Safety and Dosing Study of a Cholecystokinin Receptor Antagonist in Non-alcoholic Steatohepatitis

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High saturated fat diets have been shown to raise blood levels of cholecystokinin (CCK) and induce nonalcoholic steatohepatitis (NASH). CCK receptors are expressed on stellate cells and are responsible for hepatic fibrosis when activated. The purpose of this study was to test the safety and dose of a CCK receptor antagonist, proglumide, in human participants with NASH. An open-label single ascending dose study was conducted in 18 participants with clinical NASH based upon steatosis by liver ultrasound, elevated hepatic transaminases, and a component of the metabolic syndrome. Three separate cohorts ($N = 6$ each) were treated with oral proglumide for 12 weeks in a sequential ascending fashion with 800 (Cohort 1), 1,200 (Cohort 2), and 1,600 (Cohort 3) mg/day, respectively. Blood hematology, chemistries, proglumide levels, a biomarker panel for fibrosis, and symptom surveys were determined at baseline and every 4 weeks. Abdominal ultrasounds and transient elastography utilizing FibroScan were obtained at baseline and at Week 12. Proglumide was well tolerated at all doses without any serious adverse events. There was no change in body weight from baseline to Week 12. For Cohorts 1, 2, and 3, the median percent change in alanine aminotransferase was 8.42, −5.05, and −22.23 and median percent change in fibrosis score by FibroScan was 8.13, −5.44, and −28.87 (kPa), respectively. Hepatic steatosis as measured by controlled attenuation parameter score significantly decreased with proglumide, ($P < 0.05$). Blood microRNA biomarkers and serum 4-hydroxyproline were consistent with decreased fibrosis at Week 12 compared with baseline. These findings suggest proglumide exhibits anti-inflammatory and anti-fibrotic properties and this compound is well tolerated in participants with NASH.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Nonalcoholic steatohepatitis (NASH) is a common condition associated with obesity, high-fat diet, and metabolic syndrome that may progress to cirrhosis and liver cancer. Cholecystokinin (CCK) receptors are expressed on stellate cells and are responsible for collagen production and liver fibrosis.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Can the CCK receptor antagonist, proglumide, be safely administered to participants with NASH, and what dose is most effective in reversing the clinical manifestations of NASH?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Proglumide decreased aminotransferase levels, and decreased steatosis and fibrosis by FibroScans in a dose-related fashion and was well tolerated.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ These results provide safety and dosing information to further investigate the use of proglumide in the treatment of NASH.

Non-alcoholic steatohepatitis (NASH) is one of the leading causes of liver-related morbidity and mortality in the world, and its incidence has sharply risen since the beginning of the 21st century.[1] Unfortunately, NASH is becoming an important etiologic factor in the development of advanced liver disease and hepatocellular carcinoma (HCC).[2] Obesity, type 2 diabetes mellitus, hyperlipidemia, and a high fat diet[3] are primary conditions associated with this pandemic in both industrialized and developing countries.[4]

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In NASH, hepatic stellate cells become activated myofibroblasts and are responsible for the remodeling of the extracellular matrix of the liver[5]; however, with chronic or severe injury, fibrosis may occur.[6] Since the majority (> 90%) of HCCs develop in livers with advanced fibrosis or cirrhosis,[7] the US Food and Drug Administration (FDA) has emphasized that approval for new therapies to treat NASH should improve or prevent progression of fibrosis.

The mainstay strategy for treating obese participants with NASH includes weight loss.[8]

Weight reduction of ≥ 10% as a result of lifestyle modifications has been associated with histologic improvement in NASH[9]; however, only ~ 20% of those with initial weight loss are successful in maintaining it long-term.[10] Several pharmacological treatments have been approved to aid in weight reduction[11]; however, many have side effects or do not reduce hepatic fibrosis.[12] The initial landmark study for the treatment of NASH was the PIVENS (Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis) trial[13]; however, neither vitamin E nor pioglitazone reversed fibrosis and patients with diabetes were not included in this trial. Since abnormalities in bile acid uptake and metabolism have been associated with NASH, agents that target the farnesoid X receptor (FXR) are being investigated.[14] One FXR agonist, obeticholic acid was demonstrated in a phase III trial to decrease hepatic fibrosis at the higher dose; however, over half of the participants developed pruritus as a side effect.[15] Numerous medications have been tested in nonalcoholic fatty liver disease and NASH and the results of these trials have recently been reviewed.[16]

The gastrointestinal peptide cholecystokinin (CCK) is released from the duodenum in response to dietary fat and is responsible for gallbladder contraction and release of bile acids to aid in digestion.[17] CCK receptors are expressed on cholangiocytes[18] and respond when stimulated by decreasing bile secretion. Furthermore, CCK receptors have been identified on stellate cells[19] and therapy with CCK receptor antagonists has been shown to block collagen production.[19]

We previously tested the CCK receptor antagonist proglumide in a murine model of diet-induced NASH.[20] Proglumide was used because it is water soluble, and also because proglumide is unique among the CCK receptor antagonists in that it is the only antagonist shown to increase bile flow[21] and decrease bile acid concentration in animals. In this study, proglumide not only prevented the histologic features of NASH, but it was also able to reverse fibrosis in established NASH.[20] One of the most striking findings of our preclinical study was that proglumide prevented the development of HCC. The purpose of this current study was to test the safety and toxicity of several doses of proglumide in human participants with clinical NASH and to determine the recommended phase II dose.

