Myeloperoxidase Inhibition Improves Ventricular Function and Remodeling After Experimental Myocardial Infarction

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HIGHLIGHTS

- The inflammatory enzyme MPO is a potential therapeutic target in cardiovascular diseases.
- PF-1355 is an orally bioavailable mechanism-based inhibitor of MPO enzymatic activity. PF-1355 treatment successfully inhibited MPO in mouse models of myocardial infarction and ischemia reperfusion injury.
- Short duration oral drug treatment for 7 days attenuated inflammation and cardiac dilation during early infarct healing. However, MPO-containing cells persisted beyond 7 days.
- Prolonged 21-day treatment improved ejection fraction (~44%) and decreased end-diastolic volume (~53%) and left ventricular mass (~33%) compared with untreated control subjects.
- Better therapeutic effect was also achieved when treatment was started early (at 1 h) after the initial ischemic insult.

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PF-1355 is an oral myeloperoxidase (MPO) inhibitor that successfully decreased elevated MPO activity in mouse myocardial infarction models. Short duration PF-1355 treatment for 7 days decreased the number of inflammatory cells and attenuated left ventricular dilation. Cardiac function and remodeling improved when treatment was increased to 21 days. Better therapeutic effect was further achieved with early compared with delayed treatment initiation (1 h vs. 24 h after infarction). In conclusion, PF-1355 treatment protected a mouse heart from acute and chronic effects of MI, and this study paves the way for future translational studies investigating this class of drugs in cardiovascular diseases. (J Am Coll Cardiol Basic Trans Science 2016;1:633-43) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Myocardial infarction (MI) triggers an inflammatory cascade, where various local (ischemia, oxidative stress, endothelial, and myocardial dysfunction) and systemic factors (inflammatory cell recruitment, neuroendocrine disturbances) perform a complex interplay, which may lead to tissue fibrosis, ventricular dilation, and adverse remodeling (1), possibly culminating in sudden death or heart failure (2,3). Up to 24% (4,5) of patients after acute MI go on to develop heart failure accompanied with repeated hospitalizations, morbidity, and mortality. Successful interventional therapies have significantly improved the outcome after an acute coronary event (6) and are now a standard of care across the globe.

However, decreased mortality after acute MI has also skewed the patient population to an increased incidence of developing heart failure within both acute and chronic settings (5). Moreover, long-term survival in these patients with established therapies has not improved over the course of the last decade, and heart failure may even have slightly worsened according to a recent study (7). These factors underpin the importance of discovering new therapeutic drugs that complement current post-MI pharmacological treatment with a potential to improve left ventricular (LV) dilation, ventricular remodeling, and as a result, better long-term functional outcome (8).

Inflammation plays a central role in the evolution of pathological events after acute MI (9). Neutrophils followed by monocytes are recruited to the infarcted myocardium and deploy their inflammatory and proteolytic contents, including the enzyme myeloperoxidase (MPO), in the extracellular tissue environment (10). MPO, a highly abundant enzyme in neutrophils and inflammatory lymphocyte antigen 6C (Ly-6C)\textsuperscript{high} monocytes (11), is capable of inducing tissue oxidative damage (12). In addition to its role in various stages of atherosclerotic plaque formation, MPO is linked with continuous activation and recruitment of leukocytes to infarcted tissue (13), further potentiating the downstream inflammatory cascade. At the same time, its oxidized products (hypochlorite) activate proteolytic enzymes (14) and break down extracellular matrix with resultant adverse ventricular remodeling.

Indeed, studies involving MPO knock out mice (MPO\textsuperscript{-/-}) indicate that both the direct cytotoxic aldehyde products generated by MPO-mediated reactions (15) and leukocyte recruitment and proteolysis (13) are involved in adverse outcome after acute MI and heart ischemia reperfusion injury (IRI). As such MPO has been advocated as a prognostic and risk stratification marker in cardiovascular diseases (16). MPO plasma levels strongly predict coronary disease prevalence (17) associated with adverse outcome (18) and need of revascularization (19). These data also signify MPO as a potentially important therapeutic target (8). Thus, drugs that target MPO may help ameliorate degree of inflammation, oxidative damage, and ventricular remodeling after acute MI.

In this study, we investigated a novel MPO inhibitor, PF-1355 \textsc{(2-[6-(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl]acetamide)}, in mouse models of MI and IRI. PF-1355 is a highly selective, mechanism-based, orally administered MPO
inhibitor, which has been shown to be efficacious in a murine model of vasculitis with reduced disease severity, edema, neutrophil accumulation, and inflammation (20). PF-1355 is structurally related to a clinical candidate MPO inhibitor currently under development (21). We hypothesized that successful MPO inhibition with PF-1355 would result in decreased leukocyte recruitment and improved LV remodeling and function, and would provide insight to potential clinical applications leading to facilitate translation of this class of MPO inhibitors for cardiovascular diseases.

