European Journal of Medicinal Chemistry 100 (2015) 10–17

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Structure-activity relationship of hybrids of *Cinchona* alkaloids and bile acids with *in vitro* antiplasmodial and antitrypanosomal activities

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ARTICLE INFO

Article history:
Received 17 November 2014
Received in revised form
29 May 2015
Accepted 30 May 2015
Available online 1 June 2015

Keywords:
Cinchona alkaloids
Bile acids
Hybrids
Barton–Zard reaction
Antiparasitic activity

ABSTRACT

In this work, a series of hybrid compounds were tested as antiparasitic substances. These hybrids were prepared from bile acids and a series of antiparasitic *Cinchona* alkaloids by the formation of a covalent C–C bond *via* a decarboxylative Barton–Zard reaction between the two entities. The bile acids showed only weak antiparasitic properties, but all the hybrids exhibited high *in vitro* activities (IC_{50}: 0.48–5.39 μM) against *Trypanosoma brucei*. These hybrids were more active than their respective parent alkaloids (up to a 135 fold increase in activity), and displayed good selectivity indices. Additionally, all these compounds inhibited the *in vitro* growth of a chloroquine-sensitive strain of *Plasmodium falciparum* (3D7: IC_{50}: 36.1 nM to 8.72 μM), and the most active hybrids had IC_{50}s comparable to that of artemisinin (IC_{50}: 36 nM). Some structure-activity relationships among the group of 48 hybrids are discussed. The increase in antiparasitic activity may be explained by an improvement in bioavailability, since the more lipophilic derivatives showed the lowest IC_{50}s.

1. Introduction

Infectious diseases, such as malaria and African sleeping sickness, which are caused by the protozoan parasites *Plasmodium sp.* and *Trypanosoma sp.* respectively, are still major global health problems, especially in developing countries. According to the World Health Organisation (WHO), in 2012 malaria caused an estimated 627000 deaths, mostly among African children, and in the same year, 7197 new cases of African trypanosomiasis were reported [1]. Most of the drugs currently in use have the drawbacks of their toxicities, increased resistance, and a variable efficacy against different strains or species, making necessary the search for new antiparasitic compounds [2–5]. Actually, there are only a few efficient treatments for these diseases, and these are often based in a combination of drugs. For example, the World Health Organisation does not recommend the use of arteminisin derivatives as a monotherapy to treat malaria, since this will lead to an increase in drug resistance [1], and instead promotes the use of a combination of drugs with different modes of action. In this regard, the preparation of new bioactive substances by the use of bioconjugation can be considered an alternative approach for the discovery of new drugs. The synthesis of hybrids of bioactive compounds with the combined properties of their individual components has emerged as a fast growing methodology in medicinal chemistry [6–8]. This strategy has been employed in the search for new antimalarial compounds [9,10], and several examples of antiparasitic steroid-based hybrids have been reported [11]. On the other hand, the efficacy of a drug is closely related to its transport properties and bioavailability, which in turn are related to other processes such as intestinal absorption, further biotransformations, and the ease and mechanism of elimination [12,13]. In this sense, bile acids are of great interest in drug discovery because of their efficient transport system and their properties as adsorption enhancers [14]. All these observations led to the preparation of a new series of antiparasitic hybrids of *Cinchona* alkaloids and bile acids [15]. The strategy was to

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http://dx.doi.org/10.1016/j.ejmech.2015.05.044
0223-5234/

combine the antiparasitic properties of the natural *Cinchona* alkaloids, which are currently used to treat severe cases of malaria [16], with the known properties of the bile acids as drug transporters, especially taking into account that some bile acids also have mild antiparasitic activity [17,18]. This strategy involved the formation of a covalent C–C bond between the quinoline core of the alkaloids and the side chain of a bile acid *via* a radical Barton–Zard decarboxylation reaction, constituting the first example of this reaction using natural *Cinchona* alkaloids as substrates. The Barton–Zard reaction can be used for the formation of a new C–C bond between a nucleophilic alkyl radical, obtained by decarboxylation of an *O*-acyl ester (Barton ester), and an electron-deficient position of an aromatic substrate [19,20], such as the C-2′ position of the quinoline core of *Cinchona* alkaloids. In these compounds C-4′of the quinoline core is substituted, leaving C-2′ as the only available position for a nucleophilic attack. From a biological point of view, the incorporation of a norcholane moiety to *Cinchona* alkaloids (quinine, quinidine, cinchonine and cinchonidine), had a positive effect on their *in vitro* antiparasitic activity against *Plasmodium falciparum* and *Trypanosoma brucei* [15]. In the first group of synthesized hybrids, the degree of oxidation of the bile acid moiety had a marked influence on the activity, since the compounds derived from chenodeoxycholic acid were more active than those prepared from lithocholic acid. For this reason, and as a continuation of this work, this promising library of hybrids was expanded by the incorporation of four additional bile acid moieties (cholic, deoxycholic, hyocholic and hyodeoxycholic acids) in order to get a more complete picture of the influence of the different hydroxy groups present on the steroidal core. In the present work, 32 new hybrids were synthesised (Fig. 1) to add to the series of the 16 previous derivatives prepared from lithocholic and chenodeoxycholic acids [15]. The *in vitro* antiparasitic activities of the hybrids **1**–**12a**–**d**, the four parent alkaloids (**QN** = quinine, **QND** = quinidine, **CN** = cinchonine, **CND** = cinchonidine) and the bile acids **1**–**12** were also evaluated against *P. falciparum* and *T. brucei brucei*. The cytotoxicities of all these compounds against the WI-38 cell line (human non cancer fibroblast) were also determined. Based on previous disappointing results [15], the activity of the new hybrids against *Leishmania mexicana mexicana* was not tested. In this work, the antiparasitic activity results of the complete series of 48 hybrids are discussed, and some structure-activity relationships can now be established.