METHODS AND MATERIALS

Setting and referral process

Participants were recruited from outpatient liver clinics from two centers: Georgetown University Medical Center, and the Washington DC Veterans Affairs Medical Center. The research protocol was approved by the FDA under IND#143696 and the Institutional Review Boards at both sites, and the trial was registered on www.clinicaltrials.gov website with the registry trial number: NCT# 04152473. All participants signed and agreed to the informed consent.

Research participants and enrollment criteria

The inclusion criteria comprised male and female participants' ages 18–85 years with radiographic imaging of fatty liver disease AND elevation in serum alanine aminotransferase, (ALT) or aspartate aminotransferase (AST), AND one of the following: body mass index (BMI) > 30, hyperlipidemia, or diabetes based on an abnormal HbA1c. Eligible participants included those with evidence of mild to moderate fibrosis (F1 to F3) by transient elastography FibroScan. Participants on statins were eligible, and statins were continued at the same dose for the duration of the study.

The exclusion criteria included those with evidence of active drug/alcohol abuse, and those with chronic viral hepatitis B or C, autoimmune hepatitis, or drug-induced liver disease.

Those with evidence of cirrhosis on exam, by biopsy, by imaging, or with a kPa score on FibroScan ≥ 14, or a history of HCC were excluded. Laboratory tests that warranted exclusion included: leukocyte count < 3.5 K/UL; hemoglobin < 9.5 g/dL; blood urea nitrogen > 30 mg/dL (hydrated); creatinine > 2.0 mg/dL; ALT/AST > 5× the upper limit of normal (ULN); alkaline phosphatase > 2× ULN; abnormal total bilirubin; and abnormal prothrombin time or increased international normalized ratio or a platelet count < 150,000/mm^3. Those with a history of gall bladder disease without surgical removal were excluded. Those with an estimated glomerular filtration rate < 60 mL/minute/1.73 m^2 were excluded. Participants with type 1 diabetes mellitus, poorly controlled type 2 diabetes, with HbA1C > 8, or patients with diabetes that have not been on stable doses of antidiabetic medication for at least 90 days prior to the screening visit were excluded. Participants that were pregnant or breast feeding, those with bariatric surgery, and those with a known preexisting medical or psychiatric condition that could interfere with the participant's ability to provide informed consent or participate in study conduct were not included in the study.

Study design and objectives

This investigation was an open-label phase I study in participants with clinical NASH. The primary objective was to evaluate the safety and determine the recommended phase II dose of proglumide. The secondary objectives were to assess the effects of proglumide on serum liver transaminases, and fibrosis and steatosis scores by the FibroScan. An exploratory objective included evaluating a noninvasive blood biomarker panel for hepatic fibrosis improvement.

The study was conducted using three cohorts of participants with increasing doses of the investigational drug, proglumide. Six participants were recruited for each of the three cohorts for a total of 18 participants in this study. Each cohort was treated with proglumide by mouth for 12 weeks in sequential order as follows: the first cohort received proglumide at 800 mg/day; the second cohort was treated with proglumide 1,200 mg/day, and the third cohort received 1,600 mg/day of proglumide. After obtaining informed consent, participants were evaluated at a screening visit with a complete history and physical exam, inclusion criteria laboratory tests, abdominal ultrasound, and FibroScan. Eligible participants were scheduled for a baseline visit within 28 days where weight and BMI were calculated, an interim examination was performed, repeat liver profile was obtained to assure persistent elevation of transaminases, a medication and symptom diary were provided, and medication was dispensed. A baseline blood sample was obtained for proglumide blood levels and for research analysis of the liver fibrosis using a biomarker panel. Participants were provided a schedule with follow-up visits at Week 2, Week 4, Week 8, Week 12, and Week 16. At each of the visits weight was recorded; laboratory tests were collected for safety analysis; an interim history and physical exam were performed; the adverse events and drug accountability were

evaluated; and a new supply of medication was dispensed. At baseline and at Weeks 4, 8, and 12 blood was collected for the proglumide levels and biomarker fibrosis panel. At Week 12, an abdominal ultrasound was performed and a fasting FibroScan was obtained to determine liver stiffness by transient elastography with steatosis evaluation by controlled attenuation parameter (CAP) score. A follow-up safety visit was scheduled after stopping proglumide (Week 16) where participants had repeat chemistries and complete blood count performed.

