Autophagy Induced by Exogenous Bile Acids is Therapeutic in a Model of Alpha-1-AT Deficiency Liver Disease

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Running Title: Bile acid induced autophagy.

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Abstract

The bile acid, nor-ursodeoxycholic acid (norUDCA), has many biological actions, including anti-apoptotic effects. Homozygous PIZZ alpha-1-antitrypsin (A1AT) deficient humans are known to be at risk for liver disease, cirrhosis, and liver cancer as a result of the accumulation of the toxic, A1AT mutant Z protein within hepatocytes. This accumulation triggers cell death in the hepatocytes with the largest mutant Z protein burdens, followed by compensatory proliferation. Proteolysis pathways within the hepatocyte, including autophagy, act to reduce the intracellular burden of A1AT Z protein. We hypothesized that norUDCA would reduce liver cell death and injury in A1AT deficiency. We treated groups of PiZ transgenic mice and WT mice with norUDCA or vehicle, orally, and examined the effects on the liver. The PiZ mouse is the best model of A1AT liver injury and recapitulates many features of the human liver disease. Results: Mice treated with norUDCA demonstrated reduced hepatocellular death by compensatory hepatocellular proliferation as determined by BrdU incorporation (3.8% control, 0.88% treated, p<0.04). Ki-67 staining as a marker for hepatocellular senescence and death was also reduced (p<0.02). Reduced apoptotic signaling was associated with norUDCA, including reduced cleavage of caspases 3, 7, and 8 (all p<0.05). We determined that norUDCA was associated with a >70% reduction in intrahepatic mutant Z protein (p<0.01). A 32% increase in hepatic autophagy associated with norUDCA was the likely mechanism. Conclusions: norUDCA administration is associated with increased autophagy, reduced A1AT protein accumulation and reduced liver injury in a model of A1AT deficiency.
**Introduction**

Alpha-1-antitrypsin (A1AT) deficiency is one of the most common metabolic-genetic liver diseases (22). It occurs in 1 in 2,000-3,500 births in Caucasian populations, and is associated with chronic liver disease, cirrhosis and hepatocellular carcinoma in children and adults. The Z mutant allele of the A1AT gene (SERPINA1) is a point mutation that encodes a single amino acid substitution. This mutation is associated with the vast majority of A1AT liver disease as the classical form of homozygous, ZZ A1AT deficiency. In the liver, large quantities of A1AT protein are synthesized, and rapidly secreted in the case of the WT A1AT protein. However, the A1AT mutant Z protein folds inefficiently during biogenesis and only 15% of the mutant Z molecules are secreted by the usual pathways while 85% are retained in the ER by quality control mechanisms (20, 22). Most of the mutant Z molecules retained are degraded by ER-associated degradation (ERAD)(14, 20, 22). Some of the A1AT mutant Z molecules polymerize into a complex quaternary structure and can accumulate in the “globules” of dilated ER known to be the classical hepatic lesion in this disease. Our laboratory was the first to show that autophagy was a pathway for degradation of the A1AT mutant Z polymers and that AAT mutant Z protein was present within autophagosomes (23). The mechanism of the liver injury results directly from the accumulation within hepatocytes of the A1AT mutant Z protein (1, 11, 22). This accumulation is heterogeneous among hepatocytes and in the cells with the largest mutant Z protein burden, the accumulation triggers hepatocellular death. The death process is a cascade involving apoptosis, redox injury and mitochondrial injury, followed by compensatory regeneration and, in some patients, hepatic fibrosis and HCC result.
The modified bile acid, nor-ursodeoxycholic (norUDCA) acid has been shown to have effects on protein degradation and hepatocellular injury in vitro that might be important in A1AT deficiency (4, 5, 6). We therefore, hypothesized that norUDCA would reduce liver injury in vivo related to A1AT deficiency (4, 5, 6, 15). In this study, we investigated the effects of exogenous norUDCA administration on liver disease in the PiZ mouse, an animal model of A1AT deficiency which recapitulates many aspects of the human chronic liver disease (1, 3, 16, 23). The PiZ mouse, and homozygous ZZ humans with A1AT deficiency both have accumulation of A1AT mutant Z protein in the polymerized conformation in the liver, both have the “globules” of dilated rER filled with polymerized Z protein in some but not all hepatocytes, both have increased hepatocellular death and compensatory hepatocellular proliferation, and both develop fibrosis and HCC in late adulthood (3, 8, 11).

