FULL PAPER

Dihydroartemisinin-bile acid hybridization as an effective approach to enhance dihydroartemisinin anticancer activity

Elena Marchesi,[a] Nicola Chinaglia,[a] Massimo L. Capobianco,[b] Paolo Marchetti,[a] Tzu-En Huang,[c] Hao-Cheng Weng,[c] Jih-Hwa Guh,[c] Lih-Ching Hsu,*[c] Daniela Perrone*[a] and Maria Luisa Navacchia*[b]

[a] Dr. D. Perrone, Dr. E. Marchesi, Dr. N. Chinaglia, Dr. P. Marchetti
Department of Chemical and Pharmaceutical Sciences
University of Ferrara
Via L. Borsari, 46, 44121 Ferrara, Italy
prd@unife.it

[b] Dr. M.L.Navacchia, Dr. M.L.Capobianco
Institute of Organic Synthesis and Photoreactivity
National Research Council
Via P. Gobetti, 101, 40129, Bologna, Italy
marialuisa.navacchia@isof.cnr.it

[c] Dr. Lih-Ching Hsu, Dr. Jih-Hwa Guh, Hao-Cheng Weng, Tzu-En Huang
School of Pharmacy, National Taiwan University, No. 33, Linsen S. Road, Taipei 10050, Taiwan
lhsu@ntu.edu.tw

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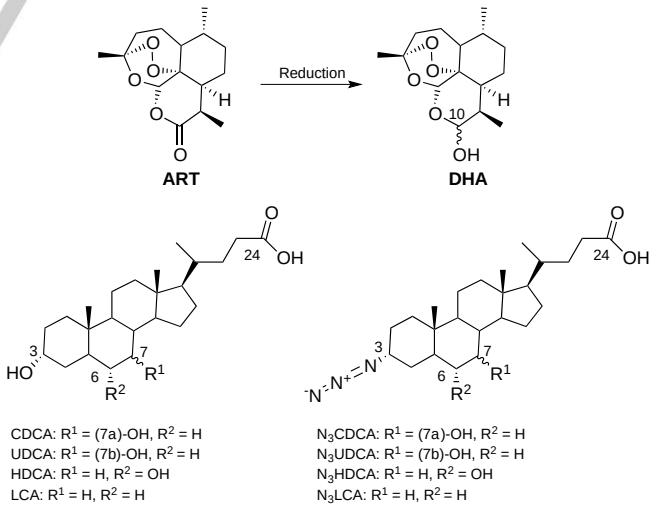
Abstract: A series of hybrid compounds based on natural products bile acids and dihydroartemisinin (DHA) were prepared by different synthetic methodologies and investigated for their in vitro biological activity against HL-60 leukemia and HepG2 hepatocellular carcinoma cell lines. Most synthesized hybrids presented significantly improved antiproliferative activities respect to DHA and parent bile acids. Compounds **2** and **13** were the most potent hybrids of the series with a 10.5- and 15.4-fold increase in cytotoxic activity respect to DHA alone in HL-60 and HepG2 cells, respectively. Strong evidence that hybrid **2** induced apoptosis was obtained by flow cytometric analysis as well as Western blot analysis.

Introduction

Despite the efforts of pharmaceutical and medical research to fight cancer, this disease is still the leading cause of death worldwide. Modern medicine has many approaches to fight cancer including surgical, radio- and chemo-therapies often used in combination. Although newer therapies have improved the survival rate and the quality of life of patients, too many advanced and metastasized cancers remain untreatable. Moreover, conventional anticancer chemotherapies are frequently associated with significant levels of toxicity and/or drug resistance. Therefore, searching for safer, more effective and selective new drugs is highly demanded. Naturally occurring cytotoxic products, being able to target multiple pathways involved in cancer cell growth through antioxidative, anti-inflammatory and apoptotic actions, are an important source of bioactive compounds and are considered as promising candidates for anticancer drug development.[1a-c] Phytochemicals from herbal medicinal plants as well as from dietary plants are also important in cancer chemoprevention and can prevent not only the initiation but also the promotion and progression of tumors through proliferation and angiogenesis inhibition.[1b] Several cytotoxic natural products have been developed as clinical drugs. For instance, taxanes, originally derived from *Taxus brevifoglia*, and vinca alkaloids from *Catharanthus roseus*, have been used to treat many types of advanced and/or metastatic cancers. Phytochemicals can also be used in combination with chemotherapeutic drugs to increase mutual anticancer activity. For instance, curcumin,[2] a natural COX-2 inhibitor, and genistein,[3] an isoflavone originated from several edible plants, were proved to enhance the cytotoxic effect of gemcitabine against pancreatic cancer cells.



Lipophilicity scale: LCA > CDCA > UDCA > HDCA

Figure 1. Molecular structures of ART, DHA, bile acids and 3-azidobile acids, and lipophilicity scale of bile acids.

In the past decades, many studies have been devoted to artemisinin (ART), a sesquiterpene lactone obtained from *Artemisia annua*, a plant of the *Asteraceae* family, and its derivatives such as dihydroartemisinin (DHA) (Figure 1). Some ART derivatives present outstanding properties against the *Plasmodium* parasites and ART-based combination therapies have become the gold standard for malaria treatment. ART bioactive features are located in the 1,2,4-trioxane ring and in the fused 6-membered α-methyl-γ-lactone ring[4] (Figure 1). DHA, a semisynthetic derivative of ART, preserves the 1,2,4-trioxane ring whereas the lactone function is modified into lactol by use of a mild hydride-reducing agent.[5] This chemical modification makes DHA a more powerful antimalarial compound respect to ART.[6] Moreover, DHA's remarkable anticancer activity towards hepatocellular carcinoma *in vitro* and in xenograft models,[7] as well as towards leukemia cells,[8a-c] has been disclosed in recent years. DHA was also proved to act as a chemosensitizer of gemcitabine against HepG2 and Hep3B hepatocellular carcinoma cells.[9]

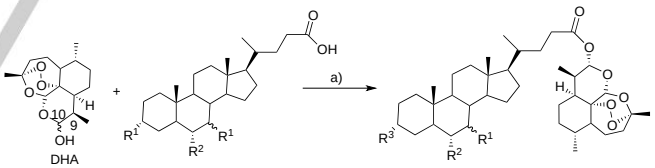
Fine chemical modifications of natural compounds can be exploited for designing new active compounds based on structure-function relationships. The tailoring of the physical-chemical properties can be addressed to improve the cytotoxicity and cytoselectivity towards target cancer cells or other properties such as bioavailability. This latter approach may represent an interesting strategy for the development of new anticancer drugs. In the past years, many C-10 derivatized DHA hybrids have been reported in literature. For example, DHA conjugated with natural active molecules such as coumarin,[10] cinnamic acid,[11] chalcone moieties,[12] *N*-aryl phenylethenesulfonamides[13], thymoquinone[14] and estradiol[15] with anticancer activity themselves, have been tested against a variety of cancer cell lines. Since our research has been mainly focused on the study of biologically active hybrids based on bile acids (BAs),[16a,c] we considered BAs as suitable partners for the preparation of novel DHA hybrids. BAs are oxidative metabolites of cholesterol with extensive biological activities.[17] The wide distribution of primary and secondary BAs in nature and the different available positions suitable for chemical modifications, such as C-3, C-6, C-7 and C-24, make BAs useful templates for drug discovery (Figure 1). From the physical-chemical point of view, BAs are amphiphilic molecules due to the presence of a concave hydrophilic α-side and a convex hydrophobic β-side. Hydroxyl group number, position (C-3, C-6, C-7) and stereochemistry (α or β) are responsible for their different lipophilicity. For instance, chenodeoxycholic bile acid CDCA and ursodeoxycholic bile acid UDCA, that differ from each other in the absolute configuration at C-7-OH, show different physical-chemical properties being CDCA more lipophilic than UDCA. Hyodeoxycholic bile acid HDCA, that differs from UDCA and CDCA for the position of one hydroxyl group, is in turn the least lipophilic compound within this series,[18] while lithocholic bile acid LCA corresponding to the 7-dehydroxylated CDCA, is the most lipophilic one (Figure 1).