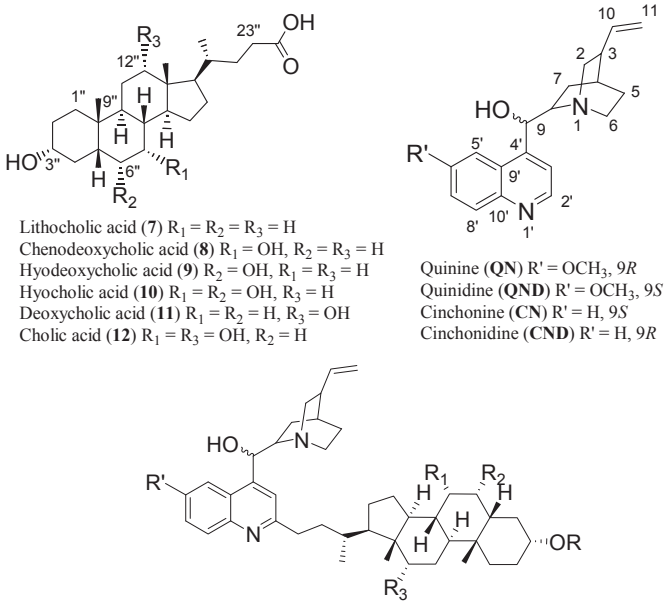


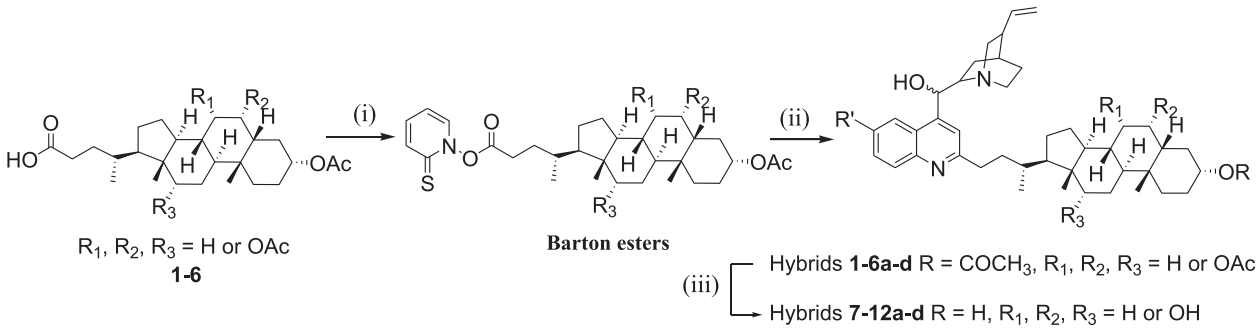
Fig. 1. Generic structure of the hybrids.

2. Chemistry

Hybrids **3**–**6a**–**d** were prepared following the synthetic pathway outlined in Scheme 1, using a Barton–Zard decarboxylation reaction as described previously for the preparation of hybrids **1**–**2a**–**d** [15]. Briefly, the Barton esters of the peracetylated bile acids **3**–**6**, obtained respectively from bile acids **9**–**12**, were prepared by reaction with 2-mercaptopyridine-*N*-oxide and DCC (*N*,*N*′-dicyclohexylcarbodiimide) in dry CH_2Cl_2 at 0 °C. These esters were then subjected to photolytic decarboxylation in the presence of a large excess (10 eq.) of a protonated *Cinchona* alkaloid acting as a radical trap, by irradiation with a 300 W tungsten lamp at 0 °C in CH_2Cl_2 under N_2 atmosphere to yield the corresponding hybrids. The purification of the final products was complicated by the large excess of the free *Cinchona* alkaloids. For this reason, the yields in Table 1 are of the final, purified compounds, and were calculated after the 2 reactions and consecutive purification steps. In general, the purification of the hybrids was achieved by normal-phase VLC, to remove the less polar side-products, such as the thioether which is the main side-product of the reaction [21], followed by reverse-phase VLC to remove most of the large excess of the parent alkaloid, and finally permeation through a Sephadex LH-20 column. The removal of the acetate protecting groups was achieved by treatment with 20% NaOH in MeOH under reflux, yielding quantitatively the corresponding deacetylated hybrids (Scheme 1). Table 1 lists the complete series of 48 hybrids, including those described herein and in the previous work in order to get a full picture of some structure-activity relationships [15].

3. Biological evaluation

The *in vitro* antiparasitic activities of the hybrids **3**–**6a**–**d** and **9**–**12a**–**d** as well as those of the parent free bile acids **7**–**12** and acetylated bile acids **1**–**6**, were evaluated against *T. brucei brucei* bloodstream forms (Tbb), and also against a chloroquine-sensitive strain of *Plasmodium falciparum* (3D7). The cytotoxicities of all these compounds were also evaluated against a human normal fibroblast cell line (WI-38). Suramin, artemisinin and campthotecin were used respectively as positive controls. In the bioassays with *T. brucei brucei* and WI-38 cells, the hybrids were evaluated at a concentration range between 0.009 μg/ml and 20 μg/ml, and the corresponding IC_{50}s (drug concentration resulting in 50% inhibition of parasite or cell growth) were calculated. In the case of *P. falciparum*, the hybrids were first evaluated in duplicate at the same range of concentrations. However, some of the compounds still showed a detectable parasite growth inhibition at concentrations as low as 0.009 μg/ml. In these cases, the evaluation was repeated (in triplicate) at lower concentrations (between 4 μg/ml and 1.8 ng/ml), in order to confirm the IC_{50} values. The selectivity indices were calculated for each parasite according to the formula: IC_{50}(WI38)/IC_{50}(parasite). Table 2 lists the *in vitro* biological activities of the complete library of hybrids, which includes the 32 new compounds described herein, as well as the 16 previously reported derivatives [15] for a better understanding of the structure-activity relationships among the compounds. The antiparasitic activities and cytotoxicities of the parent bile acids **1**–**12** were also evaluated, and those results are displayed in Table 3. In the three bioassays, the bile acids were only evaluated at 2 concentrations: 20 and 100 μg/ml. In the event of a larger than 50% inhibition at 20 μg/ml, additional bioassays were performed at lower concentrations (100–0.5 μg/ml) to determine the IC_{50}s.



