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Carriers Involved in Targeting the Cytostatic Bile Acid-Cisplatin Derivatives *cis*-Diammine-chloro-cholylglycinate-platinum(II) and *cis*-Diammine-bisursodeoxycholate-platinum(II) toward Liver Cells

OSCAR BRIZ, MARIA ANGELES SERRANO, NOEMI REBOLLO, BRUNO HAGENBUCH, PETER J. MEIER, HERMANN KOEPSELL, and JOSE J.G. MARIN

Departments of Physiology and Pharmacology (O.B., N.R., J.J.G.M.) and Biochemistry and Molecular Biology (M.A.S.), University of Salamanca, Salamanca, Spain; Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, Zurich, Switzerland (B.H., P.J.M.); and Department of Anatomy, University of Würzburg, Würzburg, Germany (H.K.)

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ABSTRACT

Molecular bases for targeting bile acid-cisplatin derivatives Bamet-R2 [*cis*-diammine-chloro-cholylglycinate-platinum(II)] and Bamet-UD2 [*cis*-diammine-bisursodeoxycholate-platinum(II)] toward liver cells were investigated. Carriers for bile acids [human Na^+-taurocholate cotransporting polypeptide (NTCP)], organic anions [organic anion transporting polypeptide (OATP)], and organic cations [organic cation transporter (OCT)] were expressed in *Xenopus laevis* oocytes (XO) and Chinese hamster ovary (CHO) cells. Drug uptake was measured by flameless atomic absorption of platinum. Rat Oatp1- or rat Ntcp-transfected CHO cells were able to take up Bamets, but not cisplatin, severalfold more efficiently than wild-type cells. This uptake was enhanced by butyrate-induced expression of both carriers. Uptake of both Bamets by Ntcp-transfected CHO cells was stimulated by extracellular sodium. The amount of Bamets, but not cisplatin, taken up by XO was enhanced when expressing OATP-A, OATP-C, NTCP, OCT1, or OCT2, a nonhepatic OCT isoform used for comparative purposes. Bamet uptake by XO was inhibited by known substrates of these carriers (glycocholate for NTCP and OATP-C, ouabain for OATP-A, and quinine for OCT1 and OCT2). Drug uptake versus substrate concentration revealed saturation kinetics (K_m was in the 8–58 μM range), with the following order of efficiency of transport (V_{max}/K_m) for Bamet-R2: OATP-C > OCT2 > OATP-A > NTCP > OCT1; and the following order of efficiency of transport for Bamet-UD2: OATP-C > OCT2 > OATP-A > OCT1 > NTCP. Increasing the generation of cationic forms of Bamets by incubation in the absence of chloride increased drug uptake by OATP-A, OCT1, and OCT2 but reduced that achieved by NTCP and OATP-C. These results suggest a role for carriers of organic anions and cations in Bamet-R2 and Bamet-UD2 uptake, which may determine their ability to accumulate in liver tumor cells and/or be taken up and efficiently excreted by hepatocytes.

The liver plays a key role in the biotransformation and excretion of a broad variety of xenobiotic and endogenous substances. Carrier proteins mediate hepatic uptake of many of these anionic, neutral, and cationic compounds from the blood. Among them, the Na^+-taurocholate cotransporting polypeptide (NTCP) is considered to be the major system accounting for the Na^+-dependent uptake of bile acids and perhaps other organic anions (Hagenbuch and Meier, 1996). A whole family of Na^+-independent carriers, the organic anion transporting polypeptides (OATPs), of which numbers of identified and cloned members are increasingly larger, contributes to this task. These transporters, expressed in different tissues, are able to carry out the uptake of a wide spectrum of structurally unrelated compounds, such as bile acids, anionic conjugated and neutral steroids, such as ouabain (Hagenbuch et al., 2000). Some of them, however, such as human OATP-A, also seem to be able to transport type-II organic cations (Van Montfoort et al., 1999; 2001). Nevertheless, such transport is probably accomplished mainly by carrier systems known as organic cation transporters (OCTs), which have been found to be involved in the hepatic clearance of many organic cations, including several chemotherapeutic drugs (Koepsell, 1998).

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ABBREVIATIONS: Ntcp, rat Na^+-taurocholate cotransporting polypeptide; Oatp, rat organic anion transporting polypeptide; OATP, human organic anion transporting polypeptide; OCT, human organic cation transporter; HMG, hydroxymethylglutaryl; Bamet-R2, *cis*-diammine-chloro-cholylglycinate-platinum(II); Bamet-UD2, *cis*-diammine-bisursodeoxycholate-platinum(II); GC, glycocholate; TEA, tetraethylammonium; TE, Tris/EDTA; CHO, Chinese hamster ovary; NTCP, human Na^+-taurocholate cotransporting polypeptide.

The use of specific transporters expressed in targeted cells has been proposed as a promising strategy for tissue-selective drug delivery (Tsuji, 1999). Regarding this, the efficiency of hepatocytes in taking up bile acids and other organic compounds, owing to the existence in the basolateral plasma membrane of the above-mentioned multispecific carrier systems, has been the basis for suggesting the use of these hepatotropic compounds or their analogs as shuttles for delivering different types of drugs to the liver (Ho, 1987). Some examples of this approach are the binding of inhibitors of hydroxymethylglutaryl (HMG) CoA reductase (Wess et al., 1994) or of cytostatic compounds (Kramer et al., 1992; Marin et al., 1998a) to bile acids. Our group has synthesized and characterized several members of a new family of antitumoral compounds by binding cisplatin to the side chain of bile acids (Criado et al., 1997a,b; 2000). They have been designated as Bamet, from "Ba," standing for the bile acid moiety, and "met," standing for the transition metal, which is the DNA-reactive moiety of these compounds (Marin et al., 1998b; Criado et al., 2000). The purpose of this approach was to obtain analogs that could be recognized by carrier proteins able to transport cholephilic compounds; this would presumably enhance their vectoriality toward the hepatobiliary system. Previous studies have shown that some of these drugs, such as [*cis*-diammine-chloro-cholylglycinate-platinum(II)] (Bamet-R2) and [*cis*-diammine-bisursodeoxycholate-platinum(II)] (Bamet-UD2), might be potentially useful in the chemotherapy of liver tumors because of their liver organotropism (Marin et al., 1998a; Macias et al., 1999), their strong cytostatic activity in vitro, and their antitumoral effect against tumors implanted in the liver of nude mice (Marin et al., 1998b; Larena et al., 2000; Dominguez et al., 2001). Previous studies carried out with rat hepatocytes in primary culture suggested an efficient uptake of Bamet-R2, probably via an Na^+-independent pathway (Macias et al., 1998). However, transport studies in intact cells fail to distinguish between the contribution of one or more carrier systems involved in the overall process, which may lead to mistaken conclusions. Elucidation of the exact route used by Bamets to enter the cells is important to further develop new and more specific derivatives. The aim of this work, therefore, was to evaluate the role of carriers of organic anions and cations in Bamet-R2 and Bamet-UD2 uptake, which may determine their ability to reach liver tumor cells and/or be taken up and excreted by hepatocytes.

