Evidence for dual effects of DNA-reactive bile acid derivatives (Bamets) on hepatitis B virus life cycle in an *in vitro* replicative system

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A liver targeting strategy to direct antiviral drugs toward hepatitis B virus (HBV) was investigated. As model drugs we used cisplatin-bile acid derivatives (Bamets) to determine the production of virions by HBV-transfected hepatoblastoma cells (HepG2 2.2.15). Drug uptake was determined using flameless atomic absorption spectrometry to measure platinum cell contents. Cytotoxic effect was determined by formazan formation and neutral red uptake tests. The release of viral surface protein was evaluated by ELISA. The abundance of HBV-DNA in the medium was determined by quantitative real-time PCR and its structure by Southern blot analysis. The uptake of Bamets by HepG2 2.2.15 cells was higher than that of cisplatin. At concentrations lower than 10 μM, distinct Bamets have no toxic effect on host cells, whereas cisplatin dramatically reduced cell viability at concentrations higher than 1 μM. All the drugs tested inhibited the release of viral proteins to the medium, but induced a marked and progressive dose-dependent increase in the amount of viral DNA in the medium. This was mainly due to the release of short fragments of HBV-DNA in the case of cisplatin. On the contrary, Bamets induced an enhanced release of circular forms of HBV-DNA. These findings suggest the existence of a dual effect of Bamets on HBV life-cycle by enhancing the production of DNA replicative intermediates but reducing the secretion of complete virions. Altogether these characteristics recommend consideration of these compounds as a useful experimental tool in the investigation of novel liver targeted therapeutic agents based on bile acid derivatives for the treatment of HBV infections, or to carry out further studies on the HBV life cycle.

Keywords: cisplatin, drug, liver, targeting, transport, ursodeoxycholic acid

Introduction

The *Hepadnaviridae* form a highly species-specific family of viruses that share some similarities regarding structure and replication characteristics, such as a circular and partially single-stranded DNA held together in a circular structure due to hydrogen bonding of a cohesive overlapping 5′ termini of variable size (Molnar-Kimber *et al.* 1984) and the existence of an endogenous DNA polymerase with reverse transcriptase activity (Fang *et al.* 1981; Lien *et al.* 1987; Marion *et al.* 1987; Murray *et al.* 1991; Wang & Seeger 1993).

Human hepatitis B virus (HBV), which belongs to the genus *Orthohepadnavirus*, is one of the members of this family. Although this hepatotropic virus is not cytopathic itself, the host immune response to viral infection can elicit a broad spectrum of clinical manifestations that may lead to chronic liver disease, cirrhosis and, eventually, hepatocellular carcinoma (Iino 2002). Alternatively, it may cause acute fulminant hepatitis (Bartholomeusz & Locarnini 2001; Kalinina *et al.* 2001). Although vaccination against this virus has been available for the past decades, HBV infection is still an important worldwide health problem. Pharmacological strategies currently being used to treat affected patients based on immunomodulatory agents, such as interferon (Hoofnagle *et al.* 1988) and on nucleoside derivatives, such as lamivudine (Dienstag *et al.* 1995), are only partially effective.

The HBV has the smallest genome (3.2 kb in the minus strand of invariable length whereas the plus strand is short-

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er and of variable length) of any virus known to infect man. Once HBV has infected the hepatocyte, its life cycle starts by translocation of HBV DNA into the nucleus of the host cell, where the plus strand is extended to form a covalently closed circular DNA (cccDNA), which adopts a supercoiled structure (Tuttleman *et al*. 1986). The minus strand of cccDNA is transcribed to RNA by the RNA polymerase of the host cell. This RNA plays a dual role as pregenome and template for viral protein synthesis. Using the pregenome as a template the viral polymerase is able to carry out reverse transcription in order to generate the minus strand of HBV DNA. Normally, the plus strand is synthesized later and completed after the formation of the nucleocapsid. The HBV has a compact and efficiently organized genome to encode more than six proteins and perform the complex steps of its life cycle (Miller *et al*. 1989). Moreover, HBV replication is cell cycle-dependent; that is, the production of viral particles is enhanced in quiescent cells. This is an important characteristic with implications in clinical practice and when investigating the biology of HBV or assessing antiviral agents.

Bile acids have been suggested as useful moieties to liver organotropic drugs (Marin *et al*. 2001). The basis of this pharmacological strategy is the existence of carrier proteins in the plasma membrane of cells derived from tissues of the entero-hepatic circuit that efficiently take up bile acids (Meier & Stieger 2002). Although cisplatin is not a useful drug to treat HBV infection, in the present study as model compounds to evaluate this pharmacological approach we have used three platinum-containing drugs: Bamet-R2, Bamet-D3 and Bamet-UD2; because they are easily detectable inside the host cells, liver organotropic and DNA-reactive, and in contrast to cisplatin, some of them, have very low toxicity. These compounds contain bile acid moieties that presumably determine their liver organotropism. These were glycocholic acid in Bamet-R2 (Criado *et al*. 1997), glycocholic acid modified by a 7-carbon spacer bound to the side chain in Bamet-D3 (Martinez-Diez *et al*. 2000), and ursodeoxycholic acid in Bamet-UD2 (Criado *et al*. 2000). In all cases, the DNA-reactive element was platinum(II). In previous studies we have shown that these compounds are recognized as substrates by several plasma membrane liver carriers, which determines their efficient uptake (Briz *et al*. 2002). This probably accounts for the enhanced vectoriality toward liver tumours, efficient elimination from the body by the liver and hence low toxic side effects (Dominguez *et al*. 2001). Moreover, these compounds are able to bind DNA and block its replication and hence cell proliferation (Marin *et al*. 1998), accounting for their strong antitumour activity (Dominguez *et al*. 2001). Given the excellent pharmacodynamic profile of Bamets and in order to investigate the potential usefulness of bile acid derivatives to deliver antiviral drugs directly to HBV-infected hepatocytes the present study was undertaken.