Previous work in this mouse model has shown that drugs which enhance autophagy are able to reduce the intrahepatic burden of the polymerized mutant Z protein and reduce liver injury. This includes studies of rapamycin and carbamazepine (9, 10). Several other agents operating by different mechanisms have also been shown to reduce liver injury in this model (19). However, none of these drug interventions has been successfully adapted to proven human drug therapy, usually due to intolerable side effects or inability to give megadoses in humans which were required for the therapeutic effect in the mouse model. Exogenous bile acids similar to norUDCA are commonly used in many liver diseases and
are typically well tolerated, suggesting a possible new, and better tolerated, therapeutic approach.
METHODS

Animals

Instructions in (NIH, 86-23) “Guide for Care and Use of Laboratory Animals” were followed, as previously described(12). Protocols were approved by the animal studies committees of St. Louis University. Mice were housed with 12h dark-light cycles and food + water ad libitum in a barrier facility. PiZ mice, described previously, were maintained on a C57Bl/6 background. NorUDCA in a dose of 425mg/kg/d was given orally in chow for periods of up to 6 weeks (age 3 weeks to 9 weeks), with standard chow as vehicle. 3 groups of 10 PiZ mice and one group of 10 WT, C57Bl/6 mice were given norUDCA and compared to equal groups of litter mate matched mice given control vehicle only. Additional groups of 5 mice were analyzed for dose-response to norUDCA. PiZ mice are transgenic for the human A1AT mutant Z gene and express hepatic and serum levels of human A1AT protein similar to homozygous ZZ humans. The endogenous murine anti-protease genes are intact, but are significantly different from human A1AT and are immunologically distinguishable, as previously published (1, 3, 9).

Liver Analysis

Sacrifice of the mice, liver sectioning, staining and examination was carried out as previously described (11, 12). BrdU labeling using 72h osmotic pumps prior to sacrifice in the PiZ mice and subsequent staining and counting of the results was performed as previously described, as was Ki67 staining and counting (11). Counting for BrdU, Ki67 and electron microscopy was done by a single examiner blinded to the experimental groups. Hepatic lysates, A1AT protein assays, monomer/polymer assays, caspase blots,
electron microscopy, and quantification were carried out as described (1, 11, 12).

Quantitative blots were repeated in triplicate and graphed by scanning as previously published (1, 11, 12). The following antibodies were used; polyclonal rabbit anti-LC3 (1:1000, Cat# NB100-2220, Novus Biologicals), mouse anti-p62 (1:2,500, Cat# 610833, BD Transduction Laboratories), polyclonal rabbit anti-β-tubulin (1:1000, Cat# sc-9104, Santa Cruz), and secondary polyclonal goat anti-rabbit IgG-HRP (1:10,000, Cat# p0448, Dako) and donkey anti-mouse IgG-HRP (1:100,000, Cat# sc-2314, Santa Cruz). β-tubulin was used as an invariant control for equal loading. For EM counting of globules with an autophagic vacoule, 25 hepatocytes per liver were examined at random in a masked fashion by a single examiner and the percent globules in membrane continuity with a multilamellar autophagic vacuoles were counted. EM counting of area of cytoplasm occupied by autophagic vacuoles was performed on standard acquired fields, randomly selected, then area of autophagic vacuoles counted by overlay grids by a single, masked examiner, as previously published (18, 23). Statistical analysis SPSS (Chicago, IL), ANOVA as appropriate.

Inflammation analysis immunohistochemistry: The following antibodies, catalog numbers and companies were used: Rat anti-mouse F4/80, MCA497GA & Rat anti-mouse F4/80-Alexa-Fluor488, MCA497A488T (AbDSerotec, Raleigh, N.C. 27609); Rat anti-mouse ly-6G, clone 1A8, 551459 & Rat anti-mouse CD45R, clone RA3-6B2, 557390 (B220) (BD Pharmingen, San Diego, CA 92121); Biotinylated rat anti-mouse Ly-6G, clone 1A8, 127603 & Dylight 594 Strepavidin, 405222 (Biolegend, San Diego, CA 92121); streptavidin –HRP, ORO3L (CalBiochem, San Diego, CA 92121); Biotinylated
Goat anti-rat, 112-065-167, Jackson Immunolabs, West Grove, PA 19390). Paraffin embedded tissue was used for initial studies of neutrophils. For Ly-6G, antigen retrieval involved heat treatment for 10 min. at 95 °C in citrate buffer. Antibody for LY-6G was used at a 1:20 dilution. Frozen tissue sections were used with a 1:20 dilution of Ly-6G or 1:30 to 1:50 dilution of F4/80, or a 1:30 dilution of CD45R (B220). Primary antibody was followed by biotinylated goat anti-rat (1:250-1:500), streptavidin HRP (1:250-1:500) and development with DAB. For immunofluorescence studies, frozen tissue sections were used. Biotinylated rat-anti-mouse Ly-6G, clone 1A8, was used at a ¼ dilution & streptavidin Dylight 594 at 1:100-1:250 dilution. Rat anti-mouse F4/80-Alexa-Fluor 488 was used as recommended, undiluted. Image J was used to adjust the intensity of images uniformly and merge the color channels. Liver tissue sections were de-paraffinized and stained for naphthol AS-D chloroacetate esterase activity (Leder stain) using the protocol in kit 91C from Sigma-Aldrich. (Sigma-Aldrich, St. Louis, MO.63103)