Materials and Methods

Chemicals. Cisplatin, sodium glycocholate (GC), ouabain, quinine hydrochloride, TEA, and cell culture medium were obtained from Sigma-Aldrich (Madrid, Spain). GC was more than 95% pure by thin-layer chromatography. Geneticin was from Roche Applied Science (Barcelona, Spain). [^{14}C]GC (55.0 mCi/mmol), [^{3}H]inulin (304.8 mCi/g), [^{3}H]ouabain (15.4 Ci/mmol), and [^{14}C]TEA (2.4 mCi/mmol) were purchased from Pacisa & Giralt (Madrid, Spain). Bamet-R2 was synthesized by binding GC to cisplatin as previously reported (Criado et al., 1997b). Bamet-UD2 was synthesized by binding two ursodeoxycholic acid molecules to cisplatin (Criado et al., 2000). All other reagents were of analytical grade and were readily available from commercial sources.

Uptake Studies in CHO Cells. Wild-type Chinese hamster ovary (CHO) cells (CHO-K1), stably SLC10A1- or Ntcp-expressing CHO 9-6 cells (Schroeder et al., 1998) as well as stably SLC21A1- or Oatp1-expressing CHO-03 cells (Eckhardt et al., 1999), were cultured as described previously (Schroeder et al., 1998). In some sets of experiments, the expression of Ntcp or Oatp1 was induced by incubating the cells with culture medium supplemented with 5 mM sodium butyrate for 24 h (Palermo et al., 1991). To determine drug uptake by these cells, experiments were carried out using four culture dishes (3 cm in diameter) for each data point. Subconfluent cell cultures were rinsed with uptake medium (116 mM NaCl or choline chloride, 5.3 mM KCl, 1.1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 11 mM D-glucose, and 10 mM HEPES/Tris, pH 7.4), and the cells were subsequently incubated in the presence of 50 μM concentrations of the desired compound at 37°C for the time indicated in Figures 1, 2, and 3 in each case. Uptake was stopped by rinsing the culture dishes four times with 2 ml of ice-cold uptake medium containing 100 μM cholic acid to reduce nonspecific extracellular binding. Cells were digested in 1 ml of 0.7% SDS. To measure platinum contents, 800-μl aliquots were mineralized as described below. Protein concentrations were measured by the method of Markwell et al. (1978).

Uptake Studies in *Xenopus laevis* Oocytes. Synthesis of cRNA for injection into oocytes was performed using recombinant plasmids containing the cDNA of NTCP (SLC10A1), OATP-A (SLC21A3), OATP-C (SLC21A6), OCT1 (SLC22A1), or OCT2 (SLC22A2). These plasmids were isolated from *Escherichia coli* using the QIAGEN Plasmid Mini Kit (QIAGEN, Barcelona, Spain) and further linearized with restriction enzymes (Roche Applied Science). cRNAs were synthesized using the T7 mMessage mMachine kit (Ambion, Austin, TX). Mature female *X. laevis* frogs from Regine Olig (Hamburg, Germany) were anesthetized by intramuscular administration (in the leg) of 12.5 mg of ketamine (Imalgène 500; Rhône Mérieux, Barcelona, Spain). Animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Unites States National Institutes of Health Publication 80-23, revised 1985). The University of Salamanca Ethical Committee for Laboratory Animals approved the experimental protocol. Harvesting and preparation of oocytes were carried out by a modification of the method described in detail by Hagenbuch et al. (1990). Briefly, a portion of ovarian tissue was removed, and individual oocytes were isolated and defolliculated by incubation with collagenase (2 mg/ml) in calcium-free buffer for 45 min at 20°C and incubated overnight at 18°C in modified Barth's solution: 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 50 mg/l gentamicin, and 15 mM HEPES, pH 7.6. Oocytes with a normal shape and color were considered healthy and were injected with 7 ng of carrier protein cRNA dissolved in 14 nl of TE buffer (1 mM EDTA and 10 mM Tris, pH 8.0), or TE buffer alone, used as a control to determine nonspecific uptake of the substrates. The oocytes were subsequently cultured in modified Barth's solution for 2 days at 18°C. To carry out uptake studies, groups of between 10 and 20 oocytes were prewashed in the uptake medium containing 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris, pH 7.5, and 100 mM choline chloride. To determine Na^+-dependent uptake by NTCP-expressing oocytes, they were incubated with medium containing either 100 mM NaCl or 100 mM choline chloride. In experiments designed to measure Bamet uptake in the absence of chloride, the composition of the uptake medium was 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 100 mM $NaNO_3$, or 100 mM KNO_3 to measure Na^+-dependent uptake in NTCP-expressing oocytes, and 10 mM HEPES/Tris, pH 7.5. The oocytes were then incubated for the desired time at 25°C in 100 μl of the uptake medium containing the compound to be tested. Uptake was stopped by the addition of 4 ml of ice-cold uptake medium containing 100 μM cholic acid. Oocytes were washed an additional three times with this solution and then collected individually, placed in individual vials, and dissolved with 200 μl of 1% SDS solution.

