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An Expandable Mechanopharmaceutical Device (1): Measuring the Cargo Capacity of Macrophages in a Living Organism

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Abstract

Purpose: Clofazimine (CFZ) is an FDA-approved, poorly soluble small molecule drug that precipitates as crystal-like drug inclusions (CLDIs) which accumulate in acidic cytoplasmic organelles of macrophages. In this study, we considered CLDIs as an expandable mechanopharmaceutical device, to study how macrophages respond to an increasingly massive load of endophagolysosomal cargo.

Methods: First, we experimentally tested how the accumulation of CFZ in CLDIs impacted different immune cell subpopulations of different organs. Second, to further investigate the mechanism of CLDI formation, we asked whether specific accumulation of CFZ hydrochloride crystals in lysosomes could be explained as a passive, thermodynamic equilibrium phenomenon. A cellular pharmacokinetic model was constructed, simulating CFZ accumulation driven by pH-dependent ion trapping of the protonated drug in the acidic lysosomes, followed by the precipitation of CFZ hydrochloride salt via a common ion effect caused by high chloride concentrations.

Results: While lower loads of CFZ were mostly accommodated in lung macrophages, increased CFZ loading was accompanied by organ-specific changes in macrophage numbers, size and intracellular membrane architecture, maximizing the cargo storage capabilities. With increasing loads, the total cargo mass and concentrations of CFZ in different organs diverged, while that of individual macrophages converged. The simulation results support the notion that the proton and chloride ion concentrations of macrophage lysosomes are sufficient to drive the massive, cell type-selective accumulation and growth of CFZ hydrochloride biocrystals.

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Conclusion: CLDIs effectively function as an expandable mechanopharmaceutical device, revealing the coordinated response of the macrophage population to an increasingly massive, whole-organism endophagolysosomal cargo load.

Keywords

Drug delivery; volume of distribution; lysosome; pharmacokinetics; clofazimine self-assembly

INTRODUCTION

In multicellular organisms, macrophages are well equipped to internalize extracellular solutes and particles through pinocytosis and phagocytosis, respectively. These processes allow them to execute a number of critical, immune surveillance functions, ranging from destruction of pathogens to removal and recycling of dead cells and aged tissue components (1–3). In addition to being highly phagocytic, the endolysosomal system of the macrophage is especially adapted for accommodating and degrading foreign material due to higher expression levels of lysosomal acidification mechanisms(4), particularly the vacuolar-type proton ATP-ase (V-ATPase)(5). However, there is no information about the extent of loading or the cargo storage capacity of macrophage populations *in vivo*, since the majority of measurements have been performed *in vitro* and in very short treatment periods by ‘feeding’ the cells with fluorescent tracer molecules(6) or inert beads of varying sizes(7)·(8) under artificial conditions.

Even though macrophages are found everywhere in the organism, their ability to influence the transport and disposition of small molecule drugs is mostly unknown. Due to their ubiquitous presence and high rates of endocytosis, pinocytosis, and phagocytosis, they are recognized to play a major role in the clearance of drug nano- or microparticles from the circulation(9, 10). Potentially, this action could be exploited for the delivery of small molecule drugs(11–13)·(14), particularly those that are poorly soluble because they are prone to form insoluble aggregates that may ultimately accumulate in macrophages(15, 16). Furthermore, cationic, amphiphilic molecules, particularly weakly basic molecules, can become trapped within lysosomes following protonation in the acidic lysosomal microenvironment(17–19). The sequestration of weakly basic, hydrophobic drug molecules within acidic subcellular (ion-trapping) compartments of macrophages is a well-documented phenomenon(17).

To study the *in vivo*, cargo storage capacity of macrophages in an intact, living organism, we have co-opted an FDA-approved and biocompatible antibiotic, clofazimine (CFZ)(15, 16). CFZ is a weakly basic anti-mycobacterial agent that is clinically used to treat leprosy and multi-drug resistant tuberculosis(20–22). It exhibits extensive bioaccumulation following oral administration, in both humans and animal models(23–25). Because CFZ is both highly lipophilic (LogP=7.66) (26, 27) and contains an ionizable amine group (apparent pK_a =6.08) (28), it is poised to accumulate in adipose tissue, intracellular membranes, and acidic organelles such as lysosomes. In both human and animal models it has been shown that, following prolonged oral dosing, CFZ forms insoluble Crystal-Like Drug Inclusions (CLDIs) within macrophage lysosomes(15).

Here, by exploiting CFZ's self-assembly into CLDIs, we decided to further probe the macrophage's response to an increasing load of drug cargo. Experimentally, CLDIs were microscopically monitored to determine the numbers of drug-sequestering macrophages in different organs. From these same organs, CLDIs were then biochemically isolated and the drug content chemically analyzed to determine the average amount of drug per CLDI-containing cell(15, 29). Additionally, we modeled the subcellular transport and precipitation properties of CFZ using a physiologically based pharmacokinetics approach to assess whether thermodynamics alone could explain the selective accumulation and stabilization of the drug in macrophage lysosomes. Based on the results, we propose that CLDIs function as an expandable mechanopharmaceutical device, which impacts the structural and functional properties of macrophages in different organs, and accounts for a drug loading-dependent increase in the volume of distribution (V_D) of the drug.