The BAs lipophilicity has also been exploited to improve the bioavailability of chemotherapeutics by conjugating drugs to endogenous BAs through a covalent linkage.[19] The discovery of growth inhibitory effects of endogenous BAs on several cancer cell lines[20] through, among others, apoptosis, membrane alterations, modulation of nuclear receptors and oxidative stress points to a potential anticancer activity. UDCA is known to exert cytoprotective effects against the toxicity of other bile acids[21] and anticarcinogenic effects against hepatocellular carcinoma and colon cancers.[22a-f] However, endogenous BAs display a relatively low cytotoxicity (IC_{50} > 100 μM), therefore the search for new derivatives of BAs with higher cytotoxic activity has been receiving much attention.[23a-b]

In order to explore the cytotoxic activity of new BA-DHA hybrids, we selected four endogenous bile acids together with their C-3-azido analogues as DHA combination partners: CDCA, a primary bile acid produced in the liver and UDCA, HDCA and LCA, secondary bile acids generated during the intestinal transit (Figure 1). The C-3-azido derivatives, namely N_3CDC-DHA, N_3UDC-DHA**,** N_3HDC-DHA**,** N_3LC-DHA**,** were tested in the light of the azido group's biological relevance. Indeed, the effect of C-3-azido substitution on BAs has been studied on cancer cell viability.[24] Moreover, it has been recently reported that the replacement of the hydroxyl group at C-3 with a polarizable zwitterion moiety, such as the azido one (Figure 1), can enable the interaction with receptor residues *via* stable H-bond.[25a-c] In this light, a panel of BA-DHA hybrids was prepared combining the four selected endogenous BAs and their corresponding C-3-azido derivatives with DHA through different linkage positions (C-24 or C-3 or C-7) and linker nature (ester moiety, triazole, succinic chain). The hybrids were tested *in vitro* against HL-60 leukemia and HepG2 hepatocellular carcinoma cells and an insight of cell death mechanism was reported.

Results and Discussion

The condensation reaction between the appropriate bile acid and DHA mediated by EDCI was successfully employed for the preparation of BA-DHA hybrids **1**-**4**. The same synthetic strategy was applied for the preparation of hybrids **5**-**8** obtained by reaction of DHA and the appropriate 3-azido bile acid (Scheme 1).

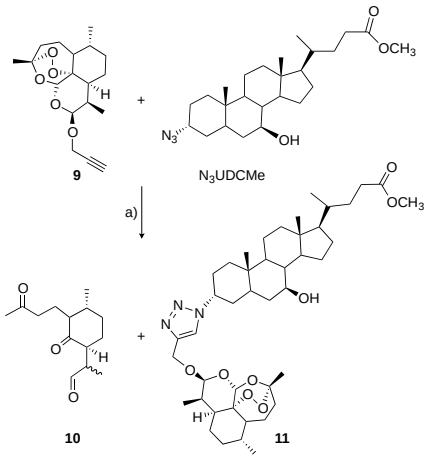


Scheme 1. Synthesis of hybrids **1-8** by condensation reaction. a): EDCI, DMAP, DMF, 25 °C, 18 h, 25-60% yield.

N_3CDCA, N_3UDCA and N_3LCA were in turn prepared as previously reported by the authors.[26] An unprecedented N_3HDCA was obtained in the same way unless in very poor yield. In all cases, the chromatographic purification allowed the isolation of the target hybrid as pure 10-α isomer whose configuration was assigned by ^{1}H-NMR analysis on the basis of $J_{H9\text{-}H10}$ value. The large $J_{H9\text{-}H10}$ found (9.7-9.8 Hz) was consistent with an anti-periplanar arrangement of H-9 and H-10 protons that led to the 10-α isomer. It is worth noting that, under the reaction conditions employed, only the α-stereoisomer was detected among the

products, even though the starting DHA was a mixture of α and β epimers.

We also explored a synthetic approach to obtaining a derivative of UDC-DHA hybrid *via* CuAAC click chemistry (Scheme 2). This coupling methodology allows introducing a triazolyl group which is known to improve the biostability, bioavailability and also the anticancer activity of bioactive compounds.[21, 22a]

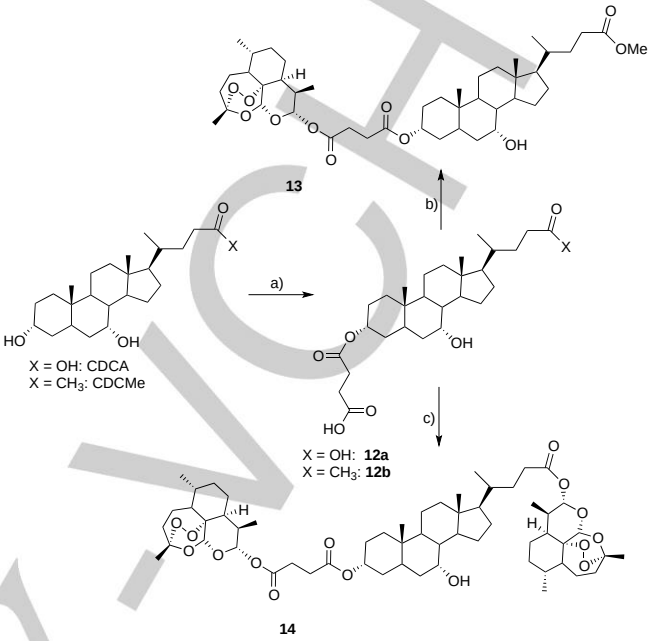


Scheme 2. Synthesis of triazolyl hybrid **11**. a): the best click chemistry reaction conditions: propargyl-DHA **9**/N_3UDCMe ratio: 1/1, CuI 0.1 eq, room temperature, 18 h, 28% yield.

Propargyl-DHA **9**,[27] was reacted in a 1:1 ratio with N_3UDCMe[24] in the presence of a Cu(I) based system catalyst. Several reaction conditions were explored by using different catalysts, solvents, temperature and reaction times (see SI table S1). The best results were obtained by using Cu(I) in the absence of ascorbate in anhydrous polar aprotic solvent such as acetonitrile at room temperature. ^{1}H-NMR analysis of the crude mixtures allowed to identify the signals of the target hybrid **11** together with signals that can be attributed to the tricarbonyl derivative **10**. We reasoned that the presence of reductive agents, such as click reagents, favors the reduction of DHA respect to the sterically hindered cycloaddition reaction. Indeed, under reductive conditions, DHA can undergo the homolytic cleavage of the peroxide ring, resulting in radical intermediates that proceed to form stable end products such as the tricarbonyl derivative **10**, already reported in literature as a DHA reduction product.[22c,d] Chromatographic purification on silica gel column allowed to obtain pure **11** in 10-β configuration in ca. 28% yield.

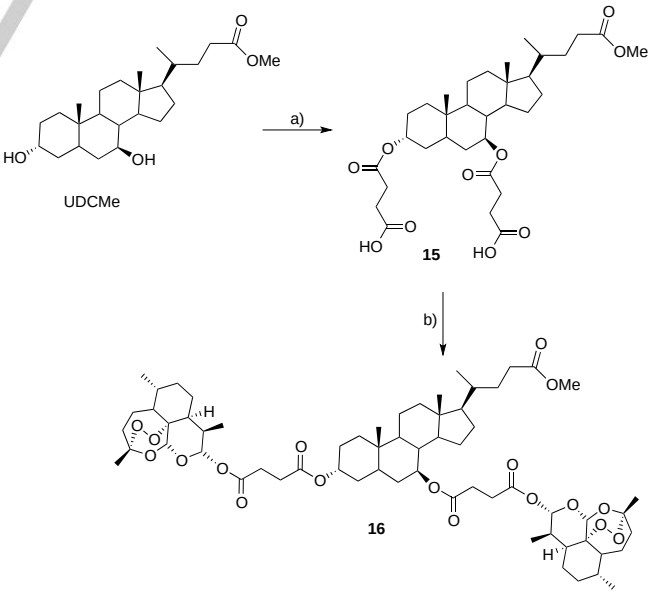
The synthesis of the target CDC-hybrid compounds **13** and **14** is described in Scheme 3. To prepare compound **13**, the C-3 hydroxyl group of CDCMe was first converted into its hemisuccinate by using succinic anhydride and DMAP. The resulting compound **12b** was condensed with DHA in the same conditions described above for the C-24 hybrids. Furthermore, we employed the hemisuccinate **12a** (obtained as for **12b**, but starting from CDCA), to achieve the bis-DHA hybrid **14** in which two residues of DHA were condensed to the position C-3 and C-24 respectively (Scheme 3).

Finally, we considered the preparation of the bis-DHA-hybrid **16,** in which two residues of DHA were condensed to UDCMe in the position C-3 and C-7 respectively, after the conversion of their hydroxyl groups into hemisuccinates by fusing UDCMe with an excess of succinic anhydride at a high temperature (Scheme 4).



