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Usefulness of Liposomes Loaded with Cytostatic Bile Acid Derivatives to Circumvent Chemotherapy Resistance of Enterohepatic Tumors

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ABSTRACT

We have investigated the sensitivity of the cisplatin-resistant enterohepatic tumor cell lines LS174T/R (human colon adenocarcinoma), WIF-B9/R (rat hepatoma-human fibroblast hybrid), and Hepa 1-6/R (mouse hepatoma) to free and liposome-encapsulated cytostatic bile acid derivatives Bamet-R2 and bamet-UD2. Expression of resistance associated genes was measured by quantitative reverse transcription-polymerase chain reaction or Western blotting. Drug uptake was determined by atomic absorption spectrophotometry. In resistant cells, overexpression of MRP1 and MRP2 was accompanied by reduced accumulation of cisplatin. The expression of MDR1 and GST-P was only enhanced in LS 174T/R. A higher expression of p53 was seen in LS 174T/R and Hepa 1-6/R cell lines but not in WIF-B9/R cells. In wild-type counterparts, uptake and cytostatic ability of Bamets were markedly higher (UD2 > R2) than that of cisplatin. Both effects were further enhanced by liposome formulation. Bamets were able to overcome cisplatin resistance in all cell lines. Cisplatin prolonged the survival time of nude mice in whose livers a Hepa 1-6 tumor had been implanted, but failed to exert a beneficial effect when the tumor was Hepa 1-6/R. In both cases, tissue distribution of cisplatin was: kidney ≫ liver > tumor. Survival was markedly longer in animals receiving Bamet-UD2, even if the implanted tumor was resistant. The accumulation of Bamet-UD2 in tissues was: liver > tumor > kidney. Liposome formulation further enhanced the beneficial properties of Bamet-UD2. Thus, the amount of drug in the tumor was increased and that in liver and kidney was reduced (tumor > liver > kidney), and life span was prolonged. In conclusion, liposomal Bamet-UD2 may be a useful tool to circumvent resistance to chemotherapy, particularly in tumors of the enterohepatic circuit.

The development of resistance is one of the factors that often limit the clinical usefulness of cisplatin-related cytostatic drugs (Canon et al., 1990). Because one of the major mechanisms of resistance is that mediated by export proteins, several inhibitors of these pumps have been investigated. However, clinically relevant chemosensitizing doses of the currently available reversal agents cannot be given to humans without significant side effects (Naito et al., 2000). An alternative to the functional blocking of exporting pumps is enhancing drug specificity toward tumor cells to reach intracellular concentrations of the active agent enough to carry out its cytostatic effect. With this aim, our group has developed novel organotropic cytostatic bile acid derivatives named "Bamets" (Marin et al., 2001). Previous studies have shown that *cis*-diamminechloro cholylglycinate platinum(II) (Bamet-R2) and *cis*-diammine bis-ursodeoxycholate platinum(II) (Bamet-UD2) (Fig. 1) can be efficiently taken up by carriers located in the cells of the enterohepatic circuit, such as transporters for anionic, cationic, and neutral organic compounds (Briz et al., 2002). This may account for their ability to accumulate in liver tumor cells and/or be taken up and efficiently excreted by hepatocytes. Thus, Bamet-R2 and Bamet-UD2 have been suggested as potentially useful in the chemotherapy of hepatic tumors, owing to their liver organotropism (Macias et al., 1998; Larena et al., 2001), their strong "in vitro" cytostatic activity, and their "in vivo" antitumoral effect (Dominguez et al., 2001). Moreover, their amphiphilic properties permit their efficient inclusion in liposomes (Briz

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ABBREVIATIONS: Bamet-R2, *cis*-diamminechlorocholylglycinate; Bamet-UD2, *cis*-diammine bis-ursodeoxycholate platinum(II); UDCA, ursodeoxycholic acid; ABC, ATP-binding cassette; GC, sodium glycocholate; FITC-GC, cholylglycylamido-fluorescein isothiocyanate; GST-P, glutathione *S*-transferase-P; HD, high density; LD, low density; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; PCR, polymerase chain reaction.

et al., 2000). Drug delivery using liposomes has been used to solubilize poorly water-soluble drugs, such as several lipophilic derivatives of cisplatin (Al-Baker et al., 1992), as well as a means to reduce the toxicity and enhance the therapeutic efficiency of drugs (Allen and Moase, 1996). Moreover, increasing local tumor exposure to the cytostatic agent by inclusion in liposomes (Papahadjopoulos et al., 1991; Gabizon et al., 1997) contributes to circumvent drug resistance of cancer cells (Gabizon, 1995). In the present study, we have extended previous in vitro investigation on Bamet-R2 (Briz et al., 2000), by determining, both in vitro and in vivo, the efficacy of liposomes loaded with Bamet-UD2, the most promising compound of the Bamet family reported to date (Dominguez et al., 2001). Although the effect of the leaving moiety in Bamet-UD2 [i.e., ursodeoxycholic acid (UDCA) (Criado et al., 2000)] on cisplatin-induced toxicity has not yet been established, the possibility that the known protective effect of UDCA against common mechanisms of cell damage such as oxidative injury (Mitsuyoshi et al., 1999; Trauner and Graziadei, 1999) may endow the complex with additional beneficial properties can be expected. These may contribute to the absence of nephrotoxicity, hepatotoxicity, myelotoxicity, and neurotoxicity observed after repeated doses of Bamet-UD2 to rats in a simulated chemotherapeutic regime (Dominguez et al., 2001).

Exposure to cytostatic drugs is known to up-regulate several mechanisms involved in tumor resistance to alkylating agents such as cisplatin: 1) The development of insensitivity to aggressive stimuli that in normal cells activates the p53-mediated tumor suppressor pathway, which plays a central role in cell cycle regulation and the induction of apoptosis (Reles et al., 2001); 2) the enhanced exporting capability of cancer cells caused by the overexpression of multidrug resistance genes that coded for several members of the ATP-binding cassette (ABC) superfamily of proteins, such as human MDR1 (*ABCB1*), MRP1 (*ABCC1*), and MRP2 (*ABCC2*) (Minemura et al., 1999; Schrenk et al., 2001); and 3) The overexpression of detoxifying systems, such as glutathione *S*-transferase-P, which inactivates cisplatin as well as other drugs and, in turn, renders them better substrates for some of the MRPs, known as GS-X pumps because of their ability to transport glutathione conjugates (Liu et al., 2001). An important aim of the present study was to explore the ability of free and liposome-encapsulated cytostatic bile acid derivatives Bamet-R2 and Bamet-UD2 to overcome cisplatin resistance that was induced in three cell lines of enterohepatic origin.

