Pharmacological inhibition of protein tyrosine phosphatase 1B (PTP1B) protects against atherosclerotic plaque formation in the LDLR−/− mouse model of atherosclerosis.

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Abstract

Cardiovascular disease (CVD) is the most prevalent cause of mortality among patients with Type 1 or Type 2 diabetes, due to accelerated atherosclerosis. Recent evidence suggests a strong link between atherosclerosis and insulin resistance, due to impaired insulin receptor (IR) signalling. Here we demonstrate that inhibiting the activity of protein tyrosine phosphatase 1B (PTP1B), the major negative regulator of the IR prevents and reverses atherosclerotic plaque formation in LDLR−/− mouse model of atherosclerosis. Acute (single dose) or chronic PTP1B inhibitor (trodusquemine) treatment of LDLR−/− mice decreased weight gain and adiposity, improved glucose homeostasis and attenuated atherosclerotic plaque formation. This was accompanied with a reduction in both, circulating total cholesterol and triglycerides, a decrease in aortic monocyte chemoattractant protein-1 (MCP-1) expression levels, and hyperphosphorylation of aortic Akt/PKB and AMPKα. Our findings are the first to demonstrate that PTP1B inhibitors could be used in prevention and reversal of atherosclerosis development and reduction of CVD risk.

Keywords:

Insulin, insulin resistance, insulin receptor, AMPK, atherosclerosis.

Clinical Perspective:

i) Background: Cardiovascular disease (CVD) is the most prevalent cause of mortality among patients with Type 1 or Type 2 diabetes due to accelerated atherosclerosis.

ii) Summary of Results: Inhibiting the activity of PTP1B prevents and reverses atherosclerotic plaque formation in LDLR−/− mouse model of atherosclerosis, and is associated with a decrease in aortic MCP-1 expression levels, hyperphosphorylation of aortic Akt/PKB and AMPKα.

iii) Potential significance: Our findings are the first to demonstrate that PTP1B inhibitors could be used in prevention and reversal of atherosclerosis.
1. Introduction

CVD is a general term used to describe all conditions affecting the heart and blood vessels and is responsible for almost a third of deaths worldwide (WHO Statistics, http://www.who.int/mediacentre/factsheets/fs317/en/). Many of the conditions that contribute to CVDs are due to a narrowing and hardening of the blood vessels through a process known as atherosclerosis, arising due to lipid accumulation which, over time, develops into plaques. Subsequently, these atherosclerotic plaques can lead to ischaemic injury by a number of mechanisms such as complete occlusion of the blood vessel or, alternatively, the plaque may become unstable and rupture resulting in thrombosis [1,2]. This process may be exacerbated by risk factors encompassing genetic aspects, lifestyle choices such as smoking, excessive drinking, physical inactivity and obesity or conditions such as diabetes [3]. Indeed, in both type 1 and type 2 diabetic patients, a high proportion of mortality is associated with CVD, where defective insulin signalling leads to endothelial dysfunction and accelerated atherosclerosis. The mechanism contributing to this pathology is somewhat unclear; however, it has been suggested that insulin resistance and hyperglycaemia results in intracellular metabolic changes leading to oxidative stress and chronic low-grade inflammation [4]. Therefore, clarification of the mechanism controlling these diseases is needed to enable the design of more targeted and effective therapeutics.

In support of a link between defective insulin receptor (IR) signalling and atherogenesis, it was found that apolipoprotein-E-deficient mice (ApoE−/−) devoid of IR in the vascular endothelium had increased plaque development [5]. Moreover, ApoE−/− mice with a heterozygous deletion of the IR and its downstream target, Insulin Receptor Substrate 1 (IRS1), also develop accelerated atherosclerosis [6], as
well as mice lacking insulin receptor substrate 2 (IRS2\(^{-/-}\)) [7]. Furthermore, decreased insulin signalling in nonhematopoietic cells, as achieved by transplantation of ApoE\(^{-/-}\) mouse model of atherosclerosis with bone marrow cells from IRS1\(^{+/+}\) IR\(^{+/+}\) ApoE\(^{-/-}\) mice, contributed to increased atherogenesis in these mice [6]. Finally, mice that were devoid of both LDLR\(^{-/-}\) and Akt2, an important downstream component of the IR signalling cascade, exhibited impaired glucose homeostasis, elevated insulin and cholesterol levels and developed more complex atherosclerotic plaques [8]. Therefore, targeting components that inhibit IR signalling could prove an effective therapeutic.