Materials and methods

Chemicals

Dulbecco's modified eagle's medium (DMEM), gentamicin, 3-amino-7-dimethylamino-2-methylphenazine (neutral red, NR), $NaHCO_3$, L-glutamine, polyethylene glycol (Molecular weight ≈8000) and dimethylsulfoxide (DMSO) were provided for Sigma-Aldrich Quimica (Madrid, Spain). Dodecyl sulfate sodium salt (SDS) was from Merck (Barcelona, Spain). Ciprofloxacine (Baycip®) was supplied by Bayer (Leverkusen, Germany). 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), proteinase K (PCR grade) and geneticin® (G418) were from Roche (Barcelona, Spain). Fetal calf serum (FCS) was obtained from Gibco BRL (Barcelona, Spain). [^{3}H]-Inuline (304 mCi/g) was purchased from PerkinElmer Life Science (Pacisa&Giralt, Madrid, Spain).

Cell culture

HepG2 2.2.15 is a stable cell line derived from hepatoblastoma HepG2 cells containing the hepatitis B virus genome (Sells *et al*. 1987). This cell line is a common *in vitro* model of HBV infection used by many investigators in their search for anti-HBV drugs (Schalm *et al*. 1995) because compounds blocking any late step of viral replication such as transcription, translation, pregenome encapsidation, reverse transcription, particle assembly and release can be examined using HepG2 2.2.15 cells. Under normal culture conditions, there is a correlation between hepatitis B surface antigen (HBsAg) in the supernatant and the production rate of mature virions (Aden *et al*. 1979), which is higher in non-dividing cells (Copeland *et al*. 1980). Thus, to reduce potential indirect artefacts due to Bamet-induced inhibition of cell proliferation that might modify the rate of virion production and counterbalance the potential antiviral effect of Bamets, these cells were used once confluence had been reached. They were seeded in Roux Flasks with DMEM culture medium supplemented with 10% FCS, 4 mM L-glutamine, 26.2 mM $NaHCO_3$, 25 mM HEPES, 20 mg/ml gentamicin and 200 mg/100ml of ciprofloxacine. After the first passage, ciprofloxacine was replaced by 150 mg/ml G418. Cells were used at the third passage when 1.5×10^5 (uptake studies) or 3.5×10^5 (rest of the studies) cells were seeded on plastic dishes (3.5 cm diameter), and maintained for two (uptake studies) or three (rest of the studies) days before any treatment, to reach approximate sub-confluence or confluence conditions, respectively. The culture medium was replaced by a fresh one every 3 days. When drugs were added, they were first dissolved in DMSO and then diluted with culture medium to reach

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final DMSO concentrations lower than 0.2% (vol/vol). The solution was filtered (Sterivex 0.2 μm pore diameter, Millipore Iberica, Madrid, Spain) and the contents in platinum were determined to adjust to the desired final drug concentration with culture medium. In dose–response studies, serial dilutions from the solution with the highest drug concentration were prepared.

Drug uptake measurement

To carry out measurements of drug uptake, cells were preincubated with drug-free uptake medium (116 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose and 10 mM HEPES). After equilibration at 37°C for 1 h, this medium was replaced by a fresh one containing 50 μM cisplatin, Bamet-R2, Bamet-D3 or Bamet-UD2 and incubated at 4°C or 37°C for 5, 15, 30, 45, 60, 90 or 120 min. To evaluate the contribution of drug dissolved in the uptake medium that contaminated the harvested cells, extracellular water was measured in some dishes from each experiment by adding [^{3}H]-inuline in ice-cold uptake medium to the cells 4 min before the harvesting procedure. At the indicated times, cells were washed four times with ice-cold uptake medium and digested with 0.7% SDS for 2 h to measure radioactivity, proteins and platinum concentrations. The concentrations of cisplatin and Bamets were determined by flameless atomic absorption spectroscopy, as previously described (Macias *et al*. 1998). Radioactivity was measured in a liquid scintillation counter (LS-6500; Beckman, Madrid, Spain). Bovine serum albumin was used as standard to determine protein concentrations (Markwell *et al*. 1987).