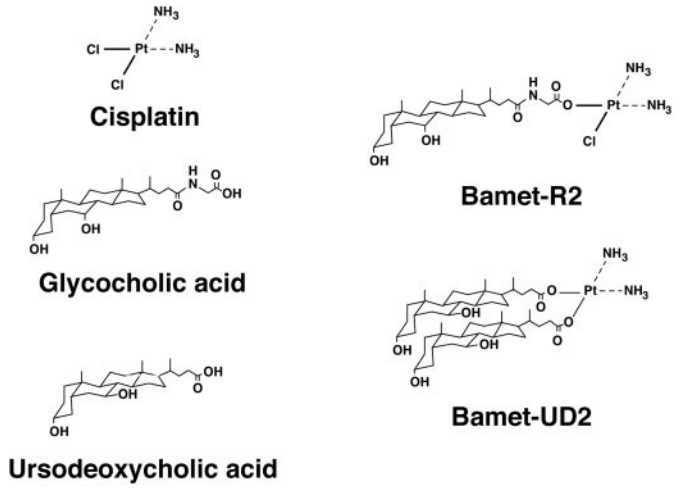

Fig. 1. Chemical structures of cisplatin, glycocholic acid, ursodeoxycholic acid, Bamet-R2, and Bamet-UD2.

Materials and Methods

Chemicals. Cisplatin, sodium glycocholate (GC), bovine serum albumin, gelatin type B, culture media, and protease inhibitors (sodium orthovanadate, 4-(2-aminoethyl) benzenesulfonyl fluoride, aprotinin, bestatin, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, leupeptin and pepstatin A) were obtained from Sigma-Aldrich (Madrid, Spain). Asolectin from soybean, L-α-phosphatidylethanolamine, and stearylamine were from Fluka (Madrid, Spain). [^{14}C]GC (55.0 mCi/mmol) was from PerkinElmer Life Science (Boston, MA). Previously published methods were used to synthesize cholylglycylamido-fluorescein isothiocyanate (FITC-GC) (Sherman and Fisher, 1987), Bamet-R2 (Criado et al., 1997), and Bamet-UD2 (Criado et al., 2000).

Cells. Mouse hepatoma Hepa 1-6 cells and human colon adenocarcinoma LS 174T cells were obtained from the American Type Culture Collection (Manassas, VA). Wild-type human lung large-cell carcinoma COR-L23 cells and the multidrug-resistant COR-L23/R clone were purchased from the European Collection of Cell Cultures (Salisbury, UK). The polarized WIF-B9 cell line, originated from the fusion of rat hepatoma Fao cells with human fibroblast WI-38 cells (Bravo et al., 1998), was kindly provided by Dr. Doris Cassio (Institut National de la Santé et de la Recherche Médicale U442, Paris, France). Cells were cultured with appropriate media in a humidified 5% CO_2/95% air atmosphere at 37°C. Cisplatin-resistant sublines (LS 174T/R and WIF-B9/R) were 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 for Hepa 1-6/R (Briz et al., 2000).

Determination of Resistance Associate Gene Expression. Changes in the amount of mRNA corresponding to the human, rat, or mouse orthologs for MDR1 and MRP1 were determined by real-time quantitative reverse transcription-PCR. In brief, total RNA (~30 μg) was isolated from ~5 $\times$ 10^6 cells using RNAeasy spin columns (QIAGEN, Izasa, Barcelona, Spain), measured using the Ribo-Green RNA-Quantitation kit (Molecular Probes, Leiden, The Netherlands), and, after DNase treatment, subjected to reverse transcription using random nonamers and the Enhanced Avian reverse transcription-PCR kit (Sigma-Genosys, Cambridge, UK). PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems, Madrid, Spain) in an ABI-Prism 5700 Sequence Detection System (Applied Biosystems) with the following thermal conditions: a single cycle of 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 60 s. Primer oligonucleotides obtained from Sigma-Genosys (Table 1) were designed with the assistance of Primer Express software (Applied Biosystems) to amplify cDNA fragments contained in described sequences, and their specificity was checked using BLAST. Detection was carried out using SYBR Green I. Nonspecific products of PCR, as detected by 2.5% agarose gel electrophoresis or melting temperature curves, were not found in any case, except for human MRP1. Therefore, detection of MRP1 amplification products was carried out using a more selective method based on the Taqman probe 5′-ACC GTG CTG CTG TTT GTC ACT GCC-3′. The results obtained from each sample were normalized using 18S rRNA, which was measured with the TaqMan Ribosomal RNA Control Reagents kit (Applied Biosystems).

Western blot analysis was used to investigate GST-P and p53 expression levels. Cells (~2 $\times$ 10^6) were washed with ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and lysed by incubation for 30 min in ice-cold radioimmunoprecipitation assay buffer (1% Nonidet

TABLE 1
Oligonucleotide primer sequences used for real-time quantitative PCR

Gene Product	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size	Position (5′–3′)	Accession no.
			bp		
Human MDR1	GCCTACTTGGTGGCACATAAC	CCAAAGACAACAGCTGAAA	74	2997–3071	AF016535
Human MRP1	CCGCAGATCTTAAAGTTGCTCA	TGCCACTGACGAAGCAGATG	151	980–1130	NM_004996
Human MRP2	GGCAGTGAAGAAGAAGACGATGA	ATTGGACCTAGAACTGCGGCT	133	2666–2798	X96395
Rat Mdr1	GCCTACTTGGTGGCACATAAC	CCAAAGACAACAGCTGAAA	75	2907–2981	L15079
Rat Mrp1	GTGAAGCTTTCCGTGTACTGGAA	TACATGATTGCACAGGAAAAGGA	90	742–831	AJ277881
Rat Mrp2	TGATCGGTTTGCTGAAGAGCT	ACGCACATTCCCAACACAAA	139	1119–1257	NM_012833
Mouse Mdr1	GCCTACTTGGTGGCACATAAC	CCAAAGACAACAGCTGAAA	75	2984–3058	NM_011075
Mouse Mrp1	GTCATGAGGGCGGCAAGAT	CCCTGACCACTGACACTGTCA	144	2518–2661	NM_008576
Mouse Mrp2	TCTAGAGACGGATAGCCTCATTCA	CGCTGTCTAGGACCATTACCTTGT	125	4398–4522	NM_013806

P-40, 0.5% sodium deoxycholate, and 0.1% SDS dissolved in phosphate-buffered saline) supplemented with protease inhibitors. Cell extracts were centrifuged (20,000*g*) at 4°C for 20 min to remove cell debris and the supernatant was stored at −80°C until use. Protein concentrations were determined using the bicinchoninic protein assay kit (Pierce, Madrid, Spain), using bovine serum albumin as standard. Samples were electrophoresed through an SDS-12% polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore) in a semidry transfer cell (Bio-Rad, Madrid, Spain). Antibodies were as follows: Mab 14.1.3, a monoclonal antibody against human and rat GST-P previously obtained by us (Monte et al., 2000); DO-1 monoclonal antibody (BD Pharmingen, Madrid, Spain) specific for human p53, and Ab7 polyclonal antibody (Calbiochem, Madrid, Spain), able to detect human, mouse, and rat p53. Protein load was determined using a specific monoclonal antibody for actin (anti-actin, clone AC-40, Sigma). A chemiluminescence detection system (Amersham Biosciences, Barcelona, Spain) was used to visualize the bands.

