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Novel bile acid derivatives (BANBs) with cytostatic activity obtained by conjugation of their side chain with nitrogenated bases

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ABSTRACT

Drug targeting may contribute to overcoming resistance to chemotherapy and to reducing side effects. Here, by conjugating a nitrogenated base (NB) to the side chain of a bile acid (BA) moiety, we have synthesized and evaluated six novel compounds, designated BANB-1 to -6, with potential cytostatic activity and vectoriality toward enterohepatic tumors. These compounds were purified by liquid chromatography and their purity was checked by TLC and HPLC before being chemically characterized using IR, $^{1}H/^{13}C$ NMR and FAB-MS. Using several cell lines – HepG2 (human hepatoblastoma), LS 174T and Caco-2 (human colon adenocarcinoma), Hepa 1-6 (mouse hepatoma), McA-RH7777 (rat hepatoma), CCRF S-180 II (mouse sarcoma) and CHO (Chinese hamster ovary) – their effect on cell viability was measured with the formazan test after drug exposure for 6 h (cytotoxic effect) or 72 h (cytostatic effect). A weak cytostatic effect of BANB-1, BANB-2 and BANB-3 was detected even in CHO cells stably transfected with rat bile acid transporters (Ntcp and Oatp1/1a1). In contrast, BANB-4, BANB-5 and BANB-6, similarly to cisplatin, showed strong cytostatic effects, together with mild non-specific toxicity. BANB-6 was effective even against Hepa 1-6/R cells, which were partly resistant to cisplatin. Treatment with BANB-6, but not cisplatin, was able to prolong the life span of Nude mice bearing tumors formed by Hepa 1-6/R cells orthotopically implanted in the liver. In conclusion, our results support the hypothesis that cytostatic bile acid derivatives such as BANB-6 may offer a useful pharmacological strategy for the treatment of tumors of the enterohepatic circuit.

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Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BA, bile acid; BANB, bile acid-nitrogenated base conjugate; CA, cholic acid; FAB, fast atom bombardment; GCA, glycocholic acid; HRMS, high-resolution mass spectrometry; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NB, nitrogenated base; NMR, nuclear magnetic resonance; NTCP or Ntcp, human or rat sodium-taurocholate cotransporting polypeptide; OATP or Oatp, human or rat organic anion-transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; UDCA, ursodeoxycholic acid

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1. Introduction

In our effort to develop cytostatic drugs with organotropism towards tumors of the enterohepatic circuit, in previous studies we have reported the synthesis, chemical characterization and evaluation of the pharmacological activity at the preclinical stage of cisplatin-bile acid derivatives named ''BAMETs'' [1]. Among these compounds, *cis*-diamminechloro cholylglycinate platinum(II) (BAMET-R2) and *cis*-diammine bis-ursodeoxycholate platinum(II) (BAMET-UD2) were found to be the two compounds with the most promising characteristics because they can be efficiently taken up by transporters for anionic, cationic and neutral organic compounds located in liver and intestinal cells [2]. This probably accounts for their ability to accumulate in liver tumor cells whereas they are taken up and efficiently excreted by normal hepatocytes. Thus, owing to their liver organotropism [3,4] their strong ''in vitro'' cytostatic activity, and ''in vivo'' anti-tumoral effect [5], BAMET-R2 and BAMET-UD2 have been suggested as potentially useful drugs in the chemotherapy of liver tumors. More recently, their beneficial cytostatic properties against intestinal tumors have been also proposed [6,7].

One of the factors that often reduce the clinical usefulness of cisplatin-related cytostatic drugs is the development of resistance to them [8], which can be due in part to an up-regulation of export pumps. Although several agents able to block the efflux of cytostatic drugs through these transporters have been developed, the pharmacologically efficient doses of currently available chemosensitizing agents are so high that they cannot be used in clinical practice owing to their noxious side effects [9]. An alternative to increasing intracellular concentrations of the drug in tumor cells even in the presence of functional export pumps, is to enhance drug vectoriality toward these cells. At this respect, BAMET-UD2 has been shown to be taken up by liver and colon cancer cells, even if these were resistant to cisplatin. As a consequence, BAMET-UD2 efficiently induces apoptosis and overcomes resistance to chemotherapy with cisplatin both ''in vitro'' and ''in vivo'' [6,10,11].

The above is consistent with the presence of bile acid transporters in tumors of the enterohepatic circuit, i.e., the liver [12–15] and the intestine [7], able to take up bile acid derivatives. This, together with the diminishing popularity of regimens including cytostatic drugs containing transition metals such as platinum, owing to their non-specific toxicity [16], prompted us to extend previous investigations in this field by searching for novel cytostatic bile acid derivatives in which the active moiety would be an organic one instead of a transition metal.

Cytotoxic nucleoside analogues and nucleobases were among the first chemotherapeutic drugs to be developed for the pharmacological treatment of both solid tumors and malignant disorders of the blood. Since an analog of deoxycytidine, cytarabine (1β-D-arabinofuranosylcytosine), was first proposed as anticancer agent in the middle of the 1960s [17], many other pyrimidine and purine nucleoside analogs have been developed, and several of them are currently used clinically as antimetabolite drugs [18]. These compounds compete with endogenous nucleosides and interact with a large number of intracellular targets, which accounts for their cytostatic effect. However, their efficacy is often limited by the pre-existence or development of resistance [19] and by the fact that noxious side effects, such as myelosuppression and cardiotoxicity, may accompany treatment with regimens including these drugs [20]. Therefore, the development of improved derivatives is needed. In the present study we carried out the synthesis, chemical characterization and preliminary evaluation of the ''in vitro'' and ''in vivo'' biological effects of six compounds, designated BANB-1 to -6 because they share the characteristic of having been obtained by conjugation to the side chain of different bile acid (BA) species of a nitrogenated base (NB) alone or forming part of a nucleoside moiety.