Protein tyrosine phosphatase (PTP)1B has been identified as the major negative regulator of the IR itself [9]. In mice, whole body PTP1B\(^{-/-}\) studies established PTP1B as a major regulator of insulin sensitivity and body mass, via inhibition of insulin and leptin signalling, respectively [10,11]. Our recent data suggested that hepatic-specific deletion of PTP1B, in addition to improving glucose and lipid homeostasis and increasing insulin sensitivity, was protective against endothelial dysfunction in response to high fat diet (HFD) [12]. This was also associated with decreased hepatic inflammation in these mice [13]. Specifically, mice lacking hepatic PTP1B exhibited decreased systolic and diastolic blood pressure, in response to HFD feeding, when compared to control littermates [12]. Furthermore, we have also shown that myeloid-specific PTP1B deletion can protect against HFD-induced inflammation and hyperinsulinaemia and is facilitated by an increase in the secretion of the anti-inflammatory cytokine interleukin (IL-10) and a decrease in pro-inflammatory TNF\(\alpha\) cytokine secretion [14]. Since atherosclerosis is regarded as a chronic low level inflammatory disease [15-18], we hypothesized that targeting PTP1B activity using a PTP1B specific inhibitor Trodusquemine [19], could prove
effective in prevention and possibly reversal of atherosclerotic plaque formation. This would enable direct testing of the translational potential of PTP1B inhibitors [20], which are in Phase II clinical trials for diabetes treatment, and Phase I clinical trials for breast cancer treatment (https://clinicaltrials.gov/ct2/show/NCT02524951), as treatments for atherosclerosis and reduction of CVD risk. To directly test this, we used the LDLR−/− mouse model of atherosclerosis, under physiological and obesogenic conditions.

2. RESEARCH DESIGN AND METHODS

2.1 Animal studies. All animal procedures were performed under a project license approved by the U.K. Home Office under the Animals (Scientific Procedures) Act 1986 (PPL 60/3951). Eight week old male LDLR−/− mice were purchased from Jackson Labs, individually housed and maintained at 22–24°C on 12-h light/dark cycle with free access to food/water. Following two weeks of acclimatisation time, mice were placed on chow or high fat diet (HFD 42% from fat, 0.2% cholesterol, Envigo, Huntingdon UK) for 12 weeks and weighed weekly to monitor weight gain.

2.2 Drug treatments. The PTP1B inhibitor Trodusquemine was obtained from Dr N Tonks (Cold Spring Harbor, USA). After 1 week HFD, 20 mice were injected intraperitoneally (I.P.) with the PTP1B inhibitor Trodusquemine (10mg/kg), followed by 4 subsequent weekly injections at 5mg/kg, as previously described for ob/ob mice [19] and a 6 week washout period. These were designated the chronic group, whereas the remaining mice were injected with saline. After 8 weeks HFD, a further 20 mice were injected with a single dose of 10mg/kg Trodusquemine and designated accordingly, followed by a 4 week washout period. At week 12 of HFD, mice were fasted for 5h and injected with either saline or insulin (10 mU/g body weight) for 10
mins prior to culling by CO$_2$ inhalation and subsequent cervical dislocation. **Trodusquemine** treatment was halted prior to the end of the study to ensure that the procedure of treatment (by intraperitoneal injection) did not affect the terminal signalling experiment by altering stress hormone levels and thus adversely affecting insulin signalling. Heart and aortic tissues were harvested and collected for further analysis. Tissues for subsequent western blot or qPCR analysis were frozen in liquid nitrogen and stored at -80°C until needed, whereas tissues for histology were immersed in formalin for 24h at 4°C, then stored at 4°C in PBS until analysed.

2.3 **Glucose Tolerance Tests.** Mice were fasted for 5h prior to commencement of glucose tolerance tests (GTT). Briefly, baseline glucose levels were sampled from tail blood using glucose meters (AlphaTRAK, Abbott Laboratories, Abbot Park, IL, USA). Subsequently mice were injected I.P. with 20% glucose (w/v) and blood glucose measured at 15, 30, 60 and 90 mins post-injection.

2.4 **Body Fat Mass Composition.** The body composition of each mouse was analysed using an Echo MRI-3-in-1 scanner (Echo Medical Systems, Houston, TX, USA).

