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Original Article

A placental growth factor-positively charged peptide potentiates the antitumor activity of interferon-gamma in human brain glioblastoma U87 cells

Yu Liu[1,3*], Naifei Chen[2,3*], Hongmei Yin[2,3], Leilei Zhang[3], Wei Li[2], Guanjun Wang[2], Jiuwei Cui[2], Bo Yang[1], Ji-Fan Hu[2,3]

[1]*Department of Neurosurgery, First Affiliated Hospital, Zhengzhou University, Zhengzhou 450001, Henan, China;* [2]*Stem Cell and Cancer Center, First Hospital, Jilin University, Changchun, Jilin 130021, China;* [3]*Stanford University Medical School, Palo Alto Veterans Institute for Research, Palo Alto, CA 94304, USA.* [*]*Equal contributors.*

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Abstract: Interferons have been marketed to treat hematological malignancies, but their efficacy in the treatment of solid tumors has been significantly hindered by low antitumor efficacy and numerous side effects. We used a "cDNA in-frame fragment" library screening method to identify short cDNA peptides that potentiate the anti-tumor activity of interferons. In this study, we synthesized a hybrid molecule by fusing a short positively charged peptide derived from placental growth factor-2 to the C-terminus of human IFNγ. Using the human brain glioblastoma U87 cell line as a model system, we found that the hybrid interferon exhibited significantly higher activity than did the wild-type IFNγ in inhibiting tumor cell growth. As compared with the unmodified IFNγ, the hybrid interferon was better at inhibiting cell invasion in a matri-gel assay and at decreasing tumor colony formation. The enhanced antitumor activity of the synthetic interferon was correlated with the activation of interferon pathway genes and the blockade of tumor cell division at the S-G2/M phase. This study demonstrates the potential of a synthetic IFNγ for use as a novel antitumor agent.

Keywords: Antitumor, interferon-gamma, synthetic interferon, positively charged peptide, tumor, apoptosis, cell proliferation

Introduction

Interferon gamma (IFNγ), a natural potent pleiotropic cytokine used to treat a variety of malignancies [1], is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells in antigen-specific immunity [2]. Binding of this cytokine to its surface receptor initiates a cascade of events that induce the phosphorylation of JAK1 (Janus kinase 1) and TYK2 (Tyrosine kinase 2), followed by the activation of the signal transducer and activation of transcription (STAT) family transcription factors [3-6]. The activated STAT complex is subsequently translocated to the nucleus, where it induces transcription of a number of genes related to cell-cycle arrest and apoptosis, resulting in both apoptotic and nonapoptotic cell death [4, 5, 7] as the molecular basis for antiviral and antitumor therapy.

Interferons are an effective treatment for chronic myeloid leukemia (CML), often leading to hematologic remission [8-10]. *In vitro* studies have demonstrated that interferon can directly inhibit CML cell growth and can induce apoptosis in CML cell lines. However, IFNγ therapy is limited by its systemic toxicity [11]. Long-term parental administration of IFNγ is required to maintain therapeutic efficacy, and this often induces high-grade toxicity and significant side effects in many patients.

To potentiate the antitumor activity of interferons, we developed a cDNA in-frame fragment library screening technology. In this approach, short cDNA fragments were fused "in frame" to the C-terminus of IFN. Using an ISRE-luciferase reporter system, we identified short cDNA fragments that enhance the anti-tumor activity of IFN ("IFN enhancer peptide", IEP). Interestingly, three IFN enhancer peptides contain a short, positively charged peptide derived from placen-

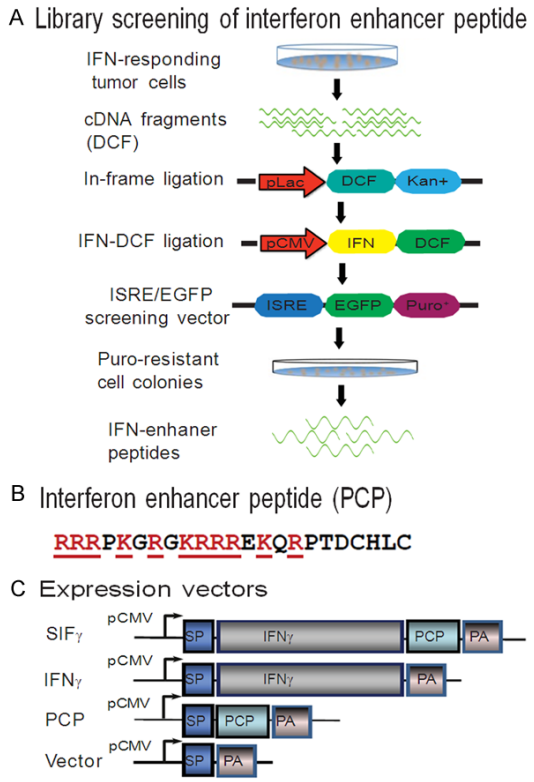

Figure 1. Interferon enhancer peptides. A. cDNA in-frame fragment library screening for short interferon enhancer peptides. Total RNAs were converted into double-strand cDNAs. Short cDNA fragments were generated by shearing and were ligated "in-frame" in the upstream of kanamycin gene. After kanamycin selection, the in-frame cDNAs were digested and fused in frame to the C-terminus of IFN. Using the ISRE-luciferase reporter system, cells were selected by puromycin, which is under the control of ISRE. The short cDNA fragments that enhance the anti-tumor activity of IFN ("IFN enhancer peptide", IEP) were then recovered by PCR using the vector primers. B. Interferon enhancer peptide that contains a short positively charged peptide (PCP) derived from placental growth factor-2 (PLGF-2). Positively charged amino acids are labeled in underlined red. R = arginine, K = lysine. C. Schematic diagram of the synthetic interferon gamma (SIFγ) vector. pCMV: CMV promoter; SP: signal peptide; PCP: PLGF-derived positively charged peptide; PA: SV40 poly A signal; IFNγ: wild type interferon gamma; vector: vector control; SIFγ: synthetic interferon gamma composed of interferon gamma-linker-PCP.

tal growth factor-2 (PLGF-2) (Guo, unpublished data), which has been known to enhance the activity of three growth factors (vascular endothelial growth factor-A, platelet-derived growth factor-BB, and bone morphogenetic protein-2) [12]. When fused to the C-terminus of human interferon alpha, this PLGF-2 peptide enhanced antitumor activity in pancreatic cancer cells [13].

