Fluorescence Tomography of Rapamycin-Induced Autophagy and Cardioprotection In Vivo

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Background—Autophagy is a biological process during which cells digest organelles in their cytoplasm and recycle the constituents. The impact of autophagy in the heart, however, remains unclear in part because of the inability to noninvasively image this process in living animals.

Methods and Results—Here, we report the use of fluorescence molecular tomography and a cathepsin-activatable fluorochrome to image autophagy in the heart in vivo after ischemia/reperfusion and rapamycin (RAP) therapy. We show that cathepsin-B activity in the lysosome is upregulated by RAP and that this allows the expanded lysosomal compartment in autophagy to be imaged in vivo with fluorescence molecular tomography. We further demonstrate that the delivery of diagnostic nanoparticles to the lysosome by endocytosis is enhanced during autophagy. The upregulation of autophagy by RAP was associated with a 23% reduction (P<0.05) of apoptosis in the area at risk and a 45% reduction in final infarct size (19.6±5.6% of area at risk with RAP versus 35.9±9.1% of area at risk without RAP; P<0.05).

Conclusions—The ability to perform noninvasive tomographic imaging of autophagy in the heart has the potential to provide valuable insights into the pathophysiology of autophagy, particularly its role in cardiomyocyte salvage. Although additional data are needed, our study supports the investigation of RAP therapy in patients with acute coronary syndromes. (Circ Cardiovasc Imaging. 2013;6:441-447.)

Key Words: apoptosis • autophagy • molecular imaging • myocardium • rapamycin

Autophagy, a biological process in which the cell digests a portion of its own cytoplasmic contents, is classically upregulated during starvation.1 The upregulation of autophagy, however, has also been documented in neurodegeneration,1 myocardial ischemia,2,3 and cardiomyopathy.4 It remains unclear, however, whether the upregulation of autophagy in the heart is protective or deleterious.5,6 It has been postulated by some that by clearing damaged mitochondria, which are a potent proapoptotic stimulus, autophagy protects the cardiomyocyte (CM) from apoptotic cell death.1,7 Others have proposed that autophagy in itself may result in cell death.8 This uncertainty is driven in large part not only by the biological complexity of the process but also by the absence of a probe-based technique to image autophagy in vivo.

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Autophagy, apoptosis, and necrosis constitute a spectrum of processes involved in cell injury and death. Although probe-based techniques have been developed to image apoptosis9–11 and necrosis,12 in the heart in vivo, current techniques for determining the autophagic state of a cell are limited to genetic constructs and the use of fluorochromes with little ability to penetrate tissue.13 A strong need thus exists for a noninvasive technique supporting the in vivo detection of autophagy in the heart to be developed. Our goal here was to exploit the upregulation of cathepsin activity in the expanded lysosomal compartment during autophagy to develop such an approach. A model of ischemia/reperfusion (IR) plus rapamycin (RAP) treatment was used to create a robust model of CM autophagy in this proof-of-principle study. RAP is a macrolide immunosuppressant known to induce autophagy via its inhibition of mammalian target of rapamycin (mTOR). RAP and its analogs have also been used to treat transplant rejection and restenosis and have an established safety profile for clinical use.1,7 A cathepsin-activatable near-infrared fluorochrome (CAF-680) and fluorescence molecular tomography (FMT) of the heart in vivo were used to demonstrate the feasibility of the approach. We further aimed to determine whether the augmentation of CM autophagy by RAP during IR would be...
associated with a reduction in CM apoptosis and whether it would generate a protective or a deleterious effect.

Methods

Animal Protocol

The upregulation of autophagy in vivo was determined by the signal emitted from the CAF-680. As shown in Figure 1, 4 groups of mice were imaged: mice with IR, mice with IR and postconditioning (IR+PC), mice with IR plus RAP treatment before coronary ligation (IR+preRAP), and mice with IR plus RAP treatment after coronary ligation (IR+postRAP). In all groups, the CAF (ProSense-680, Perkin Elmer, Waltham, MA) was injected at the onset of reperfusion, and fluorescence imaging of the heart was performed 4 hours later. An initial cohort of mice was imaged ex vivo with fluorescence reflectance imaging (FRI) to establish proof of principle. In vivo imaging was subsequently performed in cohorts of mice with IR and IR+preRAP using FMT and micro–computed tomography (CT).

