Inhibition of CD38 with the Thiazoloquin(az)olin(on)e 78c Protects the Heart against Postischemic Injury

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ABSTRACT

Inhibition of and genetic deletion of the NAD(P)⁺ hydrolase [NAD(P)ase] CD38 have been shown to protect against ischemia/reperfusion (I/R) injury in rat and mouse hearts. CD38 has been shown to enhance salvage of NADPH, which in turn prevents impairment of endothelial nitric oxide synthase function, a hallmark of endothelial dysfunction. Despite growing evidence for a role of CD38 in postischemic injury, until recently there had been a lack of potent CD38 inhibitors. Recently, a new class of thiazoloquin(az)olin(on)e compounds were identified as highly potent and specific CD38 inhibitors. Herein, we investigate the ability of one of these compounds, 78c, to inhibit CD38 and protect the heart in an ex vivo model of myocardial I/R injury. The potency and mechanism of CD38 inhibition by 78c was assessed in vitro using recombinant CD38. The dose-dependent tissue uptake of 78c in isolated mouse hearts was determined, and high tissue permeability of 78c was observed when delivered in perfusate. Treatment of hearts with 78c was protective against both postischemic endothelial and cardiac myocyte injury, with preserved nitric oxide synthase-dependent vasodilatory and contractile function, respectively. Myocardial infarction was also significantly decreased in 78c-treated hearts, with preserved levels of high-energy phosphates. Protective effects peaked at 10 μM treatment, and similar protection without toxicity was seen at 5-fold higher doses. Overall, 78c was shown to be a potent and biologically active CD38 inhibitor with favorable tissue uptake and marked protective effects against I/R injury with enhanced preservation of contractile function, coronary flow, and decreased infarction.

Introduction

Recently, much attention has focused on the discovery (Kellenberger et al., 2011; Blacher et al., 2015) and development of CD38 inhibitors (Moreau et al., 2013; Becherer et al., 2015; Hoffner et al., 2015; Li et al., 2015). This is due to the wide variety of physiologic and pathologic conditions influenced by CD38 (Malavasi et al., 2008; Quarona et al., 2013). As an enzyme, CD38 primarily functions as an NAD(P)ase, hydrolyzing NAD(P)⁺ to (2'-phospho-)ADP ribose [(2'-P)-ADPR] (Berthelier et al., 1998). CD38 is also an ADP-riboyl cyclase, producing in lesser amounts (2'-phospho-)cyclic ADP ribose [(2'-P)-cADPR] (Berthelier et al., 1998). CD38 can also convert NADPH to nicotinic acid adenine dinucleotide phosphate (NAADP) under acidic conditions in the presence of nicotinic acid (Chini et al., 2002). CD38 is also an NAADPase, hydrolyzing NAADP to 2'-P-ADPR (Graeff et al., 2006) (see Fig. 1 for a summary of CD38 enzymatic functions). Recently, we discovered that activation of CD38 in the ischemic heart is an important mechanism of myocardial and endothelial NADPH depletion (Reyes et al., 2015; Boslett et al., 2018a). This endothelial NADPH (H) depletion was shown to severely impair endothelial nitric oxide synthase (eNOS) function, limiting endothelium-dependent vasodilation in the postischemic heart.

This novel finding led to our search for better CD38 inhibitors than those used previously, with a focus on potency and efficient cellular uptake. Until recent years, there was a lack of high-affinity CD38 inhibitors suitable for therapeutic use, with known inhibitors such as α-NAD requiring millimolar levels (Reyes et al., 2015). We have observed that the natural product flavonoid, luteolinidin, inhibits CD38 in sub-10 μM concentrations and exerts potent cardiac protection after ischemia/reperfusion (I/R) in isolated rat hearts (Boslett et al., 2017). However, the multiple polar phenolic groups of this flavonoid precluded direct tissue uptake when delivered in buffer. Rather, liposomal formulations of luteolinidin were necessary to achieve rapid tissue uptake and therapeutic levels. Although luteolinidin was highly effective in conferring myocardial protection, its requirement for liposomal packaging would limit possible future uses and routes of administration in its translation to clinical use. For simple direct administration, an inhibitor would optimally have both high potency and bioavailability, respectively, limiting off-target effects and facilitating effective delivery.
One promising family of compounds recently identified as CD38 inhibitors are the thiazoloquin(az)olin(on)es. These were shown to have efficacy for in vitro CD38 inhibition down to the low nanomolar range, with favorable pharmacokinetics (Becherer et al., 2015). In this study, we test the cardioprotective efficacy of the lead compound in this class of thiazoloquin(az)olin(on)es (78c), which was selected as most promising for translation. Initially, studies were performed to determine the dose-dependent potency of CD38 inhibition using an established in vitro assay (Graeff and Lee, 2013). We then determined the total and free tissue levels of 78c in perfused mouse hearts after dose-dependent treatment with 78c. Further studies were performed in the isolated mouse heart model of global I/R injury. Measurements in myocardial tissue of CD38 substrates NAD(P)\(^1\) and its CD38-derived enzymatic products (2’-P-)ADPR were performed that demonstrated effective CD38 inhibition with 78c delivery. Lastly, myocardial protection was evaluated in this model, where it was demonstrated that CD38 inhibition confers marked cardiac protection with preserved endothelial and myocyte function with decreased infarction.