2. Materials and methods

2.1. Chemicals

Bile acids (BA) – cholic acid (CA), glycocholic acid (GCA), and ursodeoxycholic acid (UDCA) – adenine, adenosine and metformin were purchased from Sigma–Aldrich (Madrid, Spain). All other chemicals were from VWR International (Barcelona, Spain), except lamivudine, which was kindly supplied by Glaxo Wellcome Research and Development (Hertfordshire, UK). They were of high purity and were used as purchased without any further purification. Culture Medium (DMEM), gentamicin, $NaHCO_3$, L-glutamine, polyethylene glycol ($M_r \approx 8000$) and dimethylsulfoxide (DMSO) were from Sigma–Aldrich. Geneticin G-418 was from Roche (Barcelona, Spain). Foetal calf serum (FCS) was obtained from TDI (Madrid, Spain).

2.2. Synthesis and purification

The nitrogenated BA derivatives were obtained using a modified method of a previously described technique for the synthesis of amide derivatives of BAs [21,22]. In brief, BAs were activated with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in an N_2 atmosphere and then reacted with the nitrogenated base (NB), i.e., adenine, adenosine or metformin, to produce the BANB derivative. TLC on silica gel plates (60 F254, Merck) and the solvent systems described below were used to check the synthesis and separation procedures.

2.2.1. Synthesis of BANB-1, BANB-2 and BANB-3

The BAs (CA or UDCA, 1.22 mmol) in dimethylformamide (DMF) were reacted with EEDQ (1.71 mmol; i.e., a molar ratio of 1.4) for 4 h at 80 °C under nitrogen atmosphere. After activation, a suspension of the base (adenine or adenosine, 1.83 mmol; 1.50 mmol/mmol) in DMF and small amount of triethylamine (265 μL) were added and the reaction mixture was heated to 125 °C and maintained at this temperature for 10–30 h. Once cooled to room temperature, the crude reaction product was obtained by precipitation with cold (4 °C) ethyl ether (dry). The result was a complex mixture as revealed by TLC chromatography. The final products were isolated by column chromatography using silica gel and the eluent mixture indicated in each case.

2.2.1.1. BANB-1 (30% yield). This was obtained from CA and adenine. ^{1}H NMR: Table 1. ^{13}C NMR: Table 2. HRMS (FAB+, M + 1): calculated 525.3315; found 525.3299. Eluent system: butyl acetate:methanol:water (70:30:2).

Table 1 – ^{1}H NMR assignments for the most representative protons of compounds BANB-1, BANB-2, BANB-3, BANB-4, BANB-5 and BANB-6 (200 MHz) in CD_3OD

H	BANB-1	BANB-2	BANB-3	BANB-4	BANB-5	BANB-6
3	3.47 (1H, m)	3.25 (1H, m)	3.3-3.4 (1H, m)	3.37 (1H, m)	3.34 (1H, m)	3.40 (1H, m)
7	3.79 (1H, bs)	3.66 (1H, bs)	3.3-3.4 (1H, bs)	3.82 (1H, bs)	3.78 (1H, bs)	3.50 (1H, bs)
12	3.95 (1H, bs)	3.82 (1H, bs)		4.00 (1H, bs)	3.94 (1H, bs)	
18	0.71 (3H, s)	0.60 (3H, s)	0.58 (3H, s)	0.73 (3H, s)	0.69 (3H, s)	0.70 (3H, s)
19	0.90 (3H, s)	0.79 (3H, s)	0.83 (3H, s)	0.95 (3H, s)	0.90 (3H, s)	0.96 (3H, s)
21	1.07 (3H, d, J = 6.2)	0.85 (3H, d, J = 6.2)	0.83 (3H, d, J = 6.2)	1.10 (3H, d, J = 6.2)	1.03 (3H, d, J = 6.2)	1.00 (3H, d, J = 6.2)
25					4.12 (2H, s)	
2′	8.41 (1H, s)	8.19 (1H,s s)	8.18 (1H, s)			
8′	8.63 (1H, s)	8.06 (1H, s)	8.05 (1H, s)			
Me_2N5'				3.16 (6H, s)	3.10 (6H, s)	3.12 (6H, s)
1″		5.83 (1H, d, J = 6.7)	5.83 d (1H, d, J = 6.7)			
2″		4.61 (1H, dd, J = 6.7; 5.1)	4.61 (1H, dd, J = 6.7; 5.1)			
3″		4.19 (1H, dd, J = 5.1; 2.6)	4.19 (1H, dd, J = 5.1; 2.6)			
4″		4.04 (1H, ddd, J = 3 × 2.6)	4.03 (1H, ddd, J = 3 × 2.6)			
5″		3.76 (1H, dd, J = 12.4; 2.6)	3.75 (1H, dd, J = 12.4; 2.6)			
5″		3.60 (1H, dd, J = 12.4; 2.6)	3.61 (1H, dd, J = 12.4; 2.6)			

δ in ppm (J in Hz).

Table 2 – ^{13}C NMR assignments for compounds BANB-1, BANB-4, BANB-5 and BANB-6 (50.3 MHz) in CD_3OD

C		BANB-1	BANB-4	BANB-5	BANB-6
1	CH_2	36.5	36.5	35.9	38.0
2	CH_2	31.2	31.2	31.2	31.1
3	CH	72.9	72.9	72.9	72.1
4	CH_2	40.5	40.5	40.4	38.6
5	CH	43.1	43.0	42.9	44.8
6	CH_2	35.9	35.9	35.9	36.1
7	CH	69.1	69.1	69.0	71.9
8	CH	41.0	41.0	41.0	44.5
9	CH	27.9	27.9	27.8	40.7
10	C	35.9	35.9	36.5	35.2
11	CH_2	29.6	29.6	29.6	22.4
12	CH	74.1	74.0	74.0	41.6
13	C	47.5	47.5	47.5	44.0
14	CH	43.2	43.2	43.2	56.7
15	CH_2	24.3	24.2	24.2	27.9
16	CH_2	28.7	28.8	28.7	29.7
17	CH	48.1	48.2	48.2	57.5
18	CH_3	13.0	13.1	13.1	12.7
19	CH_3	23.2	23.2	23.2	24.0
20	CH	36.9	37.1	36.9	36.9
21	CH_3	17.8	17.9	17.8	19.2
22	CH_2	34.3	30.8	33.2	35.2
23	CH_2	31.4	35.2	34.1	36.1
24	C	176.0		174.7	
25	CH_2			45.3	
2′	CH	153.1			
8′	CH	146.0			
1′	C		179.7	176.8	179.5
3′	C		167.8	167.9	167.6
5′	C		166.6	166.4	166.5
Me_2N5'	CH_3		36.5	36.5	36.5

δ in ppm.

