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Hemin potentiates the anti-hepatitis C virus activity of the antimalarial drug artemisinin

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Abstract

We report that the antimalarial drug artemisinin inhibits hepatitis C virus (HCV) replicon replication in a dose-dependent manner in two replicon constructs at concentrations that have no effect on the proliferation of the exponentially growing host cells. The 50% effective concentration (EC_{50}) for inhibition of HCV subgenomic replicon replication in Huh 5-2 cells (luciferase assay) by artemisinin was 78 ± 21 μM. Hemin, an iron donor, was recently reported to inhibit HCV replicon replication [mediated by inhibition of the viral polymerase (C. Fillebeen, A.M. Rivas-Estilla, M. Bisaillon, P. Ponka, M. Muckenthaler, M.W. Hentze, A.E. Koromilas, K. Pantopoulos, Iron inactivates the RNA polymerase NS5B and suppresses subgenomic replication of hepatitis C virus, J. Biol. Chem. 280 (2005) 9049–9057.)] at a concentration that had no adverse effect on the host cells. When combined, artemisinin and hemin resulted, over a broad concentration range, in a pronounced synergistic antiviral activity. Also at a concentration (2 μM) that alone had no effect on HCV replication, hemin still potentiated the anti-HCV activity of artemisinin.

Keywords: HCV; Antiviral; Replicon; Artemisinin; Hemin

Hepatitis C virus (HCV) is an enveloped single stranded (+) RNA virus that belongs to the genus Hepacivirus of the family *Flaviviridae* [1]. HCV causes acute and chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2]. Worldwide more than 170 million people are chronically infected with HCV and are thus at increased risk of developing serious life-threatening liver disease [3]. Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin [4]. Unfortunately, this standard treatment results in only 50–60% of the patients in a sustained virological response and is associated with important side-effects. Thus, there is an urgent need for new therapeutic strategies.

Artemisinin is a sesquiterpene lactone (Fig. 1) that was isolated from *Artemisia annua* L. or Sweet wormwood [5]. In traditional Chinese herbal medicine, infusions of Sweet wormwood were the remedy for a variety of ailments and illnesses. More detailed studies revealed that artemisinin elicits antimalarial, but has also antibacterial, anti-inflammatory, and anti-angiotensin, activity, as well as antiproliferative activity against a variety of tumor cell lines [6–8]. The various biological activities of artemisinin originate from the same molecular mechanism namely a Fe(II) Fenton reaction [9], in which the endoperoxide bridge in artemisinin is cleaved leading to the generation of reactive oxygen species (such as hydroxyl radicals, superoxide anions, and carbon-centered radicals). These radical species contribute to damaging membranes and to the alkylation of proteins. Some reports suggest that heme-iron(II) and oxidative stress are not the only mechanisms by which artemisinin exerts its antimalarial activity

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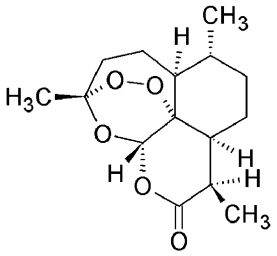

Fig. 1. Structural formula of artemisinin.

[10]. Specific inhibition of Falcipain 2 [11], a papain family cysteine protease of malaria, and the inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATP orthologue of *Plasmodium falciparum* [12] were proposed as potential targets for artemisinin.

