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# A Novel Proposal for Nonalcoholic Steatohepatitis: Ubiquitous Compound 4-Methylimidazole Promotes Both the Classic Hallmarks of NASH and a Novel Immunoediting Pathway to Elicit Immune-Mediated Liver Damage

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## ABSTRACT

Nonalcoholic Steatosishepatitis (NASH) is a chronic disease that affects 3% of Americans where the liver becomes steatotic and inflamed. 4-Methylimidazole (4-MI) is a ubiquitous byproduct of caramel coloring production, and as a result is frequently ingested. Recent studies have shown that 4-MI causes hepatic lipid and macrophage accumulation. Because of this, 4-MI's ability to regulate classic genetic hallmarks of NASH along with new potential contributors to hepatic damage in NASH was tested. HepG2 (Liver) and U937 (Immune) cells were treated with 4-MI for 24 hours. Genetic analysis was conducted via RT PCR and cellular analysis of inflammatory potency and viability was conducted via Inflammatory Attachment and Trypan Blue Assay. On the molecular level 4-MI dysregulated genes to promote NASH: disrupting homeostasis, downregulating anti-inflammatory and upregulating pro-inflammatory genes. Pro-inflammatory gene COX2 was downregulated in HepG2, however further research revealed its pivotal role in regeneration and results demonstrated downregulation of regeneration genes. Apoptotic genes were dysregulated to promote immunoediting: tumor antigens BCL2 and MCL1 were overexpressed in HepG2 while proapoptotic FASR and FASL were upregulated in HepG2 and U937 respectively, allowing detection of hepatocytes as precancerous while opening a separate apoptotic pathway. Molecular results were supported on the cellular level; inflammatory activity increased in U937. Viability increased in HepG2 but decreased pass control with the introduction of FASL protein. Overall, this data suggests that 4-MI promotes the classic pathology of NASH: promoting steatosis and chronic inflammation. Furthermore, 4-MI encourages liver damage via immunogenic apoptosis while decreasing healing efficiency.

**Keywords**: 4-Methylimidazole; 4-MI; Toll-Like Receptors; TLR; Cytokines; Nonalcoholic Steatohepatitis; NASH

## INTRODUCTION

Nonalcoholic Steatohepatitis (NASH) is an underdiagnosed liver disease thought to affect at least 2-3% of all Americans [1] [2]. Representing the deadliest form of Nonalcoholic Fatty Liver Disease, NASH is characterized by inflammation, steatosis, and damage of the liver. Although the causes of NASH currently remain unknown, it is postulated to be triggered and promoted by environmental agents.

Recent studies have suggested that chronic exposure to inflammatory substances has been linked to the onset or worsening of NASH. Therefore, identification and reduction of harmful inflammatory substances found in common food products may be a beneficial step for victims suffering from NASH.

4-Methylimidazole (4-MI) is a ubiquitous, potentially inflammatory compound that large populations are frequently exposed to. 4-MI is the toxic byproduct of the Mailard Reaction, the primary step for the creation of caramel coloring. Therefore, 4-MI prevails in products that utilize caramel coloring, including soft drinks, candies, coffee, etc. More importantly, in the liver of rats, 4-MI has been shown to induce hepatic lipid accumulation and macrophage infiltration, both of which are key pathological features of NASH. Furthermore, one of the main targets of 4-MI in the body has been shown to be the liver, where NASH occurs. Despite these aspects suggesting 4-MI's link with NASH as well as its prevalence in our daily lives, 4-MI's role in promoting NASH currently remains unclear. Therefore, our study aimed to investigate 4-MI's effects in relation to NASH using an *in vitro* model. Our study is perhaps the first to identify 4-MI as a potential contributor to NASH and reveal key mechanisms that are involved in its pathway.