**Materials and Methods**

All chemicals and reagents were purchased from Sigma, with the exception of CD38 inhibitor 78c, which was obtained as a gift from GlaxoSmithKline, and recombinant CD38, which was a generous gift from Dr. Hon-Cheung Lee (Peking University, Shenzhen, China).

**Animals.** All mice used for experiments were in the age range of 4–6 months. C57Bl/6J mice were obtained from Jackson Laboratories. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**CD38 Activity Assay.** CD38 has been reported to function primarily as an NADPase (NADP hydrolase) converting NADP to 2’-P-ADPR. A small fraction of NADP is converted by CD38 to potent calcium signaling molecule 2’-P-cADPR as a part of its ADP-ribose cyclase function. The formed 2’-P-cADPR can then be hydrolyzed to 2’-P-ADPR (cADPR hydrolase). At acidic pH and in the presence of nicotinic acid, CD38 can convert NADP to another potent calcium-mobilizing second messenger, NAADP, in the base-exchange reaction. In this exchange, nicotinamide of NADP is replaced with nicotinic acid. Formed NAADP can be broken down to 2’-P-ADPR in an NAADP hydrolase reaction (NAADPase).

**Isolated Heart Perfusion.** Isolated heart experiments were performed as described previously (Reyes et al., 2015; Boslett et al., 2018a). Male C57Bl/6 mice weighing 25–30 g were heparinized and injected with 100 mg/kg ketamine and 15 mg/kg xylazine intraperitoneally. Hearts were excised, cleaned of nonmyocardial tissue, cannulated via the aorta, and perfused retrogradely in Langendorff mode with Krebs-Henseleit buffer (119 mM NaCl, 17 mM glucose, 2 mM sodium pyruvate, 25 mM NaHCO\(_3\), 5.9 mM KCl, 1.2 mM MgCl\(_2\), 2.5 mM CaCl\(_2\), and 0.5 mM NaEDTA). A polyvinylchloride balloon...
cartridges with 10% NH4OH in water. These samples were then frozen with 1 ml 40% methanol, metabolites were eluted from the SPE cartridges twice under light vacuum. After washing with 1 ml water, nonpolar metabolites were eluted with 1 ml 40% methanol and nucleotides with 10% NH4OH in water. Eluates were immediately frozen in liquid nitrogen, lyophilized, and then resuspended in 100 µl deionized water for HPLC analysis.

**HPLC Analysis of Nucleotides.** HPLC of nucleotides [ATP, ADP, AMP, 2'-P-ADPR, NAD(P)] was measured using ion-pairing reversed phase HPLC. Ion pairing was essential to obtain adequate retention time and separation using reversed phase HPLC. HPLC was carried out in the reversed phase using a two-pump system with a Supelcosil LC-18-T column (25 cm x 4.6 mm x 5 µm) and matching guard column. Detection of nucleotides was performed with absorbance at 254 nm. The composition of MPA was 65 mM potassium phosphate/10 mM tetrabutylammonium hydrogen sulfate (ion-pairing agent) in water (pH 5.0) and MBP was identical except for a final concentration of 50 mM acetonitrile. The gradient elution of nucleotides was performed at constant flow of 1 ml/min. The gradient was performed as follows: 0–10 minutes, 0%–16% MBP; 10–20 minutes, 16%–60% MBP; 20–25 minutes, 60% MBP; 25–32 minutes, 60%–0% MBP; and 32–42 minutes, 0% MBP. Concentrations of nucleotides in heart samples were derived from standard curves prepared from the analysis of pure standards, which, in the case of (2'-P-ADPR), were prepared from the reaction of rCD38 with pure NAD(P)H. For all samples, HPLC analysis was performed with and without sample spiking to ensure proper peak identification.

