

**Novel alpha interferon (IFN-alpha) variant with improved inhibitory activity against hepatitis C virus genotype 1 replication compared to IFN-alpha2b therapy in a subgenomic replicon system.**

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

6  
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25

1 **ABSTRACT**

2

3 Hepatitis C treatment is based on the association of pegylated IFN- $\alpha$  and ribavirin. To  
4 improve the level of sustained virological response to treatment, especially in patients  
5 infected with HCV genotype 1, new IFNs with improved efficacy and toxicity profile may be  
6 developed. In this report we show that a novel and naturally occurring human IFN- $\alpha$  17  
7 variant, GEA007.1, which was discovered using an original population genetics-based drug  
8 discovery approach, inhibits HCV genotype 1 RNA replication more efficiently than IFN- $\alpha$   
9 2b, in the BM4-5 cell line harbouring an HCV subgenomic replicon. Moreover, we show that  
10 the complete viral clearance is obtained in BM4-5 cells after long-term treatment with  
11 GEA007.1, when HCV subgenomic RNA is still detected in cells treated with other IFN- $\alpha$   
12 variants or with standard IFN- $\alpha$  2b. Eventually, we demonstrate that the better inhibitory  
13 activity of GEA007.1 is likely to be due to a stronger and faster activation of the JAK-STAT  
14 signalling pathway and to a broader expression of IFN- $\alpha$  responsive genes in cells, compared  
15 to standard IFN- $\alpha$ . Our results demonstrate a superior inhibitory activity of GEA007.1 over  
16 IFN- $\alpha$  2b in the HCV replicon system. Clinical trials are required to determine if GEA007.1  
17 could be a potent 'next generation' IFN for the treatment of HCV infection, especially in non-  
18 responders or relapsing patients infected with HCV genotype 1 who currently represent a  
19 clinical unmet need.

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21

## 1 INTRODUCTION

2 Hepatitis C virus infects 170 million people worldwide and leads, in approximately 70% of  
3 cases, to chronic infection (3, 24, 26). Current treatments are based on the association of  
4 pegylated-interferon-alpha 2a or 2b (IFN- $\alpha$  2a or IFN- $\alpha$  2b) and ribavirin (5, 8, 20). This  
5 treatment induces a sustained virological response (SVR) in approximately 60% of cases. The  
6 rate of SVR varies from 30-40% in patients infected with genotype 1, to 80-90% for those  
7 infected with genotype 2 or 3. Despite a rather good efficacy, this treatment is often poorly  
8 tolerated because of side effects (5). It is therefore important to improve current molecules  
9 and carry on searching for novel antiviral agents with an enhanced anti-HCV activity to  
10 overcome the failure of current treatments, especially in patients infected with HCV genotype  
11 1 (4).

12 Until the very recent development of a complete and robust HCV replication system (19, 27,  
13 28), hepatoma cell lines harbouring HCV subgenomic replicon were a relevant model to study  
14 some aspects of HCV biology as well as the effect and mechanism of action of antiviral  
15 agents against HCV replication (1). Various interferons, including IFN- $\alpha$ ,  $\beta$ , and  $\gamma$ , were  
16 studied using this model, thus providing some insight on their antiviral effect (6, 7, 10, 18),  
17 and mechanism of action (6, 11, 12, 15). Overall it was shown that the transduction pathway  
18 following the interaction of exogenous IFNs with their cellular receptors was functional in  
19 most cases, although slight differences could exist between different Huh7 cell lines  
20 harbouring HCV subgenomic replicon. These differences may account for the variability  
21 observed in the amount of interferon used to obtain a given inhibition. Altogether, HCV  
22 replicon systems were validated as a suitable tool for the study of the efficacy and mechanism  
23 of action of interferon molecules. Interferon- $\alpha$  is known to exhibit several independent  
24 biological activities, including immunomodulatory, antiproliferative, and antiviral activities  
25 (14, 22, 23). In the context of an antiviral strategy, this pleiotropic effect of IFN- $\alpha$  may

