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Inhibition of HCV by the serpin antithrombin III

Mohammed Asmal[1,2]*, Michael Seaman[1,2], Wenyu Lin[2,3], Raymond T Chung[2,3], Norman L Letvin[1,2]^
and Ralf Geiben-Lynn[1,2]

Abstract

Background: Although there have been dramatic strides made recently in the treatment of chronic hepatitis C virus infection, interferon-α based therapy remains challenging for certain populations, including those with unfavorable IL28B genotypes, psychiatric co-morbidity, HIV co-infection, and decompensated liver disease. We have recently shown that ATIII, a serine protease inhibitor (serpin), has broad antiviral properties.

Results: We now show that ATIII is capable of inhibiting HCV in the OR6 replicon model at micromolar concentrations. At a mechanistic level using gene-expression arrays, we found that ATIII treatment down-regulated multiple host cell signal transduction factors involved in the pathogenesis of cirrhosis and hepatocellular carcinoma, including Jun, Myc and BMP2. Using a protein interactive network analysis we found that changes in gene-expression caused by ATIII were dependent on three nodes previously implicated in HCV disease progression or HCV replication: NFκB, P38 MAPK, and ERK1/2.

Conclusions: Our findings suggest that ATIII stimulates a novel innate antiviral host cell defense different from current treatment options.

Keywords: Antithrombin III, Hepatitis C virus, OR6 replicon cells, NFκB, P38 MAPK, ERK1/2

Background

Antiviral treatment of hepatitis C virus (HCV) is aimed at persistent eradication of the virus, as measured in sustained virological response (SVR). SVR rates are high with current treatment options, a combination of peg-interferon-apha (IFN-α)–ribavirin and direct-acting antiviral agent (DAA) but HCV patients infected with HIV and/or other co-morbidities might benefit less from the new treatment options [1].

HCV infection is currently one of the most clinically relevant co-morbidities in the HIV population; it affects 15–30% of the 1 million HIV-positive patients in the US [2]. Furthermore, progression to end-stage liver disease occurs six times faster in co-infected patients [3-6], with decompensated cirrhosis being one of the primary causes of hospitalization and death in this population [4,6-8]. Treatment of HCV infection in HIV-positive patients has a lower success rate [9-13] and an increased risk of hepatotoxicity from antiretroviral drugs compared to HIV-

negative patients [14-16]. Because of an increased risk of life-threatening complications during peg-IFN-α–ribavirin therapy, patients with hepatic decompensation are not typically candidates for this therapy unless easy access to orthotopic liver transplantation is available [17,18]. Furthermore, since the antiviral activity of IFN-α is mediated, at least in part, through the cytokine network, immunological abnormalities, such as those that often result from HIV infection, reduce IFN-α efficacy. This loss of efficacy has the result of higher treatment failure in HIV/HCV coinfected patients when compared to HCV monoinfected patients [19-22].

The effective treatment of HIV in persons with advanced cirrhosis may be challenging due to cirrhosis-induced alterations in the hepatic metabolism of antiretroviral drugs and the potential for increased risk of drug-induced liver injury. To prevent possible liver toxicity, drug doses may be reduced and certain otherwise preferred drugs may be avoided [23,24]. Reducing antiretroviral doses and hence plasma concentrations, however, may also lower the barrier to the emergence of drug-resistant HIV. Thus, effective therapy to eliminate HCV is necessary to optimize therapy for HIV.

* Correspondence: masmal@partners.org
^Deceased
[1]Division of Viral Pathogenesis, BIDMC, Boston, MA 02215, USA
[2]Harvard Medical School, Boston, MA 02215, USA
Full list of author information is available at the end of the article

We are investigating here antithrombin III (ATIII), a member of the serine protease inhibitor protein family (serpins), as its anti-inflammatory and anticoagulant activities were found to decrease liver damage [25]. Serpins participate in the early innate immune response to viral infection [26] and they simultaneously possess broad-spectrum anti-viral and anti-inflammatory capabilities. In the case of HIV infection, serpins reportedly interfere with viral replication at both the entry and the reverse transcription stages [27-30]. In particular, the serpins alpha-1 anti-trypsin, the secretory leukocyte protease inhibitor and ATIII have significant *in vitro* ability to inhibit HIV-1, with the latter, ATIII, being the most potent [29-34].

In HCV infections with co-morbidities new drugs with different mechanisms of action other than the DAAs are urgently needed. We hypothesized that the broad immunomodulatory and anti-viral properties of ATIII might extend to other chronic viral infections due to a different mechanism of action, in particular, since a serpin receptor, the LDL receptor-related protein 1 (LRP1), is highly expressed on hepatocytes [34] and was found to block HCV infection [35].

Therefore, we undertook an investigation of whether ATIII has the potential to inhibit HCV replication *in vitro*. We used gene-arrays to probe the molecular mechanisms underlying ATIII's immunomodulatory and antiviral properties, and uncover the signal transduction pathways that result in inhibition of viral replication.

Results

ATIII treatment augments the inhibition of HCV replication by IFN-α

IFN-α is currently part of the standard therapy for chronic HCV infection, in addition to ribavirin and an NS3-4A protease inhibitor. In certain patient subpopulations, this regimen is not always effective or is poorly tolerated. We have previously reported that the serpin ATIII has potent anti-viral activity against HIV [33,34]. We sought to determine whether ATIII might also have activity against HCV since serpin receptors are highly expressed on hepatocytes [36]. We employed the OR6 replicon system [37] expressing full-length genotype 1b virus to assess whether ATIII is capable of inhibiting HCV [38,39]. Although heparin activation augments the anti-HIV activity of ATIII we used unmodified ATIII because heparin activation also increases the off-target effects of ATIII on thrombin. Unmodified ATIII has a demonstrated favorable toxicity profile and has been used in humans for more than 20 years.

We initially explored the effect of ATIII monotherapy on HCV replication. We treated OR6 replicon cells with 7, 17 and 58 μM of ATIII for 48 h. We had previously demonstrated that these concentrations effectively inhibited HIV replication *in vitro* [40]. We quantified viral inhibition as the percentage of residual luciferase activity compared to a vehicle treated control. We observed that ATIII monotherapy inhibited HCV replication in the replicon system in a dose dependent manner, with the lowest dose of 7 μM inhibiting virus 70.2% ±8.8% (p<0.001, n=6) (Figure 1A).

For comparison, we assessed the ability of IFN-α2 monotherapy to inhibit the replicon. We tested doses of 4 and 16 IU IFN-α2, and found 71.4±10.1% and 84.4 ±8.4% inhibition of HCV, respectively. These results are similar to what has been reported previously [41]. We next sought to determine whether ATIII and IFN-α might have an additive effect on HCV replication. We treated replicon cells with 7, 17 and 58 μM ATIII and with 4 and 16 IU/ml IFN-α2 (Figure 1B/C). We observed an additive effect, as treatment with ATIII significantly decreased HCV replication compared to IFN-α2 monotherapy (P-value of between <0.05 and <0.01).
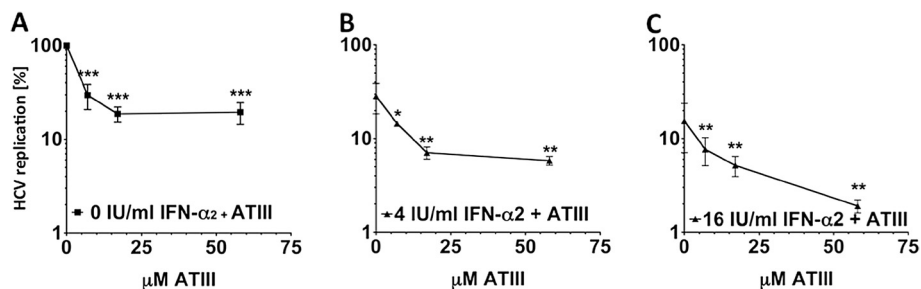
Figure 1 Additive effect of simultaneous ATIII and IFN-α treatment on HCV replication. (**A**) Effect of ATIII treatment alone on HCV replication. Significant inhibition is indicated as asteriks in compare to vehicle treated control (***, P<0.001, one-table *T*-test, n=6). (**B**) Additive effect of ATIII on 4 IU/ml IFN-α2 treatment (n=3). (**C**) Additive effect of ATIII on 16 IU/ml IFN-α2 treatment (n=3). Luciferase expression in HCV OR6 replicon cells was used as an indicator of HCV replication after treatment with IFN-α2 and/or ATIII. 5x10^4 HCV OR6 replicon cells were treated with different concentrations of IFN-α2 and ATIII. Residual luciferase activity as percentage of the vehicle treated control was used to quantify HCV replication. The additive effect of ATIII (7, 17, 58 μM) on different concentrations of IFN-α2 (in *B* and *C*) is shown by significant decrease (*, P<0,05; **, P<0.01; *** P<0.001, unpaired *T*-test) in residual luciferase activity in compare to inhibition of IFN-α2 starting concentration (4, 16 IU/ml without ATIII treatment). Error bars represent standard error of the mean.

This additive effect was already observed at the lowest dose (7 μM) of ATIII tested (Figure 1). We performed similar experiments using IFN-α5, a different subtype of IFN-α, and confirmed the additive effects of ATIII observed with IFN-α2 (data not shown).

To exclude the possibility that the antiviral effect of ATIII was due to a cytotoxic effect, we assayed for cytotoxicity using Neutral Red and Trypan Blue exclusion staining at the indicated concentrations of drugs. Neither ATIII alone or in combination with IFN-α2 or IFN-α5 showed a cytotoxic effect (data not shown).

ATIII-induced alterations in gene expression in non-replicon cells

To assess the effect of ATIII treatment on host cell gene expression in the absence of HCV protein expression, we treated Huh7.5. non-replicon cells with the highest concentration of ATIII that would be used in the subsequent gene array experiments: 24 U/ml (58 μM), which is 24-fold the physiologic concentration. We found no significant alterations in expression of genes in the array following ATIII treatment of the non-OR6 replicon cells, demonstrating that, at these concentrations and in the absence of HCV replication, ATIII has no significant effect on expression of our transcriptional pathways of interest. Using Trypan Blue exclusion staining we also found no drug-related cytotoxicity at the concentrations used (data not shown).

HCV-induced alterations of hepatocyte gene expression

To assess the effect of HCV replication on hepatocyte physiology we compared the transcriptional profile of HCV replicon cells to that of Huh7.5 non-replicon cells using the Transduction Pathfinder RT2 Profiler PCR Assay. Initially, experiments were performed in the absence of exogenous ATIII. We observed substantial differences in the transcription of multiple genes involved in the innate host cell response between cells expressing and not-expressing HCV (Tab. I). Many of these HCV-induced changes have been previously described elsewhere [42-49] confirming the validity of our system. The gene with the greatest increase in expression was Matrix Metallopeptidase 10 (MMP10) (49-fold, P = 0.006), a key mediator in the Jak-Stat pathway and part of the inflammatory response of the host cell against HCV. Additional effects of HCV on host gene expression include activation of p53 and TGF-β pathways: we found a 3-fold (P = 0.03) increase of Cyclin-dependent kinase inhibitor (CDKN) 1A and a 10-fold (P = 0.005) increase of CDKN2B. Other observations from this study that are consistent with previously described associations with HCV include findings of a 9-fold (P = 0.04) increase of Bone morphogenetic protein 4 (BMP4), part of the hedgehog pathway, and a 4-fold (P = 0.03) increase in Heat Shock Protein (HSP) 90AA2, part of the cellular stress response.

Effect of ATIII on HCV-induced changes in gene expression

We subsequently sought to determine if ATIII might modulate the effects of HCV on host gene expression. We treated replicon cells with 7 μM ATIII, a concentration at which inhibition of HCV replication was observed, and compared gene expression to untreated replicon cells (Table 1). None of the genes affected by HCV expression appeared to be substantially affected by ATIII treatment at this lowest dose.

At higher concentrations of ATIII, we found only a modest effect on HCV-induced transcriptional changes (data not shown). There was no ATIII dose-dependent

Table 1 Determination of ATIII treatment effect on gene expression in the context of HCV

Gene	Symbol	Replicon/Non-replicon Gene expression		Replicon/Replicon+7 μM ATIII Gene expression	
		Fold change	P-value	Fold change	P-value
Bone morphogenetic protein 4	BMP4	9.15	0.04	−1.68	0.09
Cyclin-dependent kinase inhibitor 1A	CDKN1A	2.61	0.03	−1.64	0.1
Cyclin-dependent kinase inhibitor 2B	CDKN2B	10.98	0.005	−1.59	0.1
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	5.70	0.002	−1.73	0.01
Fatty acid synthase	FASN	4.01	0.01	−2.87	0.9
Heat shock 27kDa protein 1	HSPB1	2.82	0.001	−1.21	0.6
Heat shock protein 90kDa alpha class A member 2	HSP90AA2	4.01	0.03	−1.14	0.6
Interleukin 4 receptor	IL4R	4.76	0.01	−1.79	0.04
Matrix metallopeptidase 10	MMP10	49.29	0.006	−2.19	0.3
Ornithine decarboxylase 1	ODC1	4.49	0.01	1.35	0.4
Retinol binding protein 1	RBP1	2.66	0.045	−1.59	0.2
WNT1 inducible signaling pathway protein 1	WISP1	6.32	0.008	−1.16	0.6

effect on expression of any of the genes in Table I. These results suggest that the mechanism by which ATIII inhibits HCV within 48 h may not involve modulation of the genes influenced by HCV infection.

ATIII-induced alterations in replicon cell gene expression

HCV infection often leads to chronic hepatitis, cirrhosis, and occasionally to hepatocellular carcinoma [50]. This progression in liver pathology is associated with increased expression in hepatocytes of the transcription factors JUN and MYC, which may play crucial roles in oncogenesis [51,52]. In order to investigate the influence of ATIII on pathways important for HCV disease progression we employed the Transduction Pathfinder RT2 Profiler PCR Assay to quantify the expression of 84 key genes belonging to 18 different regulatory pathways in the presence of different concentrations of ATIII.

To investigate whether the therapeutic use of ATIII might have an influence on gene-expression in OR6 replicon cells, we treated these cells with supra-physiologic concentration of ATIII: 2.4-fold (7 μM), 7-fold (17 μM) and 24-fold (58 μM) blood concentrations. We used supra-physiologic doses of ATIII in part because ATIII is known to accumulate in the liver - a fact which may be of therapeutic advantage.

Treatment of replicon cells with these doses of ATIIII altered expression by more than 5-fold in a group of genes when compared to vehicle treated controls (Figure 2). Interestingly, genes that were most significantly affected were all down-regulated. Among those genes found to be down-regulated following ATIII treatment were JUN and MYC, which are known to be important factors in the pathogenesis of HCV-related hepatocellular carcinoma. We found that these genes were down-regulated in a dose dependent manner, up to 931-fold for JUN (P = 0.0002), and up to 45-fold for MYC (P = 0.0007) at 58 μM.

The next largest decrease in gene expression, up to 346-fold (P = 0.009 at 58 μM), was observed for the transcription factor CAAT/enhancer binding protein (CEBPB), a protein regulated by insulin. Another gene downstream of insulin Hexokinase 2 (HK2), was down-regulated up to 14-fold (P = 0.0039). Growth arrest and DNA-damage-inducible protein (GADD45A), a gene in the p53 pathway, was down-regulated 35-fold at 58 μM (P = 0.0023). Bone morphogenetic protein 2 (BMP2), a gene of the Hedgehog pathway, was down-regulated 13-fold (P = 0.03) at 58 μM. B-cell CLL/lymphoma2-like 1 (BCL2L), a transcript belonging to the Jak/Src pathway, exhibited an approximately 10-fold decrease in expression (Figure 2). Down-regulation of these genes was specific to ATIII treated OR6 cells with ongoing HCV replication, and was not observed in the untreated OR6 replicon, nor in the ATIII-
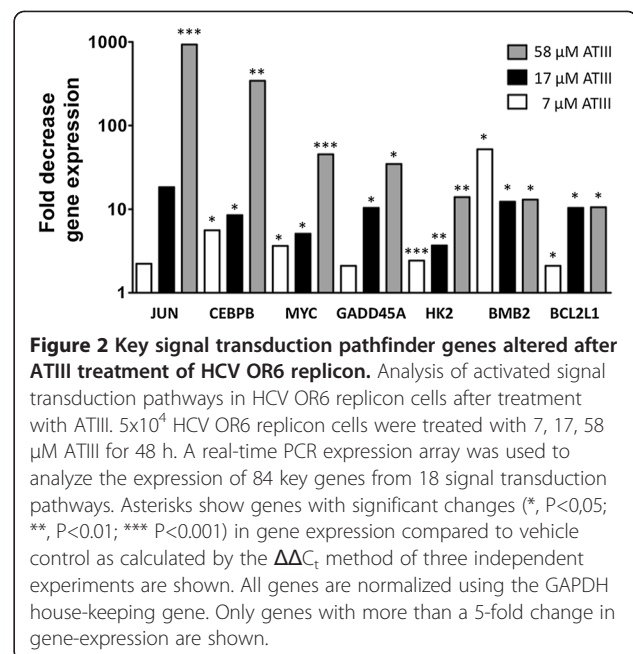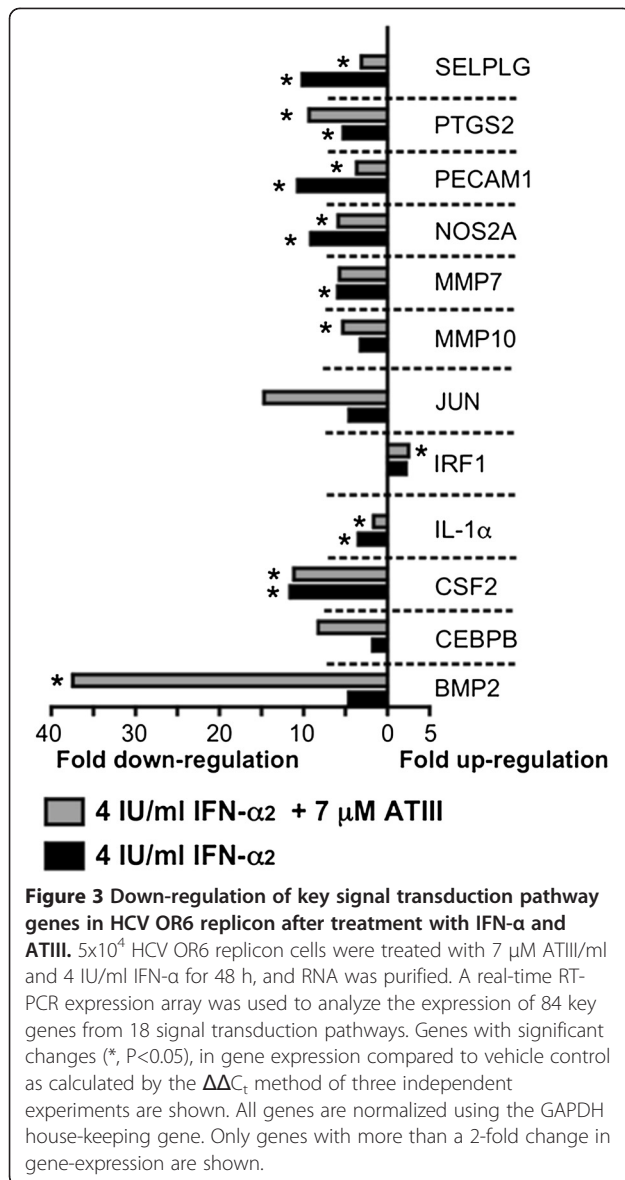

Figure 2 Key signal transduction pathfinder genes altered after ATIII treatment of HCV OR6 replicon. Analysis of activated signal transduction pathways in HCV OR6 replicon cells after treatment with ATIII. 5×10^4 HCV OR6 replicon cells were treated with 7, 17, 58 μM ATIII for 48 h. A real-time PCR expression array was used to analyze the expression of 84 key genes from 18 signal transduction pathways. Asterisks show genes with significant changes (*, P<0,05; **, P<0.01; *** P<0.001) in gene expression compared to vehicle control as calculated by the $\Delta\Delta C_t$ method of three independent experiments are shown. All genes are normalized using the GAPDH house-keeping gene. Only genes with more than a 5-fold change in gene-expression are shown.

treated Huh7.5 controls suggesting that ATIII induces a specific anti-viral gene program.

Changes in gene expression when ATIII is used in combination with IFN-α

Our data indicate that both IFN-α and ATIII stimulate the host innate antiviral response. We sought to determine whether these proteins engaged redundant cellular pathways, or had non-overlapping mechanisms of action. We applied IFN-α2 in isolation, to measure the gene expression alterations attributable to this drug. The gene expression profile induced by IFN-α monotherapy that we observed was consistent with previous reports [53,54]. At 4 IU/ml, we found Interferon regulatory factor 1 (IRF-1) up-regulated 2-fold. We also identified a set of inflammatory genes that were down-regulated: Colony stimulating factor 2 (CSF2) (inhibited 11.5-fold, P=0.01), IL-1α (inhibited 4-fold, P=0.02), MMP7 (inhibited 6-fold, P=0.04), MMP10 (inhibited 3-fold), Nitric oxide synthase 2A (NOS2A) (inhibited 9-fold, P=0.009), and Prostaglandin-endoperoxide synthase (PTGS2) (5-fold, P=0.01) (Figure 3).

When IFN-α was administered in combination with ATIII, additional genes were significantly altered, potentially explaining the additive antiviral effect of ATIII when added to IFN-α treatment. The most substantially down-regulated gene was BMP2, belonging to the Hedgehog pathway, which was decreased by 37-fold (P=0.04). JUN and PTGS2 both belonging to the Phospholipase C pathway were 14-fold (P=0.08) and 9-fold (P=0.02) down-regulated. CEBPB of the insulin pathway was 8-fold down-regulated (Figure 3).

Figure 3 Down-regulation of key signal transduction pathway genes in HCV OR6 replicon after treatment with IFN-α and ATIII. 5×10^4 HCV OR6 replicon cells were treated with 7 μM ATIII/ml and 4 IU/ml IFN-α for 48 h, and RNA was purified. A real-time RT-PCR expression array was used to analyze the expression of 84 key genes from 18 signal transduction pathways. Genes with significant changes (*, P<0.05), in gene expression compared to vehicle control as calculated by the $\Delta\Delta C_t$ method of three independent experiments are shown. All genes are normalized using the GAPDH house-keeping gene. Only genes with more than a 2-fold change in gene-expression are shown.

We repeated these experiments using IFN-α5, to exclude the possibility that our results may have been idiosyncratic to IFN-α2. We observed the same gene expression pattern with IFN-α5 treatment, with or without ATIII treatment (data not shown).

Network analysis of ATIII-induced interactomes in OR6 replicon cells

To gain further insight into the mechanism of action of ATIII in reducing HCV replication, we performed a biologic network analysis of ATIII treated OR6 replicons. This analysis method complements data generated from our gene arrays by facilitating the recognition of hierarchical gene clusters that might intersect with HCV replication. This software-supported interactome analysis is based on a vast library of gene interactions

known to regulate certain pathways and allows to identify pathways effected by a drug.
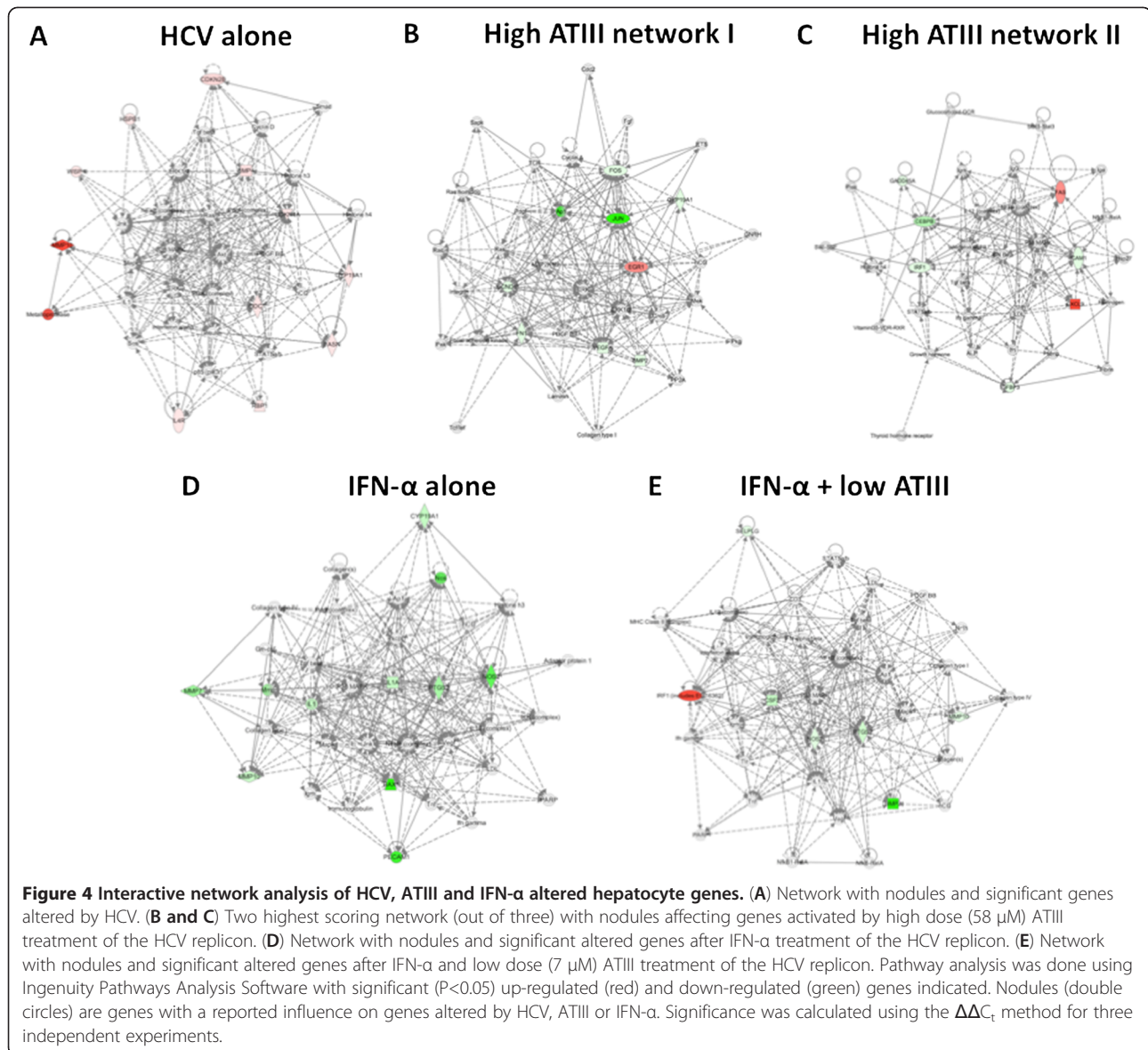
We used genes which we had observed to be significantly up-regulated by HCV replication (Table 1) to generate interactomes describing host cell transduction pathways activated by HCV. We identified nodules regulated by ERKs, AKT, PI3K, RAS, NFκB, P38, P38 MAPK and MAPK as all being activated by HCV infection (Figure 4A). We then assessed the influence of treatment with the high dose (58 μM) of ATIII on gene expression to determine which of these HCV nodules were affected by gene expression changes downstream of ATIII. We found that ATIII interacted with two independent networks that were also modulated by HCV. The highest scoring network was mostly dependent on the ERKs (Figure 4B), and the second highest scoring network interfered with NFκB and P38 MAPK (Figure 4C). These results suggested that despite our observation that ATIII and HCV alter the expression of different sets of individual genes, transcriptional programs activated by ATIII may interfere with 3 out of the 6 nodules activated by HCV. We hypothesize that this might be significant enough to counteract some of the pathologic effects of HCV.

We have demonstrated additive activity of IFN-α and ATIII in inhibiting HCV. We thus next sought to determine whether they might exhibit overlapping effects on the HCV interactome. We compared the effect of IFN-α/low ATIII dose therapy to that of IFN-α alone on HCV induced nodules. Treatment with 4 IU/ml IFN-α alone altered three HCV induced nodules: P38 MAPK, MAPK and NFκB (Figure 4D). The addition of low dose ATIII did not alter the number of nodules affected, but did increase the IRF1 response (Figure 4E) which is known to be responsible for some of the HCV inhibition and might be the reason for the additive effect on IFN-α treatment.

Discussion

The cornerstone of effective therapy for chronic hepatitis C infection has been IFN-α, a critical mediator in the innate immune response to viral infection. Even with the advent of small molecule direct inhibitors of viral enzymatic activity, IFN-α remains important for attaining sustained virologic response, perhaps because of the need to engage host antiviral programs to completely eradicate viral reservoirs. However, interferon-based therapy is not without its shortcomings, including poor tolerability or poor efficacy in certain patient populations.

We now demonstrate that a novel activator of host innate antiviral responses, ATIII, may give insight into adjunctive therapies for HCV that might augment or even replace IFN-α in cases where there are co-morbidities or genetic factors that preclude the use of IFN-α. There is

Figure 4 Interactive network analysis of HCV, ATIII and IFN-α altered hepatocyte genes. (**A**) Network with nodules and significant genes altered by HCV. (**B and C**) Two highest scoring network (out of three) with nodules affecting genes activated by high dose (58 μM) ATIII treatment of the HCV replicon. (**D**) Network with nodules and significant altered genes after IFN-α treatment of the HCV replicon. (**E**) Network with nodules and significant altered genes after IFN-α and low dose (7 μM) ATIII treatment of the HCV replicon. Pathway analysis was done using Ingenuity Pathways Analysis Software with significant (P<0.05) up-regulated (red) and down-regulated (green) genes indicated. Nodules (double circles) are genes with a reported influence on genes altered by HCV, ATIII or IFN-α. Significance was calculated using the $\Delta\Delta C_t$ method for three independent experiments.

circumstantial evidence that ATIII may play a role in the pathogenesis of HCV infection: low plasma concentrations of ATIII have previously been correlated with progression to chronic hepatitis and cirrhosis [55]. Furthermore, there is a high density of serpin receptors on hepatocytes, suggesting that serpins may have localized effects on hepatic innate immunity [56].

We used the OR6 replicon to probe how ATIII might influence HCV pathogenesis. We demonstrated that ATIII inhibited HCV replication at micromolar concentrations. While this inhibition was not as potent as that of either IFN-α or fluvastatin [41] it was many-fold greater than that of ribavirin [57].

We next investigated the mechanism of ATIII's anti-HCV activity. After more than 20 years of research, the mechanism of action of IFN-α in inhibiting HCV has only

recently been determined. HCV cell-based expression models, as the one used in this study, were used to demonstrate that IFN-α-induced signal transduction through the Jak/STAT pathway was necessary for HCV inhibition [45]. To elucidate the mechanism(s) through which serpins activate the host defense system, we employed the OR6 replicon system, and analyzed changes in gene-expression patterns of 84 key genes representative of 18 different signal transduction pathways. We found that ATIII treatment down-regulated JUN expression. It has previously been shown that the JNK inhibitor, SP600125, interferes with the oncogenic potential of HCV non-structural protein 3 [58]. In addition, we found that ATIII treatment reduced induction of the transcription factor MYC, expression of which has been associated with progression of liver disease to chronic hepatitis, cirrhosis, and

hepatocellular carcinoma [52,59]. ATIII treatment also reduced CEBPB, a transcription factor of the CCAAT/enhancer-binding protein class. These transcription factor proteins have been shown to be important for HCV inhibition: the CCAAT/enhancer-binding protein-homologous protein (CHOP) is activated by HCV envelope protein and is associated with disease progression [60]. The mechanism of action of this protein class is not fully understood, but CHOP can sensitize cells to apoptosis by down-regulation of BCL-2 expression, depletion of cellular glutathione, and exaggerated production of reactive oxygen species [61].

Our data also identified a substantial down-regulation of BMP2, a protein that regulates hepcidin [62]. Hepcidin is critical for iron homeostasis and is also a critical host cofactor involved in promoting HCV replication [63]. Chronic HCV infection has been found to be associated with increased serum iron and transferrin saturation as well as hepatic iron accumulation; moreover, hepatic hepcidin mRNA expression is increased in patients with chronic HCV infection [64]. Hepcidin transcription is stimulated by iron overload as well as by inflammation through IL-6 [65], which is elevated in patients with chronic HCV. The identification of hepcidin as a HCV replication cofactor suggests a molecular basis for the well-known clinical association between chronic HCV infection and dysregulation of iron homeostasis. Moreover, it is possible that the up-regulation of hepcidin transcription by IL-6 potentially creates a positive feedback loop between chronic inflammation and HCV replication. Together, these findings suggest that ATIII therapy may reduce the pathogenic impact of HCV infection.

Thus, our data indicate that ATIII targets several genes that are known to promote both liver disease and HCV replication. ATIII treatment may therefore alter the expression of these genes and act to simultaneously slow both HCV replication and ultimately liver degeneration. ATIII's effect on gene-expression was also observed when replicon cells were co-treated with low concentrations of IFN-α. It was during this dual drug treatment that gene expression of BMP2, CEBPB, and JUN were most dramatically down-regulated.