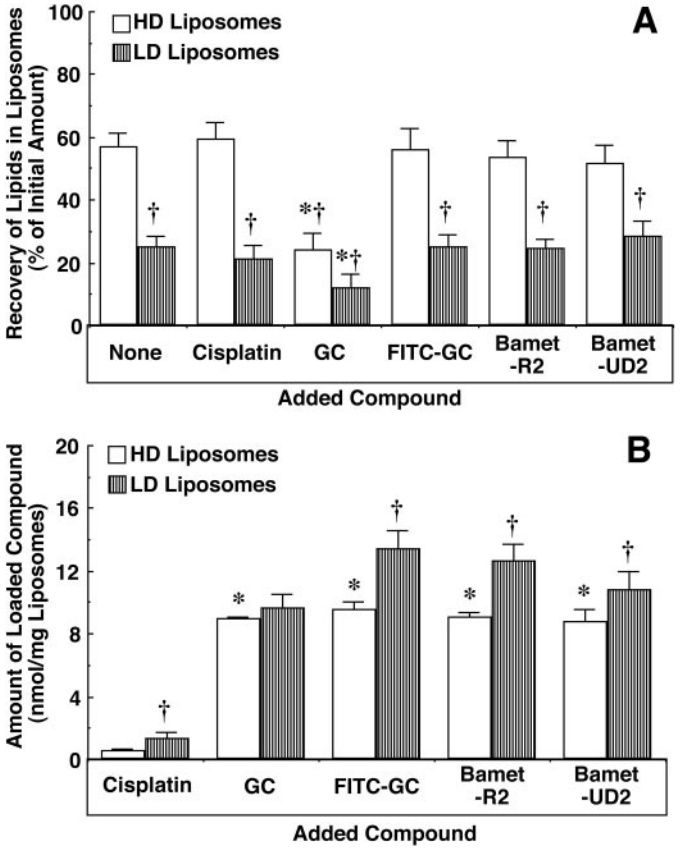

Fig. 2. Yield of the method used for liposomes formation (A) and drug encapsulation (B). Liposome fractions were obtained by serial ultracentrifugation of the initial lipid mixture at 125,000*g* for 1 h (HD, □) or by an additional ultracentrifugation at 265,000*g* for 1 h of the supernatant obtained in the first centrifugation (LD, ▥). Recovery is expressed as the percentage of the initial amount of lipids. The amount of drug encapsulated by the different types of liposomes from a 100 μM solution of cisplatin, GC, or the bile-acid derivatives FITC-GC, Bamet-R2, and Bamet-UD2 is expressed as milligrams of liposomes obtained. Values are means ± S.E.M. from three different experiments. *, $p < 0.05$; **, $p < 0.01$, compared with similar liposomes containing no drug (A) or cisplatin (B). †, $p < 0.05$, on comparing HD with LD liposomes containing the same drug.

Preparation of Liposomes. Drug-loaded liposomes were prepared as previously described in detail (Briz et al., 2000) using phospholipids that were purified from asolectin before being dissolved in chloroform. The solvent was then removed by rotary evaporation under reduced pressure to form a thin lipid film. Dry lipids were hydrated and dispersed together with the desired amount of drug in 150 mM NaCl and then sonicated under nitrogen at 25°C for 10 min. To obtain cationic liposomes, two positively charged components, such as L-α-phosphatidylethanolamine (10%) and stearylamine (10%), were included in the initial lipid mixture (Levchenko et al., 2002). Liposome size and morphology were determined in a previous study (Briz et al., 2000), which revealed that liposome preparation was formed mainly by oligolamellar and multilamellar particles with diameters smaller than 0.10 μm. In the present study, from this preparation two different fractions of drug-containing liposomes were obtained by differential ultracentrifugation and, although physical-chemical characteristics were not investigated, they were designated as high (HD) and low density (LD) liposome fractions. HD fraction was obtained by centrifugation at 125,000*g* for 1 h at 4°C and LD fraction from the supernatant of the first one by centrifugation at 265,000*g*, also for 1 h at 4°C. Both were recovered from the corresponding pellet with a syringe and a 21-gauge needle in 1 ml of 150 mM NaCl and sterilized by filtration through 0.22-μm filters before being stored under a nitrogen atmosphere at 4°C until use. The amount of liposomes was determined by the Sudan black B dye solubilization method, as reported previously (Briz et al., 2000). FITC-GC was measured fluorometrically at excitation and emission wavelengths of 354 nm and 525 nm, respectively.

Drug Uptake and Cytostatic Activity. To determine steady-state drug loads in the cells, subconfluent cultures were incubated in the presence of the desired compound at 37°C for 2 h. They were then washed four times with ice-cold fetal calf serum-free culture medium containing 100 μM cholic acid and digested in 1 ml of 0.7% SDS. [^{14}C]GC was measured on a liquid-scintillation counter. Platinum contents were determined by flameless atomic absorption spectrophotometry (Briz et al., 2002). To measure the in vitro cytostatic activity of free and liposome-encapsulated drugs, the amounts of living cells were determined by the formazan test (Promega, Madison, WI), after incubating ~5,000 cells with the desired compound for 72 h.

Antitumor Activity. Male Nude (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 ster-

ile conditions in a laminar flow hood. Animals were handled in accordance with recommendations of the University of Salamanca Ethical Committee for Laboratory Animals. Mouse hepatoma Hepa 1-6 or Hepa 1-6/R cells ($\sim 10^7$) were injected into the backs of Nude mice. After 2 weeks, tumors growing subcutaneously ($\sim$2 cm in diameter) were removed and minced into cubic fragments of $\sim$1 mm^3 that were implanted in the livers of different animals (Dominguez et al., 2001). Treatment, starting the next day, consisted of two i.p. injections of 15 nmol/g of body weight/week cisplatin, Bamet-UD2, or anionic or cationic liposome-encapsulated Bamet-UD2 dissolved in 0.5 ml of sterile 150 mM NaCl. Owing to its low solubility in this medium, free Bamet-UD2 was administered as a suspension. Control animals received only the vehicle. Animal survival was monitored three times daily. As soon as possible after death, samples of tumor, liver, and kidney were collected to measure platinum contents.

Statistical Methods. Unless otherwise stated, results are expressed as means ± S.E.M. To calculate the statistical significance of the differences between groups, the paired or unpaired Student's *t* tests were used, as appropriate. The Bonferroni method was used for multiple-range testing.