2.2.1.2. BANB-2 (20% yield). This was obtained from CA and adenosine. ^{1}H NMR: Table 1. HRMS (FAB+, M + 1): calculated 657.3738; found 657.3742. Eluent system: isoamyl acetate:propionic acid:propanol:water (4:3:2:1).

2.2.1.3. BANB-3 (6% yield). This was obtained from UDCA and adenosine. ^{1}H NMR: Table 1. HRMS (FAB+, M + 1): calculated 641.3788; found 641.3799. Eluent system: butyl acetate:methanol (75:25).

2.2.2. Synthesis of BANB-4, BANB-5 and BANB-6

In this case, the BAs (CA, GCA and UDCA, 1 mmol) were dissolved in ethanol and activated with 1.4 mmol EEDQ (i.e., a molar ratio of 1.4) in an N_2 atmosphere at 60 °C for 4 h. Then, a suspension of 500 mg metformin hydrochloride and 1,8-diazabiciclo[5.4.0]undec-7-ene (DBU, 4 mL) in ethanol (previously kept at 60 °C for 30 min) was added and the reaction mixture was maintained under reflux for 24 h. By precipitation with diethyl ether at 4 °C overnight, soluble and insoluble fractions were separated and checked by TLC. They were found to be complex mixtures both containing the conjugated products. The crude reaction was subjected to two successive chromatographic procedures on silica gel, using as eluents butyl acetate/methanol/water (80:20:2, v/v) first, and then ethyl acetate/methanol (9:1).

2.2.2.1. *BANB-4 (21% yield)*. This was obtained from CA (1 mmol) and metformin (3 mmol). IR (ν, cm^{-1}): 3342, 1634, 1564, 1524, 1450, 1400, 1224, 1077 and 816. ^1H NMR: Table 1. ^{13}C NMR: Table 2. HRMS (FAB+, *M* + 1): calculated 502.3757; found 502.3782.

2.2.2.2. *BANB-5 (20% yield)*. This was obtained from GCA (1 mmol) and metformin (2.3 mmol). IR (ν, cm^{-1}): 3480, 3240, 1659, 1634, 1570, 1530, 1463, 1403, 1336, 1270, 1223 and 1077. ^1H NMR: Table 1. ^{13}C NMR: Table 2. HRMS (FAB+, *M* + 1): calculated 559.3972; found 559.4021.

2.2.2.3. *BANB-6 (24% yield)*. This was obtained from UDCA (1 mmol) and metformin (5 mmol). IR (ν, cm^{-1}): 3346, 3191, 1560, 1521, 1457, 1397, 1221, 1010 and 815. ^1H NMR: Table 1. ^{13}C NMR: Table 2. HRMS (FAB+, *M* + 1): calculated 486.3808; found 486.3821.

2.3. Analytical methods

Mass spectrometry studies were carried out on a VG-Autospec, using L-SIMS ionization in the FAB^+ mode (Cs ion emission) and m-NBA as matrix. ^1H (200 MHz) and ^{13}C (50.3 MHz) NMR spectra were obtained in CD_3OD and DMSO-d6 solutions on a Bruker AC200 instrument. The carbon resonances were distinguished by DEPT-90 and DEPT-135 experiments. TMS was used as internal standard for ^1H and ^{13}C NMR spectra.

2.4. Evaluation of acute cytotoxic and cytostatic effects

The following cell lines obtained from the American Type Culture Collection (Manassas, VA, USA) were used: HepG2 (human hepatoblastoma), LS 174T and Caco-2 (human colon adenocarcinoma), Hepa 1-6 (mouse hepatoma), McA-RH7777 (rat hepatoma), CCRF S-180 II (mouse sarcoma). Wild-type Chinese hamster ovary cells (CHO-K1) and two sublines stably transfected with rat bile acid transporters Ntcp (CHO-9-6), and Oatp1/1a1 (CHO-03) were kindly supplied by Drs. P. Meier, B. Stieger and B. Hagenbuch (University of Zurich, Switzerland). Cisplatin-resistant subline Hepa 1-6/R was selected by double subcloning using the limiting dilution method from cultures continuously exposed to increasing concentrations (from 1 to 10 μM) of cisplatin, as previously reported [10]. Cells were cultured with appropriate media in a humidified CO_2:air (5:95%) atmosphere at 37 °C.

The test based on formazan formation from tetrazolium salt by living cells (CellTiter98®, Promega, Madison, WI) was used to evaluate drug-induced non-specific acute toxicity by measuring the reduction in cell viability after short-term (6 h) exposure to the desired drug. The formazan test was also used to determine the cytostatic effect as the reduction in amount of living cells in the culture after long-term (72 h) exposure to the drug. In both cases approximately 10^4 cells/100 μL/dish seeded in 96-wells plates were used.

2.5. Evaluation of antitumor activity

Male Nude (Ico: Swiss nu/nu) mice (Iffa Credo; Barcelona, Spain) were housed in sterile micro-isolator cages and fed with commercial mouse pelleted food from Panlab (Madrid, Spain) and water ad libitum. Temperature (20 °C) and the light/dark cycle (12 h/12 h) were controlled. All manipulations were done under sterile conditions in a laminar flow hood. Animals were handled in accordance with the recommendations of the University of Salamanca Ethical Committee for Laboratory Animals. Mouse hepatoma Hepa 1-6/R cells (~10^7) were injected into the backs of Nude mice. After 2 weeks, tumors growing subcutaneously (~2 cm in diameter) were removed and minced into cubic fragments of ~1 mm^3, which were implanted in the livers of different animals [5]. Treatment, starting the next day, consisted of two injections (i.p.) of 5 or 15 nmol/g b.w./week of cisplatin or BANB-6 in a suspension of 0.5 mL of sterile 150 mM NaCl. Control animals received only vehicle. Animal survival was monitored three times daily.

