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An Expandable Mechanopharmaceutical Device (2): Drug Induced Granulomas Maximize the Cargo Sequestering Capacity of Macrophages in the Liver

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Abstract

Purpose: Drug-induced liver injuries (DILI) comprise a significant proportion of adverse drug reactions leading to hospitalizations and death. One frequent DILI is granulomatous inflammation from exposure to harmful metabolites that activate inflammatory pathways of immune cells of the liver, which may act as a barrier to isolate the irritating stimulus and limit tissue damage.

Methods: Paralleling the accumulation of CFZ precipitates in the liver, granulomatous inflammation was studied to gain insight into its effect on liver structure and function. A structural analog that does not precipitate within macrophages was also studied using micro-analytical approaches. Depleting macrophages was used to inhibit granuloma formation and assess its effect on drug bioaccumulation and toxicity.

Results: Granuloma-associated macrophages showed a distinct phenotype, differentiating them from non-granuloma macrophages. Granulomas were induced by insoluble CFZ cargo, but not by the more soluble analog, pointing to precipitation being a factor driving granulomatous inflammation. Granuloma-associated macrophages showed increased activation of lysosomal master-regulator transcription factor EB (TFEB). Inhibiting granuloma formation increased hepatic necrosis and systemic toxicity in CFZ-treated animals.

Conclusions: Granuloma-associated macrophages are a specialized cell population equipped to actively sequester and stabilize cytotoxic chemotherapeutic agents. Thus, drug-induced granulomas may function as drug sequestering “organoids” –an induced, specialized sub-compartment– to limit tissue damage.

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Keywords

Granuloma; clofazimine; biocrystal; macrophage; TFEB

Introduction

Macrophages mediate inflammatory responses and have been implicated in a multitude of disease states, ranging from obesity (1, 2) to some types of cancers (3, 4). One particular macrophage-mediated inflammatory response, granuloma formation, has been observed almost everywhere in the body, from the skin to the lungs. A granuloma is a specialized collection of macrophages that function to compartmentalize a foreign body away from the surrounding tissues (5–7). This reaction is commonly seen in bacterial and fungal infections; for example, in leprosy, granulomas form within the skin and near nerves, causing severe neuropathy (8). In tuberculosis, granulomas form “tuber”-like structures around the bacteria, to limit their spread (9). Other inflammatory diseases of unknown origins can also be accompanied by the formation of granulomas. For example, Crohn’s disease is an inflammatory condition in which chronic inflammation induces granuloma formation throughout the gut (10). Yet another kind of granuloma are the so called “foreign body granulomas”, which are formed by macrophages attempting to engulf exogenous materials, from splinters to surgical equipment, that are accidentally introduced in the body (11).

Hepatic granulomas are often observed in human liver biopsy samples, and can arise due to tuberculosis, brucellosis (12), and, in some cases, can be the result of a drug-induced liver injury (DILI), caused by certain classes of drugs, such as sulfonamides (13) and quinidine (14). Drug-induced liver granulomas are especially perplexing, in part, because the mechanisms by which drugs trigger granuloma formation are unknown. Kupffer cells, the macrophages of the liver, are among the first cells to interact with foreign substances ingested through the gut and have been implicated in protecting the host from harmful xenobiotics (15). Kupffer cells have also been shown to play a major role in regulating the activity of drug-metabolizing enzymes such as the cytochrome P450 complex (16, 17), which can lead to drug-induced liver injury through production of toxic metabolites and inflammatory biomarkers. Drugs like acetaminophen or bacterial products like lipopolysaccharide can activate Kupffer cells (18, 19), promoting a pro-inflammatory phenotype within the liver. While the activation of Kupffer cells can lead to an inflammatory response, resulting in tissue damage and hepatotoxicity (20), activated Kupffer cells and macrophages associated within the granuloma may play different roles in drug disposition in the liver.

Here, we hypothesized that the macrophages associated with drug-induced granulomas played a specific role in ameliorating drug-induced liver damage and toxicity. To test this hypothesis, we turned to the FDA-approved antibiotic, clofazimine (CFZ), a highly lipophilic, poorly soluble, weakly basic phenazine drug to model drug-induced hepatic granulomatous inflammation. CFZ has been used to successfully treat and cure leprosy since the 1950s (21) and is currently recommended as a treatment for multi-drug resistant tuberculosis (22). Due to its lipophilicity and unique pharmacokinetic properties (23), it

bioaccumulates extensively during long term oral administration. Aside from causing skin pigmentation, prolonged oral administration of CFZ (>3 weeks) (24, 25) leads to its precipitation within liver, lung and spleen (26, 27). These precipitates can be ingested by phagocytosis (28) and accumulate within macrophage lysosomes, as microscopic, insoluble aggregates of protonated CFZ hydrochloride (29). While the soluble form of CFZ is cytotoxic *in vitro* (28), the insoluble, protonated hydrochloride salt form is significantly less toxic to cells. Importantly, the drug is well tolerated by patients and is relatively nontoxic in animal models (30, 31), despite its propensity to extensively bioaccumulate and induce granulomatous inflammation. Thus, we decided to use this drug as a model to study the mechanisms behind drug-induced granulomatous inflammation, and the role that the liver macrophage plays in this inflammatory response.

MATERIALS AND METHODS

Drug Administration to Mice

Animal care was provided by the University of Michigan's Unit for Laboratory Animal Medicine (ULAM), and the experimental protocol was approved by the Committee on Use and Care of Animals (Protocol PRO00005542). Mice (4 week old, male C57Bl6) were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimatized for 1 week in a specific-pathogen-free animal facility. CFZ (C8895; Sigma, St. Louis, MO) was dissolved in sesame oil (Shirakiku, Japan) to achieve a concentration of 3 mg/ml, which was mixed with Powdered Lab Diet 5001 (PMI International, Inc., St. Louis, MO) to produce a 0.03% drug to powdered feed mix, which was orally administered *ad libitum* for up to eight weeks. A corresponding amount of sesame oil was mixed with chow for vehicle treatment (control). Mice were euthanized via carbon dioxide asphyxiation and exsanguination at the time of organ harvesting.

Kupffer Cell Isolation

Kupffer cells were isolated from the livers of untreated mice as follows. After euthanasia, the heart was perfused with 10 mL of pre-cooled Hank's buffered salt solution (HBSS) without magnesium or calcium, with 0.5 mM EGTA and 25 mM HEPES, pH adjusted to 7.4. The portal vein was then injected with 10 mL of 1 mg/mL collagenase D (Worthington Biochemical Corporation, Lakewood, NJ) in DMEM-low glucose (Life Technologies) with 15 mM HEPES (Life Technologies). The liver was then removed, placed in a sterile petri dish, and minced into small (2–4 mm) pieces using a sterile scalpel blade. Collagenase solution (15 mL) was added, and the tissue was incubated (40 min, 37°C), with occasional pipetting to dissociate tissue. The suspension was then filtered through a 100 µm cell strainer (Fisher Scientific, Waltham, MA) and centrifuged (200 x g, 5 min). The supernatant was discarded, and the cells were resuspended in DMEM-low glucose (15 mL) with 15 mM HEPES, and centrifuged (200 x g, 5 min). This was repeated for two additional washes. After the final wash, macrophages were suspended in DMEM:F/12 (1:1) (Life Technologies) with 10% FBS and penicillin/streptomycin, were counted, and plated onto collagen-coated glass coverslips, which were placed into 12-well tissue culture plates and allowed to attach overnight (37°C). The next day, cells were washed with DMEM:F/12 to remove non-

adherent cells and immunofluorescent staining of F4/80 was performed to identify macrophages (32) from other cell types.