In this regard, our study focused on evaluating 4-MI's effects on the key hallmark mediators of NASH; steatosis, inflammation, and apoptosis. Steatosis, the intracellular lipid accumulation, can be characterized by the overexpression of fat synthesis (SREBF1, FASN) and underexpression of fat metabolism (PPARαgenes. The *dysregulation* of these factors will result in the abnormal intracellular fat retention, which will lead to disruption of homeostasis and may even lead to the bursting of the steatotic cells. The abnormal retention of lipids within the hepatocytes of the liver is a key pathological feature of NASH. Similarly, inflammation in NASH can also be characterized by the overexpression of the pro-inflammatory genes (TNFα, IFNγ, IL1β, IL6, COX2, TLR3, TLR4) and underexpression of the anti-inflammatory genes (IL4, IL10, TGFβ1). Because the liver is a weakly immunoprivileged site, disruption of liver tolerance will lead to adverse inflammatory processes damaging the liver. Liver tolerance is also induced by the *dysregulation* of these inflammatory genes, which will result in promotion of unrestricted, uncontrolled inflammation, a key pathological feature of NASH.

Steatosis and inflammation are the defining characteristics of NASH. However, apoptosis plays a paradoxical role in NASH. Although there have been many reports of pro-apoptotic actions in NASH contributing to liver damage, there have also been reports of anti-apoptotic actions in NASH leading to tumors, but also ultimately leading to liver damage. To explain this phenomenon, we applied the concept of tumor immunity, where immune cells are able to detect cancerous or precancerous cells due to their overexpression of tumor antigens and eliminate them accordingly via immunoediting. Using this concept, we tested for key pro-apoptotic and tumor-mediated death (FASR, FASL) genes as well as anti-apoptotic and tumor

antigenic (BCL-2, MCL-1) genes. Through this concept, we will be proposing a novel pathway by which 4-MI may promote immune-mediated liver damage in NASH.

Overall, the purpose of our study was to investigate 4-MI's effects on all of the aforementioned hallmark mediators of NASH on the genetic and cellular levels on human liver hepatocyte (HepG2) and human lymphocytic (U937) cell lines. The alternate hypothesis states that 4-MI would *adversely* affect all the targets on all of the molecular level to promote steatosis, inflammation, and apoptosis, the key hallmarks of NASH.

## METHODS

*Cell incubation and Treatment*

The HepG2 human liver epithelial cell line (ATCC, New York, NY) was grown *in vitro* in Eagle's Minimum Essential Medium also supplemented with 10% fetal bovine serum (Invitrogen, USA). The U937 human lymphocytic cell line (ATCC, New York, NY) was grown *in vitro* in RPMI 1640 media supplemented with 10% fetal bovine serum (Invitrogen, USA). The two cell lines were chosen due to their effectiveness as *in vitro* models for various hepatic and immune diseases, respectively. By using the cell lines dually, they served as sources for human hepatocytes and human lymphocytes, and as an *in vitro* model for NASH. The cells were grown in conditions of 95% $\boldsymbol{O_2}$/5% C$\boldsymbol{O_2}$ in a 37° incubator (NAPCO). After the cells reached approximately 70% confluency, the cells were treated with 1 and 10nM of 4-MI for 24 hours. Concentrations of 4-MI far below that found in common caramel colored products were chosen (IARC, 2012).

*Gene Expression*

To evaluate 4-MI's effects on various genetic expressions, we performed total mRNA isolation via the Tri-Reagent method following the manufacturer's protocols (Molecular Bioproducts). U937 cells were harvested via centrifugation while HepG2 cells were first detached from the flask by trypsin (Invitrogen, USA). The isolated mRNA was analyzed via spectrophotometer to normalize results. Afterwards, semi-quantitative Reverse Transcription and Polymerase Chain Reaction were performed, followed by gel electrophoresis

The mRNA products were first denatured at 95C for 5 minutes, then cooled on ice. A master mix containing reagents from Invitrogen (Dinucleotide Triphosphates, 5X First Strand Buffer, Dithiothreitol, Random Primers, RNase Inhibitor, Reverse Transcriptase) was added to each mRNA sample which were placed in a GeneAmp Thermocycler PCR System 9700 (P.E. Applied Biosystems) set to run for 60 minutes at 40°C and 10 minutes at 65°C. The RT products were added into a new master mix containing reagents and primers from Invitrogen (10X PCR Buffer, MgCL2, Dinucleotide Triphosphates, Forward and Reverse Primers). Forward and Reverse Primers included TNFα, IFNg, IL1b, IL6, COX2, TLR3, TLR4, IL4, IL10, TGFb1, PPARa, SREBF1, FASN, FASL, FASR, BCL-2, MCL-1, as well as the reference gene, b-actin. Following the addition of the master mix to each RT products, the samples were placed in the thermocycler which was run for standard PCR cycles with varying melting temperatures for each primers.