As a proof-of-concept study, we attempted to determine whether a synthetic IFNγ-IEP fusion protein, when delivered by a lentiviral vector, was able to enhance the inhibition of cancer cell proliferation and invasion. We also examined whether this synthetic interferon was able to modulate the effect of the chemotherapeutic drug temozolomide (TMZ) in a human glioblastoma U87 cell line.

Materials and methods

Cell culture

U87MG, a human brain glioblastoma cell line, was purchased from the American Type Culture Collection (ATCC, VA). The human glioblastoma cell line U138MG was obtained as a gift from Dr. Gordon Li at Stanford University Medical School [14]. Both cells lines were routinely cultivated in -MEM medium (Invitrogen, CA), supplemented with 10% fetal bovine serum, 1 × Non-Essential Amino Acid (NEAA), 1% glutamine and 100 U/ml penicillin-streptomycin at 37°C in atmosphere containing 5% CO_2.

For lentiviral packaging, 293T cells were purchased from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS, 1 × Non-Essential Amino Acid (NEAA), and 100 U/ml Penicillin-Streptomycin (Invitrogen, CA).

Interferon-enhancer peptide library screening

A cDNA in-frame fragment library screening method was used to identify short peptides that potentiate the antitumor activity of interferons (**Figure 1A**). In this approach, double-strand cDNAs derived from fetal fibroblasts were fragmented with a Branson sonicator. The gel-purified short fragments were used to construct an in frame short peptide library by inserting the fragments immediately downstream of the "ATG" of kanamycin. After transformation, only those E. coli that carry the in-frame short peptide (DCF) vectors could survive in the kanamycin LB plate.

The in-frame DCFs were digested by BamH1/EcoRV and were ligated "in frame" to the C-terminus of IFN in a lentiviral vector constructed in the lab [15, 16]. After packaging,

lentiviruses were used to transfect a 293T cell line that carries the ISRE-GFP-Puro-Reporter system (Promega, WI). After puromycin selection, cells were collected and sorted for copGFP fluorescence by FACS Sorter (BD LSR Fortessa, CA). The ISRE-responding short peptides were recovered by PCR from the sorted cells and cloned into a pJet vector (Thermo Fisher, MA) for sequencing. Using this approach, we identified short cDNA fragments that enhance the anti-tumor activity of IFN ("IFN enhancer peptide", IEP). Three IFN enhancer peptides containing a short positively charged peptide derived from placental growth factor-2 (PLGF-2) were recovered after screening (Guo, unpublished data).

IEP-plasmid construction and viral production

We then examined the role of this PLGF-2 derived peptide by fusing it to the C-terminus of human IFNγ. Specifically, the full-length human IFNγ cDNA was amplified from a mixed cDNA library derived from human spleen and leucocytes with overlapping PCR primers JH2245 (forward): 5'-TAGAAGATTCTAGAGCCGCCACCATGAAATATACAAGTTATATCTTGGC-3' and JH2246 (reverse): 5'-TGGGTCTCCTCCTCTGGGATGCTCTTCGACCTCGA-3'. The PLGF-2 PCP fragment was amplified with PCR primers JH2247 (forward): 5'-GAGCATCCCAGAGGAGGAGACCCAAGGGCAGGGGGA-3' and JH2243 (reverse): TTCGTCGACGATATCTCACAGGTGGCAGTCTGTGGGTCTC-3'.

Interferon enhanced peptides (IEPs) were identified by cDNA in-frame fragment library screening (**Figure 1A**).

A short positively charged peptide (PCP) derived from placental growth factor-2 (PLGF-2) PCP fragments (**Figure 1B**) was amplified by PCR and fused to the C-terminal of IFNγ by overlapping PCR. The ligated IFNγ-PCP was cloned into the Xba1/EcoRV site in a lentiviral vector constructed in the lab [15, 16]. To distinguish this hybrid from wild type interferon gamma, we use the term "synthetic interferon gamma" (SIFγ).

For lentivirus packaging, the constructed plasmids were co-transfected with pSPAX2 and pMD2.G packing vectors using lipofectamine 2000 (Invitrogen, CA). The viral supernatants were collected 48 h and 72 h after transfection [17, 18] and used for cell transfection.

MTT assay

For the cell proliferation assay, 96-well plates were seeded with 7500 cells/well U87 and U138 stable clone cells that carry the wild type IFNγ, the synthetic SIFγ, and the empty vector, respectively. Forty-eight hours after plating, cells were incubated with 20 μl 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, MO] per well at 37°C for 4 hours. After removal of the media, 150 μl DMSO was added, and the cells were shaken using an orbital shaker for 10 min. The cell absorbance was measured at 490 nm.

Clonogenic assay

The clonogenic assay was performed using the method as previously reported [19, 20]. Briefly, 50 cells in 0.3% agar suspension were plated over an agar underlay (2.0% agar) in a 60 mm dish to form colonies. Two weeks after seeding, colonies were fixed with 6% glutaraldehyde, stained with 0.1% crystal violet, and counted using a stereomicroscope. Cell division was evaluated by plating efficiency (PE) as the ratio of the number of colonies to the number of cells seeded.

Invasion assay

Six-well transwell inserts (Corning, MA) were coated with BD matri-gel basement membrane matrix (BD Biosciences, CA) according to the manufacturer's instructions. Cells (5×10^4) transfected with interferon were suspended in 1.5 ml basal MEM medium and added to a coated insert. Complete MEM medium (2.5 ml) was added to the well as a chemoattractant. After incubating for 16 hours, we swabbed the interior of the inserts to remove non-migratory cells. Migratory cells were detected by staining with 0.1% crystal violet in 10% ethanol solution and visualizing the cells under a microscope. Three randomly selected fields (magnification × 100) were chosen, and the stained cells were counted by image J software. The results were expressed as the percentage of the control group.

Cell cycle analysis

U87 tumor cells were fixed in ice-cold 70% ethanol and stained with PI (propidium iodide)/Triton-X100 staining solution for 30 minutes.