Scheme 3. Synthesis of hybrids **13-14** by condensation reaction. a): succinic anhydride, DMAP, 115 °C, 20 h, 48-84% yield; b) **12b**, DHA, EDCI, DMAP, DMF, 25 °C, 18 h, 48% yield; c) **12a**, DHA, EDCI, DMAP, DMF, 25 °C, 18 h, 28% yield.

In all cases, the purification by flash chromatography allowed the isolation of the hybrids **13**, **14** and **16** as pure 10-α isomers.



Scheme 4. Synthesis of bis-DHA hybrid 16 by condensation reaction. a): succinic anhydride, 200 °C, 1 h, 69% yield; b): DHA, EDCI, DMAP, DMF, 25 °C, 25 % yield.

The biological activity of DHA and BA-DHA hybrids was evaluated in a leukemia cell line HL-60 and a hepatocellular carcinoma cell line HepG2 (Table 1).

HL-60 cells were treated with various concentrations of DHA and BA-DHA hybrids for 48 h, the cell viability was then determined by the MTT assay and the antiproliferative activities were expressed as IC_{50} values. As showed in Table 1, all the mono BA-DHA hybrids (**1**-**8**, **11** and **13**) were more potent, with IC_{50} values ranging from 0.19 μM (**2**) to 1.7 μM (**7**), than DHA with an IC_{50} value of 2.0 μM. On the contrary, bis-DHA hybrids (**14** and **16**) showed a lower cytotoxicity (with IC_{50} of 7.6 μM and 3.7 μM, respectively) compared to DHA. Similar results were obtained in HepG2 cells treated with DHA and BA-DHA hybrids for 72 h. In particular, hybrid **13**, the most potent hybrid of the series against HepG2 cells, showed an IC_{50} of 1.36 μM, whereas bis-DHA hybrids **14** and **16**, the least cytotoxic hybrids, showed IC_{50} of 29 μM and 21 μM respectively, while DHA had an IC_{50} value of 21 μM. All other hybrids were found also more cytotoxic than DHA in HepG2 cells with IC_{50} values ranging from 1.7 μM (**11**) and 20 μM (**5**).

In addition, the cytotoxic activity of the most potent hybrids **1**, **3**, **4**, **6**, **8**, **11** and **13** as well as of DHA was also evaluated on normal epithelial cells (NEC)[28] by MTT assay. All the compounds tested showed a remarkable lower cytotoxicity towards normal cells respect to HL-60 and HepG2 cancer cell lines (see SI table S2).

The toxicity of the BA and DHA derivative building blocks was also tested towards both HL-60 and HepG2 cell lines (see SI Table S3). All the BA building blocks showed an $IC_{50} \geq 100$ μM in both cell lines, except for: CDCMe with IC_{50} = 45 and 66 μM in HL-60 and HepG2 respectively; UDCMe and N_3UDCMe and compound **9** that are slightly toxic only in HL-60 cells with IC_{50} = 58, 44 and 21 μM respectively.

Table 1. IC_{50} values of DHA and BA-DHA hybrids in HL-60 cells (48 h-treatment) and HepG2 cells (72 h-treatment).

		HL-60		HepG2	
Bile acid	Compound	IC_{50} (μM)[a]	DHA/Hybrid [b]	IC_{50} (μM)[a]	DHA/Hybrid[b]
	DHA	2.0 ± 0.4	-	21 ± 2	-
	1	0.50 ± 0.06	4.0	3.8 ± 0.4	5.5
CDC	5	0.68 ± 0.01	2.9	20 ± 2	1.1
	13	0.34 ± 0.03	5.9	1.36± 0.04	15.4
	14	7.6 ± 1.4	0.3	29 ± 5	0.7
	2	0.19 ± 0.03	10.5	1.8 ± 0.2	11.7
UDC	6	0.24 ± 0.02	8.3	2.2 ± 0.4	9.5
	11	0.328 ± 0.002	6.1	1.7 ± 0.2	12.4
	16	3.7 ± 0.1	0.54	21 ± 2	1.0
HDC	3	0.35 ± 0.06	5.7	2.0 ± 0.2	10.5
	7	1.7 ± 0.2	1.2	14.2 ± 0.7	1.5
LC	4	0.41 ± 0.05	4.9	5.7 ± 0.7	3.7
	8	0.35 ± 0.04	5.7	4.7 ± 0.4	4.7

[a] Data are presented as mean ± SEM of at least three independent experiments. [b] The DHA/Hybrid value was calculated as the ratio of the IC_{50} of DHA and the hybrid.

The cytotoxic activity of the combination of DHA and UDCA at a 1:1 molar ratio was also tested against HL-60 cells in comparison with that of the corresponding hybrid **2** (UDC-DHA) (Figure 2).

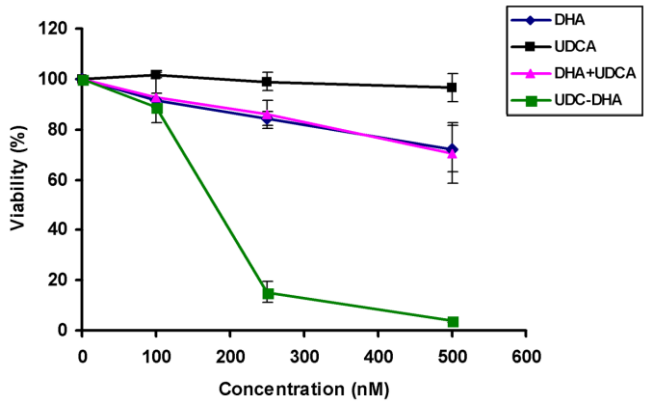


Figure 2. Hybrid **2** (UDC-DHA) is more potent than the combination of DHA and UDCA at a 1:1 molar ratio. HL-60 cells were treated with DHA or UDCA alone or in combination, or treated with hybrid **2** for 48 h. Cell viability was measured by the MTT assay. Data are presented as mean ± SEM of two to three independent experiments.

As expected, hybrid **2** was found much more potent than the two components combined (DHA+UDCA), since the combination revealed a similar cytotoxicity to that of DHA alone, confirming the importance of the hybridization to enhancing the biological activity of DHA (Figure 2).

Flow cytometry analysis and Western blot analysis were used to determine whether hybrid **2** (UDC-DHA) could induce apoptosis. HL-60 cells were treated with the vehicle control, 100 μM UDCA, 5 μM DHA or 0.5 μM hybrid **2** (UDC-DHA) in culture medium for 48 h and then harvested for propidium iodide (PI) staining and flow cytometry analysis or Western blot analysis. As illustrated in Figure 3a, the subG1 cells were significantly induced by 5 μM DHA (70.5%) and 0.5 μM hybrid **2** (90.7%) compared to the vehicle control (11.3%) or 100 μM UDCA (10.8%) ($P < 0.001$), suggesting that DHA and hybrid **2** caused cell death in HL-60 cells. In addition, subG1 population induced by 0.5 μM hybrid **2** was higher than that induced by 5 μM DHA ($P < 0.001$), consistent with results shown above that hybrid **2** was much more potent than DHA. Similar results were obtained in HepG2 cells treated with the vehicle control, 100 μM UDCA, 20 μM DHA or 2 μM hybrid **2** (UDC-DHA) for 48 h. As shown in Figure 3b, the subG1 cells were significantly induced by 20 μM DHA (28.1%) and 2 μM hybrid **2** (63.6%) compared to the vehicle control (3.88%) or 100 μM UDCA (4.89%) ($P < 0.001$ for DHA and $P < 0.01$ for hybrid **2**) in HepG2 cells. Western blot analysis revealed that DHA or hybrid **2**

treatment led to dramatic decreases in PARP and caspase 3 protein levels and significant increases in cleaved PARP and cleaved caspase 3 levels in HL-60 cells (Figure 3c) and PARP cleavage in HepG2 cells (see SI Figure S1), indicative of apoptosis. Altogether, these results confirmed that DHA and hybrid **2** (UDC-DHA) induced apoptosis in HL-60 and HepG2 cells. ART derivatives with the peroxide bridge can produce free radicals or reactive oxygen species (ROS) which are associated with the induction of oxidative DNA damage and apoptosis.[29] To determine whether BA-DHA hybrids could induce ROS, HepG2 cells were treated with 20 or 40 μM of DHA or hybrid **2** (UDC-DHA) for 24 h and DCFH-DA was included in the last 30 min of incubation. Cells were then harvested for flow cytometric analysis to measure ROS generation.[28] Results shown in Figure 3d indicated that both DHA and hybrid **2** (UDC-DHA) induced ROS in a dose-dependent manner and 40 μM of hybrid **2** induced significantly more cells with ROS production compared to 40 μM of DHA ($P < 0.05$), suggesting that ROS induction by BA-DHA may be correlated with apoptosis in HepG2 cells. In contrast, no clear ROS production was detected in DHA or hybrid **2**-treated HL-60 cells (data not shown). Lu et al. did not observe ROS induction by DHA in HL-60 cells either using the same detection method.[8b] It has been widely believed that undifferentiated HL-60 cells cannot produce ROS in response to stimuli; however, it has also been reported that ROS can be induced by stimuli and measured using a highly sensitive chemiluminescence dye L-012 in HL-60 cells.[30] More studies are required in order to clarify whether ROS play a role in apoptosis induced by BA-DHA hybrids in HL-60 cells.

