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Antiviral Effect of Artemisinin from *Artemisia annua* against a Model Member of the *Flaviviridae* Family, the Bovine Viral Diarrhoea Virus (BVDV)

Abstract

The antiviral activity versus flaviviruses of artemisinin, a safe drug obtained from *Artemisia annua* and commonly used to treat malaria, has been investigated using as an *in vitro* model bovine epithelial cells from embryonic trachea (EBTr) infected with the cytopathic strain Oregon C24V, of bovine viral diarrhoea virus (BVDV), which is a member of the *Flaviviridae* family. Antiviral activity was estimated by the degree of protection against the cytopathic effect of BVDV on host cells and by the reduction in BVDV-RNA release to the culture medium. To induce an intermediate cytopathic effect in non-treated cells, EBTr cells were first exposed to BVDV for 48 h and then incubated with virus-free medium for 72 h. Ribavirin and artemisinin (up to 100 μ M) induced no toxicity in host cells, whereas a slight degree of toxicity was observed for IFN- α at concentrations above 10 U/mL up to 100 U/mL. Treatment of infected cells with IFN- α , ribavirin and artemisinin markedly reduced BVDV-induced cell death. A com-

ination of these drugs resulted in an additive protective effect. These drugs induced a significant reduction in the production/release of BVDV virions by infected EBTr cells; there was also an additive effect when combinations of them were assayed. These results suggest a potential usefulness of artemisinin in combination with current pharmacological therapy for the treatment of human and veterinary infections by flaviviruses.

Key words

EBTr · Hepacivirus · Hepatitis · Interferon · Liver · Malaria · Ribavirin

Abbreviations

BVDV: Bovine Viral Diarrhoea Virus
HCV: Hepatitis C Virus
IFN: Interferon

Introduction

The *Flaviviridae* family includes three different genera: Pestivirus (e.g., bovine viral diarrhoea virus, BVDV); Flavivirus (e.g., Japanese encephalitis virus); and Hepacivirus (e.g., hepatitis C virus, HCV). Since all members of the *Flaviviridae* family share similar-

ities in virion structure, genome organisation, and replication machinery, some viruses, in particular BVDV, have been used as *in vitro* models for infection by these viruses [1]. Although for the study of the specific biology of these viruses the use of cell cultures to propagate other flaviviruses, such as HCV, is also preferred and, since very recently possible [2], the use BVDV is a

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History

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popular alternative experimental model as a first screening test to investigate the activity of potential antiviral agents against flaviviruses [1]. The reason for using BVDV is that this virus is less hazardous than other members of this family because it is not infective for humans and BVDV replicates efficiently in cell culture [3]. An additional advantage is that there are two biotypes of BVDV: cytopathic and non-cytopathic according to their effect on cell cultures. Cytopathic and non-cytopathic strains differ in genome size/structure owing to the insertion of cellular sequences or gene rearrangements at the junction site between non-structural protein 2 (NS2) and NS3 [4], [5], [6] and in the fact that, in contrast to infection with non-cytopathic strains, infection with cytopathic BVDV leads to lysis of the host cell and hence represents a very useful tool in the investigation of the antiviral protective effect of drugs.

Artemisinin or "qinghaosu" is an endoperoxide sesquiterpene lactone [3R,5aS,6R,8aS,9R,12S,12aR]-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one] obtained from the plant *Artemisia annua* and is currently used in the treatment of malaria. This compound and its derivatives have the ability to destroy the agent causing malaria (*Plasmodium falciparum*) by releasing high doses of free radicals that attack the cell membrane of the parasite in the presence of high iron concentrations [7]. The excellent safety and efficacy of artemisinin and its derivatives, either alone or in combination with other drugs, have allowed over one million malaria patients to be cured [8].

Since previous studies have suggested antiviral activity of artemisinin and/or its derivatives against hepatitis B virus (HBV) [9] and cytomegaloviruses [10], [11], we have carried out the present study to evaluate the activity of this drug against infection by *Flaviviridae* viruses. Owing to the fact that the mechanisms of action of the antiviral effect of IFN- α [12] and ribavirin [13], [14] against *Flaviviridae* viruses are probably different to those described for artemisinin [15], [16], there exists the interesting possibility of additive effects of these drugs, which has been also investigated in the present study.

