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# Anti-HCV activities of selective polyunsaturated fatty acids

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**Abstract**

HCV infection can lead to chronic infectious hepatitis disease with serious sequelae. Interferon-α, or its PEGylated form, plus ribavirin is the only treatment option to combat HCV. Alternative and more effective therapy is needed due to the severe side effects and unsatisfactory curing rate of the current therapy. In this study, we found that several polyunsaturated fatty acids (PUFAs) including arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) are able to exert anti-HCV activities using an HCV subgenomic RNA replicon system. The $EC_{50}$ (50% effective concentration to inhibit HCV replication) of AA was 4 μM that falls in the range of physiologically relevant concentration. At 100 μM, α-linolenic acid, γ-linolenic, and linoleic acid only reduced HCV RNA levels slightly and saturated fatty acids including oleic acid, myristic acid, palmitic acid, and steric acid had no inhibitory activities toward HCV replication. When AA was combined with IFN-α, strong synergistic anti-HCV effect was observed as revealed by an isobologram analysis. It will be important to determine whether PUFAs can provide synergistic antiviral effects when given as food supplements during IFN-based anti-HCV therapy. Further elucidation of the exact anti-HCV mechanism caused by AA, DHA, and EPA may lead to the development of agents with potent activity against HCV or related viruses.


*Keywords:* HCV; Replicon; Polyunsaturated fatty acids including arachidonic acid; Eicosapentaenoic acid; Docosahexaenoic acid

Chronic hepatitis C virus (HCV) infection, previously the most common causative agent of non-A, non-B hepatitis, is a major health problem worldwide. According to the estimation made by the World Health Organization, HCV affects 170 million individuals worldwide [1]. Although the acute phase of infection is usually associated with mild symptoms, approximate 80% of HCV infection results in chronic infection that frequently leads to severe chronic liver disease; 20–30% of infected individuals may develop cirrhosis and 1–3% may develop liver cancer [2,3]. Neither a vaccine nor specific antiviral agents are available for the treatment of HCV infection. Currently, interferon-α (IFN-α), or PEGylated IFN-α, combined with ribavirin, a guanosine analogue, is the only recommended treatment [4]. Although the combination therapy represents a major advance in the treatment of HCV, this treatment is effective only in around 50% of patients [5]. Given the high prevalence and severe clinical sequels of HCV infection, discovery, and development of molecular-based agents for HCV therapy have become a focus of intensive research. Equally important is to assess and better define the risks and benefits of alternative and nontraditional means for treating or stabilizing HCV infection [6].

HCV is a member of the family Flaviviridae. It contains a single-stranded, positive-sense RNA genome of approximate 9.5 kb encoding a unique polyprotein of approximately 3000 amino acids [7,8]. The polyprotein precursor is co- and post-translationally processed by cellular and viral proteases to yield the mature structural and nonstructural proteins arranged in the sequence of $NH_2$–C–E1–E2–P7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH. This polyprotein is consisted of at least three structural (core, E1, and E2) and seven nonstructural (NS) (p7, NS2, NS3, HS4A/B, and NS5A/B) protein coding region. It has been recently described that some HCV-encoded proteins can be produced from alternate reading frames through ribosomal frameshift [9]. The ORF is flanked

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at the 5′ end by a nontranslated region (NTR) that functions in part as an internal ribosome entry site (IRES) and at the 3′ end by a highly conserved sequence essential for replication of viral genome. In recent years, many potent antiviral agents are being developed based on viral enzymes that are essential for HCV replication.

The inability to efficiently propagate HCV in cell culture had impeded the development of antiviral agents against this virus. This obstacle was partly overcome by the development of a bicistronic subgenomic HCV replicons in Huh-7 cells [10,11]. These subgenomic replicon systems have greatly facilitated the studies of HCV replication. With the aid of the HCV subgenomic RNA replication system, we evaluated the effect of PUFAs on HCV replication. In this report, we showed that AA, DHA, and EPA are able to exert anti-HCV activity. Detailed dose-dependent studies showed that AA was effective at concentration that is achievable at normal physiological conditions. Importantly, when combined with IFN-α, AA was able to exert strong synergistic anti-HCV activity.