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

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

16

17

## 1 MATERIALS AND METHODS

2 *Identification and production of IFN- $\alpha$  variants.* Original genetic variants of 14 different  
3 human IFN- $\alpha$  protein subtypes were provided by GenOdyssee. Discovery of natural cytokine  
4 mutants with improved therapeutic utility was performed using the following process: a  
5 genomic DNA library was constituted with genomic DNA from 239 different individuals  
6 representing altogether 85% of the human ethnic diversity of the worldwide population, and  
7 screened for non-synonymous single nucleotide polymorphisms in the coding sequence of the  
8 14 different human IFN- $\alpha$  gene subtypes. The identification and selection of IFN- $\alpha$  protein  
9 mutants with functionally-relevant amino-acid mutations, was then carried out using  
10 bioinformatics tools, including sequence/structure analysis and molecular modelling. IFN- $\alpha$   
11 variants (IFN $\nu$ ), named thereafter GEA007.1, GEA009.2, GEA011.1, GEA013.1, derived from  
12 such procedure, are respectively natural mutants of the human IFN- $\alpha$  17, 21, 5, and 7  
13 (SwissProt primary accession numbers P01571, P01568, P01569, P01567). All carry a unique  
14 amino-acid mutation (G45R for GEA007.1, D95N for GEA013.1, C122S for GEA011.1, and  
15 K179E for GEA009.2), with changes in three-dimensional structure and electrostatic  
16 isopotentials at the receptor-binding surface, based on molecular modelling. The recombinant  
17 human IFN- $\alpha$  variants (IFN $\nu$ ) used in this study were produced in the methylotropic yeast,  
18 *Pichia pastoris*, with a yield of approximately 10-20 mg/l, following protocols and  
19 recommendations provided by Research Corporation Technologies, Inc. IFN $\nu$  proteins were  
20 secreted into the culture medium and purified using Cibacron and ion exchange  
21 chromatography to obtain pure preparations of the recombinant human IFN $\nu$  in a phosphate  
22 buffered solution (PBS). The purity of each preparation was greater than 95% and endotoxin  
23 content, determined by the LAL test, was less than 5IU/ $\mu$ g. IFN- $\alpha$  2b, named IFN- $\alpha$  2WT  
24 thereafter, in order to be an relevant control was synthesized and purified using the same  
25 process. In our experiments 1mg of IFN- $\alpha$  produced in yeast was equivalent to 3x10e8

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

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

14 **Protocols of administration of IFN.** BM4-5 cells were seeded in 6-well plates at a density of  
15  $2.5 \times 10^5$  cells/well, sixteen hours before starting treatments. IFN $\nu$  (GEA007.1, GEA009.2,  
16 GEA011.1, and GEA013.1) and IFN- $\alpha$  2WT were administrated to cells in complete medium  
17 in the absence of geneticin. For short-term treatments, the administration of each drug (at  
18 concentrations ranging from 0 to 3333 pg/ml) was renewed every day for three consecutive  
19 days. For long-term treatments, IFN- $\alpha$  2WT or GEA007.1 was administered every day at 333  
20 pg/mL for 21 days. In order to maintain cells in proliferation/division, cells were trypsinized  
21 and diluted four times every four days. A total of 5 passages was performed during treatment.  
22 At the end of this period of treatment, the administration of IFN- $\alpha$  2WT and GEA007.1 was  
23 interrupted and cells were kept for 5 further passages in medium containing or not geneticin  
24 to monitor a potential rebound of HCV subgenomic RNA replication.

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

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



1 ***Analysis of the signal transduction pathway induced by IFN- $\alpha$  treatment in BM4-5 cells.***

2 To evaluate the activation of intracellular signaling pathways by IFN- $\alpha$  2WT and IFN $\nu$ , BM4-  
3 5 cells (75x10<sup>3</sup> cells/well in 24 wells plate) were transfected with an Interferon Stimulating  
4 Response Element (ISRE) -luciferase plasmid (Stratagene) by using the TransIT-TKO reagent  
5 (Mirus) following the manufacturer's recommendations. Twenty-four hours after transfection,  
6 duplicate cultures in 24 well plates were either left untreated or treated for 24 hours with a  
7 range of IFN- $\alpha$  2WT and IFN $\nu$  of 0.3, 3, 33, 333 and 3333 pg/ml. Cells were then washed  
8 with cold phosphate-buffered saline (PBS), lysed by passive lysis buffer (Promega) and the  
9 luciferase activity measured as recommended by the manufacturer (Promega). The luciferase  
10 activity induced by IFN- $\alpha$  2WT for each concentration was taken as 100% and other  
11 luciferase activity were normalized accordingly.