The interest of these aims is based on the fact that *Flaviviridae* viruses constitute a major cause of disease worldwide. Thus, infection by hepatitis C virus (HCV) frequently causes chronic hepatitis that may progress to cirrhosis and hepatocellular carcinoma [17], [18]. The problem is aggravated by the absence of an efficient vaccine against HCV and because currently the standard treatment, based on pegylated IFN- α and the purine nucleoside analogue ribavirin (1 β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), in addition to having noxious side-effects, are not efficient in approximately half of the infected patients [18], [19]. This means that the search for more effective therapies is crucial [20].

Materials and Methods

Chemicals

Artemisinin, extracted from *Artemisia annua*, was obtained and chemically purified to more than 98% as described previously [10]. Gentamicin, ribavirin and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich Química (Madrid, Spain). Human re-

combinant interferon- α (IFN- α) from *E. coli* was supplied by Calbiochem BioNova (Madrid, Spain). Foetal calf serum (FCS) was from TDI, S.A. (Madrid, Spain). Trypsin was obtained from Roche (Barcelona, Spain).

Cell cultures and viral infection

Bovine epithelial cells derived from embryonic trachea (EBTr) were cultured with modified Eagle's medium (MEM) with GLUTAMAX-I containing 1% antibiotic-antimycotic mixture (Invitrogen; Barcelona, Spain), supplemented with 10% FCS and 5 mg/L gentamicin. They were plated on 96 multiwell plates (15×10^3 cells/well; 50 μ L/well) were allowed to attach for 2 h before adding 50 μ L of culture medium with the desired dilution of an initial suspension of BVDV (cytopathic strain Oregon C24V, genotype I, subgenotype b). Medium containing BVDV inoculum was removed after 18, 24 or 48 h. The cells were washed twice with virus-free medium, and incubated with fresh medium for 72 or 96 h (Fig. 1).

Antiviral effect and toxicity in host cells

EBTr cells seeded in 96 multiwell plates were incubated in the absence or presence of BVDV for 48 h and then with 100 μ L virus-free culture medium containing different concentrations or combinations of IFN- α , ribavirin or/and artemisinin, previously dissolved in DMSO (< 0.2% final concentration in culture medium) for 72 h. Cell viability at the end of the experimental period was measured by the tetrazolium salt test (CellTiter 96[®] Aqueous; Innogenetics; Barcelona, Spain). Drug-induced toxicity was determined from the reduction in cell viability in non-infected cells, whereas drug-induced antiviral protection was determined by the recovery of cell viability in cultures where this was expected to be reduced due to the BVDV-induced cytopathic effect.

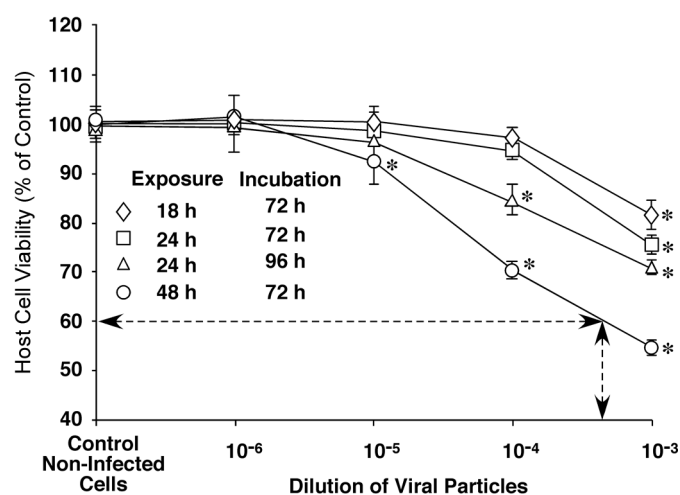


Fig. 1 Effect of varying the time of exposure to the cytopathic strain Oregon C24V of bovine viral diarrhoea virus (BVDV) and incubation in the absence of the virus on EBTr cells. The efficiency of the infection was measured by the reduction in cell viability at the end of the experimental period. Arrows and dashed lines indicate the dilution of BVDV inducing 40% cell death, which was used in subsequent screenings of the antiviral effects of the drugs. Values are means \pm SD of 24 measurements carried out in three different cultures. * $P < 0.05$ as compared to control non-infected cells by the paired t -test.