Results

Efficiency of Liposomes to Encapsulate Bile Acid Derivatives. Compared with the initial mixture of lipids, the yields of HD and LD liposome fractions were 57 and 24%, respectively (Fig. 2A). The yield was approximately 84% when both fractions were obtained together. This was not affected by the presence of cisplatin, FITC-GC, Bamet-R2, or Bamet-UD2, but was reduced by GC (Fig. 2A). Using FITC-GC, we have shown no saturation in the ability of these liposomes to encapsulate bile acid derivatives up to at least 100 nmol/mg lipid mixture (Briz et al., 2000). Accordingly, in the present study, we used the mixture 10 nmol of drug/mg of lipid (Fig. 2B). Because Bamets were encapsulated in a lower amount of lipids than that present in the initial mixture, the Bamet-to-lipid ratio in final liposome preparations were in some cases higher than the initial 10 nmol drug/mg lipid mixture. The poor encapsulation of cisplatin contrasted with the strong ability to load Bamets, which was higher in LD liposomes. One of the advantages of liposome formulation for poor water-soluble compounds is that the amount of drug that can be put in a suspension as incorporated in liposomes is much higher than that present in aqueous solution. This property was greatly enhanced in the case of Bamets.

Cisplatin-Resistant Cells. Three cell lines of enterohepatic origin from three different species [i.e., LS 174T (human colon adenocarcinoma), WIF-B9 (rat hepatoma-human fibroblast hybrid), and Hepa 1-6 (mouse hepatoma)], were used to select cisplatin-resistant sublines. For comparative purposes, a human lung carcinoma cell line with a multidrug resistance phenotype, COR-L23/R, and its parental cell line, COR-L23, were also investigated. A significant reduction in

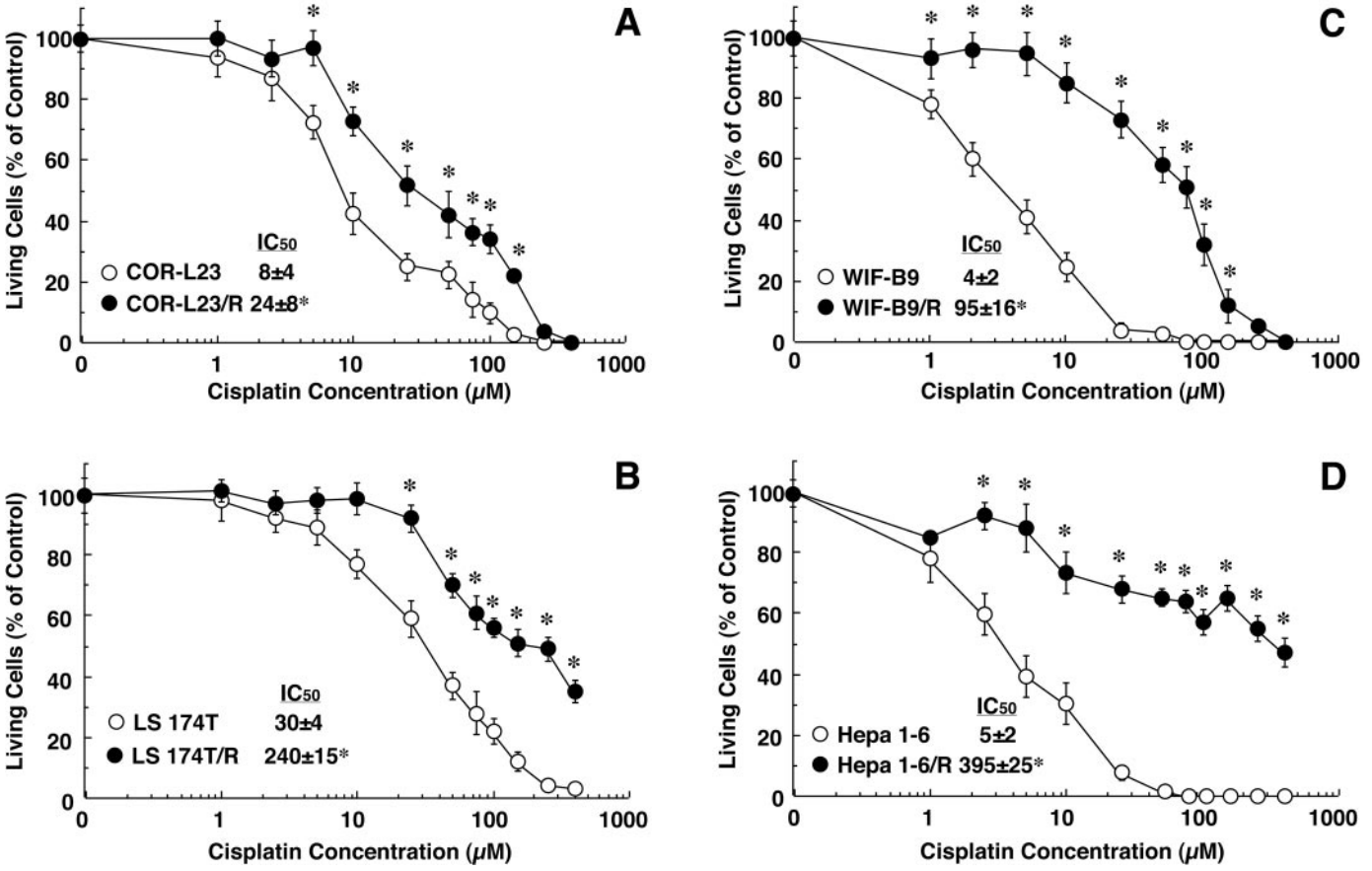

Fig. 3. Cytostatic effect of cisplatin on wild-type (○) and resistant (●) cells of the following cell lines: COR-L23 and COR-L23/R cells from human lung large cell carcinoma (A), LS 174T and LS 174T/R cells from human colon adenocarcinoma (B), WIF-B9 and WIF-B9/R rat hepatoma-human fibroblast hybrid cells (C), and Hepa 1-6 and Hepa 1-6/R cells from mouse hepatoma (D). The proportion of living cells compared with nontreated dishes was determined by the formazan test after the culture had been incubated with cisplatin for 72 h. Values are means ± S.E.M. from four different cultures carried out in triplicate. IC_{50} was defined as the drug concentration required for reducing the amount of living cells by 50%. *, $p < 0.05$ on comparing wild-type and resistant cells by the Student's *t* test.

the sensitivity of the resistant cell lines to the cytostatic effect of cisplatin was observed (Fig. 3). Although COR-L23/R cells were originally selected for resistance to doxorubicin, they were also found to be cross-resistant to cisplatin (Fig. 3A). The IC_{50} values for cisplatin (Fig. 3) indicate that the resistance was higher in enterohepatic cell lines—7.9-, 26.5-, and 74-fold in LS 174T/R (Fig. 3B), WIF-B9/R (Fig. 3C), and Hepa 1-6/R (Fig. 3D), respectively—than in COR-L23/R cells (2.9-fold).

