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Manuscript #AAC00199-06 Version 3

Novel interferon alpha variant with improved inhibitory activity against HCV genotype 1 replication compared to IFN-alpha 2b therapy, in a subgenomic replicon system

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Word count: 5002 words

Number of figures: 4

Number of tables: 2

Running title: Novel interferon-α variant with improved inhibitory activity in HCV replicon system

Key Words: HCV replicon system, variant interferon-alpha, viral clearance, antiviral therapy

ABSTRACT

Hepatitis C treatment is based on the association of pegylated IFN-α and ribavirin. To improve the level of sustained virological response to treatment, especially in patients infected with HCV genotype 1, new IFNs with improved efficacy and toxicity profile may be developed. In this report we show that a novel and naturally occurring human IFN-α 17 variant, GEA007.1, which was discovered using an original population genetics-based drug discovery approach, inhibits HCV genotype 1 RNA replication more efficiently than IFN-α 2b, in the BM4-5 cell line harbouring an HCV subgenomic replicon. Moreover, we show that the complete viral clearance is obtained in BM4-5 cells after long-term treatment with GEA007.1, when HCV subgenomic RNA is still detected in cells treated with other IFN-α variants or with standard IFN-α 2b. Eventually, we demonstrate that the better inhibitory activity of GEA007.1 is likely to be due to a stronger and faster activation of the JAK-STAT signalling pathway and to a broader expression of IFN-α responsive genes in cells, compared to standard IFN-α. Our results demonstrate a superior inhibitory activity of GEA007.1 over IFN-α 2b in the HCV replicon system. Clinical trials are required to determine if GEA007.1 could be a potent 'next generation' IFN for the treatment of HCV infection, especially in non-responders or relapsing patients infected with HCV genotype 1 who currently represent a clinical unmet need.

INTRODUCTION

Hepatitis C virus infects 170 million people worldwide and leads, in approximately 70% of cases, to chronic infection (3, 24, 26). Current treatments are based on the association of pegylated-interferon-alpha 2a or 2b (IFN-α 2a or IFN-α 2b) and ribavirin (5, 8, 20). This treatment induces a sustained virological response (SVR) in approximately 60% of cases. The rate of SVR varies from 30-40% in patients infected with genotype 1, to 80-90% for those infected with genotype 2 or 3. Despite a rather good efficacy, this treatment is often poorly tolerated because of side effects (5). It is therefore important to improve current molecules and carry on searching for novel antiviral agents with an enhanced anti-HCV activity to overcome the failure of current treatments, especially in patients infected with HCV genotype 1 (4).

Until the very recent development of a complete and robust HCV replication system (19, 27, 28), hepatoma cell lines harbouring HCV subgenomic replicon were a relevant model to study some aspects of HCV biology as well as the effect and mechanism of action of antiviral agents against HCV replication (1). Various interferons, including IFN-α, β, and γ, were studied using this model, thus providing some insight on their antiviral effect (6, 7, 10, 18), and mechanism of action (6, 11, 12, 15). Overall it was shown that the transduction pathway following the interaction of exogenous IFNs with their cellular receptors was functional in most cases, although slight differences could exist between different Huh7 cell lines harbouring HCV subgenomic replicon. These differences may account for the variability observed in the amount of interferon used to obtain a given inhibition. Altogether, HCV replicon systems were validated as a suitable tool for the study of the efficacy and mechanism of action of interferon molecules. Interferon-α is known to exhibit several independent biological activities, including immunomodulatory, antiproliferative, and antiviral activities (14, 22, 23). In the context of an antiviral strategy, this pleiotropic effect of IFN-α may

account for adverse effects that are observed *in vivo* which are a major cause of either non compliance or premature interruption of the treatment (5). One possible approach to improve the currently used IFN-α is to identify novel IFN-α entities with increased antiviral activities and potentially decreased other activities.

In this study, we evaluated and compared to standard IFN-α 2b (i.e. IFN-α 2 wild-type) the inhibitory effect of original and functionally-relevant variants of different IFN-α subtypes (IFNv), against the replication of the hepatitis C virus (HCV) using a cell line harbouring a HCV genotype 1b subgenomic replicon (BM4-5 cells). One of the studied variants, GEA007.1, was found seven times more efficient than the IFN-α 2WT molecule to inhibit HCV RNA synthesis in BM4-5 cells. Interestingly, long-term treatment with GEA007.1 was able to cure BM4-5 cells from HCV replicon in contrast to the standard IFN-α 2WT molecule tested in the same conditions. Moreover, based on mechanistic studies, we propose that the increased efficacy of the novel IFN-α 17 variant GEA007.1 against HCV replication is likely to be due to a more potent activation of the JAK-STAT transduction pathway in BM4-5 cells, following the interaction of GEA007.1 with type 1 IFN receptor.

MATERIALS AND METHODS

Identification and production of IFN-α variants. Original genetic variants of 14 different human IFN-α protein subtypes were provided by GenOdyssee. Discovery of natural cytokine mutants with improved therapeutic utility was performed using the following process: a genomic DNA library was constituted with genomic DNA from 239 different individuals representing altogether 85% of the human ethnic diversity of the worldwide population, and screened for non-synonymous single nucleotide polymorphisms in the coding sequence of the 14 different human IFN-α gene subtypes. The identification and selection of IFN-α protein mutants with functionally-relevant amino-acid mutations, was then carried out using bioinformatics tools, including sequence/structure analysis and molecular modelling. IFN-α variants (IFNv), named thereafter GEA007.1, GEA009.2, GEA011.1, GEA013.1, derived from such procedure, are respectively natural mutants of the human IFN-α 17, 21, 5, and 7 (SwissProt primary accession numbers P01571, P01568, P01569, P01567). All carry a unique amino-acid mutation (G45R for GEA007.1, D95N for GEA013.1, C122S for GEA011.1, and K179E for GEA009.2), with changes in three-dimensional structure and electrostatic isopotentials at the receptor-binding surface, based on molecular modelling. The recombinant human IFN-α variants (IFNv) used in this study were produced in the methylotropic yeast, *Pichia pastoris*, with a yield of approximately 10-20 mg/l, following protocols and recommendations provided by Research Corporation Technologies, Inc. IFNv proteins were secreted into the culture medium and purified using Cibacron and ion exchange chromatography to obtain pure preparations of the recombinant human IFNv in a phosphate buffered solution (PBS). The purity of each preparation was greater than 95% and endotoxin content, determined by the LAL test, was less than 5IU/μg. IFN-α 2b, named IFN-α 2WT thereafter, in order to be an relevant control was synthesized and purified using the same process. In our experiments 1mg of IFN-α produced in yeast was equivalent to 3x10e8