Measurement of BVDV-RNA production

BVDV-infected and non-infected cells seeded in 6 multiwell plates at 45×10^4 cells/well and treated as described above were harvested and viral RNA was purified using the QIAmp viral RNA mini kit (Izasa; Barcelona, Spain). The relative abundance of viral RNA was determined by real-time quantitative RT-PCR as previously described [9], [21]. Primers were designed for the BVDV NS4B/NS5A region using Primer Express Software (Applied Biosystems; Madrid, Spain). The forward primer sequence was 5'-TGCGGGTAGAAACCTATTACA-3' and the reverse one 5'-GAGCC-CACCCGAGTACCAT-3' (accession number: AF091605).

RT was carried out using random hexamers and avian myeloblastosis virus RT (cloned AMV first strand cDNA synthesis kit; Invitrogen). To normalise the results and compare the measurements carried out with different RT reactions, a fixed amount (77.5 pg) of exogenous RNA was added to each sample. This was accurately measured using the Ribo Green RNA-Quantitation kit (Molecular Probes, Invitrogen). As external standard, the cRNA of the rat bile salt export pump (BSEP, gene symbol *Abcb11*) was used, which was synthesised as reported elsewhere [21] using the pSPORT1 plasmid containing the appropriate cDNA kindly supplied by Dr. Peter Meier, Dr. Bruno Steiger and Dr. Bruno Hagenbuch (Department of Clinical Pharmacology, Zurich University Hospital, Switzerland). The sequence of primers used for PCR of BSEP were: forward 5'-ATCTACGCAGGAGTCGGCAT-3' and reverse 5'-TCATCTAGGACAAGTGTGGAACCA-3' (accession number: NM_031760). They were selected after confirming that they were not able to amplify any sequence in the material from RT that was carried out with RNA obtained from BVDV and host cells. To ensure the linearity of the measurement method, standard curves were generated by fitting the \log_{10} of the dilution of DNA – obtained from RT-PCR of BVDV-RNA or rat BSEP-RNA added to the non-diluted sample at a fixed amount of 77.5 pg – versus the corresponding threshold cycle (Ct), or cycle of PCR when the fluorescence due to interaction of SYBR Green I with amplification products first passed the established threshold value. RT-PCR measurements were carried out in triplicate for each sample in five samples collected from three different cultures. The values for r were 0.9967 for BVDV-RNA and 0.9961 for BSEP-RNA.

Statistical analysis

Data points were obtained in three or four different cultures, in which each condition was assayed in 8 different wells. Values are given as means \pm SD. Regression lines were calculated by the least-squares method. 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 [22].

Results

In order to accurately determine drug-induced protection, infection conditions able to induce an intermediate cytopathic effect of approximately 40% were selected (Fig. 1). The reason for using 40% cell death as control conditions was that in preliminary experiments we had observed that a higher cytopathic effect was accompanied by a very variable production of viral particles and the protective effect of the drugs assayed showed considerable

variability (data not shown). On the other hand, a lower cytopathic effect involves working with a very narrow range of drug-induced cell protection. After testing several infection and incubation times, the best results were obtained using 48 h infection time followed by 72 h incubation time (Fig. 1). These conditions were then used to calculate the required dilution of each stock solution of viral particles to achieve BVDV-induced cell death of 40% in infected non-treated cells.

