Is diminazene an angiotensin-converting enzyme 2 (ACE2) activator? Experimental evidence and implications

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Abstract

Antiprotozoal veterinary drug diminazene aceturate (DIZE) has been proposed to be an angiotensin-converting enzyme 2 (ACE2) activator. Since then, DIZE was used in dozens of experimental studies, but its mechanism of action attributed to ACE2 activation and enhanced formation of angiotensin-(1-7) (Ang-(1-7)) from Ang II was not carefully verified. The aim of this study was to confirm the effect of DIZE on catalytic activity of ACE2 and extend it to other peptidases involved in formation and degradation of Ang-(1-7). Concentration-dependent effect of DIZE on the initial rate of a fluorogenic substrate hydrolysis by human and mouse recombinant ACE2 was measured at assay conditions imitating that of the original report but no activation of ACE2 was documented. Similar results were obtained with a more physiologically relevant assay buffer. In addition, DIZE did not affect activity of recombinant neprilysin, neurolysin, thimet oligopeptidase and ACE. Efficiency of the fluorogenic substrate hydrolysis ($V_{max}/K_m$ value) by ACE2 in response to different concentrations of DIZE was also measured but no substantial effects were documented. Likewise, DIZE failed to enhance the hydrolysis of ACE2 endogenous substrate Ang II. Identity of the commercial recombinant ACE2 variants used in these experiments was confirmed by inhibition with two well-characterized inhibitors (DX600 and MLN4760), activation by NaCl and Western Blotting using validated antibodies. These observations challenge the widely accepted notion about the molecular mechanism of DIZE action and call for not ascribing this molecule as an ACE2 activator.
Significance statement

Diminazene aceturate (DIZE) has been proposed and widely used in experimental studies as an angiotensin-converting enzyme 2 (ACE2) activator. Our detailed in vitro pharmacological studies failed to confirm that DIZE is an ACE2 activator. In addition, DIZE did not substantially affect the activity of other peptidases involved in formation and degradation of angiotensin-(1-7). Researchers should refrain from calling DIZE an ACE2 activator. Other mechanisms are responsible for the therapeutic benefits attributed to DIZE.
Introduction

Angiotensin-converting enzyme 2 (ACE2) is a critical member of the renin-angiotensin system (RAS), responsible for activation of angiotensin-(1-7)/Mas receptor axis and counteracting many of the pathophysiological consequences of ACE/angiotensin II/angiotensin type 1 receptor axis (Bader et al., 2020). Since its discovery by two independent groups in 2000 (Donoghue et al., 2000; Tipnis et al., 2000), key functions of ACE2 and its critical role in pathogenesis of various cardio- and cerebrovascular, renal and endocrine disorders have been studied by multiple research groups (Marquez et al., 2021). Focus on ACE2 was further enhanced during mid-2000 by a discovery that the peptidase serves as a receptor for SARS-coronavirus (SARS-CoV) (Li et al., 2003) and most recently, by the ongoing COVID-19 pandemic caused by SARS-CoV-2 virus which also uses ACE2 to enter host cells (Hoffmann et al., 2020). This increased focus on ACE2 has led to many exciting discoveries related to understanding of various disorders and pathogenic mechanisms, as well as molecular intricacies of catalysis and modulation of ACE2 and related peptidases. Given the importance of ACE2 in the function of RAS, a number of approaches have been explored to harness the therapeutic potential of this enzyme, including use of recombinant ACE2 as a biologic agent (Marquez et al., 2021). Among these studies, an intriguing and potentially clinically impactful discovery was identification of a small molecule activator of ACE2, diminazene (DIZE) (Kulemina and Ostrov, 2011). Diminazene aceturate is an antiprotozoal agent primarily used in cattle and small ruminants but it is not approved for use in humans by the FDA (Kuriakose and Uzonna, 2014). Since the original report suggesting stimulation of ACE2 activity by DIZE, few dozen research groups used this pharmacological agent for proof-of-concept studies to document beneficial function of ACE2 in preventing and/or reversing various disease conditions, including myocardial infarction (Qi et al., 2013; Castardeli...
et al., 2018), pulmonary hypertension (Rigatto et al., 2013; Shenoy et al., 2013), ischemic stroke (Bennion et al., 2015) and Alzheimer’s disease (Kamel et al., 2018; Evans et al., 2020). Despite the widespread use of DIZE as an ACE2 activator in experimental studies, little was known about selectivity of this agent towards peptidases that belong to the same family of enzymes as ACE2 and are involved in formation and degradation of angiotensin-(1-7) (Ang-(1-7)). Therefore, the original objective of this study was to confirm enhanced catalytic activity of ACE2 in response to DIZE and evaluate the effect of this drug on activity of neprilysin (NEP), neurolysin (Nln), thimet oligopeptidase (TOP) and ACE. Here, we report the results of detailed in vitro pharmacological studies which indicate that DIZE is not a direct activator of ACE2 and challenge the previous observations and widely accepted notion about this molecule.
Materials and Methods

Materials – DIZE was purchased from Sigma Aldrich Co. (product D7770-1G, lots SLBS6137 and SLBZ7521). Recombinant human and mouse ACE2 (products 933-ZN and 3437-ZN), human NEP (product 1182-ZNC), human Nln (product 3814-ZN), human TOP (product 3439-ZN) and human ACE (product 929-ZN) were obtained from R&D Systems (Minneapolis, MN). Selective ACE2 inhibitors, DX600 (product AS-62337) and MLN4760 (product 5306160001) were purchased from AnaSpec and Millipore-Sigma, respectively. Fluorogenic substrate Mca-Pro-Leu-Gly-Pro-D-Lys(Dnp)-OH (product 4027687) was obtained from Bachem, whereas Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp) and Mca-Ala-Pro-Lys(Dnp) were purchased from Enzo Life Sciences (products BML-P227 and BML-P163).