RNA extraction and real-time PCR for autophagy

Total RNAs were extracted by TRI® Reagent (Sigma, MO, USA) from mice livers and treated with DNase I before the synthesis of the first strand of cDNA. Real-time PCR were performed as we previously described using SYBR Green Supermix. mRNA levels are expressed as fold changes after normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described(10). The primers for mice used in real-time PCR analyses were shown in Table 1.
Cell culture

HTOZ cells were modified from HeLa Tet-Off cells by transfection with the resulting pTRE1-ATZ plasmid (23). HTOZ/M were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and Pen/Strep, at 5% CO₂, 100% humidity and 37 °C. Cells cultured in medium containing doxycycline (DOX, 40ng/ml) turns off expression of α1ATZ proteins. 60-70% confluent cells were used in experiments. Cells were incubated 24h with norUDCA as indicated.

Western Blotting Analysis of Cell Culture

Whole cell lysates were prepared as previously. Proteins were separated by SDS-PAGE, transblotting and subsequent immunoreactions using chemiluminescence and antibodies goat anti- human α1AT from Diasorin (1:25,000, Stillwater, MN, RT 25 minutes) as previously described (1, 19, 23).

RESULTS

NorUDCA is associated with reduced intrahepatic accumulation of A1AT mutant Z protein in the PiZ mouse.

Groups of PiZ mice were given control vehicle chow or norUDCA drug chow as per methods above. First, liver sections were examined by H&E and PAS (periodic acid-Schiff) with digestion for histopathologic changes associated with norUDCA
administration (Figure 1a-b). Examination of the H&E sections revealed lymphocytic and polymorphonuclear infiltrate in the hepatic lobule and portal tracts with moderate steatosis, which is typical of both human ZZ liver and the PiZ mouse. There was no evidence of necrosis or cholestasis on single examiner, masked H&E examination using standard criteria (bile canicular plugs, ballooning hepatocytes, ground glass cytoplasm), which was not different in the control PiZ or treated PiZ mice. PAS with digestion is an established technique to label the intracellular accumulations of mutant Z glycoprotein red. The PAS with digestion revealed striking reduction in the intracellular accumulations of A1AT mutant Z protein (red “globules”) (p<0.001), which are known to be composed of A1AT mutant Z protein in the polymerized conformation (1). The polymerized conformation is thought to be especially injurious to the cell. We performed electronic scanning quantification of the area of the sections occupied by the globules (figure 1c) which confirmed the visual examination (p<0.001). We also measured the size of the few globules that did remain and found the mean area of the globules significantly reduced (Figure 1d) (p<0.001).

Next we duplicated the examination of the hepatic content of A1AT mutant Z protein by a second, biochemical method, since previous work has shown that a significant portion of the mutant Z protein retained in the liver is not in a visible globule (1). This method quantifies A1AT mutant Z protein molecules as either single units (“monomers”) and as the insoluble aggregates in the unique “polymer” conformation. However, once the monomer and polymer populations are separated in this technique, they are quantified by immunoblot after denaturation so that the polymers are broken up and run on the gel at
the monomeric molecular weight for comparison. It has been previously shown that
A1AT polymers exist in multiple pools, both within and outside of the globules visible by
light microscopy (1, 11). The result shows no change in the monomers but a more than
50% reduction in A1AT polymer accumulation (p<0.01) associated with norUDCA
(figures 2a-b). The monomers are typically considered newly synthesized molecules,
which adopt an abnormal conformation during biogenesis, leading to a lack of secretion,
intracellular accumulation and then over time either intracellular degradation or
polymerization.

Next we tested A1AT serum levels to detect any effect of norUDCA on secretion. PiZ
mouse A1AT mean serum levels were not different in the control and treated mice groups
at baseline (p=0.6), but post treatment there was a 22% reduction in serum level in the
norUDCA treated (p=0.006) (figure 2c). This was a novel finding, as there is no previous
report of a chemical agent which reduced the secretion of A1AT from hepatocytes. It was
unclear if the molecules were being degraded instead of secreted, or if there was reduced
synthesis. Since the overall burden in the cells was significantly lower, it was clear that
increased intracellular retention without degradation was not the cause. The lack of
change in the intracellular monomer protein at steady state suggested no change in
expression, however, it is still possible that the effect would be produced by a reduction
in A1AT mRNA. Therefore, we performed RT-PCR quantification of A1AT mRNA,
using previously established methods, and found no change between control and
norUDCA treated PiZ mice (figure 2d, p=0.4) (10).
Moreover, we also noted that the gallbladders of norUDCA mice were engorged and averaged more than 3 times the volume of gallbladders in the control PiZ animals without evidence of duct obstruction either grossly, on histopathologic examination or by biochemical indicators. This is consistent with the previously described cholerhetic effect of norUDCA (figure 3) (5).

