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Folate Receptor-Beta Expression as a Diagnostic Target in Human & Rodent Nonalcoholic Steatohepatitis

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Abstract

INTRODUCTION: Nonalcoholic steatohepatitis (NASH) afflicts 20–36% of individuals with nonalcoholic fatty liver disease (NAFLD). A lipotoxic hepatic environment, altered innate immune signaling and inflammation are defining features of progression to NASH. Activated resident liver macrophages express folate receptor beta (FR- β) which may be an indicator of progression from steatosis to NASH. The goals of this study were to characterize FR- β protein expression in human NAFLD and rodent models of NASH, and demonstrate liver targeting of an FR- β imaging agent to the liver of a rodent NASH model using FR- β .

METHODS: Rat liver lysates from methionine choline deficient (MCD) fed rats, high fat diet (HFD) and methionine choline sufficient (MC+) rat controls were analyzed for hepatic FR- β protein. The FR- β -targeted agent, Etarfolatide was injected into MCD and MC+-fed C57BL/6 mice for efficient FastSPECT hepatic imaging. Additionally, FR- β expression across the stages of human NAFLD from normal to NASH was assessed.

RESULTS: FastSPECT images show targeting of Etarfolatide to the liver of mice fed 8 weeks of MCD diet but not control-fed mice. The MCD rat model exhibited significantly increased protein expression of hepatic FR- β in contrast to HFD or normal samples. Similarly human liver samples categorized as NASH Fatty or NASH Not Fatty showed elevated FR- β protein when compared to normal liver. FR- β transcript expression levels were elevated across both NASH Fatty and NASH Not Fatty samples.

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Statement of Conflict of Interest

Drs Lake, Cherrington and Hardwick have nothing to disclose.

Dr. Low indicates relevant conflicts of interest for grants, personal fees, and stock with Endocyte Inc

Dr. Low indicates relevant financial activities outside of the submitted work: grants, personal fees and stock.

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CONCLUSION: The findings in this study indicate that FR- β expression in NASH may be harnessed to target agents directly to the liver.

Keywords

NAFLD; NASH models; folate receptor-beta; macrophages; imaging

INTRODUCTION

NAFLD is an increasingly prevalent hepatic manifestation of the metabolic syndrome. Worldwide, 11–46% of the population is afflicted with NAFLD (Ali & Cusi 2009, Berlanga et al 2014). Progression from simple steatosis to inflammatory, fibrotic nonalcoholic steatohepatitis (NASH) puts the NAFLD population at risk of developing cirrhosis, hepatocellular carcinoma and features of end-stage hepatic disease. Recent retrospective studies of pathological liver progression show that up to 36% of NAFLD patients have progressed to NASH (Pais et al 2013). The growing epidemic highlights the need for non-invasive diagnostics, biomarkers and therapeutic targets for NASH. The advancement of effective NASH therapeutics are primary goals for many pharmaceutical companies, clinicians and scientists as shown by the increases in NASH clinical trials (Issa et al 2017, Younossi et al 2017). Given the prevalence of this disease and association to obesity, type 2 diabetes and hepatocellular carcinoma the development of more targeted therapeutics for the NASH population are direly needed (Eshraghian 2017, Said & Ghufraan 2017).

The stages of NAFLD have been well characterized pathologically and while mechanisms are not clearly understood for the progression to NASH, insight into the role of the hepatic lipotoxic environment and innate immunity is growing in the field (Arrese et al 2016, Gadd et al 2014). The environment of the fatty liver characterized pathologically as steatosis, and also considered the first ‘hit’ of NAFLD, promotes increased sensitivity of the liver to additional ‘hits’ leading to the development of the inflammatory stage of the disease or NASH (Day & James 1998). The interplay of innate immunity, the inflammasome, macrophages and the gut-liver axis are contributing factors in the progression of NAFLD to NASH (Arrese et al 2016, Magee et al 2016). Markers of these mechanisms and importantly, diagnostics to identify individuals that have progressed from steatosis to NASH are lacking (Loomba 2018).