international unit (IU), (i.e. 1 IU is equivalent to 3.33 pg). Stock solutions were stored at -80°C. Dilutions of these stock solutions were done in PBS extra-temporally. Prior to activity testing of the different IFN preparations against HCV in the replicon model, the different IFN preparations were tested for their specific activity and standardized *in vitro* using a gene-reporter assay as described elsewhere (17). It was shown that the inhibitory activities of IFN-α 2WT produced in *Pichia Pastoris* and manufactured IFN-α 2b (Intron A®, Schering Plough Corporation) produced in *E. Coli* are similar, indicating that the yeast *Pichia Pastoris* production system does not affect the activity of IFN-α.

Cell Culture. BM4-5 cells, a Huh7 cell line harbouring an HCV genotype 1b subgenomic replicon, that were kindly provided by Dr C. Seeger (Fox Chase Cancer Center, Philadelphia, USA), were cultured in DMEM medium (Invitrogen) complemented with 10% FCS (Foetal Calf Serum; Perbio), antibiotics, and 500 µg/mL of geneticin (G418 Sulfate; Invitrogen) as previously described (10).

Protocols of administration of IFN. BM4-5 cells were seeded in 6-well plates at a density of 2.5x10e5 cells/well, sixteen hours before starting treatments. IFNv (GEA007.1, GEA009.2, GEA011.1, and GEA013.1) and IFN-α 2WT were administrated to cells in complete medium in the absence of geneticin. For short-term treatments, the administration of each drug (at concentrations ranging from 0 to 3333 pg/ml) was renewed every day for three consecutive days. For long-term treatments, IFN-α 2WT or GEA007.1 was administered every day at 333 pg/mL for 21 days. In order to maintain cells in proliferation/division, cells were trypsinized and diluted four times every four days. A total of 5 passages was performed during treatment. At the end of this period of treatment, the administration of IFN-α 2WT and GEA007.1 was interrupted and cells were kept for 5 further passages in medium containing or not geneticin to monitor a potential rebound of HCV subgenomic RNA replication.

Analysis of IFN toxicity. Cells were seeded in 96-well plates at a density of 12,500 cells/well. They were treated by IFNs with the same concentrations and conditions than those used for short-term inhibitory assays. The cell viability was measured by both neutral red and MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) tests. The cytotoxic concentration 50% (CC_{50}) is defined as the concentration of interferon leading to a 50% reduction in the absorbance value compared to non treated cells.

Analysis of HCV subgenomic RNA synthesis. Total RNA was extracted with the "Extract all" reagent according to manufacturer's instructions (Eurobio). Northern Blot analysis was then performed using the NorthernMax™-Gly kit according to manufacturer's instructions (Ambion). Briefly, 5 µg of total RNA were denaturated in glyoxal buffer at 50°C for 30 minutes, separated on 1,1% agarose gel, and transferred over night by capillarity onto a charged nylon membrane (HybondN+, Amersham). Hybridization was carried out with three different [^{32}P]CTP-labelled riboprobes obtained by *in vitro* transcription (Riboprobe in vitro transcription system, Promega). Two probes complementary to the NS5A region were used to detect either the negative or positive HCV subgenomic RNA strands. A third probe was complementary to the β-actin mRNA and obtained by *in vitro* transcription using the plasmid pTRI beta actin human (Ambion). First, the blot was hybridized for 16 hours at 68°C with the two riboprobes directed against the negative strand of HCV subgenomic RNA and β-actin mRNA, respectively. After hybridization, the membrane was washed twice in stringent conditions, then exposed sequentially to X-ray film and a phosphor screen for quantification. The amount of β-actin mRNA was used as an internal loading control to standardize the amount of HCV subgenomic RNA detected. The same membrane was subsequently hybridized with the negative sense riboprobe to visualise and quantify the level of positive HCV subgenomic RNA strand, using the same procedure.

Analysis of the signal transduction pathway induced by IFN-α treatment in BM4-5 cells. To evaluate the activation of intracellular signaling pathways by IFN-α 2WT and IFNv, BM4-5 cells (75x10e3 cells/well in 24 wells plate) were transfected with an Interferon Stimulating Response Element (ISRE) -luciferase plasmid (Stratagene) by using the TransIT-TKO reagent (Mirus) following the manufacturer's recommendations. Twenty-four hours after transfection, duplicate cultures in 24 well plates were either left untreated or treated for 24 hours with a range of IFN-α 2WT and IFNv of 0.3, 3, 33, 333 and 3333 pg/ml. Cells were then washed with cold phosphate-buffered saline (PBS), lysed by passive lysis buffer (Promega) and the luciferase activity measured as recommended by the manufacturer (Promega). The luciferase activity induced by IFN-α 2WT for each concentration was taken as 100% and other luciferase activity were normalized accordingly.

