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Basic Research

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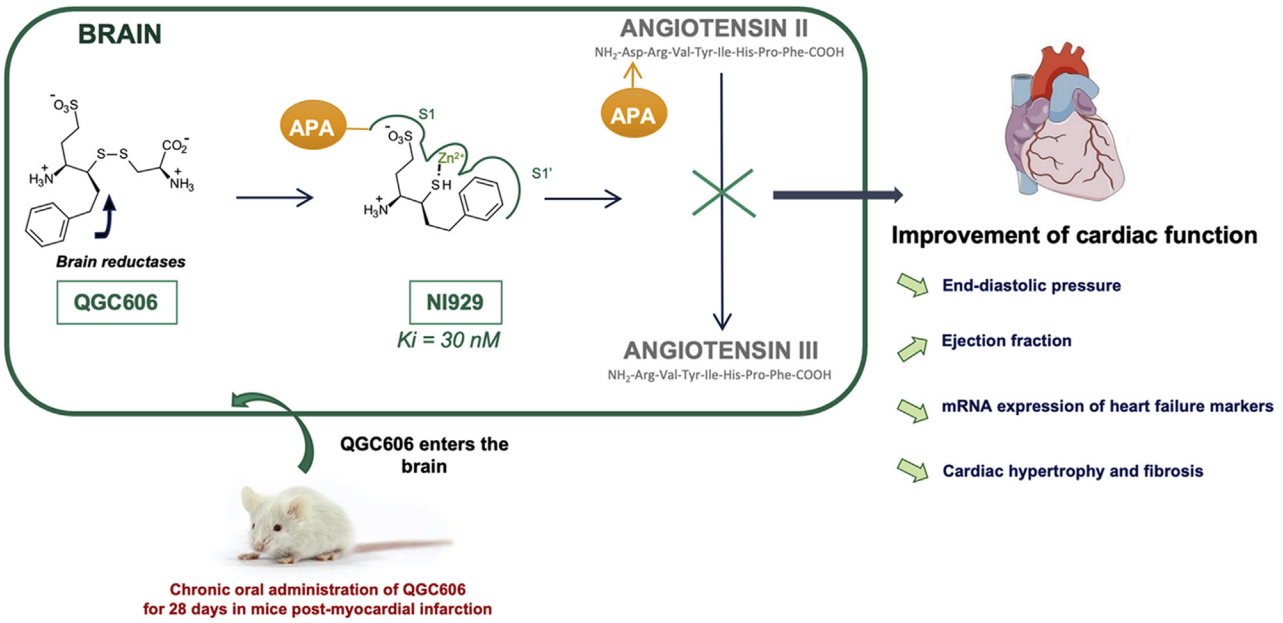

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ABSTRACT

Background: Blockade of brain renin-angiotensin system (RAS) overactivity by firibastat, the first centrally acting aminopeptidase A (APA) inhibitor prodrug, has already demonstrated its effectiveness in improving cardiac function after myocardial infarction (MI). We developed QGC606, a more potent and more selective APA inhibitor prodrug and studied its effects after long-term oral administration in mice post-MI.

Methods: Two days after MI induced by the left anterior descending artery ligation, adult male mice were randomized into 4 groups to receive oral treatment during 4 weeks with vehicle; QGC606; firibastat; or the angiotensin-I converting enzyme inhibitor ramipril, used as positive control.

Results: Four weeks post-MI, brain APA was overactivated in vehicle-treated MI mice. QGC606 treatment normalized brain APA hyperactivity to control values measured in sham-operated mice. Four weeks post-MI, QGC606-treated mice had higher left ventricular (LV) ejection fractions, significantly smaller LV end-systolic diameter and volume, significantly lower HF biomarkers mRNA expression (Myh7 and Anf) and plasma N-terminal pro B-type natriuretic peptide (NT-pro-BNP) and noradrenaline levels than saline-treated mice. QGC606 treatment significantly improved the dP/dt max and min, LV end-diastolic pressure without affecting blood pressure (BP), whereas we observed a decrease in BP in ramipril-treated mice. We observed also a reduction of cardiac fibrosis, highlighted by lower connective tissue growth factor mRNA levels and a reduction of both the fibrotic area and MI size in QGC606-treated mice.

Conclusions: Chronic oral QGC606 administration in post-MI mice showed beneficial effects in improving cardiac function and reducing cardiac remodeling and fibrosis but, unlike ramipril, without lowering BP.

RÉSUMÉ

Contexte : Le blocage de la suractivité du système rénine-angiotensine (SRA) cérébral par le firibastat, le premier promédicament inhibiteur de l'aminopeptidase A (APA) à action centrale, a déjà démontré son efficacité dans l'amélioration de la fonction cardiaque après un infarctus du myocarde (IM). Nous avons développé le QGC606, un promédicament inhibiteur de l'APA plus puissant et plus sélectif, et avons étudié ses effets après une administration orale à long terme chez des souris après IM.

Méthodes : Deux jours après un IM provoqué par la ligature de l'artère descendante antérieure gauche, des souris mâles adultes ont été réparties au hasard en quatre groupes pour recevoir un traitement oral pendant quatre semaines avec le véhicule, le QGC606, le firibastat ou le ramipril, un inhibiteur de l'enzyme de conversion de l'angiotensine I, utilisé comme contrôle positif.

Résultats : Quatre semaines après l'IM, l'APA cérébrale était suractivée chez les souris IM traitées par véhicule. Le traitement par le QGC606 a permis de ramener l'hyperactivité de l'APA cérébrale à des valeurs contrôles obtenues chez les souris ayant subi une chirurgie fictive. Quatre semaines après IM, les souris traitées par le QGC606 présentaient des fractions d'éjection du ventricule gauche (VG) plus élevées, un diamètre et un volume endo-systolique du VG significativement plus petits, une expression de l'ARNm des biomarqueurs de l'insuffisance cardiaque (Myh7 et Anf) et des taux plasmatiques du fragment N-terminal du propeptide natriurétique de type B (NT-pro-BNP) et de noradrénaline significativement plus faibles que les souris traitées par une solution saline. Le traitement par le QGC606 a amélioré significativement la dP/dt max et min, la pression endo-diastolique du VG sans affecter la pression artérielle (PA), alors que nous avons observé une diminution de la PA chez les souris traitées par le ramipril. Nous avons également observé une réduction de la fibrose cardiaque, mise en évidence par des niveaux inférieurs d'ARNm du facteur de croissance du tissu conjonctif (Ctgf) et une réduction de la zone fibrotique et de la taille de l'IM chez les souris traitées par le QGC606.

Conclusions : L'administration orale chronique de QGC606 chez des souris post-IM a montré des effets bénéfiques en améliorant la fonction cardiaque et en réduisant le remodelage et la fibrose cardiaques mais, contrairement au ramipril, sans abaisser la pression artérielle.

Myocardial infarction (MI) is the most common cause of heart failure (HF) with reduced ejection fraction (EF). Activation of the systemic renin-angiotensin system (RAS) after MI is considered to play an important role in cardiac remodeling, as inhibition of angiotensin-I converting enzyme (ACE) activity and blockade of type I angiotensin-II receptor (AT1R) have been demonstrated to improve cardiac function, remodeling, and long-term clinical outcomes: in particular, the occurrence of cardiovascular events and death.[1-5] However, the use of ACE inhibitors is often associated with hypotension,[6] limiting the ability to administer the most effective dose for full benefit on left ventricular (LV) size and function, even after progressive and careful dose titration. Thus, it appears important to find new drugs with the same efficacy as ACE inhibitors but with fewer and milder side effects.