2.5 **Immunoblotting.** Frozen aorta tissues were homogenised in 300µl of ice-cold Radioimmunoprecipitation assay (RIPA) buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA pH 8.0, 1mM NaF, 0.1% SDS, 1% Triton X-100, 1% Sodium Deoxycholate with freshly added 1mM NaVO$_4$ and protease inhibitors) using a PowerGen 125 homogeniser and lysates normalised to 1µg per 1µl. Proteins were separated on a 4-12% Bis-Tris gel by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were probed for the following targets (Cell Signaling); p-IR
(Tyr 1162/1163), IR β-chain, p-AKT (Ser 473), p-p38 (The 181/Tyr 182), total p-38 p-S6 (Ser 235/236), total S6, p-AMPKα (Thr 172), total AMPKα, PTP1B and GAPDH.

2.6 RNA extraction and qPCR. Frozen tissues were lysed in Trizol reagent (Sigma, UK) and RNA isolated using phenol/chloroform extraction according to manufacturer’s instructions. RNA was then synthesized into cDNA using tetrokits (Bioline) and subjected to qPCR analysis using SYBER and LightCycler 480 (Roche). Gene expression of ICAM-1, VCAM-1 and MCP-1 was determined relative to the reference gene ELF1.

2.7 Histology. Immediately following cervical dislocation, hearts were immersed in formalin and stored at 4°C for 24hrs, before being transferred to PBS until further analysis. Hearts were bisected to remove the lower ventricles, frozen in OCT and subsequently sectioned at 5µm intervals until the aortic sinus was reached. Sections were mounted and stained with oil red O to assess plaque formation. Images were captured using a light microscope and plaque formation quantified using Image J software.

2.8 Serum Analysis. Blood was collected during terminal procedures after fasting (5hrs) and spun at 5,000g to isolate serum, then stored at -80°C. Serum samples were subsequently analysed for insulin using ELISA (R&D Systems) or total cholesterol and triglycerides (Sigma).

2.9 Statistical Analysis. We expressed all values as mean ± S.E.M. We determined group sizes by performing a power calculation to lead to an 80% chance of detecting a significant difference (P ≤ 0.05). For both in vivo and ex vivo data, each n value corresponds to a single mouse. Statistical analyses were performed by
using one-way or Two-way ANOVA, followed by Tukey's or Dunnet's multiple-comparison tests to compare the means of three or more groups or by an unpaired two-tailed Student's t-test to compare the means of two groups. Variances were similar between groups. In all figures, \*#/p\leq0.05, **#/p\leq0.01, ####/p\leq0.001, ####/p\leq0.0001. All analyses was performed using GraphPad Prism (GraphPad Software).
3. Results

3.1 PTP1B inhibitor treatment decreases body weight and improves global glucose homeostasis.

The exponential rise in patients presenting with obesity and type 2 diabetes has resulted an increased interest from pharmaceutical companies for the use of PTP1B inhibitors as a potential therapeutics [20]. Given there is increasing evidence implicating defective insulin signalling as a major contributor to the pathogenesis of atherosclerosis, we hypothesised that PTP1B inhibition should have beneficial protective effects. To determine if global PTP1B inhibitors would attenuate plaque formation, we used the LDLR<sup>−/−</sup> mouse model of atherosclerosis [21]. We assessed whether either a single dose and/or chronic PTP1B inhibition could slow or reverse atherosclerotic plaque formation in mice fed a high-fat (HFD) diet (or chow as control). The PTP1B inhibitor trodusquemine was selected as this drug has been reported to be more specific than previously synthesised compounds, since it binds and inhibits allosterically rather than at the catalytic domain which is highly conserved between other tyrosine phosphatase family members [22]. Furthermore, this drug is currently in phase I trials in breast cancer patients (https://clinicaltrials.gov/ct2/show/NCT02524951), after previously being tested as a treatment for type 2 diabetes and obesity.