After the participants in each cohort completed the treatment, the laboratory tests, adverse events, and imaging results were provided in a blinded fashion to the Safety Monitor for evaluation. The Safety Monitor was required to confirm there were no safety issues in each cohort before recruitment into the next dosing cohort was allowed.

Intervention

Proglumide is a water-soluble CCK receptor antagonist manufactured using good manufacturing practice criteria by AMSA S.p.A. – Cosma S.p.A. (Milan, Italy). The bulk compound was > 99% pure by ultra high-performance liquid chromatography and mass spectroscopy. Proglumide was compounded into vegan capsules with each capsule containing 400 mg by Custom Scripts Pharmacy (Lancaster, PA). Participants in Cohort 1 were administered proglumide using 400 mg by mouth twice daily for a total daily dose of 800 mg. Cohort 2 was treated with proglumide 400 mg orally three times daily or 1,200 mg/day, and Cohort 3 was administered proglumide 800 mg by mouth twice daily or 1,600 mg/day. The investigational pharmacist labeled the research medication at each site and dispensed it every 4 weeks during the treatment period.

Study assessments

Laboratory blood tests including a hematology panel, a chemistry panel, a liver profile, prothrombin time/international normalized ratio, urinalysis, and a pregnancy test (as needed) were evaluated at each visit for safety analysis. Parameters were established in the clinical protocol for early stopping rules and halting of the study for certain elevations in hepatic transaminases and significant changes in other laboratory values.

Participants' drug compliance and adverse events were recorded in patient diaries and reviewed at each visit. In addition, a side effect questionnaire was performed at each visit that specifically asked if side effects over the past month included the following: loss of appetite, itching, chest pain, shortness of breath, trouble urinating, diarrhea or constipation, and headache or confusion. Adverse events and any protocol deviations were recorded with the level of severity, whether the investigation drug was held or continued, and the likelihood of whether the side effect was related to proglumide. The adverse events were reported to the safety monitor, the respective Institutional Review Boards, and the FDA at the required intervals or during the annual progress report.

Blood samples were collected to measure proglumide blood levels by mass spectroscopy in the Georgetown University Lombardi Core Metabolomics and Proteomics facility and serum 4-hydroxyproline levels were measured in Cohort 3 using the ultra-high performance liquid-chromatography–mass spectrometry. Four selective microRNAs (miRNAs) that have been shown to inhibit hepatic fibrosis (e.g., miR-185-5p, miR-378a-3p, miR708-5p, and miR-346-5p)[22–25] as potential blood biomarkers were measured by quantitative reverse transcription polymerase chain reaction as we have described previously;[26] (See details in **Supplementary Materials**).

Liver ultrasound and FibroScan were performed by a technician blinded to the baseline reports and interpreted by a staff radiologist or hepatologist, respectively, who were not involved in the research study.

Statistical analysis

Statistical analysis of deidentified data was performed by a statistician with expertise in clinical trials from another university and not involved in conducting this clinical trial. An intent-to-treat analysis was performed, in which the available data from all patients were included in the statistical analysis. Changes in liver transaminases and mean values for each cohort at Week 12 were compared with baseline values. Values for mean baseline fibrosis kPa values and CAP scores for steatosis were compared using Student's *t*-test for changes at Week 12 from baseline. Nonparametric Spearman's rho analysis was performed to determine two-tailed correlation coefficients for the percent change in serum ALT, AST, fibrosis kPa score, CAP steatosis score, proglumide blood levels, and for each miRNA.

RESULTS

Patient characteristics

Of the 18 participants enrolled in the study, there were 5 women and 13 men. The ages ranged from 32 to 67 years with a mean age of 53.7 ± 2.3 years and with no significant difference in mean ages between the three cohorts. The mean ± SEM age for Cohort 1 was 58.5 ± 2.39 years, Cohort 2 was 55 ± 4.18 years, and Cohort 3 was 47.5 ± 4.43 years. There was no change in body weight from baseline (96.7 ± 5.1 kg) to Week 12 (98.9 ± 5.8 kg). Of the participants, 13 (72%) were White non-Hispanic, 2 (11%) were White Hispanic, 2 (11%) were Black, and 1/18 (6%) was South Asian. Fourteen participants had BMI measurements > 30 upon enrollment; 10 had hyperlipidemia, and 2 had type 2 diabetes.