METHODS

Methods detailing animal models, cardiac magnetic resonance (CMR) imaging, echocardiography, MPO enzymatic activity assays, histology, flow cytometry, and statistical analysis are provided in the Supplemental Appendix.

RESULTS

PF-1355 simulant human and mouse MPO in vitro and ex vivo. We first tested the inhibitory potential of PF-1355 against purified human MPO and found the half-maximal inhibitory concentration of PF-1355 to be 0.56 μmol/l for MPO peroxidation activity (Supplemental Figure 1A). We then tested on mouse MPO extracted from MI and compared it with an intrinsic control without the drug. More than 80% of MPO control activity was reduced with a dose as little as 0.61 μmol/l (Supplemental Figure 1B). Mouse MPO obtained from bone marrow neutrophils was also inhibited with the similar potency (Supplemental Figure 1C).

Next, we determined the therapeutic efficacy of PF-1355 under biologically relevant conditions. For this purpose, mice received 50 mg/kg PF-1355 orally starting within 1 h post-surgery for 2 days, and hearts were processed to extract protein from extracellular fraction (ECF) and intracellular fraction (ICF) of the infarct tissue, as described previously (22). MPO activity was significantly reduced in both ECF and ICF as compared with vehicle-treated control subjects (p = 0.04 and p = 0.006, respectively) (Figure 1A). ECF MPO is important, as it is implicated in oxidative stress, host tissue damage (23,24), and neutrophil extracellular trap formation (25). ICF MPO (lysosomal or intragranular MPO) was also inhibited, indicating that the agent may enter inflammatory cells. Note that the infarct fractions of vehicle-treated mice had similar MPO activity when compared with untreated infarcts (Supplemental Figure 1D), indicating that the vehicle treatment was inert. Moreover, PF-1355 plasma levels measured in animals with MI on day 2 showed that the average PF-1355 exposure was maintained above 3.1 μM (Supplemental Table 1), which exceeds the half-maximal effective concentration level in LPS-stimulated human blood (20).

PF-1355 inhibits MPO in vivo in mouse MI and IRI. MPO-Gd (bis-5-hydroxytryptamide-diethylene-triaminepentaacetate-gadolinium) is an activatable magnetic resonance imaging agent for reporting extracellular MPO activity (26,27). It has been validated for noninvasive MPO-specific imaging in MI and was shown to be able to follow therapeutic response to atorvastatin post-MI (10). To evaluate the effects of PF-1355 in vivo, we performed MPO-Gd magnetic resonance imaging in mice on day 2 after MI (Figure 2A), which corresponds to the acute inflammatory phase known to have high neutrophils and inflammatory monocyte numbers (10). As expected, the imaging metric lesion activation ratio (LAR) was elevated in the infarcted tissue. LAR reflects the amount of imaging agent activated by MPO over background, and was measured as a ratio of delayed enhancement at 60 min over nonspecific early enhancement at 15 min, as described previously (27). In the treated group, the LAR significantly decreased (p = 0.0003) (Figure 1C). In addition, absolute contrast to noise ratio (CNR) at 60 min was also reduced in the treated group (p = 0.04) (Figures 1C and 1D). These results were similar to the ex vivo ECF MPO inhibition with PF-1355 and confirmed the in vivo drug efficacy noninvasively. MPO-positive infarct areas were not significantly different between the two groups (p = 0.22) (Figures 1B and 1E).

To validate in vivo MPO inhibition by PF-1355 in reperfusion injury, mice were subjected to transient coronary ligation and then treated for 2 days until MPO-Gd imaging (n = 5 to 8/group) (Supplemental Figure 2A). Both LAR and absolute CNR at 60 min were decreased significantly in the treated groups compared with the vehicle-treated control subjects (p = 0.045 and p = 0.01, respectively) (Supplemental Figure 2B). MPO-positive infarct areas were again found to be similar in both groups (p = 0.6) (Supplemental Figure 2C). These results confirmed successful in vivo MPO inhibition after IRI by PF-1355. Moreover, when CNR values were plotted as a function of time course, we observed decreased enhancement at all time points in treated groups compared with vehicle control subjects, again confirming that PF-1355 decreased imaging agent activation and retention (Supplemental Figure 2D).