MATERIALS AND METHODS

Animal Studies and Treatment Protocols

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 weeks old, male C57Bl6) were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimatized for 1 week in a specific-pathogen-free animal facility. Clofazimine (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, and orally administered *ad libitum*. Mice were fed for 2, 3, 4, and 8 weeks, which yielded estimated whole body cargo loads of 3.5mg, 5.25mg, 7mg, and 14mg, respectively. A corresponding amount of sesame oil was mixed with chow for vehicle treatment (control). For washout experiments, mice were fed with drug free, vehicle-containing diet for eight weeks, after an eight week loading period with the CFZ-containing diet. At the end of experimentation, mice were euthanized via carbon dioxide asphyxiation followed by exsanguination.

Macrophage Isolation

Following euthanasia, four different macrophage populations were isolated to study CFZ accumulation. Alveolar(29, 30) and peritoneal macrophages(15), and bone marrow monocytes(31) were all isolated using previously described techniques. To isolate Kupffer cells, the portal vein was 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 tissue 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 μ m cell strainer (Fisher Scientific, Waltham, MA) and centrifuged (200 x g, 5 min). The supernatant was discarded, and the cells were resuspended in 15 mL DMEM-low glucose with 15 mM HEPES, and centrifuged (200 x g, 5 min). This was repeated for two additional washes. After the final wash, the cells were suspended in DMEM:F/12 (1:1) (Life Technologies)

with 10% FBS and penicillin/streptomycin and macrophages were counted. For all cell groups, an aliquot was plated onto 4 or 8 chamber coverglass (#1.5, Lab-Tek II, Nunc, Rochester, NY), and for Kupffer cells, the plates were coated with Collagen 1 (Corning, Corning, New York) for imaging. The cells were allowed to attach overnight, washed with media, and imaged using a multi-parameter microscope imaging system (*vide infra*).

Multi-parameter Microscope Imaging and Analysis of Macrophage Sub-Populations

Multi-parameter polarization, brightfield, and fluorescence imaging and analyses of different macrophage subpopulations were performed using a custom-built imaging system, as previously described(29). Brightfield, fluorescence, and polarization images were acquired on adherent cells. Images were analyzed using ImageJ software(32, 33). Values for dichroism and optical density are reported as an average signal per cell, from 0–1. At least 150 cells of each type were analyzed at each time point. Following loading with 7 and 14 mg of cargo, macrophages were classified as xenobiotic sequestering based on whether or not they contained a detectable Cy5 signal from CFZ-HCl(15) using a K-means clustering analysis, with the clusters set to 2 based on of the $\text{Log}_{10}(\text{Intracellular Cy5 fluorescence})$.

Biochemical Analysis of Cellular Drug Cargo

The drug concentration in cells was calculated after measuring drug content in isolated macrophage populations and organ homogenates using established methods(15, 24, 34, 35). For isolated macrophage populations, cells were counted within each cell sample using a hemocytometer to determine the total recovered macrophage population. The cells were then centrifuged, and the media was removed. The cell pellet was suspended in 1 mL of DI water, and the drug was extracted and measured using a previously described spectrophotometric method(35–37). Drug accumulation is reported as fmol CFZ/xenobiotic sequestering cell.

Sample Preparation for Microscopy

In preparation for microscopy, portions of the organ were removed, immediately submerged in OCT (Tissue-Tek catalog no. 4583; Sakura), and frozen (-80°C). For transmission electron microscopy, organs were prepared using previously described methods(23). Immunohistochemistry of F4/80 (Abcam, 1:500 dilution) stained sections ($5\mu\text{m}$) was performed using Alexa-Fluor 488 (Abcam, 1:500 dilution).

Determination of Cargo Volume Occupancy

The volume occupied by biocrystalline drug molecules within a macrophage was estimated by using the reported crystal-packing density of 1.36 g/mL (35) for CFZ-HCl, which was then converted to a molar volume of $0.377\text{ }\mu\text{m}^3/\text{fmol}$. Cellular volume occupancy was then estimated using the measured drug content per cell and the calculated molar volume.

Determination of Vesicle Size and Shape

The size and shape of cargo loaded vesicles was microscopically measured within loaded macrophages obtained from CFZ-treated animals, using the Cy5 fluorescence channel to specifically monitor the vesicles containing CFZ-HCl. Using ImageJ(32, 33), the radius of each vesicle was determined. Vesicles which showed Cy5 fluorescence were counted as CFZ

sequestering. The vesicle volume was estimated assuming spherical shape, while CLDI volume was estimated assuming a cylindrical shape.

Analysis of Organ Macrophage Counts

To determine the macrophage population numbers in lung, liver, and spleen from animals treated with CFZ, cryosections were obtained (5 μ m). The change in the number of macrophages in a volume of tissue was determined by dividing the total F4/80 signal staining intensity between an 8 week CFZ-treated sample by the total F4/80 signal staining intensity of a vehicle-treated sample (the area and thickness of the sections analyzed were kept the same). The total macrophage population was then determined by multiplying literature reported (baseline) macrophage population values for each organ(38) by the relative expansion factor determined via increased intensity of the macrophage marker's fluorescence signal, via immunofluorescence. To determine the fraction of macrophages that contained CLDIs, the fraction of cells which were positive for Cy5 fluorescence and F4/80 staining were determined by visual inspection, using a mask of the F4/80 staining. Five images per organ per animal were analyzed for each measurement.

Measurement of Drug Accumulation based on Whole Organ Homogenates

For chemical analysis protocol development, organs were obtained from euthanized, untreated mice. Samples from the different organs (20–30 mg) were homogenized in radioimmunoprecipitation assay buffer (500 μ L; Sigma) with added protease inhibitors (Halt protease and phosphatase inhibitor cocktail and 0.5 M EDTA; Thermo Pierce, Rockford, IL). In order to determine the recovery yield, the organ homogenates were spiked with a known amount of drug. For analysis, homogenate (350 μ L) was removed, and drug was extracted as described under "Biochemical Analysis of Cellular Drug Concentrations."