**HPLC of 78c.** Compound 78c was extracted from heart tissue in either ice-cold 0.1 M HCl in 20% DMSO for measurement of total levels or ice-cold PBS for measurement of free levels. Homogenates were first centrifuged to pellet insoluble debris and were then filtered to remove protein interferences prior to HPLC. Fluorescence detection of 78c was used with excitation and emission wavelengths of 286 and 388 nm, respectively. Reverse phase HPLC was performed with a Waters Atlantis C18 column with a MPA of 100 mM potassium phosphate (pH 7.0) and a MBP of 100 mM potassium phosphate (pH 7.0) in 50% acetonitrile at a total flow of 1.2 ml/min. The gradient elution of 78c was performed with an initial percentage of MBP of 20%. Between 0 and 15 minutes, the percentage of MBP increased from 20% to 100%. Elution at 100% B occurred from 15 to 20 minutes, with the return of MBP to a baseline level of 20% from 20 to 22.50 minutes. The run ended after a 5-minute restabilization period from 22.50 to 27.50 minutes at 20% B. Peak identification and quantitation was performed using the same batch of 78c standard used for isolated heart experiments.

**Myocardial Infarct Size Measurement.** Ex vivo myocardial infarction was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining of heart sections, as reported previously (Boslett et al., 2018a). The heart was immediately removed after I/R and frozen for 20 minutes for hardening. The heart was then serially sectioned into transverse slices (1 mm) by a heart slicer (Zivic Laboratories) and incubated in 1.5% TTC in PBS for 15 minutes at 37°C to demarcate the viable (brick red) and infarcted (pale) myocardium. Heart slices were then fixed overnight in 10% neutral-buffered formaldehyde for improved color contrast and were digitally imaged. Computerized planimetry (with image-analysis software MetaVue, version 6.0) of each section was used to determine the percentage of infarction from the total cross-sectional area of the left ventricle.

**Statistical Analysis.** Results are expressed as the mean ± S.E. Statistical significance was determined by ANOVA (followed by the Holm-Sidak test) for multiple groups. Paired or unpaired t tests were used for comparison between two groups. In the case of time-dependent data, ANOVA with two-way repeated measures was used to determine significance.
Results

Potency of CD38 Inhibition. Compound 78c (Fig. 2A) was tested for its potency against rCD38. rCD38 (0.1 μg/ml) was incubated with varying concentrations of substrate etheno-NAD (0–100 μM) and 78c in the range of 0–40 nM, with the conversion of ε-NAD to ε-ADPR monitored by an increase in fluorescence. Inhibition data fit strongly to an uncompetitive inhibition model, with a $K_i$ of ∼9 nM for human CD38. An uncompetitive inhibition model was determined by carefully measuring $V_{max}$ and apparent $K_m$ with the different combinations of substrate and inhibitor concentrations, and then modeling the inhibition data with Lineweaver–Burk analysis (Fig. 2B) and nonlinear regression analysis (Fig. 2C). Consistent with uncompetitive inhibition, both $V_{max}$ and apparent $K_m$ decreased with increasing inhibitor concentration, producing parallel lines with different $X$ and $Y$ intercepts.

Fig. 2. Characterization of CD38 inhibition by 78c. (A) Structure of CD38 inhibitor 78c. Maximum rates of reaction were measured for the CD38-catalyzed conversion of substrate ε-NAD (0–100 μM) to fluorescent etheno-ADPR in the presence of 78c (0–40 nM). (B) inhibition data analyzed by Lineweaver–Burk analysis showed uncompetitive inhibition of CD38, as increasing the concentration of 78c decreased both the $V_{max}$ and apparent $K_m$. (C) Nonlinear regression of 78c inhibition data fit to the equation for uncompetitive inhibition enabled determination of the inhibitor constant ($K_i$) of 8.4 nM. Each data point represents the average of four to six experiments. RFU, relative fluorescence unit.