## Materials and methods

*Reagents and cells*. Dulbecco's modified Eagle's medium (DMEM) high glucose, Fetal calf serum (FCS), TRIZOL reagent, and G418 (geneticin) were purchased from Invitrogen (Carlsbad, CA). Arachidonic acid (AA; 20:4, n6), docosahexaenoic acid (DHA; 22:6, n3), eicosapentaenoic acid (EPA; 20:5, n3), α-linolenic acid (18:3, n3), γ-linolenic acid (18:3, n6), and linoleic acid (18:2, n6) were obtained from Cayman Chemical (Ann Arbor, MI). Oleic acid (18:1, n9), myristic acid (14:0), palmitic acid (16:0), and steric acid (18:0) were obtained from Sigma–Aldrich (St. Louis, MO). The [α-$^{32}$P]dCTP was purchased from Amersham Bioscience (Piscataway, NJ). Human hepatoma cells (Huh-7) was purchased from Japanese Collection of Research Bioresources (JCRB, JCRB0403) and Huh-7 cell clone containing HCV replicon (Ava5) was provided by Apath (St. Louis, MO). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS in a humidified atmosphere containing 5% $CO_2$. For Ava5, the culture medium was additionally supplemented with 500 μg/ml G418.

*Cytotoxicity assay*. Cell viability was determined by MTS assay that was essentially as described [12]. The principle of this convenient method is based on the conversion of tetrazolium salt (MTS) into a chromatic, soluble formazan by a mitochondria enzyme, NAD-dependent dehydrogenase, in live cells. MTS and PMS are purchased from Sigma (St. Louis, MO) or Promega (Madison, WI) as powder and prepared in DPBS (Dulbecco's phosphate-buffered saline). For a measurement with a 96-well microtiter plate, 2 ml reagent containing both MTS and PMS in the ratio of 20:1 is mixed immediately with 8 ml serum-free DMEM before adding into drug-treated cells. Drug concentration is performed with four repeats.

*Northern blotting*. Total RNA was isolated from cells using TRIZOL reagent (one-step, guanidium thiocyanate phenol–chloroform total RNA isolation reagent) according to supplier's instruction. RNA was isolated and concentration was determined by spectrophotometer. All reagents used for analysis are of ultra-pure grade. RNA samples, 10 μg each well, were loaded onto 1% TBE agarose gel and separated by electrophoresis at 10 V/cm for 1.5 h according to Kevil et al. [13]. The RNAs in the gel were then transferred to a positively charged nylon membrane, BrightStar-Plus (Ambion, Austin, TX), by a vacuum blotter (Vacu. GeneXL, Pharmacia, MI). After drying, RNA was then crosslinked to the membrane by UV irradiation (Stratagene, CA). The membrane was probed separately with the NS5B gene fragment of HCV and human glyceraldehydes-3-phosphate dehydrogenase (GADPH) fragment labeled with [α-$^{32}$P]dCTP by rediprime II random prime labeling system (Pharmacia, MI) in accordance with manufacturer's instructions. Hybridization was carried out with denatured probes in Rapid-hyb hybridization buffer (Pharmacia) for 2 h at 65 °C. After hybridization, membranes were washed once in 2× SSC–0.2% SDS for 20 min at 60 °C and once in 1× SSC–0.2% SDS for 20 min at 60 °C and twice in 0.1× SSC–0.2% SDS for 15 min at 65 °C. The results of hybridization were visualized by autoradiography.

