and synthetic bile acids (Fig. 1). We sought to enrich the panel with new hydrophobic BAs. We focused especially on compounds related to UDCA and DCA being the prototypical cytoprotective and cytotoxic BAs, respectively. We were drawn in this regard towards azido analogues since the azide group is well known to enhance lipophilicity. We evaluated the relative polarity and effect on cell viability in the human oesophageal HET-1A cell line. We chose this line because of our ongoing investigation into the role of BAs in provoking oesophageal cancer. The objective of the present study was to

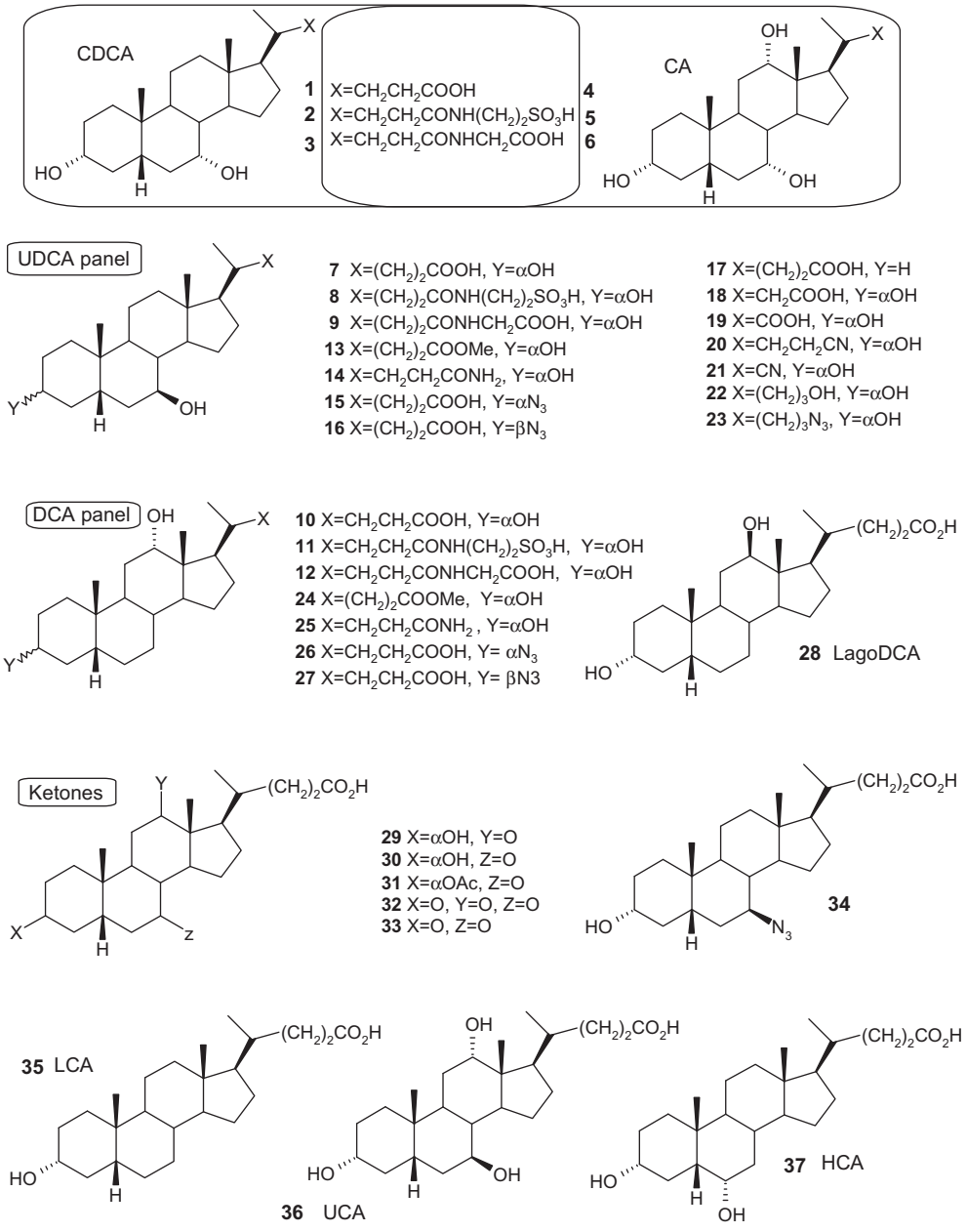


Figure 1. Structures of BAs.

characterize the relationship between cytotoxicity and polarity and to investigate if toxicity is related to functional group type and arrangement in synthetic and natural BAs.

2. Results and discussion

2.1. Synthesis

A group of 16 BAs was assembled from gifts and commercially available products. This was augmented with 21 compounds previously reported and synthesised for this study or newly prepared and characterised (compound identity synthesised using conventional methods was confirmed from references listed in Table 1). UDCA and DCA 3-azides were synthesised as outlined in Scheme 1 from the respective protected BAs **38** and **41**, which were prepared in three steps from the parent BAs. Introduction of 3α-azides (**15**, **26**) (i.e., with retention of configuration) was accomplished by generating first the β-bromides (**39**, **42**). The β-azides (**16**, **27**) could be obtained by direct SN2 substitution on the α-mesylates (**40**, **43**). Similarly, the 7-β azido analog of UDCA (**34**) was generated by treating the appropriately protected CDCA-derived mesylate (**45**) with NaN_3 in DMPU followed by deprotection in aqueous base. The 24-azido UDCA (**23**) was prepared by $LiAlH_4$ reduction of UDCA to alcohol **22** followed by regioselective mesylation of the primary alcohol in cold solvent. Chromatographic isolation yielded the 24-mesylate (**46**) (and 3α, 24 dimesyloxy UDCA) in pure form after which azide substitution with NaN_3 in DMPU afforded the 24-azide. By performing various conventional side chain (Scheme 2) and 3-OH transformations (including the 3-deoxy compound **17**), we assembled a panel of 15 UDCA analogs in which the 7-βOH group was conserved (Fig. 1). Notably, eight of these retained an ionizable side chain (**7–9**, **15–19**). For comparison, seven DCA analogs were assembled along with LagoDCA (**28**) the epimeric 12-βOH compound (Fig. 1). A third panel consisted of five oxosteroid analogues (**29–33**).