2.6. Statistical methods

The results are expressed as individual values or as means ± S.D. To calculate the statistical significance of the differences between groups, the Student's *t*-test or ANOVA analysis followed by Dunnett's Many-to-One multiple comparison test were used, as appropriate.

3. Results

3.1. Chemistry

Syntheses of derivatives of BAs with nitrogenated bases were carried out following adapted procedures for the formation of amides using EEDQ as a coupling agent [23], as previously described in the literature for the synthesis of BA amide derivatives [21,22]. Using this method BANB-1, BANB-2 and BANB-3 were synthesized. These had the expected structure, derived from the reaction between the activated carboxyl group and the more reactive nitrogen in each NB; namely, the N-9 in adenine and the NH_2-6 in adenosine [24,25]. The ^1H NMR spectra (Table 1) of these products and their MS fully agree with the depicted structures (Fig. 1).

Synthesis of the 1,3,5-triazine derivatives was carried out following the same adapted procedure described above. After different attempts to improve the yields and to obtain a less complex reaction mixture, the best results were obtained when ethanol was used as solvent and the reaction was carried out in the presence of DBU in order to neutralize the hydrochloride form of the biguanide. The isolated products, designated BANB-4, BANB-5 and BANB-6, which do not have the initially expected structure of the amides between BA and metformin, were exhaustively characterized by spectroscopic procedures and identified as those depicted in Fig. 1, whose ^1H and ^{13}C NMR data are listed in Tables 1 and 2. The formation of the 1,3,5-triazine moiety can be explained through the initial formation of the amides I between the corresponding BA and the N^5 of the metformin, which was followed by reaction between N^2 and the carbonyl at C-24 or C-26 in the BA residues to form the triazine ring (Fig. 1, inset). Although the initial amides could be expected to be part of the final products, whose ^1H and ^{13}C NMR spectra would be very

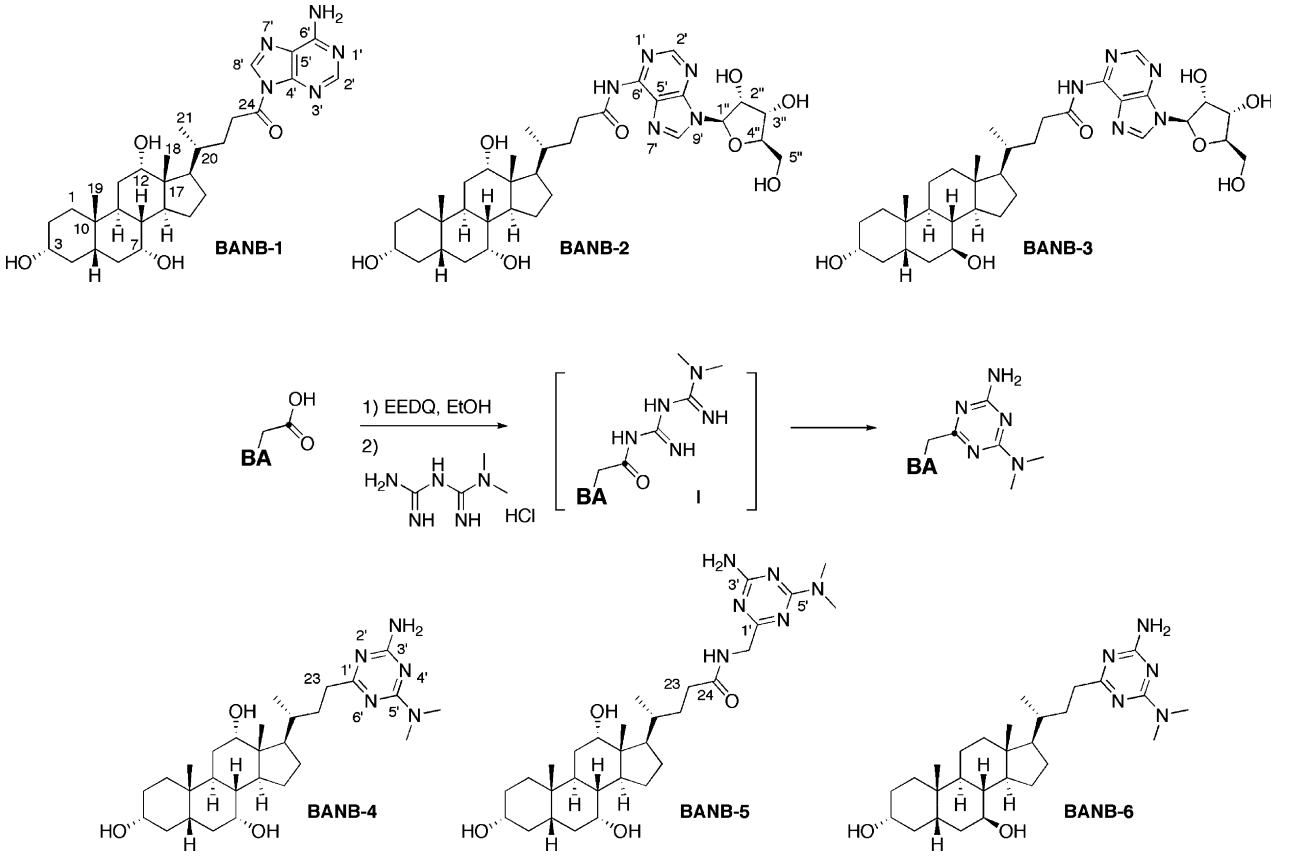

Fig. 1 – Chemical structures of bile acid derivatives containing nitrogenated bases (BANBs). Bile acid moieties were cholic acid (CA) in BANB-1, BANB-2 and BANB-4, glycocholic acid (GCA) in BANB-5 and ursodeoxycholic acid (UDCA) in BANB-3 and BANB-6. Central inset represents the mechanism of nitrogenated ring formation reaction for BANB-4, BANB-5 and BANB-6.

similar, the molecular ion obtained in the HRMS indicated the loss of a water molecule in agreement with the proposed structures. Further evidence for the cyclic system was that the final products did not form the rose-red complex with ammoniacal copper sulphate that was expected for the biguanide form [26].