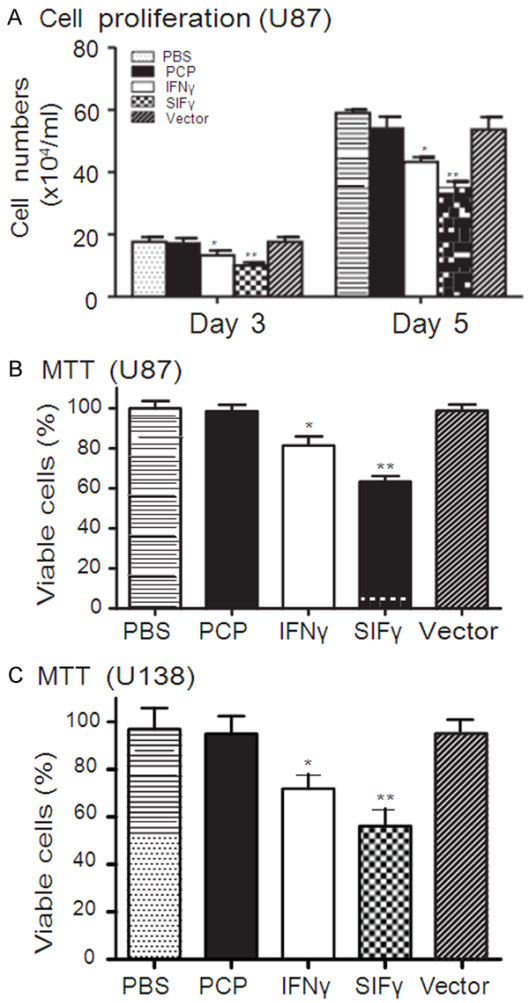

Figure 2. Inhibition of human glioblastoma U87 cell growth by the synthetic interferon SIFγ. A. Inhibition of glioblastoma U87 cell growth. Tumor cells were seeded in 96 well plates and were transfected with lentiviruses carrying IFNγ, SIFγ, and vector control. PBS was used as the negative control. Cells were collected and counted on day 3 and day 5 after viral infection. All data shown are mean ± SEM from three independent assays. ANOVA test: Day 3: * and day 5 **P < 0.05 as compared with PBS and vector controls; Day 5: *P < 0.01 as compared with PBS and vector controls; **P < 0.01 as compared with PBS and vector controls and P < 0.05 as compared with the IFNγ group. B, C. Synthetic interferon SIFγ induces cell death in U87 (B) and U138 (C) glioblastoma cells. Cells were collected on day 7 following lentiviral transfection. Cell viability of glioblastoma cells was measured by the MTT assay. ANOVA test: *P < 0.01 as compared with PBS and vector controls; **P < 0.01 as compared with the IFNγ group and the control groups.

Staining solution was prepared with 0.1% Triton-X100, 200 μg/ml RNase A and 20 μg/ml PI. The stained cells were detected by Flow cytometry (BD LSR Fortessa). ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in various phases of cell cycle.

Gene expression by real time qPCR

After removing genomic DNA contamination with DNase I (Sigma, MO), M-MLV Reverse Transcriptase (Invitrogen, CA) was used to synthesize cDNA [21, 22]. For qPCR, cDNA samples were amplified using CFX96™ real-time system (BIO-RAD) by SYBR PrimeScript™ RT-PCR Kit (Clontech, CA). The mRNA expression levels were quantitated by normalizing over β-actin (housekeeping gene) as previously described [21, 23]. The comparative *CT* method was applied in the quantitative real-time RT-PCR assay according to the delta-delta *CT* method [15, 24]. PCR primers used for qPCR are listed in Table S1.

Statistical analysis

The statistical significance of differences between treatment groups of data was assessed using SPSS 21.0 software (SPSS, Inc., IL). One-way ANOVA (Bonferroni test) was used to compare variables among treatment groups. All experiments and assays were repeated 3-5 times, and data were expressed as the mean ± SEM of independent experiments. The values were considered significantly different at $P < 0.05$.

Results

Identification of IFN enhancer peptides

Using a cDNA in-frame fragment library screening, we identified short cDNA fragments that enhance the anti-tumor activity of interferons (**Figure 1A**). Three IFN enhancer peptides (IEP) contained a consensus amino acid sequence consisting of a positively charged peptide (PCP) (Figure S1). The PCP contains a total of eight arginine residues (R) and three lysine residues (K) (**Figure 1B**). A short peptide containing this consensus sequence has been shown to enhance the function of several growth factors, including vascular endothelial growth factor-A, platelet-derived growth factor-BB, and bone morphogenetic protein-2 [12]. Thus, we were interested to learn whether this consensus PCP was also able to potentiate the therapeutic role of IFNγ in pancreatic cancer cells.

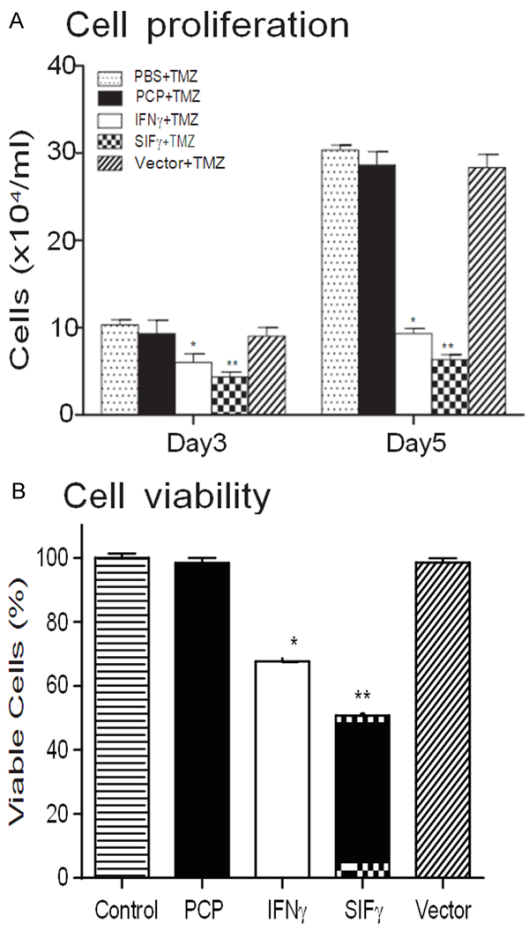

Figure 3. SIFγ potentiates the therapeutic effect of temozolomide (TMZ). A. SIFγ enhances cell growth inhibition by low dose of TMZ. Cells were transfected with interferon-containing lentiviruses and treated with the low dose of TMZ (50 nM/ml). Cells were counted on day 3 and day 5 after viral infection. All data shown are mean ± SEM from three independent assays. ANOVA test: Day 3: * and **P < 0.01 as compared with PBS and vector controls; Day 5: *P < 0.01 as compared with PBS and vector controls; **P < 0.05 as compared with the IFNγ group and P < 0.01 with the control groups. B. SIFγ potentiates the chemotherapeutic effect of low dose of TMZ. Cells were transfected with interferon-containing lentiviruses and treated with the low dose of TMZ (50 nM/ml). Cell viability was measured by a MTT assay. All data shown are mean ± SEM from three independent assays. ANOVA test: *P < 0.01 as compared with PBS and vector controls; **P < 0.01 as compared with the IFNγ group and the control groups.