12 **Western Blot analysis.** IFN- $\alpha$  2WT or GEA007.1 were administered at a concentration of  
13 3333 pg/mL or at 33 pg/mL on BM4-5 cells for 30 min, 1 h, 2h, 4h, 8h, 12h, 24h, 48h, and  
14 72h. Administration was renewed each 24h. At the indicated time, cells were washed twice  
15 with cold PBS, then lysed using a buffer (0,2% NP40, 150 mM NaCl, 20 mM Tris, 2 mM  
16 EDTA, 0,1% glycerol, 10 mM dithiothreitol) containing serine/threonine phosphatases  
17 inhibitors (50 mM NaF, 200  $\mu$ M orthovanadate), and protease inhibitor (complete protease  
18 inhibitor, Roche). Proteins of cell lysates were denaturated at 100°C in Laemmli buffer (50  
19 mM Tris pH 6,8; 2% SDS; 10% glycerol; 0,1% bromophenol blue; 1.25% 2-  
20 mercaptoethanol), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis  
21 (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham  
22 Pharmacia Biotech). Non-specific binding sites were blocked for 2 h at room temperature  
23 (RT) with 5% milk, 0,1% Tween 20 in PBS. The membrane was then incubated overnight at  
24 4°C with monoclonal antibodies raised against PKR (Santa Cruz Biotechnology), p48  
25 (Beckton Dickinson) or  $\beta$ -actin (Sigma). Membranes were then washed three times, and

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

5 ***Analysis of IFN- $\alpha$  specific gene expression using GEArray™ Q Series Array.*** BM4-5 cells  
6 were incubated in 6 well plates at 80% confluence. The cells were either left untreated or were  
7 incubated with IFN-2WT or GEA007.1 at the same concentration of 33 pg/mL. After 8 hr of  
8 incubation, total RNA was extracted with Extract all reagent (Eurobio) and stored at -80°C.  
9 For one set of experiment, six arrays (GEArray™ Q series Human Interferon  $\alpha$ ,  $\beta$  Response  
10 Gene Array–SuperArray, Bioscience Corporation) were used following manufacturer’s  
11 recommendation. For each array, 96 gene-specific cDNA fragments are printed on a 3,8 x  
12 4,8cm nylon membrane. The list of the genes is accessible on the SuperArray web site  
13 ([www.superarray.com](http://www.superarray.com); Ref. HS-054). A total of 3 $\mu$ g of each RNA sample was used as a  
14 template to generate cDNA by reverse transcription. cDNA was then amplified and labelled  
15 with biotin-16-dUTP (Roche) by a Linear Polymerase Reaction (LPR) (AmpoLabeling-LPR  
16 Kit). After a step of pre-hybridization, the membranes were hybridized overnight with the  
17 different probes, corresponding to the different treatments. The signal was revealed by  
18 chemiluminescent reaction using alkaline phosphatase-conjugated streptavidin, and detected  
19 by a scanner camera (Biorad). All the results were obtained from the analysis of non saturated  
20 images, after 2 min of exposure. Spot quantification was performed with Quantify-One  
21 software (BioRad). The mean intensity of blank spots served as negative controls and was  
22 subtracted from other values. The mean intensity of  $\beta$ -actin gene served as a positive control  
23 and was used to normalize intensities of the spots to allow relative comparisons between the  
24 different arrays. To determine which genes were up regulated, intensities of spots resulting  
25 from hybridization with cDNA obtained from total RNA extracted from untreated cells were

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

3

4

## 1 RESULTS

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

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

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

11 ***Long-term administration of GEA007.1 allows to cure cells from HCV genotype 1b***  
12 ***replicon subgenome.*** Having established that GEA007.1 had the best inhibitory activity  
13 compared to IFN- $\alpha$  2WT, with a very good EC<sub>90</sub>, we asked whether a prolonged  
14 administration of this IFN $\nu$  could induce a better sustained inhibitory effect than IFN- $\alpha$  2WT  
15 and lead to the rapid eradication of HCV replication in cells. BM4-5 cells were treated during  
16 20 days though 5 passages, with either IFN- $\alpha$  2WT or GEA007.1 at a single concentration of  
17 333 pg/mL in medium without geneticin as described in materials and methods. The results of  
18 a Northern Blot analysis presented in Figure 2 shows that HCV subgenomic RNA synthesis  
19 was inhibited more intensively by GEA007.1 than IFN- $\alpha$  2WT, thus confirming the better  
20 inhibitory activity of GEA007.1. Moreover, after 20 days of treatment and 5 passages, HCV  
21 subgenomic RNAs (both strands) were no more detectable in cells treated with 333 pg/mL of  
22 GEA007.1, whereas the positive strand HCV subgenomic RNA was still detectable in  
23 standard IFN- $\alpha$  2WT treated cells. To monitor the potential rebound of HCV replication after  
24 cessation of treatment, cells were kept untreated for at least 4 additional passages either in  
25 absence or presence of geneticin. The presence of geneticin was meant to select back more