3.2. *Cytostatic activity "in vitro"*

The cytostatic effect of BANB-1, BANB-2 and BANB-3, which contain either adenine or adenosine in their molecular structure, was compared to that of lamivudine, an antiviral nucleoside derivative that is not expected to have an antiproliferative effect and was used here as a negative control. Indeed, no significant change in the amount of cells in the culture was observed when HepG2, LS 174T, Hepa 1-6 or CCRF S-180-II cells were exposed to lamivudine for 3 days (Fig. 2). BANB-1, BANB-2 and BANB-3 were able to reduce cell culture growth only when assayed at a high concentration (500 μM). This effect was stronger in the case of BANB-2. Owing to the fact that these three compounds had only weak activity, we decided to elucidate whether their cytostatic activity could be enhanced by increasing their uptake by cells transfected with Ntcp and Oatp1/1a1. These transporters, when expressed in *Xenopus laevis* oocytes have been found to be able to transport BA derivatives also obtained by conjugation of their side chain with relatively bulky compounds, such as fluorescein isothyocianate (data not shown). The effect of BANBs was compared to that of lamivudine, which does not have a bile acid moiety. Two cell lines derived from Chinese hamster ovary cells (CHO-K1) stably transfected with the rat ortologs of the bile acid transporters Oatp1/1a1 (CHO-03) and Ntcp (CHO-9-6) were assayed (Fig. 3). As expected, this maneouver enhanced the cytostatic activity of these compounds, whereas that of lamivudine was not changed. Nevertheless, cytostatic activity was only observed at high concentrations. This suggested that these compounds should be classified as weak cytostatic drugs and that further evaluation could be ruled out.

In contrast, BANB-4, BANB-5 and BANB-6 induced a significant cytostatic (long-term) effect, which in preliminary experiments was seen to be stronger for BANB-6. Thus, BANB-6 was further assayed in several cell lines, including LS 174T, Caco-2, McA-RH7777 and Hepa 1-6 (Fig. 4). In all these cases acute toxicity (short-term effect) was moderate and similar to that caused by cisplatin (Fig. 4A, C, E and G). Moreover the cytostatic (long-term) effect was strong and of the same degree as that induced by cisplatin, which was used here as a positive control (Fig. 4B, D, F and H). When assayed in partially cisplatin-resistant Hepa 1-6/R cells, the cytostatic

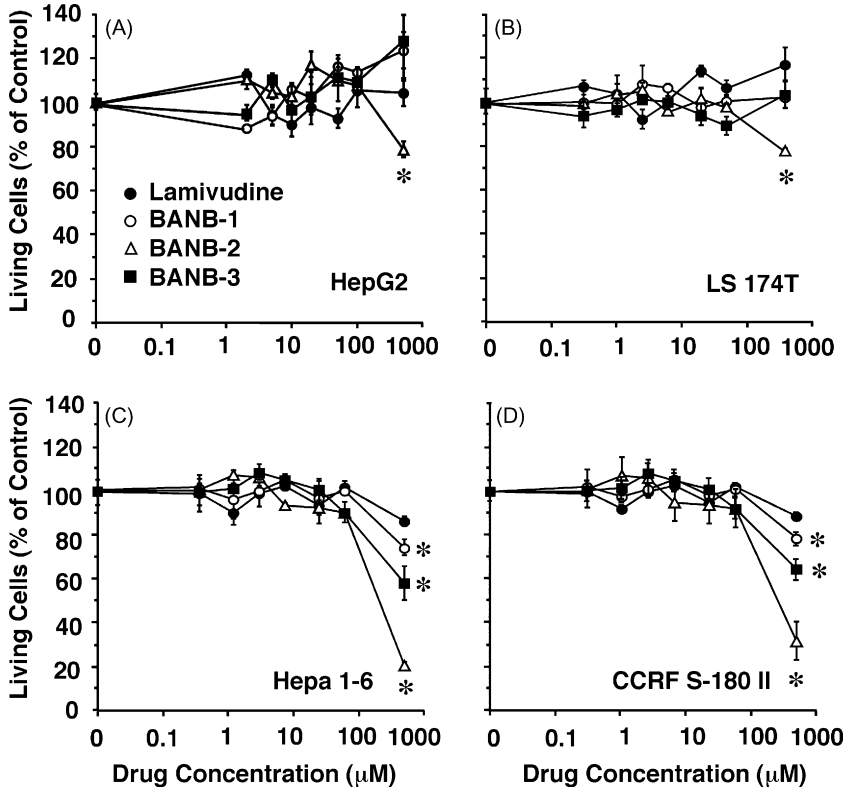

Fig. 2 – Cytostatic effect of BANB-1, BANB-2 and BANB-3 on the following cell lines: (A) HepG2 from human hepatoblastoma; (B) LS 174T from human colon adenocarcinoma; (C) Hepa 1-6 from mouse hepatoma, and (D) CCRF S-180 II from mouse sarcoma. The proportion of living cells as compared with non-treated dishes was determined with the formazan test after the culture had been incubated with the desired concentration of drug for 72 h. Lamivudine, a nucleoside derivative with no cytostatic effect, was used as a negative control. Values are means ± S.D. from four different cultures carried out in triplicate. *$P < 0.05$ on comparing with non-treated cells by Dunnett's test.

(long-term) activity was lower than that of cisplatin for BANB-4 and BANB-5 but higher than that of cisplatin for BANB-6 (Fig. 5A). Investigation of drug-induced acute toxicity (short-term effect) revealed that this was moderate and did not match the cytostatic (long-term) effect. The strongest compound regarding the inhibition of cell culture growth, i.e., BANB-6, was less toxic (short-term effect) than BANB-4 and BANB-5 at low concentrations (Fig. 5B). The results described above were consistent with the of IC_{50} values calculated for these two compounds (Table 3). Both cisplatin and BANB-6 had IC_{50} values in the same range, although always higher for BANB-6, except for Hepa 1-6/R cells, in which the opposite was found.

