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Novel artemisinin derivatives with potential usefulness against liver/colon cancer and viral hepatitis

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ABSTRACT

Antitumor and antiviral properties of the antimalaria drug artemisinin from *Artemisia annua* have been reported. Novel artemisinin derivatives (AD1–AD8) have been synthesized and evaluated using in vitro models of liver/colon cancer and viral hepatitis B and C. Cell viability assays after treating human cell lines from hepatoblastoma (HepG2), hepatocarcinoma (SK-HEP-1), and colon adenocarcinoma (LS174T) with AD1–AD8 for a short (6 h) and long (72 h) period revealed that AD5 combined low acute toxicity together with high antiproliferative effect (IC_{50} = 1–5 μM). Since iron-mediated activation of peroxide bond is involved in artemisinin antimalarial activity, the effect of iron(II)-glycine sulfate (ferrosanol) and iron(III)-containing protoporphyrin IX (hemin) was investigated. Ferrosanol, but not hemin, enhanced antiproliferative activity of AD5 if the cells were preloaded with AD5, but not if both compouds were added together. Five derivatives (AD1 > AD2 > AD7 > AD3 > AD8) were able to inhibit the cytopathic effect of bovine viral diarrhoea virus (BVDV), a surrogate in vitro model of hepatitis C virus (HCV), used here to evaluate the anti-*Flaviviridae* activity. Moreover, AD1 and AD2 inhibited the release of BVDV-RNA to the culture medium. Co-treatment with hemin or ferrosanol resulted in enhanced anti-*Flaviviridae* activity of AD1. In HepG2 cells permanently infected with hepatitis B virus (HBV), AD1 and AD4, at non-toxic concentrations for the host cells were able to reduce the release of HBV-DNA to the medium. In conclusion, high pharmacological interest deserving further evaluation in animal models has been identified for novel artemisinin-related drugs potentially useful for the treatment of liver cancer and viral hepatitis B and C.

1. Introduction

Primary and secondary liver cancers, whose most frequent origin is colon cancer, are among the main causes of death due to cancer worldwide.[1] Advances in surgery and radiotherapy permit to cure a certain number of these patients nowadays, however these approaches cannot always be applied, and pharmacological regimens are of limited usefulness because drugs available may elicit undesirable side effects, initial chemoresistance or the development of drug refractoriness during treatment.[2,3]

Hepatitis B virus (HBV), which belongs to the genus *Orthohepadnavirus* of the family *Hepadnaviridae*, causes chronic infection in the host liver that may result in cirrhosis and eventually hepatocellular carcinoma.[4] The WHO estimates that, in spite of the availability of a safe vaccine, there are 300 million people infected with HBV.[5] Currently the most commonly used drugs to treat chronic hepatitis B are pegylated interferon and nucleoside analogues, such as lamivudine and adefovir, which are not fully effective in many cases, owing to a wide range of adverse effects[6] and the appearance of viral mutant strains that are resistant to the drug.[7]

Infection by hepatitis C virus (HCV), which belongs to the genus *Hepacivirus* of the family *Flaviviridae*, is another important health problem with 130 million people affected worldwide. The probability of this people to become chronically infected is high (50–85%).[8] Unfortunately, in approximately 20% of these patients this condition may evolve to cirrhosis and hepatocellular carcinoma.[9]

Abbreviations: ART, artemisinin; ARS, artesunate; AD, artemisinin derivative; DHA, dihydroartemisinin.

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Moreover, no safe vaccine against HCV has yet been developed. Treatment of chronic hepatitis C with pegylated interferon and ribavirin lack complete efficacy and good tolerability, which may be further complicated by the emergence of strains resistant to currently available drugs. Together these factors account for a frequent failure of the pharmacological treatments of these patients.[10]

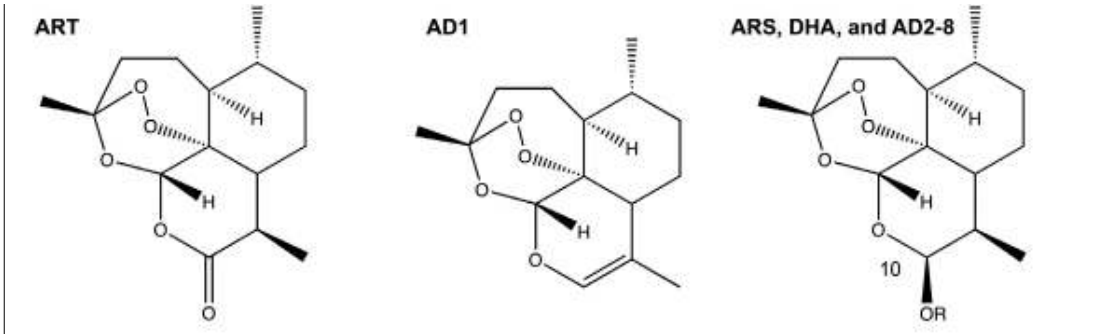
Artemisinin (ART) is a drug obtained from the plant *Artemisia annua* that has been recently recommended by the WHO in combination with other antimalaria drugs to treat drug-resistant *Plasmodium falciparum* strains, cerebral malaria and malaria in children.[11] In an attempt to improve ART bioavailability and efficacy, several derivatives have been synthesized such as dihydroartemisin (DHA), a reduced lactol that is more active but thermally less stable than ART; and artesunate (ARS), which is more active and less toxic than its parent drug. All these derivatives belong to a large family of compounds named artemisinins or artemisinin-like derivatives (ADs) that share the endoperoxide bridge and hence are expected to keep part of the pharmacological properties of ART.[12] Based on their cytotoxic activity against *Plasmodium falciparum*, ART and its semi-synthetic derivatives have shown promising results when they have been evaluated in vitro as anticancer and antiviral drugs.[13,14] More precisely, activity against viruses responsible for viral hepatitis B[15] and C[16] has been reported.