**Inflammatory infiltrate in PiZ liver is reduced after norUDCA administration.**

Histological examination of the PiZ liver shows leukocyte infiltrates are dispersed throughout the tissue, but clusters, or foci, of inflammatory cells are also present, usually in association with cells containing globules of mutant Z protein. (figure 1a and 1b, figure 4a and 4b). These foci are often localized to lobular regions. Since leukocytes involved in the inflammatory response are typically proliferating, we next used BrdU labeling to label proliferating inflammatory cells (figure 4b). The results show non-hepatocyte cells with BrdU labeling both in the immune foci and in infiltrates dispersed throughout the parenchyma of PiZ liver tissue.

Some of these labeled cells may be inflammatory leukocytes, Kupffer cells or sinusoidal endothelial cells. We next used staining for the F4/80 antigen in the liver, which labels Kupffer cells, infiltrating monocytes/ macrophages and a subset of dendritic cells (figure 4c). The results show F4/80 positive cells are significantly increased in PiZ liver compared to WT, with intense staining in inflammatory foci in PiZ liver (figure 4d). We also labeled neutrophils using immunohistochemistry for Ly6G(monoclonal 1A8), a cell
membrane protein present on neutrophils and using activity assays for naphthol AS-D chloroacetate esterase, (NCE), an activity present in cells of the granulocyte lineage. The assays reveal that foci contain a significant number of neutrophils and a few neutrophils are present as isolated infiltrates. However, when we stained for B cells using B220 antibody (figure 4f), we found scant labeling in the inflammatory foci.

To document the relative abundance and positions of various cell types in the foci, immunofluorescence staining was performed, (figure 4g). The results show that neutrophils were often in the core/center of foci and macrophages in peripheral areas. In general, neutrophils showed a C shaped or honeycomb-like staining pattern, predominant in the core. Macrophages, usually, were less prevalent in the foci, and either found at the periphery or localized to one region. Macrophages appeared as large, round brightly stained cells or narrow, spindle shaped cells.

Next, we used the methods above to quantify PiZ mice at baseline to PiZ treated with norUDCA (figure 4h). Proliferation of inflammatory cells by BrdU uptake was significantly lowered, by approximately half, in association with norUDCA administration (p<0.02). Inflammatory foci were markedly reduced in number in norUDCA treated liver, as assayed by counting by a single, masked examiner. In addition to lowering the average number of foci with norUDCA treatment, the average area of foci was diminished by almost half (figure 4h) (p<0.006).

NorUDCA is associated with reduced evidence of liver injury in PiZ liver.
The profound reduction in mutant Z protein intrahepatic accumulation associated with norUDCA, and the reduction in inflammation, led us to hypothesize that large reductions in other markers of hepatic injury would also be found, consistent with the known intracellular injury cascade associated with A1AT mutant Z protein accumulation (1, 11).

First, we examined serum ALT in the control PiZ mice compared to the norUDCA treated PiZ mice (figure 5a). Past studies have shown an approximate 2-fold increase in ALT in PiZ mice compared to WT mice (WT mouse ALT upper limit of normal in our reference lab is <65 IU/ml), and these data now show reduced ALT to approximately the WT mice level associated with norUDCA treatment (p<0.05)(7). We next examined caspase cleavage since previously published data from many labs have shown that apoptotic signaling via caspase cleavage is associated with intracellular accumulation of mutant Z protein (8, 11, 12, 13, 14, 23). Caspase 12 in mice is associated with ER stress, such as a burden of unfolded protein in the ER, and caspase 3 is a product cleaved as a late step in the apoptotic cascade. Cleavage of these species indicates increased ER stress apoptotic signaling and increased distal pathway signaling, respectively (10, 16, 23). Our results show reduced caspase 12 cleavage (p=0.01, figure 5b-c) and reduced caspase 3 cleavage (p=0.03, figure 5d-e) in PiZ mice treated with norUDCA. Previous work has shown that the caspase cleavage mainly takes place in the small number of cells with the largest mutant Z protein polymer burden (usually the largest globules) and these results are based on whole liver lysates, so while the results are significant, the magnitude of some of the readout may be diluted (11). Several previously published studies have also shown that TUNEL staining in PiZ liver is not useful, as the apoptotic bodies are cleared.
very rapidly such that the signal in PiZ mouse liver and in human liver is near the limit of
detection (1, 11).

Next, in a group of 10 PiZ control and treated mice, as per the methods, we measured
hepatocellular proliferation by 72h BrdU incorporation prior to sacrifice (16). Previously
published worked has established this as a marker for hepatocellular death in this, and in
other models (16). The results revealed reduced compensatory proliferation of
hepatocytes associated with norUDCA treatment (Figure 5f, p=0.02). We also examined
proliferation by a second method using Ki67 staining, which is a marker for proliferative
entry into the cell cycle (2, 12, 13). Previous work has shown, in both human liver and in
PiZ mouse liver, that Ki67 positive liver cells are increased in PiZ liver compared to WT
(2). Therefore, we stained and counted Ki67 in three randomly selected PiZ control mice
compared to three norUDCA treated PiZ mice and found a significant reduction in the
norUDCA treated consistent with reduced compensatory liver proliferation (p=0.04,
figure 5g).