Because of the less favorable pharmacokinetic properties, low solubility, and in order to further improve the antimalarial and antitumor activity of artemisinin, a number of first generation derivatives, i.e., artesunate, artemether, arteether, artelinate and later generation derivatives, i.e., deoxoartemisinin, artemisinin dimers, trimers, tetramers, cyanoarylmethyl-artemisinin, and others [13–15], were generated. Besides improved antimalarial and antitumoral activity, some artemisinin analogues were reported to exhibit also antiviral activity against the human cytomegalovirus (HCMV) and the hepatitis B virus (HBV) [16–18]. The anti-HCMV activity of artesunate (the prodrug of dihydroartemisinin) is probably mediated through the perturbation of cellular activation pathways (NF-κB) that play an essential role in virus replication [16]. Furthermore, it was shown that the anti-HCMV activity of artesunate can be augmented by combining artesunate with ferrous iron, i.e., Ferrosanol or holo-transferrin [17]. Similarly, the neurotoxicity of artemisinin against neuroblastoma cells can be enhanced by the addition of hemin [19,20]. Clinical data suggest that iron has a negative influence on the outcome of chronic HCV [21–23]. Iron administration was recently shown to inhibit HCV replicon replication; an effect that was explained by the fact that iron binds with high affinity in the Mg^{2+} binding pocket of the RNA-dependent RNA polymerase [24]. Thus increased intracellular iron concentrations (i) inhibit HCV replicon replication and (ii) stimulate the antiviral (and antitumoral) activity of artemisinin, and analogues. Here, we report that artemisinin inhibits the replication of HCV replicons in a concentration-dependent manner, that this inhibition is largely independent of the cytotoxic properties of artemisinin and that the antiviral activity can be potentiated by combining artemisinin with the iron donor hemin.

Materials and methods

Compounds. Artemisinin was from Sigma–Aldrich (St. Louis, USA). Hemin chloro(7,12-diethenyl-3,8,13,17-tetramethyl-21*H*,23*H*-porphine-2,18-dipropanoato(4-)-*N*(21),*N*(22),*N*(23),*N*(24))ferrate(2-)dihydrogen was purchased from Sigma (Bornem, Belgium), 2′-*C*-methyl-cytidine was synthesized by standard methods [25].

Cells and viruses. Huh 7 cells containing subgenomic HCV replicons I_{389}luc-ubi-neo/NS3-3′/5.1 (Huh 5-2) [26–29] or HuH 6 cells containing I_{341}PI-Luc/NS3-3′/Con1/ET [30] were grown in Dulbecco's modified Eagle's Medium (DMEM; Gibco, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FCS) (Integro, Zaandam, The Netherlands), 1× non-essential amino acids (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 250 μg/ml Geneticin (G418, Gibco) for Huh 5-2 cells, 1000 μg/ml G418 for HuH 6 cells.

Anti-HCV assay in Huh 5-2 cells. Huh 5-2 cells were seeded at a density of 5×10^3 per well in a tissue culture treated white 96-well view plate (Canberra, Zellik, Belgium) in complete DMEM supplemented with 250 μg/ml G418. Following incubation for 24 h at 37 °C (5% CO_2) medium was removed and 3-fold serial dilutions in complete DMEM (without G418) of the test compounds were added in a total volume of 100 μl. After 4 days of incubation at 37 °C, cell culture medium was removed and luciferase activity was determined using the Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands); the luciferase signal was measured using a Luminoskan Ascent (Thermo, Vantaa, Finland). The 50% effective concentration (EC_{50}) was defined as the concentration of compound that reduced the luciferase signal by 50%.

Anti-HCV assay in HuH 6 cells. HuH 6 cells were seeded at a density of 1×10^4 cells per well in 96-well cell culture plates in complete DMEM supplemented with 1000 μg/ml G418. Following incubation for 24 h at 37 °C cell culture medium was removed and 3-fold serial dilutions of the test compounds in complete DMEM without G418 were added in a total volume of 100 μl. After 4 days of incubation at 37 °C, cell culture fluid was removed and monolayers were washed once with phosphate-buffered saline. Cells were lysed in 100 μl Cells-to-cDNA II lysis buffer (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. Lysates were denatured for 15 min at 75 °C and diluted with 100 μl water. Thereafter, lysates were used immediately without further storage.