Mice were treated chronically with trodusquemine, or given a single dose after 8 weeks of HFD feeding (Fig. 1A). Similar to whole body PTP1B deletion [10], and treatment of ob/ob mice using these inhibitors [19], chronic global inhibition of PTP1B prevented weight gain in both, chow and HFD fed mice, when compared to saline controls (Fig. 1B,C), and led to decreased fat mass (Fig. 2A,B). Lean mass
was significantly decreased after 6 weeks of treatment in chronically treated mice (Fig. 2C, D), when compared to vehicle controls, but remained stable over weeks 8 to 10, with no significant reduction in muscle mass when compared to vehicle-treated animals. Following 8 weeks on HFD, a single dose of trodusquemine led to a 20% reduction in body weight (Fig. 1B), with a greater than 50% reduction in fat mass, that accounted for the majority of the effect in weight loss (Fig. 2A). In addition, in agreement to what has been previously shown [19], trodusquemine exposure led to reduced food intake in both HFD-fed and CHOW-fed cohorts (Supplemental Fig. 1A, B). This was evidenced at week 9 which was one week following commencement of the single dose cohort. Interestingly, even though the chronic group had ceased drug treatment after week 6, a significant reduction in food intake was still present. However, PTP1B activity assays performed on liver tissues collected at week 12 during terminal culls revealed no significant inhibition of PTP1B remained in drug treated cohorts (Supplemental Fig. 1C). The lack of inhibition observed is likely due to drug washout, since single dose and chronic cohorts had 4- and 6-weeks, respectively, in the absence of inhibitor prior to culling. Finally, in both, chronic- and single dose-inhibitor treated mice, there was also a significantly improved glucose handling in HFD-fed mice at all time-points (weeks 8, 10 and 12 (Figure 3A, C and E, respectively)) whereas, this was not evident in chow-fed mice at week 12 (Figure 3F).

Previous research has shown HFD-fed mice develop hyperinsulinaemia [23,24]. In agreement with these studies, HFD led to an increase in circulating insulin levels in saline treated mice (Figure 4A), whereas, a significant decrease in circulating insulin levels was observed in HFD-fed, but not that of chow-fed mice, treated either with a single dose or chronically with trodusquemine (Figure. 4A, B
respectively). Therefore, global inhibition of PTP1B, using PTP1B specific inhibitor, mirrored results previously observed in whole body PTP1B knockout mice, with regards to beneficial effects on body weight, adiposity and glucose homeostasis maintenance [10,11].

**3.2 PTP1B inhibitor treatment protects against and reverses obesity-induced increase in atherosclerotic plaque area.**

Increased blood lipid and lipoproteins are widely used as biomarkers to predict CVD risk [25]. To assess the lipid lowering potential of PTP1B inhibition, lipid analyses were performed. A single dose and chronic treatment with trodusquemine resulted in significantly decreased serum cholesterol (Figure 5A) and triglyceride levels (Figure 5B) in HFD-mice. A similar decrease was also measured in chow-fed trodusquemine -treated mice (Figure 5A, B). Subsequently, both single dose and chronic trodusquemine treatment resulted in attenuated plaque formation, as indicated by a decrease in total plaque area (Figure 5 C,D). Therefore, we show, for the first time, that use of global PTP1B inhibitor not only decreases weight gain and improves glucose maintenance, but also decreases and most importantly reverses atherosclerotic plaque formation in an LDLR−/− mouse model of atherosclerosis, under obesogenic HFD-feeding conditions.

Atherosclerosis is now widely regarded as a chronic, low grade inflammatory condition characterised by an increased pro-inflammatory environment and decreased anti-inflammation, pro-resolutionary signalling [26,27]. Thus, a vicious cycle ensues and a failure of the tissue to return to homeostasis. Therefore, we investigated the expression of genes important in the inflammatory response including monocyte chemoattractant protein-1 (MCP-1), intracellular cell adhesion
molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). MCP-1 is responsible for recruiting monocytes to the aortic tissue whereas both ICAM-1 and VCAM-1 enable their transmigration [7]. Although there were no changes in the expression of aortic ICAM-1 (Figure 6A) or VCAM-1 (Figure 6B), in either chronic or saline treated mice, those animals treated with a single injection of trodusquemine exhibited attenuated aortic MCP-1 expression levels (Figure 6C). Hence, suggesting less monocyte recruitment and a reduced inflammatory environment which could contribute to the reduction in plaque development.

