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Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an "in vitro" replicative system

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Abstract

The antiviral effect against hepatitis B virus (HBV) of artemisinin, its derivative artesunate and other compounds highly purified from traditional Chinese medicine remedies, were investigated. HBV production by permanently transfected HepG2 2.2.15 cells was determined by measuring the release of surface protein (HBsAg) and HBV-DNA after drug exposure (0.01–100 μM) for 21 days. The forms of HBV-DNA released were investigated by Southern-blotting. Neutral Red retention test was used to evaluate drug-induced toxicity on host cells. The compounds were classified according to their potential interest as follows: (i) none: they had no effect on viral production (daidzein, daidzin, isonardosinon, nardofuran, nardosinon, tetrahydronardosinon and quercetin); (ii) low: they were able to markedly reduce viral production, but also induced toxicity on host cells (berberine and tannic acid) or they had no toxic effect on host cells but only had a moderate ability to reduce viral production (curcumin, baicalein, baicalin, bufalin, diallyl disulphide, glycyrrhizic acid and puerarin); (iii) high: they induced strong inhibition of viral production at concentrations at which host cell viability was not affected (artemisinin and artesunate). Moreover, artesunate in conjunction with lamivudine had synergic anti-HBV effects, which warrants further evaluation of artemisinin/artesunate as antiviral agents against HBV infection.

Keywords: Artemisinin; Artesunate; Curcumin; Traditional Chinese medicine; Hepatitis B

1. Introduction

The Hepadnaviridae family is formed by a group of highly species-specific viruses, that share the presence of an endogenous DNA polymerase with reverse transcriptase activity (Fang et al., 1981; Lien et al., 1987; Wang and Seeger, 1993) and whose genome in the mature virions is formed by a circular partially double-stranded DNA (pdsDNA) in which both strands are held together by hydrogen bonding between the 5′ ends of the two strands (Molnar-Kimber et al., 1984). One member of this family, the human hepatitis B virus (HBV), is characterized by its high hepatotropism. This virus belongs to the genus *Orthohepadnavirus* and is not cytopathic itself, although it may cause acute fulminant hepatitis (Bartholomeusz and Locarnini, 2001; Kalinina et al., 2001) or chronic liver disease that may evolve to cirrhosis and, eventually, to hepatocellular carcinoma (Iino, 2002).

In spite of the availability of an effective and safe vaccine against HBV, infection by this virus is an important worldwide health problem (Beasley and Hwang, 1991; The EASL Jury, 2003). Although several pharmacological strategies are currently being implemented to treat affected patients, no effective antiviral therapy against HBV infection has yet been fully developed. Thus, new drugs to be used alone or in combination with existing treatments are needed. In this respect,

Abbreviations: DMSO, dimethylsulfoxide; HBV, hepatitis B virus; HBsAg, HBV surface antigen; NR, Neutral Red; PCR, polymerase chain reaction; QPCR, quantitative real-time PCR; Ct, QPCR, cycle at which the arbitrary fluorescence threshold is reached; TCM, traditional Chinese medicine

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"in vitro" screening of potentially active compounds, as that carried out here, is a useful step in the preclinical development of novel drugs.

A stably HBV-transfected HepG2 2.2.15 cell line was derived from hepatoblastoma HepG2 cells (Sells et al., 1987), and has been a useful "in vitro" model for evaluation of novel anti-HBV drugs, as well as to investigate several steps of the biology of HBV (Schalm et al., 1995). HepG2 2.2.15 cells produce and secrete mature HBV virions, whose amount can be determined by the abundance of viral DNA or immunoreactive viral proteins (Sells et al., 1987). Under normal culture conditions, several forms of HBV-DNA are present in HepG2 2.2.15 cultures (Doong et al., 1991; Sells et al., 1987). The nucleic acid profile is essentially the same as that found in liver tissue from HBV-infected individuals (Sells et al., 1988). These forms are pdsDNA, covalently closed circular DNA (cccDNA), relaxed circular DNA (rcDNA), integrated in the host cell genome (iDNA), single stranded (ssDNA) and fragmented (fDNA). Moreover, it has been shown that the amount of extracellular viral DNA varies proportionally to changes in the intracellular viral DNA level (Sells et al., 1988). This property was taken into account in the design of the present study.