Recently several novel ADs with different bulky groups at position C10 of DHA have been synthesized (Fig. 1). The initial aim to synthesize this group of derivatives was to mimic the ability of ARS to be transformed into DHA through the cleavage of the ester moiety at different rates. The rational was that slower release of the active agent would improve the pharmacokinetic properties of the drug. Indeed these compounds have demonstrated in vitro cytotoxic effect against leukaemia cells, anti-angiogenic activity in vivo and ability to overcome chemoresistance mediated by multidrug resistance protein 1 (MDR1).[17] In the light of these remarkable characteristics further in vitro evaluation of eight of these novel drugs was recommended. The aim of the present work was therefore to investigate their antiproliferative effect against cells derived from primary liver cancer and colon adenocarcinoma as the most frequent origins of secondary liver cancer, and their antiviral activity versus the *Hepadnaviridae* and *Flaviviridae* families, accounting for viral hepatitis B and C. Since ART exerts its antimalarial activity through activation by iron, the activity of ADs in these in vitro models has been also investigated in the presence of iron(II)-glycine sulfate (ferrosanol) and iron(III)-containing protoporphyrin IX (hemin).

2. Materials and methods

2.1. Chemicals

ART, and ARS were obtained as previously described.[18] DHA and the ADs were synthesized following the methodology published elsewhere.[17] Dulbecco's modified Eagle's medium (DMEM), gentamicin, 3-amino-7-dimethylamino-2-methylphenazine (Neutral Red), $NaHCO_3$, L-glutamine, minimum essential medium (MEM), thiazolyl blue tetrazolium bromide (MTT), hemin and dimethylsulphoxide (DMSO) were provided by Sigma–Aldrich Quimica (Madrid, Spain). MEM GLUTAMAX™ was obtained from Invitrogen (Barcelona, Spain). Dodecyl sulphate sodium salt (SDS) was from Merck (Barcelona, Spain). Ciprofloxacine (Baycip®) was supplied by Bayer (Leverkusen, Germany). 4-(2-Hydroxyethyl)-1-piperazineethansulphonic acid (HEPES), trypsin and geneticin® (G418) were from Roche (Barcelona, Spain). Ferrosanol was purchased form UCB Pharma, S.A. (Madrid, Spain). Fetal calf serum (FCS) was obtained from TDI (Madrid, Spain).



Code	Artemisinin-like derivatives	MW	R	R
ART	Artemisinin	282.33	NO	-
ARS	Artesunate	384.42	YES	$COCH_2CH_2COOH$
DHA	Dihydroartemisinin	284.33	YES	H
AD1	Anhydrodihydroartemisinin	266.33	NO	-
AD2	10-dihydroartemisinyl acetate	326.38	YES	$COCH_3$
AD3	10-dihydroartemisinyl butyrate	354.44	YES	$COCH_2CH_2CH_3$
AD4	10-(2'-butyloxy) dihydroartemisinin	340.38	YES	$CH_2CH(CH_3)_2$
AD5	10-dihydroartemisinyl 2'- propylpentanoate	410.54	YES	$COCH(C_3H_7)_2$
AD6	10-dihydroartemisinyl 2', 2'-dimethylpropionate	368.46	YES	$COC(CH_3)_3$
AD7	10-dihydroartemisinyl perfluoropropionate	430.36	YES	COC_2F_5
AD8	10-dihydroartemisinyl dimethylcarbamate	355.43	YES	$CON(CH_3)_2$

Figure 1. Chemical structure of artemisinin (ART), artesunate (ARS), dihydroartemisinin (DHA) and novel ART derivatives (AD1–AD8).

2.2. Evaluation of in vitro antiproliferative activity

The following human cell lines from the American Type Culture Collection (Manassas, VA, USA) were used: HepG2 (from hepatoblastoma, HB-8065), SK-HEP-1 (from hepatocarcinoma, HTB-52), and LS174T (from colon adenocarcinoma, CL-188). They were cultured with appropriate media in a humidified CO_2:air (5:95%) atmosphere at 37 °C. ART, ARS, ADs, ferrosanol and hemin were dissolved in DMSO, which was used at <0.2% final concentration in culture medium.

Formazan formation from the tetrazolium salt by living cells was used to evaluate the drug-induced non-specific acute toxicity (short-term incubation, 6 h) or the antiproliferative effect (long-term incubation, 72 h) by measuring the reduction of cell viability after the exposure to the desired drug. Approximately 5×10^3 or 15×10^3 cells/well (depending on the cell line) were seeded in 96-wells plates. In some experiments the effect of ferrosanol or hemin was investigated by combining one of these compounds with AD5 before being added to the culture or after preloading the cells with AD5 for 1 h to prevent potential peroxide activation in the media prior to the uptake of AD5 by the cells.

2.3. Evaluation of anti-*Flaviviridae* activity

EBTr cells were culture as previously described.[16] Cells were seeded in 96-wells plates (15×10^3 cells/well) and left to attach for 2 h before adding the desired dilution of BVDV (the cytopathic strain Oregon C24V, genotype I, sugenotype b) to reach 40% cytopathic effect in infected non-treated cells. The BVDV inoculum was removed after 48 h and the cells were incubated for 72 h with fresh virus-free culture medium containing the desired ADs to study their anti-*Flaviviridae* activity. In some experiments ferrosanol or hemin were added after incubation with the AD for 1 h. Cell viability was then measured by the MTT test to determine drug-induced toxicity in non-infected host cells and drug-induced protection in BVDV-infected EBTr cells. Determination of BVDV-RNA release to the medium was carried out by real-time quantitative PCR. As normalizer to calculate absolute values of BVDV-RNA the addition of a known amount of previously synthesized cRNA corresponding to the rat bile salt export pump (Bsep, gene symbol *Abcb11*) was carried out as reported elsewhere.[16]

2.4. Evaluation of anti-*Hepadnaviridae* activity

HepG2 2.2.15 cells permanently infected with HBV were derived from hepatoblastoma HepG2 cells.[19] They were cultured as previously described.[15] During the experimental period (21 days), the culture medium was replaced by a fresh one, without (control conditions) or with the compound to be tested, previously dissolved in DMSO (<0.2% final concentration in culture medium), every 3 days. Using the Neutral Red test[20] as previously described,[15] drug-induced cell toxicity was evaluated by measuring the amount of living cells in the culture medium after drug exposure for 21 days. To determine the abundance of HBV-DNA in the culture medium released from host cells, real-time quantitative PCR was used. The supernatant of HepG2 2.2.15 cells was collected on day 21 and the DNA was extracted using an adaptation of the alkaline digestion method as previously reported.[21]

2.5. Statistical analysis

Data points were obtained from at least 3 different cell cultures, in which each condition was assayed at least in 3-well. To calculate the statistical significance of differences the paired *t*-test was used.