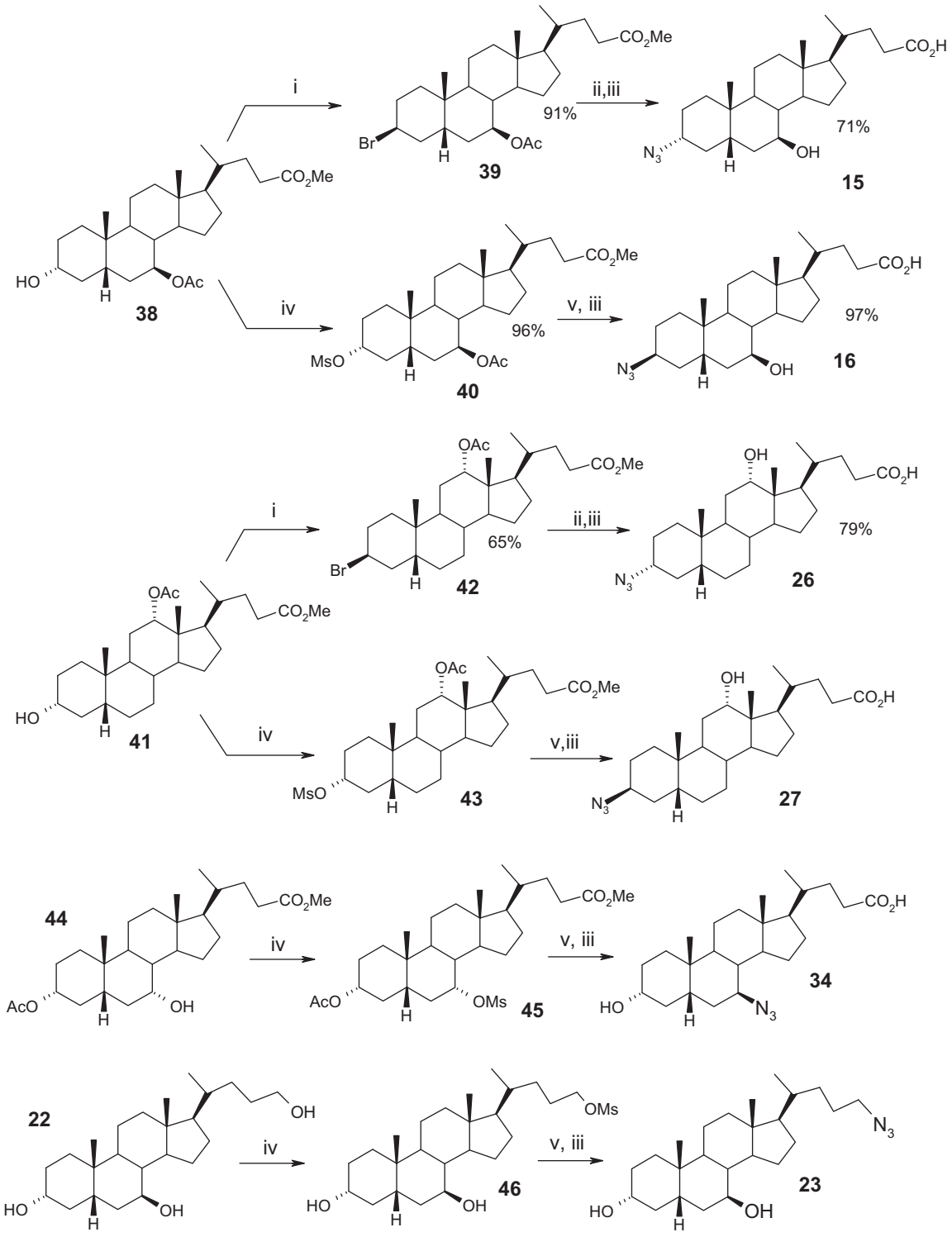
2.2. Reverse phase thin layer chromatography (RPTLC)

RPTLC has been used widely for lipophilicity determination including for BAs.[28,29] Relative to LOGP/D experiments it has the usual chromatographic advantages; relative to HPLC it has higher throughput and, for generally UV transparent analytes such as BAs, it permits simple post-chromatographic derivatization. We developed the RPTLC approach here using commercially available BAs UDCA, CDCA, CA and DCA along with their glycine and taurine conjugates (compounds **1–12**). The relationship between retention and pH was assessed in the pH range 5.4−8.4 for this group. The approach was optimised with respect to pH and solvent composition before application to the wider panel of compounds. Over four concentration levels in the organic modifier concentration range (ϕ) 50–80% the relationship with R_M was linear (r^2 >0.99) which allowed the R_{Mw} to be estimated by extrapolation:

Table 1
BAs physicochemical properties (R_{Mw}, total hydrophobic surface area (HSA), total polar surface area (PSA))

Compound number and trivial name	R_{Mw}	HSA (Å^2)	PSA (Å^2)	Cell viability at24 h 500 (μM) (HET-1A)	CC_{50} (μM) (95% CI)	Cell viability at 24 h 500 (μM) (Huh7)
1 CDCA	4.193	458	157	0.422	216 (134–346)	0.723
2 GCDCA	2.926	450	230	1.116	nd	1.146
3 TCDCA	2.597	521	233	1.300	729	1.053
4 CA	3.25	433	183	1.016	1075 (910–1270)	0.908
5 GCA	2.243	430	237	1.121	nd	0.879
6 TCA	1.908	493	254	1.052	nd	0.877
7 UDCA	3.35	452	164	0.788	1313 (997–1727)	0.740
8 GUDCA	2.124	452	234	1.073	1002 (753–1333)	1.049
9 TUDCA	1.717	520	231	1.092	nd	1.038
10 DCA	4.023	454	156	0.412	257 (199–332)	0.570
11 GDCA	2.926	455	222	1.148	nd	1.062
12 TDCA	2.529	517	229	1.113	1237	1.050
13 MeUDCA[37]	4.776	514	146	0.943	nd	0.812
14 UDCA-24NH_2[38]	4.54	447	185	0.397	161 (115–225)	0.500
15 UDCA3αN3	5.412	445	205	0.368	45 (28–70)	0.266
16 UDCA3βN3	5.452	446	194	0.303	37 (30–44)	1.009
17 3deoxyUDCA[39]	5.304	475	145	0.399	30 (22–40)	0.237
18 Nor UDCA[40]	3.395	427	158	0.888	nd	0.718
19 BisNorUDCA[41]	3.623	416	147	0.805	799	0.748
20 UDCA24CN	4.938	449	162.3	0.940	nd	0.852
21 BisNorUDCACN	3.623	413	155	0.805	nd	0.873
22 UDCA-24OH[42]	5.032	480	140	0.975	nd	0.445
23 UDCA-24N_3	5.299	476	181	0.759	688 (206–1000)	0.451
24 MeDCA[43]	4.697	512	137	0.281	46 (30–69)	0.488
25 DCA-24NH_2[38]	3.895	451	176	0.386	39 (32–49)	0.252
26 DCA3αN3	5.402	441	196	0.307	71 (59–84)	0.371
27 DCA3βN3[44]	5.454	452	193	0.354	97 (72–129)(	1.085
28 LagoDCA[45]	3.89	457	158	0.590	389 (251–601)	0.890
29 12-KetoLCA[43]	3.66	457	157	0.818	nd	0.806
30 NCA[46]	4.468	452	155	0.769	nd	0.756
31 NCA3-acetate[47]	5.638	480	172	0.472	366 (260–516)	0.777
32 3,7,12-ketone[48]	1.925	425	175	1.109	nd	0.544
33 3,7-diketone[48]	3.266	447	152	0.935	nd	0.546
34 3OH,7βN3	5.466	440	203	0.290	99 (65–147)	0.641
35 LCA	5.27	479	130	0.417	25 (17–36)	0.637
36 UCA[49]	3.011	429	186	0.808	nd	0.823
37 HCA[50]	4.407	428	188	0.882	nd	0.738

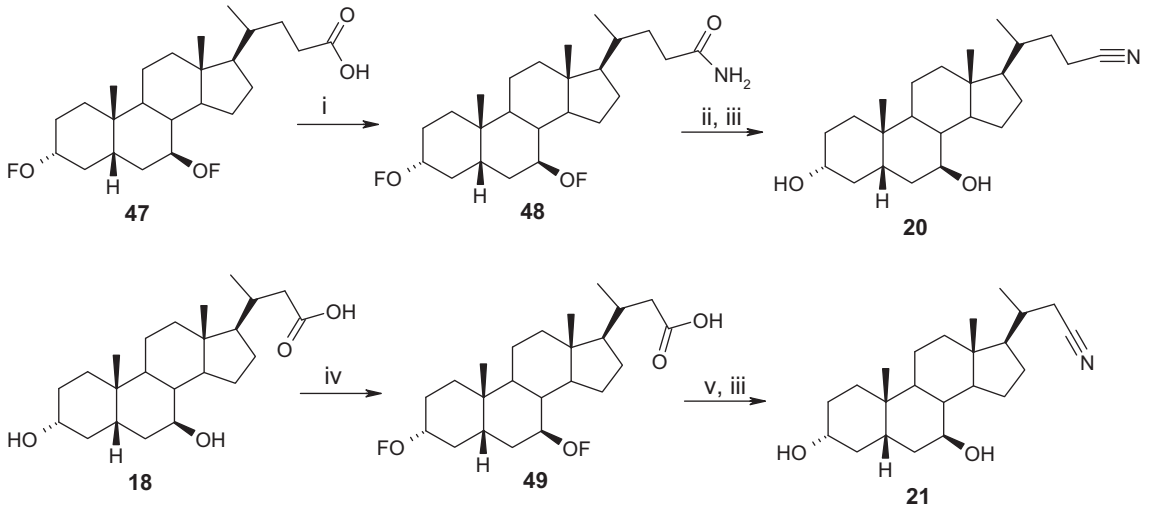
The effect on cell viability relative to control in the HET-1A cell line after 24 h incubation is shown (n = 6) along with an estimated CC_{50} for this. The effect in the hepatic Huh7 line was also evaluated. Compounds were introduced in DMSO and cell viability was normalised to control DMSO at the same concentration.