IR injury was induced in female C57Bl6 mice by a 35-minute ligation of the proximal left coronary artery and was followed by 4 hours of reperfusion. Mice in the IR+preRAP group received RAP (3 mg/kg body weight) by intraperitoneal injection 2 hours before the coronary ligation. Mice in the IR+postRAP group were injected intravenously with the identical dose of RAP during the period of coronary ligation (3 mg/kg body weight) by intraperitoneal injection 2 hours before the coronary ligation (IR+postRAP). In all groups, the CAF (ProSense-680, Perkin Elmer, Waltham, MA) was injected at the onset of reperfusion, and fluorescence imaging of the heart was performed 4 hours later. An initial cohort of mice was imaged ex vivo with fluorescence reflectance imaging (FRI) to establish proof of principle. In vivo imaging was subsequently performed in cohorts of mice with IR and IR+preRAP using FMT and micro–computed tomography (CT).

Fluorescent Reflectance Imaging

The initial assessment of CAF-680 activity in the myocardium was performed with FRI. Mice with IR (n=7), IR+PC (n=7), and IR+preRAP (n=7) were studied. The mice were euthanized 4 hours after reperfusion, and the heart was cut in the short-axis plane into apical, midventricular, and basal sections. FRI of these sections was performed on a commercial imaging system (Kodak In-Vivo FX Pro, Carestream Health, Rochester, NY) with a spatial resolution of 48 μm, a 30-second exposure, and wavelengths of 650 nm (excitation) and 700 nm (emission). FRI was also performed in a separate cohort of mice with IR (n=7), IR+preRAP (n=7), and IR+postRAP (n=7) that were injected with fluorescently labeled annexin-V (ANN-750) and fluorescent microspheres. The fluorescent microspheres (10-μm diameter, Life Technologies, Carlsbad, CA) were given by intracardiac injection 5 minutes before reperfusion. The ANN-750 probe (Annexin Vivo 750, Perkin Elmer) was injected at the onset of reperfusion. The hearts were sectioned, as described above, 4 hours after reperfusion, and FRI was performed at 750-nm excitation/800-nm emission for annexin-V and 550-nm (excitation)/600-nm (emission) for the fluorescent microspheres.

A third cohort of mice with IR (n=7), IR+preRAP (n=7), and IR+postRAP (n=7) was used to image infarct size at 24 hours by 2,3,5-triphenyltetrazolium chloride (TTC; GFS Chemicals, Powell, OH). Fluorescent microspheres were injected as described above. The mice were euthanized 24 hours after reperfusion, and the heart was sectioned in its short axis into 1-mm-thick slices. The distribution of the microspheres was imaged on a multispectral imaging platform (Kodak In-Vivo FX Pro, Carestream), and images of TTC were obtained with a flat-bed scanner (Hewlett Packard, Palo Alto, CA).

Image analysis was performed in ImageJ (National Institutes of Health, Bethesda, MD). The average fluorescence signal in the ischemic area of the myocardium (defined in those hearts not injected with microspheres as the region between the upper and lower right ventricular insertion points) was compared with that in the nonischemic septum (Figure 1B). The contrast-to-noise ratio between these 2 zones was calculated by the following equation: contrast to noise ratio = (signal ischemic−signal nonischemic)/SDnoise.15 Automated segmentation of the annexin-V and TTC images was performed in ImageJ by using a signal intensity threshold ±2 SDs above that in the nonischemic septum. The area at risk (AAR) in the fluorescent microsphere images was segmented manually.

In Vivo Imaging

FMT-CT was performed in mice with IR+preRAP (n=7) and IR (n=7) 4 hours after reperfusion and the intravenous injection of 5 nmol of CAF-680. CT imaging was performed on a commercial micro–positron emission tomography-CT system (online-only Data Supplement). The mice were transferred from the CT to the FMT system while still anesthetized and held in position in a plexiglass holder.