Western Blot analysis. IFN-α 2WT or GEA007.1 were administered at a concentration of 3333 pg/mL or at 33 pg/mL on BM4-5 cells for 30 min, 1 h, 2h, 4h, 8h, 12h, 24h, 48h, and 72h. Administration was renewed each 24h. At the indicated time, cells were washed twice with cold PBS, then lysed using a buffer (0,2% NP40, 150 mM NaCl, 20 mM Tris, 2 mM EDTA, 0,1% glycerol, 10 mM dithiothreitol) containing serine/threonine phosphatases inhibitors (50 mM NaF, 200 µM orthovanadate), and protease inhibitor (complete protease inhibitor, Roche). Proteins of cell lysates were denaturated at 100°C in Laemmli buffer (50 mM Tris pH 6,8; 2% SDS; 10% glycerol; 0,1% bromophenol blue; 1.25% 2-mercaptoethanol), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). Non-specific binding sites were blocked for 2 h at room temperature (RT) with 5% milk, 0,1% Tween 20 in PBS. The membrane was then incubated overnight at 4°C with monoclonal antibodies raised against PKR (Santa Cruz Biotechnology), p48 (Beckton Dickinson) or β-actin (Sigma). Membranes were then washed three times, and

incubated at RT for 1 hour with horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulins (Immunotech) at 1/10 000. Bound antibodies were detected using ECL technologies, ECL+ for p48 and ECL for PKR and β-actin, as specified by the manufacturer (Amersham Biosciences).

Analysis of IFN-α specific gene expression using GEArray™ Q Series Array. BM4-5 cells were incubated in 6 well plates at 80% confluence. The cells were either left untreated or were incubated with IFN-2WT or GEA007.1 at the same concentration of 33 pg/mL. After 8 hr of incubation, total RNA was extracted with Extract all reagent (Eurobio) and stored at -80°C. For one set of experiment, six arrays (GEArray™ Q series Human Interferon α, β Response Gene Array–SuperArray, Bioscience Corporation) were used following manufacturer's recommendation. For each array, 96 gene-specific cDNA fragments are printed on a 3,8 x 4,8cm nylon membrane. The list of the genes is accessible on the SuperArray web site (www.superarray.com; Ref. HS-054). A total of 3µg of each RNA sample was used as a template to generate cDNA by reverse transcription. cDNA was then amplified and labelled with biotin-16-dUTP (Roche) by a Linear Polymerase Reaction (LPR) (AmpoLabeling-LPR Kit). After a step of pre-hybridization, the membranes were hybridized overnight with the different probes, corresponding to the different treatments. The signal was revealed by chemiluminescent reaction using alkaline phosphatase-conjugated streptavidin, and detected by a scanner camera (Biorad). All the results were obtained from the analysis of non saturated images, after 2 min of exposure. Spot quantification was performed with Quantify-One software (BioRad). The mean intensity of blank spots served as negative controls and was substracted from other values. The mean intensity of β-actin gene served as a positive control and was used to normalize intensities of the spots to allow relative comparisons between the different arrays. To determine which genes were up regulated, intensities of spots resulting from hybridization with cDNA obtained from total RNA extracted from untreated cells were

used to establish the basal expression level. The up-regulation of genes under interferon treatment was reported as positive fold increase compared to untreated control samples.

RESULTS

Identification of novel IFN-α variants with improved inhibitory properties. Based on the hypothesis that natural evolution of the human genome has likely created in human populations a series of unpredictable and functionally-relevant genetic variants with various structural and biological properties, non-synonymous single nucleotide polymorphisms were searched in the genomes of 239 individuals representing altogether 85% of the current human population ethnic diversity to identify interesting novel alleles of IFN-α genes coding for IFN-α proteins with superior or novel therapeutic properties, compared to standard IFN-α 2a or 2b. Several variants of IFN-α 17, 21, 5, and 7, referred thereafter as GEA007.1, GEA009.2, GEA011.1, and GEA013.1 (IFNv), were selected following this approach, for their significantly improved (GEA007.1, GEA013.1) or decreased (GEA009.2, GEA011.1) inhibitory properties, compared to standard IFN-α 2WT, based on preliminary data obtained with both an *in vitro* model of vesicular stomatitis virus (VSV)-infected WISH cells and an *in vivo* model of EMCV infected-mice (data not shown, manuscript in preparation by Tovey *et al.*).

Anti-HCV effect of novel IFN-α variants. To evaluate and quantify the inhibitory effect of these novel IFNv, BM4-5 cells were treated for three days with increasing concentrations of GEA007.1, GEA009.2, GEA011.1, GEA013.1, as well as with IFN-α 2WT used as reference. Total RNA was extracted and subjected to Northern Blot analysis with three different riboprobes designed to detect both negative and positive HCV subgenomic RNA strands and β-actin mRNA (Figure 1). A dose dependent reduction in the amount of HCV subgenomic RNA was observed with all IFNv tested, and the greatest effect was obtained with GEA007.1. Three independent experiments were performed to obtain statistically relevant data. The quantification of the relative intensity of HCV subgenomic RNA and control β-actin signals

after exposure to phosphor screens allowed the calculation of the effective concentration to inhibit 50%, defined as the concentration required to induce a reduction of 50% of the amount of HCV subgenomic RNA detected by Northern Blot after normalisation, for each IFN tested (Table 1). Two IFNv, GEA007.1, and GEA013.1 to a lesser extent, had a better EC_{50} than IFN-α 2WT, suggesting higher efficacy of these molecules compared to control. The better inhibitory activity of GEA007.1 (x7) and GEA013.1 (x1.5) over IFN-α 2WT remained significant when the drugs EC_{90} were concerned (Table 1). This inhibitory activity was not due to toxicity as all IFNs tested did not show any cytotoxic effect on the BM4-5 cells, with CC_{50} (i.e. cytotoxic concentration 50%) always superior to 3333 pg/mL, the highest dose used in our experiments (Table 1).