Scheme 1. Synthesis of azidobile acids. Reagents and conditions: $P(Ph_3)_3$, NBS, THF, −18 °C to rt, 1.5 h; (ii) NaN_3, DMF, 60 °C, 3 d; (iii) 2 M NaOH/MeOH (pH ~14), reflux, 24 h; (iv) MsCl, NEt_3, DCM, 0 °C, 20 min; (v) NaN_3, DMPU, 50 °C.

$$R_M = -S\phi + R_{Mw} \quad \text{where } R_M = \mathrm{Log}(1/R_f - 1)$$

The slope S which is a measure of the degree of responsiveness to changes in mobile phase composition was found to be linear with R_{Mw} for the set. For the set of 12 development compounds R_{Mw} values were estimated at pH 5.4, 7.4, 8.4. In most cases, including, unexpectedly, the taurine conjugates, R_{Mw} values were higher at pH 5.4 than at the latter two pH values, which were similar. It was decided therefore to assess the retention for the whole group of 37 compounds at pH 7.4 since this was the pH at which toxicity was to be determined in the cell-based assay and variations due to shifts in pK_a in methanol mixtures had been shown to be marginal. The rank order at pH 7.4 for the initial test set of 12 was: CDCA∼DCA≫UDCA>CA>GDCA>GCDCA>TCDCA>TDCA >GCA>GUDCA>TCA>TUDCA. This is roughly as expected and in accordance with numerous studies on the relative chromato-



Scheme 2. Synthesis of nitriles: (i) $(BOC)_2O$, pyridine, NH_4HCO_3, rt, 24 h; (ii) trifluoroacetic anhydride/pyridine (1:1); (iii) NaOMe/MeOH reflux, 1 h; (iv) formic acid, perchloric acid 47 °C, 3 h; (v) THF, rt, 3 h. TFA, trifluoroacetic anhydride, $NaNO_2$ 0–5 °C (1 h), 38–40 °C (2 h).

graphic retention of these bile acids and their conjugates.[30,31] Retention in RP chromatographic systems is a function of the number of hydroxyl groups, their topological arrangement and extent of ionisation, bearing in mind the differences between glyco- and tauro-conjugates.[30] Relative retention of the BAs is also influenced by organic modifier and stationary phase identity.[31] The relative retention of unconjugated CA to UDCA was found to be pH dependent in our system but at pH 7.4 CA (3.25) was slightly more polar than UDCA (3.35) which accords with its greater hydrophilicity in other measures such as Log *D* and water solubility.[32] On the other hand the UDCA conjugates GUDCA and TUDCA were more polar than the corresponding CA conjugates. There was a significant inverse correlation between R_{Mw} and estimated polar surface area ($r^2 = 0.81$) in this set, little correlation with hydrophobic surface area ($r^2 = 0.15$) and a moderate positive correlation with the ratio of HSA to PSA ($r^2 = 0.65$). Associations with these global properties are strongly influenced by variation in molecular size and substitution pattern. For example, tauro- and glyco-conjugates have increased HSA and PSA relative to the unconjugated compounds and hence the HSA:PSA ratio does not reflect the decreased R_{Mw}. R_{Mw} data for the whole set at pH 7.4 is presented in Table 1.

The panel as a whole (**1–37**) exhibited satisfactory dispersion and range in lipophilicity as reflected in R_{Mw} (1.717(**9**)–5.466(**34**)) (Table 1). The six azido-analogs were highly lipophilic as expected. Indeed the azides with ionizable side chains were more highly retained than some of the non-azido neutral compounds. However, on the whole the neutral compounds such as the esters and amides yielded R_{Mw} values above the median. Introduction of keto (oxo) groups at the 3, 7 and 12 positions has the effect of depressing hydrophobicity by promoting water access to both faces of the steroid[28,29] and this had the expected effect on R_{Mw} through compounds **29**–**33**.

2.3. Effects on cell viability

Cell viability in the oesophageal HET-1A line was determined at an initial BA test concentration of 500 μM for all compounds in the set over 24 h (Fig. 2). This high concentration (pharmacologically) has been widely used to evaluate BA toxicity. It has been reported that 500 μM DCA causes cell death through apoptosis rather than necrosis.[19] Moreover, BAs can achieve this concentration in vivo and during UDCA treatment.[33,34] In the present case it allowed relative toxicity assessment of compounds that are moderately cytotoxic. Where we observed a significant effect on cell viability at 500 μM, the experiment was repeated at successively lower concentration in order to estimate a CC_{50} value (half maximal cytotoxic concentration). In a number of cases the experiment was repeated at increasing concentration up to 10 mM. Concentration response curves were not calculated in cases where there were poor convergence using a monophasic sigmoid function. The 500 μM cell viability values were found to be most useful therefore for comparisons and they were consistent with CC_{50} values in cases where these were estimated. It is also worth noting that while the results here for classical bile acids are consistent with reported effects on cell viability using other measures of apoptosis, the MTT assay is a reflection of mitochondrial function which could be attenuated in cells that are nonetheless alive. On the other hand BA induction of apoptosis is to a significant extent due to mitrochondrial interactions directly and indirectly.

In this context the following general structure-cytotoxicity observations can be made: (i) glycine and taurine conjugates of CA, CDCA, DCA and UDCA were not cytotoxic at 500 μM. There was in these cases a trend towards increased cell proliferation which only achieved statistical significance in the case of TDCA. Three of these compounds (**3**, **8**, **12**) did trigger cell death when the concentration was raised to >1 mM. The conjugates and least toxic unconjugated BAs caused cell death at a similar concentration suggesting that for these compounds the mechanism of cell death was not structure specific and at such high concentration attributable to detergency and necrosis. The HET-1A cell line is not known to express a BA transporter and therefore considered impermeable to the BA conjugates; (ii) CDCA, DCA and particularly LCA were potently cytotoxic as expected (Table 1); (iii) all of the ionisable azido compounds were potently cytotoxic. The UDCA and DCA 3-azido analogues were significantly more toxic than the parent compounds. Indeed the UDCA 3-azides were more toxic than the corresponding DCA compounds. The 7β-azido compound (**34**) was also cytotoxic (CC_{50} 99 μM) however the UDCA-based 24-azide had not effect on cell viability at 500 μM despite its high lipophilicity; (iv) the DCA and UDCA primary amides (**25**, **14**) markedly reduced viability (CC_{50} 39, 161 μM, respectively); (v) in contrast, the DCA methyl ester (**24**) was more toxic that DCA itself but the UDCA analogue **13** was not more toxic than UDCA. DCA methyl ester **24** was not acting as a prodrug for DCA in this context because it could be recovered unchanged from the medium at 24 h; (v) the 3-deoxy UDCA compound (**17**) (an isomer of LCA)

was highly toxic; (vi) apart from the amide **14**, the UDCA side chain analogs (**18–23**) which are neutral and hydrophobic were not cytotoxic; (vii) oxidation of the steroid secondary alcohols to ketone level was associated with a reduction in cytotoxicity. This is consistent with the reported haemolytic potential of this series.[28,29]

Overall, the most polar compounds were least cytotoxic and there was an association between lipophilicity and toxicity with some important exceptions, which are discussed below. The azido and amide analogs were stable over the time course of the experiments as evidenced by TLC/HPLC.