Safety measurements

Overall, proglumide was well tolerated at all three doses without any serious adverse events. All 18 of the participants enrolled completed the study and none dropped out due to side effects. The adverse events reported during the study are listed in **Table 1**. Only nine side effects were reported in six of the participants, and all of these events were reports in participants from Cohort 1 or Cohort 2. There were no adverse events reported in those receiving the highest dose of proglumide. Of the side effects reported, gastrointestinal side effects were most frequent, including nausea (1), loss of appetite (1), abdominal pain (2), and constipation (3). All of the reported side effects were mild and resolved without discontinuing or holding proglumide. The majority of the adverse events reported occurred within the first month of initiating therapy with proglumide.

Compared with baseline values, there were no toxicities recorded for laboratory tests for all cohorts. The mean lab values for routine chemistries and hematology performed at each visit are found for each cohort in **Table S1a–c**.

Gallbladder and liver ultrasound were performed at baseline and after 12 weeks of proglumide since acute cholecystitis has been reported with more potent, CCK-A receptor antagonists.[27] None of the participants developed cholecystitis or new gallstones during the 12-week course of therapy. By Week 12, two of the participants (from Cohort 3) no longer had radiographic evidence of hepatic steatosis by liver ultrasound.

Effects of proglumide therapy on hepatic transaminases

There were no adverse events reported involving an increase in transaminases in any of the treatment groups during the study. The median percent change in serum ALT was +8.42, −5.05, and −22.23 for Cohorts 1, 2, and 3, respectively (**Figure 1a**), with significance observed with the highest dose of proglumide ($P < 0.05$).

The median percent change in serum AST was −16.94, −14.33, and −18.52 for Cohorts 1, 2, and 3, respectively (**Figure 1b**). The baseline serum ALT value for all participants was 1.79 times the upper limit of normal. Typically participants with NASH have ALT levels 1.5 to 4 times the upper limit of normal; therefore, our population's transaminase values were characteristic of those with NASH without advanced fibrosis.

Effects of proglumide on hepatic fibrosis and steatosis by transient elastography

Transient elastography utilizing FibroScan was measured in kPa for an estimation of hepatic fibrosis. Of the 18 participants enrolled, FibroScan at baseline included $N = 12$ with F1 fibrosis, $N = 3$ with F2 fibrosis, and $N = 3$ with F3 fibrosis. The median percent change from baseline values to Week 12 of the study for liver stiffness measured in kPa using FibroScan was +8.13, −5.44, and −28.87, respectively, for Cohorts 1, 2, and 3 (**Figure 2a**). Compared with the lower dose of proglumide (800 mg/day), change in fibrosis scores of those on the highest dose of proglumide (1,600 mg/day) nearly reached significance ($P = 0.07$).

At baseline, steatosis measured by CAP scores in dB/m with FibroScan included mild ($N = 1$), moderate ($N = 5$), and severe steatosis ($N = 12$). Steatosis measurements by CAP scores improved in all three cohorts with proglumide over the duration of the study ($P = 0.047$). The median percent change in CAP scores from baseline values to Week 12 was −1.27, −13.27, and − 3.38, for Cohorts 1, 2, and 3, respectively (**Figure 2b**).

Proglumide blood levels by mass spectrometry

All of the participants' baseline blood proglumide levels were undetectable. Proglumide blood levels measured by mass spectrometry at Week 12 are plotted for each cohort (**Figure 3a**), showing that the mean levels increased with each ascending dose administered. The mean proglumide blood levels measured at baseline, and at Weeks 4, 8, and 12 are shown for each treatment cohort (**Figure 3b**). **Table S5** shows the mean values and SEM. The peak serum concentration or (C_{max}) was reached in all of the participants from Cohort 1, and five out of six of the participants in Cohort 2 by week 8. In Cohort 3, half of the participants had higher proglumide blood levels detected at Week 12 than at Week 8, suggesting that Cohort 3 had not yet reached steady state. However, this difference could be due to the fact that more of the Cohort 3 participants had afternoon appointments and their proglumide blood levels had started to decline. We previously showed[28] in participants with cirrhosis and healthy controls, peak blood levels occurred within 1 hour after injections. Once maximum, the drug concentration was reduced to half in about 2.5 hours ($C_{max1/2}$). In the prior pharmacokinetics study, proglumide blood samples were collected after a single does several times over a 24-hour period. Therefore, we believe the C_{max} values are related to the time of ingestion before the sample was collected. All three participants with stage F3 fibrosis had C_{max} proglumide values greater than 24,000 ng/mL. Two of these participants were in Cohort 3; however, neither of these participants experienced side effects or adverse events. The third participant with F3 fibrosis from Cohort 1 had a proglumide C_{max} value at week 8 of 26,219.5