Some decades ago, western medicine started to look for novel drugs derived from remedies used in traditional Chinese medicine (TCM) (Han, 1988; Wang, 2000). The vast experience of TCM accumulated over millennia has selected several therapeutic methods, which include the use of herbal therapy (Chen and Chen, 1998). Approximately 100,000 herbal treatments have been recorded. An important effort has been undertaken to identify novel drugs and applications from these potentially useful natural products, with a view to develop standardized treatments for different human diseases (Seeff et al., 2001). Regarding chronic liver disease, more than 70 herbal mixtures have been used in TCM (Chang, 1998). However, despite the isolation of numerous chemical constituents from these plants, the compounds responsible for their therapeutic properties have not yet been clearly identified. Recently, the anti-malarial, anti-cancer and antiviral activities of several compounds derived from TCM remedies, in particular artemisinin and its semi-synthetic derivative artesunate, have been demonstrated (Efferth et al., 2001, 2002a,b,c, 2003). The aim of the present study was to evaluate "in vitro" the potential usefulness of a library of structurally diverse compounds (alkaloids, lactones, steroids, flavonoids, etc.), including artemisinin/artesunate, in alternative or complementary therapies against chronic hepatitis B.

2. Materials and methods

2.1. Drugs from TCM

Except for lamivudine, that was kindly supplied by Glaxo Wellcome Research and Development (Hertfordshire, UK), the compounds tested in the present study were chemically purified to more than 95% (usually 98%), as described previously (Efferth et al., 2002a). The scientific names of plants and animals that were the source of these compounds were as follows: artemisinin was extracted from *Artemisia annua*; artesunate is a semisynthetic derivative of artemisinin; baicalein and baicalin were from *Scutellaria baicalensis*; berberine from *Hydrastis canadensis*; bufalin from *Bufo marinus* and *Bufo viridis*; curcumin from *Curcuma longa*; daidzein, daidzin and puerarin from *Pueraria lobata*; diallyl disulphide from *Allium sativum*; glycyrrhizic acid from *Glycyrrhiza uralensis*; isonardosinon, nardofuran, nardosinon and tetrahydronardosinon from *Nardostachys chinensis*; quercetin from *Tripterospermum lanceolatum,* and tannic acid from *Quercus infectoria*.

2.2. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, 3-amino-7-dimethylamino-2-methylphenazine (Neutral Red (NR)), $NaHCO_3$, L-glutamine, polyethylene glycol ($Mr \approx 8000$) and dimethylsulfoxide (DMSO) were provided by 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). Foetal calf serum (FCS) was obtained from TDI (Madrid, Spain).

2.3. Cell Cultures

HepG2 2.2.15 cells 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 2 mg/ml of ciprofloxacine. After the first passage, ciprofloxacine was replaced by 150 mg/ml G418. Cells were used at the third passage when 3.5×10^5 cells were seeded on plastic dishes (3.5 cm diameter), and maintained for 3 days before any treatment, to reach approximate confluence conditions. During the experimental period (21 days) the culture medium was replaced by a fresh one, with or without (control conditions) different concentrations of TCM compounds or lamivudine, every 3 days. Before adding these drugs, they were first dissolved in DMSO and then diluted with culture medium to reach a final drug concentration equal to the highest one of the desired range and $\leq$0.2% DMSO (v/v). Serial dilutions from this solution were prepared in order to perform dose–response studies.

2.4. Evaluation of drug toxicity in host cells

Drug-induced cell toxicity was evaluated by measuring the amount of living cells in the culture on day 21 of drug expo-

sure using the Neutral Red test (Fautz et al., 1991), which is based on the ability of the lysosomes and Golgi apparatus of living cells to take up this dye. To carry out this test, after removal of the cell supernatant and a double wash of the cells with sterile phosphate buffer saline (PBS; 137 mM NaCl, 3.0 mM KCl, 0.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) 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. After washing the cells again twice, NR was eluted with an aqueous solution containing 50% (v/v) ethanol plus 1% (v/v) acetic acid for 10 min at room temperature, and the amount of dye taken up by the cells was determined by measurement of absorbance at 540 nm.

2.5. Analysis of HBsAg release to 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 infected cells to the culture medium. Results were normalized by values found in untreated cells of the same culture, which were considered as 100%. Interference by the assayed compounds in the method of measurements was ruled out by confirming the absence of the effect of including the compounds in the analysis of culture medium from control HepG2 2.2.15 cells (data not shown).