2.4. Relationship between lipophilicity and cell viability in the HET-1A cell line

A relationship between lipophilicity and toxicity of BAs has been speculated to exist for some time based mainly on the contrasting behaviour of LCA, DCA and CDCA and more polar di- and tri-hydroxy BAs such as UDCA and CA. In the present set of 37 compounds there was a significant negative correlation between R_{Mw} and cell viability at 24 h ($r^2 = 0.6$) (Fig. 3). A similar significance level was achieved using the sparser CC_{50} values. The strength of the association was significantly increased when the neutral

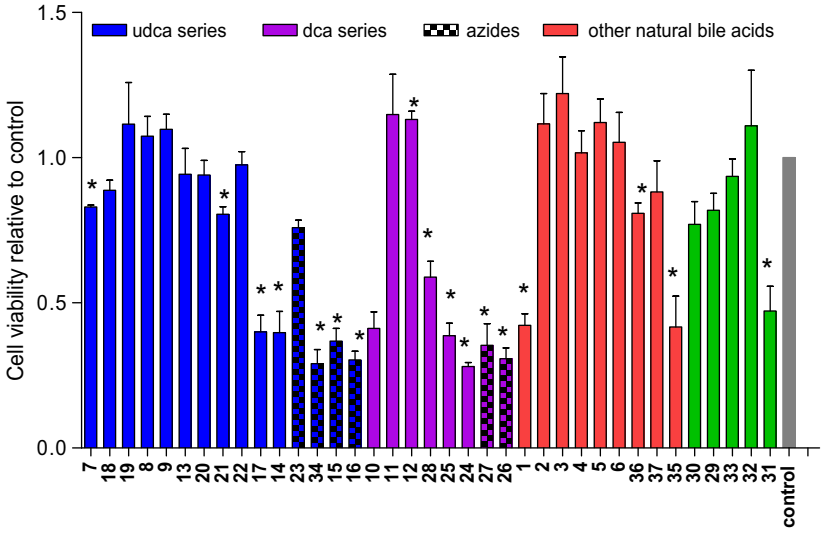


Figure 2. Effect of compounds **1**–**37** on HET-1A viability at 500 μM: $n = 6$, $^{*}p < 0.05$.

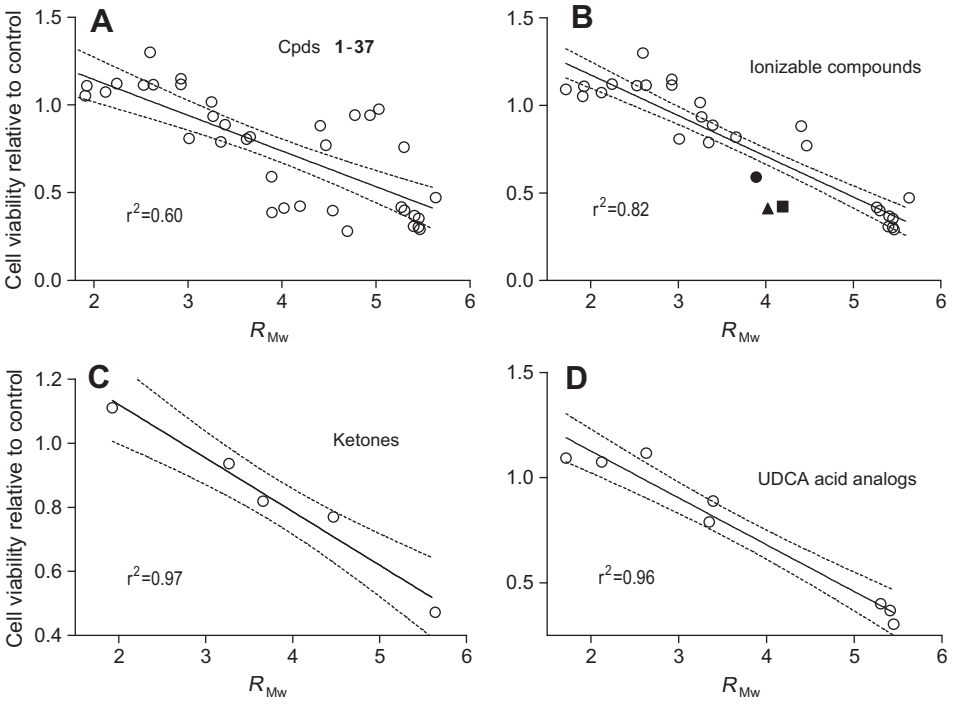


Figure 3. Linear regression lines showing 95% confidence bands for cell viability at 24 h in the HET-1A cell line and R_{Mw}. The panels show: (A) linear regression for compounds **1**–**37**; (B) linear regression for the ionisable (acidic) compounds **1**–**12**, **15**–**19**, **26**–**37**; CDCA (■) DCA (▲) and LagoDCA (●) are highlighted; (C) linear regression for the ketones **29**–**33**; (D) linear regression for the UDCA analogues with an acidic side chain.

compounds were excluded (Fig. 3B: $r^2 = 0.82$, $n = 29$). This might be explained by differences in ionization between the neutral and acid compounds that overwhelm more subtle retention interactions. However, in the group of neutral compounds ($n = 8$) there was a very weak association between R_{Mw} and cell viability ($r^2 = 0.11$). The correlation between R_{Mw} and toxicity may therefore be contingent on BA amphilicity.

It is notable that DCA, CDCA and LagoDCA are out of trend (Fig. 3B) being significantly more toxic than predicted on the basis of R_{Mw}. This may be evidence for an additional more specific mechanism for cell death induction in these cases. Interestingly, these three BAs are reported to depart from the norm in prosecretory behaviour and toxicity towards colon cells.[19,35]

Excellent correlations were obtained for the ionisable UDCA group ($r^2 = 0.96$, $n = 8$) and the ketone panel ($r^2 = 0.97$, $n = 5$) (Fig. 3C and D); focussing in this way on relatively homologous groups minimizes confounding structural factors. It would be interesting to assess correlations in even more homologous BA series with diverse lipophilicity such as fluoro- or methyl-substituted BAs. There were no significant correlations between the in silico bulk hydophobicity/polarity indices (HSA, PSA) and cell viability, consistent with the idea that (relative) BA hydrophobicity/lipophilicity is a shape dependent property[36] that is better predicted chromatographically.[28]

2.5. Relationship between lipophilicity and cell viability in the Huh7 cell line

In order to assess the generality of the findings made in the oesophageal cell line, we evaluated the effect of the compounds on the MTT signal in the Huh7 cell line at 24 h (500 μM, $n = 3$; Table 1). This cell line is a human hepatic carcinoma cell line with significantly different characteristics to the HET-1A line (hepatic versus oesophageal, cancer versus normal). Nevertheless we were able to make some interesting observations. The mean MTT signal was similar in both sets (0.75). Overall there was a weak correlation between MTT values between the two sets ($r^2 = 0.28$). There was also a weak correlation in the Huh7 set between cell viability and lipophilicity ($r^2 = 0.22$). This residual correlation appeared to be dependent on the contribution from the development set of naturally occurring BAs CA, CDCA, DCA, UDCA, their glyco- and tauroconjugates and LCA ($r^2 = 0.52$, $n = 13$). The results for these compounds correlated well between the two cell lines ($r^2 = 0.8$). Amongst the rest of the compounds ($n = 24$), mainly synthetic BAs, there was no relationship between lipophilicity and cytotoxicity in the hepatic cell line. The cytotoxicity in the hepatic cell line seemed in general to be more structure dependent and less on lipophilicity. For example, whereas in the HET-1A cell line, the 3α- and 3β-azides of UDCA and DCA were all cytotoxic, in the Huh7 line, the α- and β-compounds diverged: the α-azides of UDCA and DCA (**15**, **26**) were toxic whereas the β-azides were not. This had a significant effect on the strength of the correlation in the UDCA set in the Huh7 cell line which was otherwise strong. The 24-azide and -alcohol compounds (**23**, **22**) were significantly more toxic in the Huh7 cell line than in the HET1-A line. Whether these observations are attributable to differences in metabolic competency (and detoxification) or to intrinsic cytocidal effect they suggest prospects for selective toxicity.