Table 1 Adverse events reported by participants

Timing of event	Cohort/Participant	Description of event	Grade of toxicity	Likely related to study drug	Ongoing or resolved	Drug held or discontinued
Day 1	Cohort 1 participant 2	nausea	1 (mild)	Yes	Resolved	No
Day 1	Cohort 1 participant 2	constipation	1 (mild)	Possible	Resolved	No
Day 21	Cohort 1 participant 2	thirsty	1 (mild)	No	Resolved	No
Week 4	Cohort 1 participant 4	Itching hands	1 (mild)	Possible	Resolved	No
Day 1	Cohort 2 participant 9	constipation	1 (mild)	Possible	Resolved	No
Week 5	Cohort 2 participant 10	constipation	1 (mild)	Possible	Resolved	No
Week 2	Cohort 2 participant 11	Abdominal Pain	1 (mild)	Possible	Resolved	No
Week 7	Cohort 2 participant 11	Abdominal Pain	1 (mild)	Possible	Resolved	No
Week 2	Cohort 2 participant 12	Loss of appetite	1 (mild)	Possible	Resolved	No

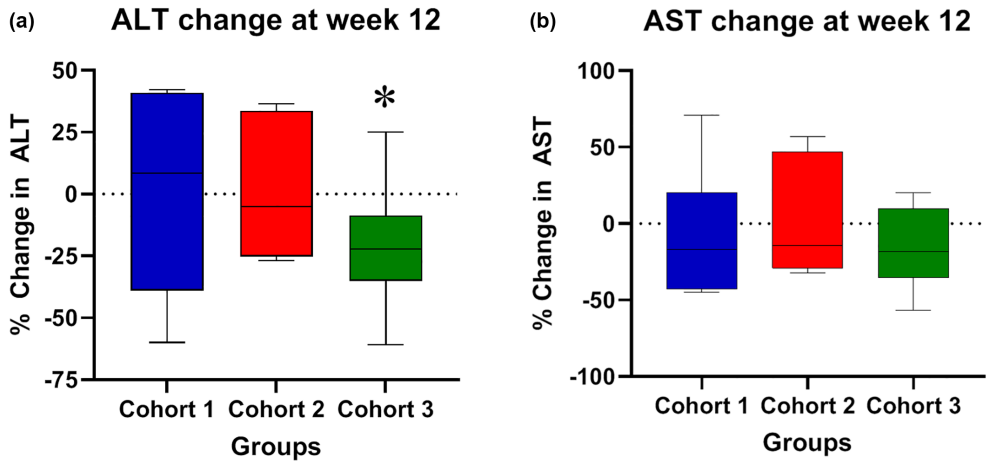

Figure 1 Percent change in liver transaminases during 12-week treatment with proglumide. (**a**) Median percent change in serum ALT values from baseline to Week 12 with range is shown according to cohort group. (**b**) Median percent change in serum AST values from baseline to Week 12 with range is shown according to cohort group. *Significantly different according to Kruskal–Wallis at $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

ng/mL. This participant (Participant 2) reported side effects with proglumide, including nausea, constipation, and increased thirst (see **Table 1**). It is uncertain whether the side effects reported by this individual were related to the higher proglumide values, because others reported gastrointestinal side effects without elevated proglumide blood levels. These results may, however, suggest that those with more advanced liver fibrosis have the potential for acquiring higher blood levels; and if side effects are reported in these participants, dose reduction may be warranted.

Blood biomarker assays with serum microRNAs and 4-hydroxyproline levels

MicroRNAs (miRNAs) are excreted in the serum and are very stable in the blood, and therefore they are being developed as biomarkers.[29] We previously found[30] a significant portion of miRNAs that were upregulated by proglumide inhibit hepatic stellate cell activation and fibrosis. Serum levels of all four of the miRNAs were found to be elevated at follow-up visits compared with baseline (**Figure 4a**). Significant differences compared with baseline measurements included miRNA-378-3p ($P = 0.004$), miRNA-708-5p ($P < 0.001$), and miRNA-346-5p ($P < 0.001$). MiRNA values appear to decrease with higher doses of proglumide, and this may be due to competition of the drug with the miRNA binding proteins such as albumin or Argonaute2.[31,32]

Changes in proline and hydroxyproline content have been shown to be significantly correlated with collagen and can be used in the assessment of normal and fibrotic tissues.[33] Five out of six participants in Cohort 3 had a decrease in the kPa liver stiffness by FibroScan, and these changes corresponded to similar decline in serum 4-hydroxyproline (**Figure 4b**). These values support our FibroScan results and microRNA values that represent decreased hepatic fibrosis.