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

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

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

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

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

14

15



## 1 **DISCUSSION**

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

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

1 variants tested in this study, GEA007.1 and to a lesser extent GEA013.1, were found  
2 significantly more potent than IFN- $\alpha$  2WT for inhibiting the replication of an HCV genotype  
3 1b subgenomic replicon in BM4-5 cells. GEA007.1 was the best molecule tested with an EC<sub>50</sub>  
4 of 22.7 pg/mL, which is 7 times lower than the EC<sub>50</sub> obtained with standard IFN- $\alpha$  2WT (i.e.  
5 154.2 pg/mL) in the same conditions (Table 1). Moreover, we found that GEA007.1,  
6 administered at 333 pg/mL every day for 20 days, was able to clear HCV subgenomic  
7 replicon from BM4-5 cells, while HCV subgenomic RNA was still detected in cells treated  
8 with standard IFN- $\alpha$  2WT in the same conditions (Figure 2). This result demonstrates the  
9 superiority of GEA007.1 over interferon- $\alpha$  2b in an HCV genotype 1b subgenomic replicon  
10 system. However, the results obtained in the HCV subgenomic replicon system may not  
11 translate into antiviral activity *in vivo*. Therefore, our results warrant further evaluation of  
12 GEA007.1 *in vitro*, using systems allowing a complete multiplication of HCV (19, 27, 28),  
13 and *in vivo*, in the frame of clinical trials.

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

1 GEA007.1 over IFN- $\alpha$  2WT, which was associated with a better inhibitory activity on HCV  
2 subgenomic RNA synthesis. Then we analysed key cell signalling proteins involved in type 1  
3 IFN-transduction pathway after BM4-5 cells exposure to GEA007.1 or IFN- $\alpha$  2WT. Western  
4 Blot analyses were performed to monitor the level of expression of some key proteins like  
5 p48 or PKR, and cDNA expression array experiments were performed to monitor the  
6 expression of IFN- $\alpha$  responsive genes. GEA007.1 induced a stronger expression of p48 than  
7 IFN- $\alpha$  2WT. This result suggests that p48 may be recruited more efficiently by the STAT1<sup>P</sup>  
8 and STAT2<sup>P</sup> heterodimer complex to form the ISGF3 complex (25), after treatment with  
9 GEA007.1 than after treatment with IFN- $\alpha$  2WT. Our results are in accordance with previous  
10 works showing that type 1 IFN signalling pathways are qualitatively functional in Huh7 and  
11 BM4-5 cell lines, as persistent HCV replication does not modify gene expression patterns  
12 after IFN- $\alpha$  treatment (13, 21). In addition, in our experimental conditions, IFN-  
13  $\alpha$  responsive genes activated in Huh7 cells harbouring HCV subgenomic replicon by both  
14 GEA007.1 and IFN- $\alpha$  2WT did not differ from those described and reported in other studies  
15 (13, 21). Lastly, a stronger and broader expression of IFN- $\alpha$  responsive genes was seen in  
16 BM4-5 cells treated with GEA007.1, compared to IFN- $\alpha$  2WT. Amongst most activated  
17 genes were found Mx1, ISG56, ISG15, ISG60, ADAR, KIAA1268, and STAT1. Mx1 (also  
18 named MxA) was the most induced gene in our system. Mx1 was also found previously as an  
19 important IFN- $\alpha$  inducible gene in IFN- $\alpha$ -treated replicon cell lines (13). A stronger  
20 expression of Mx1 was observed in cells treated with GEA007.1 which in turn could have  
21 contributed to the better inhibitory effect of GEA007.1 compared to IFN- $\alpha$  2WT. In view of  
22 the results obtained by others, showing the absence of inhibition of HCV RNA replication by  
23 Mx1 (6), it might be hypothesized that the activation of the different genes described can be  
24 only a reflect of the activation of the Jak-Stat pathway, without predicting a direct inhibitory  
25 role for a specific gene. Moreover, it is not possible at this stage to rule out the possibility that