3. Results

3.1. In vitro antiproliferative activity

The antiproliferative activity of eight ADs (see structure in Fig. 1) was determined in 3 human cancer cell lines derived from liver (HepG2 and SK-HEP-1) and colon (LS174T) cancer, and this was compared with that of ART, ARS and DHA whose antiproliferative effect has been previously demonstrated.[22–24]

ART, at least at the doses assayed here, was not able to induce a significant antiproliferative effect in these cells. Only at very high concentrations of ART a modest reduction in cell viability was observed (Fig. 2). However, treatment with increasing doses of ARS and DHA showed a significantly ability to reduce cell viability after incubation with the drug for 72 h (Fig. 2). ARS had stronger antiproliferative effect than DHA showing IC_{50} values below or equal to 20 μM in the 3 cell lines assayed (Table 1).

Regarding the antiproliferative activity of ADs, AD1 and AD7 exhibited the weakest effect (Fig. 2 and Table 1), whereas AD2, AD3 and AD8 have moderate activity (Fig. 2 and Table 1). They effect was more marked in SK-HEP-1 cells. In the rank of antiproliferative activity the next two compounds were AD4 and AD6 with similar effect on the 3 cell lines to that found for ARS and DHA. The strongest activity was found for AD5. This compound, even at very low concentrations, induced a marked reduction in the viability of the 3 cell lines assayed (Fig. 2 and Table 1).

To determine whether compounds that gave positive results in the antiproliferative test induced reduction in culture size due to drug-induced general toxicity the viability of the cells was measured after short-term (6 h) exposure to IC_{50} of these compounds (Fig. 3). This acute toxicity test revealed that only ARS, but not any AD, was mildly toxic for SK-HEP-1 and LS174T cells (Fig. 3).

To study the mechanism accounting for the AD antiproliferative activity, the effect of ferrosanol and hemin in combination with this derivative was analyzed using the most active compound (AD5) and the most sensitive cell line (SK-HEP-1) (Fig. 4). No effect of ferrosanol and hemin on the antiproliferative activity of ARS or AD5 was observed when the compounds were combined before being added to the culture medium (Fig. 4A and C). In contrast, when SK-HEP-1 cells were preloaded with ARS (Fig. 4B) or AD5 (Fig. 4D) for 1 h before adding the iron-containing compounds, ferrosanol, but not hemin, significantly enhanced the antiproliferative activity of both ARS and AD5.

3.2. Anti-*Flaviviridae* activity

When toxicity in non-infected bovine epithelial cells obtained from embryonic trachea (EBTr) host cells was investigated, as described above for human cancer cells, the ADs exhibited very low toxicity at the assayed concentrations (Fig. 5). Only DHA, AD4, AD5 and AD6 induced a moderate although significant reduction in cell viability. This was not seen, at least up to 100 μM, for AD1, AD2, AD3, AD7 and AD8 (Fig. 5). Infection with the cytopathic strain of bovine viral diarrhoea virus (BVDV) in this experimental conditions caused an approximately 40% reduction in the number of living cells (data not shown), as has been documented previously.[16]

Study of the antiviral protection induced by these drugs revealed that AD5 did not protect the host cells against the cytopathic effect of the virus (Fig. 5). In contrast, a moderate protection, as indicated by the recovery of cell viability, was observed when the cells were treated with DHA, AD3, AD4 or AD6 (Fig. 5). Additionally, AD1, AD2, AD7 and AD8 induced a strong dose-depending protective effect (Fig. 5).

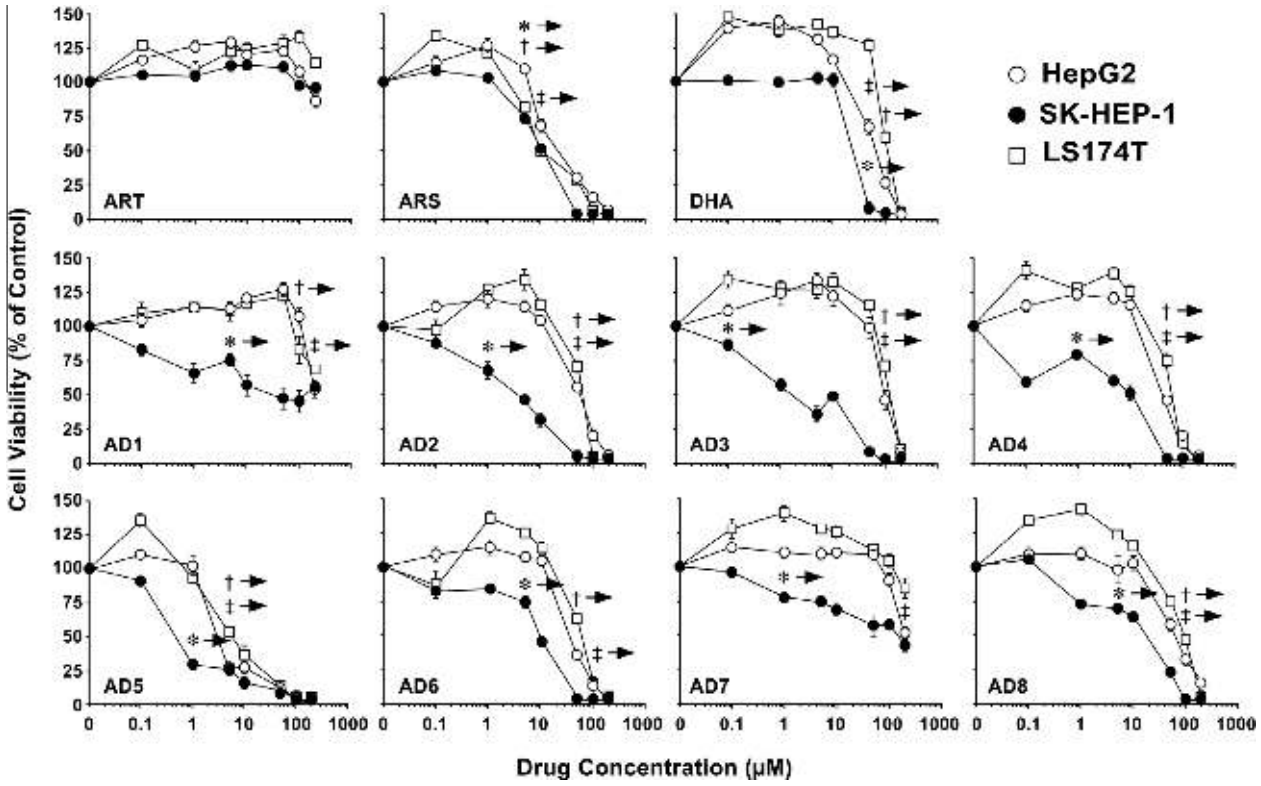


Figure 2. Effect of artemisinin (ART), artesunate (ARS), dihydroartemisinin (DHA) and novel ART derivatives (AD1–AD8) on the viability of human cells derived from hepatoblastoma (HepG2 cells; open circles), hepatocarcinoma (SK-HEP-1 cells; closed circles) and colon adenocarcinoma (LS174T cells, open squares) determined by the formazan test. Cells were incubated with the increasing doses of each compound for 72 h. Values are means ± SD of 9 wells per data point obtained in 3 separate cultures. ‡(HepG2), *(SK-HEP-1) and †(LS 174T), p <0.05 as compared with untreated cells.