Fig. 3. Contractile function recovery after ischemia. The dose-dependent effect of 78c on the recovery of contractile function was measured through the first 45 minutes of reperfusion after 30 minutes of global ischemia. 78c was protective of LVDP from 2.5 to 50 μM (A), of RPP from 5 to 50 μM (B), and of LVEDP for 5 and 10 μM (C). 78c delivered at 1 μM was not protective of any physiologic parameter measured. *P < 0.05; **P < 0.001 (mean ± S.E.M.; n = 5–9). LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; RPP, rate-pressure product.
TABLE 1
Recovery of cardiac function in 78c-treated hearts after 30-minute ischemia/30-minute reperfusion
Values are presented as the mean ± S.E.M. (n = 5–9). Values for LVDP and RPP are shown as the percentage of recovery.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LVDP (mm Hg)</th>
<th>RPP (ml/min)</th>
<th>LVEDP (mm Hg)</th>
<th>CF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>14.5 ± 2.1</td>
<td>14.3 ± 2.7</td>
<td>63.7 ± 4.2</td>
<td>69.3 ± 3.3</td>
</tr>
<tr>
<td>1</td>
<td>19.0 ± 1.9</td>
<td>14.3 ± 1.8</td>
<td>58.9 ± 3.5</td>
<td>64.1 ± 6.7</td>
</tr>
<tr>
<td>2.5</td>
<td>22.9 ± 3.2</td>
<td>17.9 ± 2.6</td>
<td>56.1 ± 5.0</td>
<td>75.5 ± 4.9</td>
</tr>
<tr>
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<td>30.0 ± 2.4</td>
<td>24.4 ± 2.6</td>
<td>52.9 ± 2.6*</td>
<td>76.5 ± 3.0*</td>
</tr>
<tr>
<td>10</td>
<td>35.0 ± 2.4**</td>
<td>30.2 ± 2.6**</td>
<td>44.8 ± 2.8**</td>
<td>80.9 ± 3.2*</td>
</tr>
<tr>
<td>50</td>
<td>33.4 ± 3.8**</td>
<td>25.4 ± 3.3**</td>
<td>54.1 ± 8.3*</td>
<td>80.6 ± 5.9*</td>
</tr>
</tbody>
</table>

The dose-dependent effects of 78c treatment on postischemic LVDP, RPP, LVEDP, and CF in hearts subjected to I/R are shown. The recoveries of LVDP, RPP, and CF were increased, and LVEDP decreased, after 30-minute ischemia/30-minute reperfusion with 78c treatment in a dose-dependent manner. LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; RPP, rate-pressure product.

*P < 0.05; **P < 0.001.

Recovery of Contractile Function. The ability of 78c to confer postischemic cardiac protection was tested dose-dependently using 1, 2.5, 5, 10, and 50 μM compound. We initially tested a dose of 10 μM, as we reasoned that this would likely well exceed the level required to achieve full CD38 inhibition throughout the postischemic period. Hearts were treated at 1:20 of CF with 200 μM 78c solution for 10 minutes prior to a 30-minute ischemia. We found that the recoveries of the left ventricular developed pressure and the rate-pressure product in each of the 10 μM–treated hearts were over double that of the vehicle-treated hearts (Fig. 3; Table 1). Consistent with this, left ventricular end diastolic pressure, a measure of diastolic relaxation, was lower in the 10 μM 78c–treated hearts.

With significant protection of cardiac function found for a 10-μM 78c dose, we performed further studies to determine the minimum cardiac protective dose of 78c and the dose dependence of cardiac protection. At 1 μM 78c, cardiac protection was completely lost, with very similar postischemic function compared with hearts receiving vehicle control. Thus, it appeared that the protective effects of 78c likely started somewhere in the range of 1–10 μM. Consistent with this, intermediate levels of protection of cardiac function were observed with infusions of 2.5 and 5 μM 78c (Fig. 3; Table 1). We also increased the 78c dose to a final concentration of 50 μM to determine whether there was any additive protective effect above the 10 μM dose or whether any toxicity was observed. At 50 μM, cardiac protection was very similar to that observed with 10 μM 78c, indicating that the protective effects of 78c were maximal at 10 μM 78c. Importantly, we did not find toxicity on acute delivery of 50 μM 78c while still maintaining cardiac protection equal to that seen with 10 μM 78c. Thus, 78c provides cardiac protection in a fairly broad range of doses with no apparent cardiac toxicity.