3. Conclusions

In BAs, azido substitution is associated with enhanced toxicity and the azide group may therefore be a useful design tool for BA-based cytotoxic agents. Azide orientation may be important in determining toxicity which could be useful in the design of selective cytocidal agents. Simple DCA and UDCA amides also hold promise in this regard. RPTLC retention extrapolated to zero organic modifier was predictive of BA toxicity in the HET-1A cell line and it could be a useful high throughput tool for cytotoxic BA discovery and development in this context. This is the first direct linear correlation between BA toxicity and a lipophilicity parameter that we are aware of.

The lipophilicity–toxicity correlation was strongest with acidic BAs and it was especially strong in structurally homologous subgroups. We are unable to say whether the association is due to a hidden common factor, membrane-perturbation effects or whether it is a reflection of a capacity to bind specifically to unidentified BA target proteins: correlations between lipophilicity and affinity/potency are well known. Significantly, the correlation broke down for non-ionizable lipophilic BAs, and it does not fully account for the toxicity associated with DCA, CDCA and LagoDCA. Apart from the naturally occurring bile acids and their conjugates, the correlation between lipophilicity and cytotoxicity did not hold in a hepatic carcinoma cell line (Huh7). Collectively, the results indicate that the relationship between lipophilicity and toxicity is complex, and probably indirect.

4. Experimental

4.1. Synthesis

Uncorrected melting points were obtained using a Stuart® melting point SMP11 melting point apparatus. Spectra were obtained using a Perking Elmer 205 FT Infrared Paragon 1000 spectrometer. Band positions are given in cm^{-1}. Solid samples were obtained by KBr disk; oils were analyzed as neat films on NaCl plates. ^1H and ^{13}C spectra were recorded at 27 °C on a Bruker Advance II 600 MHz spectrometer (600.13 MHz ^1H, 150.91 MHz ^{13}C) and Bruker DPX 400 MHz FT NMR spectrometer (400.13 MHz ^1H, 100.16 MHz ^{13}C), in either $CDCl_3$ or CD_3OD, (tetramethylsilane as internal standard). For $CDCl_3$, ^1H NMR spectra were assigned relative to the TMS peak at 0.00 δ and ^{13}C NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For CD_3OD, ^1H and ^{13}C NMR spectra were assigned relative to the centre peaks of the CD_3OD multiplets at 3.30 δ and 49.00 ppm, respectively. High resolution mass spectrometry (HRMS) was performed on a Micromass mass spectrophotometer (EI mode) at the Department of Chemistry, Trinity College. HPLC was performed on a reverse phase 250 mm × 4.6 mm Waters Spherisorb ODS-2, 5 μm column using a Waters Alliance 2695 chromatograph equipped with an autosampler, column oven and dual wavelength detector. The flow rate was 1 ml/min with a mobile phase consisting of 40% phosphate buffer pH 2.5 and 60% acetonitrile at time 0 and grading to 85% acetonitrile at 4 min. Injection volume was 20 μl, and areas determined at 254 nm. The isocratic HPLC method was aqueous phosphate buffer solution pH 2.5 40% and acetonitrile 60%. Flow rate was 1 ml/min. Flash chromatography was performed on Merck Kieselgel 60 particle size 0.040–0.063 mm. TLC was performed on silica gel Merck F-254 plates. Compounds were visually detected by absorbance at 254 nm and/or vanillin staining.

BAs **1–12** and **35** (Table 1) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). UCA and HCA (**36**, **37**) were a gift from Professor B. Natalini (University of Perugia). Identity of all BAs was confirmed with ^1H NMR, ^{13}C NMR and HRMS. Purity was confirmed by HPLC and TLC.

4.1.1. 24-Methyl 3β-bromo, 7β–acetoxy-5β-cholanoate (39)

Triphenylphosphine (0.170 g) was added to a stirred solution of **38** (0.145 g) in anhydrous THF (15 ml) and the mixture was cooled to −18 °C. *N*-Bromosuccinimide (0.115 g) was added dropwise and

the mixture allowed to warm to room temperature. After 1.5 h, when TLC analysis showed no more starting material, the reaction mixture was poured into water (50 ml) and extracted with ethyl acetate (3 × 50 ml). The organic phase was dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The product was separated by flash chromatography, using hexane/ethyl acetate 3:1 as mobile phase to yield colourless oil (0.151 g, 91%). ^{1}H NMR δ ($CDCl_3$): 4.76 (s, 1H, 3α-H), 4.69 (m, 1H, 7α-H), 3.67 (s, 3H, –O–CH_3), 2.00 (s, 3H, 7-C=OCH_3), 1.06 (s, 3H, 19-CH_3), 0.93 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.83 (C=O, 24-C), 170.81 (C=O, 7-OC=OCH_3), 74.01 (CH, 7-C), 51.64 (CH_3, OCH_3), 21.97 (CH_3, 7-C=OCH_3). IR_{vmax} (DCM): 3435.54, 2948.89, 1729.41, 1646.49 and 1243.23 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 533.2249.

4.1.2. 3α-Azido, 7β−hydroxy-5β-cholanoate (15)

To a stirred solution of **39** (1.876 g) in anhydrous *N*,*N*-DMF (40 ml) was added 5 equiv NaN_3 (1.192 g) at 60 °C. The mixture was stirred overnight then poured into saturated aqueous $NaHCO_3$ (150 ml) and extracted with ethyl acetate (3 × 100 ml). The organic phase was washed with brine (200 ml), dried over $MgSO_4$, filtered and the solvent was evaporated in vacuum. The product was separated on a flash column (hexane/ethyl acetate 9:1) to yield orange foam (1.258 g, 72%). The azide (0.150 g) was dissolved in methanol (15 ml) to which was added 2 M sodium hydroxide solution to pH ~14 and stirred at reflux for 1 day, when TLC analysis showed the hydrolysis was complete. Then the reaction mixture was poured into 1 M HCl solution (50 ml) and extracted with ethyl acetate (3 × 50 ml). The organic layer was washed with water (2 × 100 ml) and brine (1 × 100 ml), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure to give the product as a light yellow solid (0.131 g, 99%). ^{1}H NMR δ ($CDCl_3$): 3.60 (m, 1H, 7α-H), 3.30 (m, 1H, 3β-H), 0.98 (s, 3H, 19-CH_3), 0.96 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 180.19 (C=O, 24-C), 71.68 (CH, 7-C), 61.30 (CH, 3-C). IR_{vmax} (KBr): 3375.06, 2934.00, 2867.03, 2093.18, 1690.57 and 1255.27 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 440.2896.

4.1.3. 24-Methyl 3α-(methylsulfonyl)oxy, 7β-acetoxy-5β-cholanoate (40)

To a solution of **38** (1 g) and triethylamine (0.34 ml) in anhydrous dichloromethane (30 ml) was added methanesulfonylchloride (0.26 ml in 10 ml anhydrous DCM) dropwise at 0 °C and stirred for 20 min. Then, cooled water (50 ml) was added to the mixture, which was separated and the aqueous phase was extracted with DCM (2 × 40 ml). The organic phase was washed with brine (100 ml), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give colourless oil as product (1.115 g, 95%). ^{1}H NMR δ ($CDCl_3$): 4.77 (m, 1H, 7α-H), 4.62 (m, 1H, 3β-H), 3.68 (s, 3H, –O–CH_3), 3.02 (s, 3H, –OSO_2CH_3), 2.00 (s, 3H, 7-C=OCH_3), 0.99 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.02 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.82 (C=O, 24-C), 170.70 (C=O, 7-OC=OCH_3), 81.72 (CH, 3-C), 73.42 (CH, 7-C), 52.68 (CH_3, –OSO_2CH_3), 51.64 (CH_3, OCH_3), 21.92 (CH_3, 7-C=OCH_3). IR_{vmax} (DCM): 3436.82, 2950.45, 2873.89, 1729.45, 1646.30 and 1173.83 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 549.2858.