In vivo FMT was performed with the 680/700-nm excitation/emission filter setting on a commercial FMT system (Perkin Elmer). Twenty-six frontal slices of 0.5-mm thickness in the z direction with an in-plane resolution of 1×1 mm² were acquired. Data were postprocessed.
with a normalized Born forward equation to reconstruct 3-dimensional fluorochrome concentration distribution maps for quantification, as described previously. Fusion of 3-dimensional fluorescence and CT data sets was performed offline using the fiducial landmarks on the frame of the plexiglass holder (online-only Data Supplement).

Microscopy
After FMT, to determine the cellular and subcellular locations of CAF-680, the hearts of the IR+preRAP mice were excised and sectioned for confocal microscopy. Immunofluorescence staining of the lysosomal marker lysosome-associated membrane protein-2 was performed with a phycocyanin-conjugate antibody (SC-19991PE anti-mouse, Santa Cruz Biotechnology, Santa Cruz, CA) according to manufacturer instructions (online-only Data Supplement). Subsequently, CAF-680 fluorescence was excited with the 633-nm Helium-Neon laser and acquired with the 650-nm line-pass filter. Differential interference contrast images were acquired as well to visualize cellular anatomy. The nonischemic septum and the ischemic area of injury were defined by the right ventricular insertion points. Fluorescence in the ischemic region was compared with that in the nonischemic septum in the following 2 ways: (1) overall fluorescence intensity in each region normalized to area and (2) the number of hyperintense fluorescence punctates in each zone normalized to area. Colocalization of the CAF-680 and lysosome-associated membrane protein-2 signals was quantified using ImageJ.

In Vitro Studies
To further elucidate the mechanism of the enhanced CAF-680 activation by RAP, a series of experiments were performed on Chinese hamster ovary (CHO) and H9C2 cells (American Type Culture Collection, Manassas, VA). Cathepsin activity in control cells and cells exposed to RAP was determined using a commercial fluorometric assay (online-only Data Supplement).

To determine the effects of RAP on endocytosis, the cells were incubated with FER-680, which is synthesized by attaching the fluorochrome CyAl5.5 to the Feraheme (ferumoxytol, AMAG Pharmaceuticals, Lexington, MA) nanoparticle. Feraheme has a blood half-life in humans of over 10 hours and is only very slowly internalized by endocytosis in normal cells. The internalization of FER-680 was determined by fluorescence-activated cell sorting (LSRII, BD Biosciences, Franklin Lakes, NJ) and further by costaining (50 nmol/L, 1-minute incubation) the cells with the lysosomal marker LysoTracker Red (Life Technologies).

Impact on Infarct Size
Two strategies were used to investigate whether the induction of autophagy in the IR+preRAP (n=14) and IR+postRAP (n=14) mice would exert a protective or deleterious effect on infarct size. Fluorescently labeled annexin-V (ANX-750) was injected intravenously at the onset of reperfusion and used to determine the extent of cell injury by FRI 4 hours after injection. In addition, TTC staining was used to determine infarct size 24 hours after IR. The area of ANX-750 uptake and the infarct size by TTC were both normalized to the AAR, which was determined by the injection of fluorescently labeled microspheres.

Statistics
One-way ANOVA with a Tukey posttest was performed to compare the IR, IR+preRAP, IR+postRAP, and IR+PC groups. An unpaired t test with a 2-tailed P value was applied to compare the in vivo FMT data and for all other comparisons between 2 groups. All tests were performed in Prism (Graphpad, La Jolla, CA), and the results were reported as mean±SEM. A value of P<0.05 was needed to meet significance.

Results
FRI of the mice subject to IR and IR+PC showed that fluorescence activity in the hearts of these mice was not increased (Figure 1). However, a significant increase in fluorescence activity was seen in the IR+preRAP mice. The contrast-to-noise ratio between the ischemic lateral wall and the nonischemic septum was 8.8±2.0 in the IR+preRAP mice versus 0.65±2.0 in the IR mice (P<0.05). The activation of CAF-680 was thus both highly sensitive and specific for the augmentation of CM autophagy in IR by RAP.