2.6. HBV-DNA release to the culture medium

To determine the abundance of HBV-DNA in the medium of treated cells, quantitative real-time PCR was used (QPCR). The supernatant of HepG2 2.2.15 cells was collected from the culture and the DNA was extracted using an adaptation of the alkaline digestion method (Kaneko et al., 1989), as previously reported (Romero et al., 2002). The solution was neutralized with 0.12N HCl and diluted as appropriate before being used for QPCR. The sequences of the forward and reverse primer oligonucleotides and the fluorogenic Taqman® probe have been previously published (Romero et al., 2002). No interference of the compounds with QPCR was confirmed as described above for the analysis of HBsAg (data not shown). Standard curves needed to calculate the amount of DNA copies in each sample were obtained using a 904-bp HBV probe with 100% similarity with HBV subtype ayw DNA, as previously described (Romero et al., 2002). This probe was also used to carry out southern blot analysis of the HBV-DNA release, as previously reported (Romero et al., 2002). In brief, viral DNA was isolated by polyethylene glycol precipitation, purified by treatment with proteinase K, and deproteinized by extraction with phenol/chloroform (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) to carry out hybridiza-

Table 1
Host cell toxicity, antiviral effect and therapeutical index

Number	Name	Solubility[a]	CC_{50}[b]	HBsAg[c] IC_{50}	HBV-DNA[d] IC_{50}	TI[e]
1	Lamivudine	H	>100	0.2	0.3	>500
2	Artemisinin	M	160	55	>100	2.9
3	Artesunate	H	20	2.3	0.5	40
4	Berberine	L	50	11	90	4.5
5	Tannic Acid	H	17	11	>100	1.5
6	Curcumin	L	>100	72	>100	>1.4
7	Baicalein	M	>100	>100	>100	–
8	Glycyrrhizic acid	M	>100	>100	85	>1.2
9	Puerarin	H	>100	>100	78	>1.3
10	Bufalin	H	>100	>100	>100	–
11	Tetrahydronardosinon	H	>100	>100	>100	–
12	Baicalin	H	>100	>100	>100	–
13	Daidzein	L	>100	>100	>100	–
14	Daidzin	H	>100	>100	>100	–
15	Isonardosinon	H	>100	>100	>100	–
16	Nardofuran	H	>100	>100	>100	–
17	Nardosinon	M	>100	>100	>100	–
18	Diallyl Disulfide	M	>100	>100	>100	–
19	Quercetin	H	>100	>100	>100	–

[a] Solubility in culture medium at 37 °C was defined as high (H), moderate (M) or low (L) according with the absence or presence of observable turbidity in the stock solution (40–80 μl DMSO/1 ml culture medium). When added to culture medium at final concentrations no detectable precipitation was apparent in any case.

[b] CC_{50}: drug concentration (in μM) inducing 50% reduction in host cell viability.

[c] HBsAg IC_{50}: drug concentration (in μM) inducing 50% inhibition in HBsAg release.

[d] HBV-DNA IC_{50}: drug concentration (in μM) inducing 50% inhibition in HBV-DNA release.

[e] Therapeutical index (TI) was the ratio between CC_{50} and IC_{50} for the most sensitive parameter to detect decrease in HBV production (HBsAg or HBV-DNA) in each case.

tion with the ^{32}P-dCTP random-labelled 904-bp HBV-DNA probe.

2.7. Statistical analysis

Data points were obtained from at least three different cell cultures, in which each condition was assayed in triplicate. Values are given as mean ± S.D. 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. Statistical significance was set at $p < 0.05$.

3. Results

3.1. HBsAg and HBV-DNA release to the culture medium

When HepG2 2.2.15 cells were exposed to lamivudine, used here as a positive control for anti-HBV activity, the abundance of HBV-DNA released to the culture medium was significantly decreased, which was consistent with the reduction in HBsAg (Table 1).

Several of the compounds tested here were found to be able to inhibit HBsAg secretion (Figs. 1–3). Thus, artesunate was a stronger inhibitor (IC_{50} = 2.3 μM, IC_{90} = 16 μM) than the rest of compounds ($p < 0.05$), except for lamivudine. Berberine and tannic acid were also good, but less potent, inhibitors, with IC_{50} values of approximately 11 μM ($p < 0.05$ versus compounds with lower activity) and IC_{90} values between 50 and 100 μM, whereas artemisinin and curcumin had IC_{50} values between 50 and 100 μM ($p < 0.05$ versus compounds with lower activity) and IC_{90} higher than 100 μM. The rest of compounds tested were very poor inhibitors of HBsAg release or this ability was not observed at all in the range of concentrations used (data not shown).