Afterwards, the PCR products were subjected to 2% gel electrophoresis, where the band intensity was quantified via GelPro Analysis 3.1 Software (Media Cybernetics, USA). Gene expressions were normalized using the data from the reference gene, b-actin. Three trials were performed for each gene.

*Cell Attachment Assay*

To determine the inflammatory response on the cellular level through the adhesion of cells, a 24 well tissue culture plate (Costar, Cambridge, MA) was prepared. Each well was coated with 10µL of substrate fibronectin (Sigma-Aldrich, St. Louis, MO), to create a protein layer for the binding of inflammatory cells. Upon stimulation by an inflammatory agent, inflamed immune cells will bind to integrin ligands such as fibronectin, collagen, and laminin on the endothelial layer (Harburger and Calderwood, 09). Out of the integrin ligands, previous studies suggest that fibronectin produces the best inflammatory response results in the attachment assay (Weeks et al., 08). Therefore, by coating each well with fibronectin to mimic the protein on the endothelial layer, the potency of 4-MI's inflammatory response was evaluated. Cells were counted with a hemocytometer and brought up to a density of 5x106 /mL. Afterwards, 0.5 mL of suspended cells were then seeded in each well and treated with the following: 1µM 4-MI, 10µM 4-MI, 1µM TPA (12-O-tetradecanoylphorbol-13-acetate) as the positive control, and 10µL of dimethyl sulfoxide (the treatment solvent) as the negative control. Treated cells were incubated at 37ºC for 1, 24, 48, 72, and 96 hours. Following this, unattached cells and their media were removed by aspiration and the attached cells were fixed and stained with Diff Quik (Baxter Scientific).

*Cell Viability Assay*

The Cell Viability Assay measured 4-MI's ability to induce cell death in HepG2 cells. After the HepG2 cells underwent a 24 hour treatment period cells were detached and diluted with Trypan Blue dye in a 1:1 ratio of cells to 0.4% Trypan Blue solution. They were given 2 minutes to stain and the number of stained and unstained cells in a 1x1 mm square was counted using a hemocytometer.

*Statistical Analysis*

Data from all experiments were statistically analyzed (n=3) via an ANOVA followed by Post-Hoc Scheffé's test using IBM SPSS Software with significance defined as $P > 0.05$.

## RESULTS

For the PCR genetic results, 4-MI significantly up-regulated all the pro-inflammatory genes (TNFa, IFNy, IL1b, IL6, COX2, TLR3, TLR4) in both HepG2 and U937 cells with the exception of COX2 which was downregulated in the HepG2 cells (**Fig 1, Fig.2)**. As this went against the other results, upon further literature research, a paper by Martin-Sanz and his colleagues in 2010 showed that COX2 plays a major role in liver regenerative mechanisms. Therefore, the unconventional downregulation of COX2 indicates that 4-MI disrupts the expression of a key mediator of liver regeneration. Thus, for further investigation, key mediators of liver regeneration, HGF and EGFR, were also tested. 4-MI significantly downregulated all the anti-inflammatory genes (IL10, IL4, TGFb at both concentrations **(Fig. 3, Fig. 4)**. The *dysregulation* of the inflammatory genes indicates 4-MI promotes chronic, unrestricted, uncontrolled inflammation of the liver, a key pathological feature of NASH. For the HepG2 cells, lipid synthesis genes (SREBF1, FASN) were significantly upregulated while the lipid retention gene (PPARa and liver regeneration genes (HGF, EGFR) were significantly downregulated with exposure to 4-MI **(Fig. 7, Fig. 8)**. This indicates that 4-MI directly promotes steatosis, a key pathological feature of NASH as well as decreasing liver regenerative capabilities, which may lead to further liver damage.