Evaluation of drug toxicity in host cells

Drug-induced cell toxicity was evaluated by measuring the number of living cells relative to these in untreated control cultures at day 21 of drug exposure using two different tests: (1) the lysosomes and Golgi apparatus ability to take up Neutral Red (NR) (Fautz *et al*. 1991) was used extensively; and (2) the formazan formation mainly by mitochondria (Promega, Madison, Wisc., USA) was used only at high concentration-points as a confirmation method (data not shown). Because very similar results were obtained with both methods only one of them (NR uptake) is used here to express the results of cell viability. To carry out this test, after removal of the cell supernatant and washing the cells twice with sterile PBS at 37°C, 1 ml of 50 μg/ml NR in PBS was added. The cultures were incubated at 37°C in an atmosphere of 5% CO_2:95% air for 90 min to allow living cells to take up NR. After the cells had been washed again twice, NR was eluted with an aqueous solution containing 50% ethanol, 1% acetic acid for 10 min at room temperature and the amount determined by measurement of absorbance at 540 nm.

Analysis of HBsAg and HBV DNA release into the culture medium

Solid-phase ELISA, using 'ORTHO Antibody to HBsAg ELISA Test System 3' (Ortho-Clinical Diagnostics, Madrid, Spain) was used to quantify the amounts of the HBV envelope protein, surface antigen (HBsAg) released from cultures. Results were normalized by using those found in untreated cells of the same culture as 100%.

To obtain a probe for HBV DNA, HepG2 2.2.15 cells were digested using proteinase K (Kock *et al*. 1996). DNA was extracted using the phenol/chloroform-ethanol procedure and then was subjected to conventional PCR using Taq DNA polymerase (Roche) according to the conditions: 1 cycle at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min followed by annealing at 52°C for 1 min and polymerization at 75°C for 90 s, and 1 cycle of extension at 75°C for 5 min. Primers were designed with 'Primer Express' software (Perkin-Elmer Life Science). They prime a fragment of 904 bp of the HBV DNA included in the region encoding the final (3′) portion of the nucleotide sequence for protein X, complete for pre-Core, and initial (5′) for Core protein. The sequences of the primer oligonucleotides were as follows: Forward primer 5′-CCA CGG GGC GCA CCT CTC TTT A-3′; reverse primer 5′-CTG CGA CGC GGC GAT TGA GAC-3′. The result, a unique band of the expected size, was separated by 1% agarose gel electrophoresis. This was excised from the gel to extract the DNA fragment using the QIAquick Gel Extraction Kit (Qiagen, Izasa, Barcelona, Spain), and its nucleotide sequence was determined (Servicio de Secuenciacion, Instituto de Microbiologia y Bioquimica/CSIC, University of Salamanca). The results revealed, in the central region of 435 bp where no uncertainties were found, 100% similarity with HBV subtype ayw DNA (accession number V01460). BLAST analysis of both primers against this accession number also resulted in 100% matches. This information was used to design the following internal primers for this region: forward primer: 5′-TTG CCT TCT GAC TTC TTT CCT TCT-3′; reverse: 5′-TGC CTG AGT GCT GTA TGG TGA G-3′) and the fluorogenic TaqMan® probe (5′-TCG GGA AGC CTT AGA GTC TCC TGA-3′) labelled with FAM (6-carboxyfluoresceine) in 5′ and with TAMRA (6-carboxytetramethylrhodamine) in 3′. These were used to carry out quantitative real-time PCR with AmpliTaq Gold DNA polymerase (Perkin-Elmer Life Science). The conditions for this reaction were as follows: 1 cycle, hot-start at 95°C for 10 min, 50 cycles, denaturation at 95°C for 15 s, annealing/ polymerization at 59°C for 1 min. The DNA concentration in a solution of purified 904-bp probe was measured by the PicoGreen method (Molecular Probes, Leiden, The Netherlands). This was used to build up the

standard curves needed to calculate the amount of DNA copies in each sample. To carry out these determinations, a supernatant of HepG2 2.2.15 cells was collected from the culture; HBV DNA was extracted using an adaptation of the alkaline digestion method (Kaneko *et al*. 1989), and the solution was neutralized with 0.12 N HCl and diluted as appropriate before being used as template.

To carry out Southern blot analysis, viral DNA from the cell supernatant was isolated by polyethylene glycol precipitation, purified by treatment with proteinase K, and deproteinized by extraction with phenol/chloroform, as reported by Acs *et al*. (Acs *et al*. 1987). Aliquots of DNA corresponding to different experimental conditions were subjected to 1.5% agarose gel electrophoresis and subsequently transferred to a positively charged Nylon membrane (BiodineB plus, Pall Gelman, Madrid, Spain). Hybridization with the ^{32}P-dCTP random-labelled 904-pb HBV DNA probe was performed for 1 h at 60°C, using Express Hybridization solution from Clontech (BD, Madrid, Spain) and following the instructions of the manufacturer. The blot was washed under high stringency conditions and then exposed to a BioMax Kodak film (Sigma Aldrich) for 12 h.