Granuloma Isolation

Granulomas and granuloma macrophages were isolated from eight-week, CFZ-treated mice as follows. After euthanasia, the heart was perfused with 10 mL of pre-cooled Hank's buffered salt solution (HBSS) without magnesium or calcium, with 0.5 mM EGTA and 25 mM HEPES, pH adjusted to 7.4. The portal vein was then injected with 10 mL of 1 mg/mL collagenase D (Worthington Biochemical Corporation, Lakewood, NJ) in DMEM-low glucose (Life Technologies) with 15 mM HEPES (Life Technologies). The liver was then removed, placed in a sterile petri dish, and minced into small (2–4 mm) pieces using a sterile scalpel blade. Collagenase solution (15 mL) was added, and the tissue was incubated (40 min, 37°C), with occasional pipetting to dissociate tissue. Following tissue digestion, the granulomas remained intact and suspended within the Collagenase solution. The granulomas were then removed from suspension, imaged microscopically to confirm that they were granulomas (due to Cy5 fluorescence, drug content, cellularity, and fibrous superstructure). The remaining suspension was then filtered through a 100 µm cell strainer (Fisher Scientific, Waltham, MA) and centrifuged (200 x g, 5 min). The supernatant was discarded, and the remaining granuloma macrophages were resuspended in DMEM-low glucose (15 mL) with 15 mM HEPES, and centrifuged (200 x g, 5 min). This was repeated for two additional washes. After the final wash, macrophages were suspended in DMEM:F/12 (1:1) (Life Technologies) with 10% FBS and penicillin/streptomycin, were counted, and plated onto 12-well collagen-coated tissue culture plates and allowed to attach overnight (37°C). The next day, cells were washed with DMEM:F/12 and immunofluorescent staining of F4/80 was performed to identify macrophages (32) from other cell types.

Sample Preparation for Microscopy

Cryosectioning was carried out using a Leica 3050S Cryostat (Leica Biosystems Inc., Buffalo Grove, IL). Samples were sectioned to 5 µm. In preparation for cryosectioning, portions of the liver were removed, immediately submerged in OCT (Tissue-Tek catalog no. 4583; Sakura), and frozen (–80°C), or stored in 10% formalin and sent off for staining at the University of Michigan In-Vivo Animal Core for H&E, Picro-Sirius red, and Ki67 staining.

Immunohistochemistry

Following isolation and plating, cells were subjected to immunohistochemical staining and microscopic imaging to assess the presence and activation state of TFEB (Bethyl Laboratories, Montgomery, TX) LAMP1 (ThermoFisher Scientific, Waltham, MA), and LC3 (Sigma-Aldrich, St. Louis, MO) at dilutions of 1:5000, 1:500, and 1:200, respectively, following the manufacturers immunofluorescence histochemistry protocols. Immunohistochemical staining of the macrophage antigen F4/80 (Abcam, Cambridge, UK) was performed at a 1:500 dilution. The secondary antibody was Alexa-Fluor 488 (1:500 dilution) (Abcam, Cambridge, UK). Immunohistochemistry of TUNEL (R&D Systems, Minneapolis, MN) was performed using manufacturer's protocol for tissue cryosections. After staining, cells or tissues were imaged using a Nikon Eclipse Ti inverted microscope (Nikon Instruments, Melville, NY) and Nikon DS-U3 camera (Nikon Instruments) and

Photometrics CoolSnap MYO camera system (Photometrics, Tucson, AZ), under control of Nikon NIS-Elements AR Software (Nikon Instruments). Illumination for fluorescence imaging is generated using the X-Cite 120Q Widefield Fluorescence Microscope Excitation Light Source (Excelitas Technology, Waltham, MA).

TUNEL Assay and Quantification

Immunohistochemistry of TUNEL (R&D Systems, Minneapolis, MN) was performed using manufacturer's protocol for tissue cryosections to quantify cellular apoptosis. Following staining, liver cryosections were analyzed using a previously described fluorescence imaging set-up (33). Using the nuclear (DAPI) signal to generate a mask in ImageJ (34) the mean fluorescence intensity of each nucleus was measured, and any nucleus that showed a mean fluorescence intensity above background fluorescence as determined using a negative control was classified as TUNEL (+). Granulomatous regions were identified and analyzed separately due to increased cellular granularity and nuclear content.

Quantification of TFEB Activation

After staining, coverslips containing cell samples were mounted onto glass slides and imaged using the previously described imaging system (33). To determine the ratio of nuclear to cytoplasmic TFEB staining, images masks were generated using the DAPI staining for the nucleus and a brightness adjusted TFEB staining to capture the entirety of the cytoplasm. The cytoplasmic fluorescence intensity was corrected by removing the fluorescence of the nucleus, and the ratio of total fluorescence signal between the nucleus and cytoplasm was then determined. Cells were classified as either xenobiotic sequestering or not on the basis of mean Cy5 fluorescence intensity using K-Means clustering, with clusters set to two using IBM SPSS Statistics version 24.0 (IBM Software, Armonk, New York).

LC3 Quantification

After staining, coverslips containing cells were mounted onto glass slides and imaged using the previously described imaging system (33). Cell area was measured by generating a region of interest (ROI) comprising the entirety of the cell. LC (+) inclusions were counted manually within the cell, and reported as LC3 (+) inclusions/cell area to determine the impact of drug sequestration on autophagic flux within the cell. Because the sample preparation resulted in dissolution of drug inclusions from within the cell, cells were classified as xenobiotic sequestering based on the presence of a drug cavity that the insoluble drug aggregates previously occupied.

LAMP1 Quantification

After staining, coverslips containing cells were mounted onto glass slides and imaged using the previously described imaging system (33). Cellular masks were generated using a brightness adjusted LAMP1 fluorescence image, and classified as xenobiotic sequestering based on the presence of the cavity previously occupied by the insoluble drug precipitates that accumulated within the cell. Mean LAMP1 fluorescence intensity per cell was reported, to quantify lysosomal content within the cell.