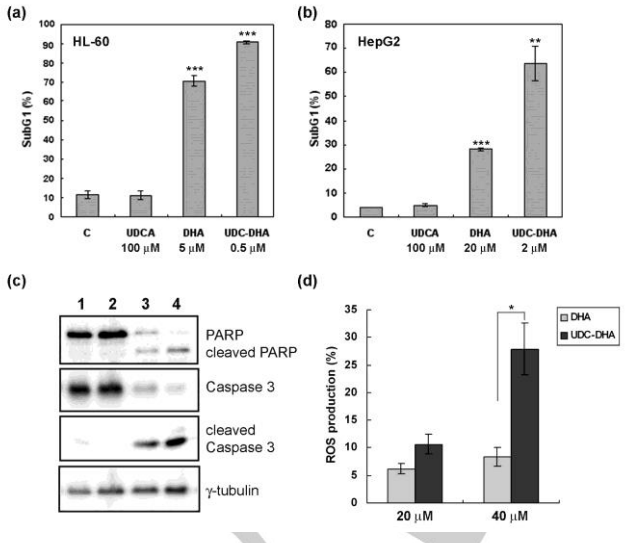


Figure 3. DHA and hybrid **2** induce apoptosis and ROS. (a) SubG1 cell populations in HL-60 cells treated with the DMSO vehicle control (C), 100 μM UDCA, 5 μM DHA and 0.5 μM **2** (UDC-DHA**)** for 48 h. Data are presented as mean ± SEM of at least three independent experiments. ***, $P < 0.001$ (compared to C or 100 μM UDCA). (b) SubG1 cell populations in HepG2 cells treated with the DMSO vehicle control, 100 μM UDCA, 20 μM DHA and 2 μM **2** (UDC-DHA**)** for 48 h. Data are presented as mean ± SEM of three independent experiments. **, $P < 0.01$. (c) Western blot analysis of HL-60 cells treated with the DMSO vehicle control (1), 100 μM UDCA (2), 5 μM DHA (3) and 0.5 μM hybrid **2** (4) for 48 h. γ-tubulin was used as a loading control. (d) DHA and hybrid **2** induced ROS in HepG2 cells. Cells were treated with 20 or 40 μM of DHA or hybrid **2** for 24 h and subjected to ROS detection by flow cytometry. Data are presented as mean ± SEM of three independent experiments. *, $P < 0.05$.

In summary, in all cases, except for bis-DHA hybrids **14** and **16**, the hybridization of DHA with BAs significantly enhanced the growth inhibitory effect of DHA in HL60 and HepG2 cells, with the IC_{50} ratios of DHA and BA-DHA hybrids ranging from 1.2 to 10.5 in HL-60 cells and from 1.1 to 15.4 in HepG2 cells. Hybrid **2** (UDC-DHA) was found the most potent hybrid with a 10.5-fold increase in biological activity against HL-60 cells, whereas hybrid **13** (CDC based hybrid conjugated through a succinic linker) was the most potent hybrid with a 15.4-fold increase in biological activity against HepG2 cells. Hybrid **11** (UDC based hybrid conjugated through a triazole linker) also exhibited a 12.4-fold increase in biological activity in HepG2 cells followed by **2** (UDC-DHA) with a 11.7-fold increase, **3** (HDC-DHA) with a 10.5-fold increase and **6** (N_3UDC-DHA) with a 9.5-fold increase (Table 1). Altogether, these results indicate that the conjugation of DHA with BA scaffolds strengthens the cytotoxicity of DHA. Remarkably the bis-DHA derivatives **14** and **16** were found both less cytotoxic than the conjugates **13** and **11** respectively. The 2:1 ratio of DHA vs BA seems to lower the cytotoxicity induced by the conjugation, but other reasons, such as differences in steric hindrance or the nature of the BA, cannot be excluded.

Figure 4 reports the hybrids with a significantly increased potency upon conjugation and highlights their cytoselectivity.

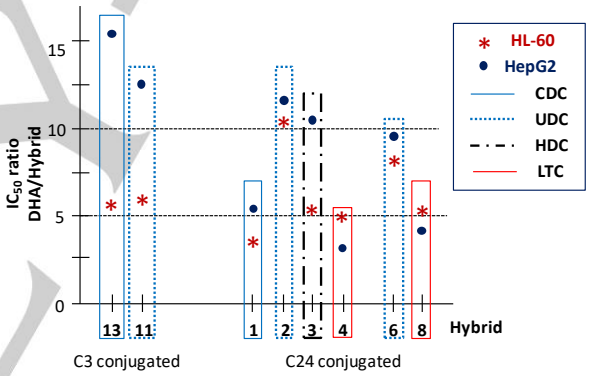


Figure 4. The improvement of antiproliferative activity expressed as IC_{50} ratio of DHA and selected hybrids in HL-60 and HepG2 cells.

Between the compounds conjugated at the C24 position, only hybrid **3** (HDC derivative) showed a marked preference for HepG2 compared to HL-60 cells. In contrast, both conjugates at the C3 position, hybrids **11** and **13**, showed a markedly higher improvement in cytotoxicity towards HepG2 respect to HL-60 despite the different linker (a triazole and a succinic linker respectively) and BA nature (UDC and CDC, respectively). These data might indicate that the conjugation at the C3 position favors the cytoselectivity towards the HepG2 cell line, possibly reflecting a more favorable uptake by hepatic cells compared to HL-60 cells (Figure 4).

Conclusions

The overall findings indicate that the conjugation of DHA with BAs can significantly improve the *in vitro* cytotoxicity in the cancer cell lines tested. This improvement is more marked in HepG2 for which DHA alone displayed a lower cytotoxicity than in HL-60

(IC_{50} 21 and 2.0 μM respectively). In HepG2 cells, four hybrids **13**, **11**, **2** and **3** were found at least 10 times more active respect to DHA alone with IC_{50} values in the low micromolar range from 1.36 μM (**13**) to 2.0 μM (**3**). In HL-60 cells, most of the tested hybrids (**1**, **2**, **3**, **4**, **5**, **6**, **8**, **11** and **13**) showed IC_{50} values in the sub-micromolar range from 0.19 μM (**2**) to 0.68 μM (**5**), but only hybrid **2** was at least 10 times more active than DHA.

Experimental Section

Materials and methods

The reactions for the synthesis of DHA-BA hybrids were monitored by TLC on pre-coated Silica Gel F_{254} plates (thickness 0.25 mm, Merck), and phosphomolybdic acid solution was used as the spray reagent to visualize the steroids. Flash column chromatography was performed on silica gel 60 Å (230-400 mesh) or with a combiflash apparatus. NMR spectra were recorded with a Varian Mercury 400 MHz instrument. ESI-MS were acquired on a Thermo Finnigan LCQ Duo Ion Trap. Elemental analyses were performed on a Perkin-Elmer 2400 microanalyzer instrument. Synthesis of intermediate N_3CDC, N_3UDC and N_3LCA were performed as previously reported.[25] Synthesis of N_3HDCA is reported in Supplementary data. Commercial DHA (from Carbosynt), CDCA, UDCA, LCA and HDCA (from ICE SpA, Italy) were used without any further purification.

General procedure for the condensation reaction

A solution of **DHA** (0.18 mmol) and the appropriate bile acid, or N_3BA derivatives, (0.27 mmol) in anhydrous DMF (1 ml) was cooled (ice bath) under Argon atmosphere, then DMAP (0.27 mmol) and EDCI (0.31 mmol) were added. After 10 min at 0 °C the reaction was warmed up to room temperature and stirred for 18h, then diluted with H_2O (15 mL) and extracted with Et_2O (3x5 mL). The organic layers were combined, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography.