Enzymatic assays – Activity of peptidases was measured in a continuous assay by documenting the increase in fluorescence signal in the result of hydrolysis of a quenched fluorescence substrate as detailed in our previous publications (Wangler et al., 2016; Jayaraman et al., 2020). For ACE2 we used fluorogenic substrate Mca-Ala-Pro-Lys (Dnp) (Kulemina and Ostrov, 2011), for ACE and NEP, Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (Johnson and Ahn, 2000; Wösten-van Asperen et al., 2008) and for Nln and TOP, Mca-Pro-Leu-Gly-Pro-D-Lys(Dnp)-OH (Jayaraman et al., 2021). As detailed in results section, the first set of experiments were carried out in 75 mM Tris-HCl 0.1 M NaCl, 0.5 μM ZnSO₄ and 0.01% Triton-X (pH 7.4) which is the same assay buffer used in the original study reporting discovery of DIZE as an ACE2 activator (Kulemina and Ostrov, 2011). To mimic a more physiologically relevant environment, specifically that of the brain due to our long-standing interests in brain peptidases (Karamyan and Speth, 2007; Karamyan et al., 2009; Rashid et al., 2014; Wangler et al., 2016),
subsequent experiments were carried out in artificial cerebrospinal fluid (aCSF; 0.1% Triton X-100, 126 mM NaCl, 26 mM NaHCO₃, 3 mM KCl, 4 mM glucose, 1.4 mM KH₂PO₄, 25 mM HEPES, 1.4 mM CaCl₂, 1.3 mM MgCl₂, 0.2 µM ZnSO₄, pH 7.2). In experiments with TOP, the assay buffer additionally contained 0.1 mM dithiothreitol (Shrimpton et al., 1997). DIZE was dissolved in deionized water to make 30 mM stock followed by dilution in the assay buffer for working stocks. DIZE (0.1 to 100 µM in 100 µl assay volume in 96-well plates) was incubated with recombinant ACE2 (0.05 or 0.065 nM human, and 0.04 or 0.065 nM mouse ACE2), ACE (0.2 or 0.5 nM), Nln (0.3 or 1.2 nM), NEP (0.12 or 0.8 nM) or TOP (0.3 or 1.2 nM) for 10 minutes at 37°C before addition of the respective fluorogenic substrate to start the reaction. In all enzymatic assays, generation of the fluorescent product was documented every 1 min in a plate reader (λₑₓ = 320, λₑᵣᵣ = 405; SynergyMX; Biotek) at initial velocity conditions where ~10% of the substrate was hydrolyzed and each sample was present in duplicate.

The inherent fluorescence quenching or enhancing property of DIZE was tested under the same experimental conditions, with the exception that 2 µM assay concentration of Mca-Pro-Leu-OH (hydrolysis product of the quenched fluorescence substrate; λₑₓ = 320, λₑᵣᵣ = 405), was present with ACE2 and different concentrations of DIZE.

NaCl dependency of ACE2 activity was measured in the same way as detailed above, except that the assay buffer was 50 mM MES, 10 µM ZnSO₄, 0.01% Triton X-100, pH 6.5 with additional 0, 0.1, 0.5 or 1 M NaCl (Vickers et al., 2002).
Kinetic experiments and enzyme inhibition – Kinetic parameters for both recombinant human and mouse ACE2 in the presence of 10 and 50 μM assay concentrations of DIZE were determined by addition of 2.5 to 100 μM fluorogenic substrate. DIZE was preincubated with human ACE2 (0.12 nM) or mouse (0.06 or 0.12 nM) ACE2 in aCSF at 37°C for 10 min before the addition of the fluorogenic substrate as described above. In inhibition assays involving DX600 and MLN4760, increasing concentrations (0.1 nM – 1 μM) of either inhibitor was preincubated with human (0.08 nM) or mouse (0.065 or 0.09 nM) ACE2 in aCSF at 37°C for 10 min followed by addition of the fluorogenic substrate.