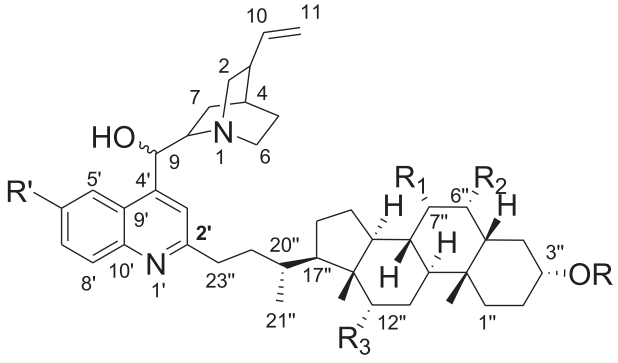
Scheme 1. Synthetic pathway for the preparation of the hybrids of *Cinchona* alkaloids and bile acids. Reagents and conditions: (i) 2-Mercaptopyridine-*N*-oxide (1.5 eq), DCC (1.5 eq), CH_2Cl_2, 0 °C; (ii) *Cinchona* alkaloids (10 eq), camphorsulfonic acid (20 eq), W-hν; (iii) NaOH 20% in CH_3OH, reflux.

4. Results and discussion

4.1. Structural analysis of compounds 3–6a–d and 9–12a–d

The structures of the synthesized compounds were confirmed by spectroscopic methods. The molecular formulae were obtained by ESIHRMS, and all the compounds were completely characterized by 1D and 2D NMR spectroscopy (See Supplementary Information for NMR assignments). The formation of the new C–C bond between C-2′ of the quinoline nucleus and C-23 of the

Table 1
Structures of the synthesized hybrids and final purified yields.



Compound	R′	OH-9	R_1	R_2	R_3	Yield (%)[a]	Deprotected compound[b]
R = $COCH_3$							R = H; R_1, R_2, R_3 = H or OHnd
1a	OCH_3	*R*	H	H	H	21[c]	**7a**
2a	OCH_3	*R*	OAc	H	H	23[c]	**8a**
3a	OCH_3	*R*	H	OAc	H	23	**9a**
4a	OCH_3	*R*	OAc	OAc	H	19	**10a**
5a	OCH_3	*R*	H	H	OAc	30	**11a**
6a	OCH_3	*R*	OAc	H	OAc	26	**12a**
1b	OCH_3	*S*	H	H	H	13[c]	**7b**
2b	OCH_3	*S*	OAc	H	H	24[c]	**8b**
3b	OCH_3	*S*	H	OAc	H	36	**9b**
4b	OCH_3	*S*	OAc	OAc	H	27	**10b**
5b	OCH_3	*S*	H	H	OAc	21	**11b**
6b	OCH_3	*S*	OAc	H	OAc	16	**12b**
1c	H	*S*	H	H	H	23[c]	**7c**
2c	H	*S*	OAc	H	H	34[c]	**8c**
3c	H	*S*	H	OAc	H	49	**9c**
4c	H	*S*	OAc	OAc	H	22	**10c**
5c	H	*S*	H	H	OAc	23	**11c**
6c	H	*S*	OAc	H	OAc	14	**12c**
1d	H	*R*	H	H	H	19[c]	**7d**
2d	H	*R*	OAc	H	H	35[c]	**8d**
3d	H	*R*	H	OAc	H	41	**9d**
4d	H	*R*	OAc	OAc	H	2	**10d**
5d	H	*R*	H	H	OAc	24	**11d**
6d	H	*R*	OAc	H	OAc	18	**12d**

[a] The yields were calculated after the two steps of the reaction and the three steps of purification, and are based on the amount of starting bile acid peracetate.
[b] Compounds obtained quantitatively after treatment of the corresponding peracetylated hybrids with NaOH in methanol under reflux. R_1, R_2, R_3 = H or OH.
[c] Compounds obtained in our previous work, see Ref. [15].

Table 2
Biological evaluation of the parent alkaloids and hybrid compounds **1a**–**d**–**12a**–**d**.