Analytical and Statistical Methods. Bamet-R2, Bamet-UD2, and cisplatin concentrations were determined by measuring platinum by flameless atomic absorption spectrophotometry (Z-8100 Polarized Zeeman apparatus with a graphite furnace; Hitachi, Tokyo, Japan). To measure platinum contents, samples were mineralized in

pure HNO_3 at 80°C for 2 h and then at 150°C until dryness. Before being measured, samples were redissolved with 20 mM HCl at 60°C for 4 h. Radioactivity was measured on a liquid scintillation counter (LS-6500; Beckman Coulter, Inc., Madrid, Spain). Unless otherwise indicated, results are expressed as mean ± S.E.M. For kinetic analyses, values were fitted to a Michaelis-Menten equation with or without an additional diffusional term. Estimations made by linear and nonlinear regression analysis were obtained using the UltraFit v2.1 software (Elsevier-Biosoft, Cambridge, UK). To calculate the statistical significance of the differences between groups, paired or unpaired Student's *t* tests were used as appropriate. The Bonferroni method was used for multiple-range testing. Statistical analysis was performed on a Power Macintosh 6400/200 (Apple, Cupertino, CA).

Results

Two different experimental models of the functional expression of membrane carrier proteins were used in this study to investigate the ability of these transport systems to mediate Bamet-R2 and Bamet-UD2 uptake. On the one hand, CHO cells stably transfected with cDNA of rat carriers were used as an initial approach to identify the role of rat carriers for cholephilic anions in Bamet uptake. Previous studies using this mammalian expression system have shown that Oatp1- and Ntcp-expressing CHO cells, but not wild-type cells, exhibit an efficient uptake of bile acids, such as GC, among several other substrates (Schroeder et al., 1998; Eckhardt et al., 1999). The results of time-course studies of Bamet-R2 and Bamet-UD2 uptake in both wild-type (CHO-K1) and Oatp1-expressing (CHO-03) CHO cells are compared in Fig. 1. Uptake by CHO-03 cells of both Bamets was approximately 6-fold higher than their levels in wild-type CHO-K1 cells. In the presence of Na^+, Ntcp-expressing CHO 9-6 cells were able to carry out the uptake of Bamet-R2 and Bamet-UD2 at amounts 5- and 8-fold higher than wild-type cells, respectively (Fig. 2). This was markedly reduced when Na^+ was removed from the incubation medium, whereas the absence of sodium did not affect drug retention by wild-type cells (Fig. 2).

The presence of sodium butyrate in the culture medium of CHO cells, which has been shown to enhance expression of recombinant proteins (Palermo et al., 1991), has been used previously to increase both Oatp1 (Eckhardt et al., 1999) and Ntcp (Schroeder et al., 1998) expression in stably transfected CHO cells. In our hands, this maneuver markedly enhanced GC, Bamet-R2, and Bamet-UD2 uptake by cells transfected with cDNA of Oatp1 or Ntcp, regarding both at steady-state (Fig. 3) and initial uptake rates (data not shown) compared with cell cultures where butyrate had been removed. By contrast, no effect of preincubation with butyrate on GC, Bamet-R2, and Bamet-UD2 uptake by wild-type CHO-K1 cells was observed (Fig. 3).

Finally, we investigated whether GC, a substrate of both Oatp1 and Ntcp, was able to inhibit Bamet uptake by CHO cells expressing one of these carriers. The results indicated that GC reduced the amount of Bamet-R2 (−75%) and Bamet-UD2 (−44%) taken up by Oatp1-transfected CHO cells. GC also induced *cis*-inhibition of Bamet-R2 (−40%) and Bamet-UD2 (−46%) uptake by Ntcp-expressing cells (Table 1).

An *X. laevis* oocyte expression system was used as an additional experimental model that permitted us to study Bamet transport by human carriers, including transporters of organic cations, and to carry out kinetic analyses. Oocytes were injected with the cRNA of the human transporters NTCP, OATP-A, OATP-C, OCT1, or OCT2. Although OCT2 is not expressed in the liver, it was included in the study for comparative purposes and because the kidney is the other organ, besides the liver, where Bamets are transiently accumulated (Dominguez et al., 2001). Before using this model, suitable expression of the carrier proteins in the *X. laevis* oocytes was confirmed in uptake experiments (data not shown) using prototypic radiolabeled substrates of these transporters, namely, conjugated cholic acid for NTCP (Meier et al., 1997) and OATP-C (Abe et al., 1999), ouabain for OATP-A (Hagenbuch and Meier, 1996), and TEA for OCT1 and OCT2 (Koepsell, 1998). Preliminary studies on the time course of functional expression (data not shown) revealed that the highest uptake rate was obtained 2 or 3 days after injecting the cRNA of each protein. Therefore, all uptake experiments were carried out at the time after injection that was most appropriate for each protein.

The amount of cisplatin taken up by oocytes expressing OATP-A, OATP-C, NTCP, OCT1, or OCT2 was not higher than that observed in oocytes injected with TE buffer, considered as the control group (Fig. 4A). Thus, no significant changes in the amount of cisplatin taken up by the oocytes were found in the presence or absence of typical substrates of the expressed transporters (Fig. 4A). These results clearly