*Synergistic statistics*. Effect of combination of drugs was evaluated using an isobologram method [14,15]. Isobologram is a generalized method for analyzing the effects of multiple drugs and for determining their additivity, synergism, or antagonism. Different doses of AA and IFN-α were combined to generate dose–response curves of inhibition of HCV replication. Cells were treated with AA (0, 0.78, 3.13, 12.5, 50, and 200 μM) and IFN-α (0, 0.78, 3.13, 12.5, 50, and 200 IU/mL) that were combined in checkerboard manner. Antiviral response for inhibitory concentration for each combination was analyzed by sigmoid regression. The resulting data were used to generate isoeffect curves (isoboles) of 50% and 90% inhibition to determine drug–drug interactions.

## Results and discussion

### *Effects of PUFAs on HCV RNA replication*

To study the effects of PUFAs on HCV replication, the HCV sub-genomic recplicon cells (Ava5) containing HCV subgenomic RNA were employed [11]. Cells were

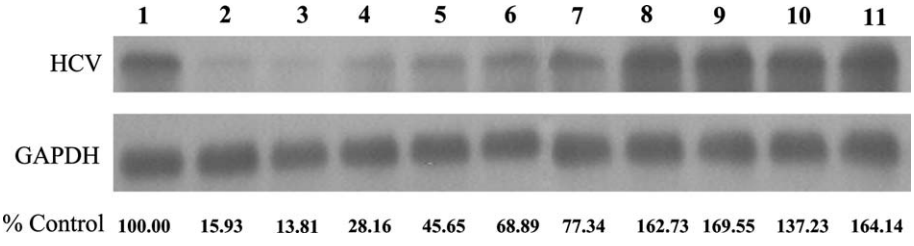


Fig. 1. Effect of fatty acids on HCV RNA replication in replicon cells containing HCV subgenomic RNA (Ava5 cells). Ava5 cells were treated with AA (lane 2), DHA (lane 3), EPA (lane 4), α-linolenic acid (lane 5), γ-linolenic acid (lane 6), linoleic acid (lane 7), oleic acid (lane 8), myristic acid (lane 9), palmitic acid (lane 10), and steric acid (lane 11) at 100 μM for 24 h. Lane 1 was mock treatment containing only solvent used for preparation of stock solutions. Cellular RNAs were extracted and analyzed by Northern blotting as described in Materials and methods. The percentage of HCV RNA remained upon each treatment is shown in the bottom of the figure.

treated with fatty acids, including polyunsaturated, monounsaturated, and saturated fatty acids, for 24 h. As shown in Fig. 1 (lanes 2–4), several PUFAs, including arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), were able to exert potent anti-HCV activities at 100 μM. At the same concentration, α-linolenic acid, γ-linolenic, and linoleic acid only reduced HCV RNA levels slightly (Fig. 1,