Ki67 Quantification

Sections stained for Ki67 were imaged using the brightfield imaging set-up previously described (33). For the CFZ-treated livers, granulomatous and non-granulomatous areas were determined based on the extent of cellular granularity, and analyzed separately. The number of Ki67 nuclei was counted manually, and is reported as Ki67 (+) nuclei per tissue area following imaging across the tissue section at 10x magnification.

TLR Staining Quantification

Using the imaging method described by Lin et al (35), the intensity of DAB staining in granulomatous and non-granulomatous areas was compared using a previously described brightfield microscopy set-up (33). To quantify DAB stained images, a red filter (655 ± 30 nm), corresponding to the absorbance of the background tissue staining, and a blue filter (480 ± 30 nm), corresponding to the absorbance peak of DAB, were used. The density of DAB staining indicates TLR staining intensity. The DAB staining density was quantified using ImageJ, and is reported as % Tissue Area TLR2 (+).

Analysis of Drug Distribution

Three livers from mice treated for 0, 2, 4, and 8 weeks were sectioned, and one section per liver was stained for F4/80 (Alexa 488; FITC channel). Ten (10x) magnification images were taken per section to capture the entirety of the liver section. When CFZ precipitates out as a hydrochloride salt, it acquires a far-red fluorescence signal that is readily visible through the standard, Cy5 filter set of the epifluorescence microscope (33, 36). Accordingly, following immunohistochemical staining, the total F4/80 staining intensity was captured by acquiring an image through the FITC channel, while the protonated CFZ fluorescence signal (36) present in the same sample was captured by acquiring a separate image through the Cy5 channel. After the images were acquired, the FITC and Cy5 intensities associated with the objects of interest present in these images were measured and analyzed using ImageJ. The relative percentage of signal intensity from the granulomas over the ten images was measured, and the percentage of total measured F4/80 and protonated drug fluorescence contributed from the granuloma was determined. Results are reported as the mean $\pm$ S.D of each of the three sections analyzed.

Transmission Electron Microscopy

Organs were submerged in fixative and cut into small (<1 mm) pieces. The organs were preserved in a glass vial with fixative and stored at 4°C. After three rinses with Sorensen's buffer (0.1 M), tissues were stained with 1% osmium tetroxide in Sorensen's buffer and washed three times in Sorensen's buffer. Dehydration was carried out with a graded ethanol-water series (50, 70, and 90% and two changes of 100%) for 15 min each. After washing with three changes of propylene oxide, the tissues were treated with Epon resin (Electron Microscopy Sciences) and polymerized at 60°C for 24 hours. The blocks were then sectioned to 70 nm using an ultramicrotome and mounted on a copper EM grid (Electron Microscopy Sciences), which was then stained with uranyl acetate and lead citrate before imaging. Samples were imaged and analyzed at the National Center for Microscopy and Imaging Research (NCMIR) at the University of California, San Diego.

Synthesis and Testing of CFZ Derivatives

For studying transport mechanisms implicated in CFZ disposition, a focused library of R-iminophenazine analogs of CFZ was previously synthesized, (38). For *in vivo* experiments, animal care was provided by the University of Michigan's Unit for Laboratory Animal Medicine (ULAM), and the experimental protocol was approved by the Committee on Use and Care of Animals (Protocol PRO00005542). Mice (4 week old, male C57Bl6) were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimatized for 1 week in a specific-pathogen-free animal facility. CFZ and its most closely related, hydrophilic analog was dissolved in sesame oil (Shirakiku, Japan) to achieve a concentration of 3 mg/ml, which was mixed with Powdered Lab Diet 5001 (PMI International, Inc., St. Louis, MO) to produce a 0.03% drug to powdered feed mix, and orally administered ad libitum for 4 weeks. Following feeding, mice were euthanized via carbon dioxide asphyxiation and exsanguination.

Macrophage Depletion Experiments

To deplete tissue macrophages, mice (n=3–4 per treatment group) were treated with liposomes containing either 7 mg/mL of clodronate or phosphate-buffered saline (PBS) (FormuMax Scientific Inc., Sunnyvale, CA). Liposomes were injected intraperitoneally, as previously described (39). Mice were initially treated with 200 μ L of liposomes followed by 100 μ L injections twice per week to ensure continual macrophage depletion. Mice were fed CFZ (CFZ) or vehicle diet (untreated) continuously for a four week period. Following two weeks of feeding, liposome administration began for two weeks. The body temperature and weight of each mouse was measured daily, prior to injections. After completing four weeks of feeding and two weeks of liposome treatment, mice were euthanized and tissues were collected. For histological analysis, formalin fixed tissue sections from the clodronate depletion experiments were stained for H&E and analyzed by a pathologist blinded to the experimental conditions at the University of Michigan In-Vitro Animal Core and scored for inflammation and necrosis on a scale of 1–4.

Biochemical Analysis of CFZ in Tissues

Following treatment with CFZ, mice were euthanized via CO₂ asphyxiation, and organs removed and weighed. Tissue (20–30 mg) was homogenized in 500 μ L of radioimmunoprecipitation assay buffer (Sigma) with added protease inhibitors (Halt protease and phosphatase inhibitor cocktail and 0.5 M EDTA; Thermo Pierce, Rockford, IL), and CFZ was extracted from tissue homogenate (350 μ L) with three passes of 1 mL of xylenes. The drug was then extracted from the xylene with three 1 mL passes of 9M sulfuric acid, as previously described (25, 40). The recovery yield was determined by spiking samples with a known concentration of CFZ. The concentration of CFZ or phenazine derivative present in the tissue was then determined using the plate reader assay (Biotek Synergy 2, Winooksi, VT) at wavelength 450 nm, and background corrected at wavelength 750 nm, with the aid of a standard curve made with solutions of known concentration.

Biochemical Analysis of Plasma Drug Concentrations

Blood was collected and centrifuged ($7,000 \times g$, 5 min). The resulting serum was extracted with acetonitrile (90% extraction efficiency) for 10 min at 4°C with vortexing. After centrifugation ($14,000 \times g$, 4°C , 10 min), the supernatant was injected into a Waters Acquity UPLC H-Class (Waters, Milford, MA) equipped with an Acquity UPLC BEH C18 column ($1.7 \mu\text{m}$, 2.1 mm [inner diameter] by 100 mm; Waters, Milford, MA). Mobile phase A was 5 mM ammonium acetate, adjusted to pH 9.9 with ammonium hydroxide, and mobile phase B was acetonitrile. The flow rate was 0.35 ml/min, with a linear gradient from 50 to 100% phase B over 1.5 min, followed by holding at 100% for 1.5 min, a return to 50% phase B, and then re-equilibration for 2.5 min. Standards were prepared by spiking untreated plasma samples with known amounts of CFZ, ranging from 0 to 15 μM . Peak area was determined using Empower 3 Software (Waters, Milford, MA).