For comparison with artemisinin, drug-induced toxicity in non-infected host cells was also investigated for IFN- α and ribavirin. Even at low concentrations (10 U/mL), IFN- α induced a slight but significant reduction in cell viability, which was not further enhanced up to 100 U/mL (Fig. 2). A similar effect has been described in MDBK cells treated with IFN- α [23]. In contrast, both ribavirin and artemisinin had no deleterious effect on non-infected host cells, at least up to the highest concentrations used in the present study (100 μ M) (Figs. 3 and 4). In studies on the combined activity of these drugs, 5 U/mL of IFN- α were used. This concentration had no toxicity when used alone but a slight reduction in cell viability of less than 15% (data not shown) was observed when it was combined with 50 μ M ribavirin. No toxicity of the combination of 50 μ M artemisinin with either 50 μ M ribavirin or 5 U/mL IFN- α was found (data not shown).

Study of the protection against BVDV induced by these drugs revealed that 25 U/mL of IFN- α were able to recover the cell viability of infected cells by approximately 80% (Fig. 2). Further increases in the dose of IFN- α failed to increase cell viability significantly. Ribavirin (Fig. 3) and artemisinin (Fig. 4) induced a dose-dependent recovery in cell viability up to 100 μ M. At concentrations lower than 50 μ M, the protective effect was stronger for artemisinin.

Use of combinations of these three drugs indicated that the protective effect of 50 μ M artemisinin with 5 U/mL of IFN- α or 50 μ M of ribavirin seemed to be additive to the effect of drugs used in-

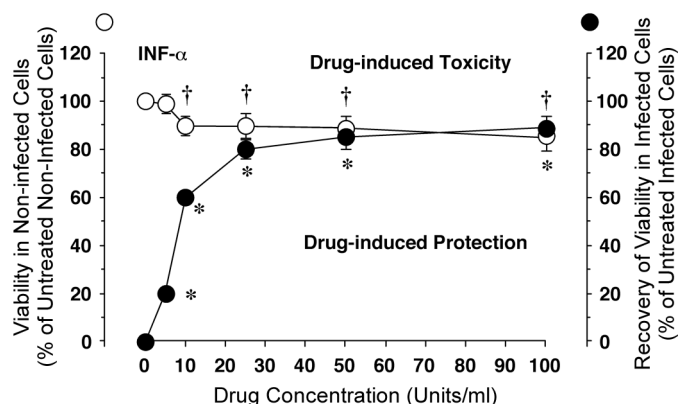


Fig. 2 Effect of IFN- α on the viability of EBTr cells. Non-infected cells were incubated with the indicated concentrations of IFN- α (open circles) for 72 h to investigate the toxicity of the drug on host cells. Similar experiments were carried out on EBTr cells that had been previously infected by exposure to BVDV for 48 h before adding the drug (closed circles). Values are means \pm SD of 32 measurements carried out in four different cultures. Using the paired t -test, comparisons were carried out in infected (* $P < 0.05$) or non-infected († $P < 0.05$) cells versus cells cultured under similar circumstances but in the absence of IFN- α .

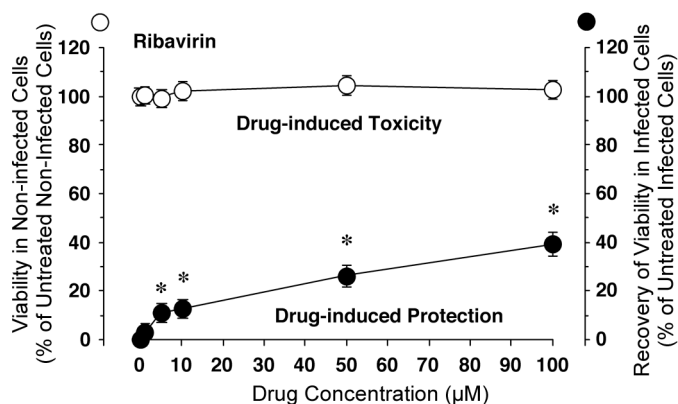


Fig. 3 Effect of ribavirin on the viability of EBT cells. Non-infected cells were incubated with the indicated concentrations of ribavirin (open circles) for 72 h to investigate the toxicity of the drug on host cells. Similar experiments were carried out on EBT cells that had been previously infected by exposure to BVDV for 48 h before adding the drug (closed circles). Values are means \pm SD of 32 measurements carried out in four different cultures. Using the paired *t*-test, comparisons were carried out in infected (* $P < 0.05$) or non-infected (in all cases $P > 0.05$) cells versus cells cultured under similar circumstances but in the absence of ribavirin.