RT-qPCR. A 25 μl RT-qPCR contained 12.5 μl of 2× reaction buffer (Eurogentec, Seraing, Belgium), 6.3 μl H_2O, 5 μl total cellular RNA extract and in the case of HuH 6 samples 300 nmol/l neo-forward primer [5′-CCG GCT ACC TGC CCA TTC-3′], 300 nmol/l neo-reverse primer [5′-CCA GAT CAT CCT GAT CGA CAA G-3′], and 300 nmol/l neo-probe [5′-FAM-ACA TCG CAT CGA GCG AGC ACG TAC-TAMRA-3′]. The RT step was performed at 48 °C for 30 min, 15 min at 95 °C and subsequent PCR amplification of 40 cycles of denaturation at 94 °C for 20 s and annealing and extension at 60 °C for 1 min in an ABI 7000 sequence detector.

Cytostatic assay. Huh 5-2 cells were seeded at a density of 5×10^3 cells per well and HuH 6 cells were seeded at a density of 1×10^4 cells per well of a 96-well plate in complete DMEM with the appropriate concentrations of G418. Serial dilutions of the test compounds in complete DMEM without G418 were added 24 h after seeding. Cells were allowed to proliferate for 3 days at 37 °C, after which the cell number was determined by means of three different assay methods: (i) by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/phenazinemethosulfate (MTS/PMS) method (Promega), (ii) by pooling the cells from three wells and counting the cells with a Z1 series Coulter counter (Beckman Coulter, Buckinghamshire, United Kingdom), and (iii) by determining the 18S ribosomal gene content by qPCR using the 18S genomic control kit (Eurogentec). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50%.

Drug combinations. The effects of drug–drug combinations were evaluated using the method of Prichard and Shipman [31]. In brief, the theoretical additive effect is calculated from the dose-response curves of individual compounds by the equation $Z = X + Y(1 - X)$, where X represents the inhibition produced by artemisinin alone and Y represents hemin alone. Z represents the effect produced by the combination of artemisinin with hemin. The theoretical additive surface is subtracted from the actual experimental surface, resulting in a horizontal surface that equals the zero plane when the combination is additive, a surface that lies above the zero plane indicates a synergistic effect of the combination, and a surface below the zero plane indicates antagonism. The antiviral assay was carried out essentially as described above for HuH 6 cells except that

compounds were added in checkerboard format. For each compound six replicate plates were used to measure the dose–response curve of each individual compound and the combinations thereof. The data obtained from all six plates were used to calculate the theoretical additive surface and the experimentally observed surface. Prior to using the data to calculate the dose-response curves the data were analyzed for variance by the ANOVA test.

Results

Antiviral activity of artemisinin and hemin in HCV subgenomic replicon containing cells

Artemisinin (Fig. 1) inhibited HCV replication in a concentration-dependent manner in Huh 5-2 cells (Fig. 2, as monitored by measuring the luciferase activity). The 50% effective concentration (EC_{50}) of artemisinin was calculated to be 78 ± 21 μM. At a concentration of 177 μM artemisinin inhibited HCV replicon replication by 65%. None of the concentrations of artemisinin tested inhibited the proliferation of exponentially growing Huh 5-2 cells