Compound 1 (CDC-DHA). Flash chromatography (AcOEt/cyclohexane 3:2 + 1‰ Et_3N): colorless syrup, yield 60%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.7 Hz), 5.53 (1H, s), 4.30 (1H, d, *J*=4.7 Hz, ex D_2O), 4.10 (1H, d, *J*=3.3 Hz, ex D_2O), 3.60 (1H, bs), 3.22-3.10 (1H, m), 2.46-2.08 (5H, m), 2.02-0.79 (45H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.58 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.08, 170.24, 103.46, 91.37, 90.46, 79.77, 70.24, 66.06, 59.68, 55.37, 51.04, 49.89, 44.51, 41.85, 41.33, 35.87, 35.80, 35.23, 34.74, 34.65, 33.63, 32.17, 31.47, 30.46, 30.36, 29.00, 27.67, 25.42, 24.11, 23.07, 22.62, 22.37, 20.92, 20.17, 19.99, 18.02, 14.00, 11.76, 11.57. MS (ESI, ES+): Calcd for $[C_{39}H_{62}O_8 + Na]^+$ 681.43; Found 681.33; 1339.40 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{62}O_8$: C, 71.09; H, 9.48; Found: C, 70.84; H, 9.83.

Compound 2 (UDC-DHA). Flash chromatography (AcOEt/cyclohexane 6:5): colorless syrup, yield 61%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.8 Hz), 5.54 (1H, s), 4.43 (1H, d, *J*=4.6 Hz, ex D_2O), 3.87 (1H, d, *J*=6.9 Hz, ex D_2O), 3.31-3.20 (2H, m), 2.47-2.37 (2H, m), 2.33-2.10 (3H, m), 2.02-0.80 (45H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.60 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.11, 103.47, 91.39, 90.46, 79.79, 69.63, 69.35, 59.68, 55.74, 54.49, 51.04, 44.50, 43.01, 42.91, 42.08, 37.64, 37.18, 35.86, 35.80, 34.74, 34.65, 33.67, 31.48, 30.46, 30.17, 28.07, 26.65, 25.43, 24.11, 23.23, 20.91, 20.76, 19.99, 18.18, 14.01, 11.95, 11.76. MS (ESI, ES+): Calcd for $[C_{39}H_{62}O_8 + Na]^+$ 681.43; Found 681.27; 1339.33 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{62}O_8$: C, 71.09; H, 9.48; Found: C, 70.94; H, 9.86.

Compound 3 (HDC-DHA). Flash chromatography (AcOEt/cyclohexane 3:2 + 1‰ Et_3N): colorless syrup, yield 37%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.7 Hz), 5.54 (1H, s), 4.40 (1H, d, *J*=4.5 Hz), 4.23 (1H, d, *J*=4.1 Hz), 3.86-3.74 (1H, m), 3.30-3.21 (1H, m), 2.47-2.10 (5H, m), 2.02-0.79 (45H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.58 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.15, 103.83, 91.45, 91.50, 79.82, 69.94, 65.84, 55.75, 55.29, 51.01, 48.08, 44.45, 42.27, 35.89, 35.28, 34.58, 34.22, 33.57, 31.46, 30.26, 29.08, 27.55, 25.39, 23.77, 23.44, 20.90, 20.28, 19.97, 17.96, 11.73. MS (ESI, ES+): Calcd for $[C_{39}H_{62}O_8 + H]^+$ 659.92, $[C_{39}H_{62}O_8 + Na]^+$ 681.91; Found 659.13, 681.20, 1339.33 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{62}O_8$: C, 71.09; H, 9.48; Found: C, 70.88; H, 9.69.

Compound 4 (LC-DHA). Flash chromatography (AcOEt/cyclohexane 3:2 + 1‰ Et_3N): colorless syrup, yield 26%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.66 (1H, d, *J*=9.7 Hz), 5.56 (1H, s), 4.45 (1H, d, *J*=4.5 Hz), 3.42-3.35 (1H, m), 2.47-2.08 (5H, m), 2.06-0.80 (47H, m), 0.77 (3H, d, *J*=7.1 Hz), 0.61 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.10, 103.47, 91.37, 90.45, 79.77, 69.75, 55.93, 55.32, 51.02, 44.48, 42.21, 41.41, 40.30, 36.20, 35.83, 35.27, 35.04, 34.62, 34.12, 33.61, 31.48, 30.30, 27.59, 26.79, 26.07, 25.42, 24.10, 23.76, 23.18, 20.90, 20.31, 20.00, 18.00, 11.77. MS (ESI, ES+): Calcd for $[C_{39}H_{62}O_7 + Na]^+$ 665.91; Found 665.27, 1307.47 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{62}O_7$: C, 72.86; H, 9.72; Found: C, 72.50; H, 10.03.

Compound 5 (N_3CDC-DHA). Flash chromatography (cyclohexane/AcOEt 6:1 + 3‰ Et_3N): white amorphous solid, yield 42%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.7 Hz), 5.54 (1H, s), 4.22 (1H, d, *J*=3.4 Hz, ex D_2O), 3.61 (1H, bs), 3.27-3.14 (1H, m), 2.46-2.05 (5H, m), 2.02-0.78 (45H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.59 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.66, 104.00, 91.92, 90.98, 80.33, 66.46, 61.09, 60.21, 55.82, 51.56, 50.35, 45.02, 42.37, 41.80, 36.38, 35.44, 35.23, 35.11, 34.88, 34.14, 32.65, 32.01, 30.86, 28.16, 26.88, 25.95, 24.63, 23.54, 23.05, 21.43, 20.66, 20.53, 18.57, 14.54, 12.29, 12.12. MS (ESI, ES+): Calcd for $[C_{39}H_{61}N_3O_7 + Na]^+$ 706.92; Found 706.33, 1389.47 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{61}N_3O_7$: C, 68.49; H, 8.99; N, 6.14; Found: C, 68.17; H, 9.37; N, 5.89.

Compound 6 (N_3UDC-DHA). Flash chromatography conditions cyclohexane/AcOEt 4:1 + 1‰ Et_3N; white amorphous solid, yield 45%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.8 Hz), 5.54 (1H, s), 3.91 (1H, d, *J*=6.9 Hz, ex D_2O), 3.43-3.20 (2H, m), 2.46-2.35 (1H, m), 2.33-2.10 (3H, m), 2.08-0.77 (46H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.60 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.64, 131.63, 110.00, 103.99, 91.91, 90.98, 80.30, 69.59, 60.55, 55.97, 54.96, 51.56, 45.02, 43.51, 43.37, 42.61, 38.96, 37.76, 36.38, 35.15, 34.14, 33.23, 32.00, 30.92, 28.58, 27.12, 26.57, 25.96, 24.64, 24.54, 23.62, 21.44, 21.30, 20.53, 18.70, 17.87, 12.47, 12.29. MS (ESI, ES+): Calcd for $[C_{39}H_{61}N_3O_7 + Na]^+$ 706.92; Found 706.40, 1389.33 $[2M+Na]^+$. Anal Calcd for $C_{39}H_{61}N_3O_7$: C, 68.49; H, 8.99; N, 6.14; Found: C, 68.21; H, 9.35; N, 5.91.

Compound 7 (N_3HDC-DHA). Flash chromatography (cyclohexane/AcOEt 3:1 + 3‰ Et_3N): white amorphous solid, yield 67%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.7 Hz), 5.54 (1H, s), 5.16-5.12 (1H, m), 4.08 (1H, d, *J*=3.0 Hz), 3.89-3.72 (1H, m), 2.46-2.09 (5H, m), 2.07-0.78 (45H, m), 0.75 (3H, d, *J*=7.1 Hz), 0.58 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.63, 140.09, 121.20, 103.99, 91.91, 90.98, 80.30, 65.52, 60.21, 58.16,

56.72, 55.65, 51.56, 49.95, 45.02, 42.79, 42.34, 37.06, 36.38, 35.14, 34.15, 33.59, 32.78, 32.00, 30.89, 28.92, 28.08, 25.96, 24.64, 24.32, 21.44, 20.78, 20.53, 19.15, 18.56, 14.54, 12.30, 12.16. MS (ESI, ES+): Calcd for $[C_{39}H_{61}N_3O_7 + Na]^+$ 706.92; Found 706.27. Anal Calcd for $C_{39}H_{61}N_3O_7$: C, 68.49; H, 8.99; N, 6.14; Found: C, 68.11; H, 9.30; N, 5.82.