Recent studies have shown that brain RAS overactivity is involved in the development of HF after MI, suggesting that its blockade could be a potential new therapeutic strategy for the prevention or treatment of HF.[7] Within the brain RAS, aminopeptidase A (APA; EC 3.4.11.7), a membrane-bound zinc metalloprotease, produces angiotensin-III (AngIII) from angiotensin-II (AngII), whereas aminopeptidase N (APN; EC 3.4.11.2), another membrane-bound zinc metalloprotease, metabolizes AngIII into angiotensin-IV.[8]

By using EC33 and PC18, 2 specific and selective APA and APN inhibitors, respectively,[9,10] we previously showed that brain AngIII is 1 of the main effector peptides of the brain RAS in the control of blood pressure (BP) and arginine-vasopressin (AVP) release in hypertensive rats.[8,11] Brain AngIII plays also a pivotal role in sympathetic hyperactivity and LV dysfunction in rats post-MI.[12]

EC33 does not cross the blood—brain barrier. Thus, for the clinical use of APA inhibitors, we developed a first-in-class orally active prodrug of EC33, RB150 (4,4′-dithio{bis[(3S)-3-aminobutyl sulfonic acid]}, obtained by dimerizing 2 molecules of EC33 through a disulfide bond,[13] which was renamed "firibastat" by the World Health Organization (WHO) (Fig.1).

In the brain, the disulfide bridge of firibastat is immediately cleaved by brain reductases, generating 2 active molecules of EC33.[13-15] Oral firibastat treatment was found to lower BP in several experimental models of hypertension[14-17] and to improve the LV ejection fraction (LVEF) after MI in rodents.[17-19] However, the oral dose of firibastat to prevent cardiac dysfunction remains high—150 mg/kg per day—underlining the interest to develop a more potent brain APA-inhibitor prodrug.

Following molecular investigations of the APA active site,[20] NI929 a more potent and more selective APA inhibitor was designed by incorporating an additional hydrophobic side chain on the EC33 scaffold[21-23] (Fig. 1). This increases the potency by > 10-fold compared with EC33. Here, we designed QGC606, a prodrug of NI929, resulting from the coupling of NI929 to L-cysteine through a disulfide bridge. Then we investigated whether QGC606, could be a potential orally active best-in-class drug candidate for the treatment of HF post-MI. We evaluated the effects of inhibiting brain APA following chronic oral treatment with QGC606 for 4 weeks post-MI and compared the effects on LV systolic and diastolic function, cardiac hypertrophy, and fibrosis with those of firibastat and ramipril, used as a positive control.

Methods

Drugs

The synthesis of QGC606 is described in the Supplemental Appendix S1. Ramipril was purchased from Clearsynth Labs Ltd (Mumbai, India).

Animals protocols

All procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or current Institutes of Health (NIH) guidelines. The project was accepted by the French Committee on Ethics in Animal Experimentation (CEEA no. 59; Ref. 2017-01#7844). We used 88 male 2-month-old Swiss mice (Janvier Labs, Le Genest St. Isle, Pays de la Loire, France). MI was obtained by the permanent surgical ligation of the left anterior descending (LAD) coronary artery. Taking into account the difficulties caused by anesthesia after surgery, and MI not yet established, it was not possible to assess the MI size and cardiac function reliably, and, thus, all the operated mice received the treatment 2 days after MI surgery. Two days after surgery, mice were randomized into 5 groups: sham-operated mice receiving vehicle (n = 19); MI mice receiving vehicle (n = 15); QGC606 at a dose of 25 mg/kg once daily (n = 20); firibastat at a dose of 150 mg/kg once daily (n = 20); and ramipril at a dose of 1.25 mg/kg once daily (n = 14). The exact number of animals per group for each measurement is detailed in Supplemental Appendix S1 and Supplemental Table S1.

The dose of ramipril (1.25 mg/kg and 2.5 mg/kg) was chosen according to Cavasin et al.[4] and Xu et al.[24] However, as we observed after treatment with ramipril at the dose of 2.5 mg/kg per day, a deleterious effect on cardiac function and animal survival (80% of mice death after 28 days of treatment), for this work, we selected the dose of 1.25 mg/kg.

Surgical procedures and pain management before, during, and after surgery and treatment procedures are detailed in Supplemental Appendix S1.

Echocardiographic measurements

Cardiac function was evaluated 2 and 4 weeks post-MI by transthoracic echocardiography with a Vevo 2100 (FUJIFILM, Visualsonics Inc., Toronto, Canada) equipped with a linear array 22−55 MHz MicroScan mouse cardiovascular transducer (MS550D), as detailed in Supplemental Appendix S1. LVEF, cardiac diameters (LV end-systolic diameter [LVESD] and LV end-diastolic diameter [LVEDD]) and volumes (LV end-systolic volume [LVESV] and LV end-diastolic volume [LVEDV]), and heart rate (HR) were measured.

Assessment of left ventricular hemodynamics

Mice were anesthetized 4 weeks post-MI, and a Millar catheter was inserted into the heart by the carotid artery for measuring various hemodynamic parameters such as systolic arterial BP (SABP) and diastolic arterial BP (DABP), dP/dt max and min, and LV peak systolic pressure (LVPSP) and end-diastolic pressure (LVEDP), as detailed in Supplemental Appendix S1. Upon completion of the measurements, the catheter was removed from the carotid artery and the animal euthanized by cervical dislocation under general anesthesia. The heart, apex, and blood were collected. The entire surgical process is detailed in Supplemental Appendix S1.

APA, APN, and ACE enzymatic activity measurements

In vitro. The enzymatic activities of recombinant mouse APA produced in our laboratory[25] and purified human APN were determined using α-L-glutamyl-β-naphthylamide (GluβNA) and L-Alanine-ß-naphthylamide (AlaßNa) as APA and APN synthetic substrates, respectively, in initial velocity conditions, as previously described[9,22] and detailed in Supplemental Appendix S1. The enzymatic activities of recombinant human ACE and recombinant mouse ACE2 were determined using the fluorescence resonance energy transfer (FRET) peptides substrates Abz-FRK(Dnp)P-OH and (7-methoxycoumarin-4-yl)acetyl-Mca-Ala-Pro-Lys (2,4-dinitrophenyl)(Dnp)-OH (Mca-APK(Dnp)-OH, respectively, as previously described[26,27] and detailed in Supplemental Appendix S1.

Ex vivo. Brain APA enzymatic activity was assessed on fresh brain homogenates 4 weeks after MI as previously described[18] and detailed in Supplemental Appendix S1. Procedures for the quantification of cardiac APA and ACE activities are detailed also in Supplemental Appendix S1.

Percentage of fibrotic area and MI size by histologic staining

After animal euthanasia, the heart was explanted and the LV and right ventricle (RV) dissected out for histologic analysis as the apex/noninfarct area for real-time polymerase chain reaction (PCR) quantification. LV and RV were frozen in protective medium Tissue-Tek (Sakura Finetek, Villeneuve d'Ascq, France) and apex-noninfarct area immediately in liquid nitrogen. Cardiac cryosections (10 µm) were stained