Table 1
Antiproliferative activity of artemisinin, artesunate dihidroartemisinin and the novel artemisinin derivatives (AD) as determined by IC_{50} values (in μM) for human hepatoblastoma (HepG2), human hepatocarcinoma (SK-HEP-1) and human colon adenocarcinoma (LS174T)

Code	Artemisinin derivatives	HepG2	SK-HEP-1	LS174T
ART	Artemisinin	>200	>200	>200
ARS	Artesunate	20	10	10
DHA	Dihydroartemisinin	70	30	120
AD1	Anhydrodihydroartemisinin	200	30	>200
AD2	10-Dihydroartemisinyl acetate	60	4	70
AD3	10-Dihydroartemisinyl butyrate	100	2	130
AD4	10-(2′-Butyloxy) dihydroartemisinin	50	10	70
AD5	10-Dihydroartemisinyl 2′-propylpentanoate	3	1	5
AD6	10-Dihydroartemisinyl 2′, 2′-dimethylpropionate	40	9	70
AD7	10-Dihydroartemisinyl perfluoropropionate	200	150	>200
AD8	10-Dihydroartemisinyl dimethylcarbamate	70	25	100

The IC_{50} was defined as the drug concentration required to reduce the amount of living cells by 50% after incubation with 0-to-200 μM drug concentration for 72 h. Values are mean ± SD from 9 wells per data point obtained in 3 separate cultures.

To study whether the antiviral protection was due to a virostatic effect, a single dose of the compounds (50 μM) was used in different culture conditions (6-well plates) to compare the degree of protection afforded to infected cells on the same plate and the release of BVDV-RNA to the culture medium as an indirect evidence of reduction in viral propagation (Fig. 6). Under similar conditions toxic effect on non-infected cells was also determined. The results regarding drug-induced toxicity in non-infected cells (Fig. 6A) and the recovery of cell viability in infected cells (Fig. 6B) were consistent with those found in dose-dependent studies carried out in 96-well plates (Fig. 5). Moreover, 3 compounds, DHA, AD1 and AD2, able to induce antiviral cell protection did also inhibit BVDV-RNA release. Another 3 compounds, AD3, AD7 and AD8 induced antiviral cell protection but without significant reduction in BVDV-RNA release, whereas AD4, AD5 and AD6 were able to inhibit BVDV-RNA release but did not induce antiviral cell protection (Fig. 6C).

To further investigate whether the effect of AD1 on BVDV was enhanced by iron, a set of experiments were performed in the presence of iron-donors using a single dose of AD1 (50 μM) in combination with hemin (0.5 μM) or ferrosanol (2.5 μg/ml) (Fig. 7). These concentrations were selected due to their absence of toxic effect on non-infected EBTr cells based on preliminary dose–response studies (data not shown). Hemin and ferrosanol were able to increase the protective effect of AD1 against the cytopathic effect of BVDV. In contrast, the anti-*Flaviviridae* effect of 50 μM ART was inhibited by hemin and ferrosanol (Fig. 7).

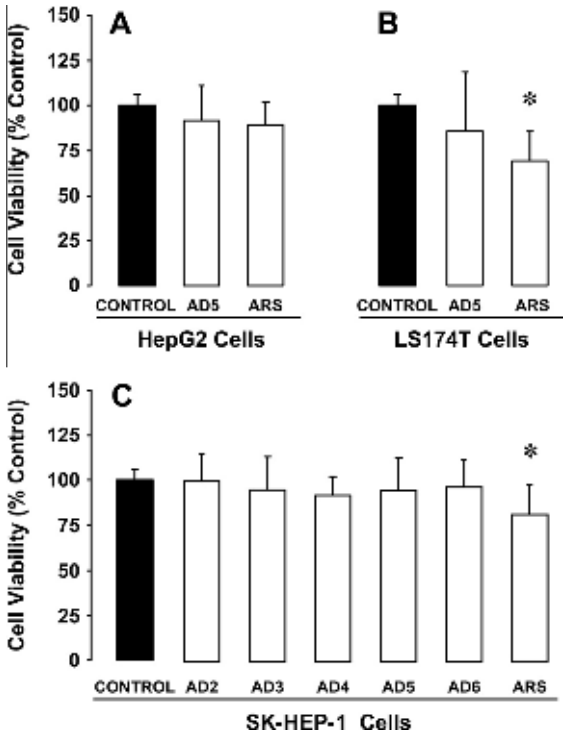


Figure 3. Acute drug-induced toxicity in human cancer cells derived from hepatoblastoma (HepG2 cells), hepatocarcinoma (SK-HEP-1 cells), and colon adenocarcinoma (LS174T cells) determined by the formazan test. Cells were incubated for 6 h with the indicated artemisinin derivative (AD) at concentrations of IC_{50} for antiproliferative effect. Values are means ± SD of 16 wells per data point obtained from 4 different cultures. *p <0.05 as compared with untreated control cells.

3.3. Anti-*Hepadnaviridae* activity

Before carrying out the study of the effect on HBV production by permanently infected HepG2 2.2.15 cells, the toxicity of AD1–AD8 on host cells was determined. Only compounds with low toxicity on HepG2 2.2.15 cells were further analyzed regarding their antiviral activity. For most ADs (except AD1 and AD4) a significant reduction in cell viability at concentrations as low as 0.1 μM (AD2, AD7 and AD8) or 1 μM (DHA, AD3, AD5 and AD6) was found (Fig. 8). In contrast, AD1 and AD4 were markedly less toxic (Fig. 9). For these two compounds the range of non-toxic concentrations was >10-fold larger. This characteristic allowed us to determine the anti-HBV activity of AD1 and AD4, which was very strong for both compounds. Both AD1 and AD4 at concentrations below 0.1 μM were able to reduce by 50% the release of HBV-DNA to the culture medium (Fig. 9). Owing to the fact that 21-days culture was required in this model, long-term iron supplementation markedly interferes with host cells biology (data not shown). Accordingly, neither hemin nor ferrosanol was tested in this in vitro model.