Mechanisms Responsible for Cisplatin Resistance. Decreased sensitivity to cisplatin (Table 2) was accompanied by a lower accumulation of this drug in resistant cells (Table 3). This was more pronounced in LS 174T/R (−75%), WIF-B9/R (−75%) and Hepa 1-6/R (−61%) than in COR-L23/R (−28%). To investigate whether this might be caused by the over-expression of members of the ABC superfamily of transporters, the expression levels of MDR1, MRP1, and MRP2 were assayed. The expression of MDR1 was detected in COR-L23 cells and, to a much lesser extent, in LS 174T cells (Table 4). A marked increase in MDR1 expression was found in COR-L23/R and, more moderately, also in LS 174T/R. However, the expression of MRP1 and MRP2 was detectable in enterohepatic cell lines but not in COR-L23, in which MRP2 was not detectable (Table 4). Moreover, both human and rat orthologs of MRP1 and MRP2 were found in rat-human hybrid WIF-B9 cells. The expression level of MRP1 was markedly enhanced in all resistant cells (Table 4), whereas that of MRP2 was higher than in the wild counterpart only in cells of enterohepatic origin. Because some of these proteins export glutathione conjugates with higher efficiency than nonconjugated substrates and because the overexpression of GST-P has been associated with the development of several enterohepatic tumors, GST-P levels were determined (Fig. 4). The results indicated that detectable levels of these proteins were present only in LS 174T cells and that enhancement of its expression appeared only in their resistant counterparts. Besides the modification in the ability of the cells to detoxify and export cytostatic drugs, the existence of changes in another important mechanism involved in the development of resistance (i.e., the p53 status) was explored (Fig. 4). The expression of p53 was clearly observed in both LS 174T cells and Hepa 1-6 cells. LS 174T/R- and Hepa 1-6/R-resistant variants expressed higher levels of p53 than their wild-type counterparts. Negligible expression of p53 was found in COR-L23, COR-L23/R, and WIF-B9 cells, whereas in WIF-B9/R cells, the presence of what seemed to be a truncated form of p53 was found.

In Vitro Cytostatic Activity of Bamet-R2 and Bamet-UD2. To determine the antiproliferative effect of Bamet-R2 and Bamet-UD2 on wild-type and resistant cells, concentra-

TABLE 2

Cytostatic effect of free and liposome-formulated compounds

Comparison of cytostatic effect on wild and resistant (*R*) sublines of the following cell lines: lung carcinoma COR-L23, human colon adenocarcinoma LS 174T, rat hepatoma-human fibroblast hybrid WIF-B9, and mouse hepatoma Hepa 1-6. The proportion of living cells compared with non-treated plates (control conditions = 100% cell viability) was determined by the formazan test after the culture had been incubated for 72 h with one of the following compounds: cisplatin, GC, Bamet-R2, Bamet-UD2, and high density (HD) or low density (LD) liposome-encapsulated Bamet-R2 or Bamet-UD2. We used drug concentrations close to the value of the cisplatin IC_{50} for each wild-type cell. These were: 10 μM for COR-L23 and COR-L23/R, 30 μM for LS 174T and LS 174T/R, and 5 μM for WIF-B9, WIF-B9/R, Hepa 1-6, and Hepa 1-6/R. The cytostatic effect was calculated as the ability to reduce the number of living cells (100 − cell viability). Values are means ± S.E.M. from four different cultures carried out in triplicate.

Formulation	GC Free	Cisplatin Free	Bamet-R2			Bamet-UD2		
			Free	HD-Liposomes	LD-Liposomes	Free	HD-Liposomes	LD-Liposomes
COR-L23	1 ± 3[a]	52 ± 5	22 ± 7[a]	46 ± 8	61 ± 5[a]	36 ± 7[a]	68 ± 7[a]	79 ± 4[a]
COR-L23/R	1 ± 3[a]	25 ± 4[b]	18 ± 6	43 ± 7[a]	57 ± 4[a]	30 ± 5	65 ± 3[a]	76 ± 3[a]
LS174T	1 ± 3[a]	51 ± 5	43 ± 9	49 ± 5	56 ± 9	56 ± 7	62 ± 4[a]	67 ± 9[a]
LS174T/R	2 ± 3[a]	13 ± 3[b]	43 ± 7[a]	54 ± 7[a]	63 ± 5[a]	51 ± 8[a]	64 ± 9[a]	74 ± 6[a]
WIF-B9	1 ± 3[a]	49 ± 5	31 ± 6[a]	40 ± 7	53 ± 9	41 ± 8	54 ± 8	64 ± 4[a]
WIF-B9/R	0 ± 2[a]	4 ± 4[b]	34 ± 6[a]	37 ± 7[a]	56 ± 5[a]	45 ± 6[a]	52 ± 4[a]	68 ± 3[a]
Hepa 1-6	2 ± 2[a]	48 ± 5	43 ± 7	54 ± 7	61 ± 7[a]	68 ± 7[a]	77 ± 8[a]	87 ± 4[a]
Hepa 1-6/R	1 ± 3[a]	26 ± 3[b]	37 ± 4[a]	48 ± 6[a]	57 ± 5[a]	64 ± 5[a]	74 ± 4[a]	79 ± 5[a]

[a] $P < 0.05$ compared with cisplatin; [b] $P < 0.05$ when comparing resistant with wild-type cells.

TABLE 3

Drug uptake of free and liposome-formulated compounds

Comparison of drug uptake (in picomoles per 2 h per milligram of protein) by wild and resistant (*R*) sublines of the following cell lines: lung carcinoma COR-L23, human colon adenocarcinoma LS 174T, rat hepatoma-human fibroblast hybrid WIF-B9 and mouse hepatoma Hepa 1-6. Drug uptake was measured after incubating cells for 2 h at 37°C in the presence of 50 μM of one of the following compounds: cisplatin, GC, Bamet-R2, Bamet-UD2, and high density (HD) or low density (LD) liposome-encapsulated Bamet-R2 or Bamet-UD2. Values are means ± S.E.M. from four different cultures carried out in triplicate.