Cytokine Measurements

Following euthanasia, sections of liver or isolated granuloma were snap frozen and stored at -80°C . Tissue (20–30 mg) or whole granuloma was homogenized in 500 μL of radioimmunoprecipitation assay buffer (Sigma) with added protease inhibitors (Halt protease and phosphatase inhibitor cocktail and 0.5 M EDTA; Thermo Pierce, Rockford, IL). Protein concentration was determined using BCA assay (ThermoScientific), and samples were loaded using equivalent protein. The supernatants were assayed for interleukin-1 receptor antagonist (IL-1RA) by enzyme-linked immunosorbent assay (ELISA; DuoSet; R&D Systems, Minneapolis, MN) in triplicate wells according to the manufacturer's instructions. The cytokine concentrations were expressed nanograms per milligram of protein.

Statistical Analysis

All data are expressed as means $\pm$ the standard deviations (SD). For multiple comparisons, statistical analyses were performed with one-way analysis of variance (ANOVA) and Tukey's post hoc comparisons. All statistical analyses were performed using IBM SPSS Statistics version 24.0 (IBM Software, Armonk, New York). P values less than 0.05 were considered statistically significant.

RESULTS

Prolonged oral CFZ administration led to extensive extracellular matrix and cytoarchitectural remodeling

Following short-term treatment (<4 weeks), no granulomas or other signs of inflammation were observed in the livers (Figure 1a, 2 wk CFZ). However, as treatment progressed, drug aggregates began to form paralleling granuloma formation, with visible signs of local inflammation (Figure 1a, 8 wk CFZ). The granulomas were primarily localized to areas near the portal vein, and were mainly comprised of macrophages (Figure 1a, Inset), surrounded by collagen fibrosis. Biochemical analysis of total drug concentration in isolated granulomas revealed the extent of accumulation was comparable to that measured in the whole organ homogenates (30.3 ± 5.4 mg CFZ/ mg protein vs. 42.9 ± 23.0 mg CFZ/ mg protein, respectively; $n=3$ whole organ homogenates or isolated granulomas, $p=0.36$).

Previously, CFZ was used to probe the mechanisms driving weak base protonation and precipitation inside cells (24, 26, 27). CFZ undergoes a fluorescence shift as it forms an insoluble hydrochloride salt inside cells, emitting a distinct signal in the far-red, fluorescence wavelength (36) (37). In protonated form, it self-assembles into ordered aggregates (Crystal-Like Drug Inclusions, or “CLDIs”), which are also detectable based on their pronounced dichroism properties when illuminated with linearly polarized light (33). This ability to microscopically monitor the distinct protonation and ordered states of the drug prompted us to ask whether CFZ differentially accumulated in granuloma-associated macrophages versus resting Kupffer cells, and if the sequestration of the drug in granulomas had any relevance to the health status of the liver. Remarkably, the preferential accumulation of the protonated form of the drug in granulomas was readily apparent, evidenced by the red shifted fluorescence of the protonated form of the drug which can be observed through the standard Cy5 filter set of the epifluorescence microscope (640 nm excitation/670 nm emission) (36, 37)(Figure 1b).

To gain more insight into the tissue remodeling observed in the liver, liver sections were analyzed for Ki67, a marker of cellular replication, and for the presence of fragmented DNA associated with apoptosis via the TUNEL assay. Within or in the vicinity of these granulomas, the dramatically greater expression of these markers provided additional evidence of extensive tissue remodeling (Figure 1c). Quantitative analysis revealed a significant increase in Ki67 (+) nuclei per tissue area within the granuloma when compared to the rest of the liver and the livers in untreated animals, with no difference observed between untreated and non-granulomatous regions ($p < 0.01$, ANOVA, Tukey’s HSD), indicative of elevated cellular division and replication within these granulomatous regions. Additionally, within the granulomas, there was a significant increase in the percentage of apoptotic cells per tissue area when compared to both non-granulomatous tissue and untreated tissue, as detected by the TUNEL assay ($p < 0.01$, ANOVA, Tukey’s HSD). Taken together, there is an over ten-fold increase in cellular turnover in these drug-loaded granulomas when compared to both the untreated organs and non-granulomatous regions, indicative of a massive underlying cellular adaptation to accommodate this drug trapping. As expected from their role in immune surveillance, granulomas exhibited greater TLR9 staining as compared to both untreated livers and non-granulomatous regions (Figure 1e).

Proceeding to quantify the distribution of protonated drug in relation to granuloma-associated macrophages and non-aggregated Kupffer cells that reside within liver sinusoids, tissue cryosections from three different animals fed 0, 2, 4, and 8 weeks were stained for the macrophage marker F4/80, and the percentage of F4/80 signal and protonated drug (as measured via Cy5 fluorescence (36)) within non-granulomatous and granulomatous regions of the liver was determined from microscopic images. In both the untreated and 2 week CFZ fed animals, there was no indication of granuloma formation, and minimal signs of drug accumulation (Figure 2a,b), resulting in 100% of both the protonated drug and F4/80 signals being localized to non-granulomatous regions of the organ (Figure 2e). After 4 weeks of treatment, protonated drug began to co-localize within the F4/80 positive cells (Figure 2c). With increased drug loading, granulomas began to form and trap a greater fraction of the protonated drug (Figure 2c, yellow arrows) in relation to the fraction trapped by the non-aggregated Kupffer cells (Figure 2c, white arrow). At this stage, 10% of the total F4/80

signal within the liver was localized within the aggregated macrophages of the granulomas, accounting for nearly 10% of the total protonated drug (Cy5 signal) (Figure 2e). As drug loading further increased between four to eight weeks of treatment, there was a significant increase in protonated drug (Cy5 signal) ($p < 0.01$, ANOVA, Tukey's HSD) and macrophages ($p < 0.001$, ANOVA, Tukey's HSD) within the granuloma. Since the far-red fluorescence was specific to the protonated, hydrochloride form of CFZ (36)(41), granuloma-associated macrophages preferentially sequestered the protonated form of the drug, while the un-ionized form of the drug was distributed mostly in non-aggregated liver macrophages and the adjacent hepatocytes.