Statistical analysis

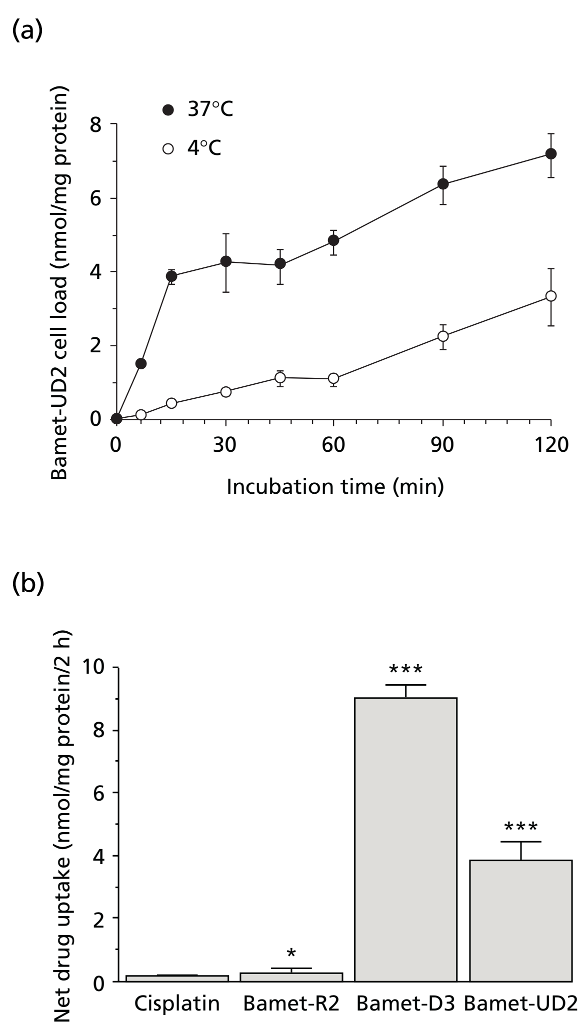
Data points were obtained in triplicate in at least three different cultures. Values are given as means ±SD. To calculate the statistical significance of differences within or among groups, the paired *t*-test or the Bonferroni method of multiple-range testing were used, as appropriate.

Results

Uptake of Bamets by HepG2 2.2.15 cells

The uptake of Bamet-UD2 (Figure 1a) as well as that of Bamet-D3, and to a lesser extent that of Bamet-R2, but not that of cisplatin (data not shown), was carried out by HepG2 2.2.15 cells in a temperature-dependent manner. When net uptake, as defined as the difference between the amount of drug taken up at 37°C and that retained at 4°C, was calculated (Figure 1b), a moderate advantage of Bamet-R2 uptake over that of cisplatin was observed, while the uptake of Bamet-UD2 and Bamet-D3 was strikingly higher than that of cisplatin.

Figure 1. Time-course of Bamet-UD2 uptake and net uptake of cisplatin, Bamet-UD2, Bamet-R2 and Bamet-D3



(a) Time-course of Bamet-UD2 uptake by HepG2 2.2.15 cells incubated with uptake medium (116 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose and 10 mM HEPES) containing 50 μM Bamet-UD2 at 4°C or 37°C for the indicated times. Cells were then washed with ice-cold uptake medium and harvested to measure Bamet-UD2 by flameless atomic absorption spectrometry. (b) Net uptake of cisplatin, Bamet-UD2, Bamet-R2 and Bamet-D3 was calculated by subtracting uptake at 4°C to that at 37°C after incubation with uptake medium containing 50 μM of one of these drugs for 120 min. Values are means ±SD from three different cultures in which each data point was the mean of three different dishes.
*$P<0.05$; **$P<0.01$; ***$P<0.001$, as compared with cisplatin.

Effect on viral protein release from HepG2 2.2.15 cells

In principle we intended to determine the amount of viral particles secreted by HepG2 2.2.15 cells by measuring the abundance of HBsAg in the culture medium every three days. Under control culture conditions this approach very probably represents an overestimation of the number of complete viral particles because not all immunoreactive proteins against the antibody used to carry out ELISA are expected to be in the supernatant of cell cultures as part of complete virions, or 'Dane particles'. Some of these proteins may be free or may form part of one of the other two types of viral particles: spherical and filamentous – both genome-free – particles (Pugh & Bassendine 1990). Furthermore, as will be described below, changes in the amount of the other component of complete viral particles,