4. Discussion

Owing to the promising pharmacological characteristics of ART, an important effort has been done in the development of ART derivatives with enhanced beneficial properties regarding their physical-chemical and biological characteristics. In this respect, changes in the molecule aimed to increase ART solubility and its circulating half-life, have resulted in several new ADs (Fig. 1) with a bulky group linked at C10 position of DHA, which is expected to reduce the hydrolysis rate and hence result in a slower DHA release. Previous studies have shown cytotoxic and anti-angiogenic properties for some of these compounds.[17] In the present study

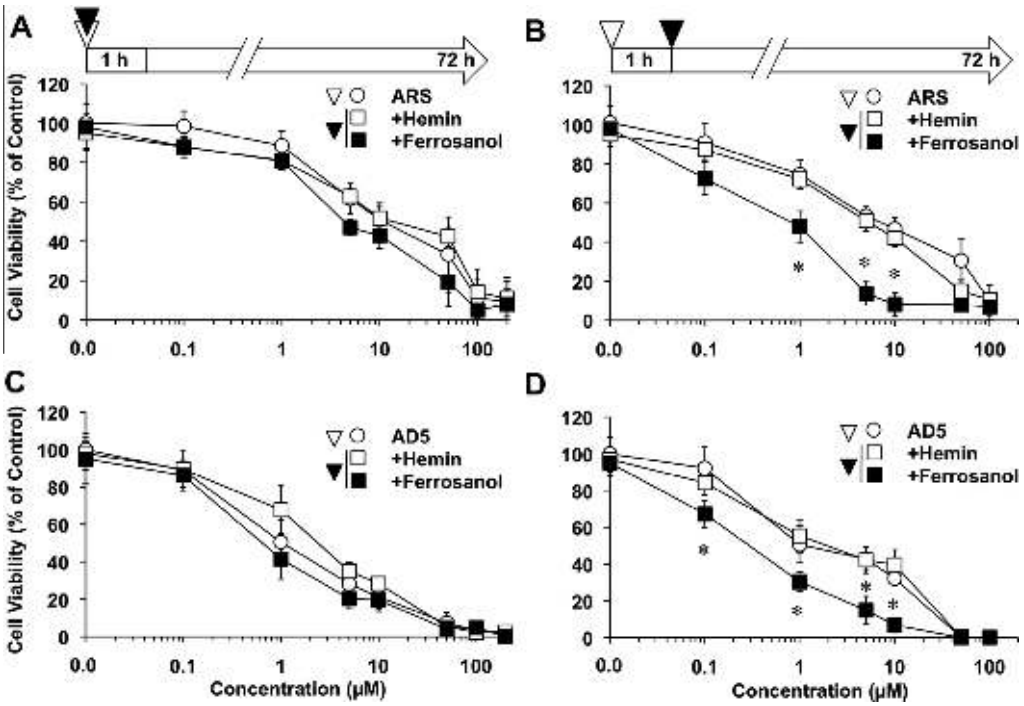


Figure 4. Effect of hemin and ferrosanol on the antiproliferative effect of artesunate (ARS) and 10-dihydroartemisinyl 2′-propylpentanoate (AD5) in SK-HEP-1 cells (derived from human hepatocarcinoma). Cells were incubated for 72 h with increasing doses of ARS (A and B) or AD5 (C and D) with or without of hemin (1 μM) or ferrosanol (5 μg/ml) added together (A and C) or after 1 h of incubation with either ARS (B) or AD5 (D). Values (means ± SD), determined by the formazan test in 12 wells per data point obtained in 3 separate cultures, are expressed as the percentage of untreated cells. *p <0.05 as compared with AD5 or ARS alone.