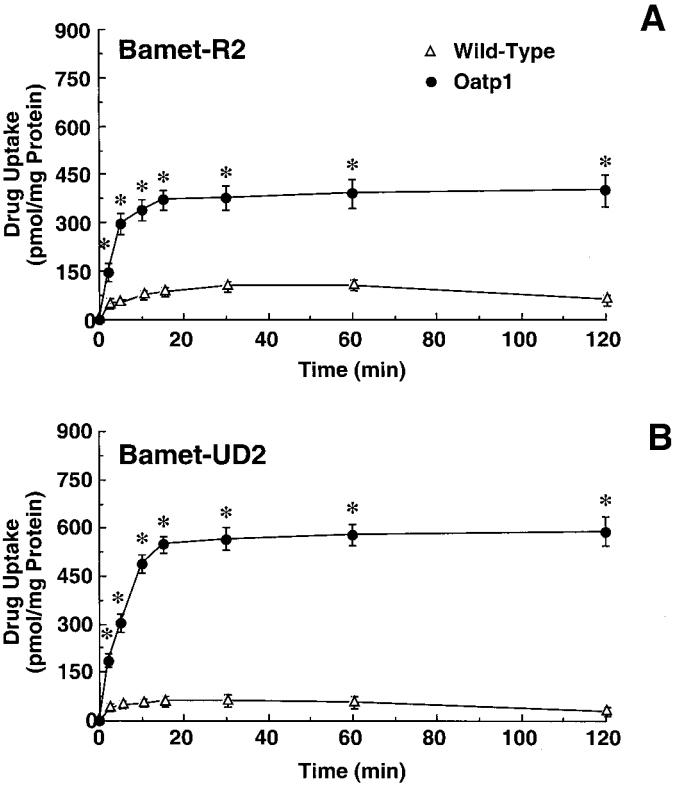

Fig. 1. Time course of Bamet-R2 (A) and Bamet-UD2 (B) uptake by Oatp1 cDNA-transfected CHO-03 cells (●) or wild-type CHO-K1 cells (△). Cells were cultured in the presence of 5 mM sodium butyrate for 24 h before carrying out uptake measurements. Then, cells were incubated with 50 μM concentrations of the desired compound in 116 mM choline chloride-containing medium at 37°C for the indicated period of time. Values are mean ± S.E.M. from three different cultures carried out in quadruplicate. *, $p < 0.05$ compared with drug uptake by wild-type CHO-K1 cells.

indicated that cisplatin is not transported by these carrier systems.

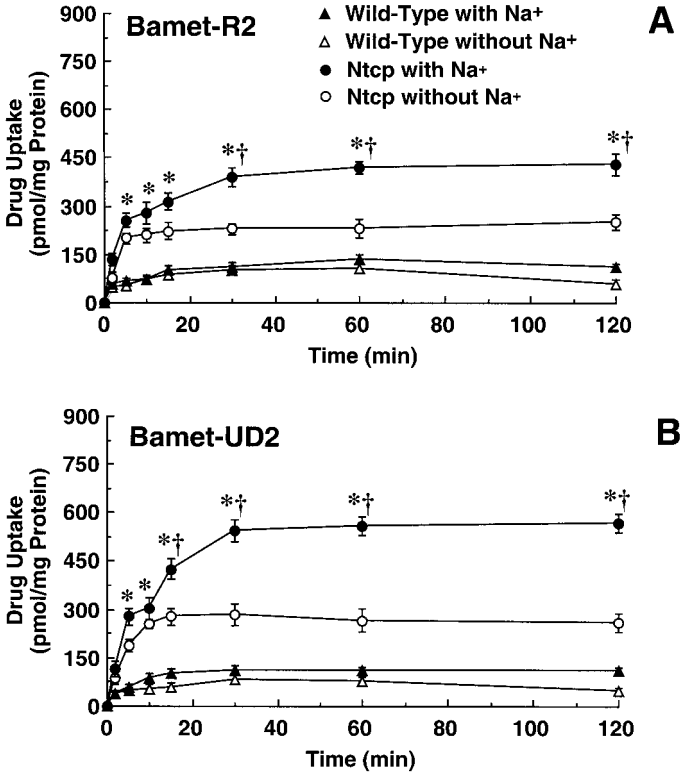

Fig. 2. Time course of Bamet-R2 (A) and Bamet-UD2 (B) uptake by Ntcp cDNA-transfected CHO 9-6 cells (circles) or wild-type CHO-K1 cells (triangles). Cells were cultured in the presence of 5 mM sodium butyrate for 24 h before carrying out uptake measurements. Then, cells were incubated with 50 μM concentrations of the desired compound in medium containing 116 mM NaCl (closed symbols) or 116 mM choline chloride (open symbols) at 37°C for the indicated period of time. Values are mean ± S.E.M. from three different cultures carried out in quadruplicate. *, $p < 0.05$ compared with drug uptake by wild-type CHO-K1 cells. †, $p < 0.05$ compared with drug uptake by CHO 9-6 cells in the absence of Na^+ in the incubation medium.

By contrast, uptake of both Bamet-R2 (Fig. 4B) and Bamet-UD2 (Fig. 4C) by OATP-A, OATP-C, NTCP, OCT1, or OCT2 cRNA-injected oocytes was higher than drug uptake by TE-injected control oocytes. In general, uptake by these carriers was higher for Bamet-UD2 than for Bamet-R2. Furthermore, the presence of known substrates of the transporters expressed in oocytes at concentrations 10-fold higher than that of the Bamet resulted in a marked inhibition of Bamet-R2 or Bamet-UD2 uptake.

To carry out kinetic studies, oocytes were incubated in the presence of several substrate concentrations for 10 min; based on the results from studies on time course uptake (Fig. 5), this was considered the right time to determine drug uptake under initial velocity conditions. Kinetic analyses revealed the existence of a saturable uptake process for Bamet-R2 (Fig. 6A) and Bamet-UD2 (Fig. 6B) by all carriers studied. Comparison of the values found for the apparent affinity constant for each carrier indicated that the order of affinity for Bamet-R2 was OATP-C ≈ OCT2 > OATP-A ≈ NTCP > OCT1 and for Bamet-UD2 was OCT2 ≈ OATP-C > OATP-A ≈ OCT1 > NTCP (Table 2). Calculation of efficiency of transport (V_{max}/K_m) revealed the following order for Bamet-R2: OATP-C > OCT2 > OATP-A > NTCP > OCT1 and the following order for Bamet-UD2: OATP-C > OCT2 > OATP-A > OCT1 > NTCP. This also indicated that Bamet-UD2 is a better substrate than Bamet-R2 for all these carriers.