Determining the Fraction of Drug Sequestered by Macrophages of Different Organs

The total drug content of lung, liver, and spleen tissues at eight weeks of treatment was determined as described above, using established protocols(34). Because CLDIs form and become stabilized exclusively within macrophages(23, 28, 35), we sought to determine the fraction of drug sequestered within macrophages of each organ. To accomplish this, tissues (n=3) were removed, weighed, and placed in a sterile petri dish, where they were manually minced and homogenized using a scalpel and syringe plunger. The resulting homogenate was filtered through a cell strainer (40 μ m) to remove larger cellular clusters and debris. The filtrate was then centrifuged (300 x g, 10 min) to pellet the CLDIs. The supernatant was removed, and the CLDI pellet was resuspended in 10% sucrose in DPBS (Life Technologies, Carlsbad, CA) without calcium chloride or magnesium chloride, pH=7.4. CLDIs were further purified using a 3-layer sucrose gradient (50%, 30%, and 10% sucrose in DPBS) centrifugation method (3200 x g, 60 min). The pelleted CLDIs were then dissolved in 9 M H₂SO₄, and the mass of drug was determined using a plate reader (Biotek Synergy 2, Winooksi, VT) at wavelength 450 nm, and background corrected at wavelength 750 nm, which was determined using a standard curve with solutions of known concentration.

Determination of the Cargo Volume Occupied per Macrophage

Using the total recovered mass of drug associated with CLDIs isolated from the liver, lung, and spleen, the volume occupied by drug within individual xenobiotic-sequestering macrophages was estimated based on the number of CLDI containing cells, using the total expanded macrophage population present in the measured tissue sample, multiplied by the fraction of cells which contained a CLDI (determined microscopically). Using literature reported values for cellular volume of the macrophage(39), the percentage of cellular volume occupied by drug was estimated by dividing the total recovered mass of drug associated within the isolated CLDIs, by the total (calculated) number of CLDI containing cells in the sample of tissue from which the CLDIs were isolated. The fraction of the phagocytic capacity occupied by cargo was calculated by dividing the measured cargo volume by the maximum phagocytic capacity of macrophages(8).

Biochemical Analysis of Drug Concentrations

The concentration of CFZ in plasma or organ homogenates was determined using a previously described method(24). In brief, blood was collected in microtainer serum separator tubes (catalog no. 3659656; Becton Dickinson, Franklin lakes, NJ) and allowed to clot at room temperature and centrifuged (5,000 x g, 5 min). Samples (20 μ l) were extracted with acetonitrile (60 μ l, 90% extraction efficiency) for 10 min at 4°C with vortexing. After centrifugation (5,000 x g, 4°C), the supernatant was injected into Agilent 1200 RRLC coupled to 6410 Triple Quad LC-MS equipped with a Waters Xbridge C18 column (2.5 μ m x, 2.1 mm x 100 mm). A standard curve was generated by extracting spiked drug samples using serum (or the organ homogenates) from a vehicle-only treated mouse mixed with CFZ stock solution from dimethyl sulfoxide, resulting in 10 different CFZ concentrations between 0 and 30 μ M. The peak area was quantified using MassHunter Quantitative Analysis software, vB.04.00. This standard curve was then used to establish the concentration of drugs in experimental samples from blood of drug-treated mice (or from organ homogenates of these mice).

Determination of the Volume of Distribution of the Drug in Different Organs (V_{OD})

Following treatment with 5.25 or 14 mg of CFZ, mice (n=3 per time point) were euthanized and the liver, spleen, small intestine, fat, kidney, and lung were removed, weighed, and the mass of CFZ within each tissue and the concentration within plasma were determined as previously described(24). The V_{OD} of the drug within macrophages, tissue, and the whole body at the different loading amounts was determined using the ratio between the total drug within tissues or individual cells and the measured plasma concentration.

Experimental Measurement of CFZ's Physicochemical Properties

Using Flynn and Kramer's methodology (40), the pH_{max} , apparent pK_a , intrinsic solubility, salt solubility, and solubility product constant (K_{sp}) of CFZ were experimentally determined as previously reported (28) Briefly, an excess of CFZ-HCl (25 mg) was placed in 15 mL of Milli-Q water, and 0, 40, 80, 120, or 200 μ L of 0.1M NaOH were added to the vials to achieve an initial equilibration pH measurement. Following a 24 hour equilibration period, 10 μ L of 0.1M NaOH was added daily over a five day period, leading to a pH range from 4.5

to 8.9. The samples were then stirred using a magnetic stirrer plate in a 25°C water bath to allow them to equilibrate for 24 hours. A 500 μ L sample was removed from each vial and filtered through a Spin-X centrifuge tube filter (0.45 μ M cellulose acetate, 2 mL polypropylene tubes, non-sterile, Costar®, Cat #8163) for 4 minutes at 10,000 RPM. The pH of the filtrate was measured (UltraBasic pH meter, Denver Instrument, Bohemia, NY) both before and after the completion of the assay, prior to measurement of solubilized CFZ. The concentration of CFZ was then determined using HPLC analysis (Waters Alliance, Separations Module 2695). Each measurement was performed in triplicate and the average used to construct the pH- dependent solubility profile. Through mathematical manipulation of the Henderson-Hasselbalch equation and inputting different combinations of the experimental, pH-dependent solubility measurements, the apparent pK_a , intrinsic free base solubility, pH_{max} , and K_{sp} were calculated, as previously described (28).