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

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

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

5  
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1 **Tables**

2 **Table 1: Inhibitory features and toxicity of various interferons**

	<b>EC<sub>50</sub> (pg/mL)</b>	<b>p<sup>a</sup></b>	<b>EC<sub>90</sub> (pg/mL)</b>	<b>p<sup>a</sup></b>	<b>CC<sub>50</sub> (pg/mL)</b>
<b>IFN-α 2WT</b>	154.2 ± 8.8		3080 ± 406		>> 3333
<b>GEA007.1</b>	22.7 ± 9.4	<0.05	471 ± 276	<0.05	>> 3333
<b>GEA009.2</b>	483.3 ± 86.6	<0.05	> 3333	>0.05	>> 3333
<b>GEA011.1</b>	187.9 ± 57.5	>0.05	> 3333	>0.05	>> 3333
<b>GEA013.1</b>	95.6 ± 86.6	<0.05	2156 ± 300	>0.05	>> 3333

3 Indicated EC<sub>50</sub>, EC<sub>90</sub>, and CC<sub>50</sub> ± standard deviation values are the means of at least three independent  
 4 experiments. <sup>a</sup> The Mann-Whitney test was used to determine whether the activities of GEA007.1,  
 5 GEA009.2, GEA011.1 and GEA013.1 on inhibiting HCV subgenomic replicon were significantly different  
 6 from IFN-α 2WT treatment.

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8 **Table 2. Relative Gene induction in BM4-5 cells after 8 hours treatment with IFN-α**  
 9 **2WT or GEA007.1**

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

10 Human Interferon α, β Response Gene Arrays (SuperArray) were performed with a concentration of  
 11 33pg/ml of IFN. Indicated values are the mean of two independent experiments.

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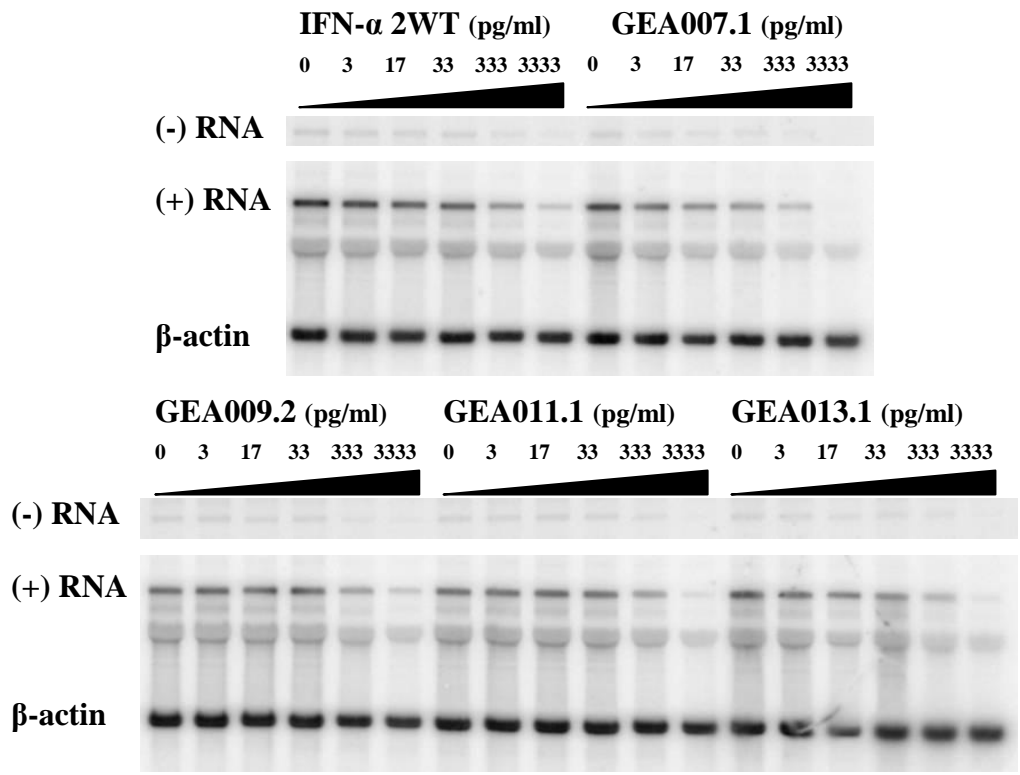
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1 **Figures**