3.3 Decrease in atherosclerotic plaque area with PTP1B inhibitor treatment is accompanied with hyperphosphorylation of aortic Akt and AMPKα1. Given the decrease in serum lipids and aortic plaque formation in trodusquemine -treated mice, we hypothesised that an upregulation of IR signalling and associated downstream pathways could account for the beneficial effects of PTP1B inhibitors in our study. However, in contrast with our hypothesis, there was no significant increase in the aortic IR phosphorylation in either of our drug treated mice cohorts (Figure 7 A, B). However, Akt phosphorylation was significantly increased in the aortas from those mice receiving a single injection of trodusquemine (Figure 7 A,C), without significant alterations in pS6 (Figure 7 A, D). There were no differences in the expression levels of aortic PTP1B in trodusquemine -treated mice when compared to the saline controls (Figure 7 E), as expected, as the inhibition of PTP1B with trodusquemine treatment has been shown to selectively inhibit PTP1B activity [19]. Interestingly, there was also a significant increase in the aortic AMPKα phosphorylation and downstream p38 (Figure 8 A,B and Figure 8 A, C respectively) in single dose trodusquemine treated mouse aortas. Finally, previous research from our lab has found deletion of hepatic PTP1B can improve the endoplasmic reticulum (ER) stress
response [13, 28], therefore, we sought to determine the effect of trodusquemine treatment on markers of ER stress. There was no significant improvement in either inositol-requiring enzyme 1α (IRE1α) nor of binding immunoglobulin protein (BiP) across treatments (see Supplemetal Fig. 2A-C). However, a single dose of trodusquemine had opposing effects, leading to increased phosphorylation of eukaryotic translation initiation factor 2a (eif2a) but a significant decrease in the expression of C/EBP homologous protein (CHOP) (Supplemental Fig. 2A, D, E). These data suggest that the beneficial effects of PTP1B inhibition cannot be attributed to direct regulation of the insulin receptor itself, but instead involves an Akt-AMPKα dependent mechanism.

4. Discussion

We demonstrate here, using the LDLR<sup>−/−</sup> mouse model of atherosclerosis, that pharmacological PTP1B systemic inhibition leads to protection against and reversal of atherosclerosis development, suggesting beneficial effects of PTP1B inhibition for the treatment of CVDs and reduction of CVD risk. We present evidence that, in addition to its improvement in glucose homeostasis and adiposity, PTP1B inhibition results in activation of aortic Akt and AMPKα1, that is independent of the effects on the insulin receptor itself. Most importantly, for the first time, we demonstrate that inhibition of PTP1B results in a decrease in circulating serum cholesterol and triglyceride levels and protection against atherosclerotic plaque formation.

Our findings complement our previous genetic research, where we demonstrated deletion of hepatic-PTP1B protected against HFD-induced endothelial dysfunction, without altering body mass or adiposity [12]. However, in
contrast to our original hypothesis, this was not a consequence of improvements in IR phosphorylation. Nonetheless, these data are in agreement with several other studies from our lab, specifically both the liver-inducible [28] and the myeloid [14] PTP1B deletion models where, although exhibiting improved glucose homeostasis, did so independently of the IR signalling cascade, suggestive of multiple targets for the beneficial effects of PTP1B inhibition. Likewise, PTP1B deletion within adipocytes was unable to improve IR signalling within this tissue [29]. Therefore, this is suggestive that PTP1B inhibition, in addition to its anti-diabetic role, may also exert its actions through different mechanism(s).

In the past few years, in support of our findings, there have been several studies supporting a beneficial role for PTP1B in endothelial dysfunction that is independent of IR signalling but, instead, dependent on that of Vascular Endothelial Growth Factor (VEGF) signalling through the negative regulation of VEGF receptor 2 (VEGFR2) (see Thiebaut et al for a recent review [30]). Pharmacological inhibition or genetic deletion of PTP1B improved heart failure due to the beneficial effects on cardiac remodelling, such as increased contractile function, and a decrease in cardiac hypertrophy in fibrosis [31]. A similar phenotype was observed in a model of sepsis, where whole body PTP1B deletion not only improved survival rate in response to septic shock, but decreased cardio dysfunction and the expression of pro-inflammatory markers such as IL1β, ICAM-1, VCAM-1, COX-2 and iNOS [32]. Furthermore, a follow up study where PTP1B was specifically deleted in endothelial cells, demonstrated again cardiac improvement exhibiting increased survival after 20 weeks post induction of heart failure [33]. Critically, these improvements were accompanied by an increase in VEGFR signalling and angiogenesis. Finally, in a model of hind limb ischaemia, deletion of PTP1B in
endothelial cells led to angiogenesis and arteriogenesis both \textit{in vitro} and \textit{in vivo}, and was mediated by enhanced VEGFR signalling [34]. Therefore, the possibility that improved VEGFR signalling is involved in the beneficial effects observed in trodusquemine treated mice, although not investigated during this study, cannot be ruled out and is worth future investigation. Likewise, the effect of trodusquemine treatment on additional cell types not limited to the vasculature, such as those involved in the immune response must also be considered, as trodusquemine acts as the global PTP1B inhibitor.

