Biodegradable nanogels for oral delivery of interferon for norovirus infection

Yunjeong Kim, Mahendra Thapa, Duy H Hua, and Kyeong-Ok Chang

Published Version Information

Citation: Kim, Y., Thapa, M., Hua, D. H., & Chang, K. (2011). Biodegradable nanogels for oral delivery of interferon for norovirus infection. Antiviral Research, 89(2), 165-173.

Digital Object Identifier (DOI): doi: 10.1016/j.antiviral.2010.11.016

Publisher's Link:
http://www.sciencedirect.com/science/article/pii/S016635421000820X

Biodegradable nanogels for oral delivery of interferon for norovirus infection

Yunjeong Kim[a], Mahendra Thapa[b], Duy H Hua[b], and Kyeong-Ok Chang[a]

[a] Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, KS 66506, USA

[b] Department of Chemistry, Kansas State University, Manhattan, KS 66506, USA

Corresponding author:

Dr. Kyeong-Ok Chang: Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, 1800 Denison Avenue, Manhattan, KS 66506; e-mail: kchang@vet.ksu.edu; Phone: 785 532-3849; Fax: 785 532-4039.

Abstract

Norwalk virus (NV) replicon-harboring cells have provided an excellent tool to the development of antivirals. Previously we demonstrated that the expression of replicon RNA and proteins was significantly reduced in the presence of various interferons (IFNs) including IFN-α and IFN-γ in a dose-dependent manner in the NV replicon-harboring cells and suggested IFNs could be therapeutic options for norovirus infection. It was also demonstrated that innate immunity including IFNs is crucial in the replication and pathogenicity of murine norovirus *in vitro* (RAW267.4 cells) and *in vivo*. IFNs have a short half life *in vitro* and *in vivo* due to low stability. Thus it is important to have a good delivery system to improve the stability of IFNs. Nanogels are nanosized networks of chemically cross-linked polymers that swell in physiologic solutions and provide improved stability and bioavailability to drugs. We have synthesized nanogels based on cross-linked polyethyleneimine (PEI)-polyethylenglycol (PEG). The PEI/PEG nanogels were further acetylated (AcNg) to reduce cellular penetration and cytotoxicity. The IFN-AcNg complex was prepared by incubating two components together at 4 ^{o}C and lyophilization. The activities of IFN alone and IFN-AcNg were evaluated in the replicon-harboring cells and against murine norovirus-1 (MNV-1) in RAW267.4 cells. The AcNg improved the stability of IFN stored at 4°C, was well tolerated in the cells. Furthermore, the activity of IFN was significantly higher when combined with AcNg in the replicon-harboring cells and against MNV-1 in RAW267.4 cells. We concluded that AcNg may be pursued further as a vehicle for oral delivery of IFNs in norovirus infection.

Keywords: antiviral; interferon; nanogel; Norwalk virus; replicon-harboring cells; murine norovirus

1. Introduction

Noroviruses are a leading cause of food- or water-borne gastroenteritis outbreaks. Studies have demonstrated that noroviruses are responsible for more than 60% of all food-water-borne gastroenteritis outbreaks (Fankhauser et al., 1998) with an estimated 23 million cases annually in the US causing 50,000 hospitalizations and 300 deaths (Mead et al., 1999). Studies of the replication of human noroviruses have been severely hampered by the absence of a cell culture system (Duizer et al., 2004). To overcome this limitation, we have previously generated Norwalk virus (NV), the prototype strain of noroviruses, replicon-harboring cells in BHK21 and Huh-7 cells (Chang et al., 2006). This replicon system has provided an excellent tool to study the replication of noroviruses and served as a platform to screen potential antiviral drugs (Chang, 2009; Chang and George, 2007b; Chang et al., 2006). Using the NV replicon-harboring cells, we have previously demonstrated that the expression of replicon RNA and proteins was significantly reduced in the presence of various interferons (IFNs) including IFN-α and IFN-γ in a dose-dependent manner and suggested IFNs could be therapeutic options for norovirus infection (Chang, 2009; Chang and George, 2007b; Chang et al., 2006). The important roles of IFN in replication and pathogenicity of noroviruses were also demonstrated using murine norovirus (MNV) *in vitro* (RAW267.4 cells) and *in vivo* (Changotra et al., 2009; Karst et al., 2003; Mumphrey et al., 2007). Among the noroviruses, only MNVs including MNV-1 strain have been successfully propagated in cell culture (Wobus et al., 2004) and provides a cell culture model for norovirus research.

IFN therapy has been a part of standard treatment regime in some infectious viral diseases such as hepatitis B and C virus infection (Foster, 2010; Liu and Kao, 2006), and its importance in respiratory viral infection including influenza virus has been studied extensively (Herzog et al., 1983; Isomura et al., 1982; Merigan et al., 1973; Phillpotts et al., 1983). However, the short half-life of IFN (up to 8.5 hr) in the serum requires repeated administration to maintain effective

concentration in targeted organs. To increase the half-life, IFN can be chemically conjugated with poly(ethylene glycol) (PEG) which is inert, water-soluble, and nontoxic and does not adversely affect the safety profile of the IFN (Foster, 2010; Kozlowski et al., 2001; Luxon et al., 2002; Sharieff et al., 2002; Shiffman, 2001). The pegylated IFN significantly increases the stability of IFN and is used for treatment of hepatitis B and C virus infection commonly in combination with ribavirin (Aghemo et al., 2010; Foster, 2010; Husa and Husova, 2001; Liu and Kao, 2006). The parental administration of high dose IFNs (international units [IU] in millions) is associated with side effects, one of the major causes of treatment failure (Negro, 2010). However, for virus infection occurring in mucosal areas, such as respiratory or gastroenteric infection, systemic administration of IFN may not be required. Therefore, oral or intranasal administration of IFNs has been explored as a way to reduce significant side effects and to introduce IFNs directly to the affected mucosal areas [review (Beilharz, 2010)].