Compound	Cytotoxicity WI-38	*Trypanosoma brucei brucei Tbb*			*Plasmodium falciparum* 3D7		
	IC_{50} ± SD (μM)	IC_{50} ± SD (μM)	Selectivity WI38/*Tbb*	Activity gain/alka	IC_{50} ± SD (nM)	Selectivity WI38/3D7	Activity gain/alka
QN	20.25 ± 0.03	21.20 ± 8.02	1.0	1.0	632.7 ± 348.8	32.0	1.0
1a[a]	23.67 ± 1.78	2.91 ± 1.61	8.0	7.3	8.7 ± 4.7 [b]	2.7	0.1
2a[a]	5.19 ± 1.76	1.62 ± 0.04	3.2	13.1	920.5 ± 255.6	5.6	0.7
3a	5.86 ± 1.79	1.49 ± 0.15	3.9	14.3	459.6 ± 437.2	13.0	1.4
4a	3.76 ± 0.08	0.45 ± 0.06	8.3	46.8	56.0 ± 19.8	67.2	11.3
5a	4.41 ± 0.38	1.47 ± 0.06	3.0	14.4	196.7 ± 52.3	22.0	3.2
6a	4.47 ± 0.27	0.48 ± 0.04	9.3	44.1	43.5 ± 24.3	103.0	14.6
7a[a]	>30.53	5.39 ± 0.29	>5.7	3.9	6.5 ± 0.7 [b]	>4.7	0.1
8a[a]	6.81 ± 2.13	0.61 ± 0.06	11.1	34.7	439.6 ± 90.9	15.5	1.4
9a	4.60 ± 0.10	0.65 ± 0.05	7.0	32.4	224.0 ± 58.9	21.0	2.8
10a	5.55 ± 1.61	0.76 ± 0.15	7.3	27.8	275.1 ± 70.7	20.0	2.3
11a	5.20 ± 1.24	0.64 ± 0.07	8.2	33.3	240.9 ± 63.3	22.0	2.6
12a	19.3 ± 4.61	1.57 ± 0.13	12.3	13.5	1.82 ± 0.75 [b]	11.0	0.3
QND	34.81 ± 1.07	25.99 ± 1.30	1.3	1.0	194.4 ± 160.5	179.1	1.0
1b[a]	11.18 ± 2.81	4.26 ± 0.37	2.6	6.1	1.2 ± 0.2 [b]	9.7	0.2
2b[a]	4.11 ± 1.47	0.58 ± 0.01	7.1	44.6	508.6 ± 102.0	8.1	0.4
3b	4.49 ± 0.68	1.47 ± 0.17	3.1	17.7	154.6 ± 41.3	29.0	1.3
4b	3.29 ± 0.58	0.56 ± 0.23	5.8	46.2	73.2 ± 20.8	45.0	2.7
5b	5.33 ± 0.88	0.69 ± 0.19	7.7	37.5	195.0 ± 49.1	27.0	1.0
6b	4.66 ± 1.16	0.53 ± 0.09	8.7	48.8	83.8 ± 46.2	56.0	2.3
7b[a]	3.95 ± 2.63	1.21 ± 0.29	3.3	21.5	1.0 ± 0.0 [b]	3.8	0.2
8b[a]	2.91 ± 1.25	0.55 ± 0.04	5.3	47.1	207.2 ± 14.9	14.0	0.9
9b	2.20 ± 0.63	0.89 ± 0.29	2.5	29.2	123.5 ± 57.4	18.0	1.6
10b	4.75 ± 0.27	0.77 ± 0.21	6.2	33.9	170.2 ± 79.9	27.9	1.1
11b	4.30 ± 1.45	0.61 ± 0.03	7.1	42.7	281.2 ± 112.5	15.0	0.7
12b	11.82 ± 1.04	1.21 ± 0.47	9.8	21.6	700.1 ± 358.0	17.0	0.3
CN	>68.03	>68.03	n.d.	1.0	292.5 ± 163.3	>222	1.0
1c[a]	10.52 ± 1.96	1.89 ± 0.01	5.6	36.0	3.4 ± 0.7 [b]	3.1	0.1
2c[a]	2.66 ± 0.08	0.59 ± 0.04	4.5	114.7	260.7 ± 86.9	10.2	1.1
3c	4.28 ± 0.37	0.64 ± 0.06	6.6	105.7	186.4 ± 40.9	23.0	1.6
4c	4.17 ± 0.78	0.51 ± 0.01	8.3	134.6	40.8 ± 4.4	102.0	7.2
5c	4.80 ± 0.72	1.51 ± 0.18	3.2	45.1	170.9 ± 56.4	28.1	1.7
6c	5.10 ± 1.35	0.55 ± 0.06	9.3	123.6	52.8 ± 23.6	97.0	5.5
7c[a]	6.35 ± 2.90	4.82 ± 0.05	1.3	14.1	1.4 ± 0.5 [b]	4.6	0.2
8c[a]	2.22 ± 0.75	0.61 ± 0.02	3.6	111.8	140.4 ± 17.2	15.7	2.1
9c	5.49 ± 1.05	0.72 ± 0.03	7.6	94.5	71.6 ± 27.6	77.0	4.1
10c	5.68 ± 1.70	1.81 ± 0.05	3.1	37.6	182.8 ± 59.6	31.0	1.6
11c	2.98 ± 1.61	0.60 ± 0.06	5.0	113.1	133.7 ± 75.6	22.0	2.2
12c	5.68 ± 2.18	1.10 ± 0.48	5.2	61.8	592.1 ± 212.2	10.0	0.5
CND	>68.03	62.18 ± 4.18	n.d.	1.0	962.6 ± 921.8	>71	1.0
1d[a]	8.37 ± 2.61	1.90 ± 0.10	4.4	32.7	1.8 ± 0.7 [b]	4.6	0.5
2d[a]	2.44 ± 0.63	0.57 ± 0.01	4.3	110.0	289.7 ± 51.0	8.4	3.3
3d	4.16 ± 0.12	1.18 ± 0.43	3.5	52.5	115.4 ± 35.0	36.0	8.3
4d	3.75 ± 0.16	0.52 ± 0.02	7.2	119.8	36.1 ± 25.2	103.7	26.6
5d	4.06 ± 0.18	0.57 ± 0.05	7.1	108.2	77.2 ± 19.3	53.0	12.5
6d	6.70 ± 1.02	1.47 ± 0.06	4.6	42.4	40.6 ± 21.3	165.0	23.7
7d[a]	14.85 ± 1.22	3.46 ± 0.51	4.3	18.0	1.7 ± 0.5 [b]	8.7	0.6
8d[a]	2.67 ± 0.84	0.62 ± 0.03	4.3	99.6	355.7 ± 31.2	7.5	2.7
9d	3.28 ± 1.68	0.53 ± 0.08	6.2	116.7	92.1 ± 42.7	36.0	10.5
10d	5.32 ± 0.43	0.62 ± 0.02	8.6	100.5	392.2 ± 38.5	14.0	2.5
11d	5.64 ± 1.73	0.66 ± 0.03	8.6	94.8	320.3 ± 85.9	18.0	3.0
12d	8.09 ± 4.68	1.75 ± 0.07	4.6	35.6	1.8 ± 0.7 [b]	4.0	0.5
Campthotecin	0.23 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Suramine	n.d.	0.03 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
Artemisinin	n.a.	n.d.	n.d.	n.d.	36.2 ± 14.2	n.d.	n.d.

SD = standard deviation; selectivity = IC_{50}(WI38)/IC_{50}(*Tbb* or 3D7); n.d. = not determined; n.a. = not active; activity gain/alka = IC_{50} (alkaloid)/IC_{50} (compound).
[a] Compounds reported in Ref. [15].
[b] IC_{50}(3D7) expressed in μM.

norcholane group could be rapidly confirmed by NMR analysis. The NMR signals corresponding to H-2′ (8.70 ppm) and CH-2′ (typically at 147–150 ppm) of the *Cinchona* alkaloids were no longer present in the spectra of the hybrids, while a new signal for a quaternary carbon at 160 ppm was evident in the ^{13}C NMR spectra. Finally, HMBC correlations between H-3′, H-22″, H-23″ and C-2′, and between H-3′ and C-23″, confirmed the newly formed C–C bond and the structure of the compounds (S29 Supplementary Information).