The activation of resident liver macrophages or Kupffer cells, are an important feature of NASH progression. Macrophage activation is a key event in triggering and amplifying inflammation in the liver in response to lipotoxic injury and is seen in NASH, hepatocellular carcinoma and hepatitis (Bility et al 2016, Dixon et al 2013, Gadd et al 2014, Kolios et al 2006, Minami et al 2018, Tosello-Tramont et al 2012). The mobilization of macrophage populations begins as early as the steatosis disease state in NAFLD progression. The macrophages gather within the portal areas of the liver however the exact stimuli for activation of these mobilized macrophages that triggers the transition from steatosis to the inflammatory NASH stage is unknown (Gadd et al 2014). Experimental studies have shown that activation of macrophages under lipotoxic conditions is linked to immunocellular components in NASH progression (Arrese et al 2016, Dixon et al 2013). Kupffer cells

differentiate into either M1 or multiple M2 subtypes which govern the functional roles of these cell types. The secretion of cytokines, chemokines and even the initiation of hepatoprotective activities as a result of activated macrophages and progression to NASH from steatosis is an initial response to combat threats to the liver. Thus, incorporating anti-inflammatory components into treatment regimens for NASH are expected to play an important role in combination therapies (Townsend & Newsome 2017).

In this study, we identify and characterize the expression of the glycosyl phosphatidylinositol (GPI)-anchored protein, folate receptor- β (FR- β) in the classical methionine and choline deficient (MCD) diet fed rat model of NASH as well as in human liver tissues pathologically classified as NASH from the NAS scoring system. As the targeting of FR- β in patients with rheumatoid arthritis has been effectively demonstrated using folate-linked agents (Kraus et al 2016), we aimed to test the targeting of a folate-linked imaging agent, Etarfolatide to inflamed livers in a NASH mouse model. Animal models are a critical aspect of NAFLD research and one in particular recapitulates the pathological profile of human NASH. The MCD diet, when fed to C57BL/6 male mice for a total of eight weeks, results in an inflammatory NASH phenotype similar to what is observed in human NASH. While the MCD diet-fed mouse model does not recapitulate the insulin resistance seen in the progression of NAFLD, the pathological profiles of NASH with inflammation and fibrosis are similar to what is observed in humans (Canet et al 2014, Hardwick et al 2010, Hardwick et al 2012, Tosello-Trampont et al 2012). Thus, for size and efficiency, an MCD mouse model was chosen to assess liver targeting of the folate-linked imaging agent, Etarfolatide using a FastSPECT live imaging system. The methionine and choline sufficient (MC+) fed mouse model of a normal liver was used as a control to assess the lack of Etarfolatide targeting to the liver.

Targeting therapeutics to activated macrophage populations in the liver of NASH patients through FR- β has the potential to effectively knock-out the inflammatory component of this disease. Furthermore, FR- β may have potential as a novel diagnostic target to distinguish inflammatory NASH from non-inflammatory NAFLD stages such as simple steatosis. Harnessing FR- β expression and targeting in NASH could be an attractive, less invasive option over liver biopsies which are the current gold standard for NASH diagnosis.

MATERIALS AND METHODS

Rodent NASH Models

8–9 week old C57BL/6 male mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Male Sprague-Dawley rats weighing 200 to 250 grams were obtained from Harlan (Indianapolis, IN). Mice and rats were provided with standard chow and water *ad libitum* during acclimation in a humidity, temperature controlled environment with a 12 hour light and dark cycle at the University of Arizona. The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-certified animal facility housed and provided care for the animals. After a week of acclimation mice were fed for 8 weeks while rats were fed for 6 weeks with a methionine and choline deficient (MCD, 518810) diet or a methionine and choline sufficient (MC+, 518754) diet. A group of rats was also fed a standard high-fat diet chose (HFD, 112280) for 6 weeks to model steatosis (Dyets Inc.,