To elucidate whether the cationic or the neutral form of these compounds was the best substrate for each carrier, uptake of both Bamet-R2 and Bamet-UD2 was studied in the absence of chloride, which is expected to increase the formation of the cationic form of these types of compounds due to replacement of chloride by an "aquo" group. Thus, the increase in the proportion of cationic forms of Bamets in the mixture had no effect on nonspecific uptake in TE-injected oocytes but significantly enhanced the uptake of Bamet-R2 (Fig. 7A) and Bamet-UD2 (Fig. 7B) by oocytes expressing OCT2, OATP-A, and, to a lesser extent, OCT1, although it

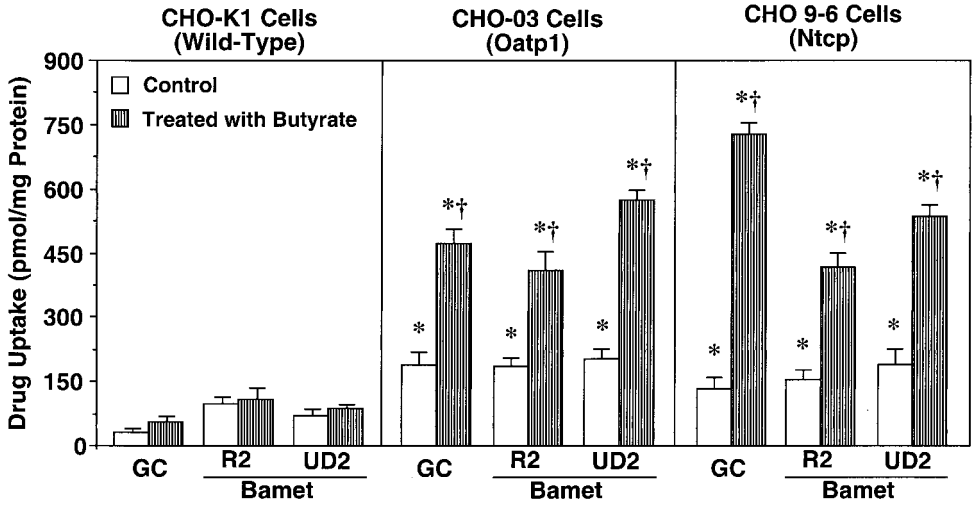

Fig. 3. Effect of the presence or absence of sodium butyrate on GC, Bamet-R2, and Bamet-UD2 uptake by wild-type CHO-K1 cells, CHO 9-6 cells expressing Ntcp, and CHO-03 cells expressing Oatp1. Cells were cultured in the absence or presence of 5 mM sodium butyrate for 24 h before carrying out uptake measurements. Then, cells were incubated with 50 μM concentrations of the desired compound in medium containing 116 mM NaCl (CHO-K1 and CHO 9-6 cells) or 116 mM choline chloride (CHO-03 cells) at 37°C for 120 min. Values are mean ± S.E.M. from three different cultures carried out in quadruplicate. *, $p < 0.05$ compared with drug uptake by wild-type CHO-K1 cells. †, $p < 0.05$ compared with cells that were not treated with butyrate.

caused a moderate decrease in Na^+-dependent uptake by NTCP and in Na^+-independent uptake by OATP-C.

Discussion

This study provides molecular bases for understanding targeting of Bamets toward normal liver and tumor cells derived from the enterohepatic circuit (Monte et al., 1999). Among other members of this family of drugs, Bamet-R2 and Bamet-UD2 exert the strongest in vitro cytostatic (Marin et al., 1998b; Martinez-Diez et al., 2000) and in vivo antitumor (Dominguez et al., 2001) effects. Although they share the antiproliferative capacity characteristic of cisplatin, both are much less toxic. This is partly because of the enhanced liver organotropism, which determines efficient excretion into bile together with lower drug exposure of other organs, including the kidney (Macias et al., 1999; Dominguez et al., 2001).

The most important structural difference among these drugs is the presence of bile acid moieties in Bamets that are absent in cisplatin. On the other hand, they share important characteristics; i.e., they are neutral compounds that in aqueous solution undergo replacement of some of the platinum(II) ligands by water. This results in the formation of "aquo" groups that are DNA-reactive (for a review, see Sundquist and Lippard, 1990). Most of this process probably occurs within cells, where chloride concentrations are lower than those in plasma. Simple diffusion of the extracellular neutral cisplatin form is believed to be the main pathway for cisplatin uptake; however, a role of mediated transport by mechanisms similar to those of neutral amino acid uptake has been suggested (Chu, 1994). Bamets are also activated to form DNA-reactive "aquo" groups in aqueous solution. In Bamet-R2, this occurs through replacement of the chloride ion present in the neutral form, thus resulting in a monocationic derivative (Criado et al., 1997b). It could be expected that, as in the case of cisplatin, this would occur within the cell. However, the transformation of Bamet-R2 is faster in 150 mM NaCl than in pure water (Criado et al., 1997b), implying that cationic forms of Bamet-R2 should be formed even in the extracellular fluid. Bamet-UD2 is also activated in 150 mM NaCl to a monocationic derivative; in this case, however, the mechanism is the release of one of the ursodeoxycholate moieties (Criado et al., 2000).

The finding that, unlike both Bamets, cisplatin was not transported by any of the rat or human carriers that have been assayed indicates that the presence of the bile acid moiety in Bamets is crucial for these compounds to be transported by these carriers. Moreover, K_m values for both drugs are similar to those found for established substrates [i.e., 17β-glucuronosyl estradiol/OATP-C, K_m = 8.2 μM (König et al., 2000); bromosulfophthalein/OATP-A, K_m = 20 μM (Kullak-Ublick et al., 1995); taurocholate/NTCP, K_m = 6 μM (Hagenbuch and Meier, 1994); methyl-4-phenylpyridinium/OCT1, K_m = 14.6 μM (Zhang et al., 1997); and TEA/OCT2: K_m = 76 μM (Gorboulev et al., 1997), which indicates that Bamet-R2 and Bamet-UD2 are good substrates for these carriers.

The ability of OCT1 and OCT2 to take up the Bamets was not surprising, because in aqueous solution, these compounds are in part as cationic forms. Moreover, overlapping substrate specificity of both carriers has been reported

TABLE 1

cis-Inhibition of Bamet-R2 and Bamet-UD2 uptake by CHO cells expressing rat Ntcp or Oatp1

Values of drug uptake at steady state (in picomoles per milligram of protein per 120 min) are means ± S.E.M. from three different cultures carried out in quadruplicate. The cells were cultured in the presence of 5 mM sodium butyrate for 24 h before the uptake experiments were performed. Then, the amount of drug retained by the cells was measured after carrying out an incubation with 50 μM Bamet-R2 or Bamet-UD2 at 37°C for 2 h. The incubation medium contained 116 mM NaCl (Ntcp) or 116 mM choline chloride (Oatp1) with or without 500 μM glycocholate (GC).