The final common pathway of liver cell death and regeneration is liver fibrosis. Fibrosis
develops in both the PiZ mouse model and in human A1AT ZZ liver, but in both cases it
is usually late in adulthood (2, 3, 9, 10, 13). In PiZ mice this is seen at about 12mo of age.
We examined our young adult mice (2 mo of age) treated with norUDCA for excess
fibrosis compared to controls (figure 5h) and found that no excessive fibrosis above
normal had yet developed in the control mice, and that this was not different from the
norUDCA treated mice. Finally, 6 mice (3 control, 3 norUDCA treated) were examined
for changes in expression of the fibrosis genes, alpha-SMA and collagen 1A1 by RT PCR of mRNA (figures 5i, 5j) (12). No difference was found, as expected, in these young mice in which hepatic fibrosis had not yet had time to develop.

Activation of autophagy in the PiZ liver is associated with norUDCA administration

Our examination of the PiZ mice treated with norUDCA was remarkable for the magnitude of the reduction in A1AT mutant Z protein retained in the liver and for the multiple markers of hepatic injury ameliorated in association with norUDCA. The effect on the accumulation of the polymer form of the A1AT mutant Z protein was especially noteworthy. Past studies from several labs have shown that agents which induce autophagy have effects very similar to these on the PiZ mouse liver (9, 10). When the A1AT mutant Z protein accumulates in cells, several intracellular processes are activated to deal with the toxic accumulation. The ubiquitin-proteasome system, termed “ER-associated degradation” (ERAD) is the primary route of disposal for newly synthesized, but abnormally folded, mutant Z protein monomers (1, 8, 11). Autophagic degradation is important in degrading the large, and often insoluble mutant Z protein polymer, both in non-dilated ER and within the globules (8, 9). Autophagy is a highly conserved intracellular process of mass disposal within unique vacuoles. For these reasons, we hypothesized that norUDCA was inducing increased autophagic degradation of A1AT mutant Z protein in the PiZ liver as the mechanism of the observed effects. To examine this, we first performed quantification by electron microscopy, as previously described and validated, of the autophagic vacuoles in the control PiZ mice compared to the norUDCA treated PiZ mice (10, 18, 19, 23). It has been previously shown in cell culture
systems, PiZ mouse liver, and in human liver that there is increased formation of
autophagic vacuoles in tissues expressing A1AT mutant Z protein compared to WT
tissues (10, 18, 19, 23). Our new results showed an additional 32% increase in autophagic
vacuoles in PiZ mice treated with norUDCA drug compared to control PiZ mice (p<0.02)
(figure 6a and 6b). Next, we confirmed this result of increased autophagic activity
associated with norUDCA with a second validated technique to quantify autophagy using
LC3 conversion (Figure 6c). LC3 is a component of the autophagic vacuole membrane
and conversion to the species LC3 II is directly related to increased numbers of
autophagic vacuoles (9). The results show increased LC3 II in PiZ liver at baseline
compared to WT mouse liver at baseline as previously reported, which is further
increased in PiZ liver with norUDCA. In preliminary dose-response experiments, the
amount of A1AT mutant Z protein in the liver affected the degree of autophagy
associated with norUDCA administration. When mice treated for short periods of time (1
week) were compared to mice treated until the majority of A1AT was no longer present
in the liver (9 weeks), but with the same dose of norUDCA, the autophagic activity was
reduced when the A1AT intracellular protein burden was reduced. The more A1AT
accumulated in the liver the more sensitive the liver was to increased autophagy
associated with norUDCA. Finally, we examined the effect of norUDCA in WT mice and
found that there was less increase in LC3 II in WT liver associated with norUDCA.