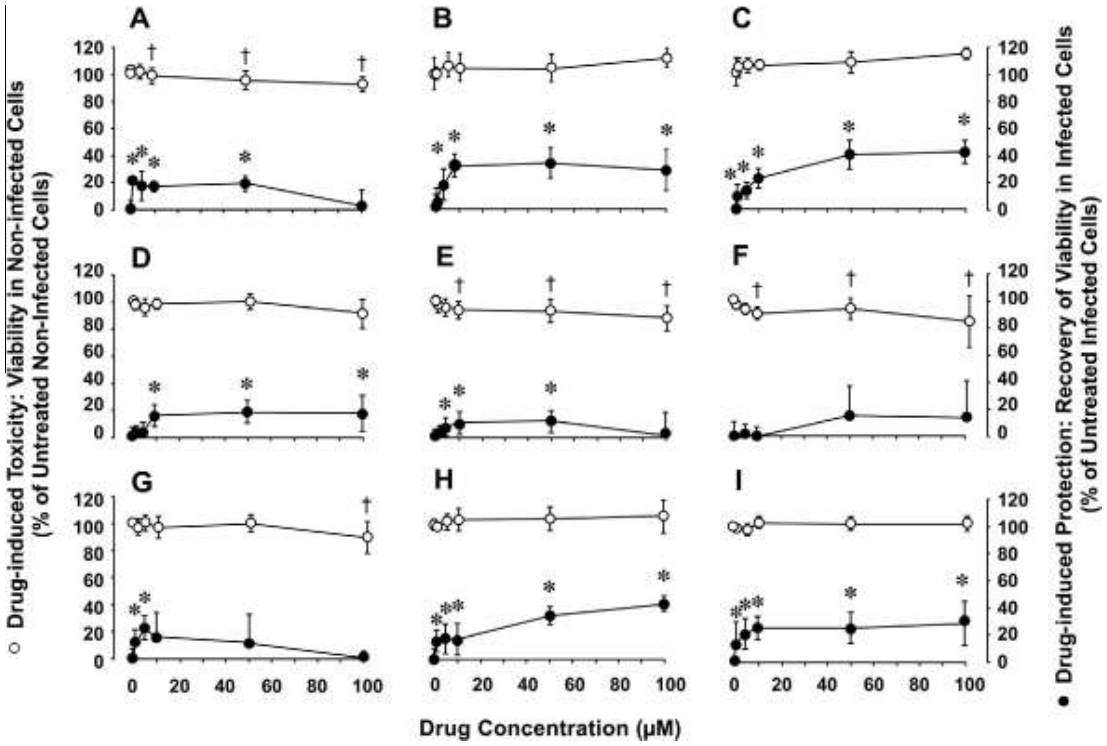


Figure 5. Effect of dihydroartemisinin (DHA) (A) and novel artemisinin derivatives: AD1 (B), AD2 (C), AD3 (D), AD4 (E), AD5 (F), AD6 (G), AD7 (H), and AD8 (I), on the viability of EBTr cells incubated in the presence or absence of bovine viral diarrhoea virus (BVDV; cytopathic strain Oregon C24V, genotype I, subgenotype b). Drug-induced toxicity on host cells was determined in uninfected (open circles) cells incubated with the indicated drug concentrations for 72 h. Similar experiments were carried out in EBTr cells previously infected by exposure to BVDV for 48 h before adding the drug (closed circles). Values are means ± SD of 12 wells per data point obtained from 3 different cultures. Comparisons with cells cultured in the absence of AD were carried out in infected ($^{*}p$ <0.05) or non-infected ($^{\dagger}p$ <0.05) cells.

we have tested in vitro their activity as potential drugs against liver/intestinal cancer and viral hepatitis B and C. The findings of the present study suggest that some of these novel ADs, mainly AD5, are promising agents to be further investigated against primary (hepatoblastoma and hepatocarcinoma) and secondary (derived from colon adenocarcinoma) liver cancer, whereas others, such as AD1 and AD4 might be useful for the treatment of HBV infections. Finally, AD1, AD2 and AD7 afford protection against BVDV, which suggest a potential usefulness against other members of this family of viruses, such as HCV (Table 2).

The pharmacological interest of ADs for the treatment of liver cancer and viral hepatitis is supported by 3 facts:

(1) The antimalarial and antiproliferative activity of ART and its derivatives is due to iron-mediated cleavage of their peroxide bridges. The radicals produced in this Fenton reaction are not only the classical hydroxyl and superoxide anions, but also carbon-centered radicals, which confers to ART alkylating properties.[25] Thus, ART forms covalent adducts with proteins, but not with DNA. Damage in cell proteins trigger apoptosis,[26,27] through a mechanism that requires the presence of heme,[27,28] which is particularly abundant in parenchyma liver cells.
(2) ART and several of its derivatives have been evaluated in large population of people suffering chemoresistant forms of malaria. The results indicate that, in general, they are safe compounds with only mild side effects. Indeed, the good tolerability of these drugs make possible their use in combined regimes for the treatment of malaria in children[29] and pregnant women.[30]
(3) There is a considerable proportion of people affected by viral hepatitis B and C as well as by liver cancer, both primary—including hepatocellular carcinoma due to hepatitis viruses B and C—or secondary—including metastasis from colon cancer—tumors who do not respond to available pharmacological regimes.[3,31]

These facts explain why over the last decade the antiproliferative activity of ART and its derivatives have been investigated against a large panel of tumors including hepatocellular carcinoma and cholangiocarcinoma.[32] Findings from the present study revealed that novel ADs were stronger inhibitors of in vitro liver cancer cell growth than ART. This may be due in part to their enhanced water solubility and/or to their slower transformation into DHA or other active product resulting from peroxide homolysis,[17] which has been proposed to determine the antiproliferative activity of ARS.[22,33,24,34] The results of the present work show IC_{50} values for the most potent AD about 10-fold lower than those found by us and others[24] for ARS in liver cancer cells. Although the antiproliferative activity of AD5 in SK-HEP-1 cells was lower than that of many chemotherapeutic drugs this was 50-fold more potent than cisplatin[35] and fivefold more potent than sorafenib,[36] which, at present is the drug of choice for these type of tumors.[37]

In the light of the marked heterogeneity regarding their acute toxicity, the magnitude of their antiproliferative effect and the selective sensitivity of hepatoblastoma, hepatocarcinoma and colon adenocarcinoma cells to these compounds, they could be classified according to their potential pharmacological interest as shown in Table 2. Thus, AD5 was considered as the most interesting compound because of its low IC_{50} in the 3 cell lines tested here as compared with ART, ARS and DHA. Moreover AD5 did not induce acute toxic effect. Since the addition of a bulky moiety at position C10 of ART decreases the rate of hydrolysis and hence DHA is slower released which results in enhanced therapeutic efficacy,[17]