	Carrier Protein Expressed in CHO Cells	
	Oatp1	Ntcp
Bamet-R2	299 ± 28	183 ± 19
Bamet-R2 + GC	105 ± 31[a]	99 ± 34[a]
Bamet-UD2	485 ± 28	309 ± 29
Bamet-UD2 + GC	271 ± 20[a]	164 ± 41[a]

[a] $p < 0.05$, compared with drug uptake in the absence of GC.

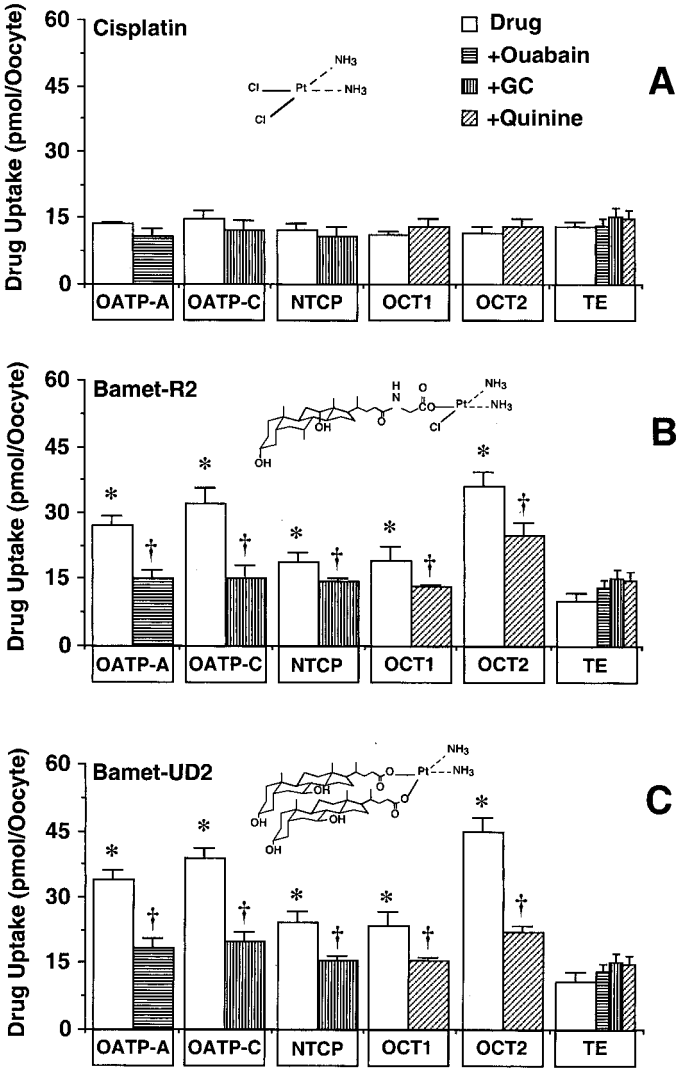

Fig. 4. Uptake by *X. laevis* oocytes expressing OATP-A, OATP-C, NTCP (Na^+-dependent uptake), OCT1, or OCT2 of cisplatin (A), Bamet-R2 (B), and Bamet-UD2 (C). Drug uptake was determined by incubating oocytes with 50 μM cisplatin, Bamet-R2, or Bamet-UD2 for 60 min at 25°C in the absence and in the presence of 500 μM inhibitor, which was ouabain for OATP-A, GC for NTCP, and OATP-C and quinine for OCT1 and OCT2. Insets, chemical structures of cisplatin, Bamet-R2, or Bamet-UD2. Values are means ± S.E.M. of 30 oocyte uptake measurements in three separate preparations. *, $p < 0.05$ compared with oocytes injected with TE buffer. †, $p < 0.05$ on comparing drug uptake in the absence and in the presence of the inhibitor.

(Okuda et al., 1999). Because OCT1 is expressed in the liver and the kidney, whereas OCT2 is expressed only in the kidney (Gorboulev et al., 1997), OCT1 may contribute to the liver uptake of these drugs, whereas both OCT1 and OCT2 may play a role in Bamet uptake by the kidney (Dominguez et al., 2001). On the contrary, cisplatin was not a good substrate for OCT1 or OCT2, which suggests that these carriers are not involved in the marked renal accumulation of cisplatin (Dominguez et al., 2001).

Although most of the known substrates for members of OATP family are organic anions, these transporters are also able to transport both organic cations (Van Montfoort et al., 1999) and neutral compounds (Hagenbuch et al., 2000). This suggests that both neutral and cationic forms of Bamets are also candidate substrates for these carriers. This is consistent with enhanced uptake of Bamets by OATP-A in the absence of chloride. However, this maneuver decreased uptake by OATP-C, which suggests that this carrier does prefer the neutral form of Bamets.

The nonsubstituted bile acid side chain with monoanionic charge is important for substrate interactions with ileal and hepatic Na^+-dependent bile acid carriers (Baringhaus et al., 1999). Moreover, it was reported that Bamet-R2 was taken up by rat hepatocytes in primary culture mainly via Na^+-independent mechanisms (Macias et al., 1998). However, the results obtained in the present work suggest that Ntcp and NTCP can mediate Bamet uptake. Two facts may partly explain this apparent controversy. First, the expression of Ntcp falls dramatically after culturing rat hepatocytes (Liang et al., 1993). Second, if the contribution of Ntcp to the overall uptake of Bamet-R2 by rat hepatocytes is low compared with other Na^+-independent pathways, which is consistent with our results, this might enhance the difficulty in observing a reduction in Bamet uptake when sodium was replaced by choline in the incubation medium. Regarding the second controversial issue, other authors have also observed that Ntcp is probably able to transport neutral steroids with lower efficiency than anionic steroids (Schroeder et al., 1998) but