Measurement of Ang-(1-7) formation from Ang II by liquid chromatography-mass spectrometry analysis – Recombinant human or mouse ACE2 (0.3 nM and 0.5 nM, respectively) were incubated with 10 or 50 μM DIZE in aCSF at 37°C for 10 min (30 µl assay volume), followed by addition of 20 μM angiotensin II (Ang II, product 002-12, Phoenix Pharmaceuticals, Inc.) and further incubation at 37°C for 10 min. These conditions ensured no more than 15% hydrolysis of Ang II. The reaction was stopped by addition of 1 µl of 1.0 N LCMS grade HCl and storage at −80°C freezer. Quantification of Ang II and its hydrolysis product Ang-(1-7) was carried out by LC-MS/MS using a SCIEX Triple Quad™ 5500+ LC-MS/MS System following a protocol modified from our recent studies (Jayaraman et al., 2021; Rahman et al., 2021). In brief, 2 µl of sample was injected onto a 50 × 2.1 mm Kinetex EVO C18 column (Phenomenex). DAMGO, a synthetic opioid (product 1171, Tocris Bioscience), was used as an internal standard and was spiked into each sample. Chromatography was performed at flow rate of 300 µl min⁻¹ and total run time of 10 min, mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient of solvent B was: linear increase from 3 to
35% during the first 5 min, increase to 85% in the next 50 sec, continuation at this level until 7 min, return to 3% in the next 50 sec and continuation at this level until 10 min. Detection of Ang II and Ang-(1-7) were carried out in the positive polarity and multiple-reaction monitoring mode (150 msec dwell times and 5500 volts). The collision energy used for Ang II and Ang-(1-7), were 29 and 30 volts, respectively. The precursor ion of charged state was calculated by mass-to-charge ratio (m/z), as M + 1, (M + 2)/2 or (M + 3)/3 primary ions, and identified and fragmented by a collision-induced dissociation (CID) voltage. The Q1 to Q3 transitions were m/z 524.1 precursor ion to m/z 263.1 for Ang II, m/z 450.4 precursor ion to m/z 110.1 for Ang-(1-7), and m/z 514 precursor ion to m/z 134.1 for DAMGO. The peak areas for Ang II and Ang-(1-7) and the internal standard were obtained through Analyst software™ (version 1.7.2) and expressed as peak area ratio (peptide peak area/internal standard peak area).

*Western Blotting* – To confirm the identity of the recombinant human and mouse ACE2 purchased from a commercial vendor, a set of Western Blotting experiments were carried out. For this, human (20 and 40 ng) and mouse (40 and 80 ng) ACE2, and human ACE (negative control, 20 and 40 ng) were resolved in a 7.5% precast polyacrylamide gel (Mini-PROTEAN TGX, product 4561023, Bio-Rad Laboratories) using conventional SDS/PAGE as described in our previous publications (Al Shoyaib et al., 2021a; Al Shoyaib et al., 2021b). The resolved samples were transferred onto a polyvinylidene difluoride (PVDF) membrane (product 88518, Thermo Scientific), blocked with 1% BSA (product BP9703-199, Fisher Scientific) solution in Tris-buffered saline with Tween-20 (TBST) and incubated with rabbit polyclonal anti-ACE2 antibody (freshly diluted in TBST containing 1% BSA at 1:10000; product RP2-ACE-2, Triple Point Biologies Inc.; also available from Abcam, product AB-38941) at room temperature.
overnight. Next, the membrane was washed five times with TBST and incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (freshly diluted in TBST containing 5% non-fat dry milk at 1:10000 dilution; product 170-6515, Bio-Rad laboratories) for 45 min. Afterwards, the membrane was washed with TBST, incubated with Super Signal™ West Pico PLUS chemiluminescent detection reagent (Thermo Scientific) and exposed to an X-ray film. Next, the membrane was incubated in Restore PLUS Western Blot Stripping Buffer (product 46430, Thermo Scientific) for 15 minutes at room temperature, washed and blocked as noted above, and incubated with rabbit polyclonal anti-ACE antibody (freshly diluted in TBST containing 1% BSA at 1:20000; product RP3-ACE, Triple Point Biologics Inc.; also available from Abcam, product AB-39172) at room temperature overnight. All subsequent steps to detect ACE via chemiluminescence were carried out as detailed above, with an exception that the secondary antibody was used at 1:20000 dilution. The original images of the films were cropped and labeled using Photoshop.

Statistical analyses – Statistical analyses and curve fitting were carried out using GraphPad Prism 9.3.1 software. The liner regression model of the software \( V_0 = \text{Slope} = \Delta \text{fluorescent intensity of the reaction product/} \Delta \text{time} \) was used to calculate slope of the line for each enzymatic reaction, which represents the initial velocity \( V_0 \) for the reaction progress curve. For calculation of \( A_{50} \) and \( A_{\text{max}} \) values, \( V_0 \) values for hydrolysis of the fluorogenic substrate in the presence of varying concentrations of DIZE were expressed as percent within each experiment, 100% being \( V_0 \) of enzymatic reaction in the absence of DIZE (i.e., basal activity with vehicle control). Curve fitting of \%V_0 \) was done by a nonlinear regression model for the three-parameter log(stimulator) vs. response equation \[ Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1+10^{\gamma((\text{Log}A_{50}-X))}) \]. \( K_m \)
and \( V_{\text{max}} \) values were calculated by fitting initial velocity values for hydrolysis of varying concentrations of the fluorogenic substrate by ACE2 in the absence or presence of 10 or 50 \( \mu \text{M} \) of DIZE into Michaelis-Menten equation \( [Y = V_{\text{max}} \cdot X/(K_m + X)] \). \( IC_{50} \) values for selective ACE2 inhibitors were calculated by fitting initial velocity values for hydrolysis of the fluorogenic substrate by ACE2 in the presence of varying concentrations of each inhibitor into a nonlinear regression model for the three-parameter log(inhibitor) vs. response equation \( [Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(X - \text{LogIC50})})] \). \( K_i \) values were calculated based on the Cheng-Prusoff equation: \( K_i = IC_{50}/(1 + S/K_m) \) where \( S \) is the substrate concentration (10 \( \mu \text{M} \) for the fluorogenic substrate in our experiments), and \( K_m \) is the \( K_m \) value for the substrate (19.4 and 9.8 \( \mu \text{M} \) for human and mouse ACE2 respectively, see Table 1). Comparison of data from experiments with several groups (Figs. 3, 4 and 6) was done by one-way ANOVA followed by Dunnett’s multiple comparison test. A p-value < 0.05 was considered statistically significant. Data are presented as mean, mean with 95% confidence intervals or mean ± S.D.
Results