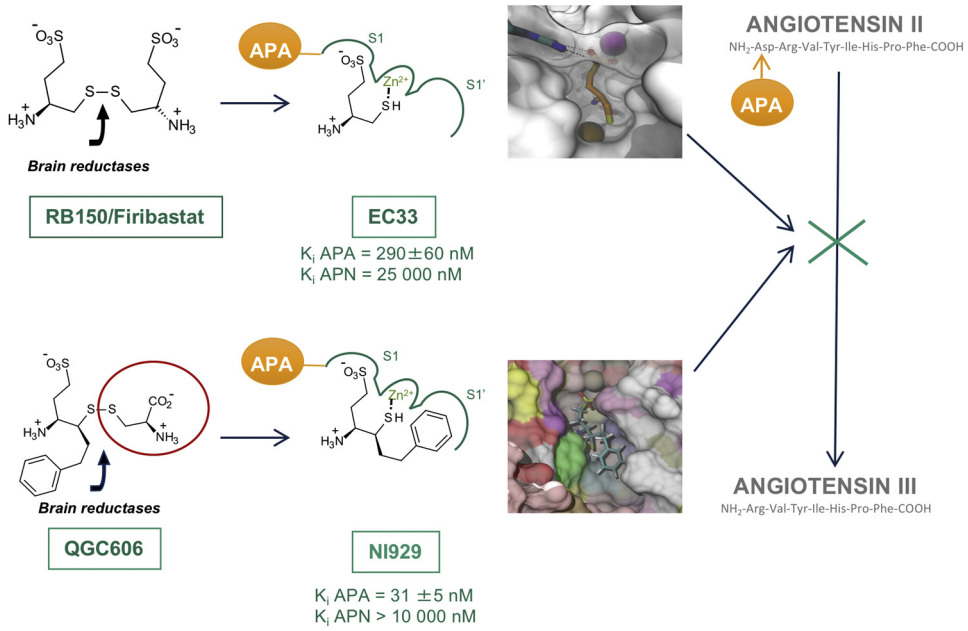

Figure 1. Pharmacologic and structural properties of the aminopeptidase A (APA) inhibitors, EC33, and NI929 and their prodrugs RB150/firibastat and QGC606. The K_i values of EC33 and NI929 were obtained from Chauvel et al.[9]; Inguimbert et al.[21]; and Keck et al.[22] The S1 subsite visualized in the 3-dimensional model of human APA after molecular docking of EC33 (**orange**) is adapted from Couvineau et al.[23] under Creative Commons Attribution 4.0 International (CC BY 4.0) license. Molecular docking of NI929 (**blue**) in the 3-dimensional model of human APA is adapted from Keck et al.[22] with permission from the American Heart Association.

with Picrosirius Red solution (VWR, Fontenay-sous-Bois, France). Percentage of fibrosis and MI size were determined as detailed in Supplemental Appendix S1.

Real-time quantification of gene expression

Real-time quantification of gene expression is detailed in Supplemental Appendix S1.

Plasma NT-pro-BNP and noradrenaline levels

N-terminal pro B-type natriuretic peptide (NT-pro-BNP) and noradrenaline levels were determined in plasma samples 4 weeks post-MI after the different treatments, as detailed in Supplemental Appendix S1.

Statistical analysis

The results are presented as the means ± standard error of the mean (SEM), with individual values for each condition. Statistical analysis was performed using GraphPad Prism 8.0.1 software (GraphPad Software, RITME, France). The significance of the differences between groups was first tested after verifying distribution normality by a Shapiro-Wilk normality test and then by 1-way analysis of variance (ANOVA), followed by Dunn's multiple comparison test (Kruskal-Wallis tests). P values ≤ 0.05 were considered statistically significant.

Results

Experimental design

The mouse model of HF was induced at day 0 by MI obtained by ligation of the LAD artery (Fig. 2). As we wanted to prevent HF development post-MI, we started chronic oral treatment of the animals with vehicle (peanut butter), QGC606, firibastat, or ramipril once daily for 4 consecutive weeks, 2 days after surgery. At 4 weeks post-MI, 2 protocols were used to avoid any pharmacologic interactions of the anesthetic agent with the *ex vivo* assessment of enzymatic activities. In study design 1, tissues and plasma were collected and the enzymatic activities of brain APA, cardiac APA, and cardiac ACE were evaluated. In study design 2, hemodynamic parameters were measured using a Millar catheter, followed by tissue dissection and plasma NT-pro-BNP and noradrenaline assays after 29 days of treatment.

Ability of QGC606 and firibastat to inhibit on recombinant APA, APN, ACE. and ACE2

The inhibitory potency (K_i) of the reduced form of QGC606, obtained under reductive conditions, in the presence of DTT, a reagent that cleaves the disulfide bridge, on recombinant mouse APA (Supplemental Fig. S1) was 2.7 ± 0.2 x 10^{-8} mol/L) and was 7.4 to 10 times greater than the value for EC33 (K_i = 2.9 ± 0.6 × 10^{-7} mol/L)[9] and that obtained in the head-to-head comparisons with firibastat obtained in the presence of dithiothreitol (DTT) (K_i = 2.0 ± 0.1 × 10^{-7} mol/L) (Supplemental Tables S3 and S4, Fig. S1). In the absence of DTT, the disulfide bridge remained intact, and QGC606, as well as firibastat, had no effect on mouse APA activity ($K_i > 10^{-5}$ mol/L). Moreover, in the presence of DTT, QGC606 is 5926-fold more active on APA than on APN (K_i = 1.6 ± 0.4 × 10^{-4} mol/L) and more selective than firibastat (60-fold) (Supplemental Tables S3 and S4, Fig. S2) and EC33 (86-fold).[9]

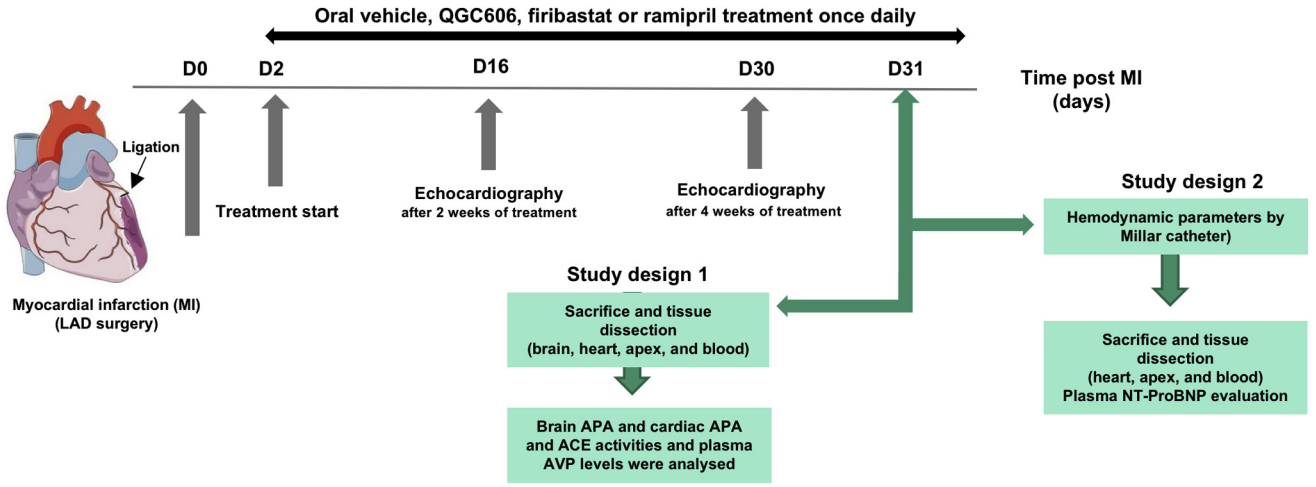

Figure 2. Schematic experimental design of the studies. Chronic oral treatment of mice post-myocardial infarction (MI) (4 weeks) with QGC606 (25 mg/kg) once daily was compared with treatment with firibastat (150 mg/kg) or ramipril (1.25 mg/kg), starting 2 days post-MI. Cardiac function was assessed by echocardiography at 2-week intervals. At 4 weeks post-MI, brain and cardiac aminopeptidase A (APA) and cardiac angiotensin converting enzyme (ACE) activities were measured in study design 1, and hemodynamic parameters were evaluated using a Millar catheter in study design 2. Size of the groups (studies 1 + 2): sham: n = 19, MI + vehicle: n = 15, MI + QGC606: n = 20, MI + firibastat: n = 20, MI + ramipril: n = 14. LAD, left anterior descending; NT-pro-BNP, N-terminal pro B-Type natriuretic peptide.

Moreover, no significant inhibition of recombinant human ACE or mouse ACE2 activity was observed with QGC606 or firibastat at concentrations up to 10^{-5} mol/L, in the absence or presence of DTT (Supplemental Table S3).