Formulation	GC Free	Cisplatin Free	Bamet-R2			Bamet-UD2		
			Free	HD-Liposomes	LD-Liposomes	Free	HD-Liposomes	LD-Liposomes
COR-L23	43 ± 15[a]	85 ± 7	235 ± 25[a]	1560 ± 106[a]	1890 ± 119[a]	328 ± 33[a]	1670 ± 145[a]	1950 ± 134[a]
COR-L23/R	45 ± 11[a]	61 ± 8[b]	247 ± 33[a]	1466 ± 133[a]	1640 ± 120[a]	333 ± 30[a]	1566 ± 141[a]	1750 ± 108[a]
LS 174T	380 ± 21[a]	175 ± 35	510 ± 32[a]	1332 ± 110[a]	1455 ± 122[a]	620 ± 41	1467 ± 139[a]	1789 ± 141[a]
LS 174T/R	420 ± 25[a]	44 ± 19[b]	490 ± 35[a]	1278 ± 128[a]	1377 ± 132[a]	580 ± 45[a]	1543 ± 125[a]	1800 ± 154[a]
WIF-B9	456 ± 28[a]	260 ± 23	955 ± 66[a]	1130 ± 99[a]	1340 ± 123[a]	1040 ± 75[a]	1255 ± 122[a]	1456 ± 118[a]
WIF-B9/R	401 ± 55[a]	65 ± 19[b]	904 ± 100[a]	1078 ± 117[a]	1244 ± 123[a]	955 ± 88[a]	1145 ± 109[a]	1345 ± 115[a]
Hepa 1-6	645 ± 57[a]	245 ± 24	1350 ± 119[a]	1485 ± 125[a]	1725 ± 110[a]	1400 ± 108[a]	1515 ± 114[a]	1855 ± 135[a]
Hepa 1-6/R	695 ± 45[a]	95 ± 20[b]	1215 ± 135[a]	1517 ± 178[a]	1687 ± 144[a]	1345 ± 145[a]	1466 ± 133[a]	1835 ± 188[a]

[a] $P < 0.05$ compared with cisplatin; [b] $P < 0.05$ when comparing resistant with wild-type cells.

tions near the value of the cisplatin IC_{50} for each wild-type cell were used (Table 2). These were: 10 μM for COR-L23 and COR-L23/R, 30 μM for LS 174T and LS 174T/R, and 5 μM for WIF-B9, WIF-B9/R, Hepa 1-6, and Hepa 1-6/R. At these concentrations, no toxic effect of GC was detected. However, a marked cytostatic effect of Bamets (Bamet-UD2 > Bamet-R2) on all cell types was observed. The cytostatic activity of these drugs was especially strong on LS 174T and Hepa 1-6 cells, in which they were equally or even more efficient than cisplatin. Inclusion in liposomes further increased the cytostatic activity of both Bamets. This was more marked in the case of LD than HD anionic liposomes. Similarly, liposomal formulation enhanced the amount of these drugs taken up by the cells (Table 3). This effect was weaker in cells able to take up free Bamets more efficiently (i.e., WIF-B9 and Hepa 1-6) than in LS 174T, in which uptake was lower, or COR-L23, in which it was even lower. The cell load of Bamets in all cell types was significantly higher than that of cisplatin and even than that of the natural bile acid GC. An important observation of the present work was that both Bamets overcame the resistance of all cell lines. In COR-L23/R, these compounds induced a cytostatic effect with an efficiency similar to that seen for cisplatin in wild-type cells, but only when they were encapsulated. By contrast, in all enterohepatic lines, free Bamets were already able to circumvent resistance, although their effect was stronger when they were loaded in liposomes.

In Vivo Antitumor Activity. To evaluate the in vivo antitumor effect of these drugs, we selected the cell line that showed the strongest resistance in in vitro studies (i.e., Hepa 1-6) and the most active and least toxic of both Bamets (i.e., Bamet-UD2). Although LD liposomes showed the highest efficiency in encapsulating Bamets as well as in reaching the highest drug uptake and in vitro cytostatic activity, owing to the low yield of this fraction, a mixture of HD and LD liposomes was used in these in vivo studies. This permitted multiplication of the yield by three, although it should be kept in mind that the efficiency of the pharmacological tool was to some extent underestimated by using this approach.

The survival of animals bearing a Hepa 1-6 tumor implanted in their livers was prolonged by free cisplatin. This was markedly longer when the animals received Bamet-UD2 (Fig. 5). The beneficial effect of this drug was further enhanced by encapsulation. This effect was more pronounced for cationic than for anionic liposomes (Fig. 5). The survival of untreated animals was similar whether they received an implanted Hepa 1-6 or a Hepa 1-6/R tumor. In animals implanted with the resistant variety of these tumors, the antitumor activity of cisplatin was almost absent. By contrast, the ability of free and liposomal Bamet-UD2 to prolong the life span of the animals persisted even though the implanted tumor was resistant to chemotherapy. At postmortem inspection, cisplatin was found mainly accumulated in the kidney, whereas Bamet-UD2 was accumulated in the normal liver tissue and in the tumor (Fig. 6). Liposomal formulation of Bamet-UD2 further reduced the amount of this drug in the kidney and in normal liver tissue but markedly enhanced accumulation within the tumor. This change in the distribution of drug between the tumor and the surrounding liver tissue was more pronounced when Bamet-UD2 was loaded in cationic liposomes.

TABLE 4

Expression levels of ABC proteins involved in multidrug resistance

Expression levels were measured in triplicate by two-steps real-time RT-PCR in RNA samples from four different cultures. Values found in wild and cisplatin-resistant cells were normalized using endogenous 18S rRNA. Values are presented as means ± S.D. from percentage of expression level found in the parent wild cell line.

	Expression Level (Percentage of Parent Wild Cell Line)		
Human	MRP1	MRP2	MDR1
COR-L23/R	753±64[a]	N.D.	2049 ± 176[a]
LS 174T/R	991±47[a]	1968 ± 134[a]	243 ± 11[a]
WIF-B9/R	2434±20[a]	990 ± 25[a]	N.D.
Rat	Mrp1	Mrp2	Mdr1
WIF-B9/R	10136±1481[a]	123 ± 10	N.D.
Mouse	Mrp1	Mrp2	Mdr1
Hepa 1-6/R	10183 ± 65[a]	1017 ± 73[a]	N.D.

N.D., not detected.
[a] $P < 0.05$ when comparing values in wild and resistant cells by Student's t-test.

Discussion

To carry out the present study, we have obtained several novel clonal cell sublines derived from enterohepatic tumors of three different species (human, rat, and mouse) that developed cisplatin resistance by culturing them in the presence of this drug. The expression levels of several proteins involved in the mechanisms responsible for drug resistance (i.e., p53, GST-P, MDR1, and two members of the MRP family with GS-X pump activity, MRP1 and MRP2) were investigated to better understand our results from the analysis of cross-sensitivity between cisplatin and Bamets.