Nanogels are nanosized networks of chemically cross-linked polymers that swell in a solvent and provide improved stability and bioavailability to drugs (Vinogradov et al., 1999; Vinogradov et al., 2006). We have synthesized nanogels based on cross-linked polyethyleneimine (PEI)-PEG, which were further acetylated to reduce cytotoxicity (AcNg). The IFN-AcNg complex was prepared by incubating two components together at 4°C in water and lyophilization. The prepared IFN-AcNg complexes of approximately 200 nM diameters were evaluated for stability of IFN and anti-norovirus effects by various assays in the NV replicon-harboring cells and MNV-1 in RAW267.4 cells. The AcNg carrier improved the stability of IFN stored at 4^{o}C, and the antiviral activity of IFN was significantly higher in IFN-AcNg formulation compared to IFN alone in the NV replicon-harboring cells or against MNV-1 in RAW267.4 cells. The preliminary study using rats suggested that IFN-AcNg did not cause side effects by oral or systemic administration, and AcNg did not cause IFN carryover to systemic circulation after oral administration of IFN-AcNg. We concluded that AcNg has a potential to be pursued as a vehicle for oral delivery of IFNs in norovirus infection.

2. Materials and methods

2.1. Cells, viruses, and reagents. The Vero cells, Huh-7, HG23 (Huh-7 based NV replicon-harboring cells), 1A7 (Huh-7 based hepatitis C virus replicon-harboring cells) and murine macrophage-like RAW267.4 cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics (chlortetracycline [25 μg/ml], penicillin [250 U/ml], and streptomycin [250 μg/ml]) (DMEM-C). Murine norovirus-1 was provided by Dr. H. Virgin (Washington University in St Louis, MO), and maintained in RAW267.4 cells. Recombinant IFN type I (human IFN-αA+D fusion protein), mouse IFN-β, and human IFN-αA were purchased from Sigma-Aldrich (St Louis, MO). The polyclonal antibody specific to NV proteinase-polymerase (ProPol) was described in a previous report (Chang et al., 2006). Antibodies specific for neomycin phosphotransferase II (NPT II) or β-actin were obtained from Santa Cruz biotechnology (Santa Cruz, CA) or Cell Signaling Technology (Danvers, MA), respectively. PEI (~25 kDa), PEG (8 kDa), dichloromethane, actonitrile, acetic anhydride, 1,1′-carbonyldiimidazole, *N,N′*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, and 1-hydroxybenzotriazole (HOBT) were purchased from Sigma-Aldrich.

2.2. Preparation of PEG/PEI nanogel. Nanogel PEG-PEI was prepared by following a method described previously (Ganta et al., 2008). Briefly, 7.0 g of PEI (MW ~25 kDa) in 20 ml of deionized water was loaded into Sephacryl S200 chromatographic column. The middle fractions (based on weight distribution) were collected and lyophilized to give 3.64 g (0.146 mmol) of PEI (molecular weight [MW] ~25 kDa). To activate PEG, 2.0 g of PEG (MW 8 kDa) in 7 ml of dry acetonitrile under argon was added to 0.41 g (2.5 mmol) of 1,1′-carbonyldimidazole, and the solution was stirred at 40°C for 2 hrs. The crude product was purified by dialysis using a MW

cut-off (MWCO) 2 kDa membrane with 10% ethanol in deionized water at 4°C for 4 hrs. The solution was lyophilized to give 1.84 g of activated PEG. Nanogel PEG-PEI was prepared by following a similar micellar method (Vinogradov et al., 2006). Synthesis started from activated PEG (~63 μmol) and PEI (~40 μmol). The PEI in 300 ml of deionized water was added dropwise to a solution of activated PEG (MW ~8 kDa) in 2 ml of dichloromethane. The reaction solution was sonicated in a water bath for 10 min, and the organic solvent was removed on a rotary evaporator resulting in a transparent solution. The solution was dialyzed with a MWCO 12K – 14K membrane in 800 ml of 10% ethanol in deionized water for 1 day at 25°C and lyophilized to give nanogel PEG-PEI (Ng). For the acetylation of Ng, 0.2 ml of acetic anhydride was added to a solution of 100 mg of Ng in 1 ml of actonenitrile under argon. The solution was stirred at 50^{o}C for 12 hrs and dialyzed with a MWCO 12 K – 14 K membrane in 100 ml of 10% ethanol in deionized water at 25^{o}C for 12 hrs. The solution was lyophilized to give 95 mg of AcNg as white solids.

2.3. Preparation of IFN-AcNg. IFN-αA+D or mouse IFN-β (for *in vitro studies)*, or human IFN-αA (for *in vivo* study) was encapsulated with AcNg. For encapsulation, 3 mg of AcNg in 0.5 ml of deionized water was mixed with the same volume of IFN (3×10^3, 3×10^4, or 10^7 IU in PBS) at 4°C for 5 min, and then the mixture was lyophilized. The lyophilized white solids were stored at -80°C. The encapsulated IFN was resuspended with 1 ml of PBS, so final concentration is 10^4 IU/μl (for *in vivo* study), or 3 or 30 IU/μl (for *in vitro* study), respectively. As a control, IFN at the same concentrations were prepared with the same method without AcNg (so final concentration is 10^4, 3 or 30 IU/μl without AcNg). For AcNg control, AcNg was prepared using the same method without IFN. The nonspecific cytotoxic effects of AcNg or IFN-AcNg in Vero, HG23 and RAW267.4 cells were monitored by the observation under a microscopy and CytoTox 96 Non-radioactive cytotoxicity assay (Promega, Madison, WI).