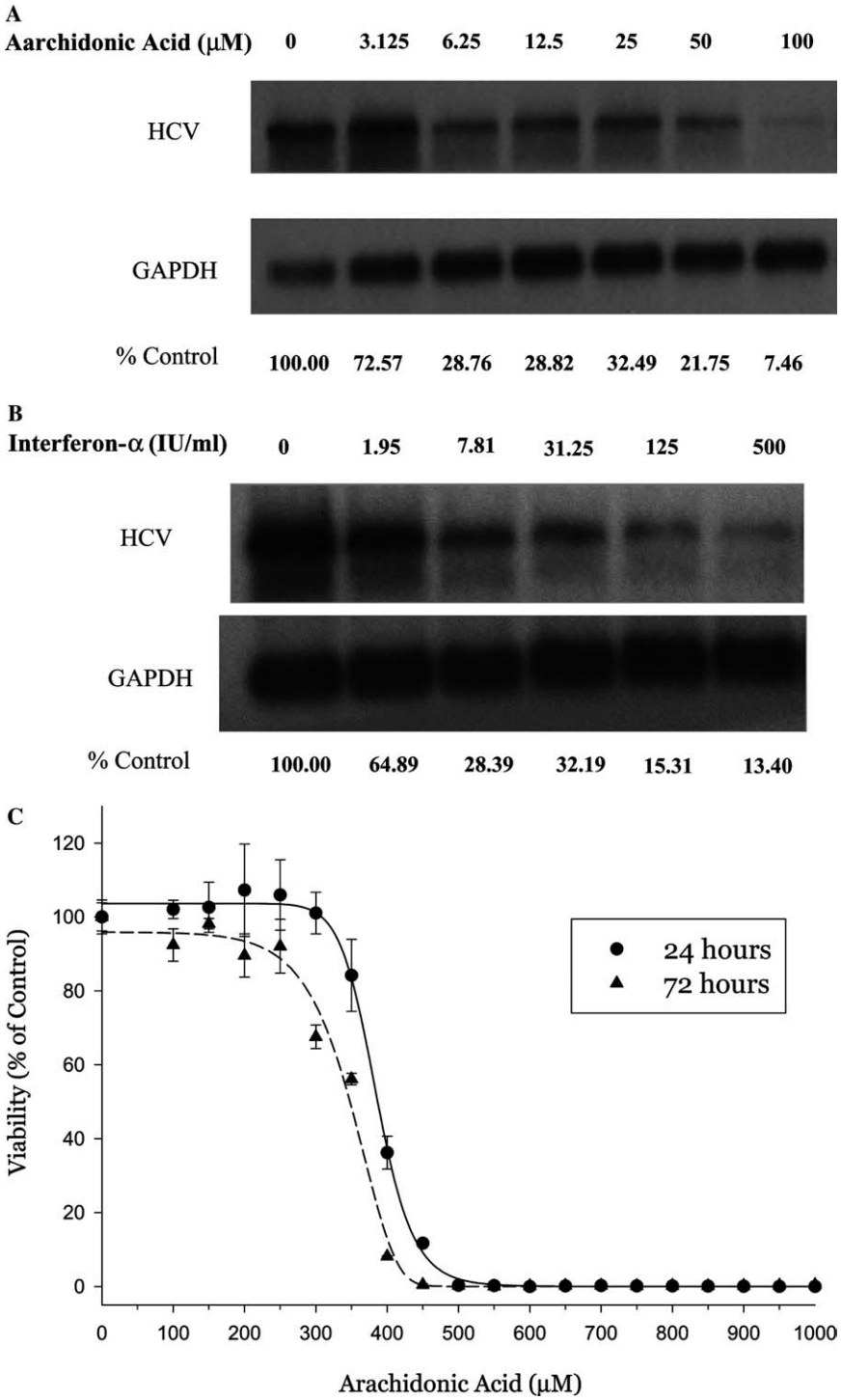


Fig. 2. Effect of AA on inhibition of HCV RNA replication and cell viability. (A) Inhibition of HCV-RNA replication by AA in a dose-dependent manner. Ava5 cells were treated with various concentrations of AA for 24 h. Total cellular RNA was extracted and analyzed for the HCV subgenomic RNA and GAPDH mRNA levels by Northern blotting. (B) As a reference standard, IFN-α was used to treat Ava5 cells and cellular RNAs were analyzed for HCV subgenomic RNA and GAPDH mRNA. (C) Viability of cells as measured by MTS assay. Cells were treated with different concentrations of AA for 24 and 72 h. Each data point was derived from six identical repeats.

lanes 5–7). In contrast, saturated fatty acids including oleic acid, myristic acid, palmitic acid, and steric acid slightly enhanced HCV RNA levels (Fig. 1, lanes 8–11). The RNA levels of GADPH, a house-keeping gene, were not affected by drug treatments.

*Arachidonic acid reduced HCV RNA level dose-dependently*

To further confirm the results, we chose to analyze the effect of AA for its anti-HCV activity in more details. Ava5 cells were treated with AA at various concentrations for 24 h. In Fig. 2A, it is clear that AA was able to suppress HCV RNA levels in a dose-dependent manner. When cells were treated with 100 μM AA, there was only 7.5% of HCV RNA left compared to untreated cells while treatment with 500 IU/mL IFN-α reduced HCV RNA level to 13.4% of the control (Fig. 2B). The $EC_{50}$ (effective concentration required to inhibit 50% of HCV RNA level) of AA is approximate 4 μM which is physiologically relevant. The plasma concentration for AA varied from 5.8 to 49.3 μM [16]. As a comparison, the anti-HCV activity of IFN-α was measured in parallel and the $EC_{50}$ of IFN-α was around 3.1 IU/ml (Fig. 2B).