Effect of DIZE on activity of human and mouse recombinant ACE2 in Tris buffer – To replicate activation of human recombinant ACE2 by DIZE, we used the same experimental conditions (including Tris HCl assay buffer, fluorogenic substrate, ACE2, etc.) reported by Kulemina and Ostrov (2011). Concentration-dependent effect of DIZE on initial velocity of fluorogenic substrate hydrolysis by ACE2 is presented in Fig. 1, indicating negligible changes in activity of the peptidase with up to 30 µM DIZE, and 20.5% (95% CI, 16.4 – 24.5%) inhibition with 100 µM of the drug. Since progression of the enzymatic reaction in these experiments was evaluated by tracking the generated fluorescence signal, we additionally evaluated the effect of DIZE on fluorescence properties of Mca (7-methoxycoumarin-4-acetyl) group in Mca-Pro-Leu-OH under the same experimental conditions. It was revealed that at high concentrations DIZE has fluorescence quenching properties, which amounts to 18.6% (95% CI, 17.9 – 19.3%) at 100 µM (Fig. 1) and is more pronounced at higher concentrations of the drug.

We repeated the same experiments with mouse recombinant ACE2 and observed nearly identical results – no changes in activity of the peptidase with up to 30 µM DIZE, 28.9% (95% CI, 20.2 – 37.3%) inhibition with 100 µM of the drug, and 17.6% (95% CI, 16.6 – 18.5%) quenching of the fluorescence signal in the presence of 100 µM DIZE (Fig. 1).

Notably, the lack of DIZE effect on ACE2 activity was contrary to our expectation because of which, we evaluated two different lots of DIZE and ACE2 but documented similar results (the complied data are presented in this manuscript).

Effects of DIZE on activity of human and mouse recombinant ACE2 in aCSF – In this set of experiments, we repeated the approach described above using aCSF instead of Tris buffer for
assays, because of our long-standing interests in brain peptidases and a need to study ACE2 in a more physiologically relevant environment. In this case too, DIZE inhibited activity of human and mouse ACE2 by 13.8% (95% CI, 12.2 – 15.3%) and 30.3% (95% CI, 27.2 – 33.3%) at 100 µM, and had negligible effect at lower concentrations (Fig. 2).

**Effects of DIZE on activity of other peptidases involved in formation and degradation of Ang-(1-7)** – To understand whether DIZE may affect activity of other peptidases related to ACE2 and by that modulate availability of Ang-(1-7), we studied its effects on catalytic activity of human recombinant NEP (neprilysin, aka neutral endopeptidase), Nln (neurolysin), TOP (thimet oligopeptidase) and ACE (Fig. 2). DIZE had negligible effect on activity of these peptidases at low concentrations, but at 100 µM it inhibited activity of NEP by 21.4% (95% CI, 17.8 – 25.7%), Nln by 18.7% (95% CI, 15.9 – 21.4%), TOP by 18.6% (95% CI, 13.1 – 24.2%), and ACE by 27.6% (95% CI, 23.9 – 31.4%).

**Effect of DIZE on catalytic efficiency of ACE2** – To determine whether DIZE may increase the catalytic efficiency of ACE2 without apparent increase in initial velocity of the substrate hydrolysis, in this set of experiments we studied its effect on hydrolysis of synthetic substrate at different concentrations. Since half-maximal activating concentration ($A_{50}$) of DIZE reported by Kulemina and Ostrov (2011) was 8 µM with $A_{max}$ value of ~300%, we used 10 and 50 µM assay concentrations of DIZE to observe unambiguous effects. In our experiments, the maximal velocity ($V_{max}$) for human ACE2 was increased by ~2% in the presence of 10 µM DIZE and decreased by ~25% in the presence of 50 µM DIZE (Fig. 3 and Table 1), with respective ~11% increase and ~35% decrease in calculated $K_m$ values. Similarly, with mouse ACE2 the calculated
V_max values decreased by ~10% and 25% in the presence of 10 and 50 µM DIZE, whereas K_m values decreased by ~2 and 14% (Fig. 3 and Table 1).

As summarized in Table 1, we observed an overall decrease in catalytic efficiency (V_max/K_m value) of ACE2 in the presence of DIZE, with exception of the human variant in response to 50 µM of DIZE where ~17% increase was documented.