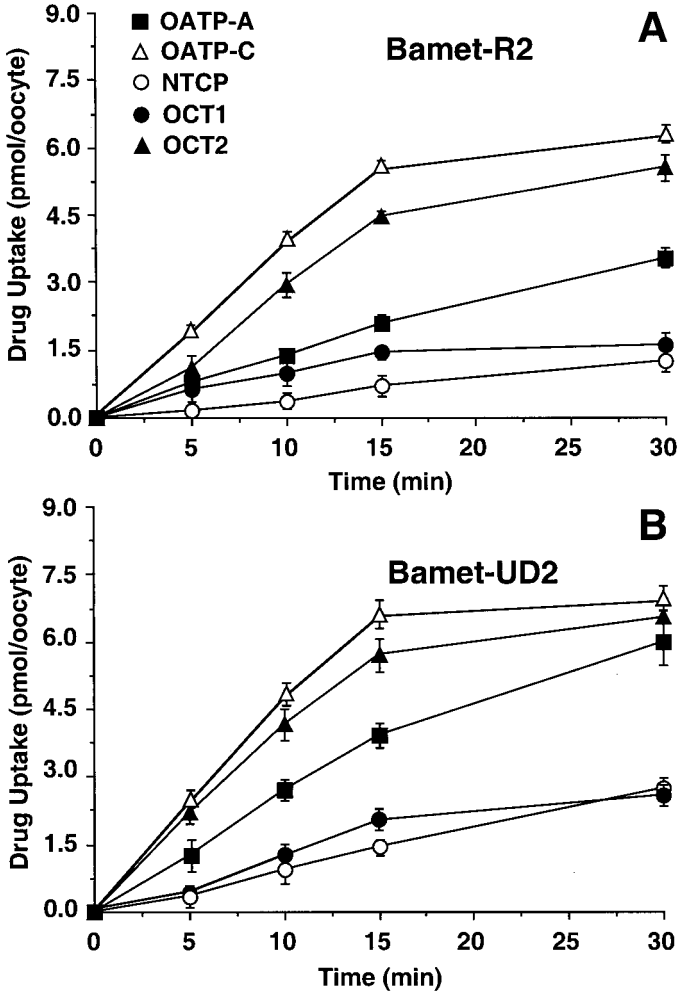

Fig. 5. Time course of Bamet-R2 (A) and Bamet-UD2 (B) uptake by *X. laevis* oocytes. Oocytes were injected with cRNA of OATP-A (■), OATP-C (△), NTCP (○; Na^+-dependent uptake), OCT1 (●), or OCT2 (▲). Uptake for the indicated incubation time with 50 μM substrate was calculated from the substration of Bamet-R2 (upper) or Bamet-UD2 (lower) retention by oocytes injected with the cRNA of the carrier and those injected with TE buffer. Between 10 and 20 oocytes were used for each data point. Values are mean ± S.E.M.

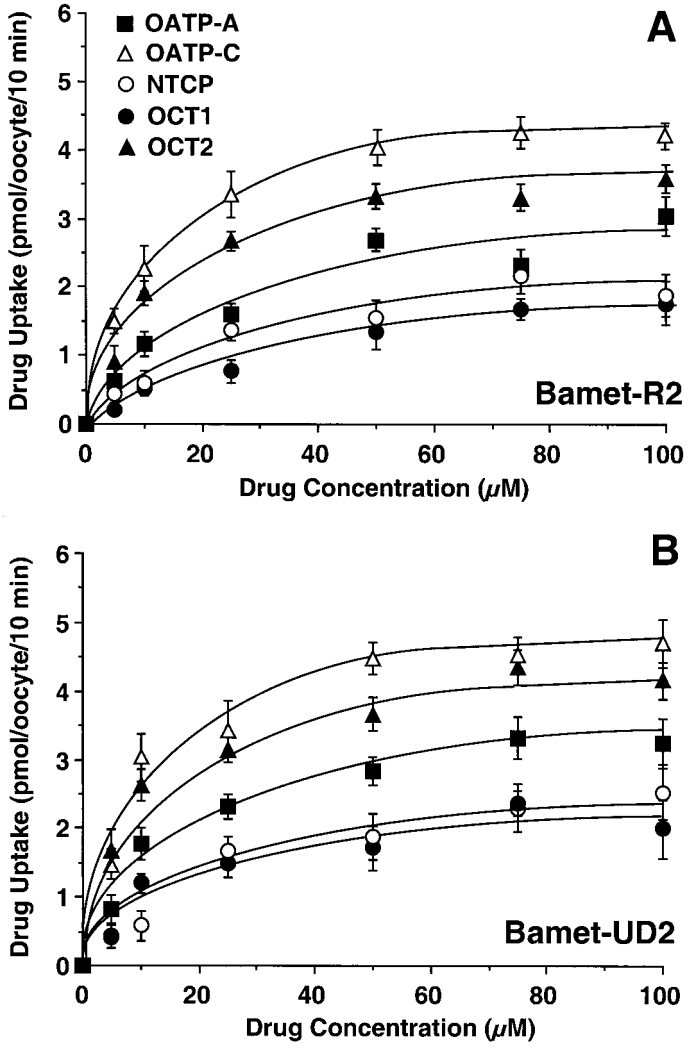

Fig. 6. Saturation kinetics of Bamet-R2 (A) and Bamet-R2 (B) uptake by *X. laevis* oocytes, which were injected with the cRNA of OATP-A (■), OATP-C (△), NTCP (○; Na^+-dependent uptake), OCT1 (●), or OCT2 (▲). Uptake of Bamets was measured at 10 min in incubation medium containing varying substrate concentrations. Between 10 and 20 oocytes were used to calculate mean values at each substrate concentration by subtracting the uptake observed in oocytes injected with TE buffer alone from that found in oocytes injected with the cRNA of the carrier. Values are mean ± S.E.M.