It should be stressed that not all immunoreactive viral proteins present in the medium formed part of complete virions—i.e., not all can be included in "Dane particles"—because some of them may be free in the medium or may form part of genome-free viral particles (Pugh and Bassendine, 1990). Therefore, although a reduction in HBsAg production must be interpreted as probably associated with lowered release of complete virions, an increase in HBsAg release does not necessarily imply an enhancement in virion production. Complementary determinations of HBV-DNA release are needed.

Thus, measurement of the amount of the HBV-DNA release to the HepG2 2.2.15 culture medium during different treatments revealed several patterns of response (Figs. 1–3). In some cases (artesunate and berberine; the later with a biphasic behaviour that will be commented below), a reduction in HBV-DNA release occurred, accompanied by a decrease in HBsAg levels in the medium (Figs. 1 and 2). In the case of baicalein, glycyrrhizic acid and puerarin, a lower release of HBV-DNA was observed together with none or weak effect on HBsAg release (Figs. 2 and 3). In cells treated with artemisinin, tannic acid, curcumin and bufalin, the abundance of HBV-DNA in the medium was increased in spite of the reduced amount of HBsAg (Figs. 1–3). In the rest of compounds, which had no effect on HBsAg release, the amount of HBV-DNA in the medium was not substantially altered (daidzin and nardosinon) (data not shown) or increased, as in the case of tetrahydronardosinon (Fig. 3), diallyl disulphide, baicalin, daidzein, isonardosinon, nardofuran and quercetin (data not shown).

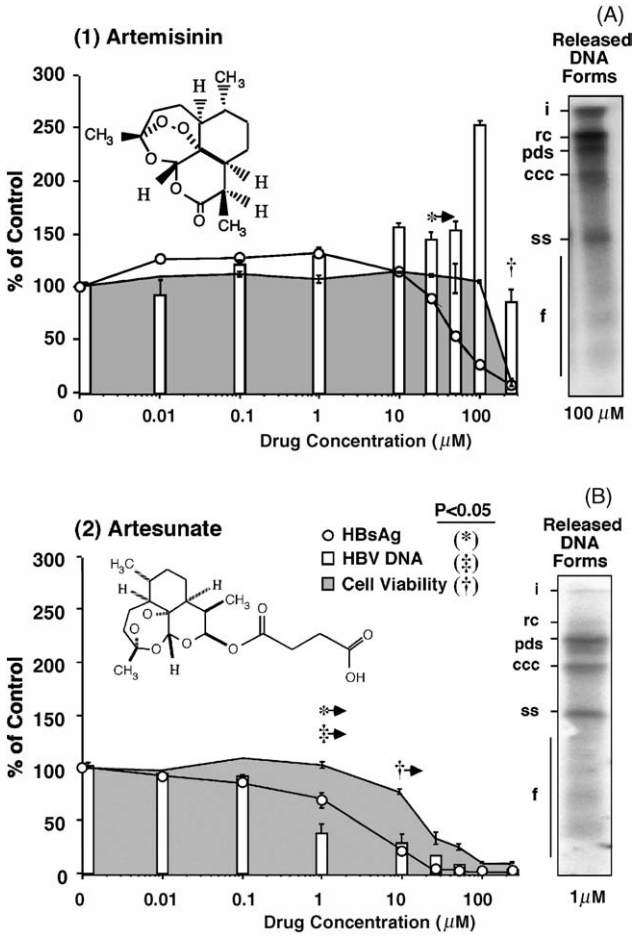

Fig. 1. Effect on cell viability and release of HBsAg and HBV-DNA to the culture medium by HepG2 2.2.15 cells after 21 days culture in the presence of the indicated concentrations of artemisinin (A) and artesunate (B). Values are mean ± S.D. from three different cultures in which each data point was the mean value from three different dishes. They are expressed as percentages of determinations carried out on dishes to which no drug had been added (0 μM or control). $^{*}p < 0.05$ on comparing the abundance of HBsAg in controls; $^{‡}p < 0.05$ on comparing HBV-DNA release with controls; $^{†}p < 0.05$ on comparing cell viability with controls. A similar statistical significance was obtained for values placed on the right of the arrow. Inset depicts the southern blot analysis of HBV-DNA found in the culture medium of the indicated drug concentration. The expected positions for known forms of HBV-DNA, i.e., integrated forms (iDNA), relaxed-circular (rcDNA), circular partially double stranded (pdsDNA), covalently-closed circular (cccDNA), single-stranded (ssDNA), and fragmented forms (fDNA) are indicated.