The activation of CAF-680 in the RAP-treated mice could also be robustly imaged in vivo. For logistical reasons, in vivo imaging was performed in the IR and IR+preRAP groups. The presence of fiducial markers in the animal cradle allowed the 3-dimensional CT and FMT data sets to be accurately coregistered (Figure 2). Volume rendering of the heart allowed fluorescence arising in the heart and liver to be definitively distinguished. Hepatic activation of CAF-680, which is a normal finding, was seen in both the IR and IR+preRAP mice imaged with FMT. CAF-680 activation in the heart, however, was seen only in the IR+preRAP mice (Figure 2).

CAF activation in the myocardium of the IR+preRAP mice could be successfully imaged in vivo in a 3-dimensional and depth-resolved manner (Figure 3). Masking the fluorescence signal in the liver, which was segmented anatomically in the CT images, was performed to optimize dynamic range. Fluorescence activity was greatest in the anterior and lateral walls of the apical half on the left ventricle (Figure 3), consistent with the location of the ischemic injury. Total fluorescence activity in the hearts...
Autophagy is a complex biological process and requires the execution of an elaborate pathway of signals and steps. However, despite the sophisticated biological understanding of the process, probe chemistries and whole-animal imaging methodologies to image autophagy have not been developed. Here, we show that the upregulation of cathepsin activity in the expanded lysosomal space allows CM autophagy to be imaged in vivo with an activatable near-infrared fluorochrome. We further show that the upregulation of CM autophagy by RAP is associated with a significant reduction in CM apoptosis and a marked cardioprotective effect.

Our broad goal of developing a probe-based tomographic technique to image autophagy in vivo required the following 3 key elements: the use of a cathepsin activatable fluorochrome (CAF-680), the use of the advanced reconstruction algorithms inherent in FMT,17 and the ability to separate autophagy-mediated activation of CAF-680 from that attributable to infiltrating leukocytes.18 Neutrophil infiltration in ischemic myocardium occurs 4 to 6 hours after injury, whereas macrophage infiltration occurs between 12 and 24 hours after injury.19 Here, by imaging CAF-680 activity within 4 hours of injury, the potential for immune-mediated probe activation was minimized. Two additional findings suggest that the CAF-680 activation seen in the myocardium at 4 hours was not immune mediated. Confocal microscopy showed that the bulk of the CAF-680 signal was localized within the lysosomes of CMs in the ischemic zone. Furthermore, CAF-680 activation was dramatically enhanced by RAP pretreatment, which exerts a significant anti-inflammatory effect. In fact, the exposure of neutrophils to RAP has been shown to attenuate their migration and chemotaxis.20,21 Taken together, these factors strongly suggest that the activation of CAF-680 within 4 hours of IR+RAP was a specific marker for the upregulation of CM autophagy.

**Impact of RAP in Ischemic Injury**
CM death within the first few hours of IR is mediated predominantly by apoptosis.22,23 Interestingly, we show here that although annexin positivity was detected in almost the entire AAR within the first few hours of IR, only a fraction of the AAR became TTC positive at 24 hours. This result is consistent with our previous

![Figure 3](http://circimaging.ahajournals.org/)

**Figure 3.** Noninvasive tomographic imaging of autophagy in the heart in vivo. **A** through **D**, Fluorescence molecular tomography–computed tomography images of a mouse after ischemia/reperfusion and rapamycin pretreatment (IR+preRAP). Hepatic fluorescence has been masked out. **A**, Left lateral view of the heart and skeleton. **B**, Posterior view of the mouse with the soft tissue structures included. The fluorescence signal in the apical third of the heart is substantially increased. Magnified views of the **C** lateral and **D** anterolateral aspects of the heart showing the increase in fluorescence activity in the injured anterior and lateral walls of the left ventricle. The tomographic nature of the fluorescence data is well demonstrated by the multiplanar reformats shown in **D, E**, Comparison of fluorescence activity in the heart in vivo after IR with rapamycin (IR+preRAP) and IR alone. *n=7 mice per group. AU indicates arbitrary units. *Statistically significant difference.
finding that the majority of annexin-positive myocardium (by magnetic resonance imaging) does not show evidence of late gadolinium enhancement (cell necrosis) 4 hours after IR.9 These results suggest that not all annexin-positive cells undergo irreversible cell death and that the expression of phosphatidylserine on the outer CM membrane is a partially reversible phenomenon.