3.3. *Antitumor activity "in vivo"*

To elucidate whether these promising results could be also extrapolated to the "in vivo" situation, cisplatin and BANB-6 were used to treat mice receiving intrahepatic implantation of a tumor previously formed with Hepa 1-6/R cells in the back of a different donor mouse. At a low dose (5 nmol/g b.w.), a tendency towards protection by BANB-6, but not cisplatin, was found (Fig. 6A). At a higher dose (15 nmol/g b.w.), the ability of BANB-6 to prolong the life span of these animals was evident (Fig. 6B). Thus, mean survival time was statistically ($P < 0.05$) increased (+41%) in comparison with the Control and cisplatin-treated animals according to the Dunnett's test for multiple comparisons. In contrast, the animals treated with cisplatin had the same mean survival times as those receiving vehicle (approximately 27 days).

4. Discussion

As indicated in Section 1, the rationale of the present study was that novel agents – BANBs, which contain a BA moiety and a nitrogenated base/nucleoside derivative moiety – could have cytostatic ability, in particular as far as tumors of the enterohepatic circuit are concerned. The results described above suggest that this could be true for some of these compounds and justify the interest in carrying out further investigation to characterize their mechanisms of action and actual drug uptake and, based on these data, to develop more potent drugs of this family.

Why did we expect BANBs to have vectorial and anti-tumor properties? One of the reasons for this was that the expression of plasma membrane proteins able to mediate the uptake of natural bile acids is restricted to only certain organs, including the liver and the intestine [27]. This offers an excellent opportunity to use BA derivatives for drug targeting to these

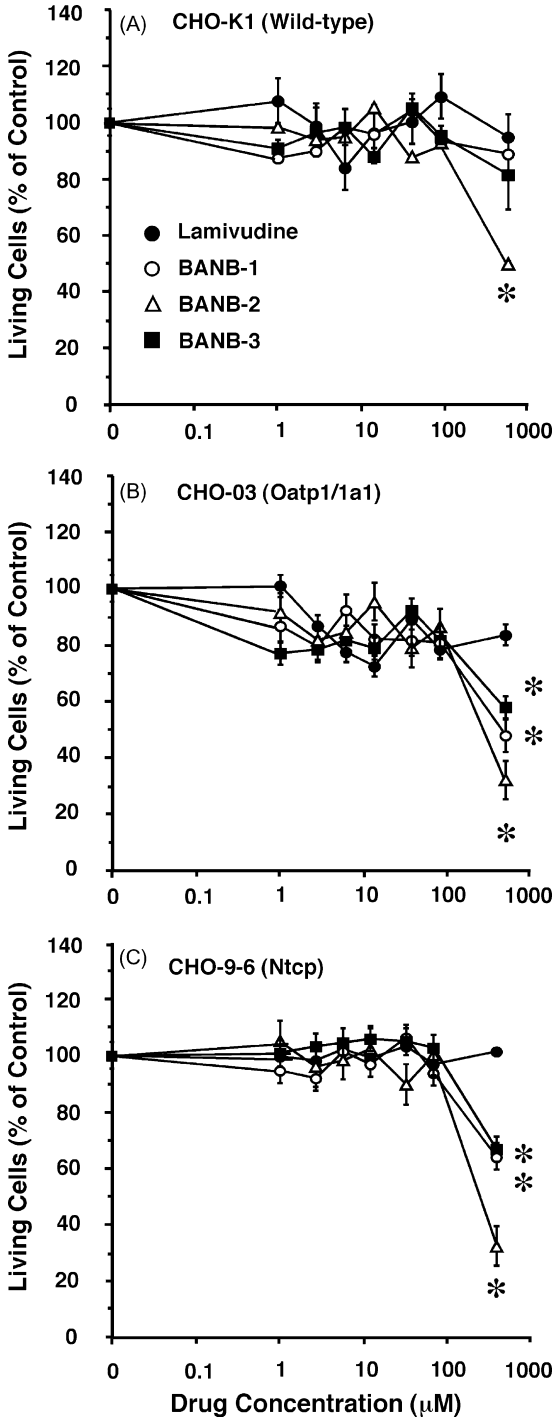

Fig. 3 – Cytostatic effect of BANB-1, BANB-2 and BANB-3 on Chinese hamster ovary (CHO) cells expressing bile acid carriers. Wild-type cells (CHO-K1) (A) were compared with cells stably transfected with the cDNA of rat Oatp1/1a1 (CHO-03) (B) or Ntcp (CHO-9-6) (C). The proportion of living cells as compared with non-treated dishes was determined with the formazan test after the culture had been incubated with the desired concentration of drug for 72 h. Lamivudine, a nucleoside derivative with no cytostatic effect, was used as a negative control. Values are means ± S.D. from four different cultures carried out in triplicate. *$P < 0.05$ on comparing with non-treated cells by Dunnett's test.

territories [28]. In this respect, since BAs were first proposed for delivering different kinds of drugs to the liver [29], many BA-drug conjugates have been synthesized [30–32]. In previous studies we have reported the interest in several members of a family of anti-tumor compounds obtained by binding cisplatin to the side chain of BAs [1]. In agreement with those studies, the present results also indicate that the expression in CHO cells of carriers, such as the rat ortholog of Ntcp and Oatp1/1a1, able to recognize BA derivatives as substrates, enhances the cytostatic activity of BANBs.

An additional reason to design BANBs was the hope that nucleobase/nucleoside derivatives bound to the BA moiety in these compounds could interact with the DNA replicative machinery by competing with natural substrates, hence inhibiting cell proliferation. This has been shown to be the case for several previously developed nitrogenated base/nucleoside analogs whose anticancer activity has been well characterized [19]. Several mechanisms of action have been suggested for these derivatives. These include: (i) incorporation into DNA and RNA macromolecules themselves inducing alteration of their structure, (ii) interference with the activity of various enzymes involved in the synthesis of nucleic acids, and (iii) modification of the metabolism of endogenous nucleosides [18]. These changes result in an inhibition of DNA synthesis, an inhibition of DNA repair and accumulation of DNA strand breaks, the induction of pro-apoptotic signals in the mitochondrial pathway, and direct binding to apoptosome or modulation of p53 expression, all leading to the activation of apoptosis, which is the main end-point of the mechanism of cytostatic action of nitrogenated base/nucleoside analogs. Moreover, since agonists to A3 adenosine receptors have recently been reported to inhibit cell growth and/or induce apoptosis in different types of tumors [33], a similar effect for other nitrogenated base/nucleoside analogs cannot be ruled out.