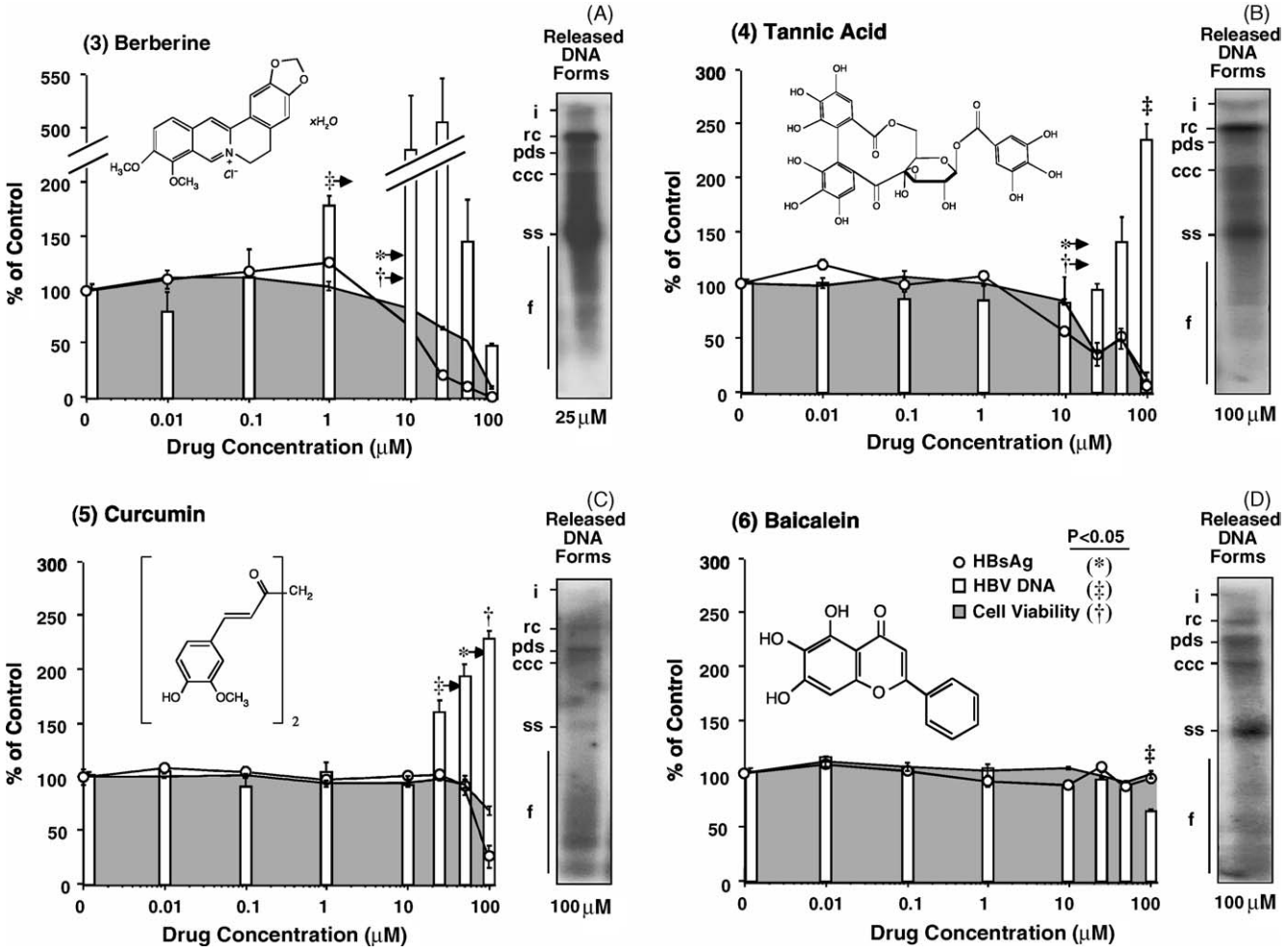

Fig. 2. Effect of berberine (A), tannic acid (B), curcumin (C) and baicalein (D) on cell viability and release of HBsAg and HBV-DNA to the culture medium by HepG2 2.2.15 cells after 21 days culture in the presence of the indicated drug concentrations (ranging from 0.01 to 100 μM). For details see Fig. 1.