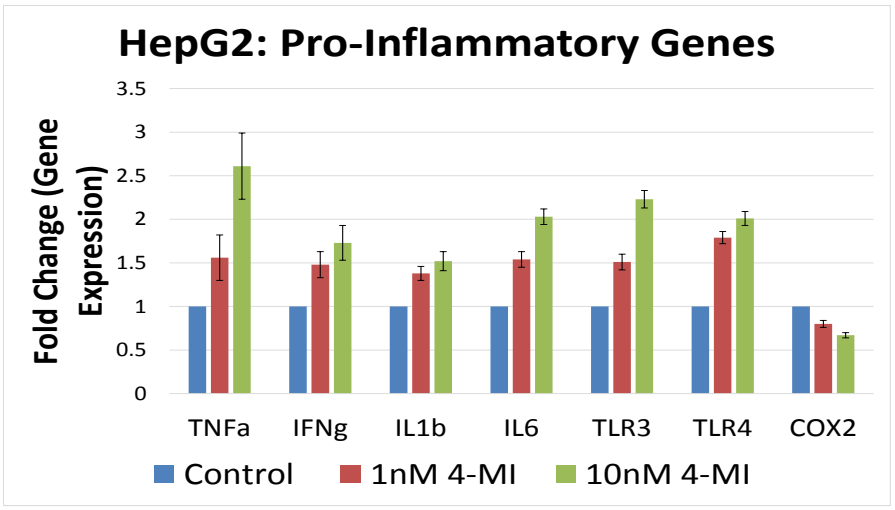



**Fig 1.** The Effect of 4-MI on pro-inflammatory genes in HepG2 is shown. All genes were significantly upregulated at both concentrations of 4-MI, except for COX2 which was significantly downregulated at both concentrations

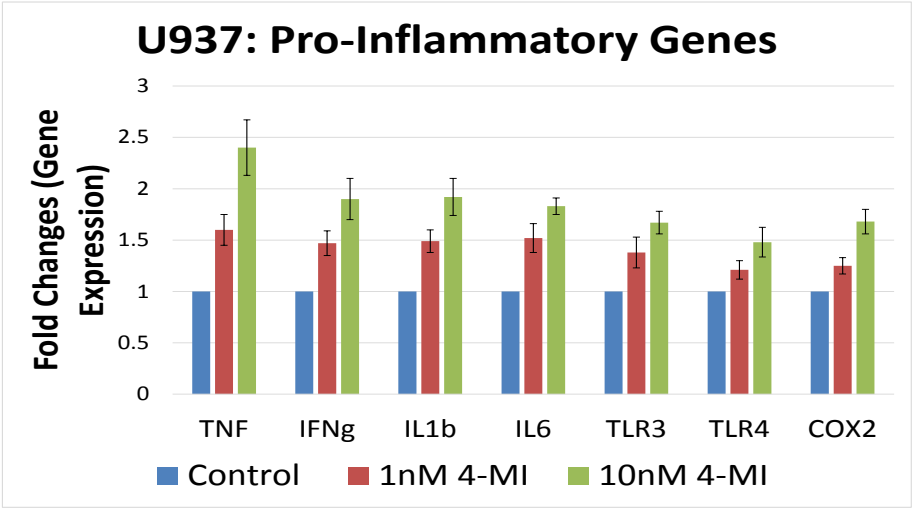



**Fig 2.**The Effect of 4-MI on pro-inflammatory genes in U937 is shown. All genes were significantly upregulated at both concentrations of 4-MI.

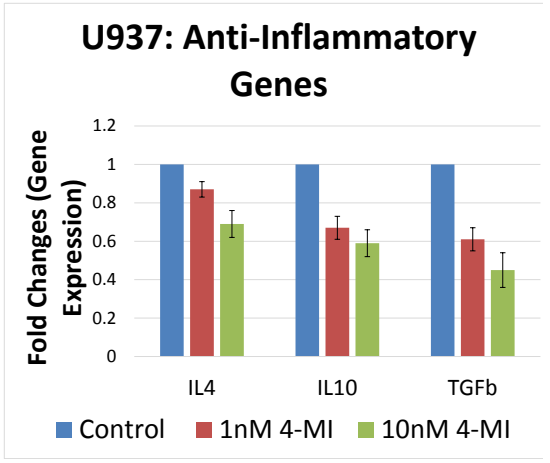



**Fig 3.**The Effect of 4-MI on anti-inflammatory genes in U937 is shown. All genes were significantly downregulated at both concentrations of 4-MI.