Total and Nitric Oxide Synthase–Dependent CF. We questioned how CD38 inhibition with 78c might impact the severe reduction in total and nitric oxide synthase (NOS)–dependent CF seen with I/R (Dumitrescu et al., 2007; Reyes et al., 2015; Boslett et al., 2018a). Total CF was measured throughout the preischemic period and through 120 minutes of reperfusion after a 30-minute ischemic period. We found that CF through the first 45 minutes of reperfusion was significantly higher (P < 0.05) in hearts treated with 5 and 10 μM 78c compared with those treated with the vehicle. Lower doses of 1 and 2.5 μM 78c did not have a significant protective effect on total CF (Fig. 4A).

As a component of total CF, we also measured NOS-dependent CF with acute Nω-nitro-L-arginine methyl ester (an NOS inhibitor) infusions to hearts after 30 minutes of reperfusion. In vehicle-treated hearts undergoing I/R, ~75% of baseline NOS-dependent CF was lost. However, 78c treatment dose-dependently preserved NOS-dependent CF after...
I/R. Although 1 μM 78c had no effect on the preservation of NOS-dependent CF, preservation was observed at 2.5, 5, and 10 μM, with values of 46.7% ± 0.9%, 78.8% ± 3.0%, and 92.0% ± 4.1%, respectively (Fig. 4B).

**Endothelial Levels of NAD(P)H.** Previously, we measured levels of NAD(P)H from the coronary endothelium of wild-type (WT) and CD38−/− hearts undergoing I/R. In those experiments, we found that levels of NAD(P)H were severely depleted in WT hearts but were largely protected in CD38−/− hearts (Boslett et al., 2018a). To determine whether 78c could prevent the NAD(P)H depletion seen in WT hearts after I/R, 78c was infused at 10 μM to ensure complete CD38 inhibition and the endothelial levels of NAD(P)H were measured. Compared with their respective nonischemic levels, postischemic levels of endothelial NAD(P)H were greatly decreased to just 25% of basal levels. This demonstrates that there is marked depletion of NAD(P)H from the coronary endothelium. With 78c, the percentage of recovery was nearly 80% of basal levels (Fig. 5). Thus, 78c treatment is highly effective in preserving endothelial NAD(P)H levels in I/R.

**Effect of 78c Treatment on Myocardial Tetrahydrobiopterin Content.** With 78c-mediated protection of total CF, NOS-dependent CF, and endothelial NAD(P)H against I/R, we sought to determine how the levels of the important NOS cofactor tetrahydrobiopterin (BH4) were affected by I/R with and without 78c treatment. In vehicle-treated hearts undergoing I/R, BH4 levels fell by ∼65% from 5.1 ± 0.3 to 1.8 ± 0.4 pmol/mg protein. With treatment of hearts with 10 μM 78c prior to I/R, postischemic BH4 levels were 3.9 ± 0.6 pmol/mg protein, demonstrating clear protection of BH4 by 78c inhibition (Fig. 6).

**Effect of CD38 Inhibition on Levels of Myocardial NAD(P)⁺ and (2′-P)-ADPR.** As another measure of CD38 activation and inhibition, we assayed the levels of CD38 substrates NAD(P)⁺ and products (2′-P)-ADPR from hearts undergoing control perfusion or I/R with and without 78c treatment. In untreated hearts, NAD(P)⁺ levels fell by ∼78% treatment. BH4 levels in hearts subjected to 30-minute ischemia/30-minute reperfusion with and without 78c treatment (10 μM) were compared with levels in nonischemic hearts (control). In untreated hearts, BH4 levels fell by ∼65% compared with nonischemic control hearts. With 78c treatment, BH4 depletion was greatly prevented (only 20% depletion). ***P < 0.001 vs. control; †P < 0.05 vs. I/R (mean ± S.E.M., n = 5).