Solubility Measurements for CFZ and CFZ-HCl in Octanol

In order to determine the solubility values for CFZ and CFZ-HCl in 1-octanol (293245; Sigma-Aldrich), both forms of the drug were introduced in great excess to 1-octanol in glass scintillation vials. A small magnetic stir bar was added to each sample, and the samples were placed on the magnetic stirrer inside the incubator (37°C). After a 24-hour equilibration period, samples were removed from the incubator and immediately filtered using Spin-X centrifuge tube filters (0.45 μ m cellulose acetate, 2 mL polypropylene tubes, Costar®) for 4 min at 5,000 x g. The concentration of the soluble drug in 1-octanol was spectrophotometrically determined (285 nm, 37°C; Synergy-2 plate reader; Biotek Instruments, Winooski, VT). For each sample, solubility measurement was performed in triplicate, and the average was reported. The standard curve was generated using CFZ crystals that were dissolved in 1-octanol at known concentrations (1–200 μ M). Pure 1-octanol was used as a baseline absorbance.

Statistical Analysis of Experimental Data

All data are expressed as mean $\pm$ standard deviation (SD). For multiple comparisons, statistical analysis was 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 or equal to 0.05 were considered statistically significant.

Physiologically-based Drug Transport Modeling and Simulation of Intracellular Drug Supersaturation

We used an established, physiologically-based cellular pharmacokinetic modeling framework(40–42) to calculate the time-dependent changes in the concentration of a monovalent weakly basic small molecule drug inside each compartment within a cell following exposure of the cell to a constant extracellular drug concentration. For the present study, we used the open-source modeling software Virtual Cell®. All of the following models can be freely accessed at <http://vcell.org>. The model used here (*awillmer: Macrophage Cargo Capacity*) was built from the established Virtual Cell model: *jsbaik: 1-Cell PK acid/base/neut total*. For boundary conditions, we used the previously published, standard eukaryotic cell parameter values as input (Table S1).

Modeling the Passive Transport Properties of a Weakly Basic Drug that Stably Accumulates and Self-Assembles in Macrophage Lysosomes

We used CFZ as a weakly basic model drug to predict its subcellular phase-transition dependent accumulation. Even though it has two ionizable groups, it can be modeled as a monobasic compound because at physiological pH, the ionization of the amine with the lower pK_a of ~ 2 will not influence the transmembrane fluxes to any significant extent. In lysosomes, CFZ accumulates as CLDIs, which mostly contain hydrochloride salts of the weak base (CFZ-HCl). Thus, using predetermined physicochemical properties of the free base as well as the salt form of the drug (apparent $pK_a = 6.08$ (28), and octanol/water partition coefficient $\log P$ ($\log K_{ow} = 7.66$ (26, 27))) as input in the Virtual Cell model, we calculated the time-dependent subcellular distribution of both neutral and ionized molecular species as a function of an extracellular total drug concentration of $10 \mu M$ (concentrations in all subcellular compartments are linearly related to the extracellular concentration). We set the extracellular volume to a high value (Table S1), so that the extracellular drug concentrations would remain nearly constant, without being affected by cellular drug uptake. In turn, the degree of supersaturation of the drug molecules in the different cellular compartments was calculated by dividing the concentrations of the protonated and unprotonated species of the drug in those compartments by the calculated solubility of the corresponding charged or neutral species in said compartments. Detailed theoretical calculations and equations used to determine the concentration of the ionized and unionized form of the weakly basic drug within subcellular compartments, and degree of supersaturation of the drug within various subcellular compartments can be found in the Supporting Information.

RESULTS

Testing the *in vivo* adaptive response of different macrophage populations to a massive, whole organism cargo load

To determine how CFZ bioaccumulation as an expandable mechanopharmaceutical device affects macrophage function, we measured the response of different macrophage subpopulations to an increasingly massive CFZ load. Experimentally, at the smallest whole body cargo load measured (3.5 mg), the drug was mostly present in alveolar macrophages (Figure 1a). This cargo was associated with red, optically dense, cytoplasmic vesicles which exhibited little dichroism signal, corresponding to a disordered, amorphous, supramolecular organization(43, 44) (Figure 1b). A 7 mg whole body cargo load resulted in a modest increase in accumulation in the alveolar macrophages (Figure 1c), and a small percentage of the drug was apparent in the peritoneal and liver macrophages (Figure 1a). However, the structure of the intracellular cargo became more organized with increasing amounts of drug as reflected in the elevated optical density (Figure 1c) and dichroism (Figure 1d) of alveolar, peritoneal, and liver macrophages. At 14 mg, cargo loads redistributed into large, highly ordered CLDIs exhibiting strong dichroism signals in all macrophages (Figure 1b). Under all loading conditions, the less mature bone marrow monocytes were free of cargo. At a population level, these trends were confirmed by multi-parameter image-based cytometric analyses(16) (Figure 1c, d).