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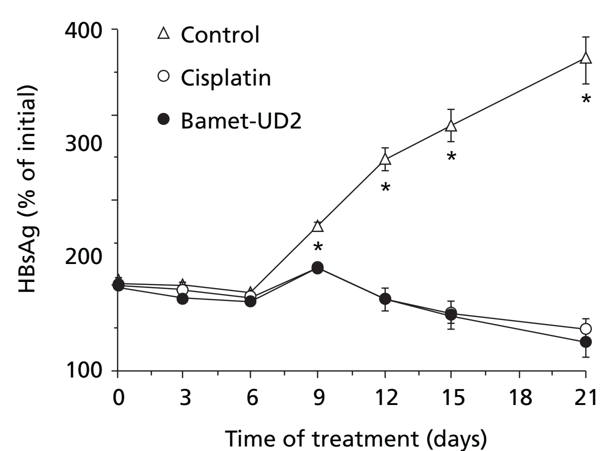
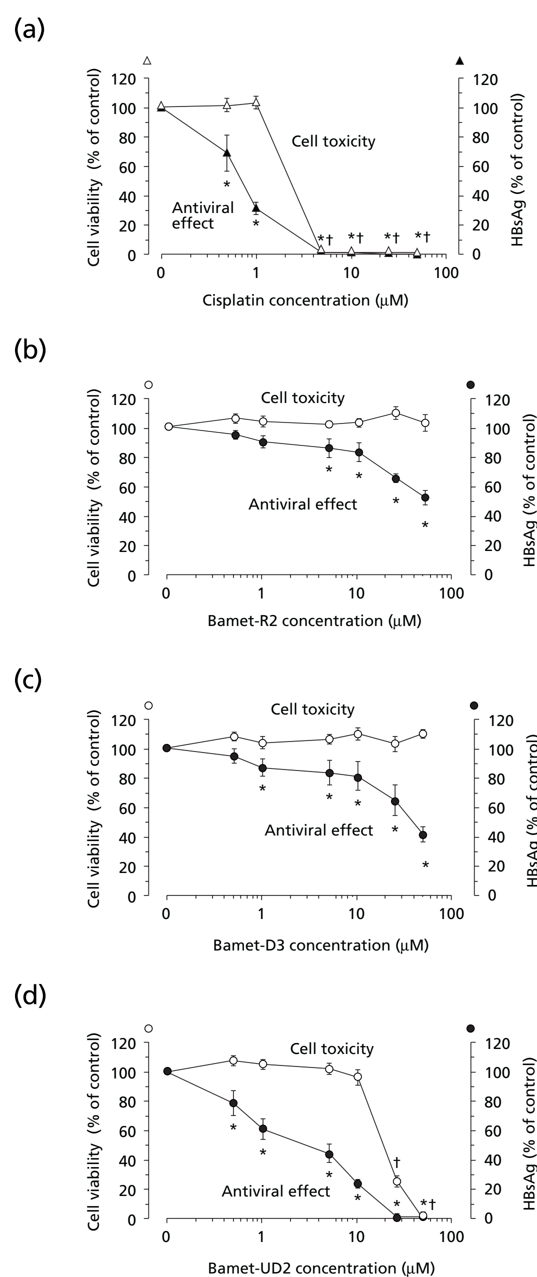
Figure 2. Time-course of HBsAg released by HepG2 2.2.15 cells into the culture medium



Cells were cultured for the indicated days with culture medium alone (Control, open triangles) or containing 1.0 μM cisplatin (open circles) or 10 μM Bamet-UD2 (closed circles). Every three days, the culture medium was replaced by similar fresh one containing the same amount of drug. The abundance of HBsAg in the collected medium was determined by ELISA. Values, expressed as percentages of initial values, are means ±SD from three different cultures in which each data point was the mean value from three different dishes.
**P*<0.05 on comparing Control with two other conditions.

Figure 3. Effect on cell viability and release of HBsAg particles by HepG2 2.2.15 cells cultured for 21 days with the indicated concentration (ranging from 0.005 to 50 μM) of cisplatin, Bamet-R2, Bamet-D3 or Bamet-UD2

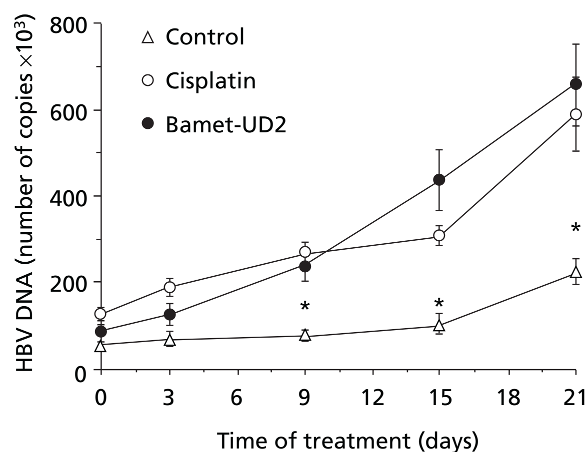


Every three days, the culture medium was replaced by a fresh one containing the same amount of drug. On day 21, cell viability was measured by the Neutral Red retention test and the amount of HBV particles in the culture medium was assessed by determining HBsAg abundance using ELISA. Values, expressed as percentages of determinations carried out on dishes in which no drug had been added (Control), are means ±SD from three different cultures in which each data point was the mean value from three different dishes.
**P*<0.05 on comparing abundance of HBsAg in Control; †*P*<0.05 on comparing cell viability with Control.

that is, DNA, revealed a marked discrepancy between abundance of HBsAg and viral DNA release after exposure to compounds assayed in the present study. This recommends expressing the results from the HBsAg analysis as viral protein release rather than as viral particles release. Under control conditions, and after a lag-time of approximately 6 days, the amount of HBsAg increased progressively throughout the culture period (Figure 2). This was significantly inhibited by cisplatin and Bamet-UD2 (Figure 2), as well as by Bamet-R2 and Bamet-D3 (data not shown).