Granuloma macrophages exhibited signs of elevated lysosomal biogenesis and autophagic activation

Next, we determined whether the aggregated macrophages present within granulomas were phenotypically distinct from the non-aggregated Kupffer cells. Due to the fact that CFZ becomes protonated in lysosomes, its lysosomal accumulation may result in activation of transcription factor EB (TFEB), which controls lysosomal biogenesis and is the master regulator of the expression of the lysosomal proton pump, V-ATPase (42). Additionally, weakly basic drugs have been shown to activate this transcription factor, resulting in the increased formation of lysosomes and the cell undergoing autophagy (43–45). Kupffer cells were isolated from the livers of untreated animals, and granulomas and granuloma-associated macrophages were isolated from the livers of 8-week CFZ treated animals, plated, and stained for TFEB. Because of the link between TFEB, the endolysosomal system, and autophagy, macrophages were also stained for LAMP1 and LC3, markers for lysosomal membrane (46) and autophagic flux (47), respectively (Figure 3a). Significantly greater TFEB activation occurred within the Cy5-positive, granuloma-associated macrophage population, as compared to the Cy5-negative macrophage population, both obtained from the same drug treated animals ($n = 30$ cells per group) ($p < 0.05$, ANOVA, Tukey's HSD) (Figure 3b). However, the level of TFEB activation did not significantly differ from the general population of Kupffer cells obtained from untreated animals. Nevertheless, granuloma-associated (Cy5-positive) macrophages also were significantly larger in size as compared to the general population of (Cy5-negative) drug-treated macrophages, and as compared to resting state Kupffer cells from untreated mice ($p < 0.05$, ANOVA, Tukey's HSD) (Figure 3c). Similarly, granuloma-associated macrophages also showed increased lysosomal-associated membrane protein (LAMP1) ($n = 25–30$ cells per group) ($p < 0.05$, ANOVA, Tukey's HSD) (Figure 3d) as well as an increase in LC3 (+) inclusions, which is a marker of autophagy ($p < 0.05$, ANOVA, Tukey's HSD) ($n = 30$ cells per group) (Figure 3e), indicating that TFEB activation due to the lysosomal sequestration of CFZ resulted in the formation of new lysosomes and the cell entering an autophagic state.

Accumulation of insoluble, intracellular drug complexes (CLDIs) accompanied granuloma formation

To determine if induction of hepatic granulomatous inflammation was a general property of phenazine compounds, or specifically stimulated by the precipitation of the poorly soluble CFZ molecules within the endolysosomal compartment of granuloma-associated macrophages, we analyzed the *in vivo* disposition of Compound 568 (Figure 4a-d), a

previously characterized derivative of CFZ that is less prone to precipitate in lysosomal pH and chloride concentrations (40). Compound 568 has the same phenazine backbone as the CFZ molecule (Figure 4a), but it has an ethyl alcohol group instead of the isopropyl group of CFZ (Figure 4e). This compound has been previously studied side-by-side with CFZ, in terms of solubility (38), intracellular accumulation (38), hydrochloride salt formation (40), and skin pigmentation (40). Through the small alterations in chemical structure, the solubility of this CFZ analog is greatly increased relative to CFZ (38). Thus, following oral treatment of mice with Compound 568, the livers were analyzed (Figure 4b-d) and compared to those of their CFZ-treated counterparts (Figure 4f-h).

Within the livers of mice treated with Compound 568, no granuloma formation was detected (Figure 4c). In the corresponding, CFZ-treated mice, granulomas were found throughout the organ, replete with CLDIs (Figure 4f, g, h). Rather than accumulating as insoluble aggregates, the fluorescence from Compound 568 was mostly localized within the bile canaliculi of the liver (Figure 4d). This indicated that the hepatocytes are able to actively eliminate Compound 568 from the organism by hepatobiliary clearance, rather than concentrating the compound within the organ. Consistent with the reduced liver accumulation and increased hepatobiliary clearance, the measured liver concentrations of Compound 568 were 10-fold lower as compared to those of CFZ (0.27 ± 0.05 mg/g tissue vs. 2 mg/g tissue (40)). Mice treated with compound 568 had 10-fold lower serum concentrations (0.77 ± 0.12 μ M), compared those treated with CFZ (26) consistent with more rapid clearance.

Inhibition of granuloma formation led to increased drug toxicity.

To obtain additional insights into the structure and function of granulomas, livers of CFZ treated mice were subjected to electron microscopy (Figure 5). Membrane-bound, CLDIs were present in the cell cytoplasm of granuloma macrophages, and neutrophils were also present in granulomas. While these cells are among the so-called “professional phagocytes” within the body, no CLDIs were observed in neutrophils, supporting the notion that CLDI stabilization mostly is a macrophage-specific phenomenon. Collagen fibers were also visible in the extracellular space, wrapping around the cells in the granulomas. One striking feature of the granuloma-associated macrophages was that there were numerous, dark deformed inclusions throughout the cytoplasm that resembled damaged mitochondria similar in morphology to the mitochondria of cultured cells treated with CFZ *in vitro* (27, 48).

Nevertheless, hepatocytes surrounded the granulomas, and the differences between the two cell types were quite striking. In the region between a small granuloma and the surrounding non-granulomatous region of the liver (Figure 5), it was evident that hepatocytes (HC) possessed intact mitochondria with no signs of drug induced alterations (Figure 5a). The border region between the granuloma and surrounding hepatocytes highlighted the differences between the two cell types (Figure 5b), with granuloma-associated macrophages containing numerous damaged mitochondria (Figure 5b, M). The region in between the granuloma and hepatocyte was also comprised of numerous membrane-bound extracellular vesicles (Figure 5b, V), wication within the liver.

Proceeding to probe the role of granulomas in relation to drug accumulation and associated toxicity, we chemically depleted the liver macrophages of mice following two weeks of treatment with CFZ using injections of liposomal clodronate, a well-studied and accepted method of macrophage depletion. After two weeks of clodronate liposome injections, CFZ-treated mice weighed an average of nearly 5 grams less than their vehicle-diet treated mice ($p < 0.001$, ANOVA, Tukey's HSD) (Figure 6a). CFZ-treated, macrophage depleted mice had a significantly lower body temperature (Figure 6b) ($p < 0.05$, ANOVA, Tukey's HSD) (Table I). The clodronate liposome-injected, CFZ-treated mice also weighed significantly less (Figure 6c) and had a significantly reduced body temperature (Figure 6d) ($p < 0.001$, ANOVA, Tukey's HSD) compared to all other control treatment groups, with no significant difference in body mass or body temperature among the controls ($p > 0.05$, ANOVA, Tukey's HSD). Taken together, this points to a deleterious effect of the combination of macrophage reduction and CFZ exposure, resulting in systemic toxicity (49).

Consistent with the functional, physiological effects of macrophage depletion on CFZ-treated mice, gross examination (Figure 6e) revealed that the livers of the vehicle-diet, PBS liposome-injected mice showed little external signs of inflammation, and appeared normal. Similarly, the livers of PBS liposome-injected, CFZ-treated mice displayed the characteristic deep red pigmentation typical of CFZ accumulation (26) with no outward signs of tissue damage. Clodronate liposome-injected, CFZ-treated mice had extensive necrosis grossly and histologically evident in the livers (Figure 6e and f) while clodronate-injected, vehicle-diet fed mice had only small areas of necrosis that were evident grossly but not histologically. Nevertheless, the macrophage-deficient, drug treated mice showed elevated scores for both inflammation and necrosis when compared to all other treatment groups. When this is combined with the reduction in body weight and temperature (Figure 6a, 6b) associated with macrophage depletion and drug therapy, and the lack of reports of systemic toxicity within the mouse model (cite Jason paper), it points to a systemic toxic event compounded by the lack of granuloma-based drug sequestration.

