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Angiotensin converting enzyme versus angiotensin converting enzyme-2 selectivity of MLN-4760 and DX600 in human and murine bone marrow-derived cells

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Abstract

Angiotensin-converting enzymes, ACE and ACE2, are key members of renin angiotensin system. Activation of ACE2/Ang-(1-7) pathway enhances cardiovascular protective functions of bone marrow-derived stem/progenitor cells. The current study evaluated the selectivity of ACE2 inhibitors, MLN-4760 and DX-600, and ACE and ACE2 activities in human (hu) and murine (mu) bone marrow cells. Assays were carried out in hu and mu mononuclear cells (MNCs) and huCD34⁺ cells or mu-lineage-depleted (muLin⁻) cells, human-recombinant (rh) enzymes, and mu-heart with enzyme-specific substrates. ACE or ACE2 inhibition by racemic MLN-4760, its isomers MLN-4760-A and MLN-4760-B, DX600 and captopril were characterized. MLN-4760-B is relatively less efficacious and less-selective than the racemate or MLN-4760-A at hu-rhACE2, and all three of them inhibited 43% rhACE. In huMNCs, MLN-4760-B detected 63% ACE2 with 28-fold selectivity over ACE. In huCD34⁺ cells, MLN-4760-B detected 38% of ACE2 activity with 63-fold selectivity. In mu-heart and muMNCs, isomer B was 100- and 228-fold selective for ACE2, respectively. In muLin⁻ cells, MLN-4760-B detected 25% ACE2 activity with a pIC₅₀ of 6.3. The racemic mixture and MLN-4760-A showed lower efficacy and poor selectivity for ACE2 in MNCs and mu-heart. ACE activity detected by captopril was 32 and 19%, respectively, in huCD34⁺ and muLin⁻ cells. DX600 was less efficacious, and more selective for ACE2 compared to MLN-4760-B in all samples tested. These results suggest that MLN-4760-B is a better antagonist of ACE2 than DX600 at 10 μ M concentration in human and murine bone marrow cells, and that these cells express more functional ACE2 than ACE.

Keywords

ACE; ACE2; MLN-4760; DX600; CD34⁺ cells; lineage-depleted cells

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1. Introduction

The classical renin-angiotensin system (RAS) consists of renin, angiotensin-converting enzyme (ACE), its major enzymatic product Angiotensin II (Ang II), and receptors that mediate biological functions of Ang II, AT1R and AT2R. Local or tissue expression of RAS with paracrine, intracrine and autocrine functions has been identified (Lavoie and Sigmund, 2003). The classical RAS is mostly pathological in cardiovascular system (de Gasparo et al., 2000; Ferrario and Strawn, 2006). The concept of protective axis of RAS has been introduced with the discovery of ACE2, a monocarboxy peptidase that generates Ang-(1-7) from Ang II (Donoghue et al., 2000). Ang-(1-7) by acting on Mas receptor produces cardiovascular protective functions and counter-regulates the detrimental effects of ACE/Ang II pathway (Santos, 2008).

With the discovery of vasoreparative propensity of bone marrow-derived stem/progenitor cells (BMSPCs), cardiovascular disease is now being viewed as the inability of these cells to repair/regenerate and restore the function of dysfunctional vasculature or myocardium (Fadini et al., 2006). Recent studies showed that local RAS modulate hematopoietic and cardiovascular reparative functions of BMSPCs (Rodgers and diZerega, 2013). We and others have shown evidence for the expression of mRNA and protein of ACE or ACE2 in human and mouse BMSPCs (Abali et al., 2002; Oliveira et al., 2010; Thatcher et al., 2011; Jarajapu et al., 2013; Singh et al., 2015).

Activity assay is a reliable measure of functional ACE or ACE2, and assays were mostly based on the propensity to cleave a surrogate substrate, fluorogenic peptides MCA-RPPGFSAFK-Dnp and 7-Mca-YVADAPK(Dnp) for ACE and ACE2, respectively (Joyner et al., 2012; Liu et al., 2010; Rice et al., 2004; Wösten-van Asperen et al., 2008; Ye et al., 2012). However these substrates are not selective and therefore use of enzyme-selective inhibitors is necessary to differentiate ACE or ACE2 versus non-ACE/ACE2-driven reaction (Carrera et al., 2014). MLN-4760 and DX600 are most frequently used ACE2 inhibitors and captopril was used as an ACE inhibitor in differentiating ACE or ACE2-specific activities. MLN-4760 is a small molecule inhibitor of ACE2 identified by high-throughput approach and reported to high affinity at human ACE2 compared with porcine ACE (Dales et al 2002). DX600 is a peptide inhibitor with nanomolar affinity to ACE2 over ACE, with mixed competitive and non-competitive inhibition (Huang et al., 2003). Subsequent studies have indeed shown species- and tissue-dependent variation in the inhibition of ACE2 by these molecules, and pH-dependency of the activity assays (Lindsey et al., 1987; Pedersen et al., 2011; Tikellis et al., 2008; Vickers et al., 2002; Ye et al., 2012). Therefore it is essential to evaluate the effects of these inhibitors in cells being studied in order to obtain reliable enzyme activities, and antagonist selectivity and potencies.

In the current study we sought to determine the ACE versus ACE2 selectivity of MLN-4760 and DX600, and to determine ACE and ACE2 activities in human and murine bone marrow-derived cells. We have carried out assays in human CD34⁺ cells, mouse lineage-negative (Lin⁻) cells, and mononuclear cells (MNCs). CD34⁺ cells are bone marrow-derived and long-known to be hematopoietic stem cells (HSCs) in humans, and are preferred population

of cells for the treatment of cardiovascular diseases (Mackie and Losordo, 2011). Mouse bone marrow Lin⁻ cells are enriched for stem/progenitors, and known to have cardiovascular reparative functions (Schattelman et al., 2010). Recombinant enzymes, MNCs, which originate from HSCs, or murine heart were used for comparison.