Long-term administration of GEA007.1 allows to cure cells from HCV genotype 1b replicon subgenome. Having established that GEA007.1 had the best inhibitory activity compared to IFN-α 2WT, with a very good EC_{90}, we asked whether a prolonged administration of this IFNv could induce a better sustained inhibitory effect than IFN-α 2WT and lead to the rapid eradication of HCV replication in cells. BM4-5 cells were treated during 20 days though 5 passages, with either IFN-α 2WT or GEA007.1 at a single concentration of 333 pg/mL in medium without geneticin as described in materials and methods. The results of a Northern Blot analysis presented in Figure 2 shows that HCV subgenomic RNA synthesis was inhibited more intensively by GEA007.1 than IFN-α 2WT, thus confirming the better inhibitory activity of GEA007.1. Moreover, after 20 days of treatment and 5 passages, HCV subgenomic RNAs (both strands) were no more detectable in cells treated with 333 pg/mL of GEA007.1, whereas the positive strand HCV subgenomic RNA was still detectable in standard IFN-α 2WT treated cells. To monitor the potential rebound of HCV replication after cessation of treatment, cells were kept untreated for at least 4 additional passages either in absence or presence or geneticin. The presence of geneticin was meant to select back more

efficiently HCV replication from the very low level of residual replication after 20 days of treatment with IFN. In the absence of geneticin, HCV subgenomic RNA remained undetectable after 5 passages post-treatment in GEA007.1 treated cells, whereas a low amount of positive strand HCV subgenomic RNA was maintained in IFN-α 2WT treated cells (Figure 2). When cells were cultured in a medium with geneticin after treatment cessation, the cells that had been treated with IFN-α 2WT could survive, but it took five more days for these cells to reach confluence, compared to untreated cells. Interestingly, cells that had been treated with GEA007.1 were no more able to survive in the presence of geneticin, indicating that GEA007.1 was able to cure the cells from HCV subgenomic RNA (Figure 2). In our experimental conditions, GEA007.1, but not IFN-α 2WT, was able to eliminate HCV replication from BM4-5 cells, thus indicating once again the better efficacy of this variant molecule over the wild type.

Stronger and faster expression of proteins of type 1 IFN signal transduction pathway in BM4-5 cells is obtained with GEA007.1, compared to IFN-α 2WT. Having established that GEA007.1 had better anti-HCV properties than IFN-α 2WT in the replicon system, we sought to determine whether the higher activity of GEA007.1 was associated with a better activation of key cell signalling proteins involved in type 1 IFN transduction pathways. To this end two set of experiments were performed. First, BM4-5 cells were transfected with a reporter plasmid containing an Interferon Stimulating Response Element upstream driving the expression of the firefly luciferase. Twenty-four hours after transfection, culture were either left untreated or treated with a range of IFN-α 2WT and IFNv of 0.3, 3, 33, 333 and 3333 pg/ml. Luciferase activity was measured 24 hours after and results obtained with IFNv for each concentration were normalized to IFN-α 2WT induced activity (Figure 3). The results showed an increased activation of the IFN stimulating pathway with GEA007.1 compared to the others IFNs with the following order GEA007.1> GEA013.1 > IFN-α 2WT = GEA011.1

> GEA009.2. A 3 and 2 fold increased activation of the IFN signaling pathways was observed with GEA007.1, by comparison with IFN-α 2WT, at the concentrations of 33 and 333 pg/ml respectively. At higher concentrations, i.e. 3333 pg/ml, the difference of activation was not significant most likely because of the saturation of the IFN receptor. Further experiments were therefore performed with low IFN concentrations of 33pg/ml (± equivalent to 10 IU) to clearly see differences between IFN-α 2WT and GEA007.1. Western Blot analyses were performed to further characterize the expression of proteins involved in the IFN signaling pathway. BM4-5 cells were treated with either GEA007.1 or IFN-α 2WT at 33 pg/mL for 30 minutes, 1, 2, 4, 8, 12, 24, 48, and 72 hours and proteins were extracted and subjected to Western Blot analysis, using antibodies directed against p48 (IRF-9) and PKR, two important proteins of the type 1 IFN transduction pathways (25). With these conditions, GEA007.1 triggered a faster and stronger expression of PKR and recruitment of p48 proteins in BM4-5 cells compared to IFN-α 2WT. The difference in activation of IFN inducible proteins was more marked for p48. In our experimental conditions, the low dose of IFN-α 2WT do not allow to visualize a significantly increase of expression of p48 and PKR proteins (Figure 4).

IFN responsive genes expression is stronger and broader after treatment with GEA007.1 than after treatment with IFN-α 2WT. To broaden results obtained by Western blotting analysis and gain a global view of the differences in transduction pathway activation activities elicited by GEA007.1 and IFN-α 2WT in BM4-5 cells, the expression of IFN-α-stimulated genes (ISGs) were analysed using cDNA expression arrays. BM4-5 cells were left untreated or treated with either 33 pg/mL of GEA007.1 or IFN-α 2WT for 8 hours. RNA were then extracted and used for RT-PCR. Labelled cDNA were used for the hybridization on arrays as described in materials and methods. Relative intensities, for the different genes and conditions of treatment, were calculated, and results from significant up-regulation in BM4-5 cells induced by the administration of either IFN are reported in Table 2. A more than 2 fold

increase in gene expression was considered as significant. Two IFN-responsive genes, MX1 and ISG56 (25), were found significantly up-regulated following IFN-α 2WT treatment, while a stronger and a broader up-regulation of genes (MX1, ISG56, ISG15, ISG60, ADAR, KIAA1268, STAT1) was found associated with GEA007.1 inhibitory activity in BM4-5 cells. The dose of IFN-α 2WT chosen in this experiment to point out differences with GEA007.1 was not high enough to trigger strong activation of gene expression, in particular for PKR and p48, at least in our experimental conditions. This result is in good correlation with the lack of increase of protein amount observed by western blotting (Figure 4). Collectively, these results confirm a better activation of type 1 IFN signal transduction in these cells with GEA007.1, compared to IFN-α 2WT at the same concentration and time of exposure. This better ability to induce a stronger, faster and broader cell signalling response was therefore associated with a superior inhibitory property of GEA007.1 over that of IFN-α 2WT observed in cells harbouring the HCV genotype 1b replicon.