Quantitative analysis of macrophage depletion was performed using F4/80 staining, to reveal a mean ($\pm$ S.D.) 42 (± 15)% reduction in macrophages in livers of CFZ-treated, clodronate-liposome injected mice, as compared to the control, CFZ-treated, PBS liposome-injected mice ($p < 0.05$, ANOVA, Tukey's HSD). This reduction in macrophages was accompanied by a mean ($\pm$ S.D.) reduction of 32 (± 6) % in the Cy5 fluorescence signal that is indicative of the accumulation of protonated drug, as compared to that of the PBS liposome injected animals of the CFZ-treated group ($p < 0.05$, ANOVA, Tukey's HSD). Thus, we inferred that less CFZ became trapped within macrophages as insoluble CLDIs, leading to increased exposure of the liver to the cytotoxic, soluble form of the drug.

Surprisingly, while clodronate liposome injection led to a 30% reduction in granuloma size relative to the PBS liposome-injected counterparts ($p < 0.05$, Student's unpaired two-tailed t-test) there seemed to be less indication of cell turnover within the granulomas of clodronate liposome-injected animals. Based on the TUNEL assay, the fraction of apoptotic cells in the granulomas of the liposomal clodronate treated animals was reduced ($p < 0.05$, ANOVA, Tukey's HSD; Figure 6g). However, the hepatocytes surrounding the granulomas displayed increased frequency of TUNEL-positive, corresponding to the regions of acute necrosis

observed in the H&E staining (Figure 6f). The reduction in TUNEL-positive apoptotic cells in the granulomas of CFZ-treated, clodronate liposome injected mice was paralleled by a reduction in the fraction of proliferating granuloma cells (detected through positive, Ki67 staining), as compared to control, PBS liposome-injected animals. While the granulomas of the clodronate liposome-injected, CFZ-treated mice had elevated Ki67 nuclei/tissue area compared to non-granulomatous regions ($p < 0.001$, ANOVA, Tukey's HSD; Figure 6h). Ki67 staining was greater in granulomas of the PBS liposome injected, CFZ-treated mice. Accordingly, highest cell turnover was indicative of the most active, drug sequestering, "functional" granulomas.

Lastly, we tested whether macrophage depletion influenced the levels of the anti-inflammatory cytokine IL-1RA (24), which was found to be upregulated following a prolonged (8 week) treatment with CFZ. Consistent with the previously reported study, the livers of the PBS liposome-injected, CFZ-treated mice showed significantly elevated levels of IL-1RA (Figure 7b; $p < 0.05$, ANOVA, Tukey's HSD). However, in the clodronate liposome-injected, CFZ-treated group, IL-1RA levels were similar to that of the untreated control groups ($p > 0.05$, ANOVA, Tukey's HSD). To further determine if the granuloma macrophages themselves were responsible for the increased IL-1RA production in CFZ-treated mice, granulomas were isolated from 8 week-CFZ fed livers and compared with whole-tissue homogenates of 8-week CFZ fed livers. The levels of IL-1RA production between the granulomas and whole-organ homogenates were both significantly higher as compared to the livers of control, untreated mice ($p < 0.05$, ANOVA, Tukey's HSD) (Figure 7c), although within treated mice, the levels of IL-1RA present in whole-organ tissue homogenates were not significantly different from that present in the granulomas ($p = 0.963$, ANOVA, Tukey's HSD).

DISCUSSION

Because the liver is the primary site of drug metabolism within the body, a multitude of drugs exert idiosyncratic, hepatotoxic side effects. We therefore postulated that drug-induced hepatic granulomatous inflammation might be a toxicity-reducing mechanism, resulting from the downstream signaling of immune cell-mediated inflammatory reactions that have the potential to interfere with liver structure and function. Indeed, many adverse drug reactions (ADRs) are associated with hepatotoxicity (50), caused by the degradation or enzymatic activation of drug molecules into reactive metabolites. In a well-known example, acetaminophen overdose depletes the substrates necessary to form non-toxic metabolites, leading to a secondary metabolism pathway which produces a hepatotoxic metabolite, damaging the liver as a result (51).

Naturally, bacteria or other pathogens trigger the formation of granulomatous inflammation, and are typically thought to serve as a means through which the immune system can encase an infectious microorganism, away from the rest of the body (9). This prompted us to begin probing the role of insoluble drug complexes in drug-induced granuloma formation, and to explore the relationship between drug-induced granulomas and drug toxicity. Using CFZ as a model drug for drug-induced granulomatous inflammation, granuloma macrophages specifically sequestered CFZ in a protonated, insoluble form that was membrane

impermeant and was therefore more readily compartmentalized and isolated from the rest of the liver, as compared to the un-ionized, free base form of the drug (28). Additional studies with a chemically similar derivative of CFZ that is less prone to form ordered crystalline aggregates within the lysosomal pH and chloride microenvironment supported the active role of granulomas in the sequestration of a poorly soluble, weakly basic small molecule drug, through an active precipitation and cell-promoted crystallization mechanism. Consistent with this notion, chemical depletion of macrophages confirmed the ability of granuloma-associated macrophages to preferentially protonate CFZ and sequester it away from hepatocytes, which may explain how granulomas can function to reduce CFZ toxicity. In the case of parasitic infection, the formation of granulomas have been shown to help prevent the spread of hepatotoxic peptides (52) produced by the parasitic eggs, and in the absence of these granulomas, there is elevated toxicity towards hepatocytes (53, 54). In the case of *Schistosoma*, the granulomas form both a physical barrier between the egg and the liver, and act to trap the toxins produced by the parasite within the granuloma, preventing neighboring tissues from becoming damaged. This hepatoprotective, barrier function of granulomas observed in parasitic and microbial infections is analogous to what we have observed with CFZ.

Based on our observations with CFZ, we hypothesize that granulomas act as integrated, dynamic “super-structures” or “organoids” comprised of specialized macrophages that effectively respond to xenobiotics that form insoluble complexes within the liver. Granuloma-associated macrophages were distinctively different from the non-aggregated, resting state Kupffer cells of the liver, based on their expression of various phenotypic markers associated with an expanded endolysosomal compartment, as well as their high rates of apoptosis and expression of Ki67, and activation of lysosomal biogenesis pathways. Xenobiotic sequestering granuloma-associated macrophages are embedded in a collagenous matrix, effectively isolating the drug from the neighboring hepatocytes. Conversely, non-aggregated, resting state Kupffer cells are primarily surrounded by hepatocytes or endothelial cells, and are localized to the sinusoidal regions of the organ. Interestingly, there are over sixty currently marketed drugs that are associated with drug-induced granulomatous inflammation, such as methyldopa (55) and phenylbutazone (56). The classes of drugs that are associated with this phenomenon are broad, ranging from antibiotics to non-steroidal anti-inflammatory agents, pointing to a variety of mechanisms that may result in the induction of inflammation. These causes may be linked to the parent-compound, or a metabolite, covalently binding to proteins within the liver, inducing precipitation or downstream inflammatory responses, or poor solubility of the drug itself within an acidic sub-cellular compartment, which is the case for CFZ and may be true for other weakly basic drugs, such as quinidine. The experimental approaches presented in this study can also be applied to these other drugs, to establish the extent to which granulomas may be ubiquitously involved in drug sequestration and detoxification, and the pathways that lead to instances of granulomatous inflammation. Of noteworthy significance, in the case of CFZ, granuloma formation and inflammation was specifically associated with the formation of insoluble precipitates comprised of CFZ hydrochloride. By slightly altering the chemical structure of CFZ, the ability of the compound to accumulate in the form of crystalline, hydrochloride salt aggregates in a lysosomal microenvironment was ablated, inhibiting