There was no cellular toxicity for cells treated with AA at this range as revealed by MTS assay (Fig. 2C) and the $IC_{50}$ (concentration required to inhibit 50% of cell viability) was measured to be around 350 and 380 μM after 24 and 72 h, respectively, of drug treatment. The cellular morphology did not change after treatment with AA for 24 h at 100 μM (data not shown). Thus, AA might exert its anti-HCV effects through a specific pathway but not because of its cellular toxicity.

*Synergistic antiviral activity of AA combined with IFN-α*

Whether AA and IFN-α combination exerts synergistic, additive, or antagonistic effects was assessed by an isobologram method [14,15]. In general, representation of an isobologram to measure drug–drug interaction is shown in Fig. 3A. It was proposed that synergy, additivity, and antagonism would be represented by concave, linear, and convex isoeffective curves (isoboles), respectively. The anti-HCV effects of AA and IFN-α in combination were evaluated. Ava5 cells were treated with these two drugs in combination in a checkerboard titration manner. HCV subgenomic RNA levels in cells were then measured. Dose–response inhibition of HCV RNA replication was evaluated for varying AA concentrations (0, 0.78, 3.13, 12.5, 50, and 200 μM) in the presence of various doses of IFN-α (0, 0.78, 3.13, 12.5, 50, and 200 IU/mL) (Table 1). The data in Table 1 were used to generate isoboles of 50% and 90% inhibition of HCV replication (Fig. 3B). AA and IFN-α exerted strong synergistic anti-HCV activities as revealed by the curvy concave plots of 50% and 90% isoboles.

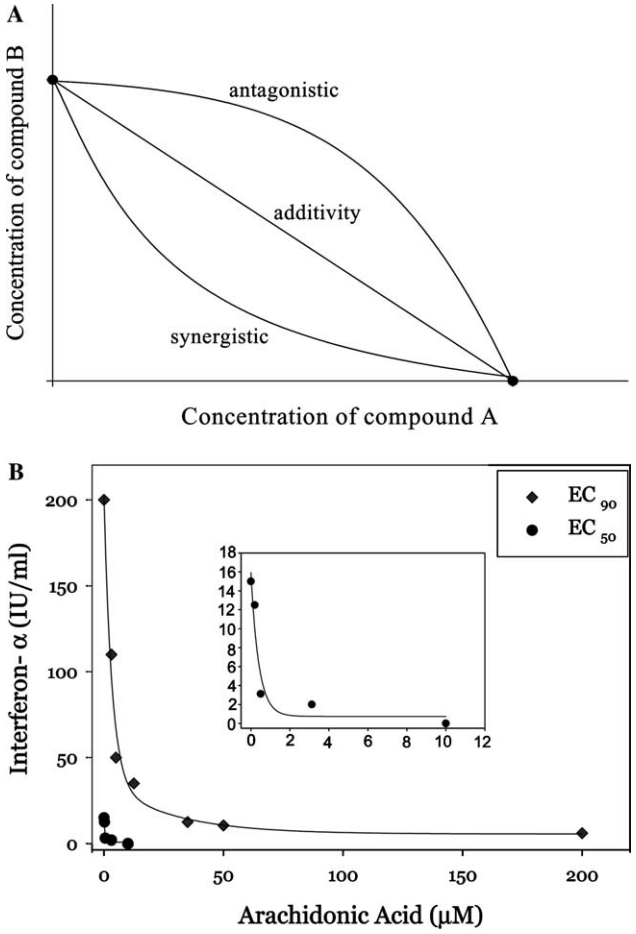


Fig. 3. Effect on HCV RNA levels by combination of AA and IFN-α. (A) In general, representation of an isobologram for measuring interaction between two drugs is shown in (A). The synergy, additivity, and antagonism would be represented by concave, linear, and convex isoeffective curves (isoboles) as shown. (B) Isoboles of 50% ($EC_{50}$) and 90% ($EC_{90}$) inhibition of HCV replication. Each data point in the isoboles was derived from interpolation of raw data from Table 1.