We constructed a synthetic interferon gamma protein (SIFγ) by fusing this positively charged peptide to the C-terminus of IFNγ (**Figure 1C**). This synthetic gene was placed under the control of a viral CMV promoter in the expression vector. For comparison, the wild type IFNγ was cloned into the same lentiviral vector. The empty lentiviral vector and the vector carrying the PCP alone were used as negative controls. After packaging, lentiviruses carrying SIFγ, IFNγ, PCP, and the empty vector were used to transfect U87 tumor cells.

Synthetic SIFγ inhibits cell growth and enhanced cell death in tumor cells

We first examined cell proliferation in U87 cells using SIFγ, IFNγ, PCP and empty vector lentiviruses. The wild type IFNγ inhibited the growth of U87 tumor cells as compared with the vector control and the PBS control. Fusion of a positively charge peptide to the C-terminus of IFNγ significantly enhanced its activity. On day 5, the synthetic interferon SIFγ showed a significantly greater inhibition of tumor cell growth than did the wild type IFNγ (**Figure 2A**, P < 0.05).

We then used the MTT assay to compare the activity of the wild type IFNγ and synthetic SIFγ. U87 tumor cells carrying the wild type IFNγ, the synthetic SIFγ, and the empty vector, respectively, were seeded in 96 well plates and were stained by MTT 48 hrs after culturing. As compared with the PBS and vector control groups, the IFNγ-treated group showed 18% cell death. The SIFγ-treated group, however, exhibited the greatest increase in cell death among the treatment groups (**Figure 2B**, 37%, P < 0.01), suggesting that addition of the PCP peptide potentiates the antitumor effect of the wild type IFNγ. SIFγ also exhibited a significantly better antitumor effect than did the wild-type IFNγ in a second glioblastoma cell line U138 (**Figure 2C**, P < 0.01).

Since interferon alpha is used as a treatment for chronic myeloid leukemia (CML), we were interested in learning if the synthetic SIFγ was also effective in CML cells. Due to low transfection efficiency of lentivirus in K562 leukemia cells, we used cell supernatants that contain equal amount of secreted SIFγ for this study. We found that the secreted IFNγ and SIFγ inhibited leukemia cell growth, but the therapeutic effect was not as great as that seen in U87 tumor cells (Figure S2). Future studies are needed to test if purified SIFγ recombinant protein or IFN-PCP would have an improved antitumor activity in leukemia cells.

The engineered SIFγ potentiates the antitumor therapy by low dose of temozolomide

The treatment of glioblastoma multiforme remains difficult in that no current treatments are curative. Temozolomide (TMZ) is recommended