2.4. Atomic force microscope (AFM) experiments. The particles of AcNg and IFN-AcNg were observed under an AFM (Veeco, Santa Barbara CA). The images were collected using a tapping mode with a high aspect ratio tip (Veeco Nanoprobe TM tips, Model TESP-HAR). A small aliquot (20 µl) of AcNg or IFN-AcNg was placed onto freshly cleaved mica, washed twice with deionized water, and dried with N_2. AFM images on different locations of the mica were then obtained from a Nanoscope IIIa SPM instrument (Veeco).

2.5. Stability of IFN with or without AcNg. The lyophilized AcNg, IFN30 (30 IU/ µl), IFN3 (3 IU/ µl), IFN30-AcNg or IFN3-AcNg was solubilized with PBS and stored at 4°C for up to 14 days. Each day, each preparation was added to one-day old, 80-90% confluent NV harboring HG23 cells at the final concentration of 3 or 30 IU/ml, and then viral RNA levels were analyzed at 24 or 48 hrs after treatment using qRT-PCR. The reduction of genome by each preparation was calculated by the comparison to that of the preparation at day 0.

2.6. Promoter-luciferase assay for IFN response element in Vero and HG23 cells. The IFN activity in IFN and IFN-AcNg preparations was assessed using the reporter assay with IFN response element in Vero and HG23 cells using plasmids, pISRE-TA-Luc, pNF-kB-TA-Luc (both from Clontech), and pRL-CMV (form Promega). One-day old (~90% confluent) Vero or HG23 cells in 12 well plates were transfected with pISRE-TA-Luc or pNF-kB-TA-Luc and pRL-CMV. The pRL-CMV (for renillar luciferase under CMV promoter) served as a control for the efficiency of the transfection and for standardization of luciferase expression levels. Cells were incubated for 4 hrs before mock-medium, AcNg, IFN (3 or 30 IU/ml, final concentration) or IFN-AcNg (3 or 30 IU/ml) was added. After an additional 18 hrs, cells were harvested for analysis of luciferase expression. The luciferase assay was carried out with Dual Glo luciferase assay system (Promega) in a luminometer (Promega). The luciferase expression (firefly luciferase) from each

reporter plasmid was normalized against the expression level of the renilla luciferase encoded in pRL-CMV.

2.7. Treatment of NV- or HCV-harboring cells with IFNα or IFN-AcNg. The effects of IFNα with or without AcNg on the replication of NV or HCV in the replicon-harboring cells were examined. Mock-medium, AcNg, IFN (3 or 30 IU/ml, final concentration) or IFN-AcNg (30 or 3 IU/ml) were added to one-day old, 80-90% confluent HG23 or 1A7 cells which were analyzed for viral protein and genome expression at 24 or 48 hr after treatment. The NV protein expression levels were examined by Western blot analysis and genome expression levels by qRT-PCR, as described below. The inhibitory effects of AgNg, IFN and IFN-AcNg on the NV or HCV replicon were compared to those of Mock-medium.

2.8. The effects of IFN or IFN-AcNg on the replication of MNV-1 in RAW267.4 cells. The effects of mouse IFN-β in IFN-AcNg preparation on the replication of MNV-1 in RAW267.4 cells were examined in comparison to IFN alone. Confluent RAW267.4 cells were inoculated with MNV-1 at a MOI of 5 or 0.05 for 1 hr, and medium was replaced with medium containing mock-medium, AcNg, IFN (30 or 3 IU/ml, final concentration) and IFN-AcNg (30 or 3 IU/ml). The virus infected cells were further incubated for up to 48 hrs, and the replication of MNV-1 was measured by $TCID_{50}$ assay with the 10-fold dilution of each sample used for virus titration (Reed and Muench, 1938), and MNV-1 ProPol was detected by Western blot.

2.9. Detection of viral RNA and proteins.

2.9.1. Real-Time qRT-PCR. The quantity of NV or HCV genome in the replicon-harboring cells was measured by real-time qRT-PCR with One-step Platinum qRT-PCR kit (Invitrogen, Carlsbad, CA), following an established protocol with specific primers and probes . For qRT-

PCR, the total RNA in cells (in 6-well plate) was extracted with RNeasy kit (Qiagen, Valencia, CA). The primer sequences for NV were: Forward 5'- CGY TGG ATG CGI TTY CAT GA-3' and reverse 5'- CTT AGA CGC CAT CAT CAT TYA C -3'. The probe sequence used was: FAM-5'-AGA TYG CGI TCI CCT GTC CA - 3'-Iowa Black. The primer sequences for HCV were: Forward 5'- CGGGAGAGCCATAGTGGTCTGCG-3' and reverse 5'-CTCGAAGCACCCTATCAGGCAGTA

-3'. The probe sequence used was: FAM-5'-GCGAGCCACCGGAATTGCCT- 3'-Iowa Black. The qRT-PCR amplification was performed in a SmartCycler (Cepheid, Sunnyvale, CA) with the following parameters: 45°C for 30 min, and 95 °C 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50 °C for 1 min and elongation at 72°C for 30 s. For quantity control, qRT-PCR for β-actin was performed as described previously (Spann et al., 2004). The relative genome levels in cells with various treatments were calculated after the RNA levels were normalized with those of β-actin.

2.9.2. Western blot analysis. Protein samples of HG23 cells or MNV-1 infected RAW 267.4 cells with various treatments were prepared in SDS-PAGE sample buffer containing 1% β-mercaptoethanol, and sonicated for 20 sec. The proteins were resolved in a 10% Novex Tris-Bis gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with guinea pig antibodies specific for NV ProPol protein and the binding of the antibodies was detected with peroxidase-conjugated, goat anti-guinea pig IgG (Sigma-aldrich). In addition, membranes were probed with rabbit antiserum specific for β-actin and peroxidase-conjugated, goat anti-rabbit IgG as a loading control. Following incubation with a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL), signals were detected with X-ray film.