3.2. Pattern of HBV-DNA forms in the culture medium

Southern blot analysis indicated that the majority of HBV-DNA present in the medium of HepG2 2.2.15 cells under control conditions (data not shown) was mainly in the form of rcDNA, ssDNA and pdsDNA, as previously described by others (Sells et al., 1988) and confirmed by us (Romero et al., 2002). However, other forms were also seen. These were: cccDNA and iDNA, probably released from detached dead cells, 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). All these forms of HBV-DNA were also observed in cells treated for 21 days with TCM drugs.

The pattern of bands was similar in most cases, except for some of the compounds able to enhance HBV-DNA release, in which southern blot analysis indicated that the higher abundance of HBV-DNA was not accounted for by an increased proportion of complete particles containing pds-DNA, but rather by enhanced release of rcDNA (in the case of artemisinin and tannic acid) and fDNA (tetrahydronardosinon > bufalin > curcumin).

An enhanced proportion of ssDNA was also seen in the culture medium of cells treated with tannic acid and baicalin. The case of berberine was peculiar in the sense that a marked increase in the amount of HBV-DNA, mainly due to an enhanced proportion of rcDNA and ssDNA forms, was observed at concentrations at which no or low cell host toxicity was found, whereas treatment with higher cytotoxic concentrations of this drug was accompanied by a marked decrease in HBV-DNA release.

3.3. Toxicity in host cells, therapeutical index and combination with lamivudine

After 21 days of treatment with different amounts of lamivudine, used as positive control, or TCM derivatives (Figs. 1–3), the viability of HepG2 2.2.15 cells was not affected or a reduction of different magnitudes in this parameter was found. Artesunate, berberine and tannic acid induced cell host toxicity at concentrations higher than 10 μM,