The initial set of hybrids was obtained as their peracetylated forms, as it was previously observed that the formation of the Barton esters was not complete when the bile acids had free hydroxy groups [22]. The choice of acetate as protective group was based on its simple protection/deprotection reactions and on the

Table 3
Biological evaluation of free and acetylated bile acids **1**–**12**.

Bile acid	Cytotoxicity WI-38			*Trypanosoma brucei brucei* Tbb			*Plasmodium falciparum* 3D7		
	% Inhibition		IC_{50} (μM)	% Inhibition		IC_{50} (μM)	% Inhibition		IC_{50} (μM)
	100 μg/ml	20 μg/ml	± SD	100 μg/ml	20 μg/ml	± SD	100 μg/ml	20 μg/ml	± SD
1	98 ± 0	27 ± 4	nd	100 ± 0	15 ± 11	nd	100 ± 1	70 ± 8	59.5 ± 28.7
2	98 ± 0	57 ± 2	16.8 ± 1.8	100 ± 0	99 ± 1	12.5 ± 1.5	90 ± 3	59 ± 24	21.0 ± 2.2
3	98 ± 0	20 ± 1	nd	100 ± 0	81 ± 5	16.3 ± 1.2	97 ± 1	73 ± 9	32.7 ± 20.8
4	96 ± 1	9 ± 6	nd	100 ± 0	83 ± 13	5.6 ± 1.4	97 ± 2	88 ± 1	8.1 ± 5.0
5	98 ± 0	10 ± 0	nd	100 ± 0	36 ± 5	nd	100 ± 6	82 ± 11	30.5 ± 15.4
6	29 ± 25	12 ± 6	nd	100 ± 0	15 ± 6	nd	100 ± 3	68 ± 17	22.5 ± 9.1
7	98 ± 0	22 ± 3	nd	100 ± 0	5 ± 6	nd	100 ± 1	85 ± 12	26.9 ± 4.4
8	94 ± 1	27 ± 9	nd	100 ± 0	11 ± 6	nd	98 ± 1	77 ± 14	18.9 ± 2.4
9	22 ± 29	21 ± 8	nd	94 ± 6	1 ± 1	nd	97 ± 2	67 ± 9	40.9 ± 22.0
10	nd	18 ± 4	nd	64 ± 45	7 ± 6	nd	92 ± 10	54 ± 44	109.0 ± 25.5
11	50 ± 22	9 ± 1	nd	100 ± 0	2 ± 2	nd	100 ± 3	59 ± 44	93.6 ± 22.8
12	nd	6 ± 0	nd	24 ± 0	20 ± 1	nd	83 ± 13	56 ± 54	132.7 ± 51.6

increased bioavailability and reduced toxicity of some acetylated compounds [23]. After deprotection under basic conditions, the structures of the deacetylated hybrids were confirmed by the disappearance of the acetyl singlets in the ^{1}H NMR spectrum (between 2.01 and 2.13 ppm), and of the C=O intense band in the IR spectra (1730 cm^{-1}), along with the appearance of a wide band at 3300 cm^{-1} corresponding to the hydroxyl groups (see Supplementary Information). In the case of compounds **11b**, **11c**, **12b** and **12c**, the NMR spectra had to be recorded in CD_3OD, due to the extensive broadening of some signals corresponding to the quinoline protons, especially H-8′, in the spectra recorded in $CDCl_3$ (S30 Supplementary Information). This broadening of the quinoline signals is probably due to conformational reasons. This behaviour was observed only in the spectra of the hybrids obtained from quinidine and cinchonine, in which the hydroxylated C-9 has an *S* configuration, bound to a norcholane moiety derived from cholic or deoxycholic acid, both of which have a hydroxy group at C-12. It is possible that in compounds **11b**, **11c**, **12b** and **12c** those two hydroxy groups may induce a conformation in chloroform which favours proton exchange, and leads to signal broadening.

4.2. Biological activities

4.2.1. Biological evaluation of bile acids *1*–*12*

Although there are several examples of antiparasitic steroids [24], to the best of our knowledge there are no previous reports of this kind of activity for the bile acids. In order to test the possible influence of the norcholane moiety, compounds **1**–**12** were evaluated against the human normal fibroblast cell line WI-38, *T. brucei brucei* and *P. falciparum* (Table 3). None of the bile acids used in the present work was significantly cytotoxic at 20 μg/ml, with the exception of the peracetate of chenodeoxycholic acid **2**, which was moderately cytotoxic against the WI-38 cell line (IC_{50} = 16.8 μM). The results of the *in vitro* bioassays revealed that the peracetylated derivatives were generally more cytotoxic against WI-38 than the free bile acids. Regarding the antitrypanosomal activity, the peracetylated bile acids **2**–**4** exhibited a moderate activity (IC_{50}s between 5.6 and 16.3 μM), while the other bile acids were inactive at 20 μg/ml. These results suggest that the acetate groups may have a positive effect on the bioavailability of the compounds due to an increased lipophilicity. Besides, the 12 bile acids had moderate *in vitro* antiplasmodial activity ($8.1 \leq IC_{50} \leq 132.7$ μM), with the peracetylated hyocholic acid **4** standing out as the bile acid with the highest activity.

4.2.2. Cytotoxicity of the hybrids

Compounds **1**–**12a**–**d** showed moderate cytotoxic activity against the WI-38 cell line with IC_{50} values between 2.20 and 23.7 μM. In general, the cytotoxicity of the *Cinchona* alkaloids was increased when a norcholane residue was attached at C-2, except for hybrids **1a**, **7a** and **12a** (IC_{50} = 23.7, >30.5 and 19.3 μM, respectively) which exhibited IC_{50}s comparable to that of quinine, their parent alkaloid (IC_{50} = 20.3 μM). Generally speaking, the hybrids with lithocholic or cholic moieties were the least cytotoxic in this series (Table 2).