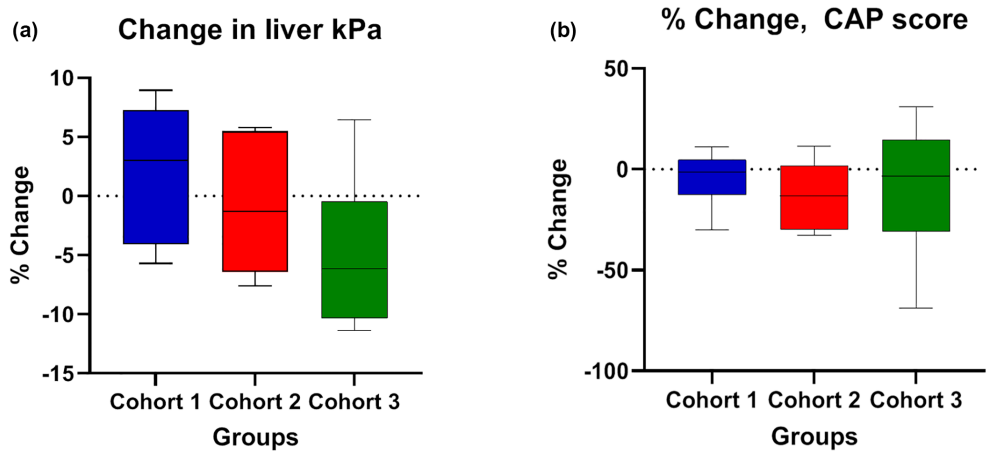

Figure 2 Change in hepatic fibrosis and steatosis by transient elastography. (**a**) Median percent change in hepatic fibrosis scores from baseline to Week 12 with range is shown according to cohort group ($P = 0.07$, Cohort 1 vs. 3). (**b**) Median percent change in hepatitis steatosis CAP scores from baseline to Week 12 with range is shown according to cohort group. Significant compare to baseline values $P = 0.047$. CAP, controlled attenuation parameter.

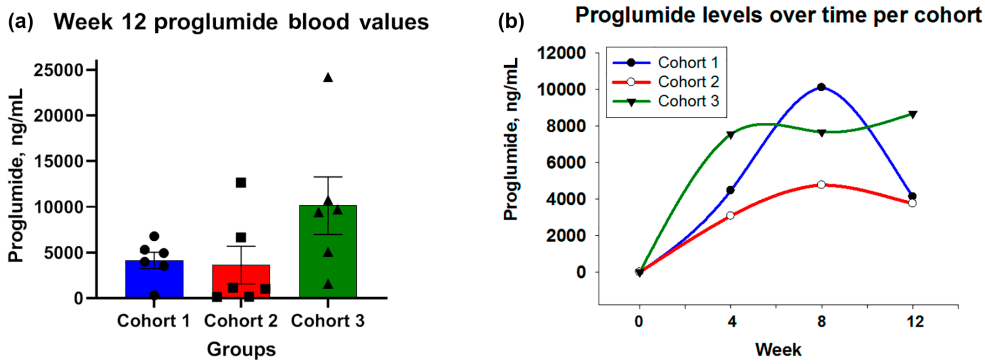

Figure 3 Proglumide blood levels per cohort measured by mass spectroscopy. (**a**) Absolute and mean ± SEM values of blood proglumide levels shown for Week 12 in the three separate cohorts. (**b**) Mean proglumide blood levels over time collected at baseline (Week 0) and Weeks 4, 8, and 12 for each cohort in the study.

DISCUSSION

This study represents a novel approach to managing the NASH epidemic by targeting the CCK receptor signaling pathway. Furthermore, the trial was based upon evidence from murine models of NASH and represents true bench-to-bedside translational research. This investigation met its primary outcome by demonstrating that oral proglumide had a broad safety profile and was well tolerated without toxicity in participants with F1–F3 fibrosis and clinical NASH. The few side effects reported were minor and none led to discontinuation of the drug. Participants were compliant with self-administration according to patient diaries and also confirmed by measurement of proglumide blood levels. We observed important changes in hepatic transaminases, fibrosis, and steatosis scores that would predict proglumide to be beneficial in proceeding with a phase II randomized placebo controlled trial. A 12-week period is probably not long enough to demonstrate significant changes in fibrosis and liver inflammation, but we gathered this information to determine the feasibility and help with sample size calculation for a phase II trial. We noted significant improvement over the 12-week period in ALT and steatosis scores and near-significant decline in fibrosis scores as determined by FibroScan. These changes observed in our study occurred without any confounding documented weight loss; in fact the mean body weight of our participants slightly increased over the study in spite of the team's recommendation to encourage healthy diets, weight loss, and exercise. Therefore, the improved transaminases and FibroScan scores most likely were the result of proglumide.