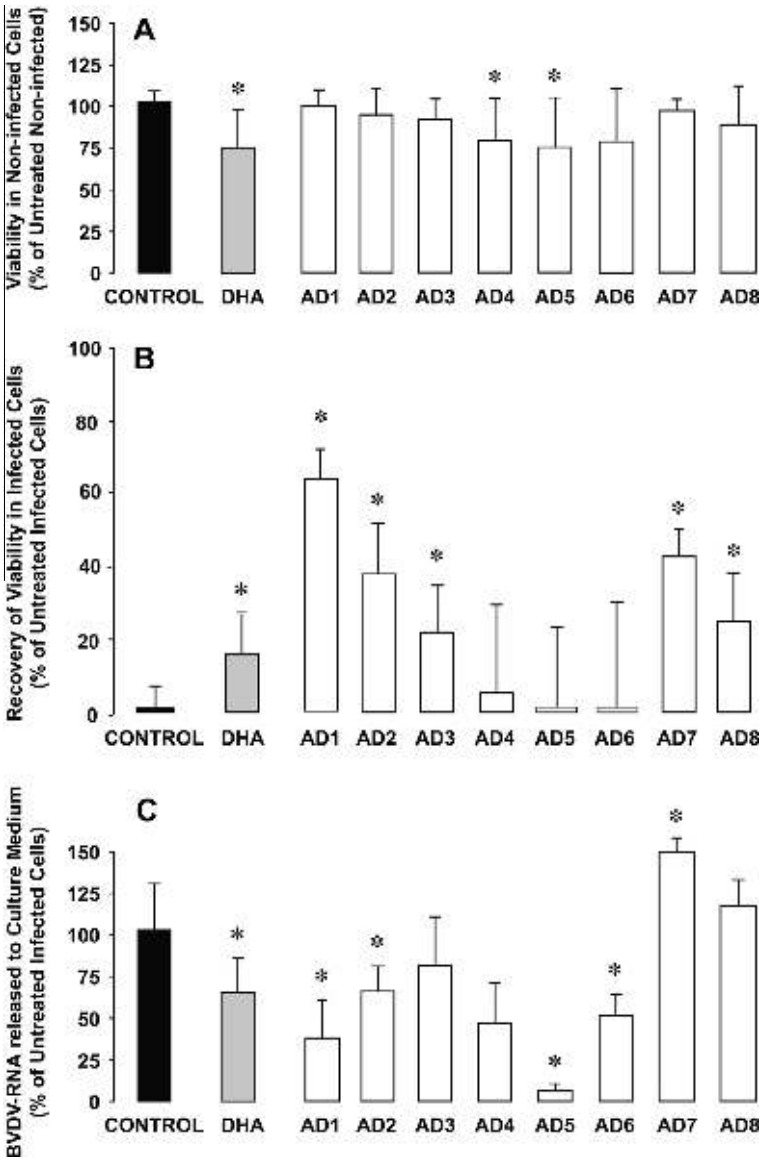


Figure 6. Anti-flavivirus effect of dihydroartemisinin (DHA) and novel artemisinin derivatives (ADs). Drug-induced toxicity was determined by measuring cell viability after incubating non-infected EBTr cells with 50 μM of drug for 72 h (A). Drug-induced protection was determined as the recovery in the viability of EBTr cells that had been previously infected by exposure to bovine viral diarrhoea virus (BVDV; cytopathic strain Oregon C24V, genotype I, subgenotype b) for 48 h before incubating with 50 μM drug for 72 h (B). Effect of treatment in infected cells with DHA and ADs on BVDV-RNA release to the culture medium (C). The abundance of BVDV-RNA was measured by real-time quantitative RT-PCR. The results were normalized by the addition to the sample of a fixed amount (77.5 pg) of an exogenous RNA (rat *Abcb11*). Values are means ± SD of 9 wells per data point obtained from 3 different cultures. $^{*}p$ <0.05 as compared with untreated control cells.

it can be suggested that the chemical properties of the R-substituent at position C10 in AD5, a propylpentanoate, could also help to increase both the alkylating properties of the molecule, once its endoperoxide bridge is cleaved, and/or to reduce the rate of DHA formation which may account for the enhanced pharmacological activity of this compound.

Regarding the effect of iron-donors on AD antiproliferative activity there are controversial data in the literature.[27,38,39] This apparent discrepancy may be due in part to differences in the expression in the in vitro models used in these studies of genes involve in heme homeostasis,[38] which are well expressed in SK-HEP-1 cells[40] used here; and to differences in the experimental design. Indeed, we have observed that the ability of ferrosanol to enhance AD5 antiproliferative activity was abolished if both compounds were combined before adding them to the culture, probably due to the lack of resulting compound to be taken up by the cells.

In previous studies we have explored the antiviral activity of ART and ARS (for a review see[14]). By reason described above, this activity could be particularly important against viruses infecting parenchyma liver cells. Indeed, using different experimental in vitro models—that is, cytophatic BVDV as an easy-to-use member of the *Flaviviridae* family[41] and the HCV replicon[42,43]—the potential interest of ART in the treatment of hepatitis C has been reported.[16,44] Interestingly, additive effect of ART to interferon and ribavirin was found.[16] In the present study AD1, AD2 and AD7 were stronger anti-BVDV agents than DHA, AD3 and AD8, whereas AD4, AD5 and AD6 were without anti-BVDV activity.

Studies on cytomegalovirus revealed that ART derivatives, such as ARS, seem to interfere with some critical points in the cell cycle regulatory process, namely NF-kB and Sp1, which are critical for the survival of the virus.[18] Moreover, the effects are increased in the presence of iron.[45] This is in agreement with results of Figure 7,

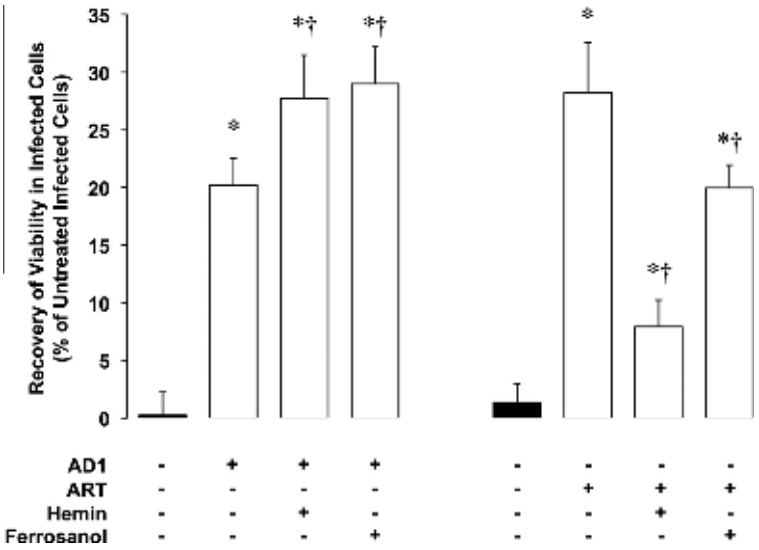


Figure 7. Effect of hemin and ferrosanol on the anti-flavivirus effect of anhydrodihydroartemisinin (AD1) and artemisinin (ART). Drug-induced protection was determined as the recovery in the viability of EBTr cells that had been previously infected by exposure to bovine viral diarrhoea virus (BVDV; cytopathic strain Oregon C24V, genotype I, subgenotype b) for 48 h before incubating with 50 μM drug in the presence or absence of 0.5 μM hemin or 2.5 μg/ml ferrosanol for 72 h. These compounds were added after preloading the cells with AD1 or ART for 1 h. Values (means ± SD) were determined by the formazan test in 12 wells per data point obtained from 3 different cultures. $^{*}p$ <0.05 as compared with untreated control cells; $^{\dagger}p$ <0.05 as compared with 50 μM of AD1 or ART.