4.2.3. Antitrypanosomal activity

All the tested hybrids displayed good activities against *T. brucei*, with IC_{50} values ranging from 0.48 to 5.39 μM and selectivity indices from 1.3 to 12.3. Moreover, the activities of the hybrids were higher than those of their corresponding parent alkaloids, as determined by the ratio: IC_{50} (alka)/IC_{50} (compound). This was especially evident in the cases of cinchonine and cinchonidine (ratios up to 100-fold and higher) (Table 2). The selectivity indices (antitrypanosomal activity vs. cytotoxicity) were all favourable, with the quinine derivative **12a** showing the largest ratio (12.3 times more active than cytotoxic, however with only a 13.5-fold increase in activity compared to quinine). Interestingly, compound **12a** was also one of the few hybrids that had a cytotoxic activity comparable to that of quinine. The absence of *in vitro* antitrypanosomal activity for the bile acids **7**–**12** is remarkable when compared to the activity of their corresponding hybrids, and suggests that this increase in activity is not due simply to an additive effect of the bile acids, but may also be related to an improved penetration capacity of the drug. Interestingly, in the case of the hybrids, the acetate groups did not have a marked influence on the *in vitro* activity, as opposed to the trend observed with the bile acids **1**–**12**. In the previous work [15], it was observed that the antitrypanosomal activity of the hybrids increased with the presence of a second oxygenated group in the bile acid moiety (IC_{50}s (**1a**–**d**, **7a**–**d**) > IC_{50}s (**2a**–**d**, **8a**–**d**)). This trend was confirmed by the present results; however the observed activities were comparable for all the dioxygenated bile acid derivatives irrespectively of the positions of the hydroxy or acetoxy groups. A third hydroxy group, as in cholic acid derivatives, did not induce an additional increase in activity. The structure of the alkaloid did not have a great influence in the antitrypanosomal activity of the hybrids. All these observations show that this activity is not as specific as expected within each series of compounds. However, the present results show some differences in the selectivity indices of the hybrids, mainly due to their variable cytotoxicities (Table 2). Fourteen of the tested hybrids had good selectivity indices (SI > 7, SI: IC_{50}WI38/IC_{50}*Tbb*). According to Pink et al. the criteria for antiparasitic hits are

IC_{50} < 1 μg/ml and SI > 10 [25]. Following these rules, the most promising hybrids would be compounds **6a**, **6c**, **8a**, **12a** and **12b** which have IC_{50}s of 0.68, 0.55, 0.61, 1.57, 1.21 μM (0.39, 0.43, 0.41, 1.08, and 0.83 μg/ml, respectively) and selectivity indices: 9.3, 9.3, 11.1, 12.3 and 9.8 respectively. It is interesting to note that except for **8a**, all the antitrypanosomal "hits" of the series have a cholic acid moiety which was also the bile acid with the lowest cytotoxicity (Table 3).

4.2.4. Antiplasmodial activity – SAR studies

Table 2 also shows the results of the *in vitro* antiplasmodial evaluation of the 48 hybrids, all of which inhibited parasite growth with IC_{50}s ranging from 36.1 nM to 8.72 μM, and selectivity indices ranging from 3 to 165. In Table 4, there is a comparison of the IC_{50} ranges of the different hybrids based on the structural features of the bile acid moiety (number and position of the oxygenated substituents), while the IC_{50}s of the individual compounds are listed in Table 2. As observed previously [15], the antiplasmodial activity increased with the number of oxygenated groups on the norcholane moiety, and most of the compounds with at least two hydroxy or acetate groups had good antiplasmodial activities. Those compounds with oxygenated groups at positions 3, 6 (compounds **3a**–**d** and **9a**–**d**) or 3, 12 (**5a**–**d**; **11a**–**d**) had higher selectivity indices ($13 \leq SI \leq 77$) compared with compounds **2a**–**d** and **8a**–**d**, which have oxygenated groups at positions 3, 7 ($6 \leq SI \leq 16$). Furthermore, those hybrids with oxygenated groups at positions 3, 6 or 3, 12 had antiplasmodial activities which were comparable or higher than those of their parent natural alkaloids (Tables 2 and 4), albeit with lower selectivity indices. The most promising compounds were those with three acetate groups, namely **4a**–**d** and **6a**–**d**, which exhibited good *in vitro* activity against *P. falciparum* with IC_{50}s < 84 nM and selectivity index values higher than 45. In particular, compounds **4c**, **4d**, **6a** and **6d** are respectively 7.2, 26.6, 14.6 and 23.7 times more active than their parent alkaloids, while their IC_{50}s (40.8, 36.1, 43.5 and 40.6 nM) and SI (102, 104, 103 and 165, respectively) are in the same range as that of artemisinin, the reference compound used in the assays (IC_{50}s = 36.2 nM). Peracetylation of the norcholane moiety had a positive influence on the antiplasmodial activity of those hybrids derived from hyocholic and cholic acids, in an opposite sense as the effect generally observed for the hybrids with one or two oxygenated groups. The same trend was also observed for the parent bile acids (Table 3). The lower activity of compounds **10a**–**d** and **12a**–**d**, compared to **4a**–**d** and **6a**–**d**, may be related to their reduced lipophilicity, due to the presence of three hydroxy groups, which could lead to a lower penetration capacity. All these observations suggest that the improvement of the antiplasmodial activity observed for several hybrids compared to the natural *Cinchona* alkaloids may be related to their lipophilicity and an increased penetration capacity, with perhaps a small additive antiparasitic effect of the bile acid moieties, which showed a moderate activity against *P. falciparum* (Table 3).

5. Conclusions

The synthesis and biological evaluation of 32 new hybrids of bile acids and *Cinchona* alkaloids is reported. These hybrids differ in their alkaloid moiety (quinine, quinidine, cinchonine or cinchonidine) and the number and position of the oxygenated groups (hydroxy or acetate) of their norcholane fragment. All the hybrids exhibited interesting *in vitro* antitrypanosomal and antiplasmodial activities, but also low cytotoxicities against the normal cell line WI-38. The activity of the hybrids against *T. brucei brucei* was considerably improved compared to that of *Cinchona* alkaloids, particularly when at least two oxygenated groups were present in the norcholane moiety. Among them, the hybrids having a 3″α,7″α,12″α-trioxygenated norcholane moiety (**6a**, **6b**, **12a**, **12b**) emerged as the most interesting trypanocidal "hits" with IC_{50}s ≤ 1.5 μM and selectivity indices > 9, due to their low cytotoxicities.