The ability of phosphatidylserine positive myocardium to remain viable has also been demonstrated by Kenis et al,23 who found after brief ischemia that the expression of phosphatidylserine on the outer cell membrane of CMs was cyclic and did not correlate with histological evidence of apoptosis or necrosis. The duration of ischemia (35 minutes) used in our study was significantly longer and likewise showed that annexin uptake did not automatically correlate with CM death. We have also previously shown that the stabilization of the apoptotic cell membrane with an annexin-labeled nanoparticle attenuates cell rupture and death.24 A certain threshold of injury may thus be needed for an annexin-positive cell to fully execute the cell death cascade.25 Further study, however, is needed to fully elucidate the fate of annexin-positive cells.

RAP treatment was associated with a significant increase in CM autophagy and significant reductions in CM apoptosis and infarct size. These data suggest that RAP-induced autophagy in IR exerts a strong protective effect. It is possible that the impact of RAP on the phosphatidylinositol 3-kinase/AKT pathway and on neutrophil migration may also play a role in its cardioprotective effect.20,21,26 However, given the dramatic rise in CAF-680 activation produced by RAP, it is likely that these effects are secondary to the protective effect of markedly enhanced autophagy.

The digestion of dysfunctional cytoplasmic organelles in autophagy is well described.1 This process provides vital nutrients and energy to an injured or starving cell. Here, we show that RAP-induced autophagy also causes cells to increase the rate at which they endocytose nutrient-containing materials such as dextran-containing nanoparticles from the extracellular space.27 This likely further enhances the ability of the cells to maintain energy homeostasis in ischemic injury and may aid CM survival.

Postconditioning has been shown to exert a cardioprotective effect in both animal models and humans.28,29 We show here, however, that this effect is not mediated via increased autophagy. CAF-680 activation in the mice exposed to postconditioning was no higher than in those with conventional IR. This suggests that the ability of postconditioning to maintain mitochondrial integrity is sufficient to negate the need for the clearance of

![Figure 4](http://circimaging.ahajournals.org/)

Figure 4. Confocal microscopy of cathepsin-activatable near-infrared fluorochrome (CAF-680) activation in autophagic cardiomyocytes. Hearts from mice (n=7) with ischemia/reperfusion (IR)+prerapamycin (preRAP) were sectioned after fluorescence molecular tomography. A, CAF-680 activation produces fluorescent punctates in the area of injury. The septum (S) is injury free and is devoid of fluorescent punctates. B, At higher magnification, the CAF-680 signal can be seen to originate within cardiomyocytes. C, An increase in both the total fluorescence and the number of hyperintense punctates was seen in the ischemic zone vs the uninjured septum. The data have been normalized to the values in the septum. D, The autophagic marker lysosome-associated membrane protein-2 (LAMP-2), shown in red, is highly expressed in the ischemic zone of the IR+preRAP mice. CAF-680 fluorescence in green (E) is present in the same subset of cells and colocalizes well with LAMP-2 fluorescence, as seen in the fused image (F). Colocalization of the CAF-680 and LAMP-2 signals in the mice exposed to IR+preRAP occurred 79±5.8% of the time. Scale bar, 0.5 mm in A and 50 μm in B and D through F. B indicates border zone. *Statistically significant difference.