2.10. Oral and intravenous administration of AcNg-IFN in Sprague-Dawley rats. Four-week old female Sprague-Dawley rats were housed and studied under Institutional Animal Care and Use Committee-approved protocols. The study was designed to determine if the oral administration (oral gavage) of 100 µl of human IFN-αA alone (10^6 IU total) or AcNg-IFN (3 µg/µl of AcNg (total 100 µg) combined with 10^6 IU of IFN-αA) leads to carry over of IFN to blood. The intravenous injection of IFN or AcNg-IFN served as a control. The animals are weighed and randomly assigned to 4 groups of 3 animals each. Each group was assigned for intravenous or oral gavage administration of AcNg or AcNg-IFN. Rats are cannulated via carotid artery to collect blood. Blood samples were collected at 30 min, 1, 4, and 24 hr and serum was separated and stored at -80°C. The concentrations of IFN in each serum were measured using IFN-α detection kit (Human IFN-α ELISA kit, Interferonsournce, Piscataway, NJ). Animals were monitored by daily behavioral observation and euthanized at 1 week after the administration, and a gross necropsy was performed.

2.11. Statistical analysis. The effects of IFN or IFN-AcNg on NV or MNV-1 replication were analyzed by Student's *t* test. Results were considered statistically significant when the *P* value was <0.05.

3. Results

3.1. Nanogel preparation. The acetylated PEG-PEI cross-linked nanogel has a molecular weight of ~23 kDa (for each mole of PEI, there is ~0.4 mole of PEG attached) determined by a size exclusion chromatography. The encapsulation of IFN by AcNg was performed in water at 4^oC for 15 min, followed by lyophilization. The lyophilized AcNg and AcNg-IFN were white powder, and easily solubilized in PBS or water. The putative scheme of acetylated Ng with IFN is shown in figure 1. The AFM images of solubilized AcNg and AcNg-IFN showed that both

contained circular aggregates up to 200 nm in diameter (Figure 2, left panels). Various concentrations of IFN were used in the encapsulation with AcNg, and their AFM images were similar in appearances. The prepared AcNg was not cytotoxic to HG23 and RAW267.4 cells at up to 10 mg/ml, compared to non-acetylated Ng toxic at 0.6 mg/ml.

3.2. The stability of IFN stored at 4°C in IFN-AcNg preparation. The anti-norovirus activity of IFN stored at 4°C without AcNg showed a steep drop (by 80%) in 3-day of incubation, while the IFN activity of the IFN-AcNg preparation decreased only by up to 17% during the same period (Figure 3). These stability results show that AcNg delayed the decrease in IFN activity. The AcNg alone did not show any antiviral effects (not shown).

3.3. The effects of IFN or AcNg-IFN treatment on ISRE-luc response in Vero and Huh-7 cells. IFN alone at 30 IU/ml without AcNg significantly increased the luciferase expression in pISRE-luc system in Vero and Huh-7 cells compared to mock-treatment, while IFN at 3 IU/ml did not (Figure 4). However, IFN at both concentrations (3 and 30 IU/ml) combined with AcNg induced significant increase in luciferase expression level in both cell lines compared to mock-treatment. Similarly, AcNg significantly increased IFN-induced luciferase expression at both IFN concentrations compared to IFN alone (Figure 4). The treatment of IFN or IFN-AcNg in the cells transfected with pNFkB-TA-luc did not induce luciferase expression (not shown).

3.4. Antiviral activity of IFN or AcNg-IFN on NV or HCV replicon-harboring cells. The treatment of IFN or IFN-AcNg (3 and 30 IU/ml IFN) for 24 or 48 hrs significantly reduced the RNA levels of NV in HG23 or HCV in GS4.1 cells (Figure 5). The longer incubation of cells with IFN or IFN-AcNg resulted in greater reduction of viral RNA in both cells. Similar to the results of the luciferase expression with pISRE-luc, IFN-AcNg was significantly more effective than IFN alone at both concentrations of 3 and 30 IU/ml (Figure 5). The NV RNA levels in HG23 cells by

3 or 30 IU/ml of IFN-AcNg were reduced on average to 71% or 35% at 24 hr and 59% or 21% of mock-treatment at 48 hr, respectively. However, with IFN alone, they were reduced to 81% or 46% at 24 hr and 69% or 35% at 48 hr, respectively (Figure 5.A). In GS4.1 cells, HCV RNA levels by 3 or 30 IU/ml of IFN-AgNg were reduced to 78% or 40% at 24 hr and 60% or 15% of mock-treatment at 48 hr. With IFN only, they were 83% or 65% at 24 hr and 65% or 40% at 48 hr, respectively (Figure 5.B). However, AcNg alone did not significantly change viral RNA level in HG23 and GS4.1 cells compared to mock-treatment (Figure 5.A and B). The western blot shows that higher dose of IFN or IFN-AcNg reduced NV protein expression compared to lower dose of IFN or IFN alone, respectively (Figure 7.A).

3.5. The effects of IFN or IFN-AcNg on the replication of MNV-1. We used mouse IFN-β to examine the effects of IFN or IFN-AcNg on the replication of MNV-1. Both concentrations of IFN (3 or 30 IU/ml) with or without AcNg significantly reduced the replication of MNV-1 up to 1000-fold at 12 or 24 hr-post infection compared to mock treatment (Figure 6). Similar to the results with NV and HCV replicon-harboring cells, the IFN activity against MNV-1 in IFN-AcNg at both IFN concentrations was significantly higher than IFN alone (Figure 6). The western blot shows that higher dose of IFN or IFN-AcNg reduced NV protein expression compared to lower dose of IFN or IFN alone, respectively (Figure 7.B).