Effect of DIZE on ACE2-mediated formation of Ang-(1-7) from Ang II – Since observations made with synthetic substrates may be misleading in identification of enzyme modulators (Jayaraman et al., 2021), in this set of experiments the concentration-dependent effect of DIZE on hydrolysis of Ang II by ACE2 was studied using mass spectrometry and no substantial differences were documented (Fig. 4). More specifically, in case of human ACE2 the relative amount (peak area of Ang-(1-7)/internal standard) of generated Ang-(1-7) in the absence of DIZE (i.e., vehicle control) was 0.32 (95% CI, 0.29 – 0.33), whereas it was 0.30 (95% CI, 0.27 – 0.32) and 0.30 (95% CI, 0.28 – 0.32) in the presence of 10 and 50 µM DIZE, respectively. Similarly, in case of mouse ACE2 the relative amount of formed Ang-(1-7) in the absence of DIZE was 0.61 (95% CI, 0.59 – 0.63), whereas it was 0.60 (95% CI, 0.58 – 0.62) and 0.59 (95% CI, 0.57 – 0.61) in the presence of 10 and 50 µM DIZE, respectively (Fig. 4). In line with this observation, the relative amount of Ang II (peak area of Ang II/internal standard) in the same samples for human ACE2 was 1.68 (95% CI, 1.64 – 1.72; vehicle), 1.68 (95% CI, 1.65 – 1.71; 10 µM DIZE) and 1.69 (95% CI, 1.65 – 1.73; 50 µM DIZE), whereas for mouse ACE2 it was 1.53 (95% CI, 1.49 – 1.56; vehicle), 1.54 (95% CI, 1.5 – 1.58; 10 µM DIZE) and 1.53 (95% CI, 1.49 – 1.57; 50 µM DIZE) (Fig. 4). As expected, the Ang-(1-7)/Ang II ratio (i.e. ratio of relative amounts) was also similar.
in these samples, being 0.187 (95% CI, 0.177 – 0.198; vehicle), 0.176 (95% CI, 0.162 – 0.189; 10 µM DIZE) and 0.177 (95% CI, 0.162 – 0.192; 50 µM DIZE) for human ACE2, and 0.40 (95% CI, 0.391 – 0.410; vehicle), 0.387 (95% CI, 0.374 – 0.401; 10 µM DIZE) and 0.387 (95% CI, 0.374 – 0.399; 50 µM DIZE) for mouse ACE2.

**Effects of MLN4760 and DX600 on activity of human and mouse recombinant ACE2** – To verify identities of human and mouse recombinant ACE2 used in the above-described experiments, here we used two well-characterized selective inhibitors of the peptidase, MLN4760 and DX600, to study the inhibition profile of the commercial ACE2. Concentration-dependent effects of both inhibitors on initial velocity of fluorogenic substrate hydrolysis by human and mouse ACE2 are presented in Fig. 5. In case of MLN4760, the calculated IC$_{50}$ values are 0.51 nM (95% CI, 0.40 – 0.64 nM; $K_i = 0.34$ nM) and 8.5 nM (95% CI, 8.02 – 9.19 nM; $K_i = 4.25$ nM) for human and mouse ACE2, respectively. In case of DX600, the calculated IC$_{50}$ value is 41.7 nM (95% CI, 35.3 – 49.2 nM; $K_i = 27.5$ nM) for human ACE2, whereas for the mouse variant, as expected, we observed low-potency inhibition (IC$_{50} > 1$ µM).

**Effect of sodium chloride on activity of human and mouse recombinant ACE2** – One of the interesting features of human ACE2 is the ability of NaCl to enhance its catalytic activity (Vickers et al., 2002). To evaluate this feature of the commercial human ACE2 used in the above-described experiments, we conducted a new set of assays using a MES-based buffer as originally described. Similar to the earlier reports (Vickers et al., 2002; Guy et al., 2003), we documented profoundly enhanced activity of human ACE2 in the presence of increasing concentrations of NaCl. The initial velocity of hydrolysis ($V_0$) was 122 (95% CI, 104 – 140) in
the absence of NaCl, and 329 (95% CI, 271 – 387), 616 (95% CI, 554 – 678), and 559 (95% CI, 510 – 609) in the presence of 0.1, 0.5 and 1 M NaCl. The same experiment was also conducted with mouse ACE2, however only slight activation was observed with 0.1 M NaCl, and inhibition was documented with 1 M NaCl; V₀ values were 466 (95% CI, 430 – 501), 549 (95% CI, 501 – 598), 414 (95% CI, 374 – 454), and 326 (95% CI, 300 – 352) for 0, 0.1, 0.5 and 1 M NaCl (Fig. 6).

**Confirmation of ACE2 identity by Western Blotting** – In this last set of experiments, we used commercial antibodies selective for ACE2 to verify the identity of the enzymes used in our study by conventional Western Blotting. As expected, these experiments confirmed identity of the recombinant ACE2 and specificity of the used anti-ACE2 antibody (Fig. 7).
Discussion