![Figure 5](http://circimaging.ahajournals.org/)

Figure 5. Mechanism of cathepsin-activatable near-infrared fluorochrome (CAF-680) activation by autophagic cells. A, A marked increase in lysosomal cathepsin-B activity was seen in CHO and H9C2 cells exposed to rapamycin (P<0.01 vs unexposed controls). B, Endocytosis of the nontargeted fluorescent nanoparticle, FER-680, from the surrounding medium was significantly increased in rapamycin-treated cells. C, Fluorescence microscopy showed that FER-680 (green, left) was internalized by autophagic cells and trafficked to the lysosome, where it colocalized strongly with the lysosome marker, LysoTracker Red (LTR, red). A fused image of FER-680 and Lysotracker Red (LTR) is shown in the right. Rapamycin exposure, thus, increases the delivery of diagnostic nanoparticles to the lysosomes of autophagic cells and simultaneously increases cathepsin activity within the lysosomes of these cells. Scale bar, 5 μm. *Statistically significant difference.
damaged mitochondria by autophagy. Preconditioning has also been shown to be cardioprotective and may exert effects on the CM similar to those of RAP pretreatment. This raises the possibility that the protective effect of preconditioning may in part be mediated by increased autophagy.

Approaches to Image Autophagy
Genetically engineered animals and cells that overexpress a fluorescent reporter protein construct are widely used to study autophagy. However, fluorochromes in the visible spectrum suffer from low tissue penetrance, high attenuation, and high background autofluorescence. These genetic reporter approaches thus are unsuited to quantitative or depth-resolved tomographic imaging of autophagy.

Although the approach presented here represents a significant advance in the imaging of autophagy, its potential for clinical translation is limited. The development of a cathepsin-activated drug and the making of a device for fluorescence tomography in humans are both formidable challenges. The development of positron emission tomography detectable agents to image autophagy in vivo will thus be of utmost importance. This offers not only a route toward clinical translation but also the potential to increase diagnostic sensitivity. The fluorescent approach described here, however, is of major preclinical value and has the potential to provide important mechanistic insights and to facilitate the development of novel pharmaceuticals.

Conclusions
The marked cardioprotective effect produced by RAP in this study is in agreement with previous observations in rats. The mice in the IR+postRAP cohort in this study were injected intravenously with RAP after the onset of myocardial ischemia and experienced a 45% reduction in infarct size. This raises the possibility of using RAP to reduce infarct size in patients presenting with acute coronary syndromes. The molecular mechanisms underlying the cardioprotective effect of RAP need to be more fully elucidated. Nevertheless, the demonstration that a well-tolerated and clinically approved agent has the potential to exert a marked cardioprotective effect is highly noteworthy.

We have shown here for the first time that quantitative tomographic imaging of autophagy can be performed in vivo. This has broad implications not only for cardiovascular disease but also for neurodegenerative disease and cancer. Serial in vivo imaging of the same animal will allow the natural history of autophagy and its pathophysiological impact to be better understood. Furthermore, our data suggest that the induction of autophagy by RAP exerts a powerful cardioprotective effect. This strategy is highly translatable and could be of significant value in reducing the potential of acute coronary syndromes to result in chronic heart failure.

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Disclosures
None.
**References**


**CLINICAL PERSPECTIVE**

Cardiomyocyte death, even in the presence of adequate reperfusion, remains a major clinical problem. Recently, a new response to ischemic stress, called autophagy, has been described. It remains unclear, however, whether autophagy in the heart is protective or deleterious. Thus, the aims of this study were the following: (1) to develop a mechanism to image autophagy in vivo to enhance the understanding of this process and (2) to investigate whether the induction of autophagy with rapamycin during ischemia/reperfusion would prove protective or deleterious. The imaging of autophagy was performed by exploiting the upregulation of lysosomal cathepsins during this process. Fluorescence imaging of a cathepsin-activatable fluorochrome was performed. In the mice injected with rapamycin, a large increase in the fluorescence signal was seen in the injured myocardium. This was not seen in the absence of rapamycin injection. The injection of rapamycin was associated with a significant reduction of apoptosis in the area at risk, as well as a reduction in final infarct size. This protective effect was seen even when the rapamycin was injected after coronary ligation. Further study is needed to determine whether the protective effects of rapamycin/autophagy are durable and are seen in large animal models as well.
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**Methods Supplement:**

**Micro-CT Imaging**

CT imaging was performed on a commercial micro-CT scanner (Inveon, Siemens Medical, Malvern PA). The mice were placed in a plexiglass holder with fiducial markers and imaged under isoflurane anesthesia. Iodinated contrast (Isovue-370, Bracco, Princeton NJ) was infused at 12.5 µl/min. The CT X-ray source operated at 80 kVp and 500 µA with an exposure time of 370 to 400 ms. The effective 3D resolution was 110 µm isotropic. The reconstruction protocol performed bilinear interpolation with a Shepp-Logan filter, and scaled pixels to Hounsfield units.