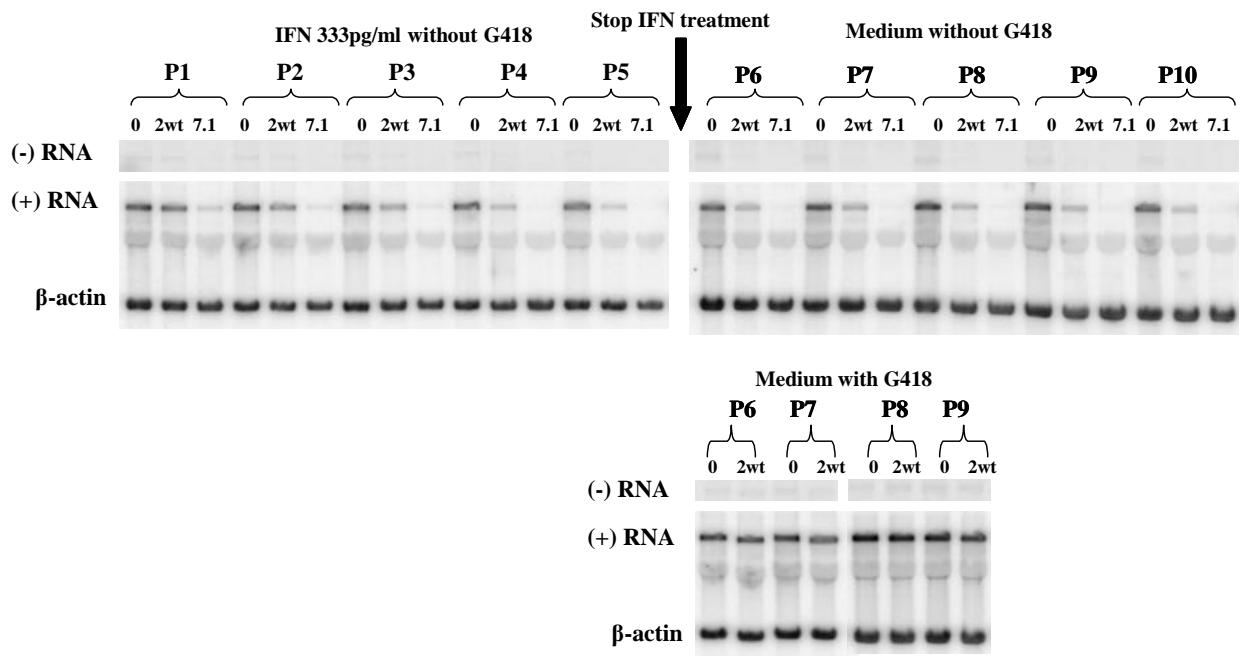
2 **Figure 1. GEA007.1 has improved inhibitory properties compared to IFN- $\alpha$  2WT and**  
3 **other IFN- $\alpha$  variants in the replicon system.** BM4-5 cells were treated during three days by  
4 IFN- $\alpha$  2WT or GEA007.1. or GEA009.2 or GEA011.1 or GEA013.1 at 0, 3, 17, 33, 333,  
5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