In the liver, increased signaling for autophagy is sometime associated with reduced levels
of p62, depending on the autophagy activation pathway involved. Therefore, we also
examined immunoblots for the mice above (figure 6c) for p62. The results showed no
decrease in p62 protein in WT mice associated with norUDCA, but there was a drop in p62 in PiZ mice associated with norUDCA. These data might indicate norUDCA was not acting via a p62 autophagy mechanism or that there was once again, evidence for a different response to norUDCA in WT mice versus PiZ mice. To further explore this question, we examined expression of a variety of genes known to be involved in autophagy (figure 6d, e, f). The ATG family of genes encodes proteins which are involved in the signaling and formation of the autophagic vacuoles. Beclin1 is part of the ATG family of autophagic regulation. Gabarap is GABAA-receptor associated protein, and is involved in vacuole formation and regulation by binding to ATG8 and other molecules. Dram1 is known to be involved in some branches of the autophagic activation pathway by interacting with p62. The results of RT PCR quantification of mRNA for these genes revealed significant changes consistent with increased autophagic activity in PiZ liver at baseline compared to WT at baseline (figure 6d), without the presence of norUDCA. When norUDCA treated WT liver was examined (figure 6e), only trends in these genes’ expression consistent with autophagic activation were seen without statistical significance. These data call into question the role of norUDCA in autophagic activation in healthy liver. However, in the PiZ mice treated with norUDCA 5 of the 7 genes had significant (p<0.05) changes in expression consistent with autophagic activation, including p62 reduced expression consistent with the drop in p62 protein, only seen in the PiZ liver. Together, these data suggest induction of autophagy by norUDCA that is uniquely seen in the presence of mutant Z protein accumulation.
Our previous work, confirmed by other investigators, has revealed that A1AT mutant Z protein accumulated in the liver in the polymerized conformation is degraded by autophagy and that administration of autophagy enhancers is associated with increased A1AT degradation and a reduced hepatic A1AT burden. We previously detected A1AT within autophagic vacuoles (18, 19, 23). In this study, we further examined the ultrastructure of the autophagic vacuoles and compared the control PiZ to PiZ treated with norUDCA (figure 6g). Typically, under transmission electron microscopy (EM) many dense globules of A1AT surrounded by rough ER (rER) can be seen, as can many multilamellar autophagic vacuoles filled with the targets of degradation. However, in PiZ liver at baseline only 4% of the globules are visualized in continuity with an autophagic vacuole. When PiZ liver treated with norUDCA is examined, 28% of the globules are in continuity with an autophagic vacuole (p<0.002). This change in ultrastructural appearance has never been previously reported in association with autophagy enhancers in liver.

Finally, we demonstrated a dose response of autophagic activation to norUDCA using a previously published in vitro system in which we could precisely control concentration and A1AT expression (19, 20, 21, 22, 23). Since we had observed that response to norUDCA in the PiZ mouse liver varied with the decreasing burden of A1AT protein in the liver over time, then a dose-response experiment in vivo would be difficult to fully control. Therefore, we employed a previously published in vitro system known to be an excellent recapitulation of the intracellular processing and degradation of A1AT mutant Z protein (9, 20, 23). We incubated cells expressing A1AT at the same level with variable
concentrations of norUDCA and assayed for changes in LC3-II as a marker directly related to the number of autophagic vacuoles present in the cells (figure 6h). The result shows increasing LC3 conversion to LC3-II with increasing dose compared to a serum starved (SS) positive control stimulus.

Discussion

A1AT deficiency liver disease has no specific treatment, beyond usual liver disease supportive care measures and liver transplant. Several liver disease treatment strategies have been evaluated in the PiZ mouse model, however, with promising results. One such strategy has been a series of studies of compounds which induce autophagy and promote increased intracellular degradation of the accumulated A1AT mutant Z protein. In the model systems, reduction of accumulated mutant Z protein has always been associated with the amelioration of liver injury. Interestingly, the studies of rapamycin and carbamazepine in the PiZ mice have required unusual dosing (9, 10). Either pulse dose schemes (rapamycin) or megadose (carbamazepine) schemes have been required to cause any change in Z mutant protein liver content, and these dosing schemes do not appear feasible in humans. In our current study, we examined the effect of norUDCA in the PiZ mouse. Our results revealed a strikingly large reduction in the accumulated mutant Z protein in the liver. This was accompanied by large reductions in many of the well-known markers of liver injury in this disease. We proposed that we had discovered another autophagy-inducing compound and further study has shown that this is the likely mechanism. This is a novel example of exogenous bile acids inducing autophagy in a way that might be more tolerable as a human therapeutic.
However, the results are interesting for another aspect of the findings, which is that the effects were only significantly powerful in the PiZ liver and not WT liver. Evidence of autophagic activation associated with norUDCA was abundant in PiZ liver, but in many ways lacking when WT mouse liver was examined, including change in p62. These data could be interpreted to question the hypothesis that norUDCA activates autophagy, but might more likely suggest that the autophagic activation of norUDCA is specific to a cellular environment in which there is already another active degradation signal. In the A1AT literature there are other examples of altered intracellular stress pathways and altered responses to agents which act in these pathways, in cells with a burden of mutant Z protein compared to those without (14, 17, 19). Some studies suggest a “cocked hammer” mechanism in which a pathway in a cell accumulating mutant Z protein is more easily activated than in an unburdened cell, but signals are not being transduced, yet. It is possible that this is the case here. NorUDCA may be a weak activator of autophagy, unless the autophagic pathway is already upregulated to deal with a burden of mutant protein, and then the response to norUDCA is more pronounced. This feature might make norUDCA even more attractive as a drug treatment. We are pursuing further studies in this, and in other model systems to determine the exact mechanism by which norUDCA activates autophagy and how this is different in cells burdened with mutant Z protein. In vitro studies will be needed to fully elucidate the molecular pathways involved in this new observation. For example, is the branch of autophagy under study here involving mTOR activation, which is the mechanism of rapamycin’s action on autophagy, or is one of the other activation pathways involved. Some of these data have been shown in
Further studies will continue to compare UDCA to norUDCA. The utility of UDCA in clinical medicine is hotly debated, and limited data is available. It is often used however, in a variety of liver diseases, including cases in which there is no disease-specific data, such as A1AT. Are the therapeutic effects which have been demonstrated in some biliary diseases based on autophagy, or is this a mechanism uniquely beneficial in the setting of A1AT deficiency? A future step could be a trial in humans of UDCA and norUDCA to examine effects on the autophagic pathway, on A1AT mutant Z protein burden in the liver and on liver injury. Such a trial would require liver biopsy to directly assay liver mutant Z protein burden and autophagic activity, since no serum marker of these readouts exists. Such a human study might face difficulties in conception, IRB approval, funding and recruitment, as is commonly the case in rare liver diseases. Rare diseases of many kinds are often deprived of therapeutic development beyond animal studies for just these reasons. We will follow and learn from the currently ongoing studies of norUDCA in primary sclerosing cholangitis (PSC) and the soon to be commenced studies in non-alcoholic fatty liver disease (NAFLD) patients with the aim of designing a practical intervention for A1AT.