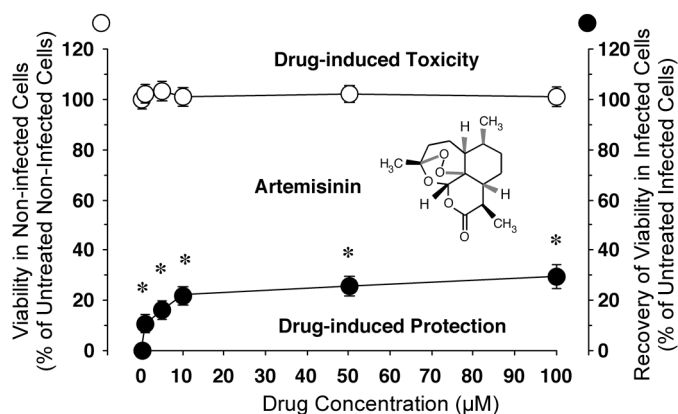


Fig. 4 Effect of artemisinin on the viability of EBT cells. Non-infected cells were incubated with the indicated concentrations of artemisinin (open circles) for 72 h to investigate the toxicity of the drug on host cells. Similar experiments were carried out on EBT cells that had been previously infected by exposure to BVDV for 48 h before adding the drug (closed circles). Values are means \pm SD of 32 measurements carried out in four different cultures. Using the paired *t*-test, comparisons were carried out in infected (* $P < 0.05$) or non-infected (in all cases $P > 0.05$) cells versus cells cultured under similar circumstances but in the absence of artemisinin. The inset depicts the chemical structure of artemisinin.

dividually (Fig. 5). The same was true for the combination of 5 U/mL of IFN- α and 50 μ M of ribavirin (Fig. 5).

To investigate whether part of the protection provided by these drugs was due to a reduction in viral propagation, as an indirect measurement, the release of BVDV-RNA to the culture medium of infected cells was determined (Fig. 6). A significant dose-dependent inhibition of the release of BVDV-RNA was observed when infected cells were treated with 5 or 10 U/mL of IFN- α , 50 or 100 μ M of ribavirin or 50 or 100 μ M of artemisinin. Moreover, two-by-two combinations of these three drugs resulted in a significantly stronger inhibitory effect than those found for each drug administered individually (Fig. 6).

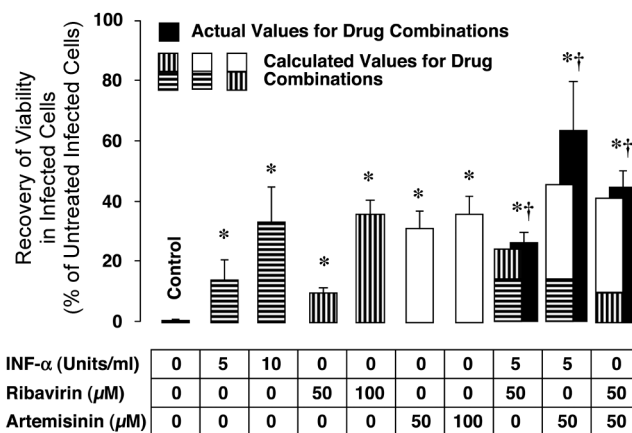


Fig. 5 Detection of the anti-viral protective effect of different combinations of IFN- α , ribavirin or artemisinin determined as the recovery in the viability of EBT cells that had been previously infected by exposure to BVDV for 48 h before adding the drug or the combination of drugs. Values are means \pm SD of 30–40 measurements carried out in five different cultures. * $P < 0.05$ as compared with non-treated cells by the paired *t*-test. † $P < 0.05$ on comparing the results of combinations (closed bars) with those obtained with each individual drug, by the Bonferroni method of multiple-range testing. Values due to individual drugs are shown again on bars corresponding to each combination.