In this study, we sought to verify the direct effect of DIZE on activity of ACE2 and extended it to closely related peptidases that are involved in formation and degradation of Ang-(1-7). DIZE, which is an antiprotozoal agent used in veterinary medicine, was identified by Kulemina and Ostrov (2011) to directly interact with ACE2 and enhance its catalytic activity. We replicated the assay conditions of the original report, but contrary to our expectation, no activation of human recombinant ACE2 was observed over a wide range of DIZE concentrations (Fig. 1). Since many studies using DIZE as an ACE2 activator have been conducted in mice, we additionally did the same experiment using mouse recombinant ACE2 but failed to document activation of the peptidase. Similar observations were documented in aCSF which was used as a more physiologically relevant assay buffer (Rashid et al., 2014) (Fig. 2). We also revealed, that at 100 µM and higher concentrations, DIZE quenches the fluorescence signal of the product generated from the hydrolysis of fluorogenic substrate in these assays. This revelation indicates overestimation of ACE2 inhibition observed with 100 µM DIZE (Figs. 1 and 2). Similarly, negligible effects of DIZE were documented on activities of recombinant NEP, Nln, TOP and ACE, peptidases involved in processing of angiotensins and Ang-(1-7) (Karamyan and Speth, 2007) (Fig. 2). We sought to clarify the concentration-dependent effect of DIZE on efficiency of fluorogenic substrate hydrolysis by ACE2 (V_{max}/K_m value) but documented minor effects (Fig. 3 and Table 1) that are substantially different from the earlier reported observations (Kulemina and Ostrov, 2011). In an effort to reproduce the original findings, we also conducted a set of experiments using Ang II as a substrate. Regretfully, in this case too we did not observe increased production of Ang-(1-7) by human or mouse ACE2 in the presence of DIZE (Fig. 4). To exclude the possibility that we experimented on a different enzyme, we additionally
authenticated the recombinant ACE2 purchased from a commercial vendor. First, we used two well-characterized selective ACE2 inhibitors and documented concentration-dependent inhibition of human and mouse ACE2 with \( K_i \) values in line with previous reports (Fig. 5). These experiments also confirmed an earlier finding that DX600 is a poor mouse ACE2 inhibitor (Pedersen et al., 2011). Since NaCl is known to enhance activity of human ACE2 (Vickers et al., 2002; Guy et al., 2003), next we evaluated this property of the peptidase. Similar to earlier reports, we observed a profound increase in activity of human ACE2 in the presence of increasing concentrations of NaCl, whereas there was a small activation or inhibition with mouse ACE2 (Fig. 6). Currently, we do not know why NaCl does not enhance activity of mouse ACE2, and we are not aware of another study investigating this question. Lastly, we confirmed the identity of human and mouse recombinant ACE2 using validated primary antibodies and conventional Western Blotting (Fig. 7).

Thus, based on these collective data and contrary to our original expectation, we conclude that DIZE does not enhance activity of ACE2. This conclusion is in contradiction with the premise of a large body of experimental studies conducted after discovery of DIZE to be an ACE2 activator. The use of DIZE in these studies was based on the therapeutic benefit attributed to ACE2 activation and enhanced formation of Ang-(1-7) from Ang II. It is important to recognize that analogues to an enzyme inhibitor, which selectively binds to an enzyme and decreases its catalytic activity, a small molecule activator would be expected to bind to the enzyme but increase its catalytic activity. To our knowledge, there are only two reports, in addition to the original study by Kulemina and Ostrov (2011), showing experimental data with DIZE directly enhancing activity of ACE2 (Shenoy et al., 2013; LA De Maria et al., 2016). These two papers
are from the same research group and appear to be related to the team of researchers discovering small molecule ACE2 activators (Hernandez Prada et al., 2008). On the contrary, several other studies also looked into confirming activation of ACE2 by DIZE in a limited set of experiments but failed to document enhanced activity of the peptidase. The first study carried out by Haber and colleagues (2014) mostly focused on ACE2 activation by another reported ACE2 stimulator, a xanthenone analog XNT, but also included focused experiments with DIZE. In experiments involving recombinant ACE2 and synthetic fluorogenic substrate or Ang II, Haber et al. did not document activation of the peptidase by DIZE. Furthermore, the authors did not observe enhanced activity of ACE2 in mouse and rat kidney lysates in response to DIZE. This was confirmed by another research group using both recombinant ACE2 and cerebral cortical lysates (Bennion et al., 2015), although no experimental details were provided. Similarly, Velkoska and colleagues assessed the direct effect of DIZE on ACE2 activity in rat cardiac (Velkoska et al., 2016) and kidney cortex (Velkoska et al., 2015) membrane preparations and did not observe activation of the peptidase. Lastly, another research group evaluated the effect of DIZE on activity of ACE2 in porcine coronary artery homogenates and failed to document activation (Raffai et al., 2014). Although, confirmation of ACE2 activation by DIZE was not the primary goal of these studies and hence only limited number of experiments were carried out, collectively they support our detailed observations suggesting that this drug does not enhance the catalytic activity of ACE2. Importantly, several of these studies also included the membrane-bound form of ACE2 in their experiments, opposite to Kulemina and Ostrov (2011) and the present study that used the soluble form of the peptidase. Nevertheless, activation of the membrane-bound ACE2 was also not observed in the presence of DIZE.
As noted above, DIZE has been successfully used in numerous experimental disease models as a pharmacological treatment, with an understanding that it is an ACE2 activator (Foureaux et al., 2013; Qi et al., 2013; Rigatto et al., 2013; Shenoy et al., 2013; Bennion et al., 2015; Souza et al., 2016; Castardeli et al., 2018; Kamel et al., 2018; Evans et al., 2020). A closer look at the published literature may explain the discrepancy between the lack of ACE2 activation by DIZE (present and above-discussed studies) and the documented beneficial effects of this drug in various disease models. One caveat with many of these studies is that activity of ACE2 was simply not measured (Foureaux et al., 2013; Rigatto et al., 2013; Souza et al., 2016; Castardeli et al., 2018). Another important detail is that many studies administered DIZE over a period of several days to weeks, and among other effects documented upregulation of ACE2 (at the level of mRNA and/or protein, e.g. (Foureaux et al., 2013; Qi et al., 2013; Goru et al., 2017; Evans et al., 2020)). In these cases, higher activity of ACE2 was also documented, which is not surprising since higher amount of enzyme affords increased catalysis and higher reaction rate within a certain concentration range (Rashid et al., 2014). In this context, it is important to note that the primary mechanism of action for the antiprotozoal activity of DIZE is explained by its ability to intercalate and bind to DNA and RNA duplexes (Pilch et al., 1995). Hence, it is not surprising that many research groups documented altered expression of ACE2 and other members of the RAS in response to chronic administration of DIZE in experimental animals. It is important to recognize that DIZE may also affect the expression of other regulatory proteins involved in modulation of ACE2. Perhaps, the best example is ADAM17 (a disintegrin and metalloproteinase 17) which controls shedding of ACE2 ectodomain from the cell membrane (Xia et al., 2013), and was shown to be downregulated by DIZE (Velkoska et al., 2016; Wang et al., 2021). ADAM17-mediated regulation of ACE2 appears to be tissues-specific (Pedersen et
al., 2015) and may be the reason why some studies failed to document DIZE-mediated upregulation of ACE2 in certain tissues (Velkoska et al., 2016). Furthermore, expression of other regulatory proteins may also be modulated by DIZE (e.g., proinflammatory cytokines, NADPH oxidase, extracellular matrix proteins (Rajapaksha et al., 2018)), which offer additional, ACE2-independent mechanisms to explain the effects of this drug in various pathophysiological conditions. Lastly, it is important to note that DIZE has been characterized by several research groups as an acid-sensing ion channel (ASIC) blocker with sub-micromolar potency (Schmidt et al., 2017; Krauson et al., 2018; Lee et al., 2018). This mechanism is likely responsible for both acute and chronic effects of DIZE, including regulation of blood pressure, inflammation and ischemic cell death, because of the well-documented role of ASICs in these and other (patho)physiological conditions (Drummond et al., 2008; Heusser and Pless, 2021).