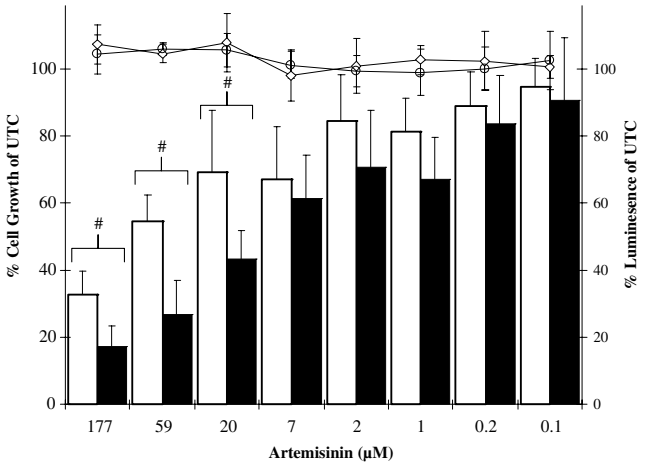

Fig. 2. Effect of artemisinin (open bars) or artemisinin combined with 2 μM hemin (filled bars) on HCV replicon replication in Huh 5-2 cells (measured as luciferase signal; bars) and the effect of artemisinin (open diamonds) or artemisinin combined with 2 μM hemin (open circles) on the proliferation of exponentially growing cells. Data are expressed as percentage of untreated controls (UTC) and are mean values ± SD of six independent experiments. $^{\#}P < 0.01$ (% luminescence obtained after treatment with artemisinin *vs* % luminescence obtained after treatment with artemisinin + 2 μM hemin).

[$CC_{50} > 177$ μM] as assessed by the MTS method. This was further corroborated by cell counting [$CC_{50} > 177$ μM] and quantifying the levels of 18S ribosomal RNA by qPCR [$CC_{50} = 124 \pm 46$ μM]. The EC_{50} of hemin for the inhibition of HCV replicon replication was 22 ± 4 μM (data not shown); the compound reduced replicon replication by 82% at a concentration of 50 μM without being cytostatic ($CC_{50} > 50$ μM). For comparative reasons the antiviral activity of a nucleoside HCV polymerase inhibitor, i.e., 2′-*C*-methyl-cytidine [25], was determined in Huh 5-2 cells. The EC_{50} value for the inhibition of HCV replicon replication by 2′-*C*-methyl-cytidine was 0.8 ± 0.2 μM and the $CC_{50} > 30$ μM. The anti-HCV activities of artemisinin and hemin were confirmed in HuH 6 cells. HuH 6 replicons have the same genetic make-up as Huh 5-2 replicons, but (i) carry different adaptive mutations, (ii) HCV replicon replication is not inhibited by IFN-γ in HuH 6 cells, and (iii) HCV replication is independent of the cell proliferation. Both artemisinin and hemin resulted in a concentration-dependent decrease in HCV RNA levels in HuH 6 cells as measured by RT-qPCR with EC_{50} values of 14 ± 21 and 4 ± 2 μM, respectively (Table 1) without being cytostatic (Table 2). A similar EC_{50} value (0.7 ± 0.5 μM) for the inhibition of HCV replicon replication by 2′-*C*-methyl-cytidine was obtained in HuH 6 cells as for Huh 5-2 cells (Table 1).

Hemin potentiates the anti-HCV activity of artemisinin

It was next studied whether hemin can potentiate the anti-HCV activity of artemisinin at a concentration of hemin (2 μM) that alone had no inhibitory effect on Huh 5-2 replicon replication (Fig. 2). Hemin (at 2 μM) resulted in a significant increase in the anti-HCV activity of those concentrations of artemisinin that had the most pronounced anti-HCV activity. Overall, the combination resulted in a 5-fold decrease of the EC_{50} value as compared to artemisinin when used alone (Table 1). The combined effect of both molecules did not result in any cytostatic or cytotoxic activities (Table 2).

Drug combinations

To further corroborate the stimulatory activity of hemin on the anti-HCV activity of artemisinin, we evaluated the combined activity over a broad concentration range of

Table 1
Inhibitory Effect of artemisinin and hemin on various HCV replicons

Cell line	Artemisinin		Hemin		Artemisinin + 2 μM hemin		2′-*C*-Me-cytidine	
	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50}(μM)	EC_{50} (μM)	CC_{50}(μM)
Huh 5-2	78 ± 21	>177	22 ± 4	>50	14 ± 4	>177	0.8 ± 0.2	>30
HuH 6	14 ± 21	>177	4 ± 2	>50	1.8 ± 0.2	>177	0.7 ± 0.5	>30

EC_{50} is the concentration of compound required to reduce the replicon content by 50% as measured by either the luciferase or the RT-qPCR assay. CC_{50} is the concentration of compound required to inhibit the exponential proliferation of the host cells by 50% as determined by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/phenazinemethosulfate (MTS/PMS) assay. Data are mean values ± SD for at least four independent experiments.