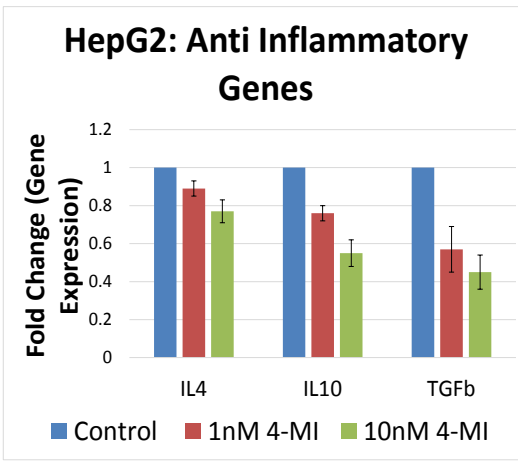



**Fig 4.**The Effect of 4-MI on pro-inflammatory genes in HepG2 is shown. All genes were significantly downregulated at both concentrations of 4-MI.

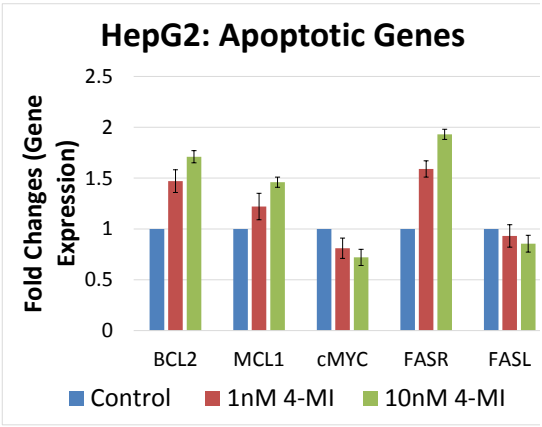



**Fig 5.**The Effect of 4-MI on apoptotic genes in HepG2 is shown. BCL2, MCL1, and FASR were upregulated, cMYC and FASL was downregulated.

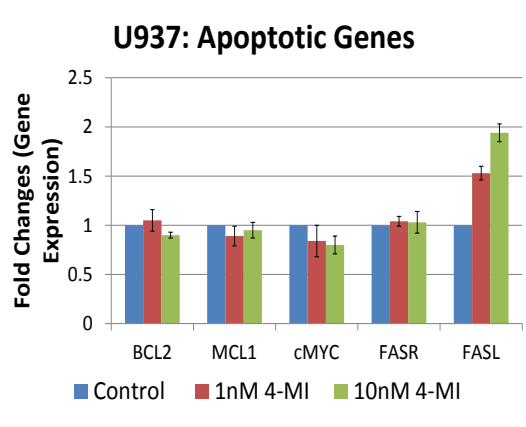



**Fig 6.**The Effect of 4-MI on apoptotic genes in U937 is shown. There was no change in expression of BCL2, MCL2, and FASR. cMYC was downregulated and FASL was upregulated.

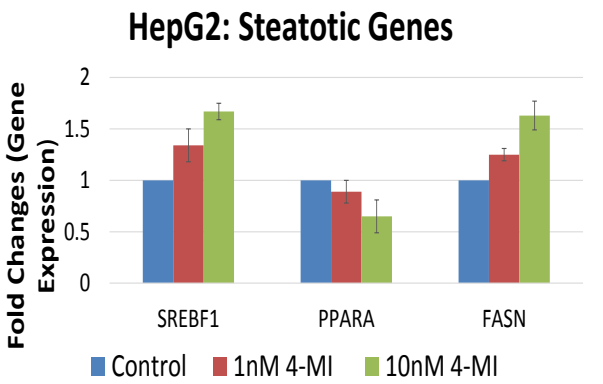



**Fig 7.**The Effect of 4-MI on steatotic genes in HepG2 is shown. SREBF1 and FASN were significantly upregulated while PPARα was significantly downregulated

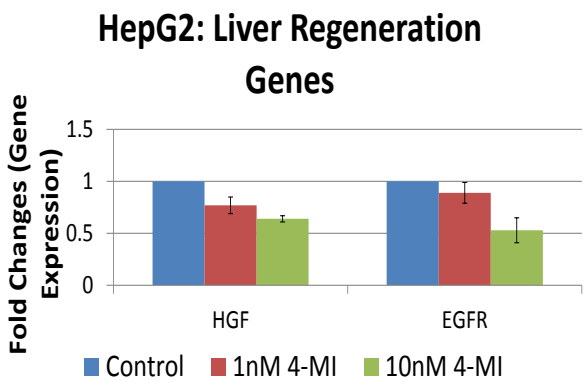



**Fig 8.**The Effect of 4-MI on regeneration genes in HepG2 is shown. All genes were significantly downregulated at both concentrations.