Bethlehem, PA). At the conclusion of the 8 week dietary phase, mice were prepared for FastSPECT imaging under anesthesia at the University of Arizona's Department of Radiology. Housing and all experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

FastSPECT Etarfolatide Imaging in a Mouse Model of NASH

Etarfolatide, formerly known as EC20, a folate-linked chelate complex of ^{99m}Tc (Kraus et al 2016) was formulated and acquired as a generous gift from Endocyte Inc. (West Lafayette, IN). The structure and information of this imaging agent are previously reported (Leamon et al 2002). One vial of EC20 was submersed in a boiling water bath for 5 minutes and 1 mL of 50 millicuries of ^{99m}Tc was directly injected into the vial and the reaction allowed to boil for an additional 15 minutes. Binding of EC20 to ^{99m}Tc was quality checked by thin layer chromatography before use. After cooling to room temperature, MCD NASH or MC+ mice were individually injected in the tail vein with an intravenous solution of the prepared EC20- ^{99m}Tc (Etarfolatide) solution under anesthesia with 2% isoflurane in oxygen. 3–5 microcuries of Etarfolatide were measured for injection at the initial timepoint. Mice were then placed on a small animal stationary FastSPECT II camera imaging system designed by the Radiology Research Laboratory at the University of Arizona for imaging of rodents under anesthesia. Images were acquired of each rodent for each minute of the first 10 minutes and every 15 minutes thereafter for a total of 3 hours while the animal was maintained under anesthesia with 0.8–1.5% isoflurane. The last two images were acquired 30 minutes apart. Still and dynamic images were acquired of MCD and MC+ mice for analysis.

Rodent Liver Immunoblotting

Sprague Dawley rats were fed a methionine choline deficient (MCD) diet for 6 weeks in order to model the pathological endpoint of steatohepatitis. While a six-week (HFD) diet was used to model steatosis. At the end of the respective diets, the rats were euthanized and livers were extracted and frozen. Whole cell liver lysates from frozen tissue were homogenized and quantified. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) as per the manufacturer's protocol. Lysates were stored at -80°C . For immunoblots, lysates were diluted in Laemmli Buffer (Bio-Rad Laboratories, Hercules, CA) and 50 microgram of protein per well were run on 10% SDS-PAGE gels. Protein was transferred overnight onto methanol activated PVDF and blocked 1 hour at room temperature in 5% nonfat dry milk (NFDM) in PBST buffer. The FOLR2 rabbit polyclonal primary antibody (ab56067) acquired from Abcam (Cambridge, MA) was diluted to 1:7000 in 5% non-fat dry milk (NFDM) in PBST buffer and incubated overnight in the at 4°C . Blots were washed 3 times for 10 minutes each in PBST buffer prior to incubation with a goat anti-rabbit IgG-HRP (sc-2004) secondary antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for one hour at room temperature in a 1:50,000 dilution of antibody in 5% NFDM in PBST. GAPDH antibody was acquired and incubated on blots for control (Abcam). The blots were imaged with the advanced ECL chemiluminescence system (GE Healthcare, Pittsburgh, PA). Quantification of relative protein expression was determined using image processing and analysis with ImageJ

software (National Institutes of Health, Bethesda, MD) and was normalized to GAPDH on control protein immunoblots.

Statistics

Statistical analysis of immunoblots and branched DNA assays were analyzed using GraphPad Prism software Version 5 (GraphPad Software, Inc., La Jolla, CA). A one-way analysis of variance to determine significant differences between groups was utilized with a post-hoc Tukey analysis. A significance level of $p < 0.05$ was used for all analyses. Asterisks (*) stand for significance compared to control or normal liver tissue while daggers represent significance in comparison to steatosis livers.