Protein interactive network analysis demonstrated that the genes that were altered by ATIII treatment were dependent on three nodes: NFκB, P38 MAPK and the ERKs. All of these nodes have been described previously as having a role in HCV replication and HCV-related liver disease [47,66-69] confirming ATIII's potential to limit HCV's destruction of the liver. These nodules are also affected in ATIII-mediated inhibition of HIV [40].

Although our replicon model can facilitate identification of substances that affect either viral genomic replication or host cell factors involved in viral genomic replication, it cannot be used to identify substances that alter other stages of the viral life cycle. Therefore, future studies using fully infectious, cell-culture-adapted HCV strains will be required to study other aspects of the HCV life cycle, such as viral entry, uncoating, viron assembly and secretion. Our data identified several genes altered by ATIII that were previously shown to be correlated with HCV disease outcome. This might explain the additive therapeutic effect when ATIII was used in combination with IFN-α. We further found that ATIII's mechanism of action is most likely multi-faceted, warranting further research into each distinct signaling pathway.

Material and methods
Cell culture
OR6 replicon cells were a gift from Dr. Nobuyuki Kato (Okayama University, Japan) and were propagated in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin, and 500 μg/ml Geneticin (Invitrogen Corp., Carlsbad, CA). Cells were cultured in a 37°C, 5% CO_2-humidified incubator for all experiments. To decrease day-to-day variability in the assay, a large homogenous population of subconfluent cells was passaged so that a similar lot of cells could be used throughout the assay.

Protein reagents
Clinical grade human ATIII (Talecris, Durham, NC) had a concentration of 6 U/mg and a purity >98%. For ATIII drug combination experiments, recombinant human IFN-α2 and IFN-α5 (PBL Interferon Source, Piscataway, NJ) was used, which had a concentration of 2.38×10^8 and 2.33×10^8 units/mg, respectively, and a purity of > 98%.

Determination of inhibitory potency
HCV replication inhibition was determined as the percentage of luciferase activity retained by the OR6 replicon after ATIII treatment, compared to a vehicle-treated control [37]. Luciferase activity was measured using the Renilla Luciferase Assay System (Promega, Madison, WI) at 1 sec. intervals using the 1420 Multilabel Counter Victor 3 (PerkinElmer, Waltham, MA). Cell viability was assessed using Neutral Red staining and Tryphan Blue exclusion staining.

Signal transduction pathway profiling
OR6 replicon cells were harvested after 48 h of ATIII and/or IFN-α treatment and total RNA was recovered using the RNeasy Kit (Qiagen) with an on-column DNAse digest (Qiagen) according to the manufacturer's protocol. Approximately 100 ng RNA was used for cDNA synthesis using the SuperArray RT^2 First Strand Kit (SABiosciences, Frederick, MD, cat. no. C-03). cDNA

was used for the RT^2 Profiler PCR Array Human Signal Transduction PathwayFinder (SABiosciences, cat. no. PAHS-014A). The genes that were investigated can be found at http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-014A.html. Three arrays of three independent experiments were performed for each treatment condition. Relative levels of transcription were determined by using the ΔC_t values for each gene obtained by subtracting the mean threshold cycle (C_t) of the GAPDH housekeeping gene from the C_t value of the gene of interest. The average ΔC_t value for 3 experiments was calculated, for each gene of interest, and the average normalized transcription was calculated as follows: $2 (-\text{average}\Delta Ct)^{-1}$. Fold increases of gene transcription, before and after treatment was calculated by dividing the average normalized transcription of each gene in the test sample by the corresponding control. Statistical significance in up- or down-regulation of transcription was determined by Student T-test (2-sample, equal variance, 2-tailed distribution), comparing the $\Delta\Delta C_t$ ($\Delta\Delta C_t = \Delta C_t$ treated - $\Delta\Delta C_t$ control). Significant differences were identified when P was less than 0.05.

Analysis of protein interactive networks and statistical analysis

Functional analysis of interacting proteins was determined using a commercial System Biology package, Ingenuity Pathways Analysis (IPA 8.0) (www.ingenuity.com) following the application protocols.

Statistical analysis

The statistical significance of differences between groups was determined using the program GraphPad Prism. A P value of <0.05 was considered statistically significant. Statistical analysis was performed using one table T-test or the unpaired T-test. Error bars represent standard error of the mean (S. E.).

Authors' contributions
MA analyzed data and wrote the manuscript. MS and WL did experiments. RTC and NLL analyzed data. RGL did experiments, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

Author details
[1]Division of Viral Pathogenesis, BIDMC, Boston, MA 02215, USA. [2]Harvard Medical School, Boston, MA 02215, USA. [3]Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114, USA.

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