Nonetheless, importantly, we observed enhancement of aortic AMPK \( \alpha1 \) phosphorylation in HFD-fed mice given a single dose of trodusquemine. This is in agreement with a similar recent study in which the PTP1B global knock out exhibited improved cardiomyocyte contractility in mice fed HFD, through an AMPK-dependent mechanism [35]. In addition, an independent study using the LDLR\(^{+/-}\) mouse model of atherosclerosis, found deletion of AMPK\( \alpha1 \) specifically in the myeloid lineage, led to hypercholesterolemia, increased macrophage inflammation and plaque infiltration and exacerbated atherogenesis [36]. Therefore, the robust phosphorylation of aortic AMPK\( \alpha1 \) observed in response to a single injection, and to some extent chronic global PTP1B inhibition with trodusquemine, and the associated protection and reversal of atherosclerotic plaque area, suggest that PTP1B inhibition may be protective through an AMPK\( \alpha1 \)-driven mechanism. It is important to note that at the time of culling, single dose and chronic drug treated mice had 4- and 6-weeks washout of drug, respectively, \textcolor{red}{this two week difference} may explain why chronic treatment did not exhibit phosphorylation of AMPK \( \alpha1 \) or Akt to the same extent as those given a single injection. \textcolor{blue}{A group of mice receiving single or chronic trodusquemine with no washout period would be required to assess if trodusquemine
could directly lead to hyperphosphorylation of AMPKα1 and Akt. However, despite this, both drug treated cohorts exhibited the same degree of decreased plaque formation.

Critically, atherosclerosis is now well regarded as a chronic low level inflammatory disease accompanied by a failure to initiate anti-inflammatory signalling, thereby preventing successful engagement of pro-resolution mechanisms and a return to tissue homeostasis. In contrast to previous therapies, including those which inhibit pro-inflammatory signalling, current research is focusing on the promotion from pro-inflammation to pro-resolution, as a means to reduce atherosclerotic plaque development, as well as other chronic inflammatory pathologies [37,38]. Our study revealed a decrease in MCP-1 expression in trodusquemine-treated mice, suggesting that PTP1B inhibition led to a less pro-inflammatory environment. Furthermore, in our model where PTP1B deletion was myeloid-specific, these mice exhibited a decrease in pro-inflammatory IL-6 and TNFα, and an increase in pro-resolution IL-10 [14]. Finally, a recent study by Zhu et al [39] found that IL-10 stimulation of AMPKα phosphorylation and subsequent downstream PI3K/Akt/mTORC1 signalling was critical for eliciting the anti-inflammatory properties of this cytokine. Therefore collectively, these data suggest that PTP1B inhibition may contribute in the switch from pro-inflammation to pro-resolution signalling, via an IL-10/AMPKα mechanism.

In conclusion, we demonstrate that global pharmacological inhibition of PTP1B, in addition to its anti-diabetic and weight loss benefits, resulted in both the reduction and reversal in atherosclerotic plaque formation under obesogenic conditions (as achieved by chronic and a single dose exposure respectively). This was achieved via an IR-independent pathway, and instead engaged Akt/AMPKα
signalling to promote a decrease in pro-inflammatory environment. Hence our data strongly suggest that PTP1B inhibitors may be used in pathologies other than type-2 diabetes and that those currently in pre-clinical trials [20], could be repurposed to target chronic inflammatory pathologies, such as atherosclerosis and help reduce CVD risk.

**Author contributions.** DT performed the experiments and wrote the manuscript, N Morrice, assisted with GTT experiments and terminal culls, LG performed the qPCR in Figure 6. N.Mody, SLM and EKL aided with terminal procedures. H.M.W suggested experiments and reviewed the manuscript. MD conceived and designed the experiments and wrote the manuscript.

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References.