3.6. Oral administration of AcNg-IFN *in vivo*. IFN was detected up to 250 pg/ml (approximately 25 IU/ml) in the blood 30 min post-administration in rats administered with IFN or IFN-AcNg intravenously. However, IFN was not detected in the blood following oral administration of IFN or IFN-AcNg up to 24 hrs, indicating that AcNg did not cause carryover of IFN to systemic circulation. There was neither significant behavioral change by daily observation or gross pathology on necropsy at 7 days post administration in all animals.

4. Discussion

Norovirus infection continues to be an important cause of gastroenteritis in humans, the leading cause of foodborne disease followed by Salmonella contamination among laboratory-confirmed single etiologic agent cases (CDC, 2010). In most cases, norovirus infection leads to acute illness, even though recent findings demonstrated that the infection could last longer than several days or even several months, especially in immunocompromised patients (Nilsson et al., 2003). Since noroviruses are very contagious and requires only a very low dose to cause an infection, it is classified as category B bioterrorism agents. However, there is no vaccine or treatments specific for norovirus infection except for supportive therapy including fluid administration to correct dehydration. Previously, we have used the recently developed NV-replicon harboring cells and demonstrated that IFN-α and γ were effective inhibitors of NV replication (Chang et al., 2006). We also suggested that NV may not have strong means to counteract IFN systems, which further suggests the potential use of IFN in the control of noroviruses (Chang et al., 2006). Other researchers have found that the replication of MNV-1 was sensitive to IFN system *in vivo* as well as *in vitro* (Changotra et al., 2009; Karst et al., 2003). These results suggest that IFN could be potential therapeutic agent for norovirus infection.

IFN treatment in various virus infections including respiratory viruses or human hepatitis viruses has been studied. Currently, the standard therapy for chronic HCV infection is the combination therapy of IFN-α and ribavirin (Aghemo et al., 2010). For treatment of HCV infection, IFNs chemically conjugated with branched or linear PEG (pegylated IFN) were recently licensed for longer half-life of IFN in parenteral administration (Jen et al., 2001). PEG is inert, water-soluble, and nontoxic and does not adversely affect the safety profile of the IFN.

PEG exists in a multitude of molecular weights, and can be attached to IFN with size up to 40kD (Sharieff et al., 2002). It was reported that higher molecular weight of PEG yields longer half-life of the conjugated IFN. However, due to physical conjugation to IFN, PEG reduces the bioactivity of IFN (Bailon et al., 2001; Monkarsh et al., 1997). The parenteral administration of high dose of IFN (in HCV infection, the standard dose for IFN is above 1 million IU) could induce significant side effects such as influenza-like syndrome with fever, chills, myalgia, and malaise (Negro, 2010).

Due to these side effects associated with the high doses of parenteral administration of IFN, oral administration of IFN has been explored by many researchers for virus infection in mucosal surfaces [review (Beilharz, 2010)]. In natural settings, humans and animals encounter various viruses and bacteria on daily basis and secret IFN in oral and nasal cavity (Beilharz, 2010). Interestingly, the oral administration of IFN was reported to be associated with no significant side effects (Dec and Puchalski, 2008). In studies of oral administration of IFN in various animals, IFN was rarely detected in the blood, which may be related to the lack of side effects. The oral IFN studies showed conflicting results on systemic effects of IFN. In humans, Witt et al (Witt et al., 1992) reported that oral administration of high dose of IFN (2.5 and 7.5 mg) to healthy volunteers did not significantly induce IFN-responding proteins in serum. However, interestingly, low oral dose of IFN (125 IU) were more effective than intramuscular dose of millions (IU) in inducing 2',5'-OAS activity in the blood (Uno et al., 2006), and oromucosal delivery of IFN at 100 IU was immunostimulating and high dose (10^7 IU) were immunosuppressive. These suggests that feedback mechanism for interferon might be important in oral administration of IFN (Beilharz, 2010).

In influenza virus infection, low dose of intranasal administration of IFN (up to 5000 IU/dose) were found effective in preventing or alleviating symptoms without serious side effects (Arnaoudova, 1976; Imanishi et al., 1980; Isomura et al., 1982). Interestingly, subsequent studies of higher doses of IFN (1000~10,000 times more per day) showed no efficacy against

influenza viruses (Hayden et al., 1983; Merigan et al., 1973; Phillpotts et al., 1984; Saito et al., 1985; Treanor et al., 1987), suggesting that the dose of IFN is an important consideration in assessing antiviral effectiveness. In animals, recent studies showed that IFN orally or intranasally delivered to mice (Beilharz et al., 2007; Grimm et al., 2007; Tumpey et al., 2007), guinea pig (Van Hoeven et al., 2009) or ferrets (Kugel et al., 2009) reduced influenza virus replication. Interestingly, oral low-dose IFN (100 IU) were effectively protected mice from a lethal challenge of influenza virus (Beilharz et al., 2007).

Nanogels are hydrogel particles of less than 1 μm in diameter formed by physically or chemically cross-linked polymer networks. These nanogels can load large amounts of small molecules and/or proteins through spontaneous electrostatic, van der Waals and/or hydrophobic interactions, and show high stability *in vitro* and *in vivo*, thus they have huge potential as drug-delivery carriers. It has been shown that nanoencapsulation of peptides and protein colloidal particles protects them against the harsh environment of the gastrointestinal tract due to their covalent nature (Lowe and Temple, 1994). One of the potential nanogel applications is to deliver functional proteins such as IFN which would be easily degraded in biological solution. Nanogels based on chemically cross-linked networks with PEG and PEI were previously synthesized and demonstrated that they are a good tool to deliver small molecules such as antisense oligonucleotides (Vinogradov et al., 1999). Despite the beneficial properties of PEG/PEI Ng, it has been associated with cell cytotoxicity *in vitro* depending on the ratio of PEG/PEI (Ganta et al., 2008). To overcome this, we acetylated PEG/PEI Ng (AcNg) to block the reactive groups (NH and NH_2) on the surface of the Ng, reducing cytotoxicity. Enteric viruses such as norovirus replicates in intestinal epithelial cells where the receptor for IFN are located on the luminal cell surface, and acetylated Ng-IFN was postulated to activate innate immunity against noroviruses in the intestinal wall without the risk of systemic toxicity. Furthermore, IFN combined with AcNg is released as an intact form, thus bioavailability of IFN is not reduced, compared with pegylated IFN (Figure 4-6). The encapsulation of IFN with AcNg requires only simple procedure:

incubation of AcNg and IFN for 5 min at 4°C before lyophilization for storage. This simple drug-loading property of AcNg offers advantages by getting rid of use of organic solvents, surfactants, or evaporation that might affect the properties of the drugs.

In our study, the incubation of the AcNg in various cells did not show cytotoxicity up to 10 mg/ml (compared to non-acetylated Ng which is toxic at 0.6 mg/ml). The round AgNg particles in the presence or absence of IFN (up to ~ 200 nM diameter) were stable (observed by AFM) for 14 days in PBS solution stored at room temperature (data not shown). Importantly, AcNg significantly increased the stability of IFN, and anti-norovirus (MNV-1 and NV-replicon harboring cells) and HCV activity of IFN in cell culture system were significantly enhanced, compared to IFN alone. In summary, these results suggest that IFN could be used as a therapeutic agent against human norovirus, and AcNg could be a potential drug carrier of IFN by oral route.

Acknowledgement

This work was supported by NIH COBRE, P20 RR016443-07. We thank David George for technical assistance.

5. References

Aghemo, A., Rumi, M.G. and Colombo, M. (2010) Pegylated interferons alpha2a and alpha2b in the treatment of chronic hepatitis C. Nat Rev Gastroenterol Hepatol.

Arnaoudova, V. (1976) Treatment and prevention of acute respiratory virus infections in children with leukocytic interferon. Virologie 27(2), 83-8.

Bailon, P., Palleroni, A., Schaffer, C.A., Spence, C.L., Fung, W.J., Porter, J.E., Ehrlich, G.K., Pan, W., Xu, Z.X., Modi, M.W., Farid, A., Berthold, W. and Graves, M. (2001) Rational design of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem 12(2), 195-202.

Beilharz, M.W., Cummins, J.M. and Bennett, A.L. (2007) Protection from lethal influenza virus challenge by oral type 1 interferon. Biochem Biophys Res Commun 355(3), 740-4.

Beilharz, M.W.C., M.J.; Bennett, A.L.; Cummins, J.M. . (2010) Oromucosal Administration of Interferon to Humans. Pharmaceuticals 3, 323-344.

CDC. (2010) Surveillance for Foodborne Disease Outbreaks --- United States, 2007 Morbidity and mortality weekly report, pp. 973-979. Vol. 59.

Chang, K.O. (2009) Role of cholesterol pathways in norovirus replication. J Virol 83(17), 8587-95.

Chang, K.O. and George, D.W. (2007a) Bile acids promote the expression of hepatitis C virus in replicon-harboring cells. J Virol 81(18), 9633-40.

Chang, K.O. and George, D.W. (2007b) Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. J Virol 81(22), 12111-8.

Chang, K.O., Sosnovtsev, S.V., Belliot, G., King, A.D. and Green, K.Y. (2006) Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology 353(2), 463-73.

Changotra, H., Jia, Y., Moore, T.N., Liu, G., Kahan, S.M., Sosnovtsev, S.V. and Karst, S.M. (2009) Type I and type II interferons inhibit the translation of murine norovirus proteins. J Virol 83(11), 5683-92.

Dec, M. and Puchalski, A. (2008) Use of oromucosally administered interferon-alpha in the prevention and treatment of animal diseases. Pol J Vet Sci 11(2), 175-86.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P. and Estes, M.K. (2004) Laboratory efforts to cultivate noroviruses. J Gen Virol 85(Pt 1), 79-87.

Fankhauser, R.L., Noel, J.S., Monroe, S.S., Ando, T. and Glass, R.I. (1998) Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J Infect Dis 178(6), 1571-8.

Foster, G.R. (2010) Pegylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b. Drugs 70(2), 147-65.

Ganta, C., Shi, A., Battina, S.K., Pyle, M., Rana, S., Hua, D.H., Tamura, M. and Troyer, D. (2008) Combination of nanogel polyethylene glycol-polyethylenimine and 6(hydroxymethyl)-1,4-anthracenedione as an anticancer nanomedicine. J Nanosci Nanotechnol 8(5), 2334-40.

Grimm, D., Staeheli, P., Hufbauer, M., Koerner, I., Martinez-Sobrido, L., Solorzano, A., Garcia-Sastre, A., Haller, O. and Kochs, G. (2007) Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. Proc Natl Acad Sci U S A 104(16), 6806-11.

Hayden, F.G., Mills, S.E. and Johns, M.E. (1983) Human tolerance and histopathologic effects of long-term administration of intranasal interferon-alpha 2. J Infect Dis 148(5), 914-21.

Herzog, C., Just, M., Berger, R., Havas, L. and Fernex, M. (1983) Intranasal interferon for contact prophylaxis against common cold in families. Lancet 2(8356), 962.