The results of chemical analyses paralleled the observed changes in loading dynamics and the redistribution of cargo among the different macrophage populations. At low (3.5 mg) cargo loading, alveolar macrophages sequestered 29.9 ± 12.4 fmol of CFZ/cell, while the other populations showed minimal loading (Figure 1e). After a whole body load of 3.5 mg, less than 0.3% of the maximal phagocytic capacity of the alveolar macrophages was reached (Figure 1f). At a larger (7 mgs) whole body cargo load, alveolar macrophages accumulated 34.0 ± 20.3 fmol CFZ/cell, which primarily remained in a disordered form as reflected in the low dichroism signal (Figure 1b, d). Peritoneal and liver macrophage populations, due to their differential accumulation pattern (Figure 1b), increased their cargo loading per cell to 105.7 ± 10.0 and 63.5 ± 16.2 fmol/cell, respectively. It is noteworthy that at the highest loads of cargo (14 mg), all tissue macrophage subpopulations exhibited similar levels of cargo loading (Figure 1e), except for monocytes. Thus, the formation of CLDIs facilitated intracellular cargo loading coincided with a systemic redistribution of cargo among all macrophage sub-populations, such that the load became more evenly distributed in the cell. In these isolated macrophages, the highest cargo volume corresponded to ~1% of the maximal phagocytic capacity of cell (Figure 1f)(8). As such, only a small fraction of the potential intracellular cargo space was occupied even at the highest whole body loads that were measured *in vivo*.

To achieve an even distribution of cargo (which was observed in the most massively loaded condition), macrophages visibly adjusted their intracellular, cytoplasmic membrane organization. For example, with a relatively low amount of loading (3.5 mg), the alveolar macrophage population accumulated an average of 21.6 ± 5.0 vesicles per cell, with each vesicle occupying a mean volume of $0.43 \pm 0.29 \mu\text{m}^3$ (Table I). Transmission electron micrographs of alveolar macrophages revealed numerous dark, lipid bound inclusions within the cytoplasm (Figure 2a). A larger (7 mg) whole body cargo load increased the number of cytoplasmic vesicles by >50% per cell ($n=30$ cells, $p<0.05$, ANOVA, Tukey's HSD), with the mean vesicular volume increasing by ~125% (Table I). At the largest whole body cargo load tested (14 mg), an expansion of membrane-bound cytoplasmic vesicles was clearly visible (Figure 2b). At this point, the number of loaded vesicles per cell decreased by >40%. The expanded, membrane bound vesicles remained stable within the macrophages as long as eight-weeks following the discontinuation of drug administration (Figure 2c).

Of noteworthy significance, the amount of cargo in the isolated macrophage populations effectively accounted for most of the total cargo load measured in whole organ homogenates (Table II). Based on the amount of cargo within each organ, the volume of blood that was cleared by the macrophages in the different organs was estimated (the organ-specific Volume of Distribution, VOD; Table III). In pharmacokinetic terms, the whole body V_D of a molecule is the ratio between the amount of drug in the organism and the plasma concentration of the drug(45). V_D represents the extent of tissue distribution(46) as reflected in the theoretical volume required to contain an administered amount of drug at the same concentration found in plasma. With an increasing cargo load, each macrophage population significantly contributed to V_{OD} and consequently, to V_D (Table III). Given that the volume of a single macrophage is ~1 pL, they contribute to V_D via the solute-to-solid phase transition that accompanies CLDI formation. At the whole organ level, there was a nearly ~100-fold increase in the V_{OD} within the liver and small intestine, a ~60-fold increase

within the spleen and ~10-fold increase within the lung, paralleling an increasing whole body cargo loading from 5.25 to 14 mg (Table IV). Adipose tissue and kidneys, neither of which contain large populations of tissue macrophages compared to other organs, had modest increases in the V_{OD} that were much lower than those observed in liver, spleen, lung, and intestine (Table IV).

The most important finding in the V_{OD} analysis pertains to the role of organ-specific macrophage subpopulations in the redistribution of cargo loads and its ultimate impact on the measured differences in the V_{OD} of the different organs. The difference in organ-specific V_{OD} (Table IV) between animals exposed to a moderate (5.25 mg) or a large (14 mg) cargo load, seemed to result in an increasingly divergent loading pattern. With a 14 mg load, for example, the spleen V_{OD} was >50 times greater than that of the kidney, while at a 5.25 mg load, the spleen V_{OD} was only ~3 times greater than kidney. However, when looking at the specific accumulation of drug in macrophages (Table II and III; and Figure 1e, f), it was apparent that the divergence in the whole organ distribution was due to differences in the number of macrophages per organ mass, since the cargo became more evenly distributed among the resident macrophages of the respective organs.

Passive transport acting in concert with a pH and chloride-dependent phase transition is sufficient to explain the function of CFZ as an expandable mechanopharmaceutical device

In order to determine whether passive transport can explain the selective precipitation and growth of CFZ hydrochloride crystals in lysosomes, we proceeded to dissect the subcellular transport and self-assembly properties of CFZ using a physiologically based, cellular pharmacokinetics modeling and simulation approach (Figure 3). Previous analyses have identified the hydrochloride salt form of CFZ as the major fraction of the bioaccumulated drug(28, 35). This hydrochloride salt form was specifically present in CLDIs, which are stabilized within acidic intracellular vesicles (as revealed by inhibiting the proton-pumping, V-ATPase which decreases CLDI formation and CFZ accumulation)(28). Without macrophages, CLDIs also failed to form and CFZ accumulated in tissues to a much lesser extent(28).