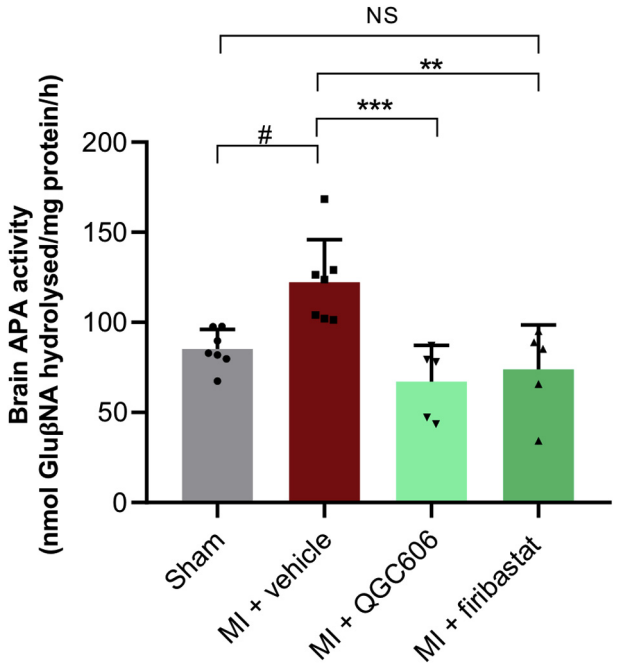

Figure 3. Effects of long-term oral administration of QGC606 on brain aminopeptidase A (APA) activity in mice post-MI. The inhibition of brain APA activity post-myocardial infarction (MI) in mice was determined 1 hour after oral QGC606 (25 mg/kg) or firibastat (150 mg/kg) treatment for 29 days and the results compared with those of mice receiving saline or sham. Mean ± standard error of the mean (SEM) of 5 to 7 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests, $^{\#}P < 0.05$ and NS, nonsignificant vs sham, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs MI + vehicle group.

Effects of oral QGC606 on mouse brain and cardiac APA activities

Brain APA activity in MI + vehicle mice was significantly higher (+43%) 29 days post-MI (122 ± 8.9 nmol GluβNA hydrolyzed/mg protein/h, $P = 0.03$) than that of sham-operated mice (85 ± 4 nmol GluβNA hydrolyzed/mg protein/h) (Fig. 3, Supplemental Table S2). Daily oral administration of QGC606 (25 mg/kg per day) or firibastat (150 mg/kg per day) for 4 weeks post-MI significantly decreased brain APA activity by 45% and 39%, respectively (67 ± 8.9 nmol GluβNA hydrolyzed/mg protein/h, $P = 0.001$ and 74 ± 11 nmol GluβNA hydrolyzed/mg protein/h, $P = 0.01$, respectively), relative to that measured in the MI + vehicle (Supplemental Table S2). In contrast, no difference in cardiac APA activity was observed between sham-operated mice and mice post-MI from all groups after 4 weeks of treatment (Supplemental Fig. S3).

Effects of oral treatment with ramipril, QGC606, and firibastat on cardiac ACE activity

Cardiac ACE activity was also determined after chronic oral treatment with ramipril, QGC606, and firibastat for 4 weeks in mice post-MI. Cardiac ACE activity in MI + vehicle mice (46,112 ± 3762 relative fluorescence unit [RFU] was significantly higher (+ 64%, $P < 0.05$) than that measured in sham-operated mice (28,140 ± 2060 RFU) (Supplemental Fig. S4). No difference in cardiac ACE activity was observed between sham-operated mice and MI + QGC006, or MI + firibastat groups. In contrast, long-term oral treatment with ramipril significantly reduced cardiac ACE activity in MI + ramipril mice (29,649 ± 2399 RFU, $P < 0.05$) compared with MI + vehicle mice (46,112 ± 3762 RFU) (Supplemental Fig. S4).

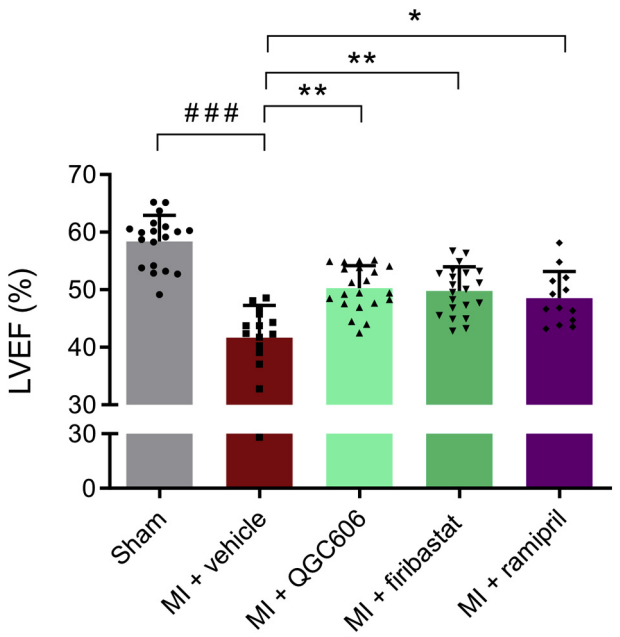

Figure 4. Effects of the long-term oral administration of QGC606, firibastat, or ramipril on left ventricular ejection fraction (LVEF) in mice post-myocardial infarction (MI). LVEF was measured by transthoracic echocardiography for sham, MI + vehicle, MI + QGC606 (25 mg/kg), MI + firibastat (150 mg/kg), and MI + ramipril (1.25 mg/kg) mice at 4 weeks post-MI. Mean ± standard error of the mean (SEM) of 14 to 20 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests, $^{\#}$ $^{\#}$ $^{\#}P < 0.001$ vs Sham, NS, nonsignificant, $^{**}P < 0.01$ vs MI + vehicle group.

Effects of oral QGC606 treatment on cardiac function post-MI in mice

Permanent LAD artery ligation induced a significant reduction of LVEF at 4 weeks post-MI (58.4 ± 1.0% for sham-operated mice vs 41.7 ± 1.5% for MI + vehicle), reflecting the deterioration of cardiac contractility after MI. Oral treatment with QGC606 or firibastat significantly increased LVEF (50.2 ± 0.9% for MI + QGC606 and 49.8 ± 0.9% for MI + firibastat, $P = 0.004$) when compared with MI + vehicle mice (41.7 ± 1.5%) (Fig. 4, Supplemental Table S2). Oral ramipril treatment also improved LVEF (50.0 ± 1.3% for MI + ramipril vs 41.7 ± 1.5% for MI + vehicle, $P = 0.03$).

Effects of oral QGC606 treatment on cardiac remodelling post-MI in mice

Permanent LAD artery ligation induced cardiac remodelling 4 weeks post-MI, with significantly greater diameters (LVEDD, Fig. 5A; $P < 0.0001$; LVESD, Fig. 5B, $P < 0.0001$) and higher volumes (LVEDV, Fig. 5C; $P < 0.0001$; LVESV, Fig. 5D; $P < 0.0001$) in MI + vehicle mice compared with sham-operated mice (Supplemental Table S2).

Oral treatment with QGC606 or firibastat for 4 weeks post-MI did not significantly reduce the LVED diameter and volume but significantly decreased the LVES diameter and volume. Notably, the LVES diameter ($P = 0.03$) and LVES volume ($P = 0.002$) of MI + firibastat mice were significantly lower than those of MI + vehicle mice (Fig. 5B and 5D). The LVES diameter ($P = 0.01$) and LVES volume ($P = 0.01$) of MI + QGC606 mice were also significantly lower (Fig. 5B and 5D). Instead, ramipril trended to moderately limit the MI-induced increase in LVED and LVES diameters and volumes (Fig. 5A-D).

Effects of oral QGC606 treatment on HF biomarkers

Significantly higher mRNA levels of HF biomarkers (Supplemental Table S2), myosin heavy chain 7 (Myh7) ($P < 0.0001$) (Fig. 6A) and atrial natriuretic factor (Anf) ($P < 0.0001$) (Fig. 6B), quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR), were found in MI + vehicle mice when compared with those in sham-operated mice. Plasma NT-pro-BNP levels were also significantly elevated ($P = 0.007$) (Fig. 6C).