For the cellular results, all of our results supported our genetic findings. The significant decrease of cell death in HepG2 cells correspond to the direct anti-apoptotic effect 4-MI induced on HepG2 cells, but the introduction of FASL protein that 4-MI promoted the release of in the U937 cells induced significant cell death **(Fig. 14)**. This indicates that the immune-mediated pro-apoptotic FAS pathway may override the intrinsic anti-apoptotic BCL2 pathway to elicit hepatocyte death, providing a potential pathway for immune-mediated liver damage. On the immune cells, 4-MI induced significant cell attachment to fibronectin, indicating direct promotion of macrophage inflammatory responses **(Fig. 15)**.

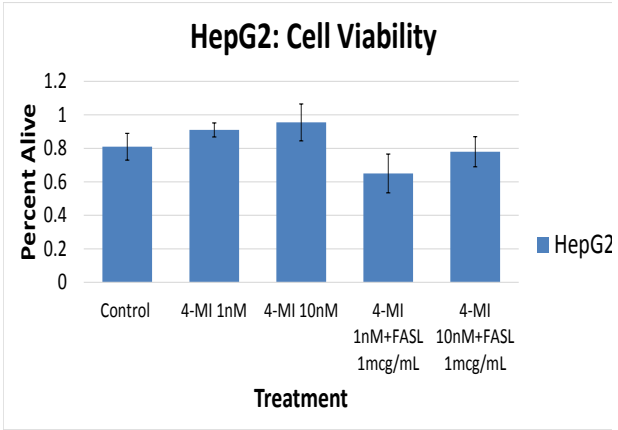



**Fig 9.**The Effect of 4-MI on cell viability in HepG2 is shown. 4-MI increased cell viability, but coupled to FASL treatment, cell viability decreased.

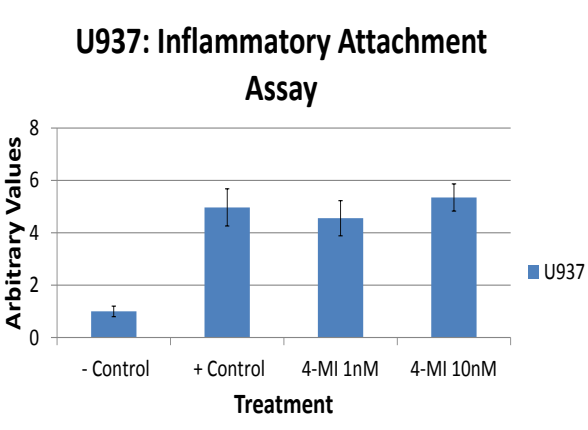



**Fig 10.**The Effect of 4-MI on inflammatory cell attachment in U937 is shown. 4-MI induced cell attachment that increased

## DISCUSSION

The goal of our study was to investigate 4-MI's effects on the key hallmarks of NASH; steatosis, inflammation, and apoptosis. Our hypothesis was supported at the molecular level as 4-MI significantly *dysregulated* all the factors in a way to promote steatosis, inflammation, and immune-mediated apoptosis. Figure 11 and 12 shows our proposed pathway by which 4-MI may promote liver damage and NASH.