4.1.4. 3β-Azido, 7β-hydroxy-5β-cholanoate (16)

Compound **40** (0.705 g) and sodium azide (0.870 g) in DMPU (20 ml) were stirred at 50 °C for 11 days (TLC hexane/ethyl acetate 3:1) then poured into water (50 ml) and extracted with ethyl acetate (3 × 50 ml). The organic phase was washed with brine (100 ml) dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give the crude product as yellow oil. This was flash columned using 5% then 10% ethyl acetate in hexane as mobile phase to yield white solid as product (0.537 g, 85%). ^{1}H NMR δ (400 MHz, $CDCl_3$): 4.74 (m, 1H, 7α-H), 3.95 (s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 2.00 (s, 3H, 7-C=OCH_3), 1.01 (s, 3H, 19-CH_3), 0.93 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.52 (C=O, 24-C), 170.52 (C=O, 7-OC=OCH_3), 73.47 (CH, 7-C), 57.94 (CH, 3-C), 51.33 (CH_3, OCH_3), 21.65 (CH_3, 7-C=OCH_3). IR_{vmax} (DCM): 3445.10, 2946.85, 2871.37, 2102.43, 1736.79 and 1248.22 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 496.3164. The crude ester azide was hydrolysed as described above (**4.1.2**) yielding the azide (**16**) as a white solid. ^{1}H NMR δ ($CDCl_3$): 3.93 (s, 1H, 3α-H), 3.55 (m, 1H, 7α-H), 1.00 (s, 3H, 19-CH_3), 0.96 (d, 3H, J = 6.02 Hz, 21-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 179.81 (C=O, 24-C), 71.54 (CH, 7-C), 58.42 (CH, 3-C). IR_{vmax} (KBr): 3330.87, 2929.30, 2867.30, 2103.92, 1687.49 and 1243.05 cm^{-1}. HRMS: Found: $(M)^-$ = 416.2913.

4.1.5. 3α,7β-Dihydroxy-5 β-cholan-24-nitrile (20)

Formyl UDCA amide (**48**) (0.42 g) was dissolved in dry THF (10 ml) at 0 °C. Pyridine (150 μl) and trifluoroacetic anhydride (270 μl) were added to this mixture. After completion of the reaction as monitored by TLC (10 h) the solvent was removed in vacuo and the residue redissolved in ethyl acetate (20 ml) which was then washed with HCl (3 × 20 ml) and water to neutrality. Chromatographic elution with ethyl acetate/hexane (1:1) afforded a white solid. Sodium (0.2 g) was added to methanol (10 ml) to form an excess of sodium methoxide. The formyl nitrile obtained in the above reaction was added to this solution which was then refluxed for 2 h. After 2 h the reaction was cooled to room temperature and added to water (20 ml). The nitrile product (**20**) was extracted with ethyl acetate. The organic layer was then washed with water (3 × 20 ml) and dried ($MgSO_4$). The solvent was removed in vacuo to yield a white solid (0.3 g, 78%). ^{1}H NMR δ (MeOD) 3.50 (m, 2-H, 3-β H, 7-α H), 2.8 (m, 1-H, 20-CH), 1.00 (d, 3-H, J = 6.02 Hz, 21-CH_3), 0.98 (s, 3-H, 19-CH_3), 0.75 (s, 3-H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 124 (CN, 22-C), 71 (3-C, 7-C). HRMS: Found: $(M-Na)^+$ 396.2867.

4.1.6. 3α,7β-Dihydroxy-24-bisnor-5β-cholane-22-nitrile (21)

Formyl protected norUDCA (3.5 g) (**18**) was stirred in trifluoroacetic acid (4 ml) and trifluoroacetic anhydride (1 ml) at 0–5 °C until dissolution was complete. Sodium nitrite (0.786 g) was then added in small portions, waiting for the salt to react between additions. After addition the mixture was stirred at 0–5 °C for 1 h. The mixture was then warmed to 38–40 °C and left to stir for another 2 h. The brown solution was cooled to room temperature and added to a mixture of water/1 M NaOH (1:1, 50 ml). The nitrile was extracted with ethyl acetate and washed with NaOH (4 × 20 ml) and water to neutrality. The ethyl acetate was dried ($MgSO_4$) and removed in vacuo to yield an off white solid. Sodium (1 g) was added to MeOH (50 ml) to form an excess of sodium methoxide. The formyl bisnornitrile obtained in the above reaction was added to this solution and refluxed for 2 h. After 2 h the reaction was cooled to room temperature and added to water (100 ml). The title compound was extracted with ethyl acetate. The organic layer was then washed with water (3 × 20 ml) and dried ($MgSO_4$). The solvent was removed in vacuo to yield a white solid (2.6 g, 94%). ^{1}H NMR δ ($CDCl_3$) 3.48 (m, 2-H, 3-β H, 7-α H), 2.8 (m, 1-H, 20-CH), 1.34 (d, 3-H, J = 7.02 Hz, 21-CH_3), 1.00 (s, 3-H, 19-CH_3), 0.76 (s, 3-H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 124 (CN, 22-C), 71 (3-C, 7-C). HRMS: Found: $(M)^-$ = 344.2590.

4.1.7. 3α,7β-Dihydroxy-5 β-cholan-24-azide (23)

3α,7β,24-Trihydroxy-5 β-cholane (0.2 g) (**22**) was dissolved in dry pyridine (4 ml) at 0 °C followed by the addition of methane sulfonyl chloride (63 μl). After 20 min the reaction was quenched by adding crushed ice and the compound was extracted with ethyl acetate (2 × 10 ml). The organic layer was washed with cold water

(2 × 20 ml), cold HCl (2 × 20 ml) and dried ($MgSO_4$) to afford a white solid (**46**). TLC of this product revealed starting material and a new product spot. Chromatographic elution with ethyl acetate afforded a white solid (0.13 g, 53%). The product was dissolved in DMPU and treated with an excess of NaN_3 at 50 °C for two days. The title compound was obtained by partitioning between ethyl acetate and water, followed by successive acid and base wash, evaporation and flash chromatography. ^{1}H NMR δ (MeOD) 3.5 (m, 2-H, 3-β H, 7-α H), 3.26 (m, 2-H, 23-CH_2), 0.99 (d, 3-H, J = 6.02, 21-CH_3), 0.95 (s, 3-H, 19-CH_3), 0.70 (3-H, s, 18-CH_3). ^{13}C NMR ppm (MeOD): 71 (3-C, 7-C), 51 (23-C), IR_{vmax} (KBr) 3341.06. 2091, HRMS: Found: $(M-Na)^+$ = 426.3096.

4.1.8. 24-Methyl 3β-bromo, 12α-acetoxy-5β-cholanoate (42)

Triphenylphosphine (0.821 g) was added to a stirred solution of **41** (1.035 g) in anhydrous THF (40 ml) and the mixture was cooled to −18 °C. *N*-Bromosuccinimide (1.210 g) was added in three parts to the reaction mixture over 1 h and it was allowed to warm to room temperature. After 1.5 h, when TLC analysis showed no more starting material, the reaction mixture was poured into 1 M HCl solution (150 ml) and extracted with ethyl acetate (3 × 100 ml). The organic phase was washed with water (2 × 100 ml) and brine (1 × 100 ml), dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The product was separated by flash chromatography twice, using 30% ethyl acetate first then 0–12% ethyl acetate in hexane as mobile phase to yield colourless semi-solid (0.766 g, 65%). ^{1}H NMR δ ($CDCl_3$): 5.10 (s, 1H, 12β-H), 4.80 (s, 1H, 3α-H), 3.68 (s, 3H, –O–CH_3), 2.09 (s, 3H, 12-C═OCH_3), 1.00 (s, 3H, 19-CH_3), 0.83 (d, 3H, J = 6.28 Hz, 21-CH_3), 0.75 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 175.07 (C═O, 24-C), 170.85 (C═O, 12-OC═OCH_3), 76.41 (CH, 12-C), 51.97 (CH_3, OCH_3), 21.78 (CH_3, 12-C═OCH_3). IR_{vmax} (KBr): 3444.74, 2940.49, 2873.22, 1736.69 and 1245.03 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 533.2252.