**CD38 Inhibition Preserves High-Energy Phosphates.** Using the HPLC method for NAD(P)⁺ and (2′-P)ADPR, we were also able to detect and quantitate ATP, ADP, and AMP levels in the nonischemic and postischemic hearts. Preischemic levels of ATP and ADP were 11.0 ± 0.9 and 5.6 ± 0.1 μmol/g tissue, respectively, with reduction after I/R to 4.38 ± 0.16 and 4.03 ± 0.20 μmol/g tissue, respectively. These magnitudes of ATP and ADP depletion are consistent with prior reports (Neely and Grotyohann, 1984; Imahashi et al., 2004). Interestingly, with 78c treatment prior to I/R, these levels were largely preserved, with levels of 8.5 ± 1.6 and 5.2 ± 0.3 μmol/g tissue, respectively, for ATP and ADP (Fig. 8B). Higher postischemic levels of ATP and ADP are consistent with the increased...
to isolated hearts were performed, as in the prior functional studies, for 10 minutes after a 20-minute period of stabilization. After either 2 minutes of additional drug-free perfusion to clear the vasculature or after 30 minutes of ischemia/30 minutes of reperfusion, hearts were frozen in liquid nitrogen for the HPLC analysis of 78c. These experiments showed that 78c infusion increased tissue 78c concentrations to a level very close to the final concentration of delivery. After the 2-minute washout period, postinfusion 78c levels in heart tissue were approximately 15.85 ± 1.43, 6.99 ± 1.34, 3.54 ± 0.80, and 2.63 ± 0.29 nmol/g tissue for 10, 5, 2.5, and 1 μM 78c treatment, respectively (Fig. 9). Thus, tissue uptake of 78c is efficient and the drug is retained in the heart.

After 30 minutes of ischemia/30 minutes of reperfusion, 78c levels decreased considerably (around 50%) to 7.91 ± 0.20, 3.49 ± 1.13, 1.93 ± 0.19, and 1.17 ± 0.04 nmol/g tissue for 10, 5, 2.5, and 1 μM 78c, respectively (Fig. 9). However, levels of 78c even at the lowest dose of 1 μM still well exceeded those required for full CD38 inhibition based on the in vitro data. With a lack of cardiac protection at 1 μM 78c, we reasoned that there were other factors, such as nonspecific protein or membrane sequestration, limiting the free levels of 78c in tissue. To test this, we performed an aqueous, pH neutral extraction of 78c from 10 μM 78c–treated hearts (control and I/R) and found that the free aqueous tissue levels of 78c were less than 5% of the total measured drug concentration, with levels of 0.68 ± 0.01 nmol/g tissue preischemia and 0.15 ± 0.02 nmol/g tissue post-I/R. With the lower doses of 78c, particularly 1 μM (where no protective effect was seen), we reason that the effective, free aqueous concentration of 78c was below that required for CD38 inhibition.

**Infarct Size.** Hearts tested for the recovery of contractile function were allowed to reperfuse for a total of 120 minutes. Hearts were then sectioned and TTC stained for delineation of viable and infarcted tissue. In vehicle-treated hearts, we measured an average infarct size of 40.9% ± 5.8%. We found that, like with the recovery of contractile function, 78c dose-dependently prevented myocardial infarction after 30 minutes of ischemia, with values of 33.7% ± 2.8%, 29.2% ± 5.1%, 21.3% ± 3.3%, 9.4% ± 2.0%, and 14.8% ± 3.0% for 1, 2.5, 5, 10, and 50 μM 78c, respectively (Fig. 10).

**Discussion**

CD38 has been implicated in many physiologic and pathophysiologic processes, including chronic lymphocytic leukaemia (Ibrahim et al., 2001), diabetes (Kato et al., 1999; Han et al., 2002), metabolic syndrome and aging (Chini, 2009; Camacho-Pereira et al., 2016), social behavior (Jin et al., 2002), metabolic syndrome and aging (Chini, 2009; Cockayne et al., 1998; Partida-Sánchez et al., 2001), stroke (Choe et al., 2011), and myocardial I/R (Reyes et al., 2015; Guan et al., 2016; Boslett et al., 2017, 2018a). Thus, there is a great need for the development of CD38 inhibitors (Choe et al., 2011; Kellenberger et al., 2011; Moreau et al., 2013; Wang et al., 2014; Becherer et al., 2015; Blacher et al., 2015; Haffner et al., 2015). Given the breadth of (patho)physiology involving CD38, the recent discovery of high-potency thiazoloquinazolinone CD38 inhibitors has great therapeutic potential (Haffner et al., 2015).