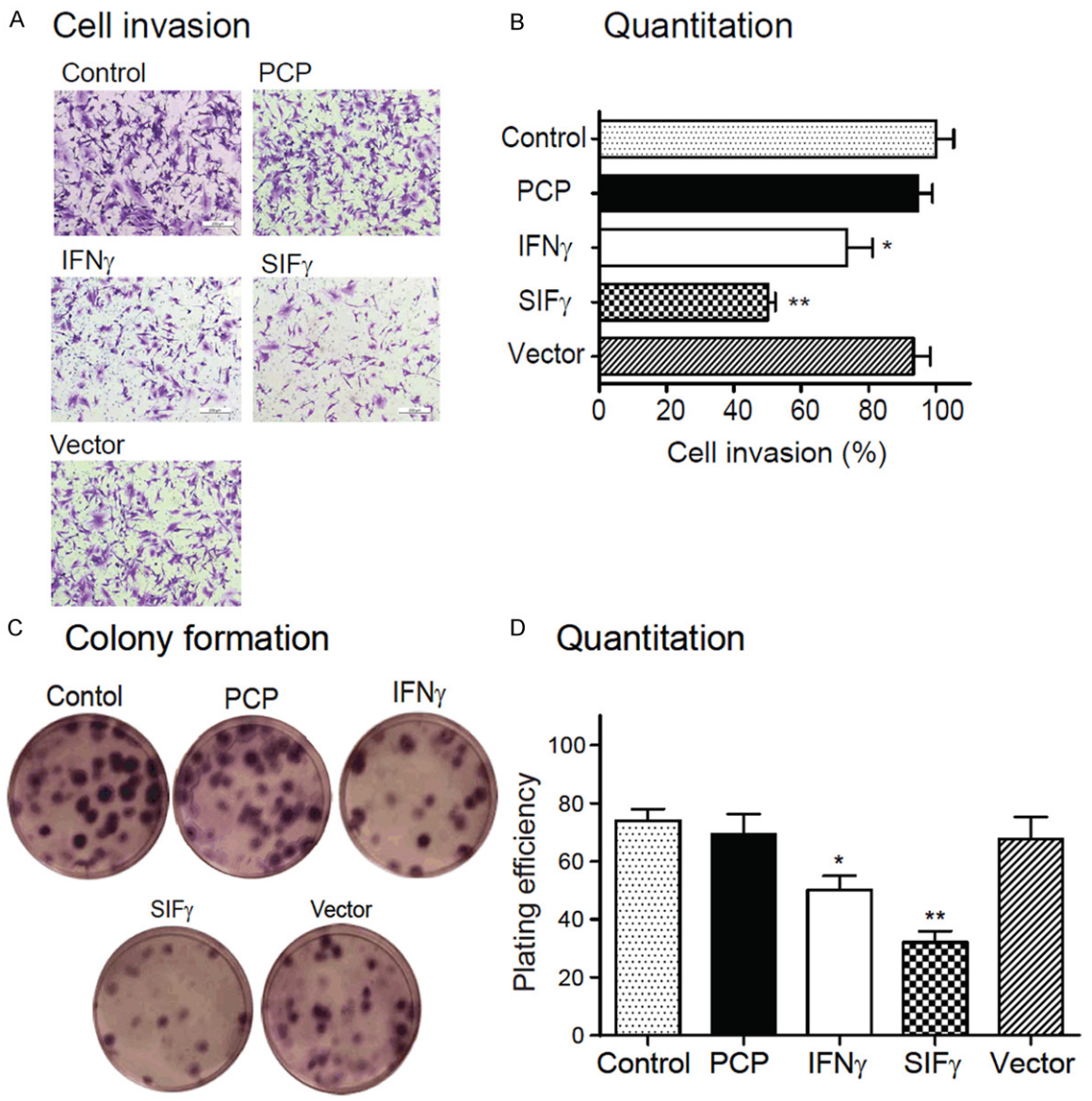

Figure 4. SIFγ inhibited invasion and colony formation in U87 glioblastoma cells. A. SIFγ inhibits cell invasion. Tumor cells infected with interferon lentiviruses were seeded in the insert for 14 hrs, stained with crystal violet solution, and visualized under a microscope. B. Quantitation of the invaded cells. The invaded cells in the insert were stained with crystal violet and counted in selected fields by imageJ software. All data shown are mean ± SEM from three independent assays. ANOVA test: *P < 0.01 as compared with PBS and vector controls; **P < 0.01 as compared with the IFNγ group and the control groups. C. SIFγ inhibits cell colony formation in U87 glioblastoma cells. Cells were stained with crystal violet solution and visualized under a microscope. D. Quantitation of tumor cell colonies. The colonies with cells > 10 were counted in selected fields by image J software. All data shown are mean ± SEM from three independent assays. ANOVA test: *P < 0.05 as compared with PBS and vector controls; **P < 0.05 as compared with the IFNγ group and P < 0.01 with the control groups.

as a first line chemotherapeutic agent, particularly in patients who have a first relapse of glioblastoma after treatment with nitrosourea chemotherapy. In order to learn if the engineered SIFγ was able to potentiate the activity of this chemotherapeutic drug in glioblastoma U87 cells, we used a dose of TMZ that led to 48% inhibition of cell growth in glioblastoma U87 cells. As seen in **Figure 3A**, the synthetic SIFγ showed a significantly greater ability than did the wild type IFNγ to potentiate the inhibition of cell growth induced by TMZ (P < 0.001).

In a cell viability assay, the wild type IFNγ, when combined with TMZ, induced cell death in U87 tumor cells (**Figure 3B**). The co-treatment of

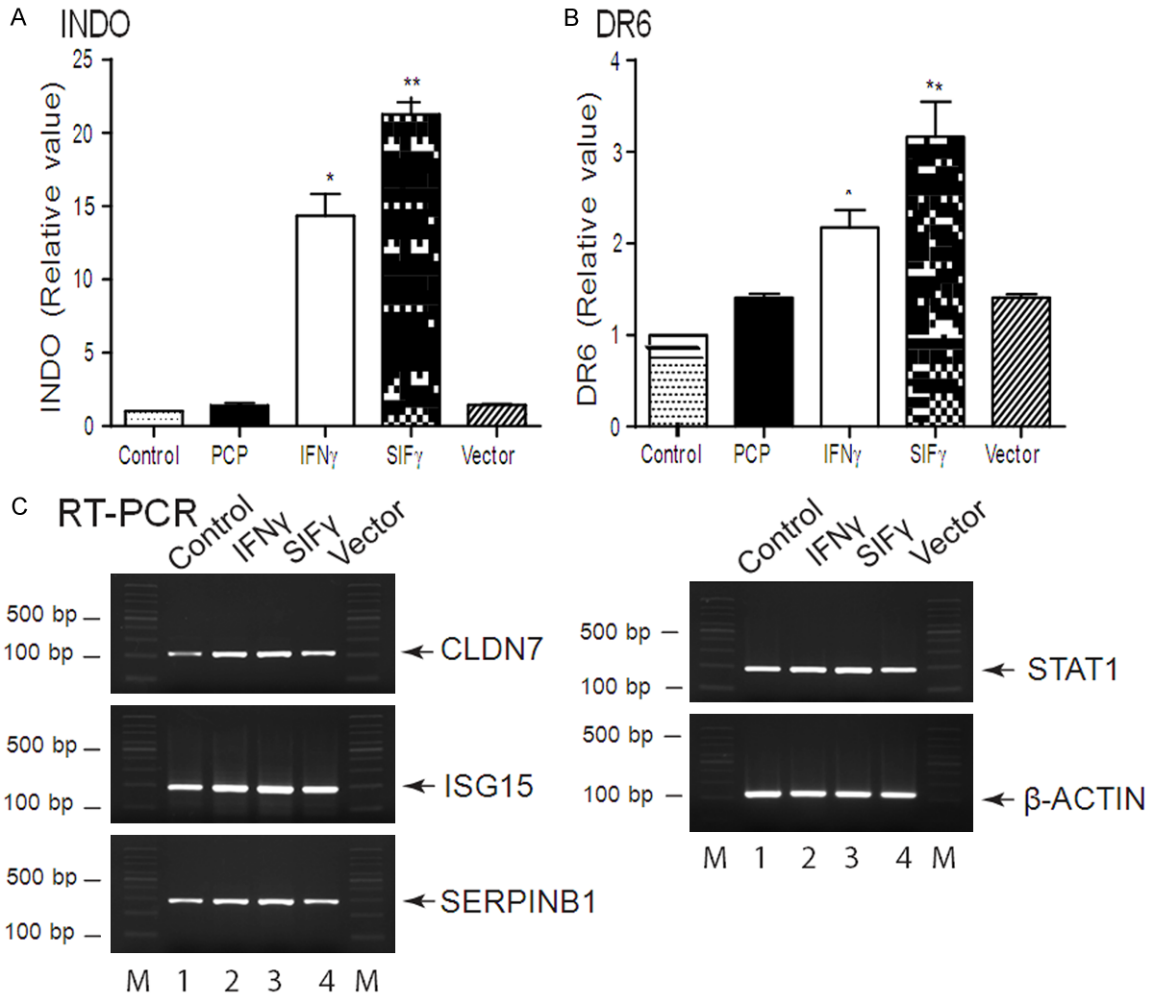

Figure 5. SIFγ activates the STAT1 signal pathway. (A, B) Quantitative expression of interferon gamma downstream target genes INDO (A) and DR6 (B). All data shown are mean ± SEM from three independent assays. ANOVA test: *P < 0.01 as compared with PBS and vector controls; **P < 0.01 as compared with the IFNγ group and the control groups. (C) RT-PCR of the STAT1 signal pathway genes. β-ACTIN was used as the internal control for PCR reaction.