Compound 8 (N_3LC-DHA). Flash chromatography (cyclohexane/AcOEt 3:1 + 3‰ Et_3N): white amorphous solid, yield 64%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.7 Hz), 5.54 (1H, s), 4.43 (1H, d, *J*=4.6 Hz), 2.46-2.06 (5H, m), 2.04-0.77 (47H, m), 0.75 (3H, d, *J*=7.0 Hz), 0.59 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.64, 149.04, 134.50, 103.99, 91.91, 90.98, 80.32, 70.29, 56.46, 55.86, 55.38, 51.56, 45.02, 42.73, 41.94, 36.38, 35.81, 35.58, 35.16, 34.65, 34.14, 32.01, 30.83, 28.13, 27.31, 26.60, 25.96, 24.64, 24.29, 23.72, 21.43, 20.84, 20.53, 18.54, 12.31.

MS (ESI, ES+): Calcd for $[C_{39}H_{61}N_3O_6 + H]^+$ 668.93; Found 667.30. Anal Calcd for $C_{39}H_{61}N_3O_6$: C, 70.13; H, 9.21; N, 6.29; Found: C, 69.91; H, 9.45; N, 6.01.

Compound 11. To a 1:1 solution of propargyl-DHA **9** (0.50 mmol) and N_3UDCMe (0.50 mmol) in anhydrous CH_3CN (1 ml), CuI (0.05 mmol) was added. After stirring at room temperature for 18 h the solvent was removed *in vacuo* and the crude material was purified by flash chromatography on silica gel (cyclohexane/AcOEt 1:1 + 3‰ Et_3N): colorless syrup, yield 28%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 8.23 (1H, s), 5.30 (1H, s), 4.81 (1H, d, *J*=3.6 Hz), 4.52-4.38 (1H, m), 3.93 (1H, d, *J*=6.9 Hz), 3.57 (3H, s), 3.34-3.27 (1H, m), 2.48-2.27 (3H, m), 2.25-0.75 (52H, m), 0.63 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 173.67, 143.50, 121.87, 103.23, 100.04, 86.90, 80.37, 68.98, 60.33, 59.53, 55.32, 54.57, 51.89, 51.13, 43.68, 42.96, 42.41, 40.29, 38.30, 37.60, 37.18, 36.61, 35.91, 34.87, 34.69, 34.13, 33.94, 33.71, 30.63, 30.26, 28.93, 28.06, 27.46, 26.59, 25.55, 24.21, 23.89, 23.07, 20.87, 20.04, 18.23, 12.64, 11.92. MS (ESI, ES+): Calcd for $[C_{43}H_{67}N_3O_8 + Na]^+$ 777.01; Found 776.47, 1529.40 $[2M+Na]^+$. MS (ESI, ES-): Calcd for $[C_{43}H_{67}N_3O_8 + Cl]^-$ 787.47; Found 788.27, 1540.87 $[2M+Cl]^-$. Anal Calcd for $C_{43}H_{67}N_3O_8$: C, 68.50; H, 8.96; N, 5.57; Found: C, 68.34; H, 9.24; N, 5.36.

Compounds 12a,b. To a solution of CDCA or CDCMe (0.25 mmol) in Py (2 ml), succinic anhydride (1.27 mmol) and catalytic DMAP were added. The mixture was warmed to reflux (115°C). After 18h, succinic anhydride (0.64 mmol) and catalytic DMAP were added again. After other 4h reflux, the reaction mixture was cooled to room temperature, diluted with AcOEt (20 ml) and washed first with 5% HCl (3x7 ml) and then with H_2O (10ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was used for subsequent reaction without any further purification. **12a.** White amorphous solid, yield 48%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 12.07 (2H, bs), 4.49-4.33 (1H, m), 4.18 (1H, bs), 3.61 (1H, bs), 2.45-2.28 (4H, m), 2.27-2.00 (2H, m), 1.96-0.79 (30H, m), 0.58 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 175.32, 173.80, 171.98, 74.51, 66.51, 55.94, 50.37, 42.36, 41.41, 40.85, 35.38, 35.13, 35.00, 34.88, 32.65, 31.18, 29.41, 29.20, 29.13, 28.22, 26.87, 23.56, 22.90, 20.67, 18.60, 12.09. MS (ESI, ES+): Calcd for $[C_{28}H_{44}O_7 + Na]^+$ 515.64; Found 515.27, 1007.20 $[2M+Na]^+$. Anal Calcd for $C_{28}H_{44}O_7$: C, 68.26; H, 9.00; Found: C, 68.01; H, 9.33. **12b.** White amorphous solid, yield 84%. ^{1}H-NMR (400MHz, $CDCl_3$): δ= 4.68-4.52 (1H, m), 3.85 (1H, d, *J*=2.6 Hz), 3.66 (3H, s), 2.74-2.49 (4H, m), 2.41-2.15 (3H, m), 2.01-0.96 (24H, m), 0.95-0.88 (6H, m), 0.65 (3H, s). ^{13}C-NMR (101MHz, $CDCl_3$): δ= 176.80, 174.79, 171.79, 74.90, 68.48, 55.75, 51.52, 50.35, 42.68, 41.13, 39.52, 39.32, 35.37, 35.11, 35.04, 34.92, 34.33, 32.73, 31.02, 30.97, 29.37, 28.94, 28.15, 26.59, 23.68, 22.71, 20.56, 18.25, 11.75. MS (ESI, ES+): Calcd for $[C_{29}H_{46}O_7 + Na]^+$ 529.67; Found 529.33, 1035.20 $[2M+Na]^+$. MS (ESI, ES-): Calcd for $[C_{29}H_{46}O_7 - H]^-$ 505.67; Found 505.13, 1011.20 $[2M-H]^-$, 1516.87 $[3M-H]^-$. Anal Calcd for $C_{29}H_{46}O_7$: C, 68.75; H, 9.15; Found: C, 68.47; 9.46.

Compound 13. The reaction was carried out as described in general procedure for the condensation reaction starting from compound **12b**. Flash chromatography on silica gel (cyclohexane/AcOEt 5:3): white amorphous solid, yield 48%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.64 (1H, d, *J*=9.8 Hz), 5.55 (1H, s), 4.43 (1H, bs), 4.17 (1H, d, *J*=3.4 Hz), 3.60 (1H, bs), 3.55 (3H, s), 2.68-2.50 (4H, m), 2.47-2.10 (5H, m), 2.09-0.72 (48H, m), 0.58 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 174.21, 171.62, 171.31, 157.61, 104.00, 99.63, 92.22, 91.01, 80.30, 74.68, 66.47, 60.21, 55.85, 51.66, 51.54, 51.28, 50.35, 45.29, 44.99, 42.36, 41.38, 36.34, 35.33, 35.11, 34.97, 34.16, 32.62, 32.09, 31.07, 30.78, 29.13, 28.21, 26.79, 25.96, 24.79, 23.55, 22.89, 21.44, 20.66, 20.51, 18.57, 14.54, 12.34, 12.08. MS (ESI, ES+): Calcd for $[C_{44}H_{68}O_{11} + Na]^+$ 796.01; Found 795.40, 1567.13 $[2M+Na]^+$. Anal Calcd for $C_{44}H_{68}O_{11}$: C, 68.37; H, 8.87; Found: C, 68.07; H, 9.10.

Compound 14. A solution of DHA (0.36 mmol) and **12a** (0.18 mmol) in anhydrous DMF (2 ml) was cooled (ice bath) under Argon atmosphere, then DMAP (0.20 mmol) and EDCI (0.61 mmol) were added. After 10 min at 0 °C the reaction was warmed up to room temperature and stirred for 18h, then diluted with H_2O (15 mL) and extracted with Et_2O (3x5 mL). The organic layers were combined, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (cyclohexane/AcOEt 5:2 + 3‰ Et_3N): white amorphous solid, yield 28%. ^{1}H-NMR (400MHz, DMSO-*d*$_6$): δ= 5.66 (2H, dd, *J*=9.8, 1.4 Hz), 5.56 (2H, d, *J*=3.5 Hz), 4.52-4.38 (1H, m), 4.19 (1H, d, *J*=3.3 Hz), 3.62 (1H, bs), 2.66-2.51 (4H, m), 2.48-2.10 (8H, m), 2.06-0.73 (66H, m), 0.61 (3H, s). ^{13}C-NMR (101MHz, DMSO-*d*$_6$): δ= 172.10, 171.07, 170.77, 103.46, 91.69, 91.37, 90.49, 79.77, 74.15, 65.93, 55.32, 51.02, 49.81, 44.48, 41.85, 40.86, 35.85, 34.68, 34.58, 33.61, 32.09, 31.57, 31.47, 30.39, 28.62, 27.66, 26.28, 25.42, 24.10, 23.02, 22.36, 20.91, 19.98, 18.04, 11.84, 11.76, 11.57. MS (ESI, ES+): Calcd for $[C_{58}H_{88}O_{15} + Na]^+$ 1048.32, $[C_{58}H_{88}O_{15} + K]^+$ 1064.42; Found 1047.33, 1063.27. Anal Calcd for $C_{58}H_{88}O_{15}$: C, 67.94; H, 8.65; Found: C, 67.79; H, 8.98.