**Fig. 11: 4-MI Promotes Classic Hallmarks: Steatosis and Inflammation**

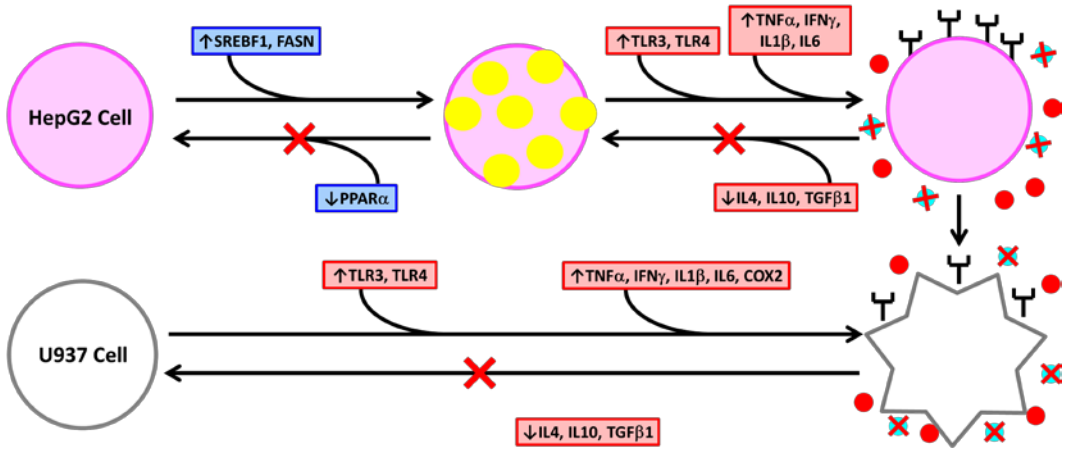
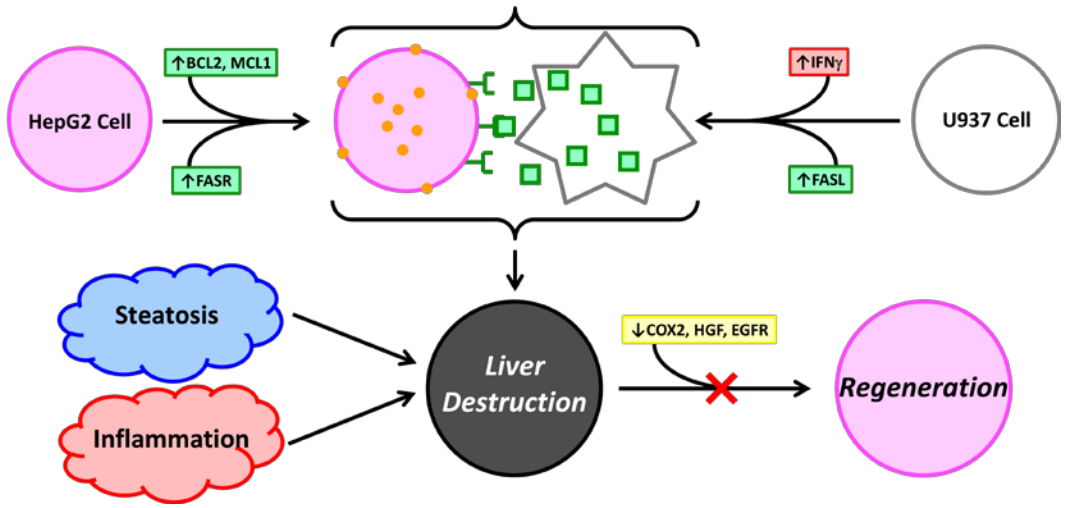



**Fig. 12: Novel Pathway by which 4-MI Promotes Liver Damage**



4-MI directly encouraged hepatic steatosis by inducing an adverse pattern of changes via the overexpression of lipid synthesis genes SREBF1 and FASN and downregulation of lipid metabolism gene PPARa. This dysregulation of lipid-regulating genes suggests 4-MI significantly promotes steatosis, which aligns with the previous literature [30]. Our results demonstrates 4-MI's ability to promote liver damage via promoting steatosis which is also one of the key hallmarks of NASH.

4-MI significantly dysregulates the expressions of genes that mediates the inflammatory response in HepG2 and U937 cells. 4-MI significantly up-regulated the expressions of harmful pro-inflammatory genes which promotes inflammation while significantly down-regulating the expressions of beneficial anti-inflammatory genes for both HepG2 and U937 cells, which prolongs inflammation. Thus, 4-MI promotes and prolongs adverse inflammatory processes in HepG2 and U937 cells, and induces effects that promotes the disruption of liver tolerance. The disruption of liver tolerance will lead to deleterious, chronic inflammatory processes and thereby, 4-MI may encourage liver damage while directly promoting one of the key pathophysiolgical symptom of NASH. The direct promotion of inflammation and steatosis, the defining characteristics of NASH, suggests 4-MI as an environmental causative factor for Nonalcoholic Steatohepatitis.