PF-1355 reduces inflammation at 7 days post-MI. To investigate the effects of PF-1355 on leukocyte recruitment and early infarct healing,
immunoreactive staining was performed on heart infarcts harvested on day 7 and compared with vehicle-treated control subjects \( (n = 5/\text{group}) \). We found a significant decrease in MPO and CD11b-positive areas within infarcts in the treated group compared with the vehicle control subjects \( (p = 0.02 \) and \( p = 0.04 \), respectively) (Figure 2A). However, CD31 (angiogenesis) and collagen I–positive areas were not different between the groups \( (p > 0.05) \) (Figure 2B). To investigate the effect of PF-1355 on early myocardial remodeling on day 7, we measured myocardial thickness at the level of the midventricular slice containing infarct. Interestingly, infarcted walls were thicker in the treated mice as early as day 7 \( (p = 0.02) \) (Figure 2C).

Flow cytometry analysis of infarct tissue leukocytes from mice treated for 7 days revealed a trend toward moderately decreased neutrophils (relative to total leukocytes) compared with vehicle control subjects \( (p = 0.1) \) (Figure 2D). Interestingly, inflammatory Ly-6Chigh monocytes were significantly decreased in the treated group \( (p = 0.04) \) (Figure 2D). However, Ly-6Clow monocytes were not affected by the drug treatment \( (p = 0.14) \), indicating that PF-1355 acts...
through a decrease in recruitment of inflammatory Ly-6C<sup>high</sup> monocytes, but numbers of reparative Ly-6C<sup>low</sup> monocytes were maintained or perhaps even increased.

**MPO-POSITIVE MYELOID CELLS ARE PRESENT IN INFARCTS BEYOND DAY 7, DURING LATE INFARCT REMODELING.** Neutrophils followed by inflammatory Ly-6C<sup>high</sup> monocytes are the main source of MPO after infarction (10). Their numbers peak during the initial pro-inflammatory phase of infarct healing (from days 1 to 3) and start to decline afterwards (28). However, continuous MPO exposure and resultant cytotoxic aldehyde products potentially mediate long-term deleterious effects on ventricular remodeling, even during chronic healing phase (15). Therefore, we also evaluated if there were increased MPO and inflammatory cell subsets during the later phase of infarct remodeling (days 14 to 15) and compared with noninfarcted hearts. On histology, we found significantly more MPO-positive cells on both days 7 and 14, although there were approximately 45% less on day 14 compared with day 7 (p < 0.05) (Figures 3A and 3B). Similar results were observed for CD11b staining (p < 0.05) (Figures 3A and 3B).
On flow cytometry, significantly higher CD11b+ myeloid cell numbers were observed in the infarcted hearts on day 7 (p < 0.01) (Figure 3C). Interestingly, myeloid cells were still elevated on day 15 (neutrophils ~4× and Ly6C<sub>high</sub> monocytes ~3.5× elevated; p < 0.01 and p < 0.05, respectively) (Figure 3C) compared with noninfarcted hearts. Consistent with tissue repair, higher numbers of macrophages (p < 0.01) and Ly-6C<sub>low</sub> cells (p < 0.05) were detected on day 15 (Supplemental Figure 3A). These results were corroborated by detection of increased neutrophils and Ly-6C<sub>high</sub> monocytes in the blood of infarcted mice (p < 0.05, p = NS, and p < 0.05, respectively) (Supplemental Figure 3B).

Taken together, elevated neutrophils and Ly-6C<sub>high</sub> monocytes on flow cytometry combined with positive staining for MPO on histology indicated a continuous presence of MPO in the infarcted heart through days 14 to 15 (although less pronounced than on day 7). Concurrently, repair and healing had also been initiated, as evidenced by increased macrophages and Ly-6C<sub>low</sub> cells.

PROLONGED PF-1355 THERAPY IMPROVES CARDIAC FUNCTION AND REMODELING. Oxidized products of MPO-catalyzed reactions have previously been implicated in late infarct remodeling observed on day 21 in mouse models of MI and IRI (13,15). Given our
data that infarcted myocardium is continually exposed to MPO beyond the first week, we decided to investigate whether PF-1355 therapy should also be continued during the late remodeling phase. For this purpose, we divided infarcted mice into 2 treatment cohorts. In the first cohort, we treated mice for 7 days (early healing phase; n = 8) and then treatment stopped until day 21 to see whether this short treatment trial during the proinflammatory phase would benefit remodeling (Figures 4A and 4B). Indeed, we found significant improvement in end-diastolic volume (EDV) compared with untreated mice with MI (p < 0.05) (Figure 4E). However, we did not see significant differences in ejection fraction (EF) (Figure 4D) and LV mass (Figure 4F), although a trend toward improvement was noted between the control and 7-day treatment groups.

In the second cohort, mice were treated for the entire 21 days (n = 5) and compared with untreated mice with MI (Figures 4A and 4C). In contradistinction to the short-term treatment group, these mice not only had decreased EDV (p < 0.001) (Figure 4E), but also improved EF (p < 0.05) (Figure 4D) and LV mass compared with untreated control subjects (p < 0.001).
In this study, we report that the MPO inhibitor PF-1355 efficiently inhibited human and mouse MPO both in vitro and in vivo in mouse models of MI and IRI. PF-1355 treatment for 7 days decreased myeloid cell recruitment and MPO presence. However, MPO-secreting neutrophils and Ly-6C<sup>high</sup> monocytes remained elevated beyond day 7 in untreated infarcts, and likely continued to promote a pro-inflammatory and oxidative environment that adversely affected healing. Indeed, prolonged (21 days) MPO inhibition improved ejection fraction, end-diastolic/systolic volume and LV hypertrophy over short (7 days) treatment. In addition, initiating treatment immediately following MI provided greater therapeutic benefit than initiating treatment 24 h post-MI, consistent with early elevation of MPO in plasma.

Improved diastolic and systolic function with MPO inhibition is associated with a decrease in recruitment of myeloid cells, in particular inflammatory Ly-6C<sup>high</sup> monocytes, as shown by our data on day 7. As they are active during both inflammatory and reparative phases, excessive numbers of Ly-6C<sup>high</sup> monocytes have a detrimental role in the final outcome of infarct healing, and successful therapeutic strategies have been employed against these cells (29–31). Interestingly, when we treated the mice for the first 7 days only (early healing phase) and then stopped treatment until day 21 (reparative/late remodeling phase), there was less beneficial effect. This strongly suggests that MPO has a detrimental role during both early and late stages of infarct evolution. Although it is most abundant during the first week post-MI, we detected continued elevated MPO levels to at least day 15, when there is also evidence for a healing environment (concomitant presence of macrophages and Ly-6C<sup>low</sup> monocytes). It is conceivable that during later stages of infarct remodeling, MPO inhibition may favorably tip the balance toward reparative cellular pathways, resulting in better chronic outcome.

When initial infarct areas were measured with magnetic resonance imaging on day 2, we did not observe significant differences between the control and treatment groups, both in MI and IRI. These findings were in line with studies on MPO knock-out mice, which indicated that MPO did not affect initial infarct size at day 3 post-IRI (15). However, MPO had a profound adverse effect on chronic LV remodeling and function that was thought to be mediated by MPO-oxidized aldehydes (15). Moreover, we found that MPO inhibition also decreased the recruitment of pro-inflammatory cells, suggesting another mechanism for MPO inhibition that may work in synergy with oxidative damage protection to improve long-term LV remodeling.

Plasma MPO levels elevate within h in patients with acute coronary syndrome (19) and predict early risk of MI and adverse outcome (18). Also, neutrophils mobilize quickly following ischemic insult, and their numbers already peak at 24 h post-MI (28), with concomitant accumulation of MPO-generated oxidants within the infarct (15). Therefore, early therapy commencement is also important to protect the myocardium from MPO released by infiltrating leukocytes. It is also noteworthy that neutrophils are

(Figure 4F). Furthermore, when compared with the 7-day treatment cohort only, we observed a significant improvement in EDV and LV mass (p < 0.05) (Figures 4E and 4F), confirming that prolonged treatment is optimal for better structural outcome. Day 2 CMR was also performed to confirm the presence and extent of the infarct, which is shown in the Supplemental Appendix. Representative videos from day 2 and day 21 CMR (Supplemental Video 1) are also included.

**Maximum Therapeutic Benefit is Achieved with Early Treatment Initiation.** Plasma MPO has been identified as one of the earliest prognostic biomarkers in patients with acute coronary syndrome (18,19). Therefore, we investigated whether the time to initiate treatment has an effect on efficacy compared with vehicle-treated control subjects. PF-1355 treatment was initiated either at 1 h post-MI (early) or 24 h post-MI (delayed), and was then continued for 28 days. Cardiac function was evaluated by echocardiography at baseline and 28 days post-surgery (Figure 5A). At the end of the study, animals in both treatment groups showed better function as measured by improved LVEF, fractional shortening, and stroke volume compared with vehicle-treated MI control subjects (p < 0.0001, p < 0.001, and p < 0.01, respectively) (Figures 5B to 5D). Importantly, the early treatment initiation group showed significantly reduced cardiac remodeling with improved LV end-systolic volume (p < 0.05), whereas the delayed treatment-initiation group did not improve relative to controls (p = NS) (Figure 5E). Functional parameters like fractional shortening and EF were also significantly better with early compared with delayed treatment initiation (p < 0.05) (Figures 5B and 5C). These data indicate that the maximal therapeutic benefit for an MPO inhibitor was realized by initiating treatment as proximal to the event as possible.