Another interesting aspect of these data is the demonstrated effect on secretion of A1AT associated with norUDCA. The intracellular trafficking of mutant Z protein is complex. There are various pools in the cell of newly synthesized Z protein monomers, small
soluble oligomers, and large insoluble polymers both within and outside of globules. It is unclear if all of these pools are in the ER spaces, or occupy other intracellular domains. Other studies of autophagy enhancers have not shown reduced secretion, which was thought to be explained by the hypothesis that polymerization and the degradation of polymer was a trafficking step very much distal to the step of biogenesis which regulates secretion. These data suggest, however, that increased autophagic degradation might not only capture mutant Z protein polymers, but perhaps also newly synthesized monomers which then get degraded rather than secreted. Another possibility is that norUDCA has other, as yet unidentified, effects on trafficking or protein degradation activities which results in reduced cellular export. These are important questions and further study may shed light not only on this specific disease, but on other basic aspects of liver cell function. Further analysis of the effects of exogenous bile acids is well justified.

Grants: This work was supported by funding from the Alpha-1 Foundation, the Saint Louis University Liver Center, and the Cardinal Glennon Foundation.

Disclosures: Authors PF and MT retain intellectual property use claims on norUDCA.

Footnotes: none


Figure 1. Histopathologic examination of PiZ mouse liver; control versus norUDCA drug treated. Panel 1a shows representative photomicrographs at high and low magnification of sections of PiZ control mouse liver (left) and norUDCA drug treated (right) stained with H&E. Panel 1b shows representative photomicrographs of sections of PiZ control mouse liver (left) and norUDCA drug treated (right) at high and low magnification, stained with PAS with digestion. Panel 1c shows the mean area of the section occupied by A1AT mutant Z protein globules by colorimetric scanning quantification (pixels of globule color per a standard field on the vertical axis) from 10 individual mice (5 fields each), control versus norUDCA drug treated (p<0.001). Panel 1d shows the mean area occupied by each individual A1AT mutant Z protein globule (number of pixels on the vertical axis) by colorimetric scanning quantification from 10 individual mice, control versus norUDCA drug treated (p<0.001, bars +/- S. D.).

Figure 2. A1AT mutant Z protein in PiZ mouse; control versus norUDCA drug treated. Panel 2a shows quantitative immunoblot for A1AT of liver from representative control PiZ mice versus norUDCA drug treated PiZ mice after separation of the A1AT mutant Z protein monomer and polymer pools. The polymers are denatured back to monomers to run at the monomeric molecule weight. Arrows indicate the 52kDa intracellular form of A1AT. Panel 2b shows quantification in arbitrary densitometry units of the mean monomer and polymer content of the PiZ control versus norUDCA drug treated livers. Monomer difference not significant, but p<0.01 for reduction of polymer in drug treated mice, bars +/- S. D. Panel 2c shows mean A1AT serum levels of control PiZ mice versus norUDCA drug treated mice in ug/ml (p=0.006). Panel 2d shows quantification of A1AT expression by RT PCR of mRNA that is not different from control PiZ compared to norUDCA drug treated liver (p=0.28).

Figure 3. Gross dissection of gallbladders from PiZ mice. Control versus norUDCA drug treated. Brackets show the extent of the gallbladder.