Consistent with these previous experimental observations(28, 35), our simulation results revealed how the unprotonated, neutral molecular species of CFZ accumulated intracellularly in the presence of a constant extracellular drug concentration (Fig. 4a). As expected, because of the higher pH (relative to the drug's apparent pK_a) of the cytoplasm and mitochondria, the unprotonated free base concentration was highest in the cytoplasm (Fig. 4b) and mitochondria (Fig. 4c), relative to lysosomes (Fig. 4d). Simulating the distribution of the ionized (protonated) molecular species of the drug over time, we observed that the protonated form remained nearly constant in the extracellular medium (Fig. 4e). The concentration of protonated drug was lowest in cytoplasm (Fig. 4f) and mitochondria (Fig. 4g), but increased dramatically in the lysosomes (Fig. 4h) due to ion trapping(47). In fact, lysosomes possessed the highest drug concentration of all cellular compartments (Fig. 4h). Because of the linearity of the model, the neutral and protonated, charged molecular species in all of the compartments proportionally decreased when the total initial extracellular CFZ concentration was reduced (for example, changing the units of micromolar to nanomolar).

In order to determine the propensity of the free base and ionized forms of the drug to precipitate in the different cellular compartments, we calculated the degree of supersaturation of both forms of the drug using their solubility properties obtained from experimental measurements (Table V). Degrees of supersaturation were calculated by determining the ratio of simulated steady state CFZ concentration to CFZ solubility (Equations 3 and 4 in the Supplementary Materials). The greatest degree of supersaturation for the ionized form of the drug was observed in the lysosomal compartment (Table V). Within the lysosomal lumen, the supersaturation of CFZ-HCl exceeded all other compartments by more than two orders of magnitude (Table V). The primary reasons for the high supersaturation in the lysosomal lumen were the high chloride concentrations and a lysosomal pH lower than the pH_{max} of CFZ-HCl. The pH_{max} represents the point of equilibrium between both the ionized and unionized compound that is in solution, as well as solid free base and crystalline salt, and when $pH < pH_{max}$, the ionized, salt form is thermodynamically favored. Related to the pH_{max} , the K_{sp} of CFZ-HCl was previously determined to be $332.3 \pm 3.71 \mu M^2$ (Supplemental Figure 5)(28). Moreover, the degree of supersaturation of CFZ-HCl in the lysosome was exceeded by 3 to 6 orders of magnitude over that of CFZ free base (Table V). This indicates that the conditions for salt precipitation would be most favorable in the lysosomal lumen, and as a result, would be the first location within the cell that precipitation would be expected to occur, as concentrations in all compartments increase following drug administration. Note that the intracellular concentrations predicted by the transport model were linearly dependent on the starting drug concentrations in the extracellular medium, so the model effectively predicted the most likely site of intracellular drug precipitation based on the lowest extracellular concentration necessary to achieve supersaturation in any intracellular compartment.

DISCUSSION

In this study, we propose that CFZ accumulation in macrophages leads to physical alterations in cell structure and function that result from CFZ's self-assembly into an expandable mechanopharmaceutical device. This expandable mechanopharmaceutical device specifically accumulates in macrophage lysosomes following prolonged oral drug administration, and its effects are posited to result from a physical space occupied by the drug, independent of the drug's pharmacological activity. As an expandable mechanopharmaceutical device, we considered CLDIs as a physical probe that can be used to measure the cargo loading capacity of different macrophage subpopulations *in vivo*, and used it to directly determine the V_D of the drug in different organs. While prior studies have determined that CLDI formation is compatible with the maintenance of normal ion homeostasis in lysosomes of macrophages(28), this is the first study to show that the mass and volume occupied by drug is specifically accumulating in lysosomes within these cells, while assessing its impact on the drug's volume of distribution (V_D)(29, 35). While it is often assumed that the V_D of a therapeutic agent is a constant, our measurements indicate that this is not necessarily the case. Furthermore, based on our mathematical modeling and simulation analysis, lysosomal pH and chloride content were found to be sufficient to account for the selective accumulation of CFZ in macrophage lysosomes accounting for the cell type selectivity of this phenomenon.

When studying the impact of macrophages on transport phenomena, fluid tracers or nanoparticles that are readily ingested by macrophages have been previously used as functional markers of macrophage-mediated clearance. To assess whole organism cargo capacity of macrophages *in vivo*, our results show how an expandable mechanopharmaceutical device that self-assembles from a small molecule building block could potentially be used to stretch the cell's cargo carrying capacity with the largest possible cargo load that can be accommodated by a living organism. This led us to consider the following question: how exactly does the organism respond to the massive build-up of a whole-body macrophage cargo load? In the experiments reported herein, the null hypothesis was that all macrophages would accumulate the drug to the same extent in all organs, and that at some point a toxicological effect may become manifested. However, our experimental results led us to reject this, in favor of an alternative hypothesis: different macrophage subpopulations initially vary in their ability to bioaccumulate drug, but gradually respond to an increasingly massive amount of cargo so that the load does not exceed the cargo capacity of the individual cells and becomes more evenly distributed across all of the macrophage populations throughout the different organs of the body. Based on theoretical considerations, a physiologically-based cellular pharmacokinetic model indicated that this phenomenon may be entirely determined by an energetically-favorable, thermodynamically-driven loading state that decreases the total free energy of the drug while maximizing the volume-to-surface area ratio of intracellular drug inclusions. The significantly different rates at which this occurs in macrophage subpopulations do not simply reflect the extent of perfusion of the different organs, nor do they reflect the partitioning of soluble drug or trapping of circulating drug particles in these organs since it varied as the whole organism drug load increased. Since undifferentiated, bone marrow monocytes did not accumulate drug to any significant extent, we infer that differences in drug accumulation kinetics reflect organ-specific variations in the differentiation of macrophages into specialized, xenobiotic sequestering cells.