Finally, it should be considered that, probably related to the requirement of substrates for active DNA synthesis, the abundance of nucleoside transporter proteins in normal tissues and tumors depends in part on the pathways that stimulate cell proliferation. Thus, in many tumor cell types an up-regulation of nucleoside transporters occurs [19], which could contribute to the vectorial properties of BANBs. These phenomena could account for some of the results obtained in the present study. Thus, when assayed in vitro BANB-6 showed a moderate non-specific toxicity similar to that of cisplatin. However, when administered to tumor-bearing mice, this compound prolonged the survival of these animals, while treatment with cisplatin failed to do so. This suggested that the mild toxicity of BANB-6 was probably mitigated by the vectorial characteristics of the compound, which presumably led to a reduced exposure to the drug of tissues located outside the enterohepatic circuit. Moreover, as already mentioned, cisplatin (at low or high doses) was unable to prolong the animals' life span due to the resistance of the implanted tumor cells, whereas BANB-6 was efficient in overcoming resistance to the chemotherapy.

We have previously shown that resistance to cisplatin by enterohepatic cells could be partly due to a reduction in the active intracellular levels of the drug as a result of combined lowered uptake (due to the down-regulation of uptake

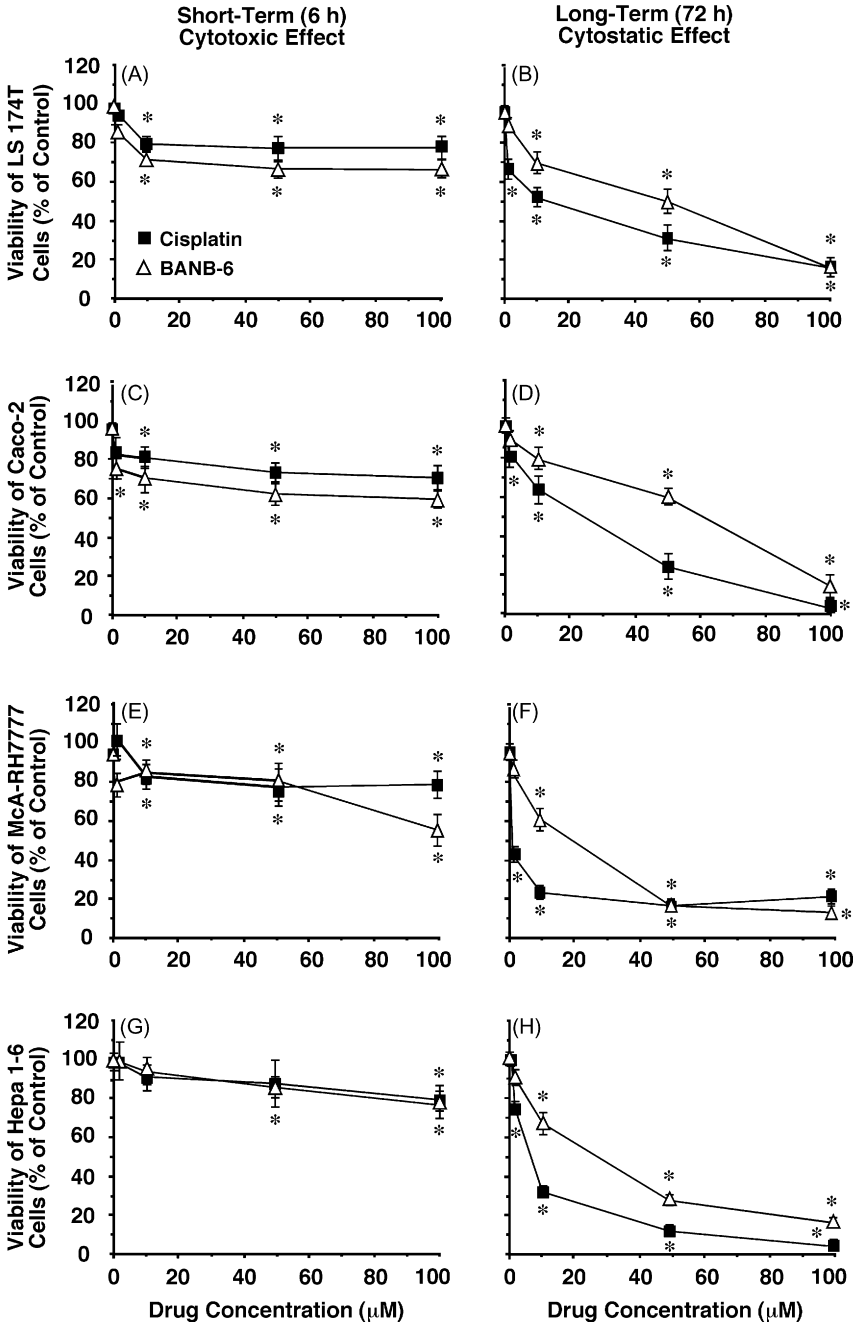

Fig. 4 – Short-term (6 h) toxic effect (A, C, E and G) and long-term (72 h) cytostatic effect (B, D, F and H) of BANB-6 on the following cell lines: (A and B) LS 174T from human colon adenocarcinoma, (C and D) Caco-2 from human colon adenocarcinoma, (E and F) McA-RH7777 from rat hepatoma, and (G and H) Hepa 1-6 cells from mouse hepatoma. Values of cell viability, determined with the formazan test, were compared with those found in non-treated dishes. Cisplatin, a well-known cytostatic agent, was used as a positive control. Values are means ± S.D. from four different cultures carried out in triplicate. *$P < 0.05$ on comparing with non-treated cells by Dunnett's test.

carriers) and enhanced efflux (due to the up-regulation of export pumps) [6]. In the present study, we did not address the drug uptake/efflux balance, which would require the synthesis of radiolabeled derivatives to be studied, however one could speculate that it is probable that the mechanism necessary for resistance to be overcome would be similar to that of previously investigated cytostatic bile acid derivatives. Thus, using atomic absorption spectrometry of platinum to determine drug uptake, we have shown that the ability of cisplatin-BA derivatives, such as BAMET-UD2, to overcome the resistance to cisplatin is in part due to enhanced uptake of these drugs by BA carriers and transporters for organic anions (OATPs) and cations (OCTs) [2], together with a lack of recognition as substrates by export pumps, which results in the reduction of drug efflux [6].