Figure Legends

**Figure 1: Global PTP1B inhibition leads to reduced body weight.** (A) Schematic representation of the experimental design. LDLR<sup>−/−</sup> male mice were divided into HFD-fed and CHOW-fed saline treated, single dose Trodusquemine (10mg/kg, I.P. at week 8) and chronic Trodusquemine (a single 10mg/kg I.P. followed by four weekly injections at 5mg/kg). (B,C) Weights of mice during the course of the experiment fed HFD (B, n=24 per group) or CHOW (C, n=4 per group). Data are represented as mean ± S.E.M. and analysed by Two way ANOVA followed by Bonferroni multiple comparison t-tests where *p≤0.05, ***p≤0.001, ****p≤0.0001 when compared to saline treated control mice.

**Figure 2: Global PTP1B inhibition leads to reduced adiposity.**

(A-D) Body composition was analysed using an Echo MRI-3-in-1 scanner where total body fat (A,B) and lean mass (C,D) were determined (HFD n=9-11 per group, CHOW, n=4 per group). Data are represented as mean ± S.E.M. and analysed by Two way ANOVA followed by Bonferroni multiple comparison t-tests where *p≤0.05, ***p≤0.001, ****p≤0.0001 when compared to saline treated control mice or #p≤0.05, ####p≤0.0001 when single dose and chronic groups were compared to each other.

**Figure 3: Global PTP1B inhibition improves glucose maintenance.** Glucose tolerance tests of saline, single dose and chronic drug treated mice fed HFD (A, C, E) or CHOW (B,D,F) diets at week 8, 10 and 12. Mice were fasted for 5h prior to basal glucose monitoring (as described in materials and methods) and subsequently mice injected I.P. with 20% glucose (w/v) and blood re-analysed at 15, 30, 60 and 90 mins post-injection (HFD n=8 per group, CHOW n=4 per group). Data are represented as mean ± S.E.M. and analysed by Two way ANOVA followed by Bonferroni multiple comparison t-tests where *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 when compared to saline treated control mice or ##p≤0.01 when single dose and chronic groups were compared to each other.

**Figure 4: Global PTP1B inhibition reduces circulating insulin levels in HFD-fed mice.**
Serum from blood collected at terminal culls was analysed for circulating insulin levels in HFD-fed (A) and CHOW-fed (B) mice using ELISA (Millipore). Data are represented as mean ± S.E.M. and analysed by unpaired t-tests where ***p≤0.001 when compared to saline treated control mice.

**Figure 5: Global PTP1B Inhibition reduces serum total cholesterol and triglycerides and prevents atherosclerotic plaque development.** Blood was collected at terminal culls and serum analysed for circulating total cholesterol (A) triglyceride levels (B) using ELISA (Sigma). (C) Representative (n=5-6 per group) aortic root sections of HFD fed mice stained with Oil Red O. (D) Quantification of plaque area as analysed using Image J software. Data are represented as mean ± S.E.M. and analysed by unpaired two tailed t-tests where *p≤0.05 or **p≤0.01 when compared to saline control.

**Figure 6: Single dose global PTP1B Inhibition reduces MCP-1 expression.**

(A-C) Genetic analysis of aortic tissues (n=6 per group) as analysed by qPCR using using SYBER and LightCycler 480 (Roche). Gene expression of ICAM-1, VCAM-1 and MCP-1 was determined relative to the reference gene ELF1. Data are represented as mean ± S.E.M. and analysed by unpaired two tailed t-tests where *p≤0.05 when compared to saline control.

**Figure 7: Global PTP1B Inhibition improves aortic Akt signalling.**

(A) Western blot analysis of aortic tissues from saline, single dose and chronic HFD cohorts injected with insulin immediately prior to culling. Quantification of p-IR (Tyr 1162/1163) (B), p-Akt (Ser 473) (C), p-S6 (Ser 235/236) (D) and total PTP1B (E) using Image J software. Data are represented as mean ± S.E.M. and analysed by one way ANOVA followed by Dunnets t-tests where *p≤0.05 when compared to saline control.