Figure 4. Inflammatory infiltrate in PiZ liver and the effect of norUDCA. Panel 4a: H&E of WT liver (A). H&E of PiZ liver with foci of leukocytes (arrows) clustered in areas of hepatocytes containing globules of A1AT mutant Z polymerized protein (B). Foci of cells in PiZ liver (C, D, E, F). Panel 4b: BrdU label of WT mouse liver (A, no signal), PiZ liver (B and C) showing proliferating leukocytes, many in foci. Panel 4c: F4/80 staining in WT mouse liver (A) and PiZ liver (B, C, D). Panel 4d: intense F4/80 staining of cells in inflammatory foci in PiZ liver (A and B). Panel 4e: Staining for
neutrophils in PiZ liver. Ly6G for neutrophils in frozen PiZ liver (A). Foci stained red by naphthol AS-D chloroacetate esterase (neutrophil NCE) activity (B). Comparison of foci in serial sections (C and D) by Ly6G (left) to NCE (right). Panel 4f: B cell staining using B220 antibody in two fields of PiZ liver (column A), compared to a negative (column B) and a positive control in WT mouse with hepatitis (column C). Panel 4g1: Examples of inflammatory foci in PiZ liver staining DAPI nuclei blue, neutrophils in red, and macrophages in green (A, B, C, D); and Panel 4g2: at higher magnification (E, F, G, H). Panel 4h: Quantification of proliferating inflammatory cells in PiZ control vehicle treated versus norUDCA liver (A, p<0.02). Number of inflammatory foci in PiZ control vehicle treated versus norUDCA liver (B, p<0.008). Panel 4i: Area of inflammatory foci in PiZ control vehicle treated versus norUDCA liver (p<0.006).

Figure 5. Markers of liver injury in PiZ mice; control versus norUDCA drug treated. Panel a shows mean ALT levels (IU/ml), p<0.05 (WT mouse normal ALT is <65 IU/ml in reference lab). Panel 5b and 5c are blot and quantification of caspase 12 cleavage (arrow 37kDa cleaved fragment) with loading control (p<0.01). Panel 5d and 5e are blot and quantification with loading control of caspase 3 cleavage (arrow 19kDa cleaved fragment) (p=0.03). Panel 5f is mean percent BrdU labeled nuclei in PiZ mouse liver, control versus norUDCA drug treated. Bars +/- S.D. p=0.02. Panel 5g shows percent Ki67 positive nuclei after counting 300 cells in each of three randomly selected PiZ control and norUDCA drug treated mice. Difference of means significant p=0.04. Panel 5h shows representative photomicrographs of control and norUDCA treated liver by Sirius Red stain. Panels 5i and 5j show quantitative RT PCR for expression of hepatic fibrosis genes, alpha-SMA and collagen 1A1, respectively, with arbitrary expression units on the vertical axis, in control and norUDCA treated PiZ liver with non-significant p values as shown.

Figure 6. Autophagic activation in WT and PiZ liver. Panel 6a shows representative transmission electron photomicrographs of a standard field used for counting of control PiZ liver compared to norUDCA treated with some of the double membrane bound autophagic vacuoles identified by arrows and expanded panels. Panel 6b shows quantification of percent cytoplasm occupied by autophagic vacuoles by transmission electron photomicrographs of control PiZ mice compared to norUDCA drug treated (p<0.02). Panel 6c shows LC3 and p62 immunoblot with loading control for WT and PiZ mouse liver as shown, in the presence of norUDCA drug. Panel 6d shows mRNA fold changes for the genes discussed in baseline WT mouse liver compared to PiZ (# for p<0.05). Panel 6e shows mRNA fold changes for WT liver without and with norUDCA drug (# for p<0.05, but none found significant). Panel 6f shows mRNA fold changes for PiZ liver without and with norUDCA drug (n=3) (# for p<0.05). Panel 6g shows transmission electron photomicrographs of liver from PiZ control mice (top row) and
PiZ+norUDCA (bottom row). Globules (G) of dilated rER engorged with polymerized mutant Z protein are shown and some are associated with multi-membrane autophagic vacuoles in association with norUDCA treatment (arrows) (bar 1um). Panel 6h. HTO/Z cells expressing A1AT Z were incubated for 24h with concentrations of norUDCA as shown, including serum starved (SS) as a positive control stimulus known to activate autophagy. Cell lysates were blotted for LC3.
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2a  A1AT Quantitative Immunoblot

Monomer

Polymer

Control  Drug

2b  Alpha-1 Monomer-Polymer Quantification

Relative Density Units (RDU)

p=<0.01

Control  Drug

2c  A1AT Post Serum Level

AAT Serum Concentration (μg/ml)

p=0.006

Control  Drug

2d  AAT Relative Gene Expression

p=0.28

Control  Drug
Gallbladders from PiZ Mice

Control

Drug
ALT  

5b Caspase 12 Cleavage  

5c Cleaved Caspase 12  

5d Cleaved Caspase 3  

5e % BrdU Positive of All Hepatocytes, Control v. NorUDCA Treated  

5f  

5g % Ki-67 Positive Hepatic Nuclei  

5h Sirius Red  

5i α-SMA Avg Ct  

5j Col1A1 Avg Ct  

Control  

norUDCA