Husa, P. and Husova, L. (2001) Treatment of chronic hepatitis C patients with combination of alpha-interferon and ribavirin, consensus and pegylated interferons. Bratisl Lek Listy 102(5), 248-52.
Imanishi, J., Karaki, T., Sasaki, O., Matsuo, A., Oishi, K., Pak, C.B., Kishida, T., Toda, S. and Nagata, H. (1980) The preventive effect of human interferon-alpha preparation on upper respiratory disease. J Interferon Res 1(1), 169-78.
Isomura, S., Ichikawa, T., Miyazu, M., Naruse, H., Shibata, M., Imanishi, J., Matsuo, A., Kishida, T. and Karaki, T. (1982) The preventive effect of human interferon-alpha on influenza infection; modification of clinical manifestations of influenza in children in a closed community. Biken J 25(3), 131-7.
Jen, J.F., Glue, P., Ezzet, F., Chung, C., Gupta, S.K., Jacobs, S. and Hajian, G. (2001) Population pharmacokinetic analysis of pegylated interferon alfa-2b and interferon alfa-2b in patients with chronic hepatitis C. Clin Pharmacol Ther 69(6), 407-21.
Karst, S.M., Wobus, C.E., Lay, M., Davidson, J. and Virgin, H.W.t. (2003) STAT1-dependent innate immunity to a Norwalk-like virus. Science 299(5612), 1575-8.
Kozlowski, A., Charles, S.A. and Harris, J.M. (2001) Development of pegylated interferons for the treatment of chronic hepatitis C. BioDrugs 15(7), 419-29.
Kugel, D., Kochs, G., Obojes, K., Roth, J., Kobinger, G.P., Kobasa, D., Haller, O., Staeheli, P. and von Messling, V. (2009) Intranasal administration of alpha interferon reduces seasonal influenza A virus morbidity in ferrets. J Virol 83(8), 3843-51.
Liu, C.J. and Kao, J.H. (2006) Pegylated interferons for the treatment of chronic hepatitis B. Recent Pat Antiinfect Drug Discov 1(1), 85-94.
Lowe, P.J. and Temple, C.S. (1994) Calcitonin and insulin in isobutylcyanoacrylate nanocapsules: protection against proteases and effect on intestinal absorption in rats. J Pharm Pharmacol 46(7), 547-52.
Luxon, B.A., Grace, M., Brassard, D. and Bordens, R. (2002) Pegylated interferons for the treatment of chronic hepatitis C infection. Clin Ther 24(9), 1363-83.
Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-related illness and death in the United States. Emerg Infect Dis 5(5), 607-25.
Merigan, T.C., Reed, S.E., Hall, T.S. and Tyrrell, D.A. (1973) Inhibition of respiratory virus infection by locally applied interferon. Lancet 1(7803), 563-7.
Monkarsh, S.P., Ma, Y., Aglione, A., Bailon, P., Ciolek, D., DeBarbieri, B., Graves, M.C., Hollfelder, K., Michel, H., Palleroni, A., Porter, J.E., Russoman, E., Roy, S. and Pan, Y.C. (1997) Positional isomers of monopegylated interferon alpha-2a: isolation, characterization, and biological activity. Anal Biochem 247(2), 434-40.
Mumphrey, S.M., Changotra, H., Moore, T.N., Heimann-Nichols, E.R., Wobus, C.E., Reilly, M.J., Moghadamfalahi, M., Shukla, D. and Karst, S.M. (2007) Murine norovirus 1 infection is associated with histopathological changes in immunocompetent hosts, but clinical disease is prevented by STAT1-dependent interferon responses. J Virol 81(7), 3251-63.
Negro, F. (2010) Adverse effects of drugs in the treatment of viral hepatitis. Best Pract Res Clin Gastroenterol 24(2), 183-92.
Nilsson, M., Hedlund, K.O., Thorhagen, M., Larson, G., Johansen, K., Ekspong, A. and Svensson, L. (2003) Evolution of human calicivirus RNA in vivo: accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. J Virol 77(24), 13117-24.
Phillpotts, R.J., Higgins, P.G., Willman, J.S., Tyrrell, D.A., Freestone, D.S. and Shepherd, W.M. (1984) Intranasal lymphoblastoid interferon ("Wellferon") prophylaxis against rhinovirus and influenza virus in volunteers. J Interferon Res 4(4), 535-41.

Phillpotts, R.J., Scott, G.M., Higgins, P.G., Wallace, J., Tyrrell, D.A. and Gauci, C.L. (1983) An effective dosage regimen for prophylaxis against rhinovirus infection by intranasal administration of HuIFN-alpha 2. Antiviral Res 3(2), 121-36.

Reed, L.J. and Muench, H. (1938) A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27, 493-497.

Saito, H., Takenaka, H., Yoshida, S., Tsubokawa, T., Ogata, A., Imanishi, F. and Imanishi, J. (1985) Prevention from naturally acquired viral respiratory infection by interferon nasal spray. Rhinology 23(4), 291-5.

Sharieff, K.A., Duncan, D. and Younossi, Z. (2002) Advances in treatment of chronic hepatitis C: 'pegylated' interferons. Cleve Clin J Med 69(2), 155-9.

Shiffman, M.L. (2001) Pegylated interferons: what role will they play in the treatment of chronic hepatitis C? Curr Gastroenterol Rep 3(1), 30-7.

Spann, K.M., Tran, K.C., Chi, B., Rabin, R.L. and Collins, P.L. (2004) Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. J Virol 78(8), 4363-9.

Treanor, J.J., Betts, R.F., Erb, S.M., Roth, F.K. and Dolin, R. (1987) Intranasally administered interferon as prophylaxis against experimentally induced influenza A virus infection in humans. J Infect Dis 156(2), 379-83.

Tumpey, T.M., Szretter, K.J., Van Hoeven, N., Katz, J.M., Kochs, G., Haller, O., Garcia-Sastre, A. and Staeheli, P. (2007) The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. J Virol 81(19), 10818-21.