Oral QGC606, firibastat or ramipril treatment for 4 weeks post-MI markedly and significantly reduced the mRNA levels of the HF biomarkers in the cardiac apex relative to those of MI + vehicle mice: Myh7 ($P = 0.001$) and Anf ($P = 0.007$) in MI + QGC606 mice; Myh7 ($P = 0.003$) and Anf ($P = 0.02$) in MI + firibastat mice; Myh7 ($P = 0.02$), Anf ($P = 0.008$) in MI + ramipril mice (Fig. 6A-C). The plasma levels of NT-pro-BNP were also significantly lower in MI + QGC606 ($P = 0.007$), MI + firibastat ($P = 0.02$) and MI + ramipril ($P = 0.02$) mice than MI + vehicle mice (Fig. 6C).

Effects of oral QGC606 treatment on hemodynamic parameters

Four weeks after MI, we measured various hemodynamic parameters in anesthetized mice such as SABP, DABP, cardiac contractility by the dP/dt max and dP/dt min, and intracardiac pressure by the LVPSP and LVEDP (Supplemental Table S2).

Compared with sham-operated mice, MI + vehicle mice showed significantly lower SABP ($P = 0.03$) (Fig. 7A); reduced cardiac contractility with significant differences in dP/dt max ($P = 0.03$) (Fig. 7C) and dP/dt min ($P = 0.005$) (Fig. 7D); and significant worsening of intracardiac pressure, highlighted by lower LVPSP ($P = 0.005$) (Fig. 7E) and higher LVEDP ($P = 0.04$) values (Fig. 7F). Chronic oral treatment with QGC606 or firibastat had no impact on SABP or DABP relative to vehicle (Fig. 7A, B). However, oral QGC606 or firibastat treatment for 4 weeks significantly improved dP/dt max ($P = 0.03$ and $P = 0.02$, respectively) (Fig. 7C) and dP/dt min ($P = 0.03$ and $P = 0.04$, respectively) (Fig. 7D) as well as LVEDP ($P = 0.04$ and $P = 0.046$, respectively) (Fig. 7F), without altering LVPSP (Fig. 7E). In contrast, ramipril significantly decreased SABP ($P = 0.046$) and DABP ($P = 0.02$) relative to that of the sham-operated group (Fig. 7A-B), as well as relative to those of MI + vehicle mice ($P = 0.04$) (Fig. 7B). Moreover, there was no significant difference in the dP/dt max or dP/dt min between MI + ramipril and MI + vehicle mice (Fig. 7C-D). Ramipril treatment induced a significant decrease in LVPSP ($P = 0.004$) (Fig. 7E) while improving LVEDP with the same efficacy as firibastat and QGC606 ($P = 0.02$) (Fig. 7F).

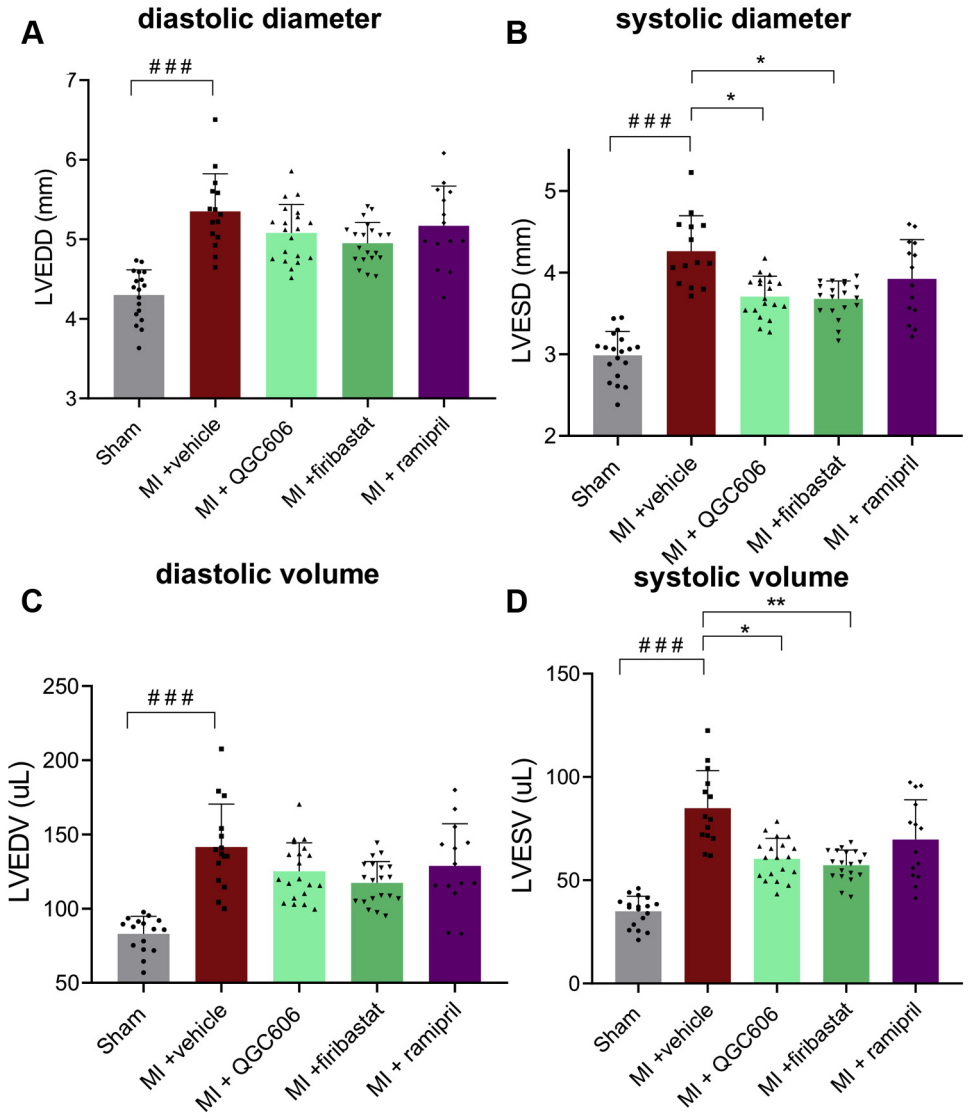

Figure 5. Effects of long-term oral QGC606, firibastat, or ramipril treatment for 4 weeks on cardiac hypertrophy in mice post-myocardial infarction (MI). (**A**) Diastolic diameter (LVEDD), (**B**) systolic diameter (LVESD), (**C**) diastolic volume (LVEDV), and (**D**) systolic volume (LVESV) in mice post-MI. At 4 weeks post-MI, cardiac diameters and volumes in mice were measured by transthoracic echocardiography and compared with those of mice receiving saline post-MI or sham. Mean ± standard error of the mean (SEM) of 14 to 20 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests, $^{\#\#\#}P < 0.001$ vs sham; NS, nonsignificant, $^{**}P < 0.01$ vs MI + vehicle group. LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVESD, left ventricular end-systolic diameter; LESV, left ventricular end-systolic volume.

Effects of oral QGC606 treatment on cardiac fibrosis

We analyzed cardiac fibrosis, which was completely established 4 weeks after MI, on heart sections after Sirius Red staining (Fig. 8A) and quantified the percentage of fibrotic tissue (Supplemental Table S2).