DISCUSSION

While waiting for the development and clinical use of new anti-HCV molecules, interferon alpha remains a major component of the anti-HCV therapy. Its use may also remain mandatory in combination with the novel HCV inhibitors to prevent resistance to the latter drugs (8). However, currently used interferon-α 2a and 2b are not efficient in all cases. Non responding and relapsing patients are observed with current pegylated IFN-α /ribavirin combination therapies. Many factors may explain this resistance of the virus to short- or long-term viral clearance activity of IFN-α 2, including host genetic and virological factors. For instance, it is well known that patients infected with HCV genotype 1 have a significantly lower response rate (5, 6), and that genetic host factors may influence the response rate to interferon based therapies (2, 16).

The main objective of this work was to determine whether better anti-HCV genotype 1 IFN molecules, compared to clinically used interferon alpha 2a and 2b, could be identified amongst the novel interferon-α variants, GEA007.1 and GEA013.1, with improved inhibitory properties on VSV and EMCV (manuscript in preparation, Tovey *et al.*,). For this study, IFN-α 2b (IFN-α 2WT) served as standard IFN-α and additional IFN-α variants with low inhibitory properties, GEA009.2 and GEA011.1, were included as negative controls. GEA007.1, GEA013.1, GEA009.2, and GEA011.1 were all identified following an original approach of natural genetic variation of cytokine genes. Basically, the genomes of 239 individuals representing altogether 85% of the human ethnic diversity were searched for new and natural functionally-relevant genetic mutants of the different human interferon-α subtypes and variants with potential superior activities compared to IFN-α 2 were selected. BM4-5 cells, a Huh7 cell line harbouring an HCV genotype 1b subgenomic replicon, were used to test the inhibitory properties of IFN-α variants. Interestingly, among the four interferon

variants tested in this study, GEA007.1 and to a lesser extent GEA013.1, were found significantly more potent than IFN-α 2WT for inhibiting the replication of an HCV genotype 1b subgenomic replicon in BM4-5 cells. GEA007.1 was the best molecule tested with an EC_{50} of 22.7 pg/mL, which is 7 times lower than the EC_{50} obtained with standard IFN-α 2WT (i.e. 154.2 pg/mL) in the same conditions (Table 1). Moreover, we found that GEA007.1, administered at 333 pg/mL every day for 20 days, was able to clear HCV subgenomic replicon from BM4-5 cells, while HCV subgenomic RNA was still detected in cells treated with standard IFN-α 2WT in the same conditions (Figure 2). This result demonstrates the superiority of GEA007.1 over interferon-α 2b in an HCV genotype 1b subgenomic replicon system. However, the results obtained in the HCV subgenomic replicon system may not translate into antiviral activity *in vivo*. Therefore, our results warrant further evaluation of GEA007.1 *in vitro*, using systems allowing a complete multiplication of HCV (19, 27, 28), and *in vivo*, in the frame of clinical trials.

The biological activity of IFN-α is related to its capacity to induce type1 IFN signal transduction pathways after interaction with its cellular receptor. Different mechanisms have been proposed to explain the inhibitory effects of IFN-α against HCV replication, including non-cytopathic and cytopathic processes (12). It was shown that the *in vitro* non cytopathic inhibition of HCV RNA replication by IFN-α in Huh7 cells is dependent on functional JAK-STAT pathway and proteasome. However, the dsRNA-dependent antiviral pathways, such as PKR and Rnase L pathways, were shown not to be involved in IFN-induced inhibition of HCV replication in Huh7 cells (12). The IFN-α induced antiviral program could i) affect the HCV IRES-directed translation and therefore cause a reduction in viral protein, and ii) could in turn inhibit viral RNA amplification as a result of a tight linkage between the translation, assembly of replication complexes and viral RNA synthesis (11). Interestingly, using an ISRE reporter assay, we showed an increased activation of the IFN stimulating pathway by

GEA007.1 over IFN-α 2WT, which was associated with a better inhibitory activity on HCV subgenomic RNA synthesis. Then we analysed key cell signalling proteins involved in type 1 IFN-transduction pathway after BM4-5 cells exposure to GEA007.1 or IFN-α 2WT. Western Blot analyses were performed to monitor the level of expression of some key proteins like p48 or PKR, and cDNA expression array experiments were performed to monitor the expression of IFN-α responsive genes. GEA007.1 induced a stronger expression of p48 than IFN-α 2WT. This result suggests that p48 may be recruited more efficiently by the $STAT1^P$ and $STAT2^P$ heterodimer complex to form the ISGF3 complex (25), after treatment with GEA007.1 than after treatment with IFN-α 2WT. Our results are in accordance with previous works showing that type 1 IFN signalling pathways are qualitatively functional in Huh7 and BM4-5 cell lines, as persistent HCV replication does not modify gene expression patterns after IFN-α treatment (13, 21). In addition, in our experimental conditions, IFN-α responsive genes activated in Huh7 cells harbouring HCV subgenomic replicon by both GEA007.1 and IFN-α 2WT did not differ from those described and reported in other studies (13, 21). Lastly, a stronger and broader expression of IFN-α responsive genes was seen in BM4-5 cells treated with GEA007.1, compared to IFN-α 2WT. Amongst most activated genes were found Mx1, ISG56, ISG15, ISG60, ADAR, KIAA1268, and STAT1. Mx1 (also named MxA) was the most induced gene in our system. Mx1 was also found previously as an important IFN-α inducible gene in IFN-α-treated replicon cell lines (13). A stronger expression of Mx1 was observed in cells treated with GEA007.1 which in turn could have contributed to the better inhibitory effect of GEA007.1 compared to IFN-α 2WT. In view of the results obtained by others, showing the absence of inhibition of HCV RNA replication by Mx1 (6), it might be hypothesized that the activation of the different genes described can be only a reflect of the activation of the Jak-Stat pathway, without predicting a direct inhibitory role for a specific gene. Moreover, it is not possible at this stage to rule out the possibility that