In myocardial I/R, we have demonstrated a role for CD38 in the degradation of myocardial and endothelial NAD(P)H (Reyes et al., 2015; Boslett et al., 2017, 2018a,b). Pharmacological...
blocking or genetic deletion of CD38 protects the heart against I/R. Since many enzymes require NAD(P)(H) as substrate, depletion of NAD(P)(H) by CD38 activation may affect several key processes, including mitochondrial respiration, reductive biosynthesis, and signaling processes. For example, sirtuins are important NAD-requiring signaling enzymes that are affected by low cellular NAD$^+$ levels caused by CD38 activation (Camacho-Pereira et al., 2016; Guan et al., 2016). Another example is glutathione reductase, which catalyzes the conversion of oxidized glutathione to two molecules of reduced glutathione at the expense of NADPH. Interestingly, CD38$^{-/-}$ mice have higher glutathione levels under normal and postischemic conditions compared with WT controls (Boslett et al., 2018a). Our studies have shown that eNOS, which requires NADPH in the vascular synthesis of vasodilatory NO, is impaired after an ischemia by CD38-mediated NADP(H) depletion (Reyes et al., 2015). The variety of potential processes affected by activated CD38 makes investigation and characterization of novel CD38 inhibitors very important.

We tested the lead compound, 78c, developed from the new class of thiazoloquin(az)olin(on)e CD38 inhibitors. We did this first in vitro with rCD38 to determine the potency and mechanism of CD38 inhibition by 78c. We then characterized the effects of 78c in a model of myocardial I/R injury with both metabolic and functional measurements. A similar study was performed previously with the flavonoid CD38 inhibitor, luteolinidin. We observed that luteolinidin, which occurs naturally in some plant species such as sorghum (Awika et al., 2004), had a $K_i$ of around 10$^{-6}$ M (Boslett et al., 2017). Using the same assay, we found that 78c is a much more potent CD38 inhibitor than the flavonoids (1000-fold), with a $K_i$ of 9 nM (Fig. 2).

Previous studies demonstrated large losses of myocardial contractile function, as well as total and NOS-dependent CF with 30-minute ischemia/30-minute reperfusion in the isolated heart (Reyes et al., 2015; Boslett et al., 2017, 2018a). Consistent with this, postischemic left ventricular function was greatly reduced after 30-minute ischemia while total and NOS-dependent CF were also significantly lower. With 78c treatment, we observed dose-dependent protection of each of these parameters from 2.5 to 50$\mu$M 78c (Figs. 3 and 4). This is consistent with studies of other CD38 inhibitors and the CD38$^{-/-}$ heart, which also demonstrated increased recovery of total and NOS-dependent CF (Reyes et al., 2015; Boslett et al., 2017, 2018a).
NADPH levels in the coronary endothelium were ~80% decreased after I/R. This was largely blocked by preischemic treatment of hearts with 10 μM 78c (Fig. 5). This large NADP(H) depletion contributes to postischemic eNOS dysfunction and its prevention by CD38 inhibition preserves eNOS-dependent vasodilatory function. This profound local NADP(H) depletion may cause impairment of NADPH-requiring enzymes, such as those in the BH4 recycling and de novo synthesis pathways. With loss of endothelial NADPH, normal CD38 substrates, NAD(P)H, NO production. Thus, CD38 activation, through endothelial NADPH depletion and subsequent contribution to low BH4 levels, acts as a trigger for endothelial dysfunction caused by impaired NOS-dependent NO production.

We assessed CD38 activation by measuring degradation of CD38 substrates, NAD(P) +, and formation of the resultant products, (2′−P)ADPR, after I/R, and tested the efficacy of 78c to block each process (Figs. 7 and 8A). In untreated hearts undergoing I/R, postischemic levels of NADP + and NADP + dropped by 30% and 50%, respectively. In these hearts, levels of the CD38 products ADPR and 2′−P−ADPR increased, consistent with CD38 activation. Treatment of hearts with 78c was effective at blocking I/R-induced CD38 activation, resulting in preserved NADP + and (2′−P)−ADPR levels near those of nonischemic hearts. Although CD38 is an enzyme that primarily produces "linear" (2′−P)−ADPR, it can also produce two calcium-mobilizing second messengers in lesser amounts: (2′−P)−cADPR (Vu et al., 1996; Kato et al., 1999; Partida-Sánchez et al., 2001; Deshpande et al., 2005; Chini, 2009) and NAADP (Chini et al., 2002) (Fig. 1). In assaying the myocardial levels of NAD(P) + and (2′−P)−ADPR, we also attempted to measure (2′−P)−cADPR and NAADP but these were undetectable, likely due to the limited sensitivity of the assay (~200 nM limit of detection) and the low concentrations of these signaling molecules. Nevertheless, production of these compounds might increase with CD38 activation in I/R. This could represent an additional mechanism contributing to I/R injury, which is partially due to perturbed Ca 2+ homeostasis of cardiac myocytes and endothelial cells (Zimmerman and Hulsmann, 1966; Ladilov et al., 2000; Garcia-Dorado et al., 2012).