The surface of ischemic tissue (MI size) and relative mRNA expression for profibrotic genes, such as connective tissue growth factor (Ctgf), were also studied in the different groups after 4 weeks of treatment. There was no significant difference in infarct size between the various treatment groups. The MI scar was approximately 15% of the total heart area for all treated groups: 16 ± 8.5% for vehicle-, 10 ± 7% for QGC606-, 13.5 ± 9% for firibastat-, and 10 ± 5% for ramipril-treated mice (Fig. 8B). The percentage of cardiac fibrosis was quantified over the entire surface of the cryosection. The percentage of fibrotic area was significantly higher in the MI + vehicle ($P < 0.0001$) than sham-operated mice, and this increase tended to be attenuated after repeated oral administrations of QGC606, firibastat, or ramipril (Fig. 8C). These observations were further confirmed by the significantly higher levels of Ctgf mRNA observed in the MI + vehicle group compared with sham-operated mice ($P = 0.007$), whereas significantly lower levels were found in MI + QGC606 ($P = 0.02$), MI + firibastat ($P = 0.03$),

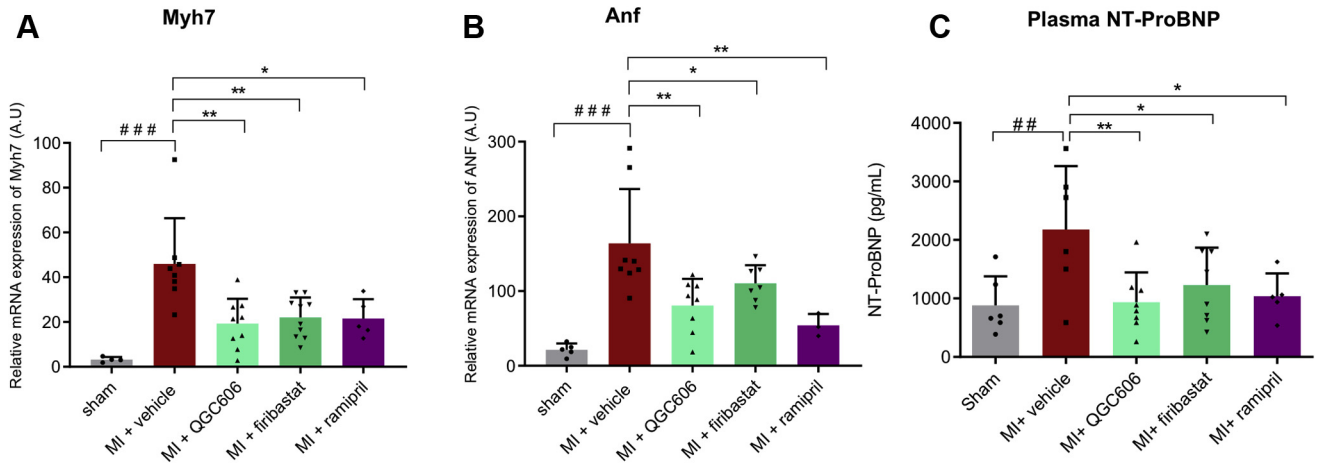

Figure 6. Effects of long-term oral QGC606, firibastat, or ramipril treatment on the mRNA levels of 2 heart failure biomarkers and on plasma N-terminal pro B-Type natriuretic peptide (NT-pro-BNP) levels in mice post-myocardial infarction (MI). Relative mRNA levels of (**A**) myosin heavy chain 7 (Myh7) and (**B**) atrial natriuretic factor (Anf) in the cardiac apex (noninfarcted area) 4 weeks post-MI, normalized against the sham-operated group (sham), with 1 housekeeping gene (HPRT) used as a reference. (**C**) Plasma brain natriuretic peptide (NT-Pro BNP) levels by enzyme-linked immunosorbent assay (ELISA) in mice post-MI. Mean ± standard error of the mean (SEM) of 5 to 8 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests, $^{\#}\,^{\#}P < 0.01$ and $^{\#}\,^{\#}\,^{\#}P < 0.001$ vs sham; $*P < 0.05$ and $**P < 0.01$ vs MI + vehicle group.

and MI + ramipril ($P = 0.01$) mice compared with MI + vehicle mice (Fig. 8D).

Effects of oral QGC606, firibastat, or ramipril treatment on plasma noradrenaline levels

Plasma noradrenaline levels were evaluated after 4 weeks of treatment. An increase of plasma noradrenaline levels was observed in MI + vehicle mice (43.1 ± 4.2 mmol/L, $P < 0.01$) compared with the sham-operated mice (33.9 ± 1.4 mmol/L) (Supplemental Fig. S5). A significant reduction of plasma noradrenaline levels was found in MI + QGC006 (37.2 ± 1.7 mmol/L, $P < 0.05$), MI + firibastat (31.1 ± 3.5 mmol/L, $P < 0.05$), and MI + ramipril (31.2 ± 4.3 mmol/L, $P < 0.05$) compared with MI + vehicle mice (Supplemental Fig. S5).

Discussion

We describe here a new brain-penetrating APA inhibitor prodrug, QGC606, which appears more potent and more selective than the first-in-class drug firibastat. We show that long-term oral treatment of post-MI mice with QGC606 for 4 weeks normalizes brain APA hyperactivity; improves cardiac function; limits cardiac remodeling; and reduces HF biomarkers expression, plasma noradrenaline levels, and fibrosis, without altering BP.

MI is known to increase circulating AngII levels, enabling them to reach and penetrate circumventricular organs, to stimulate AT1Rs located in these organs, and finally induce brain RAS hyperactivity. Brain RAS hyperactivity, observed after MI, contributes to sympathetic hyperactivity and increased release of AVP, which participates in the development of HF.[28] Transgenic rats deficient for brain angiotensinogen[29,30] demonstrate normal sympathetic activity and less LV dysfunction post-MI, strengthening the crucial role of the brain RAS in the regulation of cardiac function. We previously showed, in different experimental models of hypertension[14,15] or HF,[12,17,18] that brain APA hyperactivity is involved in brain RAS hyperactivity. Thus, blocking brain RAS hyperactivity by inhibiting brain APA activity with a centrally acting APA inhibitor appears to be an attractive strategy to limit development of HF after MI.[18]

Firibastat is an orally active centrally acting APA inhibitor prodrug of EC33, exhibiting an inhibitory potency of 290 nmol/L on APA and known to only interact with the S1 subsite of the APA active site.[13-15] To increase the potency and selectivity of EC33, an additional hydrophobic side chain was incorporated onto the EC33 scaffold, to interact with the APA S1′ subsite, generating the nonpeptide APA inhibitor, NI929, which exhibited an inhibitory potency of 30 nmol/L on APA.[21] As central nervous system bioavailability of thiol inhibitors of zinc metallopeptidases, such as neutral endopeptidase 24.11 and APN, can be enhanced when the sulfhydryl moiety is engaged in a disulfide bridge,[10] we used a similar strategy to increase the bioavailability of NI929. We generate QGC606 by coupling NI929 to L-cysteine through a disulfide bridge, allowing it to cross the blood—brain barrier. Interestingly, the presence of the L-cysteine may contribute to enhance furthermore brain penetration, thanks to potential interactions with the cerebrovascular large neutral amino acid transporter (LAT1) present at the blood—brain barrier.[31] As the thiol group of NI929 is engaged in the disulfide bridge in QGC606, it is unable to interact with the zinc atom present in the APA active site, essential for its catalytic activity.[20] However, under reductive conditions, our results showed that the disulfide bridge of QGC606 is cleaved *in vitro,* whereas *in vivo* this cleavage occurs through the action of brain reductases, generating NI929 in the brain. We showed that QGC606 inhibits *in vitro,* under reductive conditions, recombinant mouse APA activity with a K_i value of 27 nmol/L and therefore is 7.4 to 10 times more potent than firibastat (200 nmol/L) or EC33 (290 nmol/L).[9] QGC606 is also more selective than EC33 and firibastat for APA, with a selectivity