Human Liver Samples

Frozen adult human liver samples were obtained from the Liver Tissue Cell Distribution System at the University of Minnesota, Virginia Commonwealth University, and the University of Pittsburgh as reported previously (Fisher et al 2009). Donor information included with the samples included age and gender. The individual liver samples were scored by a medical pathologist at the Liver Tissue Cell Distribution System (LTCDS) using the NAFLD Activity Score (NAS) system developed (Kleiner et al 2005) as a service provided by the agency to National Institutes of Health-funded investigators. A mixture of post-mortem liver tissue and biopsies representing the full spectrum of NAFLD were acquired and stored at -80°C prior to preparing mRNA or protein lysates. Samples were diagnosed as normal (17), steatosis (10), NASH Fatty (11) or NASH Not Fatty (10) by the LTCDS pathologists scoring criteria for NASH. Tissues that histologically had $>10\%$ fat deposition within hepatocytes and no accompanying inflammation or fibrosis for steatosis were classified as steatosis samples. NASH Fatty liver samples were characterized by $>5\%$ fat deposition in the presence of inflammation and fibrosis. NASH Not Fatty samples had $<5\%$ fat deposition within hepatocytes in the presence of inflammation and fibrosis.

Human Branched DNA Analysis

Human mRNA was isolated from frozen human liver tissue samples (17 normal, 10 steatosis, 9 NASH Fatty, 8 NASH Not Fatty) with RNA-Bee solution (Tel-test, Friendswood, TX) for the purpose of directly measuring mRNA levels by branched DNA (bDNA) analysis. Concentrations were determined using UV spectrophotometry and the integrity of the two bands was assessed using gel electrophoresis with ethidium bromide staining. Probe sets for FOLR2 and GAPDH were designed and diluted in Quantigene™ lysis buffer as per recommendations in the Quantigene HV Signal Amplification Kit (Panomics, Inc., Fremont, CA). Total RNA at $1\text{ }\mu\text{g}/\mu\text{l}$; $10\text{ }\mu\text{l}$ was added to each well of a 96 well plate with capture hybridization buffer and $50\text{ }\mu\text{l}$ of each probe set. Hybridization was performed as per manufacturer's recommendations. Luminescence was analyzed on a Quantiplex™ 320 bDNA Luminometer and analyzed on Quantiplex Data Management Software Version 5.02 (Bayer, Walpole, MA).

Human Immunoblot Protein Analysis

Human liver lysates were prepared as previously described (Fisher et al 2009, Hardwick et al 2010) for 17 normal, 10 steatosis, 11 NASH Fatty and 10 NASH Not Fatty samples. A total of 50 micrograms of protein were measured and run on 10% SDS-PAGE gels prior to transfer onto PVDF membranes overnight at 4°C. Membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk (NFDM) in PBST buffer followed by overnight incubation at 4°C in a 1:5000 dilution of primary FOLR2 (Novus Biologicals) monoclonal antibody in 5% NFDM in PBST buffer. Blots were washed 3 times for 10 minutes each in PBST, and incubated in a secondary anti-mouse antibody (sc2032) and diluted to 1:40,000 in 5% NFDM in PBST for 1 hour at room temperature. PBST washes were repeated and the blots imaged with the advanced ECL chemiluminescence system (GE Healthcare, Pittsburgh, PA). Approximately, 45 second exposures of FOLR2 blots on film were developed and scanned for densitometry analysis with ImageJ software (NIH, Bethesda, MD). Control protein blots with ERK (ERK1, Santa Cruz Biotechnology, sc-93) were generated and analyzed as control protein.

RESULTS

Etarfolatide Imaging in an MCD Mouse Model

At the conclusion of 8 weeks of MCD diet, C57Bl/6 mice were anesthetized and injected by tail vein with Etarfolatide for targeting to activated macrophages and imaging on a FastSPECT II camera to assess distribution of the compound to the liver. The acquired still and dynamic images show Etarfolatide in the liver, kidneys and bladder (Figure 1 & Supplementary Video) in MCD diet mice, indicative of liver targeting and expected renal elimination. In contrast MC+ diet control mice did not exhibit any targeting of Etarfolatide to the liver although the compound can be seen clearing through the kidneys and bladder (Figure 1 and Supplementary Video). The images demonstrate the ability to target folate-linked compounds to the liver of a classical NASH rodent model.