4.1.9. 3α-Azido, 12α-hydroxy-5β-cholanoate (26)

To a solution of **22** (0.150 g) in methanol (15 ml) was added 2 M sodium hydroxide solution to pH ~14 and stirred at reflux for 1 day, when TLC analysis showed the hydrolysis was complete. Then the reaction mixture was poured into 1 M HCl solution (50 ml) and extracted with ethyl acetate (3 × 50 ml). The organic layer was washed with water (2 × 100 ml) and brine (1 × 100 ml), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure to give the product as a light yellow solid (0.131 g, 99%) mp 88 °C. ^{1}H NMR δ ($CDCl_3$): 4.01 (s, 1H, 12β-H), 3.35 (m, 1H, 3β-H), 1.01 (d, 3H, J = 6.03 Hz, 21-CH_3), 0.94 (s, 3H, 19-CH_3), 0.70 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 179.80 (C═O, 24-C), 73.29 (CH, 12-C), 61.40 (CH, 3-C). IR_{vmax} (KBr): 3440.00, 2941.49, 2866.14, 2090.46, 1709.85, 1679.00 and 1246.00 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 440.2878.

4.1.10. 24-Methyl 3α-azido, 7β−acetoxy-5β-cholanoate (22)

To a stirred solution of **19** (1.876 g) in anhydrous *N*,*N*-DMF (40 ml) was added 5 equiv sodium azide (1.192 g) at 60 °C. The mixture was stirred overnight then poured into std. $NaHCO_3$ (150 ml) and extracted with ethyl acetate (3 × 100 ml). The organic phase was washed with brine (200 ml), dried over $MgSO_4$, filtered and the solvent was evaporated in vacuo. The product was separated on a flash column (hexane/ethyl acetate 9:1) to yield orange foam (1.258 g, 72%). ^{1}H NMR δ ($CDCl_3$): 4.76 (6, 1H, J_1 = 5.52 Hz, J_2 = 5.53 Hz, J_3 = 5.01 Hz, 7α-H), 3.68 (s, 3H, –O–CH_3), 3.28 (m, 1H, 3β-H), 2.00 (s, 3H, 7-C═OCH_3), 0.99 (s, 3H, 19-CH_3), 0.94 (d, 3H, J = 6.53 Hz, 21-CH_3), 0.69 (s, 3H, 18-CH_3). ^{13}C NMR ppm ($CDCl_3$): 174.52 (C═O, 24-C), 170.40 (C═O, 7-OC═OCH_3), 73.31 (CH, 7-C), 60.58 (CH, 3-C), 51.32 (CH_3, OCH_3), 21.63 (CH_3, 7-C═OCH_3). IR_{vmax} (KBr): 3430.05, 2929.21, 2871.64, 2099.67, 1745.61, 1722.74, 1256.74 and 1164.61 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 496.3159.

4.1.11. 3α-Hydroxy, 7β-azido-5β-cholanoate (34)

^{1}H NMR δ (MeOD) 3.6369 (m, 1-H, 3 β-H), 3.0519 (m, 1-H, 7 α-H), 0.9583 (s, 3-H, 19-CH_3), 0.9407 (d, 3-H, J = 6.02, 21-CH_3), 0.6986 (s, 3H, 18-CH_3). ^{13}C NMR ppm (MeOD): 180 (C═O, 24-C), 71 (3-C), 61 (7-C). IR_{vmax} (KBr) 3340.06, 2915.00, 2105.01, 1695.34 and 1235.21 cm^{-1}. HRMS: Found: $(M-Na)^+$ = 440.2889.

4.2. Reverse phase TLC

TLC was performed on pre-coated C18 reversed-phase HPTLC (20x10, F254) plates (Merck, Darmstadt, Germany). Test solutions were applied in DMSO. The plates were then dried at 40 °C for 1 h. Plates were developed in a closed chamber at room temperature across a development distance of 15 cm. Methanol: aqueous ammonium acetate (15 mM) adjusted to the required pH with acetic acid was used as the mobile phase. After development, the plates were dried under ambient conditions and stained with a vanillin solution to visualize spots.

4.3. Cell culture

The human oesophageal squamous epithelial cell line HET-1A was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in bronchial epithelial cell basal medium (Lonza Group Ltd, Switzerland) supplemented with triiodothyronine, insulin, transferrin, retinoic acid, hydrocortisone, human recombinant epidermal growth factor, epinephrine and bovine pituitary extract.

The human hepatoma cell line, Huh7 was a kind gift from Dr. Ralf Bartenschlager (Department of Molecular Virology, University of Heidelberg, Germany). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum and 100 U/ml penicillin/streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

4.4. Calculation of physicochemical descriptors

UDCA was extracted from the X-ray crystal structure bound to AKR1C2 (PDB entry 1IHI) and visualized in molecular operating environment (MOE 2007.09, Chemical Computing Group, Montreal, Canada). Energy minima for the other test bile acids were generated from this by modification in MOE using sequential SD (100 steps) and TN (1000 steps or to an RMS gradient of <0.01 kcal/(mol*A)) using the PM3 algorithm, Stochastic searches were performed for the conjugates using PM3 over 1000 steps. Physicochemical descriptor parameters including hydrophobic surface area (ASAH) and polar surface area (ASAP) were calculated based on the minimised steroid molecules following a stochastic conformational search procedure using the database functions of MOE.

4.5. MTT assay

Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96 well plates at a concentration of 8×10^4 cells/ml (100 μl/well). After 24 h the cells were treated with various bile acids at concentrations ranging from 4 μM to 10 mM for a further 24 h in supplement free medium. Test compounds were maintained as 200 mM stock solutions in DMSO. The compounds were diluted to the required concentrations with medium. No change in pH was observed on addition of bile acids to the medium. Control wells were treated with BEBM without supplements or

with PMA 1 μg/ml as a positive control for cell death. Vehicle control wells were treated with 1% DMSO. After 22 h cells were incubated with 20 μl of MTT solution for a further 2 h. The medium was aspirated from the wells and DMSO (100 μl) added to each well to lyse the cells. The plates were shaken for 10 min to dissolve the formazan crystals and then read on a multiwell spectrophotometer at a wavelength of 570 nm. The absorbance of vehicle controls was set as 100% survival and of PMA treated wells as 0%. Cell survival rates were determined by calculating: $(T - B)/(V - B)$, where T (treated) is the absorbance of bile acid treated cells, B (blank) is the absorbance of media plus MTT and V (vehicle) is the absorbance of vehicle control cells. Values represent the mean ± SEM of three experiments performed in triplicate. CC_{50} values were estimated from concentration–effect curves generated using nonlinear regression models in GraphPad Prism5.

4.6. Statistical analysis

Statistical comparison between groups was carried out using a one-sample *t*-test to examine differences between groups. Data are graphically represented as the mean ± standard error of the mean (SEM). All data were analysed using GraphPad Prism5. $p < 0.05$ was considered to be statistically significant.

Acknowledgements

We are most grateful to Dr. John O'Brien for NMR spectroscopy. We would also like to thank Dr. Annemarie Byrne and Dr. David Prichard for their many helpful comments and suggestions.

Funding: This work was supported by the Health Research Board [TRA/2007/11] and the Embark Initiative (IRCSET Award to Sharma, 2006).