Conjugation with glutathione is a major detoxification route for cisplatin (Litterst et al., 1982). However, neither

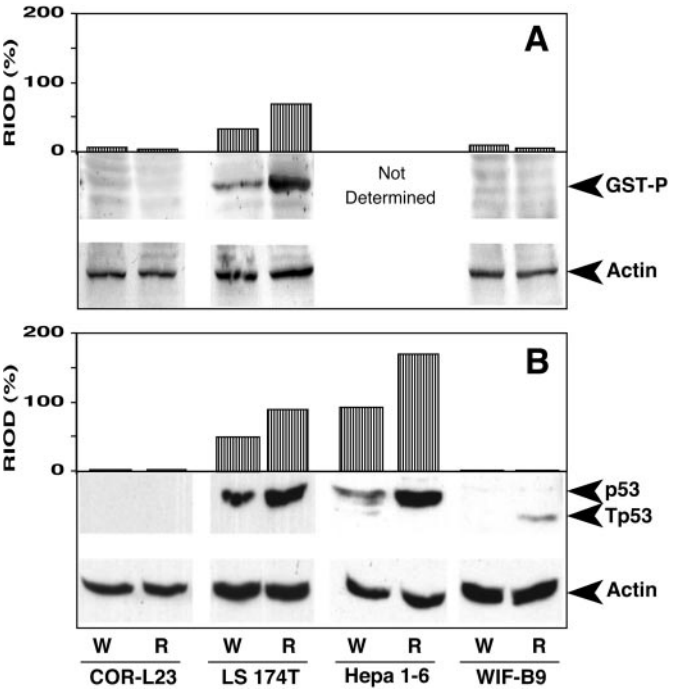

Fig. 4. Western blot and densitometric analysis of GST-P (A) and p53 (B) and wild-type (W) and resistant (R) cells. Proteins (30 μg) extracted from these cells were electrophoresed in 12% acrylamide gel and transferred to polyvinylidene fluoride membranes. mAb 14.1.3, a monoclonal antibody against human and rat GST-P; p53, a specific DO-1 monoclonal antibody able to detect human p53, and the Ab7 polyclonal antibody able to detect human, mouse, or rat p53 were used. Western blot for actin was performed to compare protein loads. Results are expressed as relative integrated optic density for the relationship between the intensity of the target protein band and that of actin (100%) in the same sample. Tp53 is presumably the truncated form of p53 in WIF-B9/R cells.

Bamet-R2 (Macias et al., 1998) nor Bamet-UD2 (Larena et al., 2001) is efficiently eliminated by the liver with no major biotransformation, despite the very high conjugating activity of hepatocytes. This is consistent with the present results showing that overexpression of GST-P accompanies the development of cisplatin-resistance in LS 174T/R cells, whereas there was no change in the sensitivity of these cells to Bamets.

Alteration in the p53 status correlates with the development of resistance to platinum-based chemotherapy in several human tumors (Litterst et al., 1982). Our results do not support a role for this pathway in the transition from COR-L23 to COR-L23/R or that from WIF-B9 to WIF-B9/R. Moreover, the induction of resistance did not reduce the amount of this protein clearly detected in both LS 174T and Hepa 1-6 cells but instead enhanced it. In nontumor cells, in which the nonactive form of the wild-type tumor suppressor protein p53 is expressed at very low levels, DNA damage caused by radiation or drugs, such as cisplatin, induces post-transcriptional modifications, mainly phosphorylation and acetylation, that increase p53 stability and trigger its activation (Palacios et al., 2000; Persons et al., 2000). Normally, therefore, wild-type p53 is rarely detectable by Western blot in many cell types, although its expression becomes detectable when it is stabilized in response to DNA damage or, alternatively, when a mutant variant of p53 is present (Righetti et al., 1996). One alternative to explain the high levels of p53 in resistant variants of LS 174T and Hepa 1-6 cells deals with the possibility that cisplatin induces mutations in the p53 protein that affect its normal structure and renders a modified protein that is easy to detect but has an impaired apoptotic function (Solary et al., 2001; Bargonetti and Manfredi, 2002). Moreover, a loss of function in proteins involved in the p53 degradative pathway may also account for the accumulation of immunoreactive p53 protein found in LS 174T/R and Hepa 1-6/R cells. Our results are in agreement with previous findings by other authors, who reported that, in several cell lines, resistance to cisplatin correlates with prolonged p53 protein stabilization and accumulation (Yazlovitskaya et al., 2001). It is noteworthy that a loss of wild-type p53 function in tumor cells could contribute to an up-regulation of the MDR1 (Thottassery et al., 1997) and MRP genes (Wang and Beck, 1998).

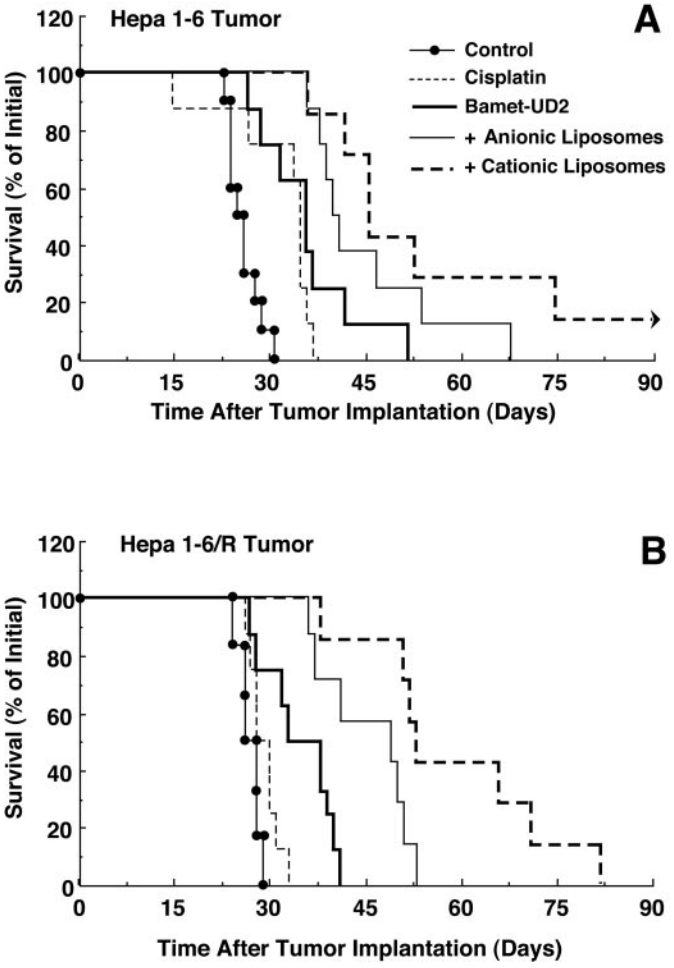

Fig. 5. Kaplan-Meier curves for the survival of nude mice after orthotopic implantation of a fragment of approximately 1 mm^3 of mouse hepatoma tumor previously grown from Hepa 1-6 (A) or Hepa 1-6/R (B) cells subcutaneously implanted into different mice. Treatment (15 nmol/g of body weight of cisplatin or Bamet-UD2—either free or encapsulated within anionic or cationic liposomes—administered i.p. twice a week) started the day after implantation of the tumor into the liver. The control group received only the vehicle (i.e., saline solution). Treatment was maintained throughout the life of the tumor-bearing mice. Values are expressed as percentages of the initial numbers of mice in each group (6–10).