Current IFN-based therapy for treating HCV infection is not satisfactory and development of more effective drugs has not been very fruitful over the past few years. In fact, many HCV patients often seek other complement and alternative medicine (CAM) and some may even avoid or abandon standard IFN-based therapy and seek other types of therapy [17,18]. Many forms of CAM have shown scientific evidence as cytoprotective agents. However, few were known to possess specific antiviral activity. PUFAs such as AA, DHA, and EPA were all recognized as essential nutrients in human diet. The metabolites of PUFAs also play numerous important roles in normal physiological conditions and progression of diseases [19]. In this study, AA was found to be able to inhibit HCV replication at physiologically relevant concentration. Further research is needed to evaluate the therapeutic role of PUFAs in the clinical

Table 1
Effects of combination of arachidonic acid and IFN-α on HCV replication[a]

| IFN-α concentration (IU/mL) | Arachidonic acid concentration (μM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.78 | 3.13 | 12.5 | 50 | 200 |
| 0 | 100 ± 9.3 | 88.0 ± 8.3 | 65.8 ± 5.1 | 38.0 ± 1.1 | 17.5 ± 3.0 | 11.6 ± 0.9 |
| 0.78 | 98.6 ± 5.6 | 71.6 ± 7.3 | 63.1 ± 4.0 | 41.6 ± 4.5 | 20.4 ± 0.7 | 10.4 ± 0.3 |
| 3.13 | 56.9 ± 4.2 | 47.3 ± 3.3 | 23.6 ± 2.6 | 19.0 ± 3.0 | 15.7 ± 1.5 | 9.8 ± 0.5 |
| 12.5 | 51.0 ± 3.2 | 38.0 ± 8.3 | 24.5 ± 5.0 | 14.5 ± 0.8 | 7.0 ± 1.0 | 7.0 ± 0.4 |
| 50 | 16.9 ± 0.6 | 18.5 ± 1.1 | 12.3 ± 2.0 | 6.8 ± 0.3 | 4.2 ± 0.3 | 2.0 ± 0.08 |
| 200 | 9.8 ± 0.6 | 8.4 ± 0.4 | 6.7 ± 0.4 | 2.8 ± 0.4 | 4.1 ± 0.2 | 2.8 ± 0.1 |

[a] Relative levels of remaining HCV RNA in cells (% of control).
Results are expressed as means ± SD of three experiments.

management for HCV-infected patients. It may be possible that these fatty acids can be both as adjunctive or complementary treatment to benefit HCV patients through dietary control. In this study, we also found that antiviral activity of IFN-α can be accentuated by AA and probably also by other PUFAs. Thus, further studies are warranted if management of AA or other PUFAs through dietary control could increase the effectiveness of current IFN-based treatment as antiviral therapy.

Polyunsaturated fatty acids (PUFAs) are important for many physiologic functions [20–22]. AA, the precursor of eicosanoids, can be catalyzed by at least three types of enzymes in cells, cyclooxygenases (COXs), lipoxygenases (LOXs), and P450 epoxygenase (CYPs), to generate numerous metabolites that can mediate diverse physiological and pathological responses such as blood pressure, inflammation, phagocyte activation, pain, and fever [22–25]. The mechanism of action of PUFAs in inhibition of HCV replication is not clear. Nevertheless, this study provides a potentially favorable observation of drug–food interactions. A human trial is mandatory to understand the clinical value of PUFAs in HCV therapy. It is also important to elucidate of the exact anti-HCV mechanism caused by the PUFAs identified this study. Such understanding may lead to the development of agents with potent activity against HCV or related viruses.

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