Rat FR- β Protein Expression Analysis

FR- β protein expression was significantly increased in samples from 6 week MCD-fed rat liver whole cell lysates. FR- β protein expression in the HFD samples was not significantly elevated above MC+ control rat liver lysates. FR- β protein expression levels were compared to GAPDH control protein levels from the same immunoblots. A representative blot of FR- β expression is shown for samples from each rat dietary model Control (n=6), HFD (n=5), MCD (n=4) (Figure 2).

Human FR- β Protein and mRNA Expression

Branched DNA analysis (bDNA) was used to directly quantitate mRNA expression levels of FOLR2 in normal, steatosis, NASH Fatty, and NASH Not Fatty human liver samples. The bDNA assay has been used previously on clinical liver samples and is highly accurate and reproducible (Fisher et al 2009, Hardwick et al 2010). A significant ($p < 0.05$) increase in FOLR2 gene expression by this method was observed for NASH Fatty and NASH Not Fatty samples in a one way ANOVA with post hoc Tukey testing when compared against normal

and steatosis samples (Figure 3A). Protein expression levels of FR- β demonstrated significant increases for both NASH Fatty and NASH Not Fatty liver samples as compared to normal liver controls. No significant FR- β protein expression changes were observed for steatosis samples as compared to controls (Figure 3B). A representative blot of FR- β protein expression in each stage of human NAFLD (n=4 samples per diagnosis) shows the band intensities in comparison to control protein (ERK) of the same samples (Figure 3C).

DISCUSSION

Inflammation and innate immune system signaling are critical drivers in the progression of NAFLD towards NASH (Magee et al 2016, Tomita et al 2006). While the pathological stages of NAFLD are well characterized, markers that delineate the transition from simple steatosis to inflammatory NASH are not as well understood (Day & James 1998). As a result, the development of an effective diagnostic for NASH has been elusive. FR- β is an proven marker of activated macrophages in inflammatory disease states such rheumatoid arthritis, systemic lupus erythematosus, and many cancers (Kraus et al 2016, Lu & Low 2012, Lu et al 2015, Lu et al 2011, Varghese et al 2007). FR- β is also an important mediator of macrophage homing and migration mechanisms (Machacek et al 2016), and is a well-characterized target for pharmaceutical intervention for inflammatory diseases and cancers (Low & Kularatne 2009, Segal & Low 2008, Varghese et al 2007). Therefore, the present study includes an assessment of FR- β selective expression in human NASH and in rodent models of NASH while assessing the ability to image inflamed livers in MCD diet-fed mouse models of NASH with a FR- β targeted imaging agent compared with healthy controls (Figure 4).

Increased mRNA and protein FR- β levels (Figure 3) in human liver tissues designated as NASH Fatty (n=11) and NASH Not Fatty (n=10) indicate that FR- β expression is a selective component of the disease stage. In both NASH Fatty and NASH Not Fatty human samples, FR- β protein expression was significantly increased when compared to normal (n=17) livers. Significance was not seen for elevated protein in NASH Not Fatty samples (Figure 3B) when compared against steatosis (n=10) samples. The limited human sample sizes should also be noted which may contribute to variation in the significance of the protein expression. However, FR- β mRNA levels are clearly elevated significantly in both NASH Fatty (n=9) and NASH Not Fatty (n=8) sample sets when compared against the steatosis samples (n=10) (Figure 3A). FR- β transcript levels in both pathological designations of human NASH are elevated regardless of liver fat content. Therefore, FR- β expression distinguishes individuals with NASH in this study. A host of published research on FR- β expression in immune cells (Lu et al 2015, Reddy et al 1999, Shen et al 2015, Shillingford et al 2012) as well as reports of high expression levels of FR- β in arthritis, lupus and several cancers (Low & Kularatne 2009, Varghese et al 2007) supports the findings we have noted in this study. The activation of macrophages and subsequent FR- β expression in NASH may well be a promising indicator for both diagnostic and therapeutic purposes as it has for other inflammatory disease (Baffy 2009, Gadd et al 2014).

Studies have investigated the utility of targeting activated macrophages previously as a pharmacologic target. In osteoarthritis patients, Etarfolatide was targeted to FR- β expressing

immune cells in the knees of patients (Kraus et al 2016). As a treatment for systemic lupus erythematosus, a folate-hapten-targeted immunotherapy demonstrated efficacy in improving animal survival and decreased SLE-symptoms and tissue damage by harnessing FR- β as a target on activated but not quiescent macrophages (Varghese et al 2007). FR- β expression on cancer and stromal cells was found to positively correlate with the stage of the cancer and metastases (Shen et al 2015). Thus, it has been demonstrated that a range of inflammatory diseases have the potential to benefit from FR- β expression on activated macrophages, suggesting that the selective expression of FR- β may also be utilized in hepatic inflammatory disease states such as NASH.

In the present study, we have demonstrated that FR- β protein expression is significantly elevated in human and rat NASH liver samples in contrast to normal, healthy livers (Figure 2, 3). Rat MCD diet-fed liver samples were utilized in the protein expression of FR- β due to the incompatibility of the FOLR2 antibodies in mouse liver samples. The rat MCD-diet model also shows identical pathological characteristics of human NASH (Hardwick et al 2012) therefore, for protein expression immunoblot assessments, rat MCD diet liver samples are reported for protein expression but not the mouse (Figure 2). Importantly, FR- β protein expression in the human liver samples (NASH Fatty and NASH Not Fatty) shown in Figure 3 and in the rat MCD-diet samples showed consistent expression. As newer reagents and antibodies develop, future assessment of FR- β protein expression mouse liver may someday be possible.

A previous clinical study showed early mobilization of Kupffer cells into portal regions of the liver in steatosis (Gadd et al 2014), demonstrating the significant role of macrophage activation in NAFLD progression to NASH. Due to the identification of FR- β expression in activated, but not quiescent macrophages (Kraus et al 2016), it can serve as a marker of at-risk patients with activated macrophage liver phenotypes. Activated macrophage populations in progressive hepatitis C and B have been identified and upregulated expression of FR- β in hepatocellular liver carcinoma patient liver samples has been shown in concordance with expression of M2 macrophage markers (Bility et al 2014, Bility et al 2016, Minami et al 2018). As a proof of concept for NASH, we conducted imaging studies in an 8-week fed MCD mouse model to investigate the targeting of the FR- β targeted imaging agent, Etarfolatide (Leamon et al 2002) to the liver of these diseased mice. In collaboration with the University of Arizona, Department of Radiology, mice were imaged on a FastSPECT camera after tail-vein injections of the radio-labeled imaging agent, Etarfolatide. Images from the MCD mice showed hepatic accumulation of the imaging agent that are not observed in MC+ control mice which show no accumulation or uptake of Etarfolatide into the liver (Figure 3). High accumulations of the imaging agent in the kidneys is also clearly observed in the images of both MCD and MC+ control mice indicating that this agent undergoes renal elimination in the model and that it possesses favorable pharmacokinetic parameters (Figure 3, Supplemental Video).

Now used in the clinic for the detection of folate receptor positive tumors, Etarfolatide has been shown previously to be targeted to the finger and knee joints, or regions of accumulated activated macrophages, in patients with rheumatoid arthritis (Kraus et al 2016). Etarfolatide has been used in many cancer patients to image folate receptor positive tumors and is also

reported to undergo clearance through the kidneys in an unmetabolized form (Leamon et al 2002, Sega & Low 2008). Overall, there is great potential for the imaging agent Etarfolatide to detect the inflammatory stage of NAFLD or NASH as a less intrusive diagnostic compared to the current standard of needle biopsy.