GEA007.1 may also induce unknown IFN inducible genes responsible for an enhanced inhibitory activity. The ability of GEA007.1 to trigger a strong antiviral response in HCV infected BM4-5 cells is most likely due to either a better interaction of this IFN to the cellular receptor or a better activity of the complex IFN–receptor in transmitting the antiviral signal. Affinity of IFN to its receptor is a major issue in terms of efficacy. For instance, it was shown that different pegylation patterns of IFN could lead to different efficacy of binding to its cellular receptor and activation of the corresponding cell signalling pathway (9). One may hypothesize that GEA007.1 has a better affinity to the type 1 IFN receptor (IFNAR) and/or that the complex formed between GEA007.1 and IFNAR has a better capacity of signal transduction than the complex formed between IFN-α 2 and IFNAR. Work is in progress to confirm this hypothesis. The difference in binding properties of GEA007.1 compared to standard IFN-α 2WT could be due to the differences observed in the amino acid sequence of GEA007.1. In particular, GEA007.1 possesses a unique G45R mutation with significant change in three-dimensional structure and electrostatic isopotentials at the receptor-binding site, compared to IFN-α 17WT protein or IFN-α 2WT protein.

In conclusion, this study shows that the IFN-α variant GEA007.1 exhibits a more potent and sustained inhibitory activity on HCV genotype 1b subgenomic replicon replication than standard IFN-α 2b. This enhanced inhibitory activity is associated with a more efficient induction of the JAK STAT cell signalling pathway. These results warrant further evaluation to determine if this enhanced activity in the HCV subgenomic replicon system could translate into a clinical benefit, especially in naïve HCV genotype 1b infected patients who do not respond well to the currently available IFN-α2/ribavirin combination therapies as well as in non responder patients who represent a growing population of difficult to treat patients. Clinical trials are required to determine the potential of GEA007.1 to fulfil this unmet clinical need. It will be also interesting to see the inhibitory effect of GEA007.1 on other HCV

genotypes both *in vitro* and *in vivo*. Furthermore, an IFN-α variant with an enhanced anti-HCV genotype 1 activity may be of utmost clinical relevance in the perspective of the new generation treatment regimens, combining IFNs with specific small molecule HCV inhibitors in current development (4).

ACKNOWLEDGEMENT

This work was supported in part by a grant of the European Community and is part of the activities of the European network of excellence viRgil (contract LSHM-CT-2004-503359).

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Tables

Table 1: Inhibitory features and toxicity of various interferons

	EC_{50} (pg/mL)	p[a]	EC_{90} (pg/mL)	p[a]	CC_{50} (pg/mL)
IFN-α 2WT	154.2 ± 8.8		3080 ± 406		>> 3333
GEA007.1	22.7 ± 9.4	<0.05	471 ± 276	<0.05	>> 3333
GEA009.2	483.3 ± 86.6	<0.05	> 3333	>0.05	>> 3333
GEA011.1	187.9 ± 57.5	>0.05	> 3333	>0.05	>> 3333
GEA013.1	95.6 ± 86.6	<0.05	2156± 300	>0.05	>> 3333

Indicated EC_{50}, EC_{90}, and CC_{50} ± standard deviation values are the means of at least three independent experiments. [a] The Mann-Whitney test was used to determine whether the activities of GEA007.1, GEA009.2, GEA011.1 and GEA013.1 on inhibiting HCV subgenomic replicon were significantly different from IFN-α 2WT treatment.

Table 2. Relative Gene induction in BM4-5 cells after 8 hours treatment with IFN-α 2WT or GEA007.1

Gene name	IFN-α 2WT Fold increase compared to no treatment	GEA007.1 Fold increase compared to no treatment
MX1	**17.83**	**25.92**
ISG-56	**2.055**	**4.455**
ISG-15	**1.7**	**4.125**
ISG-60	**1.27**	**3.985**
ADAR	**1.38**	**2.91**
KIAA1268	**1.19**	**2.35**
IFI6-16	**0.795**	**2.13**
STAT1	**1.38**	**2.08**
MAPKK1/MEK1	**0.65**	**1.72**
PKR	**0.89**	**1.535**
P48/IRF-9	**0.82**	**1.365**

Human Interferon α, β Response Gene Arrays (SuperArray) were performed with a concentration of 33pg/ml of IFN. Indicated values are the mean of two independent experiments.

Figures

Figure 1. GEA007.1 has improved inhibitory properties compared to IFN-α 2WT and other IFN-α variants in the replicon system. BM4-5 cells were treated during three days by IFN-α 2WT or GEA007.1. or GEA009.2 or GEA011.1 or GEA013.1 at 0, 3, 17, 33, 333, 3333 pg/mL. Total RNA was isolated from the cells and analysed by Northern Blot for negative and positive HCV subgenomic RNA. The β-actin serves as internal control for loading of cellular RNA.