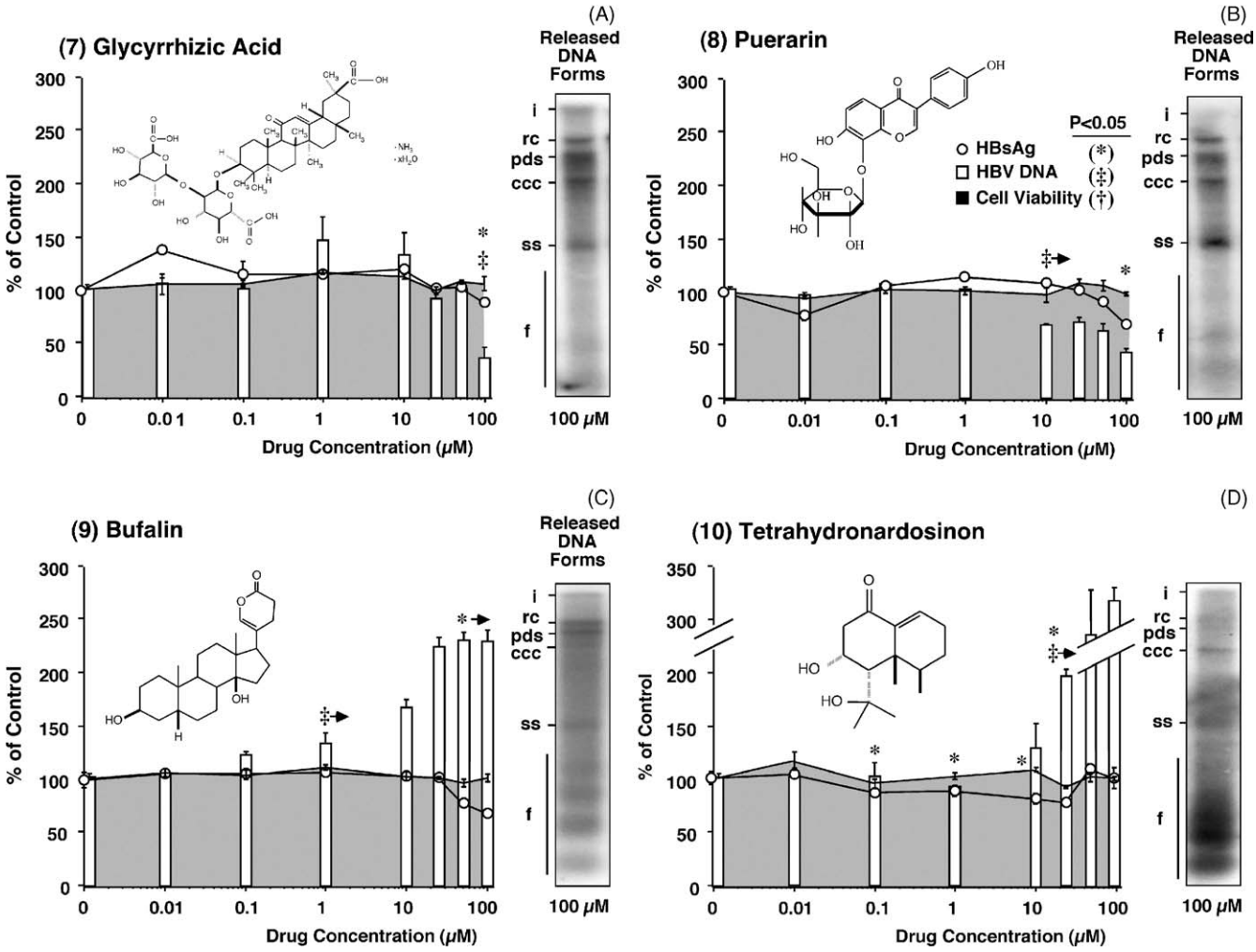

Fig. 3. Effect of glycyrrhizic acid (A), puerarin (B), bufalin (C) and tetrahydronardosinon (D) on cell viability and release of HBsAg and HBV-DNA into the culture medium by HepG2 2.2.15 cells after 21 days culture in the presence of the indicated drug concentrations (ranging from 0.01 to 100 μM). For details see Fig. 1.

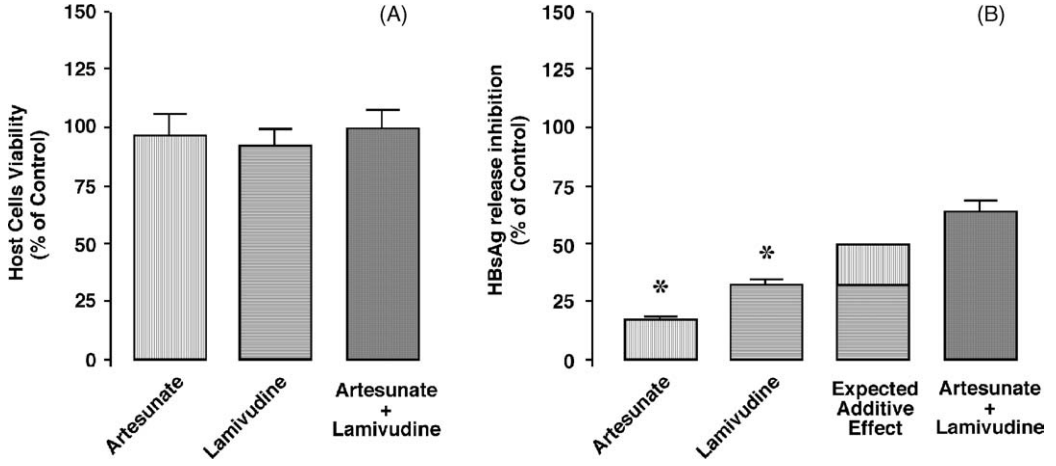

Fig. 4. Host cell viability (A) and inhibition of HBsAg release into the culture medium (B) of HepG2 2.2.15 cells incubated with artesunate and lamivudine (0.02 μM) alone or in conjunction, for 21 days. $^{*}p < 0.05$, as comparing the effect of each drug with that due to the combination of both drugs.

whereas cytotoxicity was found at concentrations close to 100 μM for curcumin and artemisinin (Table 1). For the other compounds no toxicity was found in the range of concentrations used in this study. The highest value of therapeutical index (Table 1), defined as the ratio between CC_{50} (drug concentration inducing 50% reduction in host cell viability) and IC_{50} (drug concentration inducing 50% reduction in HBsAg or HBV-DNA release), was found for artesunate. Therefore, this drug was further investigated in conjunction with lamivudine. When both compounds were administered together (20 nM each) no toxicity was induced (Fig. 4A), but a synergic inhibitory effect in HBsAg release was found (Fig. 4B).