2. Materials and methods

2.1. Characteristics of subjects

This study was approved by Institutional Biosafety Committee of North Dakota State University (protocol # B12017). The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Human leucocyte samples were obtained from United Blood Services (Fargo, North Dakota). Healthy volunteers included both males and females of age ranging from 48 to 75 years. All volunteers are Caucasians. Leucocytes were collected in chambers of Leukoreduction system (LRS chambers) following apheresis carried out by using Trima Accel system (80440).

2.2. Isolation of cells

Human CD34⁺ cells were isolated from leucocytes as described before (Jarajapu et al., 2011). Peripheral blood mononuclear cells (MNCs) were separated from total leucocytes by gradient centrifugation using Ficoll (Ficoll-Paque, GE Healthcare Biosciences). Plasma was completely excluded from the cell fraction by a series of washings using phosphate-buffered saline with 2% fetal bovine serum (FBS) and 1 mM EDTA, and centrifugation at 120 g. These cells were enriched for lineage-negative (Lin⁻) cells by using a negative selection kit (StemCell Technologies, Inc.) as per supplier's instructions. Lin⁻ cells were then enriched for CD34⁺ cells by using an immunomagnetic selection kit (Easysep, Human CD34 positive selection kit, StemCell Technologies, Inc.) as per the manufacturer's instructions. MNCs, Lin⁻ cells or CD34⁺ cells were used for different experimental protocols. Freshly isolated cells were either used for assays or cell pellets were snap-frozen in liquid nitrogen, and stored at -80°C for later use.

2.3. Preparation of murine cells

All the animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of North Dakota State University. Male C57Bl/6 mice (Harlan Laboratories) at approximately 8-10 weeks of age were euthanized by cervical dislocation under isoflurane anesthesia followed by thoracotomy. Heart was isolated, snap-frozen in liquid nitrogen and preserved at -80°C. Femora and tibiae were excised and flushed with 5 ml of phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) and 2 mM EDTA using a 25-gauge needle. Red blood cell lysis was performed by treating with hypotonic ammonium chloride buffer. Total bone marrow cells were washed by centrifugation at low speed (120 g, 10 min) for three times and the cells were resuspended in PBS. Lin⁻ cells that are enriched with primitive stem/progenitor cells were isolated by negative immunomagnetic selection (Easysep, Mouse Hematopoietic cell enrichment kit, StemCell Technologies, Inc.) as per supplier's instructions. Cell pellets were snap-frozen in liquid nitrogen and preserved at -80°C for later use.

2.4. ACE and ACE2 activity

Cells were lysed by using lysis buffer containing 20mM Tris, 100mM EDTA and 0.5% Triton X-100. Protease Inhibitor was also included in the lysis buffer (Halt Protease Inhibitor cocktail, Thermo scientific) Cell lysates were centrifuged at 9223 g for 10 min at 4°C and the supernatant was harvested. Mouse heart samples were homogenized in a buffer (50mM HEPES, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.025mM ZnCl₂), and then the protein was extracted as described above. Protein content was determined by BCA assay.

ACE-2 activity was determined following incubation with intramolecularly quenched synthetic ACE-2 specific substrate 7- Mca-YVADAPK (Dnp) (R&D systems). ACE activity was determined following incubation with intramolecularly quenched synthetic ACE specific substrate Mca-RPGFSAFK (Dnp)-OH (R&D systems). In the case of cell lysates, 10 µg of total protein was assayed for activity in a buffer with the following composition: 50mM MES (4-morpholineethanesulphonic acid), 300mM NaCl, 10µM ZnCl₂ and 0.01% Triton X-100, pH 6.5. Reaction was initiated by the addition of 5×10⁻⁵ M substrate. Where applicable, recombinant enzymes were used at a concentration of 0.01 µg per reaction. The fluorescence measurements were performed in the black microtiter plates (Costar) in a total volume of 100 µl. The plates were read using a fluorescence plate reader SpectraMax M5 (Molecular Devices) at an excitation wavelength 320 nm and emission wavelength 405 nm. Fluorescence resulting from the substrate hydrolysis increased with time, and achieved maximum by one h with recombinant enzyme however with cell/tissue lysates the maximum fluorescence was observed by four h of incubation. Therefore fluorescence recorded at one and four h of reaction time was taken for calculation of percent enzyme inhibition, when using recombinant enzymes and cell/tissue lysates, respectively. ACE2 activity was defined as the ACE2 inhibitor-sensitive fluorescence and ACE activity was defined as the ACE inhibitor, captopril-sensitive fluorescence, and were expressed as percent inhibition. Unless specified, MLN4760 was used in concentration ranging from 0.01 nM to 300 µM, and DX 600 was used in a concentration range of 0.01 nM - 30 µM. V_{max} (maximum velocity) and K_m (Michaelis-Menten constant) values were determined by monitoring hydrolysis of 5 - 75 µM substrate and analyzing the data as described below (section 2.6).

2.5. Synthesis of MLN 4760

The synthesis of MLN4760 was performed according to the reported procedures (Dales et al., 2002). The purification and analysis of the diastereomers were performed using automated flash chromatography (Fig 1). Racemic mixture showed 75:25 ratio for Isomer A: Isomer B. Isomer B, (2S)-2-[[[(2S)-3-[3-[(3,5-dichlorophenyl)methyl]imidazol-4-yl]-1-hydroxy-1-oxