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Research Article

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Vitamin D Metabolites Inhibit Hepatitis C Virus and Modulate Cellular Gene Expression

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Abstract

Background and Aims: Previous studies suggest that low serum 25-hydroxyvitamin D [25(OH) D] levels are associated with reduced responsiveness to interferon and ribavirin therapy. We investigated the impact of vitamin D metabolites on HCV and cellular gene expression in cultured hepatoma cells.

Methods: HCV Replicon cell lines stably expressing luciferase reporter constructs (genotype 1b and 2a replicon) or JC1-Luc2a were incubated in the presence of vitamin D2, vitamin D3 or 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). Presence of HCV was quantified by a luciferase reporter assay and immunoblot of the Core protein. Synergy of interferon-alpha A/D (IFN-α) and 1,25(OH)2D3 was evaluated using the Chou-Talalay method. Cellular gene expression by microarray analysis using Illumina Bead Chips and real-time quantitative PCR.

Results: Vitamin D2, D3 and 1,25(OH)2D3 each demonstrated anti-HCV activity at low micro molar concentrations. In vitro conversion from D3 to 25(OH)D3 was shown by LC/MS/MS. Combination indices of 1,25(OH)2D3 and IFN-α demonstrated a synergistic effect (0.23-0.46) and significantly reduced core expression by immunoblot. Differentially expressed genes were identified between Huh7.5.1 cells in the presence and absence of 1,25(OH)2D3 and HCV. Genes involved with classical effects of vitamin D metabolism and excretion were activated, along with genes linked to autophagy such as G-protein coupled receptor 37 (GPR37) and Hypoxia-inducible factor 1-alpha (HIF1a). Additionally, additive effects of 1,25(OH)2D3 and IFN-α were seen on mRNA expression of chemokine motif ligand 20 (CCL20).

Conclusions: This study shows that vitamin D reduces HCV protein production in cell culture synergistically with IFN-α. Vitamin D also activates gene expression independently and additively with IFN-α and this may explain its ability to aid in the clearance of HCV in vivo.

Keywords: Hepatitis C Virus; Ribavirin therapy; Interferon

Introduction

The hepatitis C virus (HCV) is the leading cause of death from viral hepatitis in this country and over 160 million persons are chronically infected worldwide [1,2]. The current standard of care for chronic HCV infection is rapidly changing and new Direct Acting Antiviral (DAAs) has made HCV treatment more efficacious. During the interferon era, an intense search was undertaken to identify risk factors for treatment failure. HCV genotype, insulin resistance, obesity, cirrhosis, and race all emerged is important predictors of sustained virologic response (SVR). Surprisingly, data also suggested that vitamin D status could also affect treatment outcomes in HCV when using pegylated-interferon and ribavirin (PEG-IFN/RBV).

Vitamin D2 comes from non-animal sources, while vitamin D3 is synthesized by humans and other mammals when 7-dehydrocholesterol is exposed to UV light. Both vitamin D2 and vitamin D3 can be obtained from the diet and from supplements. Vitamin D2 and D3 can be hydroxylated by cytochrome P450 enzymes in hepatocytes to 25-hydroxycholecalciferol (25(OH) D) [3]. Higher levels of 25-hydroxyvitamin D appear to enhance the control of viral and bacterial infections [4]. The active form of vitamin D, 1,25-dihydroxycholecalciferol (1,25(OH)2D), is believed to mediate its effects through the vitamin D receptor (VDR) and have beneficial effects on the innate immune system, although un-hydroxylated vitamin D and 25(OH) D are also expected to be weaker agonists of the VDR.

Patients with liver disease often have vitamin D deficiency [5-12], which the Institute of Medicine defines as a serum 25(OH) D level ≤ 20 ng/mL. The cause of deficiency in this group is likely multi-factorial but may be attributed to inflammation, decreased hepatic synthesis of 25(OH) D, malabsorption of fat soluble vitamins and/or decreased exposure to UV radiation in chronically ill patients [6,13].

Several studies support supplementation of HCV patients with vitamin D prior to anti-HCV therapy. Low baseline serum 25(OH) D levels are independently associated with a poor response to interferon/ribavirin-based therapy [14] while patients in whom 25(OH) D levels were greater than 20ng/mL had an increased odds ratio for achieving an SVR [15]. Lange and colleagues also found that vitamin D deficiency was a risk factor for HCV treatment failure [16]. In contrast, a trial of treatment-naïve HIV/HCV co-infected patients did not find an association between 25(OH) D levels and SVR [17]. A randomized study of vitamin D supplementation versus no supplementation in HCV genotype 1 patients treated with PEG-IFN/RBV showed significantly higher SVR rates in the supplemented group (86%) vs. controls (42%, P<0.001) [18]. The same investigators randomized patients with genotype 2 and 3 HCV who were being treated with IFN/RBV to vitamin D supplementation versus no supplementation and found similar significant improvements in SVR (95% vs. 77%, p<0.001) [19].

In vitro studies also indicate that vitamin D may inhibit HCV replication, providing a possible molecular basis of the observed associations between higher vitamin D status and more favorable treatment outcomes. Exactly which forms of vitamin D inhibit HCV expression in cell culture remains a subject of controversy. Using a full-length genotype 1b replicon system, Yano et al. demonstrated that vitamin D2 (but not vitamin D3) inhibited HCV RNA replication with an EC50 of 3.8 μM (1600 ng/mL) [20]. A subsequent study showed that a MEK1/2 inhibitor abolished the anti-HCV effect [21]. Gal-Tanamy et al. found similar results using the inter-genotypic

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HJ3-5 chimeric HCV virus and also demonstrated a synergy when combining vitamin D3 or 1,25(OH)2D with interferon-α(IFN-α) [22]; however, vitamin D2 was not tested. Most recently, Matsumura et al. found that 25(OH) D3 and not D3 or 1,25(OH)2D3 inhibited HCV in culture [23].

Despite recent advances in HCV treatment, failure in clinical practice may be higher than expected and there remains interest in optimizing patient factors, such as correcting vitamin D deficiency that might improve outcomes. Additionally gaining further insight into the actions of vitamin D and its interplay with antiviral and immunomodulatory pathways is of scientific interest. In this study, we systematically examined vitamin D's effect in vitro against HCV by testing vitamin D2, D3 and 1,25(OH)2D3 alone and in combination with IFN- α, in well-established HCV cell culture and replicon systems. Additionally, modulation of cellular gene expression by vitamin D was examined to determine potential mechanisms for vitamin D's anti-HCV effect.

Methods

Vitamin D Metabolites

Vitamins D2, D3and 1,25-dihydroxyvitamin D3 were obtained from Sigma-Aldrich (Saint Louis, MO). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) with a final concentration of less than 0.5%. 25(OH) D3 levels in supernatant were determined by liquid chromatography with tandem mass spectrometry (LC/MS/MS) to measure conversion of vitamin D3. Cytotoxicity of these compounds was determined in Huh7.5.1 cells [24] (from Francis Chisari, Scripps Research Institute, La Jolla, CA) using a colorimetric assay cell viability assay (CellTiter 96 AQ; Promega).

Cell culture, HCV models and HCV anti-viral activity

Huh7.5.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids, and 1x penicillin-streptomycin-glutamine. The HCV genotype 1b replicon BM4-5 FEO has been previously described [25] and contains a firefly luciferase-neomycin phosphotransferase fusion (FEO) protein. The SGR-JFH1/Luc plasmid also has been previously described [26] and is based on the full-length JFH-1 genotype 2A HCV virus[27] containing a bicistronic replicon with the HCV internal ribosome entry site directing translation of the firefly luciferase gene and an encephalomyocarditis virus internal ribosome entry site identical to the one found in the BM4-5 replicon[28]. This replicon was modified by restriction digestion and ligation to express the FEO gene resulting in the SGR-JFH-1 FEO replicon. In vitro transcription and generation of cell lines stably expressing the BM4-5 FEO and SGR-JFH-1 FEO replicons were accomplished by electroporation of human hepatoma Huh-7.5.1 and selection with 300 μg/ml of G-418 as we have previously described [25]. The plasmid pFL-J6/JFH, which contains a chimeric full-length 2a genome, carries the JFH-1 sequence for the 5'-untranslated region (5' UTR), the J6 sequence for core through NS2, and the JFH-1 sequence for NS3 through the end of the genome, as previously described [29]. The virus derived from pFL-J6/JFH plasmid is called JC1-Luc2a. Supernatant from JC1-Luc2a electroporated cells was collected 9 days after electroporation, concentrated and titered as previously described [30].

Compound activity assays were carried out using 10,000 replicon or JC1-Luc2a transfected cells per well on 96-well plates [25]. JC1-Luc2a infectious virus assays were carried out with 10,000 naïve cells infected at an MOI of 0.01. Compounds were added 24-120 hours after transfection or infection with supernatant. Cells and compounds were then incubated for 72 or 120 hours with all conditions run in triplicate and each experiment was performed on three separate occasions.

Synergy

Isobolograms were generated for IFN-α A/D (Sigma Aldrich catalog # I4401) and 1,25 $(OH)_2D_3$ to test for synergism in BM4-5 FEO (genotype 1b) replicon. 1,25$(OH)_2D_3$ and IFN-α were assayed separately and in combination at 0.25x, 0.5x, 1x, 2x and 4x their respective EC50s (1 ?M and 1 IU/mL) and RLUs were measured after 4 hours. Combination indices (CI) were determined with Calcusyn (Biosoft) at the EC_{50}, EC_{70}, and EC_{90} of the combination. In total, six combinations were evaluated with three replicates per condition. By convention, a combination index of <0.9 is considered synergistic, a combination index of ≥0.9 or ≤1.1 is considered additive, and a combination index of >1.1 is considered antagonistic.

Gene expression

Cells used in the microarray studies were prepared as follows: Huh7.5.1 cells were transfected with JC1-Luc2A and cultured for four days. A mock transfection without JC1-Luc2a under identical conditions as transfected cells was also performed and the cells were passed at the same time points as infected cells. On day 4, infected and mock-transfected cells were plated in 10 cm dishes at 500,000 and 250,000 cells per dish respectively. Mock-transfected Huh7.5.1 cells had a higher proliferation rate and thus required fewer cells to become confluent at 120 hours. 1,25(OH)2D3 at 4 μM was added to infected and mock-transfected cells at 0, 48, 96, 108 and 114 hours (resulting in incubations of 120, 72, 24, 12, and 6 hours, respectively). Untreated infected and mock-transfected cells were maintained for the same period of time. Cellular RNA was harvested at 120 hours, which was equivalent to day 9 after transfection, using RNeasy kit (Qiagen).

The Ambion "Illumina Total Prep RNA Amplification Kit" produced amplified biotinylated cRNAs by the "Eberwine" protocol with an input of 250 ng of Total RNA. RNA was quantified using a NanoDrop spectrophotometer and quality assessed using an Agilent 2100 Bioanalyzer. The Illumina hybridization protocol was followed for the hybridization of 750 ng cRNA on HumanHT-12_v4 slides in "HYB" buffer for 18-20 hours at 58°C with gentle rocking followed by standard Illumina washes and streptavidin-Cy3 staining. An Illumina Bead Array Reader was used on green channel only. Quality control analysis and normalization of microarray gene expression data was performed as previously described [31]. Briefly, several quality control assessments including boxplots and MA plots were applied to assess the microarray data quality. Unsupervised clustering was also performed to detect any batch effects. All arrays passed quality control filters and no outliers were found. Differentially expressed genes were identified with 95% confidence of no more than 1% false positive using multivariate permutation tests implemented in BRB array tools (version 4.2.1; http://linus.nci.nih.gov/BRB-ArrayTools.html). Microarray gene expression was confirmed by RT-qPCR using the TaqMan®Gene Expression Assays (Applied Biosystems (ABI), Foster City, CA) for gene target *CYP24A1* with the same samples used in the microarray as shown in Figure 5.

For selected genes (CCL20, GPR37, HF1a, and SLC30A10) RT-qPCR was performed to compare mRNA expression in HCV infected cells and those treated with 1,25(OH)2D3, IFN-α, or both.

Confirmation of mRNA levels was performed according to the same protocol as above, except that two time points were examined (6 and 72 hours) and the effect of 1,25(OH)2D3 (4 or 10 μM) was compared to IFN-α (1 or 10 IU/mL) alone or in combination. Data is only shown for the high concentration treatments. Each cell culture condition was performed in triplicate and GAPDH was used to normalize data. Samples were reverse transcribed using qScriptTM cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD) and each reaction was performed in duplicate. Fold changes were obtained using DataAssist software version 3.0 (ABI) using the 2-ΔΔCT¬ method [32].

Immunoblots

Immunoblots were performed as previously described [33]. Briefly, transfection and plating of JC1-Luc2a was carried out as above. After being incubated in the absence or presence of 10 μM 1,25 $(OH)_2D_3$, 10 IU/mL IFN-α or combination of 1,25$(OH)_2D_3$ and IFN-α for 72 hours, confluent plates were trypsinized, counted to be divided and then lysed in RIPA buffer in equal volumes. Each cell culture condition was performed in triplicate. Proteins were fractionated in 12%Bis-Tris SDS-polyacrylamide gels and transferred to Invitrogen nitrocellulose membranes (Carlsbad, USA). The primary antibodies, anti-core 2M (epitope, amino acids 7-50, Alexis) and anti-β-actin from Genescript (Piscataway, NJ), were detected using BioRad (Hercules, USA) goat anti-mouse IgG (catalog #170-6521/170-6520). Immunoblot band density was calculated with the analytic software ImageJ.

Data analysis

Luciferase activity was determined using a micro plate luminometer (Veritas micro plate luminometer; Turner Biosystems) according to the manufacturer's instructions (One-Glo; Promega). Relative light units (RLU) for each condition were used to generate a dose response curve for each compound by using Prism (version 4; GraphPad Software). Values for 50% effective concentration (EC_{50}) and the 50% cytotoxicity concentration (CC_{50}) were calculated for each test compound by fitting a sigmoidal (variable-slope) dose response-curve, using data combined for all treated cultures. Illumina Genome Studio Software extracted raw intensity gene expression data which was normalized using modified LOESS. A heat map was generated from the microarray data showing the top 100 modulated genes based on fold change. Hierarchical clustering of genes was performed by calculating distances using the Pearson correlation metric and then clustering these distances using the average linkage method using Cluster 3.0, and visualized using Java Tree View. (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm)

Results

Vitamin D2, D3, and 1,25(OH)2D3 demonstrate anti-HCV activity

Vitamin D_2, D_3 and 1,25$(OH)_2D_3$ all demonstrated anti-HCV activity on replicons harboring HCV genotype 1b and 2a strains at micro molar concentrations well below the CC_{50}. The EC_{50} after 72 or 120 hours of exposure to each compound is shown in (Table 1).

Vitamin D metabolites also demonstrated anti-HCV activity in Huh7.5.1 cells that were either transfected with JC1-Luc2a RNA or infected with supernatant carrying infectious JC1-Luc2a. Consistent results were seen across replicates with R2 values greater than 0.9 (Figure 1).

Cytotoxicity and Conversion of D3 to 25(OH) D3

Cytotoxicity (CC_{50}) was assessed for vitamin D_2, D_3 and 1,25 $(OH)_2D_3$ in Huh7.5.1 cells. The mean CC_{50} after 120 hours of incubation for vitamin D_2, D_3 and 1,25$(OH)_2D_3$ was 42 μM, 55 μM, and 23 μM, respectively. Similar results were seen at 72 hours (Table 2).

EC50 concentrations were well below the CC50. Conversion of 1 μM, 5 μM and 25 μM vitamin D3 to 25(OH) D3 as measured by LC/MS/MS was found to be 2 ng/mL, 24 ng/mL, and 191 ng/mL which demonstrates the physiologic activity of vitamin D3 in our cell culture system, along with the presence of 25(OH)D3 in Huh 7.5.1 cells exposed to vitamin D3.

1,25(OH)2D3 Synergy with IFN-α

Cells stably expressing the BM4-5 FEO (genotype 1b) replicon were treated with IFN-α and 1,25(OH)2D3to assess for synergistic inhibition of HCV as previously described [25]. The dose response curve for each compound alone and in combination is shown in Figure 2. Combination Indices (CI) with standard deviations are as follows: CI50 0.68 (± 0.35), CI70 0.59 (± 0.38), and CI90 0.51 (± 0.28) (R2>0.99). These results are indicative of strong synergism between 1,25(OH)2D3 and IFN-α.

Decreased core protein in HCV infected cells treated with 1,25 $(OH)_2D_3$ and IFN-α

Huh7.5.1 cells were electroporated with JC1-Luc2a and cultured for 72 hours with or without 1,25(OH)2D3, IFN-α or both. HCV core expression was examined by immunoblot with an anti-core antibody and the housekeeping protein β-actin (Figure 3), panel A.

Mild reduction in core protein expression was seen in both cells treated with 1,25(OH)2D3or IFN-α alone compared to untreated cells; however, a marked reduction of core was seen in cells that were treated with both 1,25(OH)2D3and IFN-α. Levels of β-actin relatively stable across all samples. Protein expression was quantitated as described in the Figure 3 legend and shown in Figure 3, panel B. Incubation of HCV infected cells with 1,25(OH)2D3 and IFN-α each individually reduced core expression. Combined treatment with both 1,25(OH)2D3 and IFN-α appeared to be synergistic and was significantly different than with either treatment alone.

Vitamin D alters gene expression of HCV uninfected and infected Huh7.5.1 cells

Gene expression was compared in 12 samples using an Illumina Bead Chip. The gene expression of uninfected, untreated Huh7.5.1 cells was used as a control for comparison to cells treated with 1,25 (OH)2D3 at various time points. Similarly, gene expression of untreated cells infected with JC1-Luc2a was used as a control for comparison to the cells treated with 1,25$(OH)_2D_3$at various time points. Cells were treated with 2.5 μM of 1,25(OH)2D3 for 6, 12, 24, 72 and 120 hours to capture genes that might be activated early or later after exposure to vitamin D. The EC50 of 1,25(OH)2D3 was 2.92 μM and 2.95 μM in cells electroporated with JC1-Luc2a after 72 and 120 hours, respectively. Thus, the concentrations used in this experiment were close to the EC50 and well below cytotoxic levels.

Differential gene expression was observed between uninfected and HCV JC1-Luc2a infected cells and the top 100 activated or suppressed genes reported (Figure 4).

Since only a single data point was obtained for each time point, the gene expression information obtained was not amenable to statistical

	Vitamin D2 (μM)	Vitamin D3 (μM)	1,25(OH)2D3 (μM)
1b Replicon 72 hours	12.79 (± 3.17)	6.1 (± 2.08)	3.19 (± 0.56)
1b Replicon 120 hours	14.25 (± 1.08)	3.8 (± 1.35)	1.1 (± 0.3)
2a Replicon 72 hours	14.25 (± 2.07)	12.32 (± 2.8)	5.43 (± 1.05)
2a Replicon 120 hours	13 (± 0.6)	8.45 (± 0.93)	4.71 (± 0.69)
JC1-Luc2a (electroporation) 72 hours	18.64 (± 1.37)	13 (± 3.2)	2.92 (± 0.45)
JC1-Luc2a (electroporation) 120 hours	21.4 (± 3.37)	12.57 (± 8.73)	2.95 (± 0.65)
JC1-Luc2a (infection) 72 hours	5.72 (± 1.61)	3.44 (± 0.74)	1.19 (± 0.52)
JC1-Luc2a (infection) 120 hours	4.41 (± 0.94)	2.78 (±1.29)	1.07 (± 0.27)

Table 1: The EC50s of vitamin D2, D3 and 1,25 (OH)2D3 after 72 and 120 hours are shown in genotype 1b and 2a replicons. The EC50 of HCV expressing cells transfected with JC1-Luc2a by electroporation or infected with JC1-Luc2A supernatant (MOI 0.01) are shown at 72 and 120 hours.

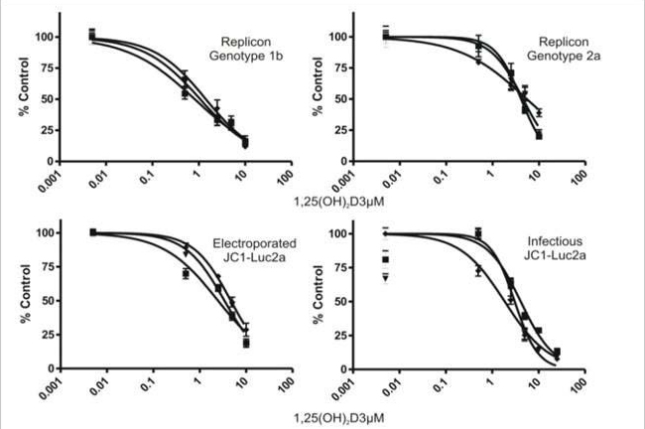

Figure 1: Dose response curves in HCV replicons (genotype 1b and 2a) and JC1-Luc2a (electrophoresed and samples exposed to infectious supernatant) incubated with 1,25(OH)2D3 for 120 hours. Each line with a square, triangle or arrowhead represents triplicates of a single experiment with standard deviation. Results are presented as a percentage of luciferase output to untreated controls

	CC50 μM (±S.D)	
Vitamin D Metabolite	72 hours	120 hours
Vitamin D2	53.9 (±4.61)	41.89 (±6.78)
Vitamin D3	50.34 (±14.45)	54.9 (±17.14)
1,25(OH)2D3	17.3 (±1.12)	22.8 (±4.95)

Table 2: Cytotoxicity of vitamin D_2, D_3, and 1,25 $(OH)_{2D3}$ in Huh7.5.1 cells.

analysis; however, it provides preliminary data of gene candidates for future experiments. The comparison of cells with and without vitamin D and HCV exposure allowed us to identify candidate genes in which vitamin D actions opposed HCV's. For example, *IL-8* mRNA expression was strongly up regulated in Huh7.5.1 cells over the duration of exposure to vitamin D, while its up regulation was relatively diminished in HCV infected cells. IL-8 is an inflammatory chemokine which may alter the outcome of HCV treatment [34]. *CYP24A1* mRNA was observed to have the highest fold increase in expression in treated cells. The enzyme encoded by *CYP24A1* is responsible for the 24-hyroxylation which targets vitamin D to excretion pathways [35]. To show correlation of the microarray data presented here, RT-qPCR was performed to assess *CYP24A1*levels on the same samples used for microarray analysis. Good correlation was seen between these two methods of assaying gene expression as shown in Figure 5.

In both uninfected and infected cells 1,25(OH)2D3 increased the expression of SLC30A10 (apical sodium dependent bile acid transporter). CLEC2Dencodes a member of the natural killer cell receptor C-type lectin family was activated in uninfected cells. In HCV infected cells, CLEC2D showed early down-regulation (6 and 12 hours), but was progressively activated with prolonged exposure to vitamin D. CCL20 (also known as liver activation regulated chemokine or macrophage inflammatory protein-3), a member of the CC chemokine family, displayed higher gene expression shortly after treatment with 1,25(OH)2D3, and showed down regulation late in HCV infected cells. CCL20 has been linked to macrophage recruitment during IFN therapy [36]. GPR37, a G-protein-coupled receptor that induced cellular autophagy [37] was also markedly

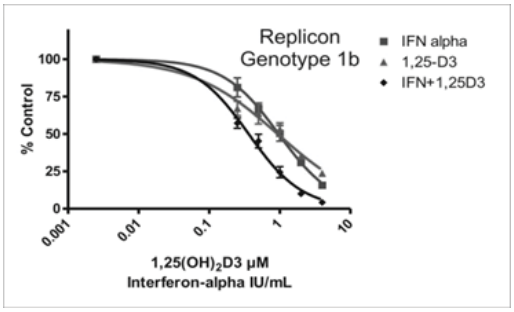

Figure 2: The dose response curve for 1,25 (OH)2D3 and IFN-α are shown with a square and triangle on the line, respectively. The combination effect of 1,25(OH)2D3 and IFN-α is the bottom line with diamonds

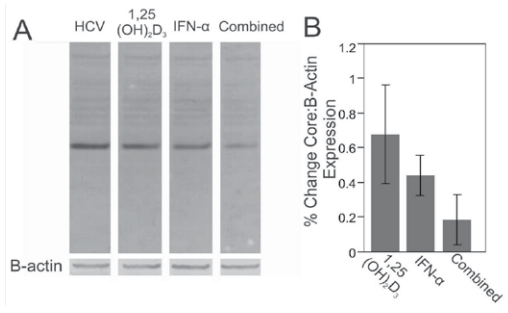

Figure 3: Combined treatment with 1,25(OH)2D3 and IFN-α reduces core protein expression. HCV core and β-actin expression in Huh7.5.1 cells infected with JC1-Luc2a was assayed by immunoblot and is shown in Panel A. Infected cells were untreated (HCV) or treated with 1,25(OH)2D3 (10 μM) or IFN-α(10 IU/mL) or both (Combined). The core band and β-actin are shown. Core expression is reduced in the 1,25(OH)2D3, and IFN-α, but is markedly decreased in the cells that were incubated with both agents. The β-actin band appears similar in all lanes. Panel B: Core and b-actin levels were quantitated with ImageJ and normalized to the control (HCV) bands, and then the normalized core to β-actin ratios was calculated in each lane (performed in triplicate). The graph in panel B shows significant decrease in core expression in cells treated with both 1,25(OH)2D3 and IFN-α compared to either of these treatments individually (p=0.0008; p=0.0191, respectively by student t-test).

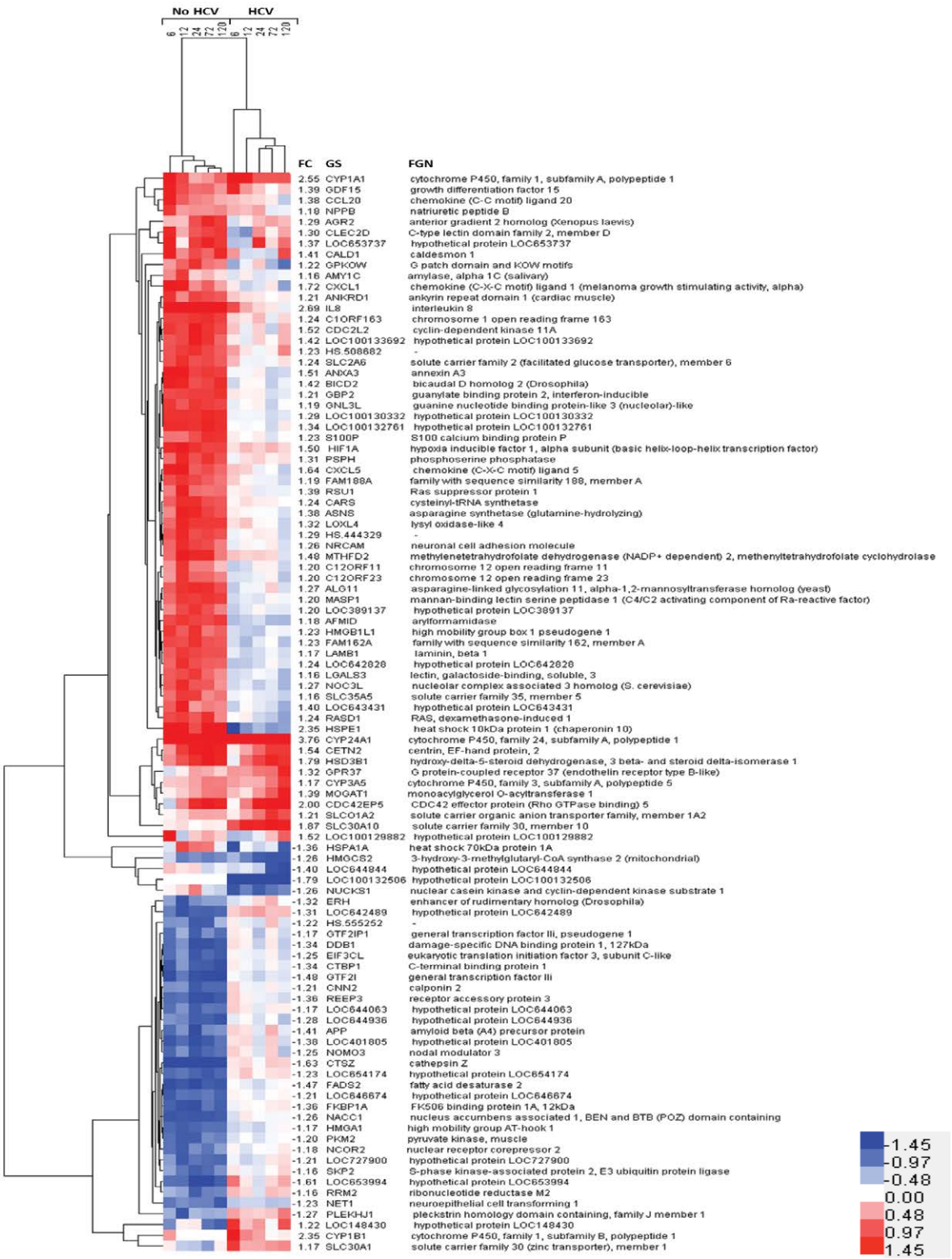

Figure 4: The top 100 differentially expressed genes are shown in a heat map. HCV uninfected, 1,25(OH)2D3 untreated cells were used as controls and were compared to uninfected huh 7.5.1 exposed to 1,25(OH)2D3 in culture for 6 hours (lane 1),12 hours (lane 2), 24 hours (lane 3), 72 hours (lane 4) and 120 hours (lane 5). Also compared to uninfected, 1,25(OH)2D3 untreated were HCV infected Huh 7.5.1 cells exposed to 1,25(OH)2D3 in culture for 6 hours (lane 6), 12 hours (lane 7), 24 hours (lane 8), 72 hours (lane 9) and 120 hours (lane 10). Red boxes are up regulated and blue are down regulated. The peak fold change (FC) is shown to the left of each gene symbol (GS)

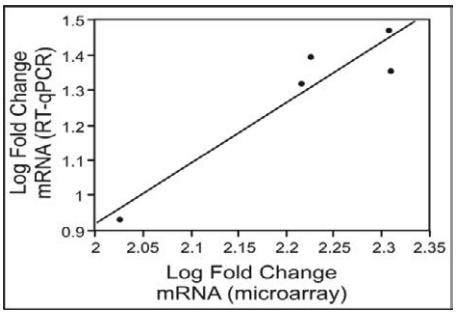

Figure 5: Confirmatory RT-qPCR of microarray data plotted on a log transformed regression. Log transformed fold changes of mRNA obtained from HCV infected cells treated with 1,25(OH)2D3 over a 120 hour time course is plotted against RT-qPCR of the same samples. A line of fit showed excellent correlation (R2 =0.89, p=0.015) of microarray and RT-qPCR data.

up regulated with progressive in vitro exposure to 1,25(OH)2D3. Hypoxia inducible factor 1 α (HIF1a), a gene necessary for autophagy and essential for response to hypoxia was strongly up regulated in HCV naïve cells, but showed reduced activity in HCV infected cells. HIF1a is known to be modulated by vitamin D and HCV [38, 39].

Real-time PCR was performed on selected genes in an attempt to corroborate microarray findings and to determine whether genes displayed additive expression when treated with 1,25(OH)2D3, and IFN-α (Figure 6).

CCL20 expression showed a trend towards an additive effect when treated with both 1,25(OH)2D3, and IFN-α at 72 hours. Again, in GRP37 (at 6 hours) there appeared to be an additive effect of mRNA expression in cells that were treated with both agents. Otherwise, no clear trends were seen with GPR37 or HIF1a. Although no statistically significant trends were seen at the highest dose (10 μM, 10 IU/mL) concentrations of 1,25(OH)2D3, and IFN-α; however, at the lower dose (1 μM, 4 IU/mL) concentration there was a highly significant fold change increase in SLC30A10 mRNA levels at 72 hours(data not shown, $p<0.0001$). SLC30A10 up-regulates expression of zinc transporter and allows for adequate intracellular zinc levels which appear to activate NF-κB and help maintain proper innate immune function [40].

Conclusion

Vitamin D has long been recognized for its importance in cholestatic liver disease due to the significant risk of osteomalacia in this patient population. Epidemiologic evidence has also linked low serum 25(OH) D levels to decreased response to interferon and ribavirin based therapy. Recent trials also support vitamin D supplementation during anti-HCV therapy with IFN/RBV [18,19,22]. This paper demonstrates the anti-HCV properties of vitamin D_2, D_3 and $1,25(OH)_2D_3$ at low micro molar concentrations in genotype 1b and 2a replicons and in HCVcc with JC1-Luc2a.

All forms of vitamin D used in this study uniformly inhibited HCV expression, in contrast to the findings Yano et. al. who found that D_2 and not D_3 inhibited HCV replicons [20]. Both D_2 and D_3 are expected to undergo 25-hydroxylation in hepatocytes. Studies have shown that 25(OH)D can activate the vitamin D receptor (VDR); albeit with decreased potency compared to the active metabolite $1,25(OH)_2D$ [41]. Thus, it is expected that D_2 or D_3 or their derivatives (hydroxylated forms) would be able to activate the VDR in the Huh7.5.1 cells used in our studies.

We demonstrated decreased HCV core production in HCVcc after exposure of cells to vitamin D_3 by immune blotting in this paper (see Figure 4). We also described efficient conversion of vitamin D_3 to 25(OH)D as assessed by LC/MS/MS. Therefore, activity of D_2 and D_3 may be attributed to 25(OH) D or the precursor forms. This is in contrast to a recent study which showed that 25(OH)D and not D_3 reduced expression of HCV core [23].

Gal-Tanamy et al. showed inhibition of HCV using a different HCV strain (HJ3-5) and precursor cell sub clones (Huh 7.5). HCV inhibition at lower concentrations in their study compared to this one may be explained by the less efficient replication of HJ3-5 [22]. Furthermore, Gal-Tanamy also pretreated naïve cells prior to HCV infection which may have altered the permissiveness of the cells and the amount of $1,25(OH)_2D_3$ needed to inhibit HCV. In our study, the EC_{50} of $1,25(OH)_2D_3$ was similar in both the HCVcc and replicon systems. This study is offers a comprehensive and systematic evaluation of vitamin D metabolites in well-established and robust HCV models. As expected based on what is known of vitamin D physiology and action at the vitamin D receptors, all metabolites tested in this study decreased HCV expression.

Vitamin D was first identified to be an antimicrobial agent in the treatment of cutaneous Mycobacterium tuberculosis (Mtb) infection over 100 years ago [42]. Mtb activates pattern recognition receptors (PRRs) that lead to the increased expression of the vitamin D receptor and conversion of vitamin D to its active metabolite ($1,25(OH)_2D$) [43]. In this study, $1,25(OH)_2D_3$ demonstrated strong anti-HCV synergy when combined with IFN-α in cells harboring genotype 1b replicons. Vitamin D may accomplish this by enhancing the cellular antiviral response and thus increasing the effectiveness of IFN-α. Clinically, these findings are supported by results of clinical trials that show improved SVR rates in patients treated with vitamin D[18,19,44]. Vitamin D reportedly has the ability to decrease inflammation and activate the innate immune systems. Monocytes treated with $1,25(OH)_2$ D_3 demonstrate suppression of effectors functions of IFN-gamma [45,46]. Similar results along with decreased expression of IFN-gamma were also seen in endometrial cells treated with vitamin D [47]. Less is known about the *in vitro* effects of vitamin D on IFN-α; however, the lessons learned from Mtb infection may provide some clues.

Vitamin D is believed to modulate Mtb infection by activation of the innate immune system through autophagy via class III phosphoinositide 3-kinase (PI 3-kinase) complex [48] and up regulation of the antimicrobial peptide cathelicidin [49]. Autophagy is a lysosome-mediated catabolic pathway involved in processes of adaptation and survival through the recycling of cyto plasmic proteins and organelles in an attempt to promote cell survival and function. HCV and other RNA viruses are known to subvert autophagy by reducing expression of immunity-associated GTPase family M [50]. Studies have shown that an HCV viral protein modulates autophagy to facilitate viral replication and production of the virion [51]. IFN-α has also been shown to modulate trafficking of HCV proteins to lysomes [52]. Thus, IFN-α and vitamin D may operate synergistically to enhance autophagy of HCV. Our data suggest an additive anti-HCV effect in cells treated with both $1,25(OH)_2D_3$ and IFN-α up regulation of GPR37.

Our data from HCV-naïve cells treated with $1,25(OH)_2D_3$ had increased expression of *HIFa* and a significantly increased mRNA

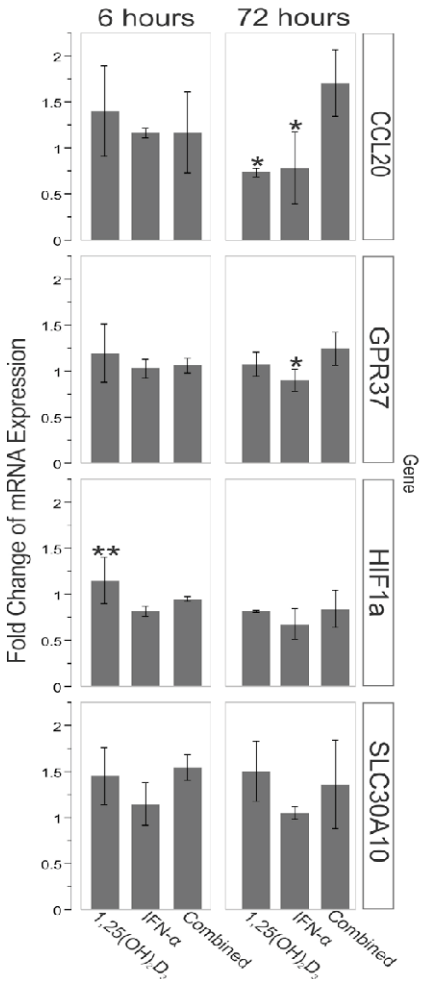

Figure 6: Vitamin D and IFN-α effect on mRNA expression of selected genes. Real- time PCR fold change of CCL20, GPR37, H1F1a and SLC30A10 are shown and compared with a student t-test. HCV cell culture without treatment was the control and all conditions were performed in triplicate. Statistically significant differences between 1,25(OH)2D3 or IFN-α treated cells compared to combined were marked with a single asterisk and between 1,25(OH)2D3 and IFN-α with two asterisk. Cells were treated with 10 μM of 1,25(OH)2D3, 10 IU/mL of IFN-α, or both for 6 (left) or 72 (right) hours. Mean values are shown with 1 standard deviation. CCL20 appeared to have an additive effect when cells were treated with both 1,25(OH)2D3 and IFN-α compared to alone (p=0.0084; p=0.0106, respectively) at 72 hours. Otherwise, 1,25(OH)2D3 treatment significantly lead to up-regulation of HIF1a of mRNA expression over IFN-α treated cells (p=0.032). No significant difference in mRNA expression was seen for SLC30A10 at 6 or 72 hours.

fold change compared to IFN-α treated cells shown in Figure 6. *HIF1a*has been shown to be necessary for induction of autophagy in HeLa cells [53]. Finally, our microarray data suggest that *CCL20* (macrophage inflammatory protein-3alpha) expression may be up regulated by vitamin D. Intriguingly, elevated serum levels of *CCL20* two weeks after initiation of IFN were shown to correlate with a virologic response to therapy [36]. Our data shown in Figure 6 suggests an additive effect of vitamin D and IFN-α on *CCL20* mRNA expression. Activation of *CCL20* may be another potential mechanism by which vitamin D might augment interferon-based HCV treatment responses. Notably was the strong up-regulation of *SLC30A10* in vitamin D treated cells, which facilitates zinc transport into cells. Zinc deficiency is common in patients with advanced liver disease, and zinc has also been shown to inhibit HCV replication in cell culture [54]. The exact role that zinc and its transporters plays in the immune system is still unclear but it appears that deficiency of zinc results in dysfunction of the innate and adaptive immune system [40].

In summary, we demonstrated that vitamin D_2, D_3 and $1,25(OH)_2D_3$ all possess modest to strong anti-HCV activity. We measured conversion of vitamin D_3 to 25(OH) D_3 in our cell culture system, and this appeared efficient and comparable to the physiologic responses expected in hepatocytes. Vitamin D showed strong synergy with IFN-α which may be explained by enhanced innate immunity leading to increased immune response in HCV infected cells through genes such as *CCL20*. Future research should target the genes activated by vitamin D in HCV towards a better understanding of this agent's antiviral potential.

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