synthetic SIFγ with the chemotherapeutic drug TMZ, however, induced significantly more cell death than did IFNγ (P < 0.01). Two control groups (PCP and vector) did not significantly affect cell viability as compared with the TMZ+PBS control.

Inhibition of cell invasion and tumor colonies by the engineered IFNγ

We then tested the activity of the synthetic interferon gamma SIFγ to alter cell invasion using a Transwell assay. Matrigel, a solubilized basement membrane preparation derived from the Engelbreth-Holm-Swarm mouse sarcoma, was used to test tumor invasiveness. Tumor cells were seeded on a Matrigel-coated transwell chamber and the number of cells that passed through the Matrigel to the bottom of the transwell was quantified as a measure of cell invasion. As shown in **Figure 4A**, U87 cells exhibited the malignant phenotype of invading across the Matrigel membrane. Treatment with the PCP and vector viruses did not significantly reduce the degree of cell invasion. Both the wild type IFNγ and the synthetic SIFγ significant reduced cell invasion. However, the SIFγ treatment showed a significantly greater activity than did the IFNγ treatment in attenuating the ability of cancer cells to pass through the Matrigel (**Figure 4B**, P < 0.01).

We then tested the ability of the synthetic SIFγ to inhibit tumor formation (**Figure 4C**). U87 tumor cells were allowed to form colonies in soft agar containing 10% FBS. After two weeks

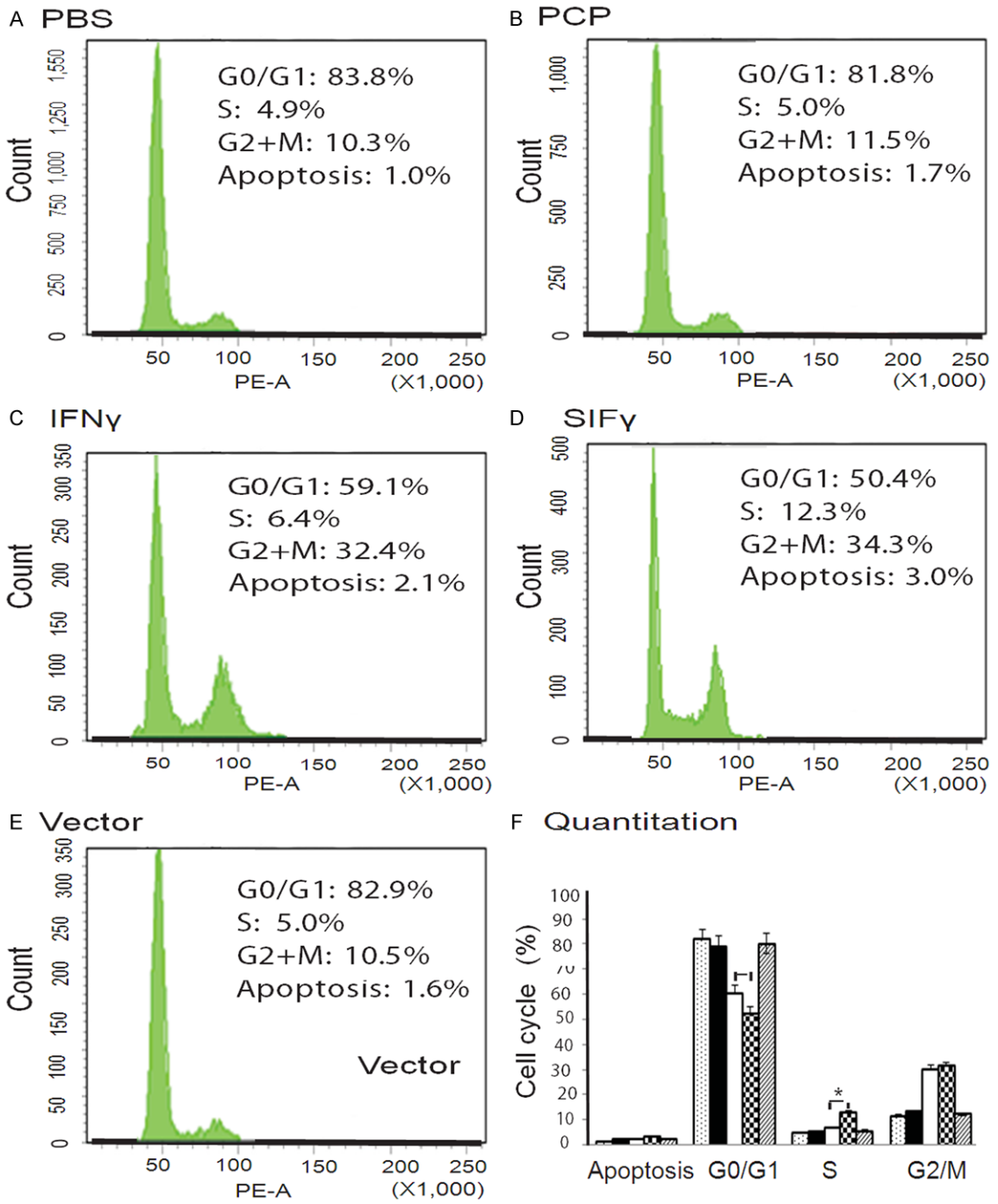

Figure 6. SIFγ induces S-G2/M phase blockade and apoptosis. U87 tumor cells were transfected with interferon-containing lentiviruses and submitted for cell cycle analysis (A-E). Three independent cell cycle analyses were performed and averaged for each treatment group (F). *P < 0.05 as compared with the wild type IFNγ group.

of culture, cell colonies were stained with 0.1% crystal violet. Wild type IFNγ inhibited the formation of tumor colonies. SIFγ was more potent than IFNγ in reducing the formation of tumor colonies. (**Figure 4D**, P < 0.05). Two controls (PCP and vector) did not significantly interfere with colony formation.