In terms of how these findings impact pharmacokinetics, CFZ induced changes in V_D which counters the underlying assumptions about the mechanistic underpinning of this pharmacokinetic parameter. V_D is usually considered the apparent volume that a systemically administered drug distributes in to reach a measureable concentration in the blood. For many poorly soluble, weakly basic drugs, the V_D can be in the order of thousands of liters, which greatly exceeds the volume of body water (60–100 liters). Large V_D is generally thought to be due to the preferential partitioning of hydrophobic drug molecules into adipose tissue or cellular membranes and lipids. For poorly soluble drugs that are administered at high doses for prolonged periods of time, if the drug molecules are metabolically stable and eliminated very slowly, bioaccumulation could eventually lead to a phase transition, with insoluble aggregates forming throughout the organism, expanding the V_D . Accordingly, the experimental and theoretical analysis presented in this study suggests that the simple, thermodynamic partitioning mechanisms underlying the general concept of V_D may need to be revisited, especially for poorly soluble weakly basic drugs like CFZ which massively bioaccumulate in the organism.

Indeed, the experimental results presented in this study add support to the role of macrophage lysosomal pH and chloride ion regulation in contributing to, and accounting for,

most of the accumulation and distribution of CFZ *in vivo* (28, 48). The results of our physiologically based model was consistent with CFZ precipitation occurring selectively in macrophage lysosomes, because the stability of CFZ hydrochloride precipitates in this organelle surpassed that of all other sites in the cell, as well as in the extracellular environment, by more than three orders of magnitude. Macrophages possess highly acidic lysosomes which can expand to accommodate large cargo loads, and the macrophage population itself is able to undergo changes in structure and function to adapt to the cargo. In the future, the mathematical model could be used to predict the intracellular concentration and precipitation of ionized vs. neutral molecular species of other weakly basic small molecule drugs and to identify other small molecule chemical agents that have similar subcellular disposition characteristics as CFZ.

Regarding our use of the word “device”, it may sound unusual if one only considers the formation of a precipitate as a side-effect or as a toxic phenomenon arising from poor pharmacokinetics or inadequate dosing. In this particular case, the precipitation behavior of the drug is being purposefully co-opted as a volumetric instrument. It is being introduced as a tool to expand the macrophage cargo capacity, and to probe how the organism responds to an increasing endophagolysosomal cargo load. In the scientific literature, one can find many references in which drugs are referred to as pharmacological “probes” or “tools” when used as inhibitors or stimulators of endogenous molecular targets or pathways, with the goal of obtaining information about how these targets or pathways mediate biochemical signaling or physiological responses in living organisms (49–52). The word “device” was purposefully chosen to highlight how CFZ was used as a self-assembling building block to construct a physical object to study the “cargo capacity” of macrophages. To use CLDIs as measurement “devices”, we have developed methods to isolate and stabilize CLDIs (34) as well as microscopy instruments to monitor their optical (29), photoacoustic (53) and biomechanical properties (54). Instead of engineering a new chemical compound to measure the cargo capacity in a living organism, CFZ was repurposed and administered to the animals to operate in this very physical manner, independently of the antimicrobial, redox, and other pharmacological interactions of freely soluble CFZ molecules.

CONCLUSION

To conclude, the results presented herein suggest a physical stimulus-dependent, macrophage-mediated biological response mechanism that is activated by the function of CFZ as a building block of an expandable mechanopharmaceutical device that accumulates within these cells. Macrophages actively stabilize intracellular CFZ as insoluble complexes that are trapped within their endophagolysosomal compartment. To accommodate increasingly massive loads of cargo, macrophages increased in numbers and adapted their intracellular membrane organization to expand the intracellular cargo space; this resulted in a gradual but dramatic increase in V_D . While resident macrophages of different organs demonstrate significant variations in their response to increasing loads of cargo, all differentiated macrophage populations were capable of sequestering very large loads. Of noteworthy significance, to our knowledge this is the first time that changes in the V_D of a small molecule drug has been directly linked to an adaptive, immune system-mediated, xenobiotic sequestration response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CFZ	Clofazimine
CFZ-HCl	Clofazimine Hydrochloride
CLDI	Crystal-Like Drug Inclusions
Cy5	Cyanine 5
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ULAM	University of Michigan's Unit for Laboratory Animal Medicine
V-ATPase	Vacuolar-type proton ATP-ase
V_D	Volume of Distribution
V_{OD}	Organ-Specific Volume of Distribution

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Figure 1: Microscopic imaging cytometry and quantitative chemical analysis indicate variations in cargo loading dynamics of different macrophage sub-populations.

(a) Brightfield images of isolated macrophage and monocytes with increasing whole-body cargo loading. (b) Linear diattenuation images of isolated macrophage and monocytes with increasing whole-body cargo loading. Optical density (c), linear diattenuation (d), cargo loading per xenobiotic-sequestering cell (e), and percent of maximal cargo loading (f) of alveolar macrophages (black), peritoneal macrophages (red), bone marrow monocytes (green), and Kupffer cells (blue) with increasing cargo loading. Data are the mean (SD) of 150 cells per cell type and condition, for imaging studies, n=3 mice per cargo treatment for drug accumulation, (*=p<0.05, ANOVA, Tukey's HSD) (Scale bar = 10 μ m).

Figure 2: Macrophage cargo accumulation induces reorganization of internal membrane architecture.

(a) Following whole-body cargo loading of 3.5 mg, small vesicles that fill the cytoplasm are apparent in alveolar macrophages. (b) A higher cargo load (14mg) results in accumulation of crystal-like drug inclusions (CLDIs) throughout the cytoplasm. (c) CLDIs remain stable even following an eight week washout period. Black arrows denote cargo-laden vesicles and red arrows denote cavities left from CLDIs removed during sample preparation. Scale bar is 2000 nm.