Regarding the activity against *P. falciparum*, an increase in activity was observed with the number of oxygenated groups of the norcholane backbone, and some of the hybrids displayed better activities than the natural *Cinchona* alkaloids. In particular, those hybrids prepared from peracetylated cholic and hyocholic acids were the most promising compounds of the series, with IC_{50}s in the same range as artemisinin.

In summary, the addition of a norcholane moiety to *Cinchona* alkaloids had a favourable effect on the antiparasitic activity for some of the compounds. This work produced several candidates, namely compounds **4a**–**d** and **6a**–**d**, for further biological studies such as *in-vivo* bioassays. These compounds can also be further modified for structure-activity improvement. This increase in activity may be related mostly to an enhanced bioavailability and cell penetration capacity of the hybrids compared to the natural alkaloids, since the bile acids *per se* did not display potent antiparasitic activities. This hypothesis needs to be confirmed by additional experiments. However, since the newly formed C–C bond connecting both parts in the hybrids is not hydrolysable, it is probable that the bile acid moiety may also play a role in molecular recognition, and, for this reason, compounds **1**–**12a**–**d** should not be considered prodrugs. Also, in a future work, the hybrids will be evaluated against other chloroquine-resistant strains of *P. falciparum*, in order to estimate their effect on the resistance phenomenon. In conclusion, in this work, the strategy of bioconjugation was used for the design and synthesis of promising antiparasitic hybrids, with improved activity compared to the parent alkaloids that can be possibly explained in terms of a better bioavailability.

Table 4
Comparison of *in vitro* antiplasmodial activities.

Num oxygenated groups	Type	Positions	Compounds	IC_{50} (3D7)	Selectivity Index	Activity gain vs. natural alkaloid
1	OAc	3	**1a**–**d**	1.2–8.7 μM	3.1–9.7	No
	OH		**7a**–**d**	1.0–6.5 μM	3.8 - > 8.7	No
2	OAc	3, 7	**2a**–**d**	260.7–920.5 nM	5.6–10.2	0.4–3.3
		3, 6	**3a**–**d**	115.4–459.6 nM	12.7–36.0	1.3–8.3
		3, 12	**5a**–**d**	77.2–196.7 nM	22.4–52.5	1.0–12.5
	OH	3, 7	**8a**–**d**	140.4–439.6 nM	7.5–15.8	0.9–2.7
		3, 6	**9a**–**d**	71.6–224.0 nM	17.8–76.7	1.6–10.5
		3, 12	**11a**–**d**	133.7–320.3 nM	15.3–22.3	0.7–3.0
3	OAc	3, 6, 7	**4a**–**d**	36.1–73.2 nM	44.9–103.7	2.7–26.6
		3, 7, 12	**6a**–**d**	40.6–83.7 nM	55.6–165.0	2.3–23.7
	OH	3, 6, 7	**10a**–**d**	170.2–392.2 nM	13.6–31.0	1.1–2.5
		3, 7, 12	**12a**–**d**	592.1 nM–1.8 μM	4.4–16.9	No

6. Experimental section

6.1. General

Bile acids (cholic, deoxycholic, hyocholic and hyodeoxycholic acids) and *Cinchona* alkaloids (quinine, quinidine, cinchonine, cinchonidine) were obtained from commercial sources and used as such or recrystallized prior to use when necessary. The peracetylated bile acids were obtained by standard procedures, using Ac_2O/DMAP/Pyridine. 2-Mercaptopyridine-N-oxide, *N,N′*-dicyclohexylcarbodiimide (DCC) and camphorsulfonic acid were purchased from Aldrich. All the solvents were distilled prior to use, and CH_2Cl_2 used for reactions was bidistilled from phosphorous pentoxide. NMR spectra were recorded in $CDCl_3$ or CD_3OD on Bruker AC-200 (200.13 MHz) and Bruker Avance II (500.13 MHz) spectrometers, using the signals of the residual nondeuterated solvent as internal reference (δ_H 7.26, δ_C 77.0 for chloroform and δ_H 3.31, δ_C 49.0 for methanol). All 2D NMR experiments (COSY, DEPT-HSQC, HMBC, and NOESY) were performed using standard pulse sequences. HRMS were recorded on a Bruker micrOTOF-Q II spectrometer. IR spectra were obtained on an FT-IR Nicolet Magna 550 instrument, optical rotations on a Perkin Elmer Polarimeter 343 (at 589 nm), and melting points on a Fisher-Johns apparatus. TLCs were carried out on Merck Sílicagel 60 F254 plates. TLC plates were sprayed with 2% vanillin in concentrated H_2SO_4 or with Dragendorff's spray reagent (Aldrich). Merck Silicagel (230–400 mesh) and RP-18 (Aldrich) stationary phases were used for Vacuum Column Chromatography. Sephadex LH-20 (GE Healthcare) was used for exclusion chromatography.

6.2. Chemistry

6.2.1. General procedure for the formation of hybrids ***3–6a–d***

In a flask protected from light with aluminium foil, the peracetate of a bile acid (**3**–**6**, around 0.2–0.3 mmol) and 2-mercaptopyridine-N-oxide (1,5 equivalents) were dissolved in 4 ml of dry CH_2Cl_2, and the solution was cooled to 0 °C. Then, 1.5 eq.eq. of DCC were added, the solution was stirred at 0 °C for 2–3 h, and the reaction was monitored by TLC. The solution was filtered through a cotton plug, to remove dicyclohexylurea, into a flask containing a *Cinchona* alkaloid (**QN**, **QND**, **CN** or **CND**, 10 eq.) and camphorsulfonic acid (20 eq.) dissolved in 4 ml of dry CH_2Cl_2. The solution was cooled to 0 °C, placed under N_2, and subsequently irradiated using a single 300 W tungsten lamp during 1h15′. The reaction was quenched with an aqueous solution of NaOH (2 N) and extracted 3 times with CH_2Cl_2. The organic layers were combined, washed with water, dried over Na_2SO_4 and evaporated yielding a residue. The latter was quickly separated in 4 fractions by VLC on silica, eluting with cyclohexane/EtOAc/methanol 1/1/0, 0/1/0, 0/95/5 and 0/9/1 respectively. Fractions 3 and 4 were pooled together to be further purified by VLC on reversed-phase (Rp-18), eluting with mixtures of H_2O/MeOH from 1/0 to 0/1, increasing the proportion of methanol by steps of 10%. Fractions eluted with 100% MeOH, contained the hybrid, together with a small amount of the parent alkaloid. These fractions were taken to dryness and submitted to a final purification by Sephadex LH-20 (MeOH) to yield the desired hybrids **3**–**6a**–**d**.