Activation of the STAT1 pathway by the engineered IFNγ

To delineate the mechanism underlying this potentiation by SIFγ, we used quantitative PCR to examine the expression of interferon pathway genes. Transfection with the empty vector

lentivirus did not affect the expression of the INDO and DR6 genes. However, both the IFNγ and SIFγ treatments significantly increased the transcription of INDO and DR6. The synthetic SIFγ exhibited a significantly higher activity than IFNγ in activating INDO and DR6 (**Figure 5A**).

We also used RT-PCR to examine the expression of the STAT1 pathway genes, including CLDN7, ISG15, SERPINB1, and STAT1 (**Figure 5B**). In all cases, we found that SIFγ exhibited a significantly greater ability than IFNγ in activating the STAT1 pathway genes.

Block of the S-G2/M transition by synthetic SIF

After treatment with SIFγ, U87 tumor cells were subjected to FACS analysis. As seen in **Figure 6**, IFNγ inhibited U87 cells by blocking cells primarily at the G2/M phase (32.4% for IFNγ vs 10.3% for PBS). SIFγ, on the other hand, induced a significantly greater blockage of U87 cells at the S phase than did the wild type IFNγ (12.3% for SIFγ vs 6.4% for IFNγ, $P < 0.01$). Both IFNγ and SIFγ increased cell apoptosis, but the differences between the two treatments were not statistically significant.

Discussion

Although IFNγ is a natural cellular cytokine that protects against viral infection and tumors, its clinical application in tumor therapy has been greatly restricted due to its toxicity and low biological activity. Using a cDNA in-frame fragment library screening assay, we identified three short "IFN enhancer peptides" (IEPs) that contain a consensus positively charged peptide derived from placental growth factor-2 (PLGF-2) (**Figure 1B**). When fused to the C-terminus of IFNγ, this short peptide significantly potentiates the antitumor activity of IFNγ in glioblastoma cells. As compared with the wild-type IFNγ, the synthetic interferon gamma (SIFγ) exhibits a significantly greater biological activity in inhibiting tumor cell growth, invasion and tumor colony formation.

The mechanism underlying the enhanced antitumor activity of IFNγ is still unknown. Previously, Hubbell and his colleagues showed that PLGF-2123-144 peptide was able to enhance the function of several growth factors, including vascular endothelial growth factor-A, platelet-derived growth factor-BB, and bone morphogenetic protein-2 [12]. It is assumed that the activity of PLGF-2123-144 peptide is related to its extraordinarily strong and promiscuous interaction with extracellular matrix (ECM) components, thus facilitating its localization and spatially regulating the signaling of the growth factors [12]. IFNγ functions through the binding with its target cell membrane receptor [25, 26]. Functionally active IFNγ receptors consist of an alpha subunit required for ligand binding and signal transduction, and a beta subunit required primarily for signaling. We found that three IFNγ enhancer peptides isolated from the cDNA in-frame library screening contain the PLGF-2123-144. Thus, it is possible that they may use a similar mechanism of ECM binding to potentiate the antitumor therapy mediated by IFNγ.

It is noteworthy that the PLGF-2 peptide is a positively charged peptide, with 11 arginine (K) and lysine (L) residues enriched at its N-terminus (**Figure 1B**). Therefore, we believe that the potentiation of IFNγ antitumor activity by this PLGF-2 PCP peptide may be related to its positively charged amino acids. The activity of growth factors is orchestrated by their binding to the extracellular matrix (ECM) [27]. The ECM bound IFNγ is more active than its soluble counterpart [28]. Through the binding of its negatively charged components in the extracellular matrix, the PLGF-2 peptide may facilitate SIFγ's access to its membrane receptor for enhanced antitumor activity. Further studies are needed to address the role of this positively charged domain in activating signal pathways mediated by growth factors and cytokines.

Several positively charged peptides have been used as cell permeable peptides (CPPs) to facilitate the ability of recombinant proteins to cross cell membranes [29-31], including the HIV-1 TAT [32], MPG [33] and herpes simplex virus-type 1 virus VP22 proteins [34]. Attaching CPPs to a recombinant protein helps deliver the cargo into living mammalian cells by direct penetration and endocytosis [29, 35-37]. PLGF-2 PCP is a typical short peptide carrying multiple arginine and lysine residues. It will be interesting to learn whether PLGF-2 IEP also functions as a novel cell permeable peptide that guides interferon to enter the target cell.

SIFγ was delivered to glioblastoma U87 cells using a lentivirus vector. Therefore, the activity observed in our *in vitro* study is probably related to its direct antitumor effect. Intralesional

delivery of IFN by adenovirus has been shown to reduce tumor size by a direct antitumor effect and through activation of innate and adaptive immune responses. We suggest that the engineered SIFγ may yield even more potent antitumor activity *in vivo* by combining the activation of innate and adaptive immune responses.

It should be emphasized that this is a proof-of-concept study to examine the role of SIFγ. Since the lentivirus system provides a fast tool to deliver SIFγ to tumor cells, we used a lentivirus carrying the synthetic SIFγ to test the antitumor effect in human brain glioblastoma cells. Future studies are needed to confirm if SIFγ, when delivered in the form of purified recombinant proteins, is able to potentiate the antitumor activity. Most importantly, the antitumor effect of SIFγ should be validated in an in vivo study, such as a xenograft tumor test in nude mice, before it can be recommended for preclinical and clinical testing.