TABLE 2

Kinetic parameters of Bamet-R2 and Bamet-UD2 uptake by *X. laevis* oocytes injected with the cRNA of human NTCP, OATP-A, OATP-C, OCT1, or OCT2

Apparent K_m and V_{max} values were obtained by fitting the results of drug uptake by *X. laevis* oocytes incubated at varying drug concentrations (from 0 to 100 μM). Efficiency of transport (Et) was calculated from V_{max}/K_m. Two days before carrying out uptake studies, the oocytes were injected with TE buffer, either alone or containing 7 ng of cRNA of human NTCP, OATP-A, OATP-C, OCT1, or OCT2. Between 10 and 20 oocytes were used to calculate mean values at each substrate concentration by subtracting the uptake observed in oocytes injected with TE buffer alone from that found in oocytes injected with the cRNA of the carrier. Values are means ± S.D.

	Bamet-R2			Bamet-UD2		
	K_m	V_{max}	Et	K_m	V_{max}	Et
	μM	pmol/oocyte/10 min	nl/oocyte/10 min	μM	pmol/oocyte/10 min	nl/oocyte/10 min
NTCP	27.0 ± 8.9	2.6 ± 0.3	96	33.2 ± 8.5	3.3 ± 0.3	99
OATP-A	23.8 ± 8.1	3.5 ± 0.4	147	14.1 ± 2.4	3.7 ± 0.2	262
OATP-C	10.0 ± 1.5	4.5 ± 0.2	450	9.7 ± 2.0	5.5 ± 0.3	567
OCT1	57.7 ± 12.0	2.8 ± 0.3	48	15.4 ± 5.6	2.5 ± 0.3	162
OCT2	13.1 ± 2.3	4.1 ± 0.2	313	8.0 ± 2.4	4.4 ± 0.3	550

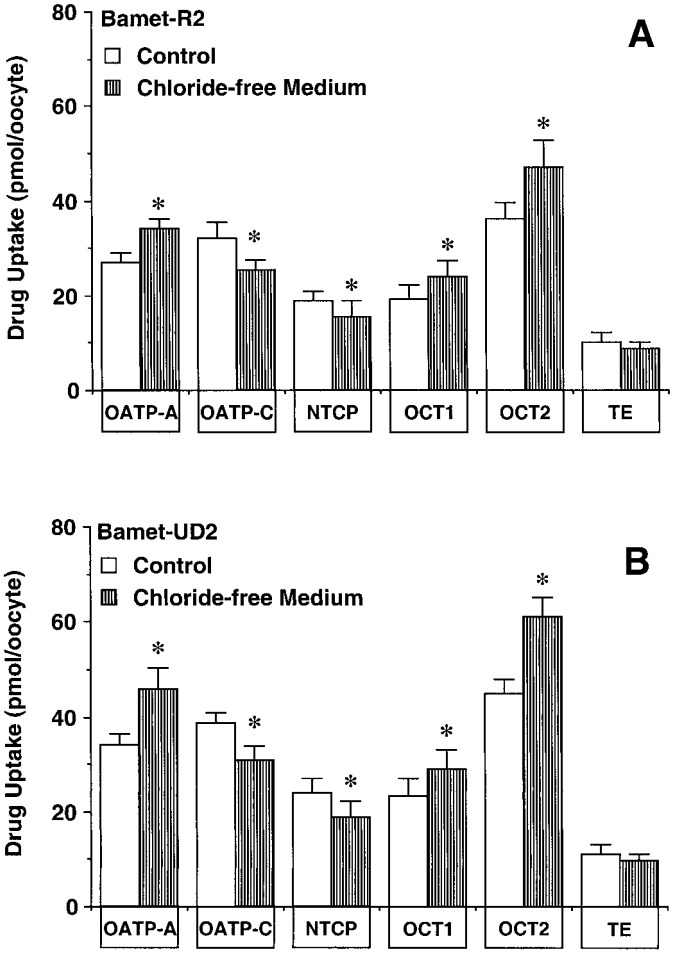

Fig. 7. Bamet-R2 (A) and Bamet-UD2 (B) uptake by *X. laevis* oocytes expressing OATP-A, OATP-C, NTCP (Na^+-dependent uptake), OCT1, or OCT2 in the presence (control) or absence of chloride in the incubation medium. Drug uptake was determined by incubating oocytes that had been injected with the desired cRNA or with TE buffer alone with 50 μM Bamet for 60 min at 25°C in uptake medium with or without chloride. Values are mean ± S.E.M. of 30 oocyte uptake measurements in three separate preparations. *, $p < 0.05$ on comparing uptake in the presence (control) and in the absence of chloride.

not cationic bile acid derivatives (Anwer et al., 1985). This suggests, but does not prove, that the form of the Bamets transported by this carrier may be the neutral one. This is in agreement with the fact that displacement toward the cationic forms by the removal of chloride ions from the incubation medium induced a reduction in Bamet uptake by NTCP.

Certain cell lines derived from liver tumors have, to a certain extent, lost their ability to take up bile acids. However, molecular evidence for the expression of both NTCP and a member of the OATP family in human hepatocellular carcinoma cells has been found (Kullak-Ublick et al., 1997). Moreover, the ability to take up bile acid derivatives is still present, although lower than that of hepatocytes, in human (Kullak-Ublick et al., 1997) and rat liver tumor cells (Monte et al., 1999). Moreover, the expression of OCT1 is preserved in chemically induced rat malignant liver lesions (Lecureur et al., 1998). Those findings, together with these of the present work, support the hypothesis that coupling of drugs to the side chain of bile acids may be a useful pharmacological strategy to target them toward liver tumor cells. However, to obtain a complete picture of this issue, screening of the presence and preserved functionality of appropriate carriers in the plasma membrane of cells collected from human tumors is needed.

In sum, these results indicate that marked liver organotropism of Bamet-R2 and Bamet-UD2 probably exists because these compounds are efficiently transported by several carrier systems for organic anions and cations expressed in the plasma membrane of hepatocytes. However, it should be kept in mind that organ specificity for a given substrate is probably generated by a combination of factors that include the expression levels of each contributing carrier and the functional balance between import carriers and export pumps.

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Address correspondence to: Dr. Jose J.G. Marin, Departamento de Fisiología y Farmacología, Campus Miguel de Unamuno, ED-S09, 37007 Salamanca, Spain. E-mail: jjgmarin@gugu.usal.es