6.2.2. Deacetylation of hybrids ***3–6a–d***

A solution of NaOH in methanol (20%) was added to compounds **3**–**6a**–**d** to reach a concentration of 1 mg/ml, and the solution was then refluxed overnight. The solvent was removed under reduced pressure and the residue dissolved in H_2O before extraction with dichloromethane. Evaporation of the organic layer yielded quantitatively the deacetylated compound (**9**–**12a**–**d**).

6.2.3. Compounds ***1–2a–d*** and ***7–8a–d***

See Ref. [15].

6.2.4. Compounds ***3–6a–d*** and ***9–12a–d***

See Supplementary Information.

6.3. Biological assays

6.3.1. Parasites, cells and media

Trypanosoma brucei brucei (strain Lister 427) bloodstream forms were cultured *in vitro* in HMI9 medium containing 10% heat-inactivated foetal bovine serum [26]. Parasites were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

P. falciparum (3D7, was originally isolated from a patient living near Schipol airport, The Netherlands) asexual erythrocytic stages were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37 °C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 [27]. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/l glucose (Sigma–Aldrich), 44 mg/ml hypoxanthin (Sigma–Aldrich), 100 mg/l gentamycin (Gibco) and 10% human pooled serum (A or O Rh^+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The human non-cancer fibroblast cell line WI38, was cultivated *in vitro* in DMEM medium (Gibco) containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and penicillin–streptomycin (100 UI/ml to 100 μg/ml). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

6.3.2. In vitro antitrypanosomal activity

The *in vitro* tests were performed in 96-wells microtiter plates as described by Hoet et al. (2004) [28]. Suramin (a commercial antitrypanosomal drug) was used as positive control. First, stock solutions of the compounds were prepared in DMSO at 4 mg/ml for the hybrids and for suramin, and at 10 mg/ml for the bile acids. The highest concentration of solvent to which the parasites were exposed was 0.5%, which was shown to have no measurable effect on parasite viability. Bile acids **1**–**12** were tested at 20 and 100 μg/ml in duplicate (six wells/concentration) and in addition bile acids **2**–**5** were tested in triplicate in an eight serial three-fold dilutions (final concentration range: 100–0.05 μg/ml, two wells/concentration). Suramin and hybrids **3**–**6a**–**d** and **9**–**12a**–**d** were tested in eight serial three-fold dilutions (final concentration range: 20–0.009 μg/ml, two wells/concentration), at least in triplicate.

6.3.3. In vitro antiplasmodial activity

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the methods described by Makler et al. [29]. The *in vitro* tests were performed in 96-wells microtiter plates as described by Murebwayire et al. [30]. The parasitaemia and the haematocrit were 2% and 1%, respectively. Artemisinin (Sigma) was used as positive control in all experiments with an initial concentration of 100 ng/ml. First stock solutions of the compounds were prepared in DMSO at 4 mg/ml for the hybrids and 10 mg/ml for the bile acids. The highest concentration of DMSO to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Bile acids **1**–**12** were tested at 20 and 100 μg/ml in duplicate (four wells/concentration) and then in duplicate in an eight serial three-fold dilutions (final concentration range: 100–0.05 μg/ml, two wells/concentration). Hybrids **3**–**6a**–**d** and **9**–**12a**–**d** were tested in two eight serial three-fold dilutions (final concentration range: 20–0.009 μg/ml

and 4000–1.8 ng/ml, two wells/concentration), in duplicate or triplicate for each range of concentrations.

6.3.4. Cytotoxicity assay

The cytotoxicity of compounds on WI38 cells was evaluated as described by Stevigny et al. [31], in 96-well microtiter plates, using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by mitochondrial dehydrogenases in viable cells [32]. Camptothecin was used as positive cytotoxic reference compound. First stock solutions of the compounds were prepared in DMSO at 4 mg/ml for the hybrids and camptothecin and at 10 mg/ml for the bile acids. The highest concentration of solvent to which the cells were exposed was 1%, which was shown to be non-toxic. Bile acids **1**–**12** were tested at 20 and 100 μg/ml in duplicate (four wells/concentration) and in addition compound **2** was tested in triplicate in a eight serial three-fold dilutions (final concentration range: 100–0.05 μg/ml, two wells/concentration). The solutions of hybrids **3**–**6a**–**d** and **9**–**12a**–**d** and of camptothecin were tested in eight serial threefold dilutions with a final concentration range of 20–0.009 μg/ml (two wells/concentration), at least in triplicate.

Acknowledgement

Research at the University of Buenos Aires was supported by Grants from CONICET (PIP No. 516-2009), UBACYT (20020100100123, Prog. 2011-2014), and ANPCYT (PICT 2010-1808). A.L. thanks CONICET for a postdoctoral fellowship. We thank Dr. Gabriela Cabrera (UMYMFOR–CONICET) for recording the mass spectra and Eng. José Gallardo and Lic. Gernot Eskuche (UMYMFOR–CONICET) for some of the NMR spectra. This work was partly supported by the Belgian Fund for Scientific Research (grants N°3.4533.10, FRFC2.4555.08 and T.0190.13). The authors thank MINCyT (Argentina) and FNRS (Belgium) for the Bilateral Cooperation Project N° V4/325M-NR/DeM-14.702 – BE1208.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.05.044.

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