**Figure 8: Global PTP1B Inhibition improves aortic AMPK signalling.**

(A) Western blot analysis of aortic tissues from saline, single dose and chronic HFD cohorts injected with insulin immediately prior to culling. Quantification, p-AMPKα (Thr 172) (B) and p-p38 (Thr 180/Tyr 182) (C) using Image J software. Data are represented as mean ± S.E.M. and analysed by one way ANOVA followed by Dunnets t-tests where *p≤0.05 when compared to saline control.
Figure 1
Figure 2
Figure 3
Figure 4
**Figure 5**

### A. Serum Cholesterol

- **CHOW:**
  - Saline
  - Single dose
  - Chronic

- **HFD:**
  - Saline
  - Single dose
  - Chronic

### B. Serum Triglyceride

- **CHOW:**
  - Saline
  - Single dose
  - Chronic

- **HFD:**
  - Saline
  - Single dose
  - Chronic

### C. Total Plaque Area

- **Saline**
- **Single dose**
- **Chronic**

### D. [Area]²

- **Saline**
- **Single dose**
- **Chronic**
Figure 6

A. ICAM-1 relative expression to ELF1

B. VCAM-1 relative expression to ELF1

C. MCP-1 relative expression to ELF1
A. Figure 7

B. p-IR/total IR

C. p-Akt/total Akt

D. p-S6/total S6

E. PTP1B/GAPDH

Figure 7
Figure 8

A.

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B.

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C.

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<td>Fold Change (relative to saline)</td>
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Supplemental Figures

Pharmacological inhibition of protein tyrosine phosphatase 1B (PTP1B) protects against atherosclerotic plaque formation in the LDLR\(^{-/-}\) mouse model of atherosclerosis.

D Thompson\(^1\), N Morrice\(^1\), L Grant\(^1\), S Le Sommer\(^1\), EK Lees\(^1\), N Mody\(^1\), HM Wilson\(^1\) & M Delibegovic\(^1\).

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**Supplemental Methods.**

**Food Intake**

Food intake of all cohorts of mice was analysed during weeks 8 and 9 of the study.

**Liver PTP1B Activity Assay**

Liver from saline, single dose or chronically trodusquemine treated mice were homogenised in PTP1B lysis buffer (130mM NaCl, 20mM Tris-HCl (pH 7.5), 5mM EDTA, 1% Triton X-100, 0.5% NP-40, protease inhibitor cocktail) and normalised to 1mg of protein in 1ml of buffer. Subsequently 2μg of mouse PTP1B antibody was added and the samples incubated for 2hrs at 4°C with top-over-end mixing before being incubated for a further hr with protein A sepharose beads. Next, beads were washed twice in lysis buffer and twice in PTP1B activity buffer (100mM HEPES (pH 7.6), 2mM EDTA, 150mM NaCl and 0.5mg/ml BSA) before being incubated at 30°C for 30 mins with shaking in activity buffer containing 1mM DTT and 200μM pp60c-Src C-terminal phospho-regulatory peptide (Enzo Life Sciences). Following incubation, 100μl on Biomol green reagent (Enzo Life Sciences) was added to 40μl of sample and after 30 mins incubation, the free phosphate was measured at 620nm.
**Immunoblotting of ER Stress markers**

Frozen aorta tissues were homogenised in 300µl of ice-cold Radioimmunoprecipitation assay (RIPA) buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA pH 8.0, 1mM NaF, 0.1% SDS, 1% Triton X-100, 1% Sodium Deoxycholate with freshly added 1mM NaVO₄ and protease inhibitors) using a PowerGen 125 homogeniser and lysates normalised to 1µg per 1µl. Proteins were separated on a 4-12% Bis-Tris gel by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were probed for the following targets; p-eif2α (Ser51), total eif2α, BiP, pIRE1α (Ser 727), CHOP and GAPDH.

**Supplemental Figure 1: Trodusquemine treatment reduces food intake.** Food intake of HFD (A) and CHOW-fed (B) cohorts at week 9. PTP1B activity data of liver tissue from HFD-fed saline, single dose or chronically trodusquemine treated cohorts following terminal culls as assessed by inhibition of free phosphate production (C, n=4-5 per group). Data are represented as mean ± S.E.M. and analysed by unpaired two tailed t-tests where *p≤0.05 or ****p≤0.0001 when compared to saline control groups.

**Supplemental Figure 2: Trodusquemine treatment does not improve ER stress.** (A) Western blot analysis of aortic tissues from saline, single dose or chronically treated HFD-fed mice injected with either saline or insulin immediately prior to culling. Quantification of p- pIRE1α (Ser 727) (B), BiP (C) p-eif2α (Ser 51) (D) and CHOP (E) represented in (A) Data are represented as mean ± S.E.M. and analysed by unpaired two-tailed t-tests.
Supplemental Figure 1
Supplemental Figure 2