Published in final edited form as:

Gastroenterology. 2011 March ; 140(3): 830–839.e3. doi:10.1053/j.gastro.2010.09.010.

S-adenosyl methionine improves early viral responses and interferon-stimulated gene induction in hepatitis C nonresponders

Jordan J. Feld^{1,2,*}, Apurva A. Modi^{1,*}, Ramy El-Diwany¹, Yaron Rotman¹, Emmanuel Thomas¹, Christopher Koh¹, Vera Cherepanov², Theo Heller¹, Marc G. Ghany¹, Yoon Park¹, Jay H. Hoofnagle¹, and T. Jake Liang¹

¹ Liver Diseases Branch, National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD

² Toronto Western Hospital Liver Center, University Health Network, University of Toronto, Toronto, Canada

Abstract

Background & Aims—Fewer than half of patients infected with Hepatitis C virus (HCV) achieve sustained viral clearance after peginterferon and ribavirin therapy. S-adenosyl methionine (SAME) increases interferon signaling in cell culture. We assessed the effect of SAME on the kinetics of the early anti-viral response and interferon signaling in patients that did not respond to previous therapy (nonresponders) and investigated its mechanisms.

Methods—Nonresponders with HCV genotype-1 were given 2 weeks of peginterferon alfa-2a and ribavirin (Course A, baseline/control). After a 1-month period, patients received SAME (1600 mg daily) for 2 weeks and then peginterferon and ribavirin for 48 weeks (Course B; completed by 21 of 24 patients). Viral kinetics and interferon-stimulated gene (ISG) expression in peripheral blood mononuclear cells (PBMCs) were compared between courses.

Address Correspondence to: T Jake Liang, Liver Diseases Branch/NIDDK/NIH, Building 10, Room 9B16, 10 Center Drive MSC 1800, Bethesda MD 20892-1800, Phone: 301-496-1721, Fax: 301-402-0491, JakeL@intra.niddk.nih.gov.

*indicated authors contributed equally to the study

Author Contributions: JJF – study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of manuscript, statistical analysis

AAM - study concept and design, acquisition of data, analysis and interpretation of data, critical revision of manuscript

RED - acquisition of data, analysis and interpretation of data, critical revision of manuscript

YR - acquisition of data, analysis and interpretation of data, critical revision of manuscript, technical support

ET - acquisition of data, analysis and interpretation of data, critical revision of manuscript, technical support

CK - critical revision of manuscript, technical support

VC – technical support

TH - analysis and interpretation of data, critical revision of manuscript

MGG - acquisition of data

YP - acquisition of data, study coordination

JHH - study concept and design, acquisition of data, analysis and interpretation of data, critical revision of manuscript, study supervision

TJL - study concept and design, acquisition of data, analysis and interpretation of data, critical revision of manuscript, technical support, material support, study supervision

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Results—The decrease in HCV RNA from 0 to 48 hours (phase 1) was similar before and after administration of SAME. However, the slope increased for the second-phase decrease in HCV between courses A and B (Course A= $0.11 \pm 0.04 \log_{10}$ IU/mL/week, Course B= 0.27 ± 0.06 ; $P=0.009$); 11 patients (53%) achieved an early virological response and 10 (48%) had undetectable HCV RNA by week 24. Induction of ISGs in PBMCs was significantly greater after Course B. In cultured cells, SAME increased induction of ISGs, compared with only peginterferon and ribavirin, and the antiviral effects of interferon by increasing STAT1 methylation, which might promote binding of STAT1 to DNA.

Conclusions—The addition of SAME to peginterferon and ribavirin improves the kinetics of the early anti-viral response and induces ISGs in patients with HCV genotype 1 that do not respond to interferon therapy. SAME might be used with peginterferon-based therapies in patients with chronic HCV infections.

Keywords

Interferon signaling; transcription factor; chronic hepatitis; SAME; interferon-stimulated genes (ISGs); viral kinetics; hepatitis C; gene induction; STAT1; methylation; PIAS1

Introduction

Despite major improvements in therapy for chronic hepatitis C virus (HCV) infection, approximately half of patients do not achieve a sustained response to treatment with peginterferon and ribavirin^{1, 2}. Attempts at retreatment in non-responders have yielded poor results, leaving such patients with few therapeutic options^{3–7}. Although new targeted antivirals look very promising, they require concomitant treatment with peginterferon and ribavirin, to prevent rapid emergence of antiviral resistance^{8, 9}. As a result, development of strategies to improve interferon responsiveness remains a priority.

To establish and maintain persistent chronic infection, HCV must block or avoid host immune responses¹⁰. An important early component of the antiviral immune response is the production of type I interferons¹¹. Once produced, interferon acts in an autocrine and paracrine manner, binding to the interferon receptor and leading to signaling through the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway. STAT1 and STAT2 bind to the intracellular domain of the interferon receptor where they are in turn phosphorylated by JAK1. Once in their active form, phosphorylated STAT1 and 2 (pSTAT1/2) form homo or heterodimers and combine with interferon regulatory factor 9 (IRF9) to form a complex known as interferon-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus and binds to the interferon-stimulated response element (ISRE), a promoter upstream of a large number of genes with antiviral, antiproliferative and immunoregulatory properties, collectively known as interferon-stimulated genes (ISGs)¹². HCV has evolved strategies to interfere with the interferon cascade at almost every level of the pathway from interferon production to inhibition of individual antiviral ISGs¹⁰.

As with most cellular processes, negative regulators of interferon signaling and ISG induction act to keep the antiviral immune response in check. The protein inhibitor of activated STAT (PIAS) family of proteins, including PIAS1, 3, X and Y, was discovered in yeast two-hybrid experiments as negative regulators of the STAT proteins¹³. PIAS1 is present in the nucleus of cells and binds p-STAT1 to prevent the interaction of ISGF3 with the ISRE, thus preventing ISG transcription¹⁴. When arginine 31, the putative PIAS1 binding site on STAT1, is methylated, binding is inhibited¹⁵. As a consequence, the methylation status of STAT1 is an important determinant of ISG expression. Duong et al.

have demonstrated that HCV exploits the down-regulation of ISG expression by interfering with STAT1 methylation and thus facilitating the interaction with PIAS116.

Expression of HCV proteins or natural HCV infection leads to up-regulation of protein phosphatase 2a (PP2A), which directly inhibits the enzymatic function of protein arginine methyl transferase (PRMT1), the major regulator of STAT1 methylation¹⁶. Reduced PRMT1 activity leads to decreased methylation of STAT1. Upon interferon stimulation, hypomethylated p-STAT1 is more readily bound by PIAS1, resulting in decreased STAT1-DNA binding and reduced ISG transcription, a favorable state for HCV. To determine if increasing STAT1 methylation improves ISG expression, Duong and colleagues treated cells expressing HCV proteins with S-adenosyl methionine (SAME), a potent methyl donor, and were able to restore ISG expression. They also showed that SAME improved the antiviral effects of interferon in a mouse model¹⁷.

Whether SAME improves interferon signaling and antiviral responses in HCV infection is currently unknown. The goal of this study was to evaluate the effect of the addition of SAME to peginterferon and ribavirin on interferon signaling and early virological responses in patients with chronic hepatitis C who had previously failed to respond to an adequate course of interferon-based therapy.

Methods

Patients

Patients with a documented history of non-response to combination treatment with standard interferon or peginterferon and ribavirin, were eligible for enrollment. Inclusion criteria included: age ≥ 18 years, documented genotype 1 chronic HCV infection, and a history of at least 3 months of combination antiviral therapy (with interferon or peginterferon plus ribavirin) and failure to achieve an early virological response (EVR) defined as a $2\text{-log}_{10}\text{IU/mL}$ decline in HCV RNA levels by week 12 of therapy. Exclusion criteria included decompensated liver disease, hepatocellular carcinoma, evidence of other forms of liver disease, human immunodeficiency virus (HIV) co-infection, active drug or alcohol abuse, any contraindication to peginterferon, ribavirin or SAME therapy and pregnancy or refusal to use adequate contraception during therapy.

All patients gave written, informed consent, and the study protocol and consent forms were approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) at the Clinical Center of the National Institutes of Health and registered in ClinicalTrials.Gov (#NCT00475176).

SAME was provided as 400 mg tablets by Pharmavite LLC (Northridge, CA) under a Clinical Trial Agreement with the NIDDK. Pharmavite had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Administration of SAME was conducted under an Investigational New Drug Application (IND # 72,595) held by the authors (Sponsor: JHH).

Treatment

To establish baseline early viral kinetics without SAME, patients were treated with peginterferon alfa-2a (180 μg subcutaneously weekly) and weight-based ribavirin (1000 mg for $<75\text{kg}$ and 1200 mg for $\geq 75\text{kg}$, by mouth daily) for 2 weeks (Course A). Therapy was then withheld for a 1-month washout period, whereupon patients received a loading regimen of oral SAME 800 mg twice daily for 2 weeks. SAME loading was followed by Course B. Peginterferon and ribavirin were restarted while SAME was continued for a planned 48 week course (Course B).

Standard early stopping rules and definitions of response were used¹⁸. A rapid virological response (RVR) was defined as HCV RNA levels <15 IU/mL by week 4 of treatment. Early virological response (EVR) was defined at treatment week 12 as partial (pEVR) if HCV RNA was detectable but had declined by at least 2-log₁₀IU/mL compared to baseline; EVR was considered complete (cEVR) if HCV RNA was no longer detectable (<15 IU/mL) at week 12. Patients who did not achieve a pEVR were given the option to stop therapy or continue for another 12 weeks. Therapy was discontinued in all patients who had detectable HCV RNA in serum at week 24. Patients for whom HCV RNA became undetectable beyond 12 weeks were given the option to extend therapy to 72 weeks. A sustained virological response (SVR) was defined as lack of detectable HCV RNA in serum 24 weeks after stopping treatment. Relapse was defined as reappearance of detectable HCV RNA after treatment.

Early Viral Kinetics

HCV RNA levels were measured using Cobas TaqMan real-time PCR (Roche Diagnostics, Palo Alto, CA), with a lower limit of detection of 15 IU/mL. Viral RNA levels were measured at baseline and at 8, 24, 48 hours, 7, 9 and 14 days of both Course A and B. After the first 2 weeks of Course B, HCV RNA levels were tested weekly until week 4, followed by every 2 weeks until week 8 and then monthly to week 24 and every two months thereafter. Viral kinetic parameters were determined as described in the supplementary methods.

Interferon Stimulated Gene Expression

Peripheral blood mononuclear cells (PBMC) were extracted from whole blood as described in the supplementary methods. mRNA expression levels for the ISGs, Viperin (RSAD2), Myxovirus resistance protein 1 (Mx1) and Interferon Stimulated Gene 15 (ISG15) were determined using TaqMan quantitative real-time PCR and normalized for 18S ribosomal RNA expression. Fold induction was calculated using the delta delta Ct method with the time 0 sample as the baseline¹⁹. Time 0 samples for all patients were then run on the same TaqMan assay to allow for comparison of ISG expression between patients. For a subset of patients, PBMC were lysed in RIPA buffer and protein expression was assessed by immunoblot for RSAD2, ISG15, DMA and actin. Induction was calculated as the ratio of expression at 24 hrs to that at time 0, normalized for actin expression, during Course A and Course B. Immunoprecipitation (IP) for STAT1 followed by immunoblot for DMA was also performed on PBMC samples at 0 and 24 hrs of Course A and Course B as described below.

Cell Culture and SAME/Interferon Treatment

Huh7.5.1 cells were grown in cell culture in complete Dulbecco's medium (DMEM). Cells were treated with increasing doses of SAME (Sigma, St Louis, MO) (0, 200, 400, 800, 1600 nM) with or without interferon- α 100 units/mL (PBL InterferonSource, Piscataway, NJ). Cells were treated with SAME for 2 hours prior to interferon treatment and then harvested in Trizol 4 hours after interferon treatment and stored at -80°C. RNA extraction, cDNA synthesis and ISG expression by TaqMan real-time PCR were performed as described for PBMC.

HCV Cell Culture

Huh7.5.1 cells were infected with the Japanese Fulminant Hepatitis 1 (JFH1) HCV clone at a multiplicity of infection of 0.05 FFU/cell. After 24 hours of infection, cells were treated with increasing doses of SAME with or without interferon. SAME was added 2 hours prior to interferon. Cells were harvested 24 and 72 hours after interferon treatment and intracellular HCV RNA was quantified as described in supplementary methods.

Immunoprecipitation and Immunoblot

Huh7.5.1 cells were treated with SAME 800 nM, interferon- α 50 U/mL or the combination of both in 10 cm dishes. SAME was added 2 hours prior to interferon. One hour after interferon treatment, cells were harvested in 200 μ l of RIPA buffer and processed as described in the supplementary methods. Experiments were repeated in the setting of HCV infection. Cells were infected with JFH1 HCV for 24 hours and then treated with SAME, interferon or the combination. Immunoblotting and co-immunoprecipitation were repeated.

Statistical Analysis

The primary outcome of the study was the change in first and second phase viral decline with the addition of SAME. Viral kinetic parameters, and changes in ALT levels, hemoglobin and ISG expression were compared between Course A and B using either the paired t-test or the non-parametric Wilcoxon Matched Pairs Test for non-normally distributed data according to the Shapiro Wilk test²⁰. The area-under-the-curve (AUC) of gene induction was estimated by an arithmetic sum of the fold expression values at all measured time points. Analyses were performed with Stata Version 9.2 (College Station, TX) and Prism Graphpad version 4.0 (La Jolla, CA).

Results

Twenty-four patients were enrolled (Table 1) between October 2007 and April 2009. Eighteen (75%) had a liver biopsy available, which showed advanced fibrosis ($\geq$ Ishak 3) in 8 (44%) and cirrhosis in 5 (21%). Baseline viral levels ranged from 5.0 to 7.9 log₁₀IU/mL. Three patients (13%) completed Course A only: one developed a severe rash attributed to ribavirin, one relapsed to benzodiazepine abuse during the initial course, and one patient withdrew from the study for personal reasons. These 3 patients were not included in the analysis. A fourth patient completed both Course A and B but missed blood draws in both Courses, precluding calculation of accurate first and second phase kinetic parameters. This patient was excluded from analysis of early viral kinetics and ISG expression but was included in analysis of treatment outcome.

SAME was well tolerated in most patients with 4 reporting mild gastrointestinal symptoms thought to be due to lactose in the tablet preparation, 3 of whom improved with lactase supplementation. Seventeen of 21 (81%) patients reported fewer interferon-related side effects with the addition of SAME, however patients were not blinded to SAME treatment. There were no serious adverse events.

Viral Kinetics

First phase decline in HCV RNA (0 to 48 hours) was similar between Course A and Course B (Table 2, Figure 1a). Similarly, calculated interferon efficacy (ϵ) did not differ between Course A and B (Table 2). In contrast, there was significant improvement in second phase viral decline with the addition of SAME, the slope of decline increasing from 0.11 ± 0.04 log₁₀ IU/mL/week during Course A to 0.27 ± 0.06 log₁₀ IU/mL/week during Course B ($p=0.009$)(Figure 1b). In keeping with their non-responder phenotype, during Course A 17 of 20 (85%) patients had a second phase slope of <0.35 log₁₀ IU/mL/week, below the minimum value associated with SVR²¹ and 6 patients had a negative second phase slope (viral load increased on treatment). With the addition of SAME, 13 patients (62%) had a second phase slope <0.35 log₁₀ IU/mL/week ($p=0.14$) and only 1 patient had a negative phase 2 slope ($p=0.038$). Overall 14 (70%) patients had improved second phase slopes in Course B compared to Course A. The second phase slope calculated from day 7 to 14 correlated well with the standard second phase slope calculation from day 7 to 28 ($r=0.80$,

$p < 0.0001$) (Supplementary Figure 1). HCV RNA nadir was lower in Course B than Course A (Table 2).

During the 2 weeks of SAME monotherapy, HCV RNA levels increased by $0.15 \log_{10}$ IU/mL, a modest but statistically significant amount ($p = 0.01$) (Figure 1c). Nevertheless, the baseline HCV RNA level was similar at time 0 of Course A and B (6.29 vs $6.35 \log_{10}$ IU/mL) ($p = 0.46$). The average ALT level declined during SAME loading from 99 ± 15 to 83 ± 10 U/L, ($p = 0.05$); however, ALT changes during peginterferon and ribavirin therapy did not differ between courses. Similarly there was no difference in decline in hemoglobin with the addition of SAME.

Treatment Response

Of the 21 previous non-responders who started Course B, 1 (5%) had an RVR, 4 (19%) had a cEVR and 11 (53%) achieved a pEVR. All but one of the 11 with a pEVR went on to have undetectable HCV RNA during the treatment (48% of all patients). To date, 13 patients have finished 24 weeks of follow-up after the end of treatment, of whom 4 achieved an SVR (39%) and 3 relapsed (3/10 30%). Of the remaining 8 patients, 5 have not responded and 3 had end-of-treatment responses. If all 3 undetectable patients relapse, the SVR rate for the cohort will be 19%. If they all go on to achieve SVR, the rate of SVR will be 33%.

ISG Expression

All three ISGs measured (ISG15, RSAD2 and Mx1) showed greater induction with the addition of SAME than with peginterferon and ribavirin alone. ISG15 induction was greater at all time-points in Course B than Course A and the difference was statistically significant at the 8 hour time-point (Figure 2a, $p = 0.035$). The area under the curve for total ISG15 expression was greater in Course B than Course A (Table 2). Similarly, Mx1 and RSAD2 expression were increased at all but the day 14 time-point in Course B compared to Course A. By day 14, expression levels for most patients had returned to near baseline (Figure 2b–c). The area under the curve for total induction of both RSAD2 and Mx1 was also significantly higher in Course B than Course A (Table 2). As with ISG15, the difference in RSAD2 expression between Course A and B was most pronounced at the 8-hour time-point, which was also time of the peak level of induction for most patients. For Mx1, the greatest difference in induction between Course A and B occurred at the 48-hour time-point, in keeping with its known later peak expression level in response to interferon²². RSAD2 and ISG15 induction at 8 hours was greater in all but 3 patients in Course B than Course A, all of whom were non-responders to treatment with peginterferon, ribavirin and SAME. Notably, ISG expression for all 3 genes was increased after SAME loading compared to the baseline in Course A and this difference was statistically significant for RSAD2 ($p = 0.02$). At baseline, mean expression of all 3 ISGs was higher in non-responders than in those with a cEVR, however the differences were not statistically significant (Supplementary Figure 2). Protein expression on a subset of patients mirrored mRNA levels. Blots from a representative patient are shown in Figure 2d. Induction at 24 hours compared to baseline was increased when patients received SAME (Course B) compared to peginterferon and ribavirin alone (Course A) (Figure 2d, panel 1). Methylation was increased with SAME loading and after an initial dose of peginterferon and ribavirin, as assessed by total DMA expression (Figure 2d panel 2). Immunoprecipitation of STAT1 followed by Western blotting (WB) for DMA showed increased STAT1 methylation (Figure 2d, panel 3).

SAME improves interferon signaling and antiviral effects *in-vitro*

To clarify how SAME improves interferon signaling and early viral kinetics, cell culture models were used. Similar to the results seen in patients, ISG expression in response to interferon was enhanced in Huh7.5.1 cells when pretreated with SAME. SAME alone had

minimal effect on ISG expression. When combined with interferon, increasing doses of SAME led to increased Mx1 expression (Figure 3a). Similar results were seen with ISG15 and RSAD2 for all experiments (Supplementary Figure 3). The effect of SAME plateaued at the 800 nM dose, with no further induction seen with higher doses. Serum concentrations of SAME in the range of 800 nM have been reported with oral treatment²³. The addition of SAME to interferon also increased ISG expression in primary human hepatocytes (Supplementary Figure 4). The effect of SAME on ISG induction was similar in the presence or absence of HCV infection (Figure 3b). Unlike in patients, SAME alone had no effect on ISG induction even in the presence of HCV infection, likely due to the defective interferon production pathways in Huh7.5.1 cells which significantly limits ISG induction in response to viral infection²⁴.

When infected cells were treated with SAME and interferon, the pattern of viral decline was similar to that observed in patients. At a very early time-point (4 hours), there was a modest increase in HCV RNA levels after exposure to SAME alone and no effect of interferon alone or in combination with SAME (Figure 4a). By 24 hours of treatment, interferon treatment significantly reduced viral replication, however the addition of SAME had no appreciable additive antiviral effect (Figure 4b). When cells were harvested 72 hours after treatment, an effect of SAME was apparent. With increasing doses of SAME, greater viral inhibition was seen (Figure 4c).

SAME affects methylation of STAT1

To evaluate whether the methylation of STAT1 or PIAS1²⁵ accounted for the observed effects of SAME on ISG induction, the methylation status of both molecules and their interaction was evaluated. Immunoprecipitation (IP) with STAT1 followed by WB for DMA showed increased methylated STAT1 in the cells treated with SAME, with or without interferon. STAT1 methylation was also increased with interferon alone, as has been reported previously¹⁶ (Figure 5a, top panel). There was no increase in STAT1 levels with interferon treatment because the IP was done 1 hour after interferon treatment, too early to see an effect on protein levels. A modest increase of PIAS1 methylation was also observed with SAME treatment, with or without interferon treatment (Figure 5a, middle panel). IP with PIAS1 followed by STAT1 WB showed decreased PIAS1-STAT1 interaction after exposure to SAME (Figure 5a, bottom panel). The experiment was repeated in the presence of HCV infection. SAME decreased PIAS1-STAT1 binding with and without interferon treatment (Figure 5b).

Discussion

Peginterferon and ribavirin are likely to remain the backbone of antiviral treatment for chronic HCV for many years, even once new direct antivirals become available^{8, 9}. As a result, improving the response to interferon-based therapy remains a major priority. Results from this pilot study show that addition of SAME to standard therapy improves early viral kinetics, likely by improving interferon signaling.

Previous *in-vitro* data suggested that SAME may improve interferon signaling and antiviral effects through increased methylation of STAT1 leading to enhanced STAT1-DNA binding and thus greater ISG expression. This effect is particularly relevant in HCV infection because of the ability of HCV to induce PP2A, which may interfere with STAT1 methylation^{16, 17}. This study was undertaken to determine if the effects of SAME observed in model systems would actually lead to improved treatment responses in patients with HCV infection.

The study design allowed patients to serve as their own controls to tease apart the effects of SAME on early viral kinetics in a cohort of genotype 1 non-responders. The addition of SAME to peginterferon and ribavirin had no effect on the first phase but improved the second phase slope of viral decline, which is thought to represent clearance of infected cells²⁶. SAME treatment was also associated with greater induction of ISGs in PBMCs. The augmentation of ISG induction might be expected to affect the first phase decline, which is thought to be a measure of the effectiveness of interferon at blocking the production of new virions²⁶. However, no early effect was seen with the addition of SAME. The consistency of the findings in different ISGs with different kinetics suggests that the effect of SAME on ISG induction is likely broad, which also fits with the proposed mechanism of improved STAT1-DNA binding, a common event for known ISGs¹⁶. Which ISGs are actually responsible for HCV clearance is currently unknown. It is possible that genes with later peak induction or delayed antiviral effects are responsible for the improvement in second phase kinetics. Ribavirin has recently been postulated to work through induction of a subset of ISGs and, like SAME, ribavirin treatment leads to an improvement in second phase slope with no effect on first phase decline^{27–29}. Notably, in the *in vitro* experiments, the effect of the addition of SAME on viral clearance was not apparent until 72 hours after interferon treatment despite an effect on ISG mRNA expression as early as 4 hours. Clearly an improved understanding of how ISGs lead to viral clearance and specifically which ISGs affect which phases of viral decline, is needed.

In numerous retreatment trials, response rates among previous non-responders have been very poor. In the REPEAT trial, the largest retreatment study to date, Jensen and colleagues reported that 41% of previous non-responders receiving standard therapy achieved a pEVR and 13% had undetectable HCV RNA at week 12 (cEVR)⁵. In the lead-in phase to the HALT-C trial, patients were retreated with peginterferon and ribavirin and 33% had undetectable HCV RNA by week 20. In this study with the addition of SAME, 10 of 21 (48%) patients had a pEVR and 19% achieved a cEVR, including 1 patient with an RVR. By week 20, 10 of 21 patients (48%) had undetectable HCV RNA. These results suggest that SAME can improve viral response rates in patients who are relatively resistant to interferon therapy. The important question, however, is whether improvement in these early responses will translate into higher rates of SVR. However it is important to acknowledge that this study was not designed nor powered to detect a difference in SVR but rather to explore whether SAME improved interferon signaling and early viral kinetics.

Clearly with SVR rates of 19–33%, the addition of SAME will not be sufficient on its own for the majority of non-responders. However ISG data from patients as well as the *in-vitro* mechanistic data suggest that SAME works by improving interferon signaling and ISG induction. Improved interferon signaling may be particularly relevant for the treatment of non-responders as we enter the era of targeted antivirals. There has been great concern about the use of direct antivirals in prior non-responders because of the risk of developing resistance with ‘effective monotherapy’ due to the lack of effect of peginterferon and ribavirin. Even a marginal improvement in interferon efficacy may be enough to prevent the development of resistance, potentially leading to improved rates of viral clearance and ultimately of SVR. This strategy will have to be evaluated directly in clinical trials. SAME may also improve outcomes in treatment naïve patients, although this was not addressed in this study.

The cell culture data provide support for the proposed mechanism of action of SAME. Improved ISG induction was seen at physiologic concentrations of SAME added to interferon. The co-immunoprecipitation data support the notion that SAME increases STAT1 methylation *in-vitro* and *in-vivo*. Treatment with SAME with or without interferon reduced the interaction of PIAS1 and STAT1. Most importantly, SAME pretreatment led to an

enhancement of the antiviral effect of interferon, particularly at later time-points, very similar to what was observed in study patients.

This study has some notable limitations. Second phase slope was assessed at 2 weeks rather than the standard 4 weeks²⁶. Asking patients to undergo therapy for a full 4 weeks before stopping treatment during the control course was felt to be problematic. Importantly, the 2-week viral kinetic data correlated very well with 4-week data in Course B suggesting that this is a reasonable surrogate (supplementary figure 1). In addition, ISG induction was measured in PBMCs rather than liver, due to the impracticality of obtaining repeated liver specimens. The mechanism by which SAME improves interferon signaling is not likely organ specific, however given that HCV affects interferon signaling differently between compartments³¹, it is possible that the effect of SAME on STAT1 methylation would be more pronounced in HCV-infected hepatocytes. It is notable that ISG induction in PBMCs correlates with treatment outcome³².

In summary, the addition of SAME to peginterferon and ribavirin led to improved early viral kinetics, enhanced ISG induction in PBMCs and higher rates of early and sustained viral clearance in a cohort of previous genotype 1 non-responders. SAME was well tolerated and *in-vitro* data suggest that it works by increasing STAT1 methylation, leading to improved STAT1-DNA binding, and ultimately to enhanced ISG expression. Thus SAME represents the first interferon sensitizing agent with *in-vivo* efficacy. SAME appears to be a useful adjunct to interferon-based therapy, a factor that may be particularly important in the era of direct antivirals for HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This research was supported by the Intramural Research Program of NIDDK. JJF reports serving on the advisory board of Roche Pharmaceuticals and Schering-Plough (Merck) Pharmaceuticals. None of the other authors has any financial interest or conflict of interest related to this research. Pharmavite provided SAME for the study but had no involvement in study design, data analysis or manuscript preparation.

Abbreviations

HCV	hepatitis C virus
SAMe	S-adenosyl methionine
RVR	rapid virological response
pEVR	partial early virological response
cEVR	complete early virological response
SVR	sustained virological response
ISG	interferon stimulated gene
STAT1	signal transducer and transcription 1
IRF9	interferon regulatory factor 9
ISRE	interferon stimulated response element
PIAS1	protein inhibitor of activated STAT1
DMA	dimethyl arginine

ISG15	interferon stimulated gene 15
Mx1	Myxovirus resistance protein 1
RSAD2	viperin
PCR	polymerase chain reaction

References

1. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975–82. [PubMed: 12324553]
2. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;358:958–65. [PubMed: 11583749]
3. Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, Lee WM, Lok AS, Bonkovsky HL, Morgan TR, Ghany MG, Morishima C, Snow KK, Dienstag JL. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N Engl J Med* 2008;359:2429–41. [PubMed: 19052125]
4. Camma C, Cabibbo G, Bronte F, Enea M, Licata A, Attanasio M, Andriulli A, Craxi A. Retreatment with pegylated interferon plus ribavirin of chronic hepatitis C non-responders to interferon plus ribavirin: a meta-analysis. *J Hepatol* 2009;51:675–81. [PubMed: 19665247]
5. Jensen DM, Marcellin P, Freilich B, Andreone P, Di Bisceglie A, Brandao-Mello CE, Reddy KR, Craxi A, Martin AO, Teuber G, Messinger D, Thommes JA, Tietz A. Re-treatment of patients with chronic hepatitis C who do not respond to peginterferon-alpha2b: a randomized trial. *Ann Intern Med* 2009;150:528–40. [PubMed: 19380853]
6. Moucari R, Ripault MP, Oules V, Martinot-Peignoux M, Asselah T, Boyer N, El Ray A, Cazals-Hatem D, Vidaud D, Valla D, Bourliere M, Marcellin P. High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. *J Hepatol* 2007;46:596–604. [PubMed: 17218037]
7. Taliani G, Gemignani G, Ferrari C, Aceti A, Bartolozzi D, Blanc PL, Capanni M, Esperti F, Forte P, Guadagnino V, Mari T, Marino N, Milani S, Pasquazzi C, Rosina F, Tacconi D, Toti M, Zignego AL, Messerini L, Stroffolini T. Pegylated interferon alfa-2b plus ribavirin in the retreatment of interferon-ribavirin nonresponder patients. *Gastroenterology* 2006;130:1098–106. [PubMed: 16618404]
8. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J, Muir AJ. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009;360:1827–38. [PubMed: 19403902]
9. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourliere M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839–50. [PubMed: 19403903]
10. Gale M Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005;436:939–45. [PubMed: 16107833]
11. Kawai T, Akira S. Innate immune recognition of viral infection. *Nat Immunol* 2006;7:131–7. [PubMed: 16424890]
12. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 2005;436:967–72. [PubMed: 16107837]
13. Liu B, Liao J, Rao X, Kushner SA, Chung CD, Chang DD, Shuai K. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A* 1998;95:10626–31. [PubMed: 9724754]
14. Shuai K. Modulation of STAT signaling by STAT-interacting proteins. *Oncogene* 2000;19:2638–44. [PubMed: 10851063]

15. Mowen KA, Tang J, Zhu W, Schurter BT, Shuai K, Herschman HR, David M. Arginine methylation of STAT1 modulates IFN α /beta-induced transcription. *Cell* 2001;104:731–41. [PubMed: 11257227]
16. Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastroenterology* 2004;126:263–77. [PubMed: 14699505]
17. Duong FH, Christen V, Filipowicz M, Heim MH. S-Adenosylmethionine and betaine correct hepatitis C virus induced inhibition of interferon signaling in vitro. *Hepatology* 2006;43:796–806. [PubMed: 16557551]
18. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009;49:1335–74. [PubMed: 19330875]
19. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques* 2005;39:75–85. [PubMed: 16060372]
20. Shapiro SSWM. An analysis of variance test for normality (complete samples). *Biometrika* 1965;52:591–611.
21. Lindh M, Alestig E, Arnholm B, Eilard A, Hellstrand K, Lagging M, Wahlberg T, Wejstal R, Westin J, Norkrans G. Response prediction and treatment tailoring for chronic hepatitis C virus genotype 1 infection. *J Clin Microbiol* 2007;45:2439–45. [PubMed: 17581934]
22. Lanford RE, Guerra B, Lee H, Chavez D, Brasky KM, Bigger CB. Genomic response to interferon-alpha in chimpanzees: implications of rapid downregulation for hepatitis C kinetics. *Hepatology* 2006;43:961–72. [PubMed: 16628626]
23. Goren JL, Stoll AL, Damico KE, Sarmiento IA, Cohen BM. Bioavailability and lack of toxicity of S-adenosyl-L-methionine (SAME) in humans. *Pharmacotherapy* 2004;24:1501–7. [PubMed: 15537554]
24. Li K, Chen Z, Kato N, Gale M Jr, Lemon SM. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. *J Biol Chem* 2005;280:16739–47. [PubMed: 15737993]
25. Weber S, Maass F, Schuermann M, Krause E, Suske G, Bauer UM. PRMT1-mediated arginine methylation of PIAS1 regulates STAT1 signaling. *Genes Dev* 2009;23:118–32. [PubMed: 19136629]
26. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;282:103–7. [PubMed: 9756471]
27. Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, Schweigler LM, Theodore D, Zacks SL, Liang TJ, Fried MW. Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response. *Hepatology*. 2007
28. Dixit NM, Layden-Almer JE, Layden TJ, Perelson AS. Modelling how ribavirin improves interferon response rates in hepatitis C virus infection. *Nature* 2004;432:922–4. [PubMed: 15602565]
29. Feld JJ, Lutchman GA, Heller T, Hara K, Pfeiffer JK, Leff RD, Meek C, Rivera M, Ko M, Koh C, Rotman Y, Ghany MG, Haynes-Williams V, Neumann AU, Liang TJ, Hoofnagle JH. Ribavirin improves early responses to peginterferon through improved interferon signaling. *Gastroenterology* 2010;139:154–62. e4. [PubMed: 20303352]
30. Everson GT, Shiffman ML, Hoefs JC, Morgan TR, Sterling RK, Wagner DA, Desanto JL, Curto TM, Wright EC. Quantitative tests of liver function measure hepatic improvement after sustained virological response: results from the HALT-C trial. *Aliment Pharmacol Ther* 2009;29:589–601. [PubMed: 19053983]
31. Huang Y, Feld JJ, Sapp RK, Nanda S, Lin JH, Blatt LM, Fried MW, Murthy K, Liang TJ. Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C. *Gastroenterology* 2007;132:733–44. [PubMed: 17258724]
32. Taylor MW, Tsukahara T, Brodsky L, Schaley J, Sanda C, Stephens MJ, McClintick JN, Edenberg HJ, Li L, Tavis JE, Howell C, Belle SH. Changes in gene expression during peginterferon and ribavirin therapy of chronic hepatitis C distinguish responders from non responders to antiviral therapy. *J Virol*. 2007

Figure 1. Effects of SAME on HCV RNA levels and on first and second phase kinetic responses to peginterferon and ribavirin

Viral kinetics were compared between Course A (peginterferon and ribavirin alone) and Course B (peginterferon, ribavirin and SAME) showing the effect of SAME on a) the first phase viral decline, b) the average second phase slope and c) on HCV RNA levels as monotherapy. P-values are based on Wilcoxon matched pairs test.

Figure 2. Effects of SAME on ISG induction in PBMCs of patients receiving peginterferon and ribavirin

Expression of a) ISG15, b) Mx1 and c) RSAD2 mRNA levels in PBMC were compared between Course A (peginterferon and ribavirin alone) and Course B (peginterferon, ribavirin and SAME) at six time points. Values reflect the average fold-induction compared to the baseline expression [Time 0] for each patient. The area under the curve (AUC) reflects the mean fold-induction over all time-points measured. Mean AUC values were compared using the non-parametric Wilcoxon matched pairs test. d) Protein expression of RSAD2, DMA and actin in PBMC was compared between baseline and 24 hours in Course A and after SAME loading at baseline and 24 hrs in Course B. STAT1 methylation was assessed by IP for STAT1 followed by WB for DMA. Results are shown for a representative patient. Relative units (RU) compared using time 0 for each Course as baseline. * p<0.05 for comparison.

a. Huh7.5.1 cells

b. HCV-infected Huh7.5.1 cells

Figure 3. Effects of SAME on ISG induction *in-vitro*

a) Huh7.5.1 cells were treated with SAME alone or SAME combined with interferon- α (50 IU/mL). Cells were incubated with SAME for 2 hours before addition of interferon and were harvested for assessment of gene induction 4 hours after interferon treatment. ISG expression (Mx1) was evaluated by real-time PCR. b) Huh7.5.1 cells infected with the JFH1 clone of HCV (for 24 hours) were subjected to the same treatment using SAME alone or combined with interferon. Note the scales of Mx1 induction are different with SAME alone and SAME + interferon. Mean results from 3 independent experiments shown. * $p < 0.05$ for comparison with interferon- α alone.

Figure 4. Effects of addition of SAME to interferon on HCV RNA levels *in-vitro*

Huh7.5.1 cells were infected with the JFH1 HCV for 24 hours and then treated with SAME alone or SAME (for 2 hours) and interferon- α (50 IU/mL). Cells were harvested at different time-points after interferon treatment and intracellular HCV RNA was measured by real-time PCR. Mean results from 3 independent experiments shown. * $p < 0.05$ for comparison with interferon- α alone.

Figure 5. Effects of SAMe on STAT1 methylation

a) Huh7.5.1 cells were treated with SAMe (800nM) for 2 hours, interferon- α for 1 hour or the combination (SAMe pretreatment followed by interferon- α) and then harvested in RIPA buffer. IP was performed with anti-STAT1, anti-PIAS1 or anti-DMA antibodies and then WB was performed with each individual antibody. The effect of SAMe treatment with or without interferon on STAT1 methylation (first panel), PIAS1 methylation (second panel) and the interaction between PIAS1 and STAT1 (third panel) is shown. **b)** The experiment was repeated in the presence of HCV infection. IP, immunoprecipitation; WB, western blot; RU, relative units.

Table 1

Baseline Characteristics

	n=24 n (%) / average $\pm$ sd
Male	16 (67%)
Race	
Caucasian	21 (88%)
African American	2 (8%)
Hispanic	1 (4%)
Median Age [years]	52.5 (range 41–65)
Mean BMI (kg/m ²)	27.2 $\pm$ 3.4
Median Ishak Fibrosis (0–6)	2 (range 0–6)
Cirrhosis	5 (21%)
Previous Therapy	
Interferon/Ribavirin	3 (12%)
Peginterferon/Ribavirin	21 (88%)
Genotype	
1	13 (54%)
1a	9 (38%)
1b	2 (8%)
Baseline HCV RNA level [log ₁₀ IU/mL]	6.3 $\pm$ 0.57

Table 2

Early Viral Kinetic Parameters

	Course A Peginterferon + Ribavirin (n=20)	Course B Peginterferon + Ribavirin + SAmE (n=20)	p-value
Baseline HCV RNA Level [$\log_{10}$ IU/mL]	6.3 ± 0.1	6.4 ± 0.1	0.46
First Phase Decline [$\log_{10}$ IU/mL]	0.56 ± 0.09	0.53 ± 0.07	0.74
ϵ	0.59 ± 0.06	0.62 ± 0.05	0.57
Second Phase Slope [$\log_{10}$ IU/mL/week]	0.11 ± 0.04	0.27 ± 0.06	0.009
HCV RNA Nadir [$\log_{10}$ IU/mL]	5.73 ± 0.3	5.54 ± 0.2	0.045
ISG15 Expression (AUC)	109 ± 62	192 ± 95	0.007
RSAD2 Expression (AUC)	72 ± 17	171 ± 67	0.038
Mx1 Expression (AUC)	43 ± 18	103 ± 32	0.040

Abbreviations: SAmE, S-adenosyl methionine; HCV, hepatitis C virus; IU, international units; ISG, interferon stimulated gene; RSAD2, virperin; Mx1, myxovirus resistance 1; AUC, area under the curve.