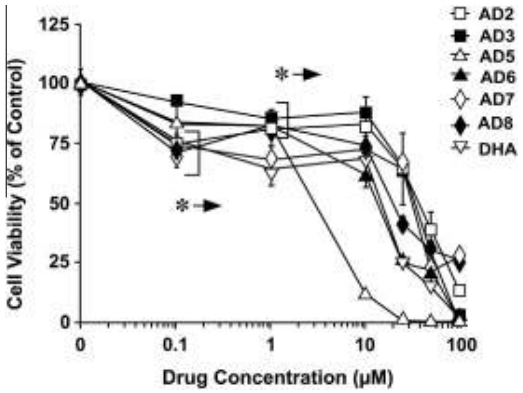


Figure 8. Toxic effect of dihydroartemisinin (DHA) and novel artemisinin derivatives (ADs) in HepG2 2.2.15 cells. These were cultured for 21 days in the presence of increasing concentrations (from 0.1 to 100 μM) of DHA, AD2, AD3, AD5, AD6, AD7 and AD8. The culture medium was replaced every 3 days by a fresh one containing the same amount of drug. On day 21, cell viability was measured by the Neutral Red retention test. Values are means ± SD of 9 wells per data point obtained from 3 different cultures. $^{*}p$ <0.05 on comparing cell viability with non-treated Control cells.

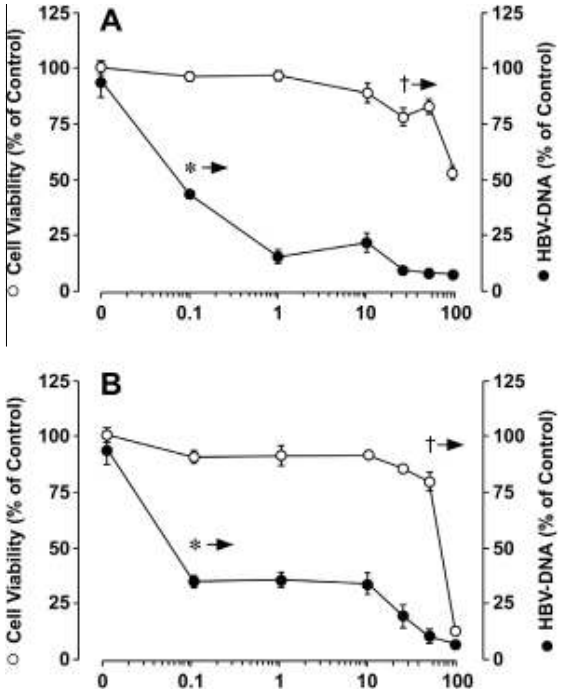


Figure 9. Toxic effect on host cells and antiviral effect on the replicative model of hepatitis B virus (HBV) in HepG2 2.2.15 cells of novel artemisinin derivatives (ADs). Effect on cell viability (open circles) and on the release of HBV-DNA to the culture medium (closed circles) after 21 days of culture in the presence of increasing concentrations (from 0.1 to 100 μM) of AD1 (A) or AD4 (B). The culture medium was replaced every 3 days by a fresh one containing the same amount of drug. On day 21, cell viability was measured by the Neutral Red retention test and HBV-DNA in the culture medium was determined by real-time quantitative PCR. Values are means ± SD of 9 wells per data point obtained from 3 different cultures. Comparisons with non-treated cells regarding HBV-DNA release ($^{*}p$ <0.05) and cell viability ($^{\dagger}p$ <0.05) were carried out.

which demonstrated that hemin improves AD1 cytoprotection against BVDV. In contrast, antiviral effect of ART was reduced in the presence of hemin (Fig. 7), which differs from previous results found by other authors using HCV replicon as an experimental model.[44] Our results indicate that antiviral effect could be associated with a strong inhibition of viral propagation (DHA, AD1 and AD2). However, protection against the cytopathic activity of BVDV was also seen even in absence of any decrease in the release of BVDV-RNA to the medium (AD3, AD7 and AD8). This suggests that in the case of some ADs the maturation of infective virions could be impaired by ADs in absence of changes in the number of BVDV-RNA copies, whereas other ADs may affect more profoundly viral life cycle by impairing genome replication. These differences in the mechanism of action could be due to the nature of a bulky group at C10 position. Thus, shorter and less hydrophilic side chain in AD1 and AD2 could determine a more effective interference with viral life cycle than longer (AD3) or more hydrophilic (AD7 and AD8) variants. Furthermore, in some cases (AD4, AD5 and AD6),

Table 2
Classification of the potential pharmacological interest of novel artemisinin derivatives (AD) to be selected for further characterization and in vivo preclinical studies

	Pharmacological interest of artemisinin derivatives		
	High	Medium	Low
Hepatoblastoma	AD5	–	AD2, AD4, AD6
Hepatocellular carcinoma	AD5 >AD2, AD3, AD4, AD6	AD1, AD8	–
Colon Adenocarcinoma	AD5	–	AD2, AD4, AD6
Viral Hepatitis B	AD1, AD4	–	–
Viral Hepatitis C	AD1	AD2, AD7	AD3, AD8

in spite the reduction of BVDV release no protection was observed, which could be due in part to the toxicity of these compounds on host cells.

Recently, the anti-HBV properties of ART and ARS have been demonstrated in an in vitro model of HepG2 2.2.15.[15] In the micromolar range (≈10 μM), similar to those described for ARS to be active against cytomegalovirus,[18] both ART and ARS were able to reduce the amount of HBsAg released to the culture medium. Interestingly, synergic effect with lamivudine was found.[15] The present results revealed that AD1 and AD4 have stronger anti-HBV activity than previously described ART derivatives. These improved characteristics could be due to the chemical properties conferred by the substituents at position C10. Thus strong antiviral properties observed can be accounted for in terms of the absence of a bulky group (AD1) or the presence of an ether group instead an ester group with a branched short chain (AD4). In addition to factors that can likely affect the activity of these compounds, such as water solubility and the ability to cross the plasma membrane of the host cells, changes in the molecular structure could influence the conversion in liver cells of the AD into the activated agent via different cytochromes[46] or could generate different types of carbon centered radicals with different therapeutic efficacies.[47]

The fact that some AD analogues were more potent than DHA itself does not rule out that these cannot be transformed into DHA to become active. Several factors may account for this difference, which may include the differential ability of the drug and prodrug to be taken up by the target cells, the differential effect of initial high concentrations versus slower release over incubation time and the marked instability of the lactol group in DHA.[12] In addition, it is also possible that these derivatives generate other active agents different to DHA by cleavage of the endoperoxide bridge.[26]

In conclusion, high pharmacological interest deserving further evaluation in animal models has been identified for novel artemisinin-related drugs potentially useful for the treatment of liver cancer and viral hepatitis B and C.

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6. Disclosure statement

The authors disclose they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence their work.

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