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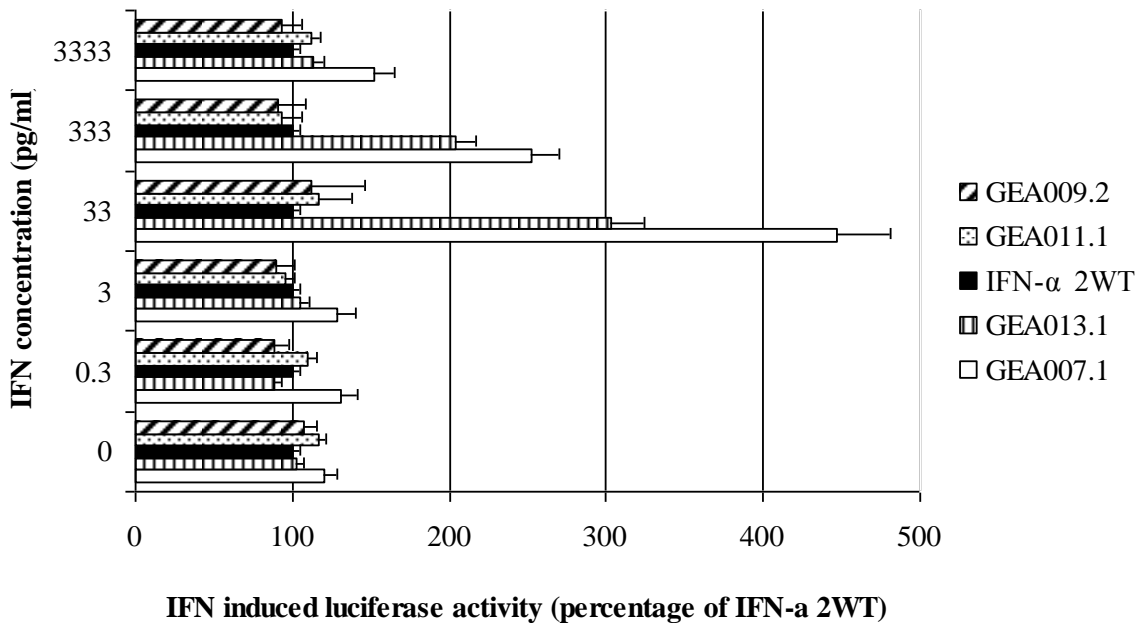


1 **Figure 2. GEA007.1 is able to clear HCV from BM4-5 cells after prolonged**  
 2 **administration**

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

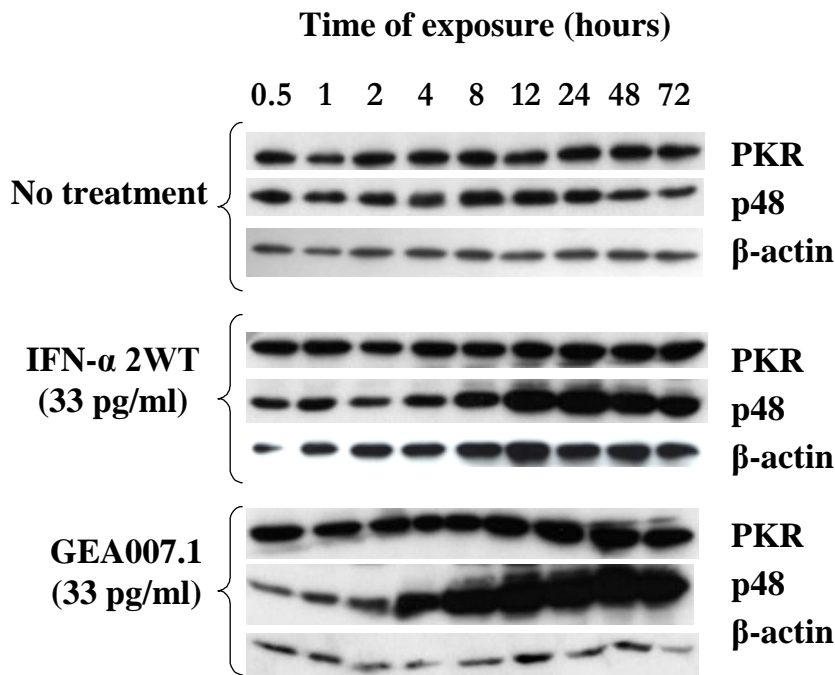


1 **Figure 3. GEA007.1 is a better inducer of the IFN signaling pathways compared with**  
2 **IFN- $\alpha$  2WT.** BM4.5 cells were transfected with an ISRE luciferase construct. 24 h after  
3 transfection the cells were incubated without or with IFN- $\alpha$  2WT t and GEA007.1 for 24h.  
4 The cells were then harvested and assayed for luciferase activity. The luciferase activity  
5 induced by IFN- $\alpha$  2WT for each concentration was taken as 100% and other luciferase  
6 activity were normalized accordingly. Mean values  $\pm$  S.D. of three independent experiments  
7 are shown. The absolute luciferase values obtained with increasing concentration of IFN- $\alpha$   
8 2WT (0 - 0,3 - 3 - 33 - 333 - 3333 pg/ml) are: 205 - 234 - 226 - 217 - 798 - 2004,  
9 respectively.



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



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