4. Discussion

Taken together, our results indicate that several of the assayed compounds show interesting anti-HBV activity with no or minor effect on host cells. Among them, artemisinin, and, in particular, its semisynthetic derivative artesunate displayed the most interesting properties. Although their effect in the "in vitro" system used here was weaker than that of lamivudine, a stronger effect of these drugs "in vivo" cannot be ruled out. Moreover, their interest is enhanced by the existence of synergic effects with lamivudine in absence of drug-induced toxicity in host cells, which may be an important characteristic due to the frequent problem in clinical practice of infection by lamivudine-resistant HBV strains.

The plant known in TCM as qinghao, which contains artemisinin, has been used for centuries in TCM as a remedy for chills and fever (Nosten and Price, 1995). Its antimalarial properties were discovered in China in 1972 (Jiang et al., 1982). Since then, artemisinin and its derivatives have been used against multidrug-resistant *Plasmodium falciparum* strains (Meshnick, 2002). The antimalaria activity of artemisinin is due to iron-mediated cleavage of its peroxide bridge and generation of three organic free radicals (Zhang et al., 1992). Artemisinin has been reported to form covalent adducts with proteins, but not with DNA, through a mechanism that requires the presence of heme (Yang et al., 1994), which is abundant in liver cells.

The range of concentrations at which artesunate was active against HBV (>10 μM) was quite similar to that previously reported for its activity versus human cytomegaloviruses (Efferth et al., 2002b). Interestingly, these levels are close to the drug concentrations reached in the plasma of patients when this drug is used in antimalaria treatments (≈7 μM) (Batty et al., 1996)

The potential pharmacological interest of artemisinin and its derivatives is enhanced by the fact that owing to their antimalaria properties these drugs have been evaluated in large populations with no evidence of serious side effects (Price et al., 1999), even during pregnancy (McGready et al., 1998), except for quite rare neurological problems that have been observed in two cases but which might have been unrelated to artemisinin treatment (Dayan, 1998). Studies carried out on laboratory animals have confirmed that the neurotoxic effect of artemisinin-related drugs appears only at drug concentrations well above therapeutic ranges for humans (Nosten and Price, 1995).

Similarly to artemisinin and artesunate, the model compound lamivudine induced a pronounced inhibition of HBsAg release and/or viral DNA at concentrations at which host cell viability was not affected. This effect was similar to that previously reported by other authors in HepG2 2.2.15 cells (Placidi et al., 2001). The anti-HBV effects observed for the rest of drugs assayed were diverse, which determines their potential interest. This can be considered low for berberine and tannic acid (with a strong ability to reduce viral production, accompanied by toxic effect on host cells), as well as for curcumin, baicalein, baicalin, bufalin, diallyl disulphide, glycyrrhizic acid and puerarin (with no toxic effect on host cells but with only a moderate ability to reduce viral production). However, at least in this experimental model, daidzein, daidzin, isonardosinon, nardofuran, nardosinon, tetrahydronardosinon and quercetin did not display interesting characteristics as anti-HBV drugs, because they had no effect on viral production by HepG2 2.2.15 cells.

Some compounds induced a toxic effect that affected both host cell survival and the machinery for viral protein production. This impairment also seemed to affect the release of viral DNA, which was reduced or increased. When DNA release was enhanced, this was mainly due to a marked increased in ssDNA (tannic acid and berberine) or fDNA (tetrahydronardosinon) forms. The rest of compounds shared the characteristic of having no toxic effect on host cells at the concentration range assayed. Nevertheless, the release of HBsAg and/or HBV-DNA was impaired in some of them. These results suggest a specific interference of compounds included in these groups with the biology of HBV, without modifying cell host viability. Because the secretion of complete HBV particles involves an increase in the release of both viral components (proteins and DNA), a marked decrease in the amount of one of them in the culture medium could be explained by a reduction in the secretion of complete virions.

Thus, although artesunate induced a parallel inhibition in HBsAg and HBV-DNA secretion, artemisinin-induced dose-dependent inhibition in HBsAg secretion was initially accompanied by an enhanced release of HBV-DNA (mainly rcDNA forms). This paradoxical effect has been previously observed when HepG2 2.2.15 cells were treated with DNA-reactive drugs, such as Bamet-UD2 or cisplatin (Romero et al., 2002). A similar behaviour, observed under different experimental circumstances, has been suggested to be due to the inhibition of complete HBV production associated with the intracellular accumulation of HBV DNA intermediates and their subsequent release to the medium (Chouteau et al., 2001).

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