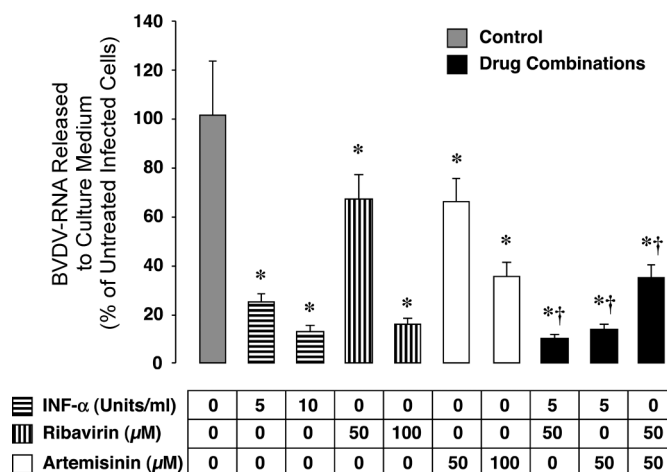


Fig. 6 Detection of the effect of different combinations of IFN- α , ribavirin or artemisinin on BVDV-RNA release to the culture medium of EBT-infected cells by exposure to BVDV for 48 h before adding the drug or the combination of drugs. Relative abundance of BVDV-RNA was measured by real-time quantitative RT-PCR, which was normalised by the addition to the sample of a fixed amount (77.5 μ g) of an exogenous RNA (rat BSEP). Values are means \pm SD of 30–40 measurements carried out in five different cultures. * $P < 0.05$ on comparing with non-treated control cells (grey bar) by the paired *t*-test. † $P < 0.05$ on comparing results of combinations (closed bars) with those obtained with each individual drug, by the Bonferroni method of multiple-range testing.

Discussion

The findings of the present study suggest that artemisinin is an inhibitor of the production of *Flaviviridae* viruses and that its effect is additive to those of IFN- α and ribavirin. The pharmacological interest of artemisinin and its derivatives for the treatment of infections by these viruses is enhanced by two facts: i) A large

proportion of people infected with HCV do not respond to available pharmacological regimes (ranging from 20% of patients infected with genotype 3 to 80% of those infected with genotype 1b), [18], [19], [20], and ii) owing to the antimalaria properties of artemisinin and its derivatives, such properties have been evaluated in large populations in which these drugs have been found to have an excellent safety profile [24], even during pregnancy [25]. Although some mild side effects have been reported, artemisinin and its derivatives induce no relevant toxicity [7], [24], [26], except for quite rare neurological problems that were observed in two cases but which might have been unrelated to the artemisinin treatment [27]. 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 [26].

Moreover, owing to the fact that the mechanisms of action of IFN- α [12], [14], [28], and ribavirin [13], [14] against *Flaviviridae* viruses are probably different to those described for artemisinin [15], [16], there exists the possibility of additive effects of these drugs, which, indeed, were observed in the present *in vitro* study. IFN binds to cell surface receptors and stimulates signal pathways that lead to the activation of cellular enzymes that repress viral replication [12], [28], whereas ribavirin, in addition to its immunomodulatory properties, has direct antiviral activities that can be ascribed to several possible mechanisms. These include the inhibition of the HCV RNA-dependent RNA polymerase NS5B and the recently described activity as an RNA mutagen able to impair viral replication [29].

Regarding the mechanism of action of artemisinin, this was not investigated in the present descriptive study, but the similarity of previously reported antimicrobial effect of this agent on different systems, suggest that very probably this is also shared in the case of the anti-BVDV effect. Thus, the antimalaria activity of artemisinin has been reported to be due to iron-mediated cleavage of its peroxide bridge (see inset of Fig. 4) and the generation of three organic free radicals [16], which confer the activated forms of artemisinin its alkylating properties. Thus, artemisinin forms covalent adducts with proteins, but not with DNA, through a mechanism that requires the presence of haeme [15], which is abundant in liver cells. Moreover, a similar mechanism accounting for the ability of artemisinin to inhibit the production of HBV virions has been suggested [9].

In sum, these results suggest a potential usefulness of artemisinin in combination with current pharmacological therapy for the treatment of human and veterinary infections by flaviviruses.

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