In conclusion, the experimental observations reported in this study indicate that DIZE does not enhance catalytic activity of recombinant ACE2 and challenge the widely accepted notion that this drug is an ACE2 activator. The pharmacological effects of DIZE observed in numerous publications are likely because of its ability to modulate the expression of various regulatory proteins and/or blockade of ASICs. Based on this, we urge the scientific community to refrain from calling DIZE an ACE2 activator. Given that ACE2 is a promising therapeutic target, studies focusing on identification of small molecule ACE2 activators remain critically important. A potential lead in this regard could be a recent discovery reporting enhancement of ACE2 activity by the SARS-CoV-2 spike protein, mediated by binding of its receptor binding domain (RBD) (Lu and Sun, 2020). Notably, the suggested conformational effects of the spike protein/RBD on
ACE2 may help to better understand the activation mechanism of ACE2 and design novel small molecule activators of the peptidase.
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Author contributions

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Performed data analysis: Hadi Esfahani and Karamyan.

Wrote or contributed to the writing of the manuscript: Hadi Esfahani, Jayaraman, and Karamyan.
References


Heusser SA and Pless SA (2021) Acid-sensing ion channels as potential therapeutic targets. *Trends in pharmacological sciences.*


Footnotes

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Figure legends

Figure 1. Effect of DIZE on activity of human and mouse recombinant ACE2 in Tris buffer. Panels a and b: representative reaction progress curves of fluorogenic substrate hydrolysis (10 µM) by recombinant human and mouse ACE2 in the presence of different concentrations of DIZE in Tris HCl assay buffer used by Kulemina and Ostrov (2011). Panels c and d: concentration-dependent effect of DIZE on hydrolysis of the fluorogenic substrate under the same experimental conditions (mean ± SD, n = 6 independent experiments with duplicate samples for each condition). Note that the initial velocity of hydrolysis in the absence of DIZE corresponds to 100% on the vertical axis (basal activity). Panels e and f: representative, concentration-dependent effect of DIZE on fluorescence signal of Mca-Pro-Leu-OH, under the same assay conditions. The only difference was presence of McaPro-Leu-OH, instead of the fluorogenic substrate, in the assay at 2 µM final concentration. Each data point represents the average fluorescence signal measured every minute for a 10 min period.

Figure 2. Effect of DIZE on activity of peptidases in aCSF. All panels document concentration-dependent effect of DIZE on hydrolysis of a respective fluorogenic substrate under the same experimental conditions described for Fig. 1, with the exception that aCSF was used as assay buffer (mean ± SD, n = 6 independent experiments with duplicate samples for each condition): Mca-Ala-Pro-Lys (Dnp) at 10 µM for human and mouse ACE2, Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH at 10 µM for neprilysin (NEP) and angiotensin converting enzyme (ACE), Mca-Pro-Leu-Gly-Pro-D-Lys(DNP)-OH at 15 µM for neurolysin (Nln) and thimet oligopeptidase (TOP). Note that the initial velocity of hydrolysis in the absence of DIZE corresponds to 100% on the vertical axis (basal activity).
Figure 3. The effect of DIZE on catalytic efficiency of human and mouse recombinant ACE2. Hydrolysis of different concentrations of fluorogenic substrate (QFS) by human and mouse recombinant ACE2 in the absence or presence of DIZE (10 and 50 µM) is presented (mean ± SD, n = 6 independent experiments with duplicate samples for each condition, FLU – fluorescence unit).

Figure 4. The effect of DIZE on hydrolysis of Ang II by ACE2. Human or mouse recombinant ACE2 (0.3 and 0.5 nM, respectively) was incubated with Ang II (20 µM) in the absence or presence of DIZE (10 or 50 µM). Relative amounts of Ang-(1-7) and Ang II were documented by mass spectrometry analysis (n = 6 independent experiments with duplicate samples for each condition; p > 0.05 for respective ACE2 alone vs. ACE2 with either concentration of DIZE; the black line within the scattered dots indicates the mean). Analyte denotes Ang II or Ang-(1-7), whereas IS denotes internal standard.