granuloma formation and leading to reduced hepatic accumulation and increased biliary excretion (Figure 4d). Because the hydroxyethyl group is only slightly electron withdrawing, the pKa will only be slightly lower than CFZ. Therefore, the protonated compound should still be protonated and accumulate in the lysosomes through ion trapping. The greatest impact of this modification will be on the intrinsic solubility of the free base and on the crystal lattice energy of the HCl salt. Nevertheless, this then resulted in an enhanced clearance and a reduction in net accumulation within the liver, as well as a reduced overall exposure of the compound to the organism, as detected through its reduced plasma concentration.

At a subcellular level, the changes that occur are just as striking as what is observed macroscopically within the liver. Within hepatic granulomas, there is significantly elevated cellular replication and death, detected through increased Ki67 (+) nuclei and TUNEL (+) nuclei, respectively. The granulomas are the site of massive cellular turnover, with macrophages constantly being recycled to help keep the insoluble drug trapped and sequestered away from the rest of the organ. In terms of the transcriptional regulatory mechanisms that are likely involved in drug-induced granuloma formation in the case of CFZ and other weakly basic therapeutics, TFEB activation emerged as a likely candidate (Figure 3b). TFEB is a major regulator of lysosomal biogenesis, and is one of the main transcription factors that is activated by weakly basic drugs that accumulate in lysosomes of cells *in vitro*. Based on our *in vivo* experiments, TFEB activation mostly was observed in granuloma macrophages, with these cells having accumulated large, crystalline aggregates of CFZ hydrochloride. Therefore, it can explain the expanded endolysosomal compartment of these macrophages, as compared to the drug-exposed non-aggregated macrophages that reside in the liver sinusoids of same, drug-exposed animals, as well as the inactivated Kupffer cells of control, untreated animals. TFEB activation can also explain the increased LAMP1 and LC-3 staining (Figure 3e), as TFEB is also a regulator of lysosomal and autophagic flux. By activating TFEB, lysosomal protein production increases greatly, allowing for the increased trapping of drug within the cell, while the increased autophagic flux allows for increased recycling and breakdown of membranes, necessary for both the trapping of the drug and formation of new cells within this environment of increased cellular turnover. Indeed, activation of TFEB can explain increased endolysosomal membrane biogenesis, as well as increases expression of the lysosomal acidification mechanism, namely vacuolar-type proton ATP-ase overexpression (57, 58) in response to weakly basic drug bioaccumulation, promoting a sort of positive feedback loop where, as more drug accumulates within the cells of the granuloma, the cells become better adapted to handle this increased drug load.

Of noteworthy significance, elevated TFEB activation within the xenobiotic sequestering granuloma-associated macrophages, but not within the non-aggregated liver macrophage also implicates TFEB as a key component of a putative, hepato-protective, response pathway. Up until this study, TFEB activation and its role in cell survival has been mostly studied as a drug resistance mechanism in cancer cells, which is detrimental to the survival of the organism and therefore cannot represent the natural, adaptive function of this transcription factor (59, 60). When granuloma formation is inhibited through chemical depletion of hepatic macrophages, the cytotoxic soluble form of CFZ is able to freely diffuse

throughout the liver. However, the reduction in the population of macrophages and the subsequent reduction in cells capable of TFEB activation limits the ability of the drug to become concentrated as the significantly less cytotoxic and membrane-impermeant hydrochloride form of the drug within granulomas, resulting in a reduction in cellular turnover and systemic toxicity in the mice. Unlike previous studies done on cancer cells (61) (45), our experiments are the first indication that TFEB activation may be a natural, protective mechanism, allowing the host organism to limit exposure of a cytotoxin through the mechanism of granuloma formation, thus preventing drug-induced liver injury, and ultimately, promoting the survival of the host.

CONCLUSION

To accommodate the massive bioaccumulation and precipitation of CFZ, the liver undergoes extensive remodeling through the development of large granulomas comprised of aggregated macrophages with distinctly different properties, as compared to neighboring non-aggregated macrophages that reside amongst hepatocytes and endothelial cells that form the liver sinusoids. Following eight weeks of treatment, the majority of the protonated drug was found sequestered within these massive “organoids”, with significantly less found in non-aggregated cells. These drug-induced granulomas were structurally and functionally distinct multicellular compartments that effectively functioned as a drug sequestering “organoid” within an organ – a collection of specialized immune cells that actively functioned to limit to the exposure of the surrounding hepatocytes to the drug. Granuloma-associated macrophages increased local cell turnover but reduced functional damage to the surrounding hepatocytes. Presumably, the granuloma-associated macrophages restricted the cytotoxic activity of the drug by sequestering it intracellularly, shielding the surrounding hepatocytes from exposure to the drug. This mechanism may act in a similar fashion to granulomas that form to trap the eggs of *Schistoma*, helping to limit the spread of toxins that damage hepatocytes. As a potential master regulator of the drug-induced, xenobiotic sequestering macrophages of granulomas, TFEB activation can explain the expanded lysosomal compartment, membrane turnover and acidification mechanisms that are involved in stabilizing the insoluble, intracellular aggregates formed by the protonated, hydrochloride salt form of CFZ. Confirming the protective role of hepatic granulomas, inhibition of granuloma formation reduced hepatic IL-1RA production, and led to greater, systemic drug toxicity. Thus, drug-induced granulomatous inflammation may be a hepato-protective adaptation put in place to trap a harmful xenobiotic, causing these granulomas to function in a similar manner to granulomas induced by bacteria or other pathogens.

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Abbreviations:

TFEB: Transcription Factor EB

CFZ:	Clofazimine
CLDI:	Crystal-Like Drug Inclusion
ADR:	Adverse Drug Reaction
DILI:	Drug-induced liver injury
TLR9:	Toll-Like Receptor 9
LC3:	Microtubule-associated protein 1A/1B-light chain 3
LAMP1:	Lysosomal Associated Membrane Protein 1
IL-1RA:	Interleukin-1 Receptor Antagonist
TUNEL:	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling

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Figure 1: CFZ treatment remodels the liver through granuloma formation.