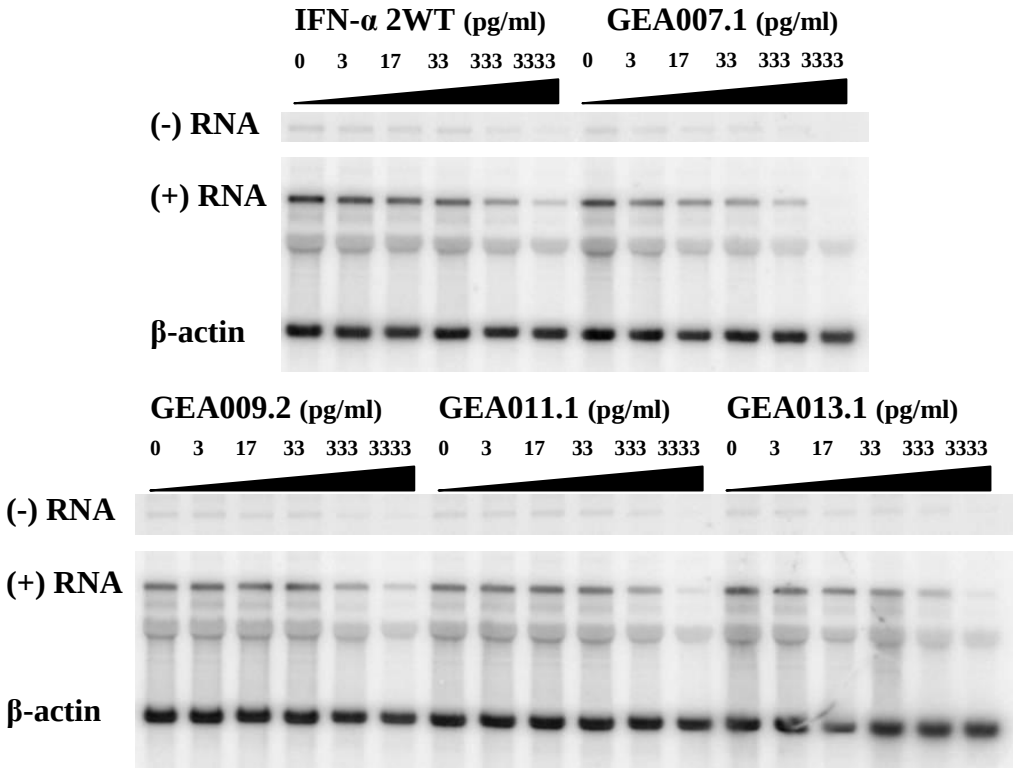


Figure 2. GEA007.1 is able to clear HCV from BM4-5 cells after prolonged administration

BM4-5 cells were left untreated or treated every day with either IFN-α 2WT or GEA007.1 at 333 pg/mL in the absence of geneticin (upper panel left). At confluence, cells were passaged by 1:4 dilution. After five passages, the treatment was stopped, and cells were further passaged by 1:4 dilution in medium free of geneticin (upper panel right) or containing geneticin (lower panel right). In the latter case no result are presented for GEA007.1 as cells died in the presence of geneticin (See text for detail). At each passage, total RNA was isolated from cells, separated on agarose gel, and analysed by Northern Blot for the detection of negative and positive RNAs. The β-actin served as internal loading control. P means cell passage, O means no IFN treatment, 2wt means treatment with IFN-α 2WT, 7.1 means treatment with GEA007.1.