Figure 5. Inhibition profile of ACE2 by DX600 and MLN4760. Concentration-dependent effect of DX600 and MLN4760 on hydrolysis of the fluorogenic substrate by human (a) and mouse (b) recombinant ACE2 (mean ± SD, n = 6 independent experiments with duplicate samples for each condition). Note that the initial velocity of hydrolysis in the absence of the inhibitors corresponds to 100% on the vertical axis (basal activity).

Figure 6. Influence of NaCl on activity of ACE2. Concentration-dependent effect of NaCl on hydrolysis of the fluorogenic substrate by human (a) and mouse (b) recombinant ACE2 (n = 6
independent experiments with duplicate samples for each condition; FLU – fluorescence unit; **p<0.01, ***p<0.001 in comparison to respective ACE2 activity in the absence of NaCl; the black line within the scattered dots indicates the mean).

Figure 7. Identification of ACE2 by Western Blotting. Representative immunoblotting experiment documenting identity of the commercial, recombinant human (h) and mouse (m) ACE2 used in this study. Human (20 and 40 ng) and mouse (40 and 80 ng) ACE2, and human ACE (negative control, 20 and 40 ng) were subjected to SDS-PAGE analysis followed by conventional Western Blotting to first detect ACE2 and then (after stripping the anti-ACE2 antibody), ACE. Migration of molecular weight standards (25 to 250 kDa) are presented on the left side of the image.
### Table 1. Calculated $V_{\text{max}}$ and $K_m$ values for experiments presented in Figure 3.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>$V_{\text{max}}/K_m$</th>
<th>Fold change</th>
</tr>
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<tr>
<td></td>
<td>(FLU/ng protein per min)</td>
<td>(µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human ACE2</td>
<td>791.2 (745.3 – 837)</td>
<td>19.4 (16.2 – 22.6)</td>
<td>40.7</td>
<td>---</td>
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<tr>
<td>hACE2 + 10µM DIZE</td>
<td>808.2 (742 – 874.4)</td>
<td>21.6 (16.7 – 26.5)</td>
<td>37.4</td>
<td>0.92</td>
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<tr>
<td>hACE2 + 50µM DIZE</td>
<td>595.8*** (549.3 – 642.4)</td>
<td>12.5* (9.3 – 15.6)</td>
<td>47.8</td>
<td>1.17</td>
</tr>
<tr>
<td>Mouse ACE2</td>
<td>560.5 (525.9 – 595.1)</td>
<td>9.8 (7.7 – 12)</td>
<td>56.7</td>
<td>---</td>
</tr>
<tr>
<td>mACE2 + 10µM DIZE</td>
<td>509.2 (460.5 – 557.9)</td>
<td>9.6 (6.4 – 12.8)</td>
<td>52.8</td>
<td>0.93</td>
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<tr>
<td>mACE2 + 50µM DIZE</td>
<td>418.3*** (378.5 – 458.2)</td>
<td>8.4 (5.5 – 11.2)</td>
<td>49.9</td>
<td>0.88</td>
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</table>

$V_{\text{max}}$ units are in FLU/ng protein per min (FLU – fluorescence unit), $K_m$ units are in µM. Data are presented as means together with 95% confidence intervals in parenthesis (n = 6 independent experiments with duplicate samples for each condition; *p<0.05, ***p<0.001 in comparison to respective ACE2 values).
Figure 1.

(A) Fluorescence units (FLU) vs. Time (min) for human ACE2 with different concentrations of DIZE (0µM, 0.1µM, 0.3µM, 1µM). The graph shows a dose-dependent increase in fluorescence units.

(B) Fluorescence units (FLU) vs. Time (min) for mouse ACE2 with different concentrations of DIZE (0µM, 0.1µM, 0.3µM, 1µM). The graph shows a dose-dependent increase in fluorescence units.

(C) % V_0 vs. [DIZE], log M for human ACE2. The graph shows a decrease in % V_0 with increasing concentration of DIZE.

(D) % V_0 vs. [DIZE], log M for mouse ACE2. The graph shows a decrease in % V_0 with increasing concentration of DIZE.

(E) Fluorescence units (FLU) vs. [DIZE], log M for human ACE2. The graph shows a decrease in fluorescence units with increasing concentration of DIZE.

(F) Fluorescence units (FLU) vs. [DIZE], log M for mouse ACE2. The graph shows a decrease in fluorescence units with increasing concentration of DIZE.
Figure 2.
Figure 3.

(A) Human ACE2

(B) Mouse ACE2

**QFS (μM)**

**V_o (FLU/ng protein per min)**

- 0 μM
- 10 μM
- 50 μM
Figure 4.

A. Ang-(1-7), relative amount (peak area analyte/IS) vs. DIZE (µM) for no ACE2, human ACE2, and mouse ACE2.

B. Ang II, relative amount (peak area analyte/IS) vs. DIZE (µM) for no ACE2, human ACE2, and mouse ACE2.

C. Ang-(1-7) / Ang II (ratio of relative amounts) vs. DIZE (µM) for no ACE2, human ACE2, and mouse ACE2.
Figure 5.
Figure 6.
Figure 7.