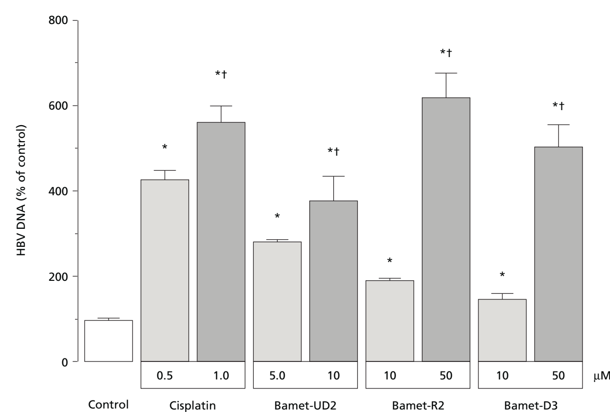
This effect was dose-dependent with dissimilar activity for each compound, as will be described below, in comparison with the effect on cell viability. Thus, cisplatin induced a very powerful inhibition of HBsAg production, but also displayed a marked reduction in the number of cells in the culture (Figure 3a). By contrast, both Bamet-R2 (Figure 3b) and Bamet-D3 (Figure 3c) had a moderate anti-HBV effect but did not affect cell viability, at least in the concentration range used in the present study. Bamet-UD2 showed intermediate behaviour. This compound induced a marked inhibition in the amount of HBsAg released into the medium and reduced cell survival only at concentrations higher than 10 μM (Figure 3d). Thus, if anti-HBV activity is defined as the ratio between the concentrations at which cell survival was not challenged but the inhibition

Figure 4. Time-course of HBV DNA release by HepG2 2.2.15 cells into the culture medium



Cells were cultured for the indicated days with culture medium alone (Control, open triangles) or containing 1.0 μM cisplatin (open circles) or 10 μM Bamet-UD2 (closed circles). Every three days, the culture medium was replaced by similar fresh one containing the same amount of drug. The abundance of HBV DNA in the culture medium was determined by quantitative real-time PCR. Values, expressed as means ±SD from three different cultures in which each data point was the mean value from three different dishes. *P<0.05 on comparing Control with two other conditions.

Figure 5. Effect of cisplatin, Bamet-R2, Bamet-D3 or Bamet-UD2 on HBV DNA release by HepG2 2.2.15 cells into the culture medium



Cells were cultured for 21 days with the indicated concentrations of one of these drugs. Every three days, the culture medium was replaced by a fresh one containing the same amount of drug. On day 21, the abundance of HBV DNA in the culture medium was determined by quantitative real-time PCR. Values, expressed as percentages of determinations carried out in dishes to which no drug had been added (Control), are means ±SD from three different cultures in which each data point was the mean value from three different dishes.
*P<0.05 as compared with Control; †P<0.05 as compared with a lower dose of the same compound.

of HBsAg production was maximal, divided by the magnitude of this inhibition, the results indicate that the order of the tested compounds regarding this parameter is: cisplatin>Bamet-UD2>Bamet-D3>Bamet-R2.

Effect of Bamets on HBV DNA release from HepG2 2.2.15 cells

Determination of the time-course of the release of viral DNA into the medium revealed that in the control group a progressive increase occurred after an initial lag-time (Figure 4). This, together with the results on HBsAg release commented above (Figure 2), is consistent with the known ability of these cells to produce and release complete HBV virions (Sells *et al*. 1987). However, in spite of a progressive decrease in the amount of viral protein released (Figure 2), when these cells were exposed to cisplatin or Bamet-UD2 the amount of HBV DNA in the culture medium increased progressively and dramatically (Figure 4). This ability to stimulate the release of HBV DNA was also shared by Bamet-R2 and Bamet-D3 (Figure 5). For all four drugs the magnitude of this release was dose-dependent. Thus, when a lower drug concentration of each compound, still able to inhibit HBsAg release but with no effect on cell viability, was used the amount of HBV DNA released into the culture medium was reduced, although it was still higher than the amount found in the control group (Figure 5).

Southern blot analysis (Figure 6, right panel, lanes 1 and 2) indicated that the majority of HBV DNA present in the medium of HepG2 2.2.15 cells was in the form of nicked relaxed circular forms (rcDNA) and single-stranded DNA (ssDNA). However, other forms, probably released from detached dead cells, could be seen. These were typically intranuclear circular covalently closed DNA (cccDNA), high molecular weight forms corresponding to integrated HBV DNA (iDNA) and forms of low molecular weight probably corresponding to incomplete copies of the genome of different sizes and/or fragmented nucleosomes containing part of the HBV DNA genome (fDNA). Indeed, a considerable amount of DNA of low molecular weight was present in the supernatant of control HepG2 2.2.15 cells probably due to apoptotic internucleosomal fragmentation (Figure 6, left panel, lanes 1 and 2). Only part of it was recognized by the HBV probe (Figure 6, right panel, lanes 1 and 2). These fragments were also observed in cells treated with cisplatin (Figure 6, left panel, lanes 3 and 4). In this case, hybridization with the HBV DNA probe was more marked (Figure 6, right panel, lanes 3 and 4).