Table 2
Effect of artemisinin and hemin on host cell proliferation and 18S ribosomal RNA content

Cell line	CC_{50} (μM)								
	Artemisinin			Hemin			Artemisinin + 2 μM hemin		
	MTS	Cell counts	qPCR	MTS	Cell counts	qPCR	MTS	Cell counts	qPCR
Huh 5-2	>177	>177	124 ± 46	>50	>50	22 ± 18	>177	>177	106 ± 53
HuH 6	>177	>177	>177	43 ± 7	>50	>50	>177	>177	>177

CC_{50} is the concentration of compound required to inhibit the exponential proliferation of the cells by 50% as determined by (i) the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/phenazinemethosulfate (MTS/PMS) assay or (ii) by cell counts or (iii) the levels of 18S ribosomal RNA determined by a qPCR assay. Data are mean values ± SD for at least four independent experiments.

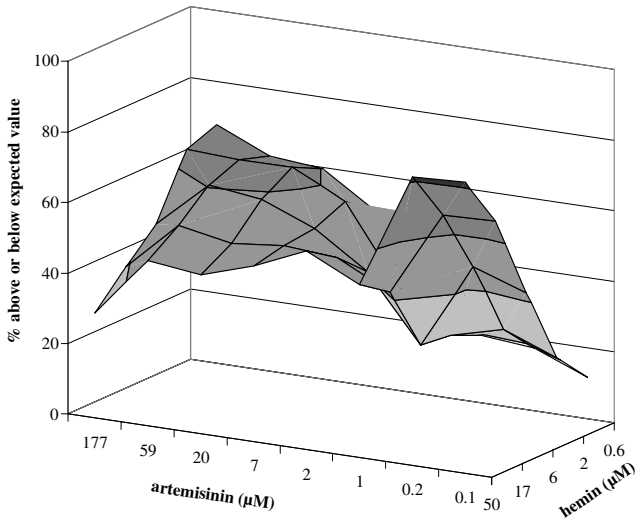

Fig. 3. Antiviral effect of drug combinations in HuH 6 cells: artemisinin (*X*-axis) and hemin (*Y*-axis). The different colors represent different ranges of values: white: 0–20%, light grey: 20–40%, grey: 40–60%, dark grey: 60–80%, and black: 80–100%. A volume below the zero plane would indicate an antagonistic effect.

both molecules (0.1–177 μM for artemisinin and 0.6–50 μM for hemin) in HuH 6 cells using the method of Prichard and Shipman [31]. All combinations resulted in an antiviral effect that was 25–80% above the expected theoretical value and thus led to a synergistic antiviral activity (Fig. 3). Prior to analysis we subjected the entire dataset to ANOVA. The dataset showed significant ($P < 0.05$) statistical interaction which indicates the dependence of the observed effect of a certain artemisinin concentration on the amount of hemin present in that particular condition.

Discussion

We report the inhibitory activity of artemisinin on subgenomic HCV replicon replication [32]. It was recently reported that (the iron sequestered in) hemin is able to suppress HCV subgenomic replicon replication by an inhibitory effect on the HCV RNA-dependent RNA polymerase [24]. Although both artemisinin and hemin when used alone exhibit in our hands a moderate antiviral effect, their combination results in a synergistic anti-HCV activity. This synergism suggests a role of iron in the anti-HCV activity of artemisinin, as is the case for the antimalarial activity of artemisinin. This peculiar mechanism of action could be advantageous in anti-HCV drugs regime. Choi et al. reported that reactive oxygen species disrupt active HCV replication complexes and rapidly inhibit HCV RNA replication in human hepatoma cells [33]. The anti-HCV activity of artemisinin may thus be directly related to the production of reactive oxygen species and alkylation of HCV proteins. HCMV replication is indirectly inhibited by artemisinin through interference with the host cell kinase cascade, i.e., NF-κB [16,17]. Expression of HCV NS5A results in an alteration of intracellular events and the constitutive activation of NF-κB [34–36]. Waris and colleagues suggested that the perturbation of the cellular environment by HCV leads to an alteration in gene expression to facilitate HCV replication [35]. It cannot be excluded that artemisinin interferes with such altered gene expression, thus hampering HCV replication.