Compound 15. UDCMe (0.61 mmol) and succinic anhydride (4.92 mmol) were warmed to fusion (200°C). After 1h, the mixture was cooled to room temperature, dissolved in DCM (25 ml) and extracted with aqueous $NaHCO_3$ (3x10 ml). The aqueous layers were combined and acidified to pH=5 by 10% HCl. The product was then extracted by DCM (3x10 ml). The organic layers were combined, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was used for the next reaction without any further purification. White amorphous solid, yield 69%. ^{1}H-NMR (400MHz, $CDCl_3$): δ= 9.47 (2H, bs), 4.84-4.74 (1H, m), 4.73-4.62 (1H, m), 3.65 (3H, s), 2.71-2.46 (8H, m), 2.40-2.13 (2H, m), 1.98 (1H, d, *J*=12.5 Hz), 1.88-0.99 (23H, m), 0.96 (3H, s), 0.90 (3H, d, *J*=6.3 Hz), 0.66 (3H, s). ^{13}C-NMR (101MHz, $CDCl_3$): δ= 178.08, 177.98, 174.77, 171.67, 171.59, 74.31, 74.05, 55.11, 54.92, 51.51, 43.54, 41.99, 39.90, 39.37, 35.20, 34.43, 33.94, 32.67, 31.05, 30.95, 29.48, 29.22, 29.01, 28.84, 28.35, 26.30, 25.68, 23.20, 21.19, 18.36, 12.05. MS (ESI, ES+): Calcd for $[C_{33}H_{50}O_{10} + Na]^+$ 629.74; Found 629.33, 1235.20 $[2M+Na]^+$, 1840.73 $[3M+Na]^+$. MS

(ESI, ES-): Calcd for $[C_{33}H_{50}O_{10} - H]^-$ 605.75; Found 605.20, 1211.00 $[2M-H]^-$, 1818.07 $[3M-H]^-$. Anal Calcd for $C_{33}H_{50}O_{10}$: C, 65.33; H, 8.31; Found: C, 65.04; H, 8.54.

Compound 16. The reaction between DHA and compound **15** was carried out as described above for hybrid **14**. Flash chromatography (cyclohexane/AcOEt 5:3). White amorphous solid, yield 25%. ^{1}H-NMR (400MHz, DMSO-d_6): δ= 5.65 (2H, dd, *J*=9.8, 4.8 Hz), 5.55 (2H, s), 4.71-4.48 (2H, m), 3.55 (3H, s), 2.70-2.38 (8H, m), 2.36-0.69 (74H, m), 0.61 (3H, s). ^{13}C-NMR (101MHz, DMSO-d_6): δ= 174.15, 171.72, 171.49, 171.31, 171.17, 103.99, 92.19, 91.01, 80.28, 73.87, 73.76, 54.76, 51.66, 51.55, 44.96, 43.51, 41.62, 39.05, 36.35, 35.04, 34.27, 34.15, 34.00, 32.96, 32.83, 32.08, 31.03, 30.83, 30.69, 29.36, 29.13, 28.91, 28.46, 25.95, 24.78, 24.64, 23.27, 21.43, 21.18, 20.51, 18.66, 12.43, 12.24, 12.21. MS (ESI, ES+): Calcd for $[C_{63}H_{94}O_{18} + Na]^+$ 1162.42; Found 1161.33. Anal Calcd for $C_{63}H_{94}O_{18}$: C, 66.41; H, 8.32; Found: C, 66.25; H, 8.62.

Biological evaluation

Cell culture, drug treatment and cell viability assays. The HL-60 human promyelocytic leukemia cells (ATCC CCL-240) were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1x Antibiotic-Antimycotic containing 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Fungizone™ (HyClone, Logan, UT), and the HepG2 human heptocellular carcinoma cells (ATCC HB-8065) were cultured in low-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1x Antibiotic-Antimycotic at 37°C in a humidified 5% CO2 atmosphere. Cells were seeded in 96-well plates (3×10^4 HL-60 cells/well and 5×10^3 HepG2 cells/well) and subjected to drug treatment for the indicated time periods followed by the MTT assay to measure cell growth as described previously.[28] Absorbance was measured at 570 nm with a reference wavelength of 690 nm.

PI staining and flow cytometric analysis. Cells were treated with drugs, harvested and fixed in methanol for at least 30 min on ice and then subjected to PI staining and flow cytometric analysis. At least 10,000 cells were analyzed for each sample using CellQuest Pro software (BD Biosciences, San Jose, CA).

Western blot analysis. After drug treatment, cells were harvested and lysed in 1x SDS sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1.4% 2-mercaptoethanol and 0.001% bromophenol blue, and boiled for 5 min. Lysates were then subjected to 10% SDS-PAGE and Western blot analysis as previously described.[31] Primary antibodies used were PARP (BD Biosciences, San Jose, CA), caspase 3 (BioCarta, San Diego, CA) and γ-tubulin (Sigma-Aldrich, St. Louis, MO).

Detection of ROS. 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used for the detection of ROS. DCFH-DA is nonfluorescent in the reduced state and is hydrolyzed by intracellular esterases to 2,7-dichlorodihydrofluorescein, which is then oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. The fluorescence intensity of DCF is then used to measure intracellular ROS by flow cytometry. HepG2 cells were seeded in 12-well plates, treated as indicated for 24 h and 10 µM DCFH-DA was added for the last 30 min of incubation at 37°C. Cells were then harvested by trypsinization, resuspended in ice-cold PBS and subjected to flow cytometirc analysis. Cells with ROS production was quantified.

Statistical analysis. Results were presented as mean ± standard error of the mean (SEM). Statistical significance was assessed with two-sided *t*-tests, and *P*-values less than 0.05 were considered statistically significant.

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Keywords: dihydroartemisinin • bile acids • natural product hybrids • anticancer activity • hepatocellular carcinoma

References:

[1] a) L. Shu, K.-L. Cheung, T. O. Khor, C. Chen, A.-N. Kong, *Cancer and Metastasis Reviews* **2010**, *29*, 483-502; b) H. Wang, T. Oo Khor, L. Shu, Z.-Y. Su, F. Fuentes, J.-H. Lee, A.-N. Tony Kong, *Anti-Cancer Agents in Med Chem* **2012**, *12*, 1281-1305; c) C. N. Ramirez, W. Li, C. Zhang, R. Wu, S. Su, C. Wang, L. Gao, R. Yin, A.-N. Kong, *AAPS J* **2017**, *20*, 19.

[2] S. Lev-Ari, A. Vexler, A. Starr, M. Ashkenazy-Voghera, J. Greif, D. Aderka, R. Ben-Yosef, *Cancer Invest* **2007**, *25*, 411-418.

[3] S. Banerjee, Y. X. Zhang, S. Ali, M. Bhuiyan, Z. W. Wang, P. J. Chiao, P. A. Philip, J. Abbruzzese, F. H. Sarkar, *Cancer Res* **2005**, *65*, 9064-9072.

[4] M. Chadwick, H. Trewin, F. Gawthrop, C. Wagstaff, *Int J Mol Sci* **2013**, *14*, 12780-12805.

[5] Y. Li, P. Yu, Y.-X. Chen, L.-Q. Li, Y.-Z. Gai, D.-S. Wang, Y.-P. Zheng, *Chin Sci Bull (Reprinted from Kexue Tongbao).* **1979**, *24*, 667-669.

[6] G. H. Posner, I. H. Paik, W. Chang, K. Borstnik, S. Sinishtaj, A. S. Rosenthal, T. A. Shapiro, *J Med Chem* **2007**, *50*, 2516-2519.

[7] C. Z. Zhang, H. T. Zhang, J. P. Yun, G. G. Chen, P. B. S. Lai, *Biochem Pharmacol* **2012**, *83*, 1278-1289.

[8] a) S. Finaurini, N. Basilico, Y. Corbett, S. D'Alessandro, S. Parapini, P. Olliaro, R. K. Haynes, D. Taramelli, *Toxicology* **2012**, *300*, 57-66; b) J. J. Lu, L. H. Meng, Y. J. Cai, Q. Chen, L. J. Tong, L. P. Lin, J. Ding, *Cancer Biol Ther* **2008**, *7*, 1017-1023; c) Z. Wang, W. Hu, J. L. Zhang, X. H. Wu, H. J. Zhou, *FEBS Open Bio* **2012**, *2*, 103-112.