A) Increased CFZ treatment results in formation of large granulomas throughout the organ comprised of immune cells. B) CLDIs accumulate extensively within the granuloma, and retain their intrinsic Cy5 fluorescence. C) Granulomatous areas of the liver exhibit higher Ki67 activation when compared to both non-granulomatous areas and untreated livers. D) Granulomatous areas of CFZ treated livers show elevated apoptotic nuclei detected via TUNEL assay when compared to both non-granulomatous areas and untreated livers. E) CLDI accumulation in granulomas results in elevated TLR2 expression within the granuloma, but not the surrounding tissue. Scale bar is 50 μm.

Figure 2: Increased drug loading results in macrophages becoming concentrated within granulomas, and increased drug accumulation mainly within the granuloma.

A-D) Untreated, 2, 4 and 8 week immunofluorescence images of liver cryosections stained for the macrophage marker F4/80 (green) and CLDI fluorescence (red). E) The total percentage of F4/80 or Cy5 fluorescence signal coming from the granuloma compared to the non-granulomatous tissue area was determined, revealing a significant increase from four to eight weeks of treatment. Yellow arrows point to granuloma macrophages loaded with CLDIs, while white arrows point to Kupffer cells with CLDIs. Scale bar is 100 μ m. (n=3 animals per time point, 10 images per liver cryosections) (*= p <0.01, ANOVA, Tukey's HSD, #= p <0.001, ANOVA, Tukey's HSD).

Figure 3: Granuloma-associated macrophages are phenotypically different from both untreated and drug treated macrophages.

A) Images showing untreated Kupffer cells, drug-treated macrophages and granuloma-associated macrophages isolated from 8 week CFZ-treated liver stained for TFEB, LAMP1 and LC3. B) Granuloma-associated macrophages show significantly elevated TFEB translocation to nucleus compared to untreated and drug-treated macrophages. C) Granuloma-associated macrophages are significantly larger than their untreated and drug-treated macrophage counterparts. D) Granuloma-associated macrophages show significantly elevated expression of LAMP1 within the cell, indicating increased lysosomal content within the cell. E) Granuloma-associated macrophages show increased punctate LC3 staining within the cytoplasm of the cell compared to untreated and drug-treated macrophages, indicating increased autophagic flux within the cell, which may be necessary to form new membranes to trap the CLDI. Scale bar is 15 μm . (n=30-35 cells per group, *=p<0.05, ANOVA, Tukey's HSD).

Figure 4: The accumulation of insoluble aggregates and granuloma formation is dependent on the isopropyl group in the CFZ molecule.

A) The derivative 568 replaces the isopropyl group with an ethyl alcohol, limiting its ability to form insoluble aggregates within the liver and does not induce granulomas as is seen in CFZ. B) H&E staining of a liver from a mouse treated with 568 shows no signs of granuloma formation. Scale bar is 200 μ m. C) The fluorescence of derivative 568 can be detected in the bile canaliculi of the liver, showing that the liver may be actively removing the chemical and eliminating it from the body. Scale bar is 200 μ m. D) Zoomed in region of the bile canaliculi showing the fluorescence of derivative 568. Scale bar is 50 μ m. E) Chemical structure of CFZ. F) H&E staining of CFZ treated liver shows extensive granuloma formation. G) CFZ treatment leads to insoluble aggregate and hydrochloride salt accumulation. H) Zoomed in region of CLDIs within the liver. Scale bar is 50 μ m. Note: Brightness for derivative 568 fluorescence images have been elevated to show biliary localization of drug.

Figure 5: TEM of 8-week CFZ treated liver showing granuloma surrounded by hepatocytes.

A) A healthy hepatocyte located on the periphery of the granuloma shows normal mitochondria unaffected by the drug. B) Border region between the granuloma and healthy liver tissue. The granuloma macrophage is loaded with CLDIs, and as a result, shows damaged mitochondria. In between the granuloma macrophage is region of cellular vesicles, which may limit exposure of toxic drug to the tissue. The neighboring hepatocyte shows healthy mitochondria. (M: Mitochondria, HN: Hepatocyte nucleus, HC: Hepatocyte, V: Vesicles, CLDI: Crystal-Like Drug Inclusion)

Figure 6: Chemical depletion of macrophages leads to signs of toxicity in animals fed CFZ. A, B) Daily mouse body mass and temperature taken during final week of liposome injection. C, D) Box plot of mouse body weight and temperature taken on final day of experiment. (*= $p < 0.001$, ANOVA, Tukey's HSD). E) Macroscopic images of livers removed from mice treated with PBS or clodronate liposomes, fed either a control or supplemented diet. The clodronate CFZ fed livers showed signs of necrosis upon gross examination. F) H&E staining of liver sections from macrophage-depleted and healthy animals. The clodronate-CFZ treated livers showed signs of necrosis radiating outward from the hepatic portal vein, while the PBS-CFZ treated mice only showed minor inflammation localized to the granulomatous areas. Scale bar is 100 µm. G) TUNEL staining of liver cryosections from clodronate experiments. Apoptosis in the PBS-CFZ treated groups was localized primarily to the granulomatous regions, while the necrotic, clodronate-CFZ livers showed reduced apoptosis within the granuloma and elevated apoptosis in small, localized regions of the

liver, consistent with the previously observed necrosis. Scale bar is 25 μm . H) Immunohistochemical staining for cellular replication marker Ki67 within liver sections. Granulomatous regions of PBS-CFZ treated livers showed significantly elevated cellular replication compared to the granulomas of the clodronate-CFZ treated livers. Scale bar is 200 μm .

Figure 7: Formation of granulomas are necessary for the increased IL-1RA production within the liver of CFZ-treated mice.

A) Liver cryosections of PBS or clodronate treated mice fed a control diet or CFZ-supplemented diet stained for F4/80 (green) and showing CLDI fluorescence. Treatment with clodronate reduces hepatic macrophages, resulting in reduced granuloma size and CLDI accumulation within the granuloma. B) IL-1RA production is inhibited within clodronate-CFZ treated mice, while it is significantly elevated in the PBS-CFZ treatment group. C) The granulomas which form ultimately are responsible for the production of IL-1RA, producing similar levels of IL-1RA to whole-organ homogenates. Scale bar is 50 μ m. (n=3-4 liver homogenates or isolated granulomas per treatment group) (*= $p < 0.05$, ANOVA, Tukey's HSD)

Table I:

Mean body mass and temperature of moss groups during final week of liposome treatment

Treatment	Average Body Mass (g) (n=3-4 mice per group)	Average Body Temperature (°C) (n=3-4 mice per group)
PBS + Control Diet	26.3 ± 1.4	34.2 ± 0.5
PBS + CFZ Diet	25.7 ± 0.8	34.3 ± 0.3
Clodronate + Control Diet	27.4 ± 1.1	34.4 ± 0.6
Clodronate + CFZ Diet	21.2 ± 1.4 *	33.1 ± 0.9 *

*
=p<0.05, ANOVA, Tukey's HSD