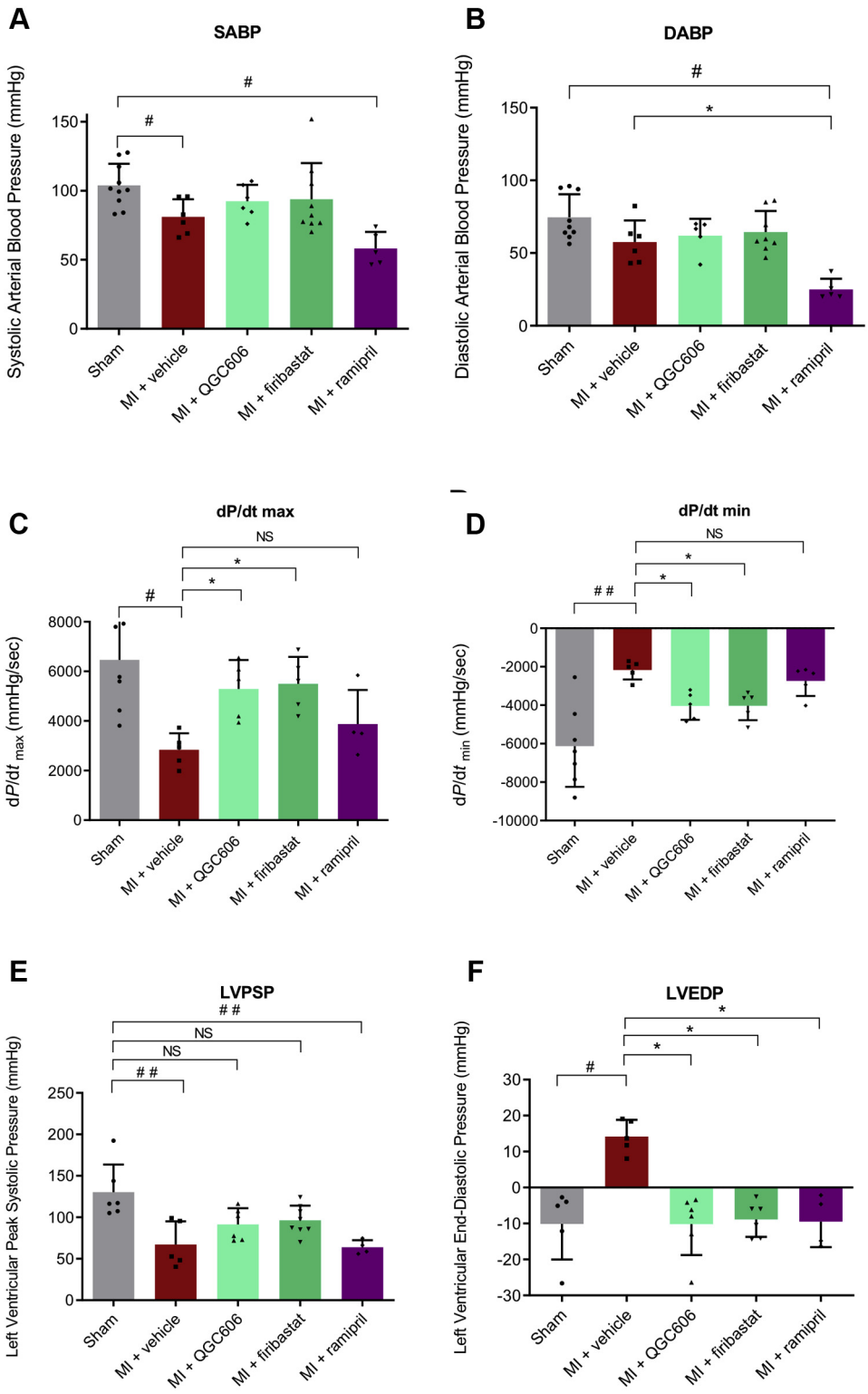

Figure 7. Effects of long-term oral administration of QGC606, firibastat, or ramipril on (**A**) systolic arterial blood pressure (SABP), (**B**) diastolic arterial blood pressure (DABP), (**C**) dP/dt max, (**D**) dP/dt min, (**E**) left ventricular end-diastolic pressure (LVEDP), and (**F**) left ventricular peak systolic pressure (LVPSP). Hemodynamic parameters measured in anaesthetized mice using a Millar catheter for sham, myocardial infarction (MI) + vehicle, MI + QGC606 (25 mg/kg), MI + firibastat (150 mg/kg), and MI + ramipril (1.25 mg/kg) mice at 4 weeks post-MI. Mean ± standard error of the mean (SEM) of 5 to 9 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests NS, nonsignificant, $^{\#}P < 0.5$, and $^{\#\,\#}P < 0.01$ vs sham; $^{*}P < 0.5$ vs MI + vehicle.

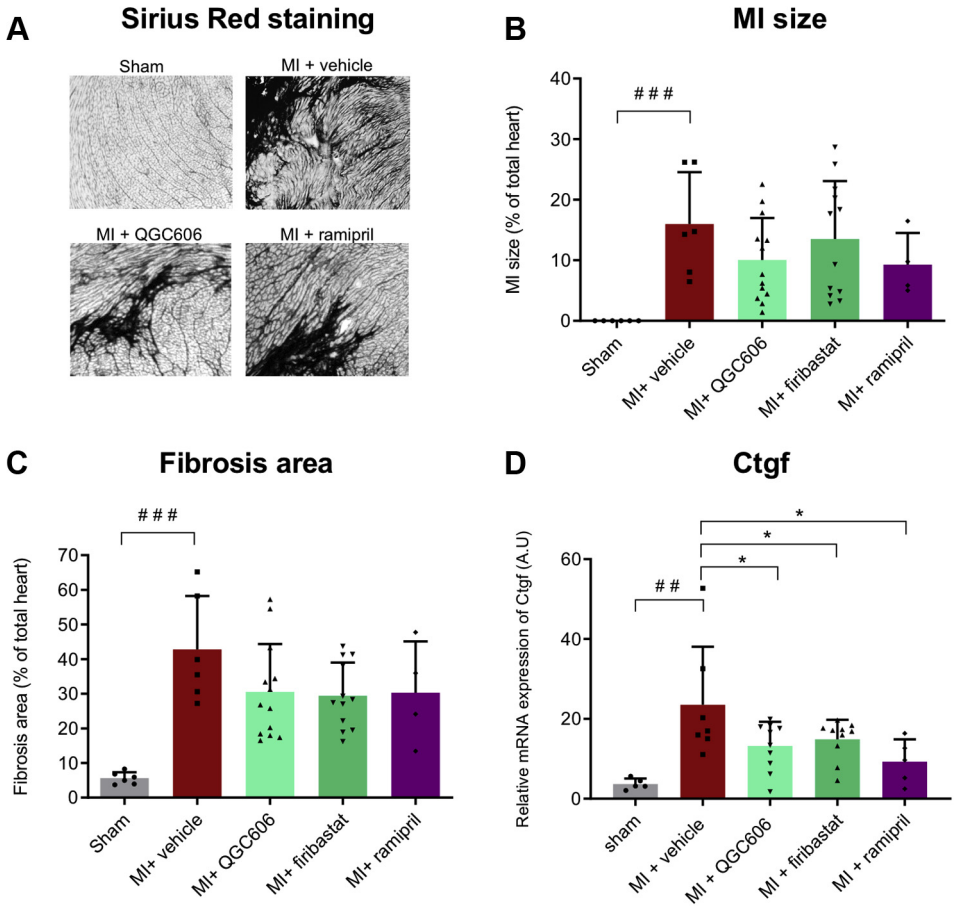

Figure 8. Analysis of fibrosis in mice at 4 weeks post-myocardial infarction (MI) after long-term oral administration of QGC606, firibastat, or ramipril. (**A**) Fibrosis observed in cardiac tissue with Sirius Red staining of the peri-infarct area. (**B**) Evaluation of MI size. (**C**) Quantification of the percentage of fibrotic area in total heart. (**D**) Relative mRNA levels for the fibrosis biomarker connective tissue growth factor (Ctgf) 4 weeks post-MI in the cardiac apex, normalized against those of sham, with 1 housekeeping gene (HPRT) as a reference. Mean ± standard error of the mean (SEM) of 5 to 13 animals for each set of conditions. Kruskal-Wallis followed by Dunn's tests, $^{\#\#\#}P < 0.001$ vs sham, $^{*}P < 0.05$ vs MI + vehicle.

for APA vs APN that is 73 times higher than that of EC33[9]/firibastat, The selectivity of QGC606 toward APA was also shown by its lack of affinity for other zinc metalloproteases involved in the production or metabolism of vasoactive peptides such as ACE and ACE-2.