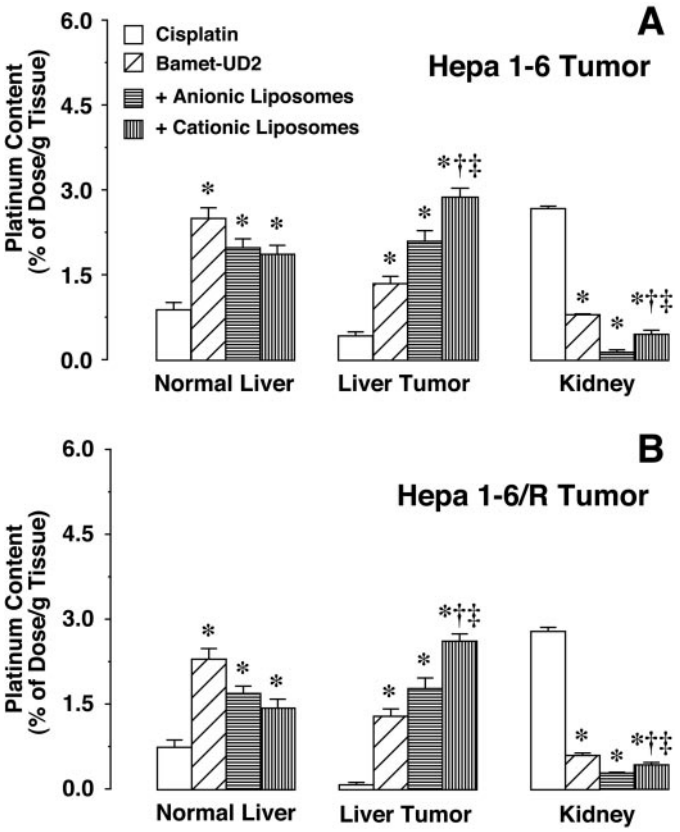

Fig. 6. Platinum content in normal liver tissue, tumor tissue, and kidney. Samples were collected as soon as possible on the day Nude mice died after undergoing orthotopic implantation of a fragment of approximately 1 mm^3 of mouse hepatoma tumor previously grown from Hepa 1-6 (A) or Hepa 1-6/R (B) cells subcutaneously implanted into different mice. Treatment (15 nmol/g of body weight of cisplatin or Bamet-UD2—either free or encapsulated within anionic or cationic liposomes—administered i.p. twice a week) started the day after implantation of the tumor into the liver. Values are means ± S.E.M. from between six and eight mice in each experimental group. *, $p < 0.05$ compared with cisplatin in the same tissue; †, $p < 0.05$ on comparing cationic with neutral liposomes; ‡, $p < 0.05$ on comparing the effect of cationic liposomes among tissues.

MDR1 seems to play an important role in drug resistance in COR-L23/R cells, in which its expression level is increased 20-fold compared with wild-type cells. However, this protein seems less relevant in resistance induced in sublines of enterohepatic origin. Thus, expression levels increased 2.5-fold in LS 174T/R cells, whereas no protein was detectable in WIF-B9 or Hepa 1-6 or in their resistant counterparts.

By contrast, the expression levels of MRP1 and MRP2 were increased between 10- and 100-fold in resistant sublines of enterohepatic origin. A lower enhancement in MRP1 was also observed in COR-L23/R cells, whereas the expression of MRP2 was not detectable in these cells.

Owing to the complexity involved in defining the exact contribution of several transporters to drug resistance in different cancer cell types, some controversy exists in the numerous studies that have been undertaken to elucidate the role of ABC proteins in extruding cisplatin and its conjugates from cancer cells. In general, it seems that cisplatin resistance is associated with elevated expression of MRP2 but not of MRP1 or MRP3 (Taniguchi et al., 1996; Cui et al., 1999). Our results suggest that whatever the export pumps involved, mechanisms responsible for reductions in cisplatin accumulation in resistant cells are not able to efficiently extrude Bamets. Moreover, it should be taken into account that drug load is the overall result of the balance between uptake and efflux mechanisms. In previous studies (Briz et al., 2002), the ability of several carriers of organic anions and cations as well as of neutral compounds located in the membrane of enterohepatic cells to transport Bamets was shown. Additionally, the possible ability of other families of carriers to transport these compounds cannot be ruled out. The question arises as to whether tumor cells are able to carry out this uptake. The probability of this being so is high, because in aqueous media, there is an equilibrium of Bamet forms with different charges; hence, if one or several carriers belonging to the families SLC10A (sodium-taurocholate cotransporter polypeptides), SLC21A (organic anion transporter polypeptides), or SLC22A (some organic cation transporters) are expressed in the target cells, they may mediate the efficient uptake of Bamet-R2 and Bamet-UD2. Thus, in all cell types used in the present study, Bamet uptake was higher than that of cisplatin, although these compounds were taken up more efficiently by cell lines of enterohepatic origin and, in particular, by lines derived from liver tumors. Moreover, despite induction of resistance to cisplatin, which was associated with a reduction in the accumulation of the drug, no changes in the cell load of Bamets occurred.

In addition to the enhanced ability to reach tumor cells and be retained even if they are resistant to the parent drug cisplatin, the presence of the amphiphilic bile acid moieties in the molecule permits efficient load in liposomes, which further enhances the ability of these compounds to be taken up by tumor cells. The use of macromolecular drug delivery systems, such as liposomes (Warren et al., 1992), has a potential to overcome multidrug resistance (for review, see Kopecek et al., 2000). The fact that liposomal Bamet had stronger cytostatic effect than the free drug also in resistant sublines—not far stronger, except for nonenterohepatic COR-L23/R cells, in which uptake of free drug was lower—can be explained by the rational on which this strategy is based (i.e., the exclusion of the drug-loaded particle from the cytoplasm of the tumor cells should render the efflux pumps ineffective). An additional beneficial effect of liposomal Bamet could be expected if Bamet molecules that are released from the particle within tumor cells are not good substrates for the exporting proteins over-expressed in resistant sublines. In agreement with other authors, the present study indicates that to target Bamets, the most efficient liposomes are those with low-density, presumably small oligolamellar particles (Goren et al., 1990) and cationic components (Blau et al., 2000). These characteristics enhance the ability of liposomes to reach the tumor, to be taken up by endocytosis, and to release the loaded drug that is accumulated within cancer cells. This preferential distribution, together with the vectorial properties of free molecules of Bamet-UD2 released from the liposomes, as well as the potential beneficial effect of the UDCA (Trauner and Graziadei, 1999) moiety that leaves the Bamet-UD2 molecule during its activation in aqueous solution (Criado et al., 2000), might account for the selective targeting to liver-implanted tumor, for the low amount that was taken up by the kidney and for the absence of Bamet-UD2-induced toxicity (Dominguez et al., 2001). All these characteristics explain why mice treated with this drug loaded in cationic liposomes survived much longer than those treated with cisplatin, even though the tumor implanted in the liver was cisplatin-resistant. This suggests that liposomal Bamet-UD2 could be a useful pharmacological tool to circumvent resistance to chemotherapy, particularly in tumors of the enterohepatic circuit.

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