CD38 inhibition also preserved the levels of high-energy phosphates, ATP and ADP, with significantly higher levels after I/R with 78c treatment compared with vehicle (Fig. 8B). This might occur indirectly through maintained mitochondrial respiration, which would be expected with protected NAD(H) levels and higher CF and increased O2 delivery. The increased contractile function seen with 78c treatment is consistent with this, as is the greatly lessened left ventricular infarct size also seen with 78c treatment (Fig. 10). Compared with a previous study with CD38 −/− hearts, treatment of hearts with 78c at a dose of 10 μM prior to I/R similarly protects the heart from infarction (Boslett et al., 2018a).

For physiologic protection, higher concentrations (2.5–50 μM) were required than expected from the in vitro data. We measured tissue uptake and levels of 78c in the heart and found that tissue uptake was efficient, as tissue drug concentrations essentially matched the delivered 78c concentration. Washout in the reperfusion period (~50%) occurred, but adequate levels for inhibition remained even after 30 minutes of reperfusion. We questioned whether the delivered drug was bound or sequestered within the tissue and not in a freely available form in the aqueous phase. Thus, we measured total and free drug concentrations in hearts receiving 10 μM 78c (Fig. 9), and we determined that much of the drug (at least 95%) was in a nonfree state, likely protein bound, sequestered in membranes, or both. Overall, we determined that 78c is a cell-permeable compound with favorable rapid absorption properties. Previously, the group that discovered the thiazoloquinazolinones as CD38 inhibitors determined 78c to be a lipophilic compound (with a ChromLog D value of 3.22 at pH 7.4 based on the octanol/water partition), which likely contributes to its high permeability (Haffner et al., 2015).

The thiazoloquinazolinones such as 78c represent the highest affinity class of CD38 inhibitors discovered. As such, they should prove invaluable in studies determining the role of CD38 in physiology and disease (Aksoy et al., 2006; Barbosa et al., 2007; Camacho-Pereira et al., 2016). Although the CD38 −/− mouse is a powerful tool to explore CD38 function, there can be unanticipated compensatory mechanisms for maintaining homeostasis in genetically modified mice. Thus, the availability of a high-affinity specific inhibitor is of immense value. As an example of this, the effect of CD38 on the aging process was demonstrated initially using a genetic approach to show that CD38 −/− mice were resistant to aging compared with WT mice (Camacho-Pereira et al., 2016), which was then confirmed by a pharmacologic approach demonstrating a robust anti-aging effect of 78c (Tarrago et al., 2018).
Overall, we demonstrate the ability of the highly potent CD38 inhibitor 78c to effect cardiac protection in an ex vivo model of I/R. Consistent with previous studies with the flavonoid CD38 inhibitor, luteolinidin, and the CD38−/− mouse (Boslett et al., 2017, 2018a), we observed protection of postischemic contractile function and NOS-dependent endothelial vasodilatory function as well as decreased infarct size with 78c treatment. The results suggest that 78c could be of great value as a tool to study the role of CD38 in disease, with potential for clinical translation in cardiovascular and other metabolic diseases.

Authorship Contributions


References


Boslett J, Hemann C, Christofi FL, and Zweier JL (2018b) Characterization of CD38 inhibitor, luteolinidin, and the CD38−/− mouse (Boslett et al., 2017, 2018a), we observed protection of postischemic contractile function and NOS-dependent endothelial vasodilatory function as well as decreased infarct size with 78c treatment. The results suggest that 78c could be of great value as a tool to study the role of CD38 in disease, with potential for clinical translation in cardiovascular and other metabolic diseases.