In summary, by combining cDNA in-frame fragment library screening with IFN-responding ISRE-luciferase reporter system, we have identified a short positively charged peptide (PCP) that, when fused to the C-terminus of interferon gamma, significantly potentiates the antitumor activity in glioblastoma cells. SIFγ significantly inhibits tumor cell growth, invasion and tumor colony formation. Future studies will be directed to enhance its activity by optimizing the positively charged domain in this short interferon enhancer peptide.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ji-Fan Hu, Stanford University Medical School, Palo Alto Veterans Institute for Research, Palo Alto, CA 94304, USA. Tel: 165-0493-5000, x63175; Fax: 165-0849-12-13; E-mail: jifan@stanford.edu; Bo Yang, Department of Neurosurgery, First Affiliated Hospital, Zhengzhou University, Zhengzhou 450001, Henan, China. Tel: +86-0371-6691-3114; E-mail: yangbo96@126.com; Jiuwei Cui, Stem Cell and Cancer Center, First Hospital, Jilin University, 71 Xinmin Street, Changchun, Jilin 130021, China. Tel: +86 431-8878-2178; E-mail: cuijiuwei@vip.qq.com

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Table S1. PCR primers used for viral packaging gene DNA and RNA

β-ACTIN	J880	CAGGTCATCACCATTGGCAATGAGC
	J881	CGGATGTCCACGTCACACTTCATGA
SERPINB1	JH3019	TCATCTCTCCCTTCAGCATTTC
	JH3020	CAGAGGCATGCTGAAAATCCAC
CLDN7	JH3021	CACGATGGGCATGAAGTGCAC
	JH3022	TGCCCAGCCAATAAAGATGGCAG
STAT1	JH3023	TGGAAATCAGACAGTACCTGGC
	JH3024	CTGAAGATTACGCTTGCTTTTCC
ISG15	JH3025	GAGCTTGTGCCGTGGCCCA
	JH3026	ACCGCTCGGGTGGACAGCCA

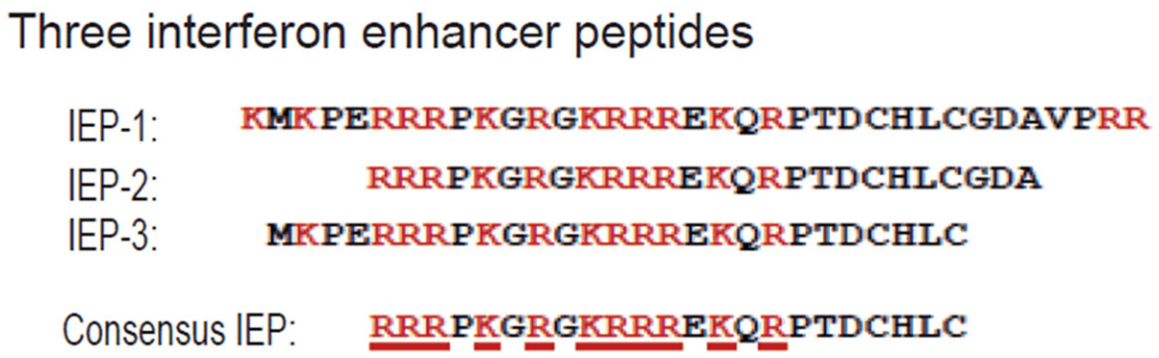

Figure S1. Screening of interferon enhancer peptides (IEP). Double-strand cDNAs (DCF) from fetal heart mesenchymal cell-derived fibroblast like cells are ligated in frame with translation initiation code "ATG" of kanamycin. The "in-frame" DCFs are selected by kanamycin and are fused to the C-terminus of IFN. Using the ISRE/copGFP/Puro+ reporter system, IEPs are identified and cloned for testing their antitumor activity. Three identified IEPs share a consensus stretch of positively charged amino acids (red).

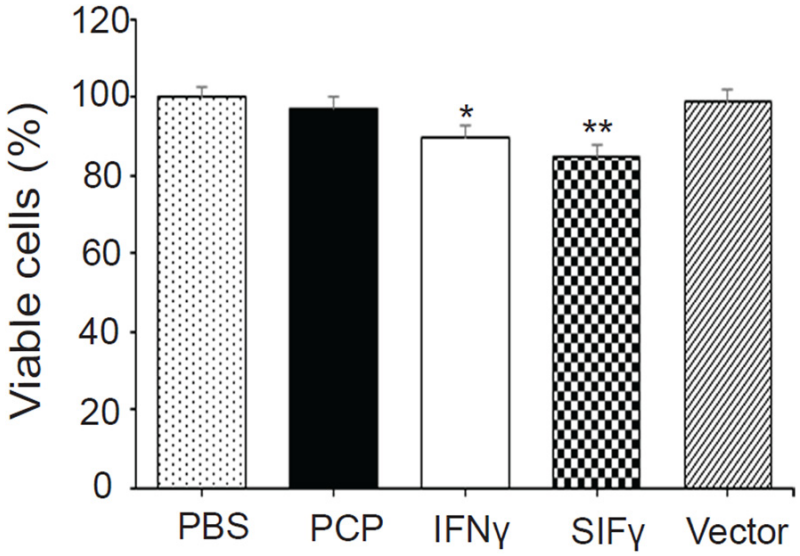

Figure S2. Inhibition of cell growth of a CML cell line. All data shown are mean ± SEM from three independent assays. ANOVA test: *p<0.01 as compared with PBS and vector controls; **p<0.01 as compared with PBS and vector controls and p<0.05 as compared with the IFNγ group. Method: We examined if the synthesized SIFγ also had the same therapeutic effect on the CML K562 cell line. Due to low viral transfection in K562 cells, the supernatants containing equal amount of secreted SIFγ and IFNγ proteins were used to treat the CML cells. For this, we expressed SIFγ and IFNγ in 293T cells. The supernatants containing the secreted interferons were collected and passed through Amicon Ultra-15 centrifugal filter Unit (MW cut off 30 KD, EMD Millipore, CA) to get rid of large molecules. The flow-through containing the interferons was loaded onto Amicon Ultra-15 centrifugal filter Unit (MW cut off 10 KD, EMD Millipore, CA) to remove proteins with small molecule size. The secreted interferons were measured with ELISA kit (PBL, CA). For the cell proliferation assay, K562 cells were seeded at a density of 2×10^4 cells/well in 24-well plates and treated with supernatants containing equal amount of secreted interferons. Seventy-two hrs after treatment, cell proliferation and cell viability were evaluated by the Trypan blue staining test. Trypan blue (0.4%) was added to 0.5ml of cell suspension for 3 min and non-viable cells were stained. Cells were counted under a microscope using a hemocytometer. The cell viability was calculated using the following equation: Cell viability (%) = number of unstained cells/total cell number × 100%.