Fusion of the CT and fluorescence tomography (FMT) datasets was performed using the custom-built Medsquare software platform being developed in our group. The FMT datasets were resampled to match the resolution of the CT data. Thereafter, the fiducial markers were used to fuse the FMT and CT datasets. Segmentation of the CT images was performed using thresholds based on the gradient of signal intensity. A mask was applied to the fluorescence data to either include or exclude the signal from the liver. Volume rendering of the heart was performed using the segmented CT data. The fluorescence signal within the cardiac volume was then integrated, normalized by volume and mapped to an arbitrary scale.
Cathepsin and LAMP-2 Detection

Immunofluorescence staining of the lysosomal marker LAMP-2 was performed with a PE-conjugate antibody (SC-19991PE anti-mouse, Santa Cruz Biotechnology, Santa Cruz CA). 8-µm frozen sections were prepared from the hearts cryopreserved after FMT-CT imaging. The slides were fixed, permeabilized and stained per manufacturers instructions. Fluorescence microscopy was performed on a laser scanning confocal microscope (Zeiss LSM510, Carl Zeiss AG, Oberkochen, Germany). PE fluorescence was excited with the 561 nm Diode laser and acquired with the 575-615 nm band-pass filter.

The measurement of cathepsin activity in vitro was performed with a direct enzymatic assay. To induce autophagy, 5 µM rapamycin was added to CHO and H9C2 cells in minimal growth media. In the control wells, no rapamycin was added and cells were grown in complete growth media. After a 2-hour incubation (37°C, 5% CO₂) the cells were removed with a cell scraper and cathepsin-B activity was measured per manufacturers instructions. Briefly cell lysates were collected and normalized to protein concentration (BCA kit, Thermo Fisher Scientific, Waltham MA). After 1 hour incubation with the substrate Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-AMC) prepared in 0.1% Brij 35 solution (Sigma-Aldrich, St. Louis MO) at room temperature, fluorescence measurements were taken on a commercial detection system with 365 nm band-pass excitation and 410-460 nm long-pass emission filters (GLOMAX Multi+, Promega, Fitchburg WI).
Preparation of Feraheme-680

Amine-modified ferumoxytol (500 mg of iron, AMAG Pharmaceuticals, Lexington MA) and 1.21 mg of CyAl-NHS ester (synthesized in house) were mixed in 10 ml of PBS buffer. The mixture was reacted for 2 hours at room temperature under shaking. Conjugation products were purified by centrifugal filtration (Amicon Ultra 50K, Millipore, Billerica MA) and gel filtration (PD-10 column, GE Healthcare, Little Chalfont, UK). The number of CyAl per ferumoxytol particle was measured by spectrophotometry (Evolution 300, Thermo Scientific). The concentration of ferumoxytol was determined by absorbance at 300 nm. The concentration of CyAl was determined by absorbance at 674 nm with an extinction coefficient of 130,000 mM\(^{-1}\)cm\(^{-1}\). The final FER-680 nanoparticle has a hydrodynamic diameter of 16.6 nm.

The endocytosis of FER-680 was assessed in CHO and H9C2 cells. The cells were cultured for 2 hours (with and without rapamycin) as described above. Thereafter, FER-680 (2 \(\mu\)g iron) was added to 1 ml of medium and after an additional 4 hr incubation the cells were collected and washed 3 times with either minimal (experimental group) or complete (control group) growth media. Flow cytometry was performed to determine the number of cells labeled with FER-680.