To demonstrate the mode of action of QGC606, we evaluated *ex vivo* brain APA activity after long-term oral treatment with QGC606 in a mouse model of HF post-MI. Four weeks post-MI, brain APA activity was significantly higher than that measured in sham-operated mice, as previously shown.[18] Four weeks of oral QGC606 treatment (25 mg/kg per day) in mice post-MI, normalized brain APA hyperactivity to control values like firibastat (150 mg/kg per day). Moreover, we previously showed[12] that chronic firibastat treatment for 4 weeks post-MI has no significant effect on the brain APA protein amount compared with MI rats treated with saline using SDS-PAGE Western Blot Analysis (Novus Biologicals, Littleton, Colorado USA). Consequently, the reduction in brain APA activity in firibastat-treated animals is caused by the presence of firibastat in these samples and not to a decrease in APA protein expression. This shows that there is no tolerance to the inhibitory action of firibastat on APA activity after long-term treatment. A similar conclusion may be proposed after QGC606 treatment.

The normalization of brain APA hyperactivity by QGC606, decreasing brain RAS hyperactivity observed after MI, should attenuate sympathetic hyperactivity as previously shown after treatment with firibastat.[12] This conclusion is supported by the significant decrease in plasma noradrenaline levels, observed after long-term oral treatment with QGC606 or firibastat for 4 weeks post-MI, reflecting a reduction of sympathetic tone. This effect was also observed after long-term treatment with ramipril (1.25 mg/kg), the efficacy of which is demonstrated by inhibition of cardiac ACE activity (−55%) similar to that measured after enalapril treatment (1 mg/kg) given under the same experimental conditions.[18] No change in cardiac ACE activity was observed with QGC606, highlighting, furthermore, its high selectivity toward APA. Also, in agreement with its central mode of action, no cardiac APA activity reduction was observed.

Echocardiographic analysis showed a significant reduction of LVEF in MI + vehicle mice 4 weeks after MI. In contrast, MI mice treated with QGC606, firibastat, or ramipril showed a significant increase in LVEF relative to MI + vehicle mice.

Previous kinetic studies performed on brain APA activity following firibastat treatment in mice post-MI,[18] and the current data obtained on LVEF after firibastat or QGC606 treatment suggest that 2 weeks of QGC606 treatment, similar to firibastat, are required to normalize brain APA hyperactivity and recovery of LVEF to values close to those observed in sham-operated animals.

Improvement of cardiac function after QGC606 or firibastat treatment was also supported by the significantly lower expression of HF biomarkers, Myh7, Anf, and plasma NT-pro-BNP levels, all similar to those found in MI + ramipril mice and all significantly lower than those found in MI + vehicle mice.

Interestingly, several other beneficial effects of chronic brain APA inhibition after MI were observed in our study. First, although cardiac hypertrophy observed in MI + vehicle mice was reduced with all drug treatments, only firibastat and QGC606 induced a significant decrease in both systolic diameter and systolic volume. These changes induced by QGC606 and firibastat treatment reflect a reduction of global cardiac hypertrophy.

Second, measurement of hemodynamic parameters in anaesthetized mice 4 weeks post-MI showed that the SABP and DABP were not modified after chronic oral administration of QGC606 or firibastat but significantly decreased after treatment with ramipril.

Third, QGC606 and firibastat treatments significantly improved the dP/dt max and dP/dt min compared with MI + vehicle group. Finally, when compared with the sham-operated group, QGC606 and firibastat tend to normalize LVPSP, whereas ramipril significantly altered it, consistent with the decrease in SABP that it induces.

After 4 weeks post-MI, QGC606, firibastat, and ramipril treatments tended to decrease cardiac fibrosis as shown by the reduced percentage of fibrotic tissue in the total heart area and a significant decrease in Ctgf mRNA levels relative to those found in MI + vehicle group. Ischemia induced by MI generates cardiomyocytes death and fibroblast recruitment to the ischemic zone to colonize the collagen, resulting in scarring.[32] With time, the propagation of fibrotic tissue is observed. Although the cardiac antifibrotic effect of ACE inhibition is well known,[33] we report here that QGC606 and firibastat treatments also tended to similarly limit the propagation of fibrosis post-MI in remote regions of myocardium in which reactive fibrosis is responsible of LV systolic and diastolic function alteration. Similar observations have been reported for losartan, an AT1R antagonist, after chronic oral administration in rats post-MI.[19] The lower effects of QGC606 and also of firibastat on cardiac fibrosis compared with cardiac function could be explained by the central mode of action of these APA inhibitors, which improved cardiac function by 3 different mechanisms: a decrease in AVP release, a reduction in sympathetic neurons activity, and an improvement of the baroreflex function. The mechanism of action of APA inhibitors on fibrosis could only be secondary to the beneficial effect of these compounds on cardiac remodeling, but this remained to be demonstrated.

Hypotension is a well-known adverse effect of ACE inhibitors, and careful dose titration is strongly recommended when initiating ACE-inhibitor treatment in patients with acute MI. This hypotensive effect is caused not only by the decrease in circulating AngII formation but also to a reduction in bradykinin degradation, resulting in overactivation of the bradykinin B2 receptor.[34] Acute B2 receptor blockade was shown to attenuate the deleterious hemodynamic effects of the ACE inhibitor treatment in mice significantly,[34] although there are data supporting the opposite.[24,35] In contrast, APA inhibitors have the advantage of improving cardiac function without lowering systolic BP and LVPSP. This is because centrally acting APA inhibitors are antihypertensive and not hypotensive agents, decreasing BP only in hypertensive but not in normotensive animals or in human volunteers.[14,36,37]

On the other hand, we recently described an additional mechanism of action of firibastat in the control of BP, involving ACE2 and angiotensin 1-7 (Ang1-7).[38] Our findings suggest that, in the brain, the increase in ACE2 activity resulting from APA inhibition by firibastat treatment, subsequently increasing conversion of brain AngII into Ang1-7 and activating the Mas receptor while blocking brain AngIII formation, contributes to the antihypertensive effect and the decrease in release of AVP induced by firibastat. Firibastat treatment thus constitutes an interesting therapeutic approach to improve control of BP in hypertensive patients by inducing in the brain RAS, hyperactivity of the beneficial ACE2/Ang1-7/MasR axis, while inhibiting that of the deleterious APA/AngII/AngIII/ATI receptor axis. This mechanism could be also involved in the effect of QGC606 or firibastat on cardiac functions, as an increase in Ang1-7 in the nucleus of the tractus solitarius was shown to facilitate arterial baroreflex function in rats with impaired baroreflex sensitivity.[39] Moreover, higher brain Ang1-7 levels in the paraventricular hypothalamic nucleus (PVN) may further inhibit sympathetic hyperactivity and thereby attenuate LV dysfunction and remodeling post-MI.[40]

Conclusions and Perspectives

Our study shows that long-term oral QGC606 treatment after MI—at a dose 7 times lower than that of firibastat and with higher selectivity—normalizes brain APA hyperactivity and prevents cardiac dysfunction by improving EF, cardiac contractility and intracardiac pressure, and attenuating cardiac hypertrophy and fibrosis. In contrast to ramipril, no hypotension was associated with QGC606 treatment. Therefore, QGC606 could represent a best-in-class drug candidate worth exploring for its safety and efficacy in patients after acute MI.

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Disclosures

S.B., M.K., Y.M., and F.B. are full-time employees and shareholders of Quantum Genomics SA. C.L.-C. is the inventor on patent WO/2004/007441, held by Quantum Genomics, which includes RB150, renamed firibastat by WHO. The remaining co-authors have no conflicts of interest to disclose.

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Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at www.onlinecjc.ca and at https://doi.org/10.1016/j.cjca.2022.01.019.