[9] J. M. Hou, D. S. Wang, R. W. Zhang, H. Wang, *Clin Cancer Res* **2008**, *14*, 5519-5530.

[10] Y. Tian, Z. Liang, H. Xu, Y. H. Mou, C. Guo, *Molecules* **2016**, *21*, 758.

[11] C. C. Xu, T. Deng, M. L. Fan, W. B. Lv, J. H. Liu, B. Y. Yu, *Eur J Med Chem* **2016**, *107*, 192-203.

[12] F. J. Smit, R. A. van Biljon, L. M. Birkholtz, D. D. N'Da, *Eur J Med Chem* **2015**, *90*, 33-44.

[13] Y. J. Liu, Z. J. Liu, J. Y. Shi, H. M. Chen, B. Mi, P. Li, P. Gong, *Molecules* **2013**, *18*, 2864-2877.

[14] T. Frohlich, C. Reiter, M. E. M. Saeed, C. Hutterer, F. Hahn, M. Leidenberger, O. Friedrich, B. Kappes, M. Marschall, T. Efferth, S. B. Tsogoeva, *ACS Med Chem Lett* **2018**, *9*, 534-539.

[15] T. Frohlich, A. Kiss, J. Wolfling, E. Mernyak, A. E. Kulmany, R. Minorics, I. Zupko, M. Leidenberger, O. Friedrich, B. Kappes, F. Hahn, M. Marschall, G. Schneider, S. B. Tsogoeva, *Acs Med Chem Lett* **2018**, *9*, 1128-1133.

[16] a) D. Perrone, O. Bortolini, M. Fogagnolo, E. Marchesi, L. Mari, C. Massarenti, M. L. Navacchia, F. Sforza, K. Varani, M. L. Capobianco, *New J Chem* **2013**, *37*, 3559-3567; b) M. L. Navacchia, A. Fraix, N. Chinaglia, E. Gallerani, D. Perrone, V. Cardile, A. C. E. Graziano, M. L. Capobianco, S. Sortino, *ACS Med Chem Lett* **2016**, *7*, 939-943; c) M. L. Navacchia, E. Marchesi, L. Mari, N. Chinaglia, E. Gallerani, R. Gavioli, M. L. Capobianco, D. Perrone, *Molecules* **2017**, *22*, 1710.

[17] J. A. R. Salvador, J. F. S. Carvalho, M. A. C. Neves, S. M. Silvestre, A. J. Leitao, M. M. C. Silva, M. L. S. E. Melo, *Nat Prod Rep* **2013**, *30*, 324-374.

[18] M. J. Monte, J. J. G. Marin, A. Antelo, J. Vazquez-Tato, *World J Gastroentero* **2009**, *15*, 804-816.

[19] V. Sreekanth, S. Bansal, R. K. Motiani, S. Kundu, S. K. Muppu, T. D. Majumdar, K. Panjamurthy, S. Sengupta, A. Bajaj, *Bioconj Chem* **2013**, *24*, 1468-1484.

[20] J. A. R. Salvador, J. F. S. Carvalho, M. A. C. Neves, S. M. Silvestre, A. J. Leitao, M. M. C. Silva, M. L. S. E. Melo, Nat Prod Rep 2013, 30, 324-374.

[21] M. Marzioni, H. Francis, A. Benedetti, Y. Ueno, G. Fava, J. Venter, R. Reichenbach, M. G. Mancino, R. Summers, G. Alpini, S. Glaser, *Am J Pathol* **2006**, *168*, 398-409.

[22] a) L. Zhu, L. J. Shan, Y. J. Liu, D. Chen, X. G. Xiao, Y. Li, *J Digest Dis* **2014**, *15*, 684-693; b) T. Ide, Y. Kitajima, A. Miyoshi, T. Ohtsuka, M. Mitsuno, K. Ohtaka, Y. Koga, K. Miyazaki, *Int J Cancer* **2006**, *119*, 2750-2759; c) H. Kohno, R. Suzuki, Y. Yasui, S. Miyamoto, K. Wakabayashi, T. Tanaka, *Clin*

Cancer Res **2007**, *13*, 2519-2525; d) D. S. Alberts, M. E. Martinez, L. M. Hess, J. G. Einspahr, S. B. Green, A. K. Bhattacharyya, J. Guillen, M. Krutzsch, A. K. Batta, G. Salen, L. Fales, K. Koonce, D. Parish, M. Clouser, D. Roe, P. Lance, P. T. G. Networks, *J Natl Cancer I* **2005**, *97*, 846-853; e) A. Patel, A. Seetharam, *J Clin Hepatol* **2016**, *6*, 311-318; f) T. Ikegami, Y. Matsuzaki, *Hepatol Res* **2008**, *38*, 123-131.

[23] a) D. Brossard, L. El Kihel, M. Clément, W. Sebbahi, M. Khalid, C. Roussakis, S. Rault, *Eur J Med Chem* **2010**, *45*, 2912-2918; b) S. W. Mao, H. Chen, L. F. Yu, F. Lv, Y. J. Xing, T. Liu, J. Xie, J. Tang, Z. F. Yi, F. Yang, *Eur J Med Chem* **2016**, *122*, 574-583.

[24] F. Majer, R. Sharma, C. Mullins, L. Keogh, S. Phipps, S. Duggan, D. Kelleher, S. Keely, A. Long, G. Radics, J. Wang, J. F. Gilmer, *Bioorgan Med Chem* **2014**, *22*, 256-268.

[25] a) C. Festa, S. De Marino, A. Carino, V. Sepe, S. Marchiano, S. Cipriani, F. S. Di Leva, V. Limongelli, M. C. Monti, A. Capolupo, E. Distrutti, S. Fiorucci, A. Zampella, *Front Pharmacol* **2017**, *8*, 162; b) C. D'Amore, F. S. Di Leva, V. Sepe, B. Renga, C. Del Gaudio, M. V. D'Auria, A. Zampella, S. Fiorucci, V. Limongelli, *J Med Chem* **2014**, *57*, 937-954; c) F. S. Di Leva, C. Festa, B. Renga, V. Sepe, E. Novellino, S. Fiorucci, A. Zampella, V. Limongelli, *Sci Rep-Uk* **2015**, *5*.

[26] C. Massarenti, O. Bortolini, G. Fantin, D. Cristofaro, D. Ragno, D. Perrone, E. Marchesi, G. Toniolo, A. Massi, *Org Biomol Chem* **2017**, *15*, 4907-4920.

[27] M. N. Aminake, A. Mahajan, V. Kumar, R. Hans, L. Wiesner, D. Taylor, C. de Kock, A. Grobler, P. J. Smith, M. Kirschner, A. Rethwilm, G. Pradel, K. Chibale, *Bioorganic Med Chem* **2012**, *20*, 5277-5289.

[28] Y. H. Lin, B. Y. H. Chen, W. T. Lai, S. F. Wu, J. H. Guh, A. L. Cheng, L. C. Hsu, *Naunyn Schmiedebergs Arch Pharmacol* **2015**, *388*, 19-31.

[29] Z. Li, Q. Li, J. Wu, M. Wang, J. Yu, *Molecules* **2016**, *21*, E1331.

[30] S. Muranaka, H. Fujita, T. Fujiwara, T. Ogino, E.F. Sato, J. Akiyama, I. Imada, M. Inoue, K. Utsumi, *Antioxid Redox Signal* **2005**, *7*, 1367-1376.

[31] L. C. Hsu, R. L. White, *Proc Natl Acad Sci USA* **1998**, *95*, 12983-12988.

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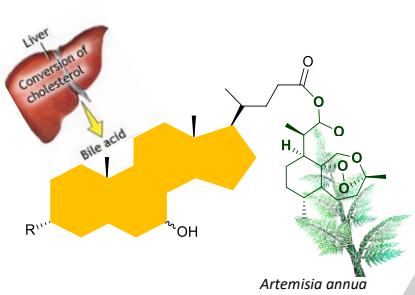

A new series of hybrids integrating two natural molecules such as dihydroartemisinin and selected bile acids were prepared through highly stereoselective one pot reactions. The hybridization efficiency was clearly highlighted by the enhanced cytotoxicity and cytoselectivity of the hybrids respect to the parent molecules alone. The biological study was targeted towards HL-60 leukemia and HepG2 hepatocellular carcinoma cell lines.