Most interesting was the effects 4-MI exposure induced onto the apoptotic genes BCL-2, MCL-1, FASL, FASR. While 4-MI up-regulated FASL on U937 immune cells, it showed no significant change on HepG2 liver cells. Similarly, while 4-MI up-regulated FASR on HepG2 liver cells, it showed no significant change on U937 immune cells. Furthermore, anti-apoptotic tumor antigens BCL-2 and MCL-1 showed significant overexpressions on the HepG2 liver cells, which was coupled with a decrease in the hepatocyte cell death. However, the introduction of FASL recombinant protein significantly increased the cell death well above the control level. This pattern of change suggests 4-MI activated the immunogenic FAS apoptotic pathway that may override the intrinsic BCL-2 pathway to elicit liver hepatocytes' death. Additionally, BCL-2 and MCL-1 have been proven to be very effective tumor antigens, allowing immune cells to trigger immune responses and kill the cells overexpressing the tumor antigens [31]. According to the well accepted theory of tumor immunity, the overexpression of BCL-2 and MCL-1 in the HepG2 liver cells may provide a targeting mechanism for the immune cells, who are also driven to the site due to promotion of alternative inflammatory responses. Where the activation of the FAS apoptotic pathway may directly encourage for hepatocyte death, the forced overexpression of the tumor antigens may indirectly encourage hepatocyte death by providing the immune cells with a hepatocyte targetting mechanism. Through this novel proposal for a tumor immune pathway, 4-MI may encourage immune-mediated liver damage.

One contradictory aspect was the downregulation of pro-inflammatory gene COX-2. Nonetheless, the major role COX-2 plays in liver regeneration suggests 4-MI disrupts, and may even decrease major liver regenerative mechanisms. The downregulation of imperative liver regeneration genes HGF and EGFR supports this; their downregulation suggests 4-MI may not only promote liver damage via inflammation and steatosis, but may also decrease the efficiency and potential for liver regeneration post-damage. This may provide an explanation for cirrhosis in chronic liver diseases such as NASH; the scarring of the liver due to defective regeneration processes elicited by exposure to toxic environmental agents, as in this case may

be 4-MI (this im actually not sure). Furthermore, previous literature demonstrated that COX-2 expression in hepatocytes protects the cells from FAS immunogenic apoptosis. Thus, 4-MI's ability to downregulate the expression of COX-2 may increase hepatocytes' susceptibility to the immunogenic FAS apoptosis which has been activated due to 4-MI exposure, which may further encourage additional liver damage [32].

A limitation in our study is that we tested for relatively short-term effects of 4-MI (≤24 hours) when in the real world, individuals are continuously exposed to 4-MI for years or decades before severe effects may occur. If all these effects occurred at nanomolar concentrations within 24 hours, what may occur over decades with continuous exposure to 4-MI?

## CONCLUSION

Our study demonstrated 4-MI's effects in relation to NASH and revealed key mechanisms that may be involved in its pathway. Our study demonstrated the followings: 1) 4-MI's ability to promote steatosis and chronic inflammation to human hepatocyte and immune cells 2) Proposal for a novel pathway for NASH, through an immune-mediated anti-tumor pathway 3) 4-MI's ability to decrease liver regenerative mechanisms to further encourage liver damage.

Overall, our study suggests 4-MI as a potential environmental, causative factor for NASH by promoting its classic hallmarks; steatosis and chronic inflammation. Additionally, 4-MI may further contribute to liver damage via a novel immunoediting pathway and decreasing liver regenerative mechanisms. Therefore, exposure to 4-MI may adversely promote liver damage to increased exposed individuals' susceptibility to NASH and/or worsen existing conditions.

In the future, we would like to conduct more genetic assays via a DNA Microarray to broaden our understanding of the etiology of NASH. Furthermore, more protein assays through ELISAS or Western Blots would strengthen our proposal of 4-MI as a contributor to NASH. Lastly, more experiments are needed to shed light on the mechanisms behind how 4-MI's exerting its effects. This may lead to new prevention methods for 4-MI exposed individuals that may already be suffering from NASH. Finally, the next steps of this study should involve investigation of 4-MI's effects in relation to NASH using *in vivo* models.

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