Figure 3: Diagrammatic representation of the integrated, transport and precipitation modeling approach used to determine the most likely site of intracellular CFZ precipitation.

The definition of each parameter value is given in Table S1 in the Supporting Information.

Figure 4: Time-plot simulations of intracellular concentrations of neutral and protonated species of clofazimine (CFZ) in different subcellular compartments.

(a) Extracellular drug accumulation of neutral CFZ remains constant during the time course of the simulation; (b) neutral drug molecules enter and reach steady state in cytoplasm; (c) the mitochondrial compartment has the highest free base drug concentration due to a pH higher than the cytosol and significant lipid content; (d) lysosomes have low concentrations of the neutral species due to their low pH; (e) extracellular concentrations of ionized CFZ (CFZH^+) are much lower than neutral CFZ species, and remains constant; (f) due to the physiological pH of the cytoplasmic compartment, there is very little protonated drug, as well; (g) the high pH of the mitochondria leads to a low concentration of protonated free base, although the concentration is higher than in the extracellular medium due to the mitochondrial membrane potential-dependent uptake; (h) the lysosomal compartment has the highest ionized drug concentration due to ion trapping.

Table I:

Vesicle number, size, and volume occupancy in alveolar macrophages following cargo loading.

Cargo Loading	Mean number of vesicles per cell $\pm$ SD (n=30 cells)	Mean vesicle volume $\pm$ SD (n=50 vesicles)	Mean total volume occupied by vesicles $\pm$ SD (n=50 vesicles)
3.5 mg	21.6 $\pm$ 5.0	0.43 $\pm$ 0.29 μm^3	9.2 $\pm$ 6.7 μm^3
7.0 mg	32.6 $\pm$ 6.8 *	0.97 $\pm$ 0.96 μm^3	31.7 $\pm$ 32.1 μm^3
14.0 mg	18.4 $\pm$ 11.1	13.8 $\pm$ 10.4 μm^3 *	253.8 $\pm$ 244.5 μm^3

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

Table II:

Estimated cargo loads of liver, spleen, and lung macrophages, at a 14 mg whole body load. Data represent the mean $\pm$ S.D., n=3 mice.

Macrophage Population	Cargo mass (mg)	Percent Xenobiotic Sequestering	Total Xenobiotic Sequestering Cells ($\times 10^6$)	Fmol Cargo/Xenobiotic Sequestering Cell	% Cell Volume Occupied by Cargo
Liver	4.57 $\pm$ 0.78	88.5 $\pm$ 3.3%	80 $\pm$ 19	120.9 $\pm$ 35.3	2.11 $\pm$ 0.62%
Spleen	3.23 $\pm$ 0.27	83.9 $\pm$ 12.5%	22 $\pm$ 5.9	310.5 $\pm$ 86.9	5.42 $\pm$ 1.52%
Lung	0.32 $\pm$ 0.06	81.1 $\pm$ 3.2%	3.7 $\pm$ 1.7	183.2 $\pm$ 91.5	3.20 $\pm$ 1.60%

Table III:

Volume of distribution in liver, lung, and spleen macrophage, at a 5.25 and 14 mg whole-body loads

Macrophage Population	5.25 mg cargo V _{OD} (nL/macrophage)	14 mg cargo V _{OD} (nL/macrophage)
Liver	0.66 ± 0.08	33.51 ± 16.43 *
Spleen	0.49 ± 0.03	64.25 ± 32.48 *
Lung	5.51 ± 0.16	42.73 ± 31.58 *

*
(=p<0.05, Two-tailed Student's t-test)

Table IV:

Volume of distribution within specific organs, at a 5.25 and 14 mg whole-body load

Tissue (n=3 per treatment)	5.25 mg cargo V _{OD} (L/kg tissue)	14 mg cargo V _{OD} (L/kg tissue)
Liver	33.9 ± 13.1	2232.5 ± 958.2 *
Spleen	118.8 ± 85.4	6139.3 ± 2637.0 *
Lung	91.2 ± 12.0	902.3 ± 524.1
Fat	91.8 ± 15.2	129.1 ± 61.1
Jejunum and Ileum	11.1 ± 6.3	1085.2 ± 606.2 *
Kidney	31.1 ± 7.3	125.6 ± 55.5

*
(=p<0.05, Two-tailed Student's t-test)

Table V:

Steady-state degree of supersaturation of free base CFZ and CFZ-HCl based on a simulated, initial extracellular concentration of 10 μM

Compartments	Estimated degree of supersaturation of free base CFZ at steady state	Estimated degree of supersaturation of CFZ-HCl at steady state
Extracellular	^(b) 1.98×10^1	0 ^(a)
Cytosol	^(b) 4.95×10^4	0 ^(a)
Mitochondrial lumen	^(b) 5.36×10^4	0 ^(a)
Lysosomal lumen	^(b) 2.25×10^3	^(c) 1.36×10^7

^(a) Because the local pH is higher than the pH_{max}, only the free base form is predicted to accumulate as the most stable precipitate.

^(b) The simulated, steady state concentration of CFZ free base in the given aqueous compartment was divided by the experimentally measured, intrinsic aqueous solubility of CFZ ($0.48 \pm 4.05 \times 10^{-6} \mu\text{M}$)(28).

^(c) Based on this degree of supersaturation calculated with Equation 4 in the Supplementary Materials, the solubility of CFZ-HCl in lysosomes, in the presence of 110 mM chloride at pH= 4.5, was 3 nM.