We selected three doses of proglumide to use in this study; a low, medium, and high dose. Proglumide was developed years ago for the treatment of peptic ulcer disease[34]; however, commercialization was halted with the development of the more potent proton pump inhibitors. These early trials included over 600 participants in 15 clinical trials that confirmed the broad safety profile of this compound. Pharmacokinetic studies performed in rats and human participants showed the metabolism and kinetics in the rats and humans were comparable and that the recommended oral dose of proglumide in people was 400 mg three times daily or 1,200 mg/day.[35] We recently completed a pharmacokinetic single-dose study testing proglumide in participants with Child-Pugh A and B cirrhosis and found the C_{max} and time to reach maximum plasma concentration (T_{max}) were comparable to that of healthy controls.[28] Since the current study was the first clinical trial in participants with NASH, we selected a proglumide dose that was lower and a dose higher than the 1,200 mg/day dosing used in earlier peptic ulcer disease studies. Although all three doses were well tolerated in participants with NASH, we observed the greatest decline in ALT and liver stiffness by FibroScan using the highest dose. Furthermore, there were no reported adverse events in participants administered the highest dose of proglumide; therefore, 1,600 mg/day would most likely be the selected dose to move forward with a phase II trial. Measurement of proglumide blood levels was beneficial in this study because they not only confirmed patient compliance and a dose-related effect, but we found that three participants with advanced F3 fibrosis experienced the highest C_{max} proglumide values. Our protocol that accurately measures proglumide blood levels with mass spectrometry will be useful in future trials, in the event that side effects occur, thus allowing for dose reduction if necessary.

The CCK peptide is a trophic hormone and has been shown to stimulate proliferation and DNA synthesis in the pancreas.[36,37] Proglumide is unique among this class of receptor inhibitors in that it is a nonselective CCK receptor antagonist with affinity to both the CCK-A and CCK-B receptors.[38] Proglumide may exert its effects in NASH by several different mechanisms of action. The most obvious mechanism is the antagonism at the CCK receptors which are expressed on fibroblasts to prevent the deposition of collagen.[19] We previously showed that livers of mice fed a NASH-inducing diet have up-regulation of the CCK-B receptor and this receptor is also over-expressed in murine and human HCC.[30] Blockade of the CCK-B receptor on hepatocytes and HCC cancer cells decreases the mitogenic actions of CCK and the prevention of HCC.[20] Proglumide treatment in animal models has also been shown to decrease inflammation[39] and also alter immune cell signatures in tumors.[40] CCK receptors and chemokine receptors are both G-protein coupled receptors (GPCRs). GPCRs are known to "cross-talk" or influence the action of other GPCRs, either by sensitizing or desensitizing the intracellular signaling or downstream pathways or by the formation of heterodimers.[41] Since proglumide reduces cytokine and chemokine expression in murine NASH livers,[30] proglumide