The proportion of small size fragments was much lower in cells treated with Bamet-UD2 (Figure 6, left panel, lanes 5 and 6), where hybridization with the HBV DNA probe was very weak (Figure 6, right panel, lanes 5 and 6). The proportion of high size HBV DNA forms also changed in

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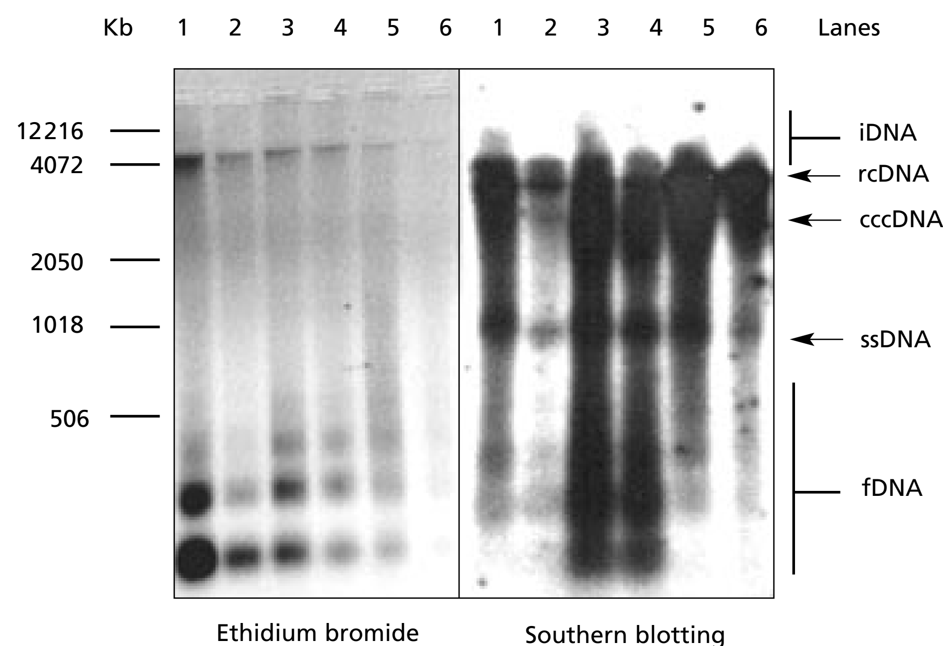
Figure 6. Southern blot analysis of HBV DNA released by HepG2 2.2.15 cells into the culture medium



Cells were cultured for 21 days with the indicated concentrations of one of the following drugs: cisplatin, Bamet-R2, Bamet-D3 or Bamet-UD2. Every three days, the culture medium was replaced by a fresh one containing the same amount of drug. On day 21, HBV DNA was purified from the collected medium, analysed by 1.5% agarose gel electrophoresis, stained with ethidium bromide (left panel), transferred to a nitrocellulose membrane, and hybridized with a probe specific for HBV DNA subtype ayw (right panel). Lanes 1 and 2 correspond to Control (no drug added); lanes 3 and 4 to 1 μM cisplatin, and lanes 5 and 6 to 10 μM Bamet-UD2. Of the 50 μl obtained in the DNA purification procedure of each sample, 40 μl was loaded in lanes 1, 3 or 5 and the remaining 10 μl in lane located to their right, that is, 2, 4 or 6. Results for molecular weight standard expressed in Kb are shown on the left. The expected positions for known forms of HBV DNA, that is, relaxed-circular (rcDNA), covalently-closed circular (cccDNA), single-stranded (ssDNA), integrated forms (iDNA) and fragmented forms (fDNA) are shown on the right.

medium conditioned by cells exposed to both drugs, but in a different way. Thus, whereas the proportion of ssDNA was high in cells treated with cisplatin, when they were treated with Bamet-UD2 these forms were less abundant than in control cells, as compared with the proportion of circular forms.

Discussion

The major finding of this study was that several members of the family of Bamets induced profound alterations on the life cycle of HBV leading to a paradoxical dissociation between a reduced release of viral proteins, presumably by inhibiting the production of completely mature virions by HBV-transfected liver cells and an enhanced release of normal intracellular forms of viral DNA, with no signs of increased cell death or release of fragmented nucleosomal particles containing part of HBV DNA.

Prior to comment our results is necessary to remind two important facts: on one hand a decrease in one of the mature virions – either proteins or DNA – should necessarily be interpreted as a decrease in the secretion of complete particles, even though the secretion of the rest of components could be maintained or even elevated. On the other hand, the amount of virions secreted by infected cells is cell cycle-dependent, that is, viral replication is inhibited during the S phase of the cell cycle, implying the existence of an inverse correlation between host cell proliferation and HBV replication (Ozer *et al*. 1996). This supports the clinical concept of enhanced viral multiplication in quiescent hepatocytes. Moreover, when using *in vitro* models in which cell proliferation is arrested, the expression of HBV mRNA is increased. This is accompanied by an increase in the intracellular level of replicative intermediates of HBV DNA, which in turn leads to higher abundance of HBV DNA in the cell supernatants. Interestingly for the interpretation of our results, it has been shown that the amount of extracellular viral DNA varies proportionally to changes in intracellular viral DNA level (Guidotti *et al*. 1994).

With these concepts in mind, several explanations for our findings could be explored. The possibility that Bamet-induced enhancement in the release of HBV DNA could

be due to the antiproliferative effect of these compounds on host cells can be ruled out, because enhancement in the release of viral DNA occurred at drug concentrations at which the number of living cells in the cultures remained unaffected.