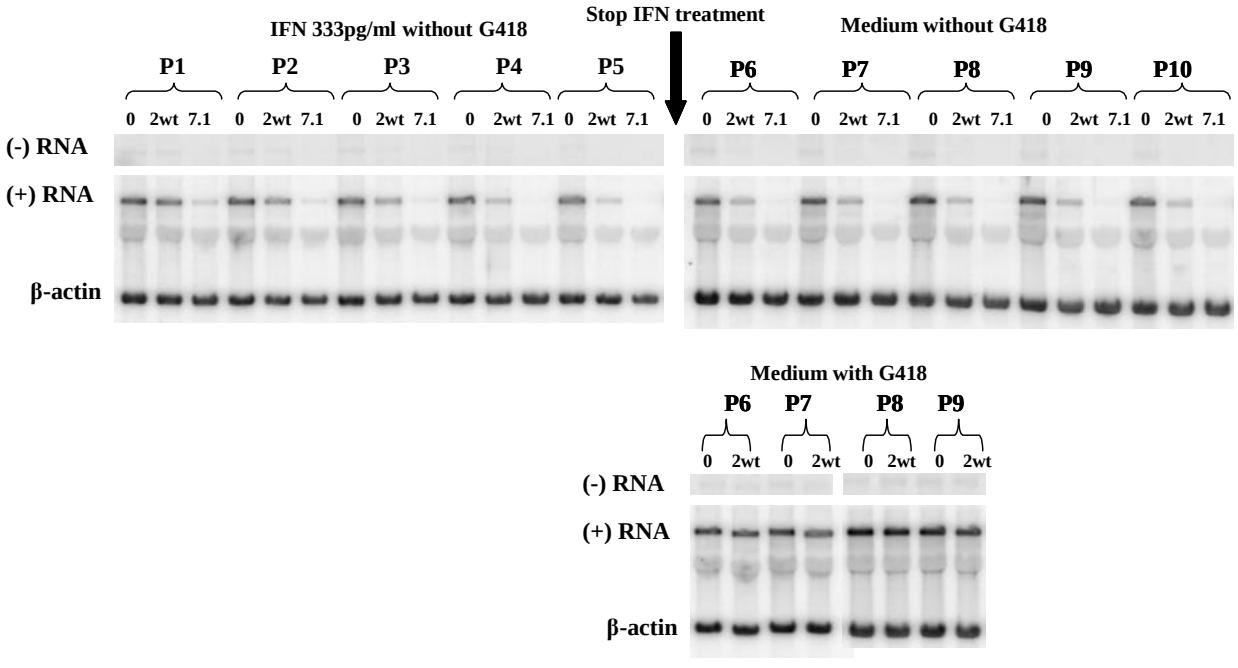


Figure 3. GEA007.1 is a better inducer of the IFN signaling pathways compared with IFN-α 2WT. BM4.5 cells were transfected with an ISRE luciferase construct. 24 h after transfection the cells were incubated without or with IFN-α 2WT t and GEA007.1 for 24h. The cells were then harvested and assayed for luciferase activity. The luciferase activity induced by IFN-α 2WT for each concentration was taken as 100% and other luciferase activity were normalized accordingly. Mean values ± S.D. of three independent experiments are shown. The absolute luciferase values obtained with increasing concentration of IFN-α 2WT (0 - 0,3 - 3 - 33 - 333 – 3333 pg/ml) are: 205 - 234 - 226 - 217 - 798 – 2004, respectively.

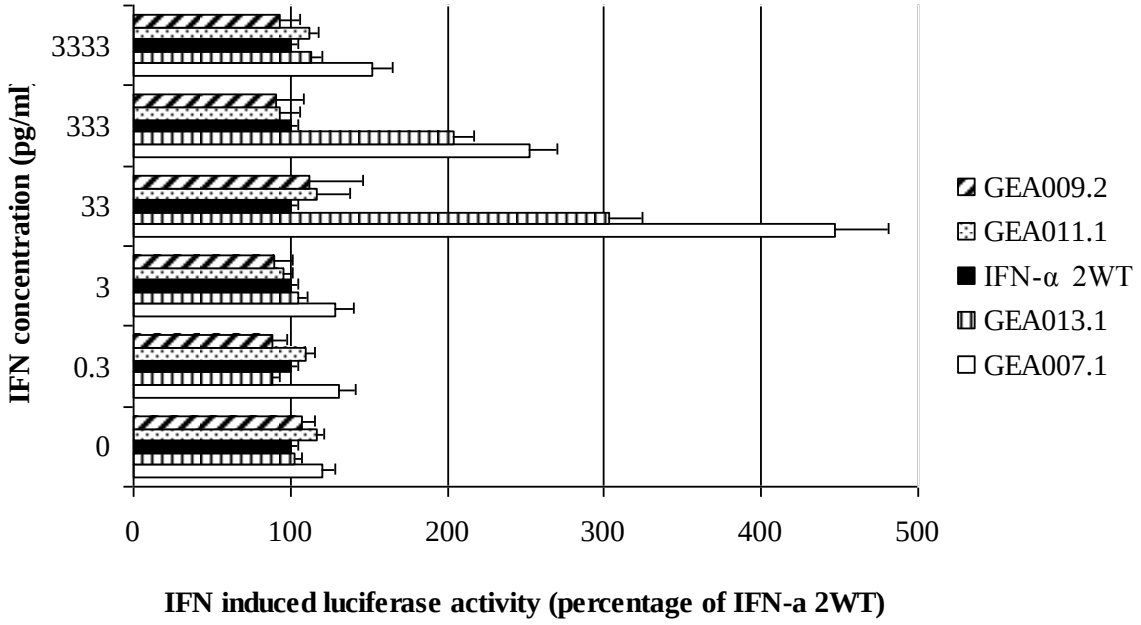
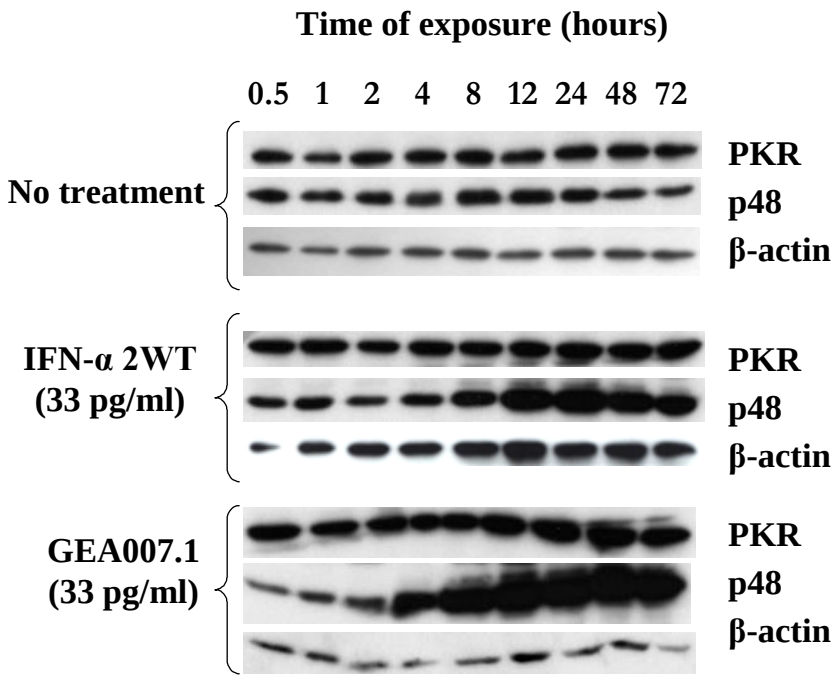


Figure. 4. Effect of GEA007.1 on PKR and p48 (IRF-9) protein expression. BM4-5 cells were cultured in absence or presence 33 pg/mL of IFN-α 2WT or GEA007.1. At different time points following IFNs administration, total cellular proteins were extracted and subjected to Western Blot analysis using anti-PKR or anti-p48 antibodies. As loading control, Western Blot was performed with anti-actin antibodies.



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