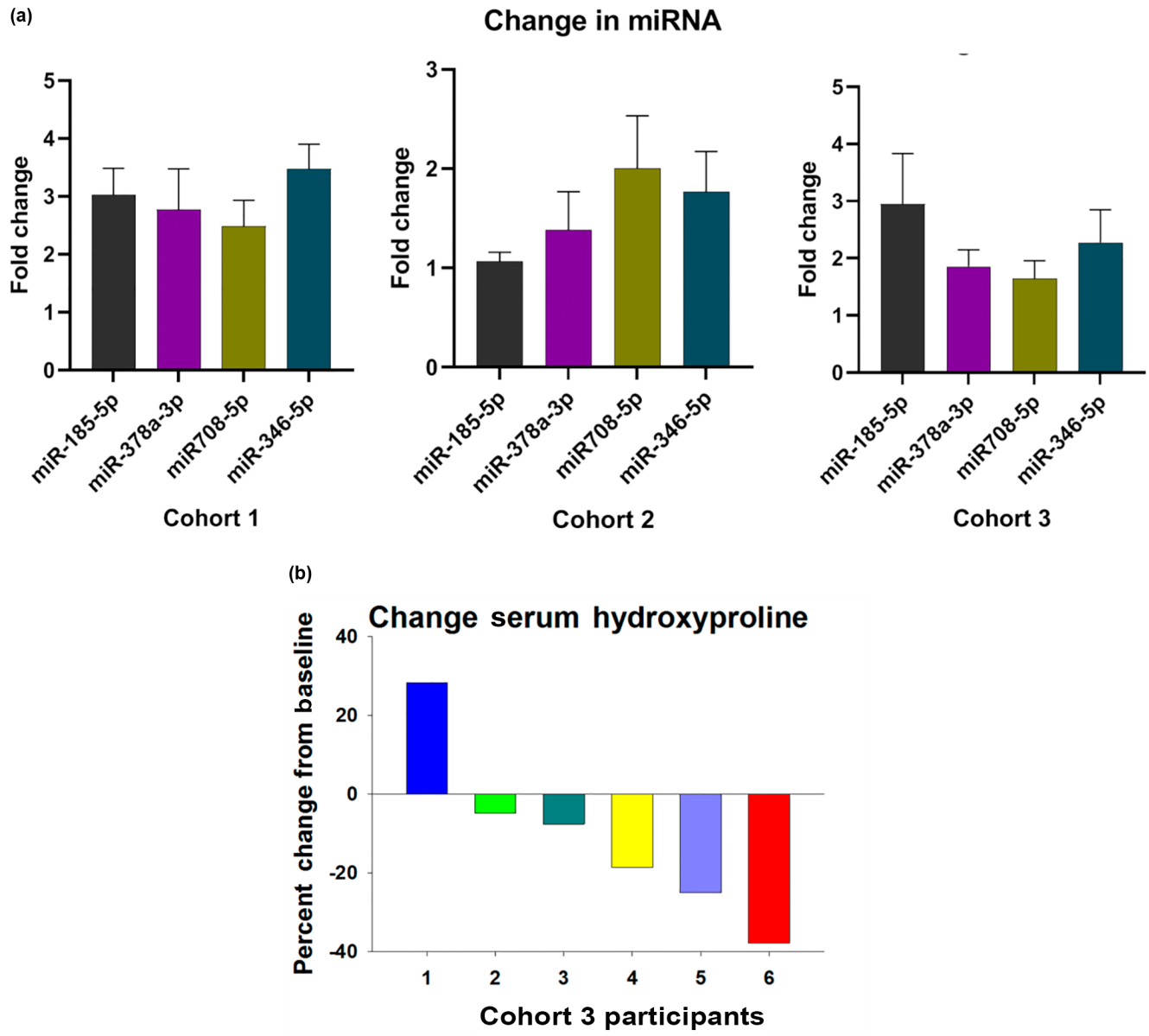

Figure 4 Blood biomarkers for change in fibrosis. (**a**) Four miRNAs known to inhibit fibrosis and hepatic stellate cell activation including miR-185- 5p, miR-378a-3p, miR708-5p, and miR-346-5p were measured at baseline and with proglumide therapy. All four miRNAs increased in serum of participants treated with proglumide as measured by quantitative polymerase chain reaction. Significant differences compared with baseline values using a two-sided Student's *t*-test included miRNA-378-3p, $P = 0.004$; miRNA-708-5p, $P < 0.001$, and miRNA-346-5p, $P < 0.001$. (**b**) Changes (%) in serum 4-hydroxyproline levels as measured by mass spectrometry at Week 12 compared with baseline are shown. Each column represents an individual participant from Cohort 3. miRNA, microRNA.

may exert its anti-inflammatory effects in the liver by cross-talk with chemokine receptors and reducing cytokines. Proglumide is the only CCK receptor antagonist that increases bile flow[21] and decreases bile acid concentration in animal models; therefore, we studied the interaction of proglumide with FXR using a human luciferase reporter assay and demonstrated that proglumide interacted with FXR as a partial agonist.[42] Since bile acids are affected by the gut microbiome, chronic proglumide therapy in mice also altered the microbiome, rendering it less hepatotoxic and increasing beneficial bacteria species.[42] These reasons are some of the myriad potential mechanisms through which proglumide may interact to decrease NASH.

Numerous investigations are being done to explore noninvasive biomarkers or that predict response to therapy to avoid liver biopsy. Although liver biopsies are generally safe in those with mild liver disease, they are not without risk[43] and also deter some participants from participation in research trials. Due to their stability in the circulation,[31] either protein-bound or exosome-encapsulated microRNAs have shown promise in evaluation of fibrosis in NASH.[44] In our mouse models,[30] we found a significant portion of miRNAs that were upregulated by proglumide in tissues and blood (e.g., miR-185-5p,[25] miR-378a-3p,[22] miR708-5p,[24] and miR-346-5p,[45]) had previously been shown to inhibit fibrosis, inflammation, or both from activated hepatic stellate cells. A

significant fold increase in the expression of these miRNAs would imply there was fibrinolysis or cessation of fibrogenesis in the livers of our study participants while administered proglumide. Further investigation of these miRNAs in combination with other noninvasive tests including transient elastography, hydroxyproline, and protein biomarkers may help determine the response to therapy without performing a liver biopsy.

In conclusion, therapy with the oral CCK receptor antagonist, proglumide, is safe and well tolerated in participants with clinical NASH. Due to its safety, tolerability, and potential efficacy, these results warrant further investigation to determine the efficacy of proglumide in those with NASH and its role in reversing hepatic fibrosis.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

Georgetown University owns intellectual property for the use of CCK receptor antagonists in NASH and J.P.S. is an inventor. All other authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

A.R., M.D.G., and J.P.S. wrote the manuscript. S.B., A.C., N.S., and J.P.S. designed the research. A.R., M.D.G., N.S., H.C., S.N., C.I.S., J.H.L., S.B., A.C., and J.P.S. performed the research. N.S., S.B., A.C., and J.K. analyzed the data.

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