Although the platinum(II) atom present in Bamets, as well as in the parent drug cisplatin, is able to form adducts with DNA by interaction at different possible binding sites, the major binding site is thought to be the N7 of guanines (Sundquist & Lippard 1990). This induces profound and irreversible alterations in DNA structure that result in a reduction in DNA replication and in the arrest of cell cycle progression in replicating eukaryotic cells (Donaldson *et al*. 1994; Marin *et al*. 1998). The question arises as to how the formation of cisplatin- or Bamet-HBV DNA adducts might cause the observed alterations in the viral life cycle. One possibility is that the structural modification induced in viral DNA might impair the progression of the viral life-cycle to completion by preventing the normal assembly of modified DNA within the nucleocapsid. If this does account for the Bamet-induced increased release of HBV DNA, one would expect to observe intermediate replicative forms in the medium. The results obtained in southern blot analysis are consistent with a role of this mechanism in Bamet-UD2 antiviral activity. In this sense, the inhibition of HBV production by iron-depletion in HepG2 2.2.15 cells has also been associated with the accumulation of high levels of viral DNA intermediates (Chouteau *et al*. 2001).

Another alternative, which does not exclude the previous one, assumes that, on the one hand, altered DNA would not be able to behave as a correct template for transcription, and hence would be unable to generate the proteins required for the formation of complete virions. On the other hand, intracellular DNA, not included in viral particles, might be able to function as a template for partial replication before polymerase is halted by the presence of DNA adducts. Thus, the expected results of this process would be a reduced synthesis and release of proteins but a high abundance of viral DNA of many different sizes. The latter is consistent with the results obtained in cultures exposed to cisplatin. However, the fact that bands of small size were similar to those found in untreated cells and that they have the size that could be expected for internucleosomal fragmentation, suggest that they are probably the results of spontaneous (untreated) or induced (cisplatin) apoptosis. In any event, the proportion of viral DNA in these bands in cells treated with Bamets was very low.

Yet another process that could be involved in the dual effect of Bamets on the release of viral protein and DNA should be considered. Using the duck hepatitis B virus (DHBV), it has been shown that the size of the cccDNA pool in infected cells is regulated by the amount of surface proteins via negative feedback (Summers *et al*. 1990). In addition, a mutation in the S gene of the HBV causes a severe defect in viral particle secretion, with no intracellular accumulation of surface proteins, while the replicative rate is enhanced (Kalinina *et al*. 2001). The possibility of the formation of Bamet-DNA adducts in this region of the HBV genome, which might act as multiple point mutations, is not unlikely. Previous studies have shown that interaction with Bamets induces profound alterations in plasmid cccDNA (Marin *et al*. 1998; Martinez-Diez *et al*. 2000). Moreover, the ability of bile acids, particularly ursodeoxycholic acid, to reach the nucleus of liver cells during regeneration or during carcinogenesis has been reported (Mendoza *et al*. 2002; Monte *et al*. 2002). Although no direct measurements of Bamets in the nuclei have been carried out, indirect evidence based on their ability to form DNA-adducts and to inhibit DNA synthesis (Marin *et al*. 1998; Martinez-Diez *et al*. 2000) suggests that the nucleus of HepG2 2.2.15 cells might be an important place where their antiviral effect could be exerted. The difference in the behaviour of cisplatin and Bamet-UD2 regarding their reactivity with respect to DNA is not unexpected because cisplatin is able to form bi-dentate adducts (Sundquist & Lippard 1990), which cause profound structural alterations that lead to the fragmentation of plasmid DNA (Marin *et al*. 1998; Martinez-Diez *et al*. 2000), while Bamet-UD2 is expected to form mono-dentate adducts (Criado *et al*. 2000), with milder effects on DNA structure (Martinez-Diez *et al*. 2000).

One of the most interesting aspects of the results of this work is the suggestion of the potential usefulness of bile acids to target agents with antiviral activity toward liver cells. Transport systems for cholephilic organic anions, such as bile acids, in HepG2 cells have been characterized molecularly and functionally (Kullak-Ublick *et al*. 1996; Marchegiano *et al*. 1992). Although the expression of these carriers in HBV-infected cells has not been investigated, the indirect results obtained in the present study suggest that the expression of these carriers would not be abolished in HepG2 2.2.15 cells, because their ability to take up Bamets was much higher than that seen for cisplatin. Moreover, it should be stressed that Bamet-UD2, the member of this family of compounds with the strongest anti-HBV effect, is also the least toxic of all of them *in vivo* (Dominguez *et al*. 2001). Bamet-UD2 also contains two ursodeoxycholic moieties, one of which is released, leaving the remaining molecule DNA-reactive. Thus, while this could exert its antiviral effect, the released ursodeoxycholic acid moiety might play its well-known cytoprotective role (Trauner & Graziadei 1999).

In conclusion, these results suggest the existence of a dual effect of Bamets on HBV life cycle by reducing the secretion of complete virions while the production of DNA replicative intermediates was enhanced. They recommend

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consideration of these compounds as a useful experimental tool in the investigation of novel liver targeted therapeutic agents based on bile acid derivatives for the treatment of HBV infections or to carry out further studies on the HBV lifecycle.

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