References and notes

1. Monte, M. J.; Marin, J. J.; Antelo, A.; Vazquez-Tato, J. *World J. Gastroenterol.* **2009**, *15*, 804.
2. Zimber, A.; Gespach, C. *Anticancer Agents Med. Chem.* **2008**, *8*, 540.
3. Fiorucci, S.; Mencarelli, A.; Palladino, G.; Cipriani, S. *Trends Pharmacol. Sci.* **2009**, *30*, 570.
4. Debruyne, P. R.; Bruyneel, E. A.; Li, X.; Zimber, A.; Gespach, C.; Mareel, M. M. *Mutat. Res.* **2001**, *480–481*, 359.
5. Kim, N. D.; Im, E. O.; Choi, Y. H.; Yoo, Y. H. *J. Biochem. Mol. Biol.* **2002**, *35*, 134.
6. Amaral, J. D.; Viana, R. J.; Ramalho, R. M.; Steer, C. J.; Rodrigues, C. M. *J. Lipid Res.* **2009**, *50*, 1721.
7. Perez, M. J.; Briz, O. *World J. Gastroenterol.* **2009**, *15*, 1677.
8. Liu, H.; Qin, C. K.; Han, G. Q.; Xu, H. W.; Ren, W. H.; Qin, C. Y. *Cancer Lett.* **2008**, *270*, 242.
9. Choi, Y. H.; Im, E. O.; Suh, H.; Jin, Y.; Lee, W. H.; Yoo, Y. H.; Kim, K. W.; Kim, N. D. *Int. J. Oncol.* **2001**, *18*, 979.
10. El Kihel, L.; Clement, M.; Bazin, M. A.; Descamps, G.; Khalid, M.; Rault, S. *Bioorg. Med. Chem.* **2008**, *16*, 8737.
11. Kim, N. D.; Im, E.; Yoo, Y. H.; Choi, Y. H. *Curr. Cancer Drug Targets* **2006**, *6*, 681.
12. Choi, Y. H.; Im, E. O.; Suh, H.; Jin, Y.; Yoo, Y. H.; Kim, N. D. *Cancer Lett.* **2003**, *199*, 157.
13. Heuman, D. M. *Ital. J. Gastroenterol.* **1995**, *27*, 372.
14. Hofmann, A. F. *Arch. Intern. Med.* **1999**, *159*, 2647.
15. Garidel, P.; Hildebrand, A.; Knauf, K.; Blume, A. *Molecules* **2007**, *12*, 2292.
16. Asamoto, Y.; Tazuma, S.; Ochi, H.; Chayama, K.; Suzuki, H. *Biochem. J.* **2001**, *359*, 605.
17. Vlahcevic, Z. R.; Gurley, E. C.; Heuman, D. M.; Hylemon, P. B. *J. Lipid Res.* **1990**, *31*, 1063.
18. Gonzalez, B.; Fisher, C.; Rosser, B. G. *Mol. Cell. Biochem.* **2000**, *207*, 19.
19. Akare, S.; Martinez, J. D. *Biochim. Biophys. Acta* **2005**, *1735*, 59.
20. Strupp, W.; Weidinger, G.; Scheller, C.; Ehret, R.; Ohnimus, H.; Girschick, H.; Tas, P.; Flory, E.; Heinkelein, M.; Jassoy, C. *J. Membr. Biol.* **2000**, *175*, 181.
21. Vyvoda, O. S.; Coleman, R.; Holdsworth, G. *Biochim. Biophys. Acta* **1977**, *465*, 68.
22. Krahenbuhl, S.; Fischer, S.; Talos, C.; Reichen, J. *Hepatology* **1994**, *20*, 1595.
23. Martinez-Diez, M. C.; Serrano, M. A.; Monte, M. J.; Marin, J. J. *Biochim. Biophys. Acta* **2000**, *1500*, 153.
24. Rao, Y. P.; Stravitz, R. T.; Vlahcevic, Z. R.; Gurley, E. C.; Sando, J. J.; Hylemon, P. B. *J. Lipid Res.* **1997**, *38*, 2446.
25. Powell, A. A.; LaRue, J. M.; Batta, A. K.; Martinez, J. D. *Biochem. J.* **2001**, *356*, 481.
26. Katona, B. W.; Rath, N. P.; Anant, S.; Stenson, W. F.; Covey, D. F. *J. Org. Chem.* **2007**, *72*, 9298.
27. Katona, B. W.; Anant, S.; Covey, D. F.; Stenson, W. F. *J. Biol. Chem.* **2009**, *284*, 3354.
28. Sarbu, C.; Kuhajda, K.; Kevresan, S. *J. Chromatogr., A* **2001**, *917*, 361.
29. Posa, M.; Kuhajda, K. *Steroids* **2010**, *75*, 424.
30. Roda, A.; Minutello, A.; Angellotti, M. A.; Fini, A. *J. Lipid Res.* **1990**, *31*, 1433.
31. Natalini, B.; Sardella, R.; Camaioni, E.; Macchiarulo, A.; Gioiello, A.; Carbone, G.; Pellicciari, R. *J. Pharm. Biomed. Anal.* **2009**, *50*, 613.
32. Heuman, D. M. *J. Lipid Res.* **1989**, *30*, 719.
33. Stadler, J.; Stern, H. S.; Yeung, K. S.; McGuire, V.; Furrer, R.; Marcon, N.; Bruce, W. R. *Gut* **1988**, *29*, 1326.
34. Hess, L. M.; Krutzsch, M. F.; Guillen, J.; Chow, H. H.; Einspahr, J.; Batta, A. K.; Salen, G.; Reid, M. E.; Earnest, D. L.; Alberts, D. S. *Cancer Epidemiol. Biomarkers Prev.* **2004**, *13*, 861.
35. Keely, S. J.; Scharl, M. M.; Bertelsen, L. S.; Hagey, L. R.; Barrett, K. E.; Hofmann, A. F. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2007**, G290.
36. Costantino, G.; Wolf, C.; Natalini, B.; Pellicciari, R. *Steroids* **2000**, *65*, 483.
37. Liliang, H.; Hua, Z.; Xiaoping, X.; Chunchun, Z.; Yu-Mei, S. J. *Organomet. Chem.* **2009**, *694*, 3247.
38. Bellini, A. M.; Quaglio, M. P.; Guarneri, M.; Cavazzini, G. *Eur. J. Med. Chem.* **1983**, *18*, 191.
39. Takashi, I.; Frederic, C. C. *J. Org. Chem.* **1983**, *48*, 1194.
40. Schteingart, C. D.; Hofmann, A. F. *J. Lipid Res.* **1988**, *29*, 1387.
41. Batta, A. K.; Datta, S. C.; Tint, G. S.; Alberts, D. S.; Earnest, D. L.; Salen, G. *Steroids* **1999**, *64*, 780.
42. Kihira, K.; Yoshii, M.; Okamoto, A.; Ikawa, S.; Ishii, H.; Hoshita, T. *J. Lipid Res.* **1990**, *31*, 1323.
43. Gao, H.; Dias, J. R. *Eur. J. Org. Chem.* **1998**, *4*, 719.
44. Anelli, P. L. B. M.; Morosini, P.; Palano, D.; Carrea, G.; Falcone, L.; Pasta, P.; Sartore, D. *Biocatal. Biotransform.* **2002**, *20*, 29.
45. Batta, A. K.; Aggarwal, S. K.; Salen, G.; Shefer, S. *J. Lipid Res.* **1991**, *32*, 977.
46. Gil, R. P.; Martinez, C. S. P.; Manchado, F. C. *Synth. Commun.* **1998**, *28*, 3387.
47. Snopek, J.; Smolkova-Keulemansova, E.; Jelinek, I.; Dohnal, J.; Klinot, J.; Klinotova, E. *J. Chromatogr.* **1988**, *450*, 373.
48. Bortolini, O.; Fantin, G.; Fogagnolo, M.; Forlani, R.; Maietti, S.; Pedrini, P. *J. Org. Chem.* **2002**, *67*, 5802.
49. Takashi, I.; Frederic, C. C. *J. Org. Chem.* **1982**, *47*, 2972.
50. Pedrini, P.; Andreotti, E.; Guerrini, A.; Dean, M.; Fantin, G.; Giovannini, P. P. *Steroids* **2006**, *71*, 189.