Uno, K., Suginoshita, Y., Kakimi, K., Moriyasu, Y., Nakano, K., Nakamura, N., Fujita, T., Horino, Y., Sato, T. and Kishida, T. (2006) Clinical utility of 2',5'-oligoadenylate synthetase activity measurement: using whole blood as a highly sensitive method to detect the effects of IFN. J Virol Methods 136(1-2), 185-92.

Van Hoeven, N., Belser, J.A., Szretter, K.J., Zeng, H., Staeheli, P., Swayne, D.E., Katz, J.M. and Tumpey, T.M. (2009) Pathogenesis of 1918 pandemic and H5N1 influenza virus infections in a guinea pig model: antiviral potential of exogenous alpha interferon to reduce virus shedding. J Virol 83(7), 2851-61.

Vinogradov, S., Batrakova, E. and Kabanov, A. (1999) Poly(ethylene glycol)–polyethyleneimine NanoGel™ particles: novel drug delivery systems for antisense oligonucleotides Colloids and Surfaces B: Biointerfaces 16(1-4), 291-304.

Vinogradov, S.V., Kohli, E. and Zeman, A.D. (2006) Comparison of nanogel drug carriers and their formulations with nucleoside 5'-triphosphates. Pharm Res 23(5), 920-30.

Witt, P.L., Goldstein, D., Storer, B.E., Grossberg, S.E., Flashner, M., Colby, C.B. and Borden, E.C. (1992) Absence of biological effects of orally administered interferon-beta ser. J Interferon Res 12(6), 411-3.

Wobus, C.E., Karst, S.M., Thackray, L.B., Chang, K.O., Sosnovtsev, S.V., Belliot, G., Krug, A., Mackenzie, J.M., Green, K.Y. and Virgin, H.W.t. (2004) Replication of Norovirus in Cell Culture Reveals a Tropism for Dendritic Cells and Macrophages. PLoS Biol 2(12), e432.

Figure Legend

Figure 1. Schematic drawing of acetylated nanogel and entrapped interferon. The cross-link of PEI and PEG molecules in aqueous solutions form nanogel (A). The nanogels are acetylated at the active sites (NH and NH_2) to prevent cellular penetration (B). Incubation of AcNg with interferon at 4ºC for 1 min produces interferon entrapment in AcNg.

Figure 2. Atomic force microscope (AFM) images of PEG-PEI acetylated-nanogel (AcNg) only (A) and AcNg-IFNα complexes (B). The AFM image (left) of each panel shows AcNg particles with or without IFN, and its height and width are shown in the right images (up to 200 nm in diameters). The scale of y-axis (height) is in nanometer.

Figure 3. Stability of IFN or IFN-AcNg. The lyophilized AcNg, IFN30 (30 IU/ml), IFN3 (3 IU/ml), IFN30-AcNg or IFN3-AcNg were solubilized with PBS and stored at 4°C for up to 14 days. Every day, each preparation was added to one-day old, 80-90% confluent NV harboring HG23 cells which were analyzed for viral RNA level at 24 or 48 hr after treatment using qRT-PCR. The reduction of genome by each preparation was calculated by the comparison to that with each preparation at day 0.

Figure 4. Induction of luciferase activity under the control of IFN response element using Vero and Huh-7 cells. One-day old (~90% confluent) Vero or HG23 cells in 12 well plates were transfected with reporter plasmid (pISRE-TA-Luc) and pRL-CMV. At 4 hr-post transfection, the cells were incubated with mock, AcNg, or IFN (3 or 30 IU) with or without AcNg for 18 hrs at which time luciferase expression was measured. Data presented as fold increase of luciferase expression. The luciferase expression (firefly luciferase) from reporter plasmid was normalized against the expression level of the renilla luciferase encoded in pRL-CMV. Luciferase

expression by IFN-AcNg that were significantly increased (P<0.05) compared to that of IFN of same concentration without AcNg are indicated by asterisks. Bars represent standard deviations of at least 3 independent experiments.

Figure 5. Effects of IFN with or without AcNg on NV or HCV replication in NV and HCV replicon harboring cells. One-day old, semiconfluent NV replicon-harboring HG23 or HCV replicon-harboring 1A7 cells were incubated with mock, AcNg, or IFN (3 or 30 IU/ml) with or without AcNg for 24 or 48 hrs, and then total RNA was prepared for real-time qRT-PCR to detect the NV genome (A) or HCV genome (B). The reduction of genomes by various treatments was calculated by the comparison to that with mock (medium) treatment. Error bars represent standard deviations from at least three independent experiments. RNA levels for cells subjected to a treatment with IFN-AcNg that were significantly reduced (P<0.05) compared to the RNA level of treated cells with IFN without AcNg are indicated by asterisks.

Figure 6. The effects of mouse IFN-β in IFN-AcNg preparations on the replication of MNV-1 in RAW267.4 cells were examined in comparison to IFN alone. Confluent RAW267.4 cells were inoculated with MNV-1 at a MOI of 5 or 0.05 for 1 hr, and medium was replaced with medium containing mock-medium, AcNg, IFN (3 or 30 IU/ml) and IFN-AcNg (3 or 30 IU/ml). The virus infected cells were further incubated for up to 48 hr, and the replication of MNV-1 was measured by $TCID_{50}$ assay. MLV-1 titers in cells treated with IFN that were significantly reduced (P<0.05) compared to the MNV-1 titer in mock-treated cells are indicated by asterisks. Error bars represent standard deviations from at least three independent experiments.

Figure 7. Effects of various IFN treatments on the expression of NV and MNV-1 proteins in HG23 (A) and RAW267.4 (B) cells, respectively. Cell lysates were prepared after incubation mock medium, AcNg alone, IFN 3 or 30 IU/ml with or without AcNg for 48 hrs in NV-harboring

HG23 cells or following MNV-1 infection in RAW267.4 cells. Western blot analysis of cell lysates was performed with antibodies against NV or MNV-1 ProPol proteins and β-actin.