In conclusion, this study demonstrates that expression of FR- β in human and rodent models is specific to liver samples diagnosed as NASH Fatty or NASH Not Fatty but not simple steatosis or normal samples. Furthermore, the utilization of FR- β expression in NASH rodent models for imaging and diagnostics is shown by the detection of the folate-linked diagnostic agent, Etarfolatide. Harnessing folate-linked imaging and therapeutic agents to target activated macrophages in the liver represents a seminal opportunity in the treatment of NASH. Overall, this study provides evidence that the selective expression of FR- β can be used to differentiate the stages of NAFLD stages in humans and NASH models while revealing the potential for novel targeted therapeutic and diagnostic options for NASH. Challenges still remain for patients with more than one type of advanced liver disease, for example FR- β expression may not differentiate NASH from hepatocellular carcinoma due to the evidence that FR- β expression and M2 macrophage population activation is clearly detectable in hepatocellular carcinoma human liver samples (Minami et al 2018). Additional biomarkers and diagnostics may be needed to differentiate patients with NASH from those with advanced hepatitis and hepatocellular carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- NASH inflammation is closely associated with macrophage activation
- Etarfolatide, a folate-linked imaging agent is targeted to activated macrophages
- Targeting of Etarfolatide to the liver occurs in mice with MCD diet-induced NASH
- Etarfolatide targeting to the liver in control mice fed an MC+ diet does not occur
- FR- β protein and mRNA expression is increased in human NASH

Figure 1. Targeted Imaging of NASH using Radiolabeled Etarfolatide in an MCD and MC+ Mouse Model

Mice fed an MCD diet for 8 weeks to develop pathological NASH were imaged while anesthetized on a FastSpect II camera imaging system. Accumulation of Etarfolatide in the MCD livers is observed (white arrows). Clearance of Etarfolatide through the kidney is shown (dashed arrows). For MC+ control-diet fed mice representing normal livers, no accumulation of Etarfolatide was observed in the liver.

Figure 2. Hepatic Protein Expression of FR- β in Rats Fed the MCD Diet.

Frozen liver sample tissue lysates from rats fed the MCD Diet, MC+ control diet or a high fat diet for 6 weeks were assessed by immunoblots for expression levels of FR- β (37 kilodaltons). GAPDH protein expression was utilized as control protein. Relative protein expression of FR- β is significantly increased in the liver lysates of MCD-fed rats compared to MC+ controls or even high fat diet-fed rats. Asterisk (*) represents significance by one way ANOVA with posthoc Tukey test as compared against MC+ control-fed rat liver lysates ($p < 0.05$).

Figure 3. Human Liver FR- β mRNA and Protein Expression

Messenger RNA levels of FOLR2 (A) were assessed in human liver samples categorized as normal, steatosis, NASH Fatty and NASH Not Fatty by the bDNA assay. Folate receptor β mRNA levels are expressed as relative light units (RLU) per 10 micrograms of total RNA. Asterisks (*) represent significance compared to normal liver samples ($p < 0.05$) by one-way ANOVA with post hoc Tukey test. Daggers represent significance as compared to steatosis samples ($p < 0.05$). FR- β protein expression levels (B) for normal, steatosis, NASH Fatty and NASH Not Fatty human liver samples are shown. Samples from all human tissues were randomized, run on polyacrylamide gels and assessed by immunoblot analysis using human ERK as control protein. Asterisks (*) represent significance with respect to normal liver samples ($p < 0.05$) as analyzed by one way ANOVA with post hoc Tukey test. Daggers represent significance as compared to steatosis samples ($p < 0.05$). A representative blot of FR- β and ERK control protein expression (C) illustrates the immunoblot profiles for each

human liver diagnosis category and include normal (n=4), steatosis (n=4), NASH Fatty (n=4) and NASH Not Fatty (n=4).

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Figure 4. Etarfolatide Targeting to Activated Macrophages by FR- β

Activated resident liver macrophages expressing increased levels of FR- β are the target for the imaging compound Etarfolatide. The targeting of Etarfolatide to FR- β on activated macrophages in human and rodent NASH shows the potential application for diagnostic or therapeutics.