A concern, when evaluating potential anti-HCV molecules in the replicon system, is that the activity is indirectly caused by cytostatic activities. It was shown that the HCV replicon replication rate is dependent on the metabolic state of the host cell; minor toxic effects could result in a similar reduction in replicon replication [37,38]. Since artemisinin is known to have antitumoral activity, we employed multiple methods to measure the potential adverse effects of artemisinin, hemin or their combination on the host cell. Furthermore, we employed HuH 6 cells in which HCV replicon replication is independent of the metabolic state (cell cycle) of the cells. Overall, both compounds or the combinations thereof had little or no adverse effect on the host cell.

Since both artemisinin and hemin are extensively used in the clinical setting (artemisinin is used against malaria and hemin is used in the clinical setting to correct heme deficiency in the liver and repress the production of porphyrin precursors), the use of these drugs could be considered as a supplemental therapy with interferon and ribavirin in future therapies for HCV. However, patients with cirrhosis may already have elevated iron levels [39] and intrahepatic iron could be implicated in a poor response to interferon therapy [21] and in worsening the liver damage due to HCV infection [22,23]. Hemin could thus be contraindicated in HCV-infected patients with progressive liver disease.

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References

[1] B.D. Lindenbach, C.M. Rice, Unravelling hepatitis C virus replication from genome to function, Nature 436 (2005) 933–938.
[2] F. Zoulim, M. Chevallier, M. Maynard, C. Trepo, Clinical consequences of hepatitis C virus infection, Rev. Med. Virol. 13 (2003) 57–68.
[3] Anonymous, Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium, J. Viral. Hepat. 6 (1999) 35–47.
[4] A. Craxi, A. Licata, Clinical trial results of peginterferons in combination with ribavirin, Semin. Liver Dis. 23 (Suppl. 1) (2003) 35–46.
[5] D.L. Klayman, Qinghaosu (artemisinin): an antimalarial drug from China, Science 228 (1985) 1049–1055.
[6] R.X. Tan, W.F. Zheng, H.Q. Tang, Biologically active substances from the genus Artemisia, Planta Med. 64 (1998) 295–302.
[7] T. Efferth, Mechanistic perspectives for 1,2,4-trioxanes in anti-cancer therapy, Drug Resist. Updat. 8 (2005) 85–97.
[8] T. Efferth, A. Sauerbrey, A. Olbrich, E. Gebhart, P. Rauch, H.O. Weber, J.G. Hengstler, M.E. Halatsch, M. Volm, K.D. Tew, D.D. Ross, J.O. Funk, Molecular modes of action of artesunate in tumor cell lines, Mol. Pharmacol. 64 (2003) 382–394.
[9] P.M. O'Neill, G.H. Posner, A medicinal chemistry perspective on artemisinin and related endoperoxides, J. Med. Chem. 47 (2004) 2945–2964.
[10] S. Parapini, N. Basilico, M. Mondani, P. Olliaro, D. Taramelli, D. Monti, Evidence that haem iron in the malaria parasite is not needed for the antimalarial effects of artemisinin, FEBS Lett. 575 (2004) 91–94.
[11] A.V. Pandey, B.L. Tekwani, R.L. Singh, V.S. Chauhan, Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite, J. Biol. Chem. 274 (1999) 19383–19388.
[12] U. Eckstein-Ludwig, R.J. Webb, I.D. Van Goethem, J.M. East, A.G. Lee, M. Kimura, P.M. O'Neill, P.G. Bray, S.A. Ward, S. Krishna, Artemisinins target the SERCA of *Plasmodium falciparum*, Nature 424 (2003) 957–961.
[13] S. Ekthawatchai, S. Kamchonwongpaisan, P. Kongsaeree, B. Tarnchompoo, Y. Thebtaranonth, Y. Yuthavong, C-16 artemisinin derivatives and their antimalarial and cytotoxic activities: syntheses of artemisinin monomers, dimers, trimers, and tetramers by nucleophilic additions to artemisitene, J. Med. Chem. 44 (2001) 4688–4695.
[14] C.H. Lee, H. Hong, J. Shin, M. Jung, I. Shin, J. Yoon, W. Lee, NMR studies on novel antitumor drug candidates, deoxoartemisinin and carboxypropyldeoxoartemisinin, Biochem. Biophys. Res. Commun. 274 (2000) 359–369.