**Discussion**

In this study, we report that the MPO inhibitor PF-1355 efficiently inhibited human and mouse MPO both in vitro and in vivo in mouse models of MI and IRI. PF-1355 treatment for 7 days decreased myeloid cell recruitment and MPO presence. However, MPO-secreting neutrophils and Ly-6C<sup>high</sup> monocytes remained elevated beyond day 7 in untreated infarcts, and likely continued to promote a pro-inflammatory and oxidative environment that adversely affected healing. Indeed, prolonged (21 days) MPO inhibition improved ejection fraction, end-diastolic/systolic volume and LV hypertrophy over short (7 days) treatment. In addition, initiating treatment immediately following MI provided greater therapeutic benefit than initiating treatment 24 h post-MI, consistent with early elevation of MPO in plasma.

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important orchestrators of post-MI infarct evolution. A dramatic decrease in neutrophils with anti-Ly6G antibody pre-treatment has been shown to impair infarct remodeling, with increased fibrosis and αSMA immunostaining (32). PF-1355 only moderately decreased neutrophil recruitment to infarct at day 7 post-MI. It did not affect neutrophil survival, as blood neutrophils were still similar between groups (p = 0.66) (Supplemental Figure 4A). Concurrently, collagen I and αSMA positive areas on histology were similar between 2 experimental groups (αSMA: p = 0.35) (Supplemental Figures 4B and 4C), indicating that moderate neutrophil decrease did not adversely affect cardiac healing. Another important difference was that in our study, we administered PF-1355 after MI, but in the previous study, the anti-Ly6G antibody was administered 1 day before MI. Although the MPO molecule can attract neutrophils independent of its catalytic activity (33), this effect is not expected to be altered by PF-1355, which is an inhibitor of MPO’s enzymatic activity and does not directly affect MPO concentration.

Percutaneous coronary intervention is the standard primary therapy in ST-segment elevation MI. However, sudden restoration of blood supply induces more oxidative stress to the already ischemic myocardium, and incoming leukocytes potentiate injury by releasing their granular contents containing MPO. Importantly, the ability of PF-1355 to inhibit MPO in a reperfusion injury model confers another
potentially translatable clinical advantage: it may be used in conjunction with percutaneous coronary intervention and protect against reperfusion injury. Drug efficacy can also be monitored noninvasively with MPO molecular imaging.

In addition to MI, MPO and its oxidized products play an important role in a variety of cardiovascular diseases. During the development of atherosclerosis lesions, MPO confers its deleterious effects by causing endothelial dysfunction (34) and both low- (35) and high-density lipoprotein modification (36,37). It also contributes to vulnerable plaque formation and rupture, and acute thrombosis (12). Clinical studies predict that MPO is associated with both diastolic and systolic dysfunction as well as increased mortality (38,39). Due to these broad-spectrum detrimental effects, MPO has been increasingly considered an important therapeutic target for other cardiovascular diseases as well (40). Whether or not MPO inhibition has a protective role against various stages of atherogenesis is under investigation, and it will be very interesting to see the results of these studies.

STUDY LIMITATIONS. It is important to mention that only young adult mice were used in the study. Old age is an important risk factor in cardiovascular diseases, and we do not know yet whether it affects PF-1355 therapy. Future studies utilizing older mice may shed more light upon this.

CONCLUSIONS

This study demonstrates that PF-1355 is a highly effective oral inhibitor of MPO enzymatic activity in vitro and in mouse models of MI and IRI. MPO inhibition for 7 days resulted in decreased MPO and CD11b expression, as well as inflammatory Ly-6C<sup>high</sup> monocytes. Because MPO is secreted beyond the early inflammatory phase, continuous MPO inhibition for 21+ days significantly improved ejection fraction, EDV/end-systolic volume, and LV mass on CMR. Our findings also support that MPO inhibitor treatment should be initiated soon after infarction to maximize therapeutic benefit. Upon successful translation, this class of drugs could be of great benefit to patients after an acute coronary event, both with and without percutaneous intervention.

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