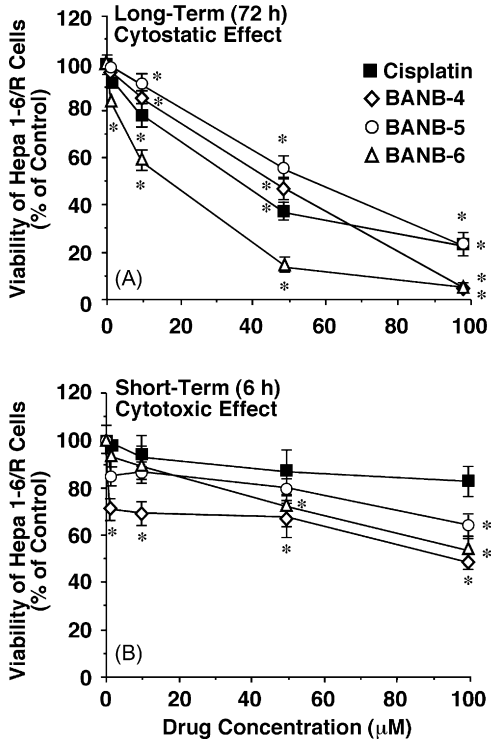

Fig. 5 – Long-term (72 h) cytostatic effect (A) and short-term (6 h) toxic effect (B) of BANB-4, BANB-5 and BANB-6 on partially cisplatin-resistant Hepa 1-6/R cells from mouse hepatoma. Values of cell viability, determined with the formazan test, were compared with those found in non-treated dishes. Cisplatin, a well-known cytostatic agent, was used as a positive control. Values are means ± S.D. from four different cultures carried out in triplicate. * $P < 0.05$ on comparing with non-treated cells by Dunnett's test.

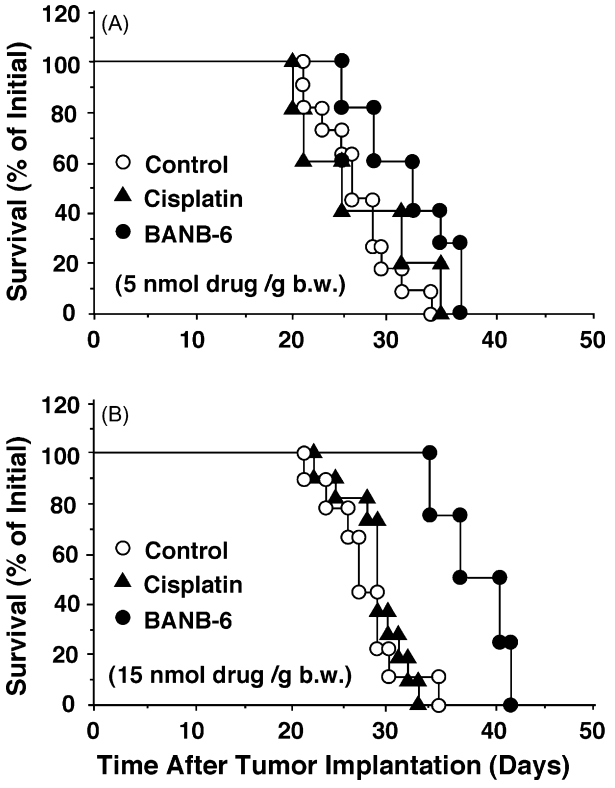

Fig. 6 – Kaplan–Meier curves for the survival of Nude mice following orthotopic implantation in the liver of a fragment of approximately 1 mm^3 of mouse hepatoma tumor previously grown from the cisplatin-resistant subline Hepa 1-6/R from mouse hepatoma. Treatment with 5 nmol/g b.w. (A) or 15 nmol/g b.w. (B) of cisplatin or BANB-6, administered i.p. twice a week was started the day after implantation of the tumor into the liver. Control groups received only the vehicle, i.e., saline solution. Treatment was maintained throughout the life span of the tumor-bearing mice. Values are expressed as percentages of the initial numbers of mice in each group (6–10).

In conclusion, our findings indicate that BANBs, in particular BANB-6 and others that may be obtained in the future based on the results of the present study, do not have the drawback of including a transition metal atom in the molecule, as was the case of BAMETs, but retain the advantages of enterohepatic targeting enabled by the presence of a BA moiety. This further supports the concept that the use of BAs as shuttles might be a useful pharmacological strategy to deliver cytostatic drugs to tumors of the liver, the biliary system, and the intestine. This is of great clinical relevance because a large proportion of all cancers is constituted by those affecting tissues included in the enterohepatic circuit [34–36] and these tumors usually have a poor prognosis, which is further aggravated by the lack of response to the available chemotherapy.

Table 3 – Comparison of cytostatic activity of BANB-6 and cisplatin on wild-type and cisplatin-resistant tumor cell lines of different origins

Cell line	IC_{50} (μM)	
	Cisplatin	BANB-6
LS 174T	18.2 ± 3.7	59.7 ± 4.7[a]
Caco-2	23.7 ± 5.1	74.6 ± 2.0[a]
McA-RH7777	3.5 ± 1.2	20.5 ± 4.5[a]
Hepa 1-6	5.0 ± 0.4	28.6 ± 2.9[a]
Hepa 1-6/R	68.3 ± 5.9	30.3 ± 5.8[a]

IC_{50} was defined as the drug concentration (in μM) required to reduce the amount of living cells by 50%. Values are mean ± S.D. from four different cultures carried out in triplicate.
[a] $P < 0.05$ on comparing with cisplatin by the Student's t-test.

Conflict of interest statement

In the period of research leading up to this publication we have not received any financial support that may affect in any way the conclusions of our article. Moreover, the authors have no direct or indirect commercial interest in any company that might be financially affected by the conclusions of the present article.

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