[15] W. Hofheinz, H. Burgin, E. Gocke, C. Jaquet, R. Masciadri, G. Schmid, H. Stohler, H. Urwyler, Ro 42-1611 (arteflene), a new effective antimalarial: chemical structure and biological activity, Trop. Med. Parasitol. 45 (1994) 261–265.
[16] T. Efferth, M. Marschall, X. Wang, S.M. Huong, I. Hauber, A. Olbrich, M. Kronschnabl, T. Stamminger, E.S. Huang, Antiviral activity of artesunate towards wild-type, recombinant, and ganciclovir-resistant human cytomegaloviruses, J. Mol. Med. 80 (2002) 233–242.
[17] S.J. Kaptein, T. Efferth, M. Leis, S. Rechter, S. Auerochs, M. Kalmer, C.A. Bruggeman, C. Vink, T. Stamminger, M. Marschall, The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo, Antiviral Res. 69 (2006) 60–69.
[18] M.R. Romero, T. Efferth, M.A. Serrano, B. Castano, R.I. Macias, O. Briz, J.J. Marin, Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an in vitro replicative system, Antiviral Res. 68 (2005) 75–83.
[19] S.L. Smith, J. Fishwick, W.G. McLean, G. Edwards, S.A. Ward, Enhanced in vitro neurotoxicity of artemisinin derivatives in the presence of haemin, Biochem. Pharmacol. 53 (1997) 5–10.
[20] S.L. Smith, J.L. Maggs, G. Edwards, S.A. Ward, B.K. Park, W.G. McLean, The role of iron in neurotoxicity: a study of novel antimalarial drugs, Neurotoxicology 19 (1998) 557–559.
[21] D.H. Van Thiel, L. Friedlander, S. Fagiuoli, H.I. Wright, W. Irish, J.S. Gavaler, Response to interferon alpha therapy is influenced by the iron content of the liver, J. Hepatol. 20 (1994) 410–415.
[22] C. Hezode, C. Cazeneuve, O. Coue, F. Roudot-Thoraval, I. Lonjon, A. Bastie, C. Duvoux, J.M. Pawlotsky, E.S. Zafrani, S. Amselem, D. Dhumeaux, Liver iron accumulation in patients with chronic active hepatitis C: prevalence and role of hemochromatosis gene mutations and relationship with hepatic histological lesions, J. Hepatol. 31 (1999) 979–984.
[23] C. Andant, J. Lamoril, J. Edery, et al., Hepatic iron concentration (HIC) and serum iron parameters in patients with chronic hepatitis C, Hepatology 9 (1996) 497–503.
[24] C. Fillebeen, A.M. Rivas-Estilla, M. Bisaillon, P. Ponka, M. Muckenthaler, M.W. Hentze, A.E. Koromilas, K. Pantopoulos, Iron inactivates the RNA polymerase NS5B and suppresses subgenomic replication of hepatitis C Virus, J. Biol. Chem. 280 (2005) 9049–9057.
[25] S.S. Carroll, J.E. Tomassini, M. Bosserman, K. Getty, M.W. Stahlhut, A.B. Eldrup, B. Bhat, D. Hall, A.L. Simcoe, R. LaFemina, C.A. Rutkowski, B. Wolanski, Z. Yang, G. Migliaccio, R. De-Francesco, L.C. Kuo, M. MacCoss, D.B. Olsen, Inhibition of hepatitis C virus RNA replication by 2′-modified nucleoside analogs, J. Biol. Chem. 278 (2003) 11979–11984.
[26] J.M. Vrolijk, A. Kaul, B.E. Hansen, V. Lohmann, B.L. Haagmans, S.W. Schalm, R. Bartenschlager, A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C, J. Virol. Methods 110 (2003) 201–209.
[27] V. Lohmann, F. Korner, A. Dobierzewska, R. Bartenschlager, Mutations in hepatitis C virus RNAs conferring cell culture adaptation, J. Virol. 75 (2001) 1437–1449.
[28] K.J. Blight, J.A. McKeating, C.M. Rice, Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication, J. Virol. 76 (2002) 13001–13014.
[29] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.
[30] M.P. Windisch, M. Frese, A. Kaul, M. Trippler, V. Lohmann, R. Bartenschlager, Dissecting the interferon-induced inhibition of hepatitis C virus replication by using a novel host cell line, J. Virol. 79 (2005) 13778–13793.
[31] M.N. Prichard, C. Shipman Jr., A three-dimensional model to analyze drug–drug interactions, Antiviral Res. 14 (1990) 181–205.
[32] B. Sas, J. Van hemel, J. Vandenkerckhove, E. Peys, Use of endoperoxides for the treatment of infections caused by flaviviridae, including hepatitis C, bovine viral diarrhea and classical swine fever virus (2005) US20050059647 A1.
[33] J. Choi, K.J. Lee, Y. Zheng, A.K. Yamaga, M.M. Lai, J.H. Ou, Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells, Hepatology 39 (2004) 81–89.
[34] G. Gong, G. Waris, R. Tanveer, A. Siddiqui, Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B, Proc. Natl. Acad. Sci. USA 98 (2001) 9599–9604.

[35] G. Waris, K.D. Tardif, A. Siddiqui, Endoplasmic reticulum (ER) stress: hepatitis C virus induces an ER-nucleus signal transduction pathway and activates NF-kappaB and STAT-3, Biochem. Pharmacol. 64 (2002) 1425–1430.

[36] G. Waris, A. Livolsi, V. Imbert, J.F. Peyron, A. Siddiqui, Hepatitis C virus NS5A and subgenomic replicon activate NF-kappaB via tyrosine phosphorylation of IkappaBalpha and its degradation by calpain protease, J. Biol. Chem. 278 (2003) 40778–40787.

[37] T. Pietschmann, V. Lohmann, G. Rutter, K. Kurpanek, R. Bartenschlager, Characterization of cell lines carrying self-replicating hepatitis C virus RNAs, J. Virol. 75 (2001) 1252–1264.

[38] L.J. Stuyver, T.R. McBrayer, P.M. Tharnish, A.E. Hassan, C.K. Chu, K.W. Pankiewicz, K.A. Watanabe, R.F. Schinazi, M.J. Otto, Dynamics of subgenomic hepatitis C virus replicon RNA levels in Huh-7 cells after exposure to nucleoside antimetabolites, J. Virol. 77 (2003) 10689–10694.

[39] I.S. Silva, R.M. Perez, P.V. Oliveira, M.I. Cantagalo, E. Dantas, C. Sisti, C. Figueiredo-Mendes, V.P. Lanzoni, A.E. Silva, M.L. Ferraz, Iron overload in patients with chronic hepatitis C virus infection: clinical and histological study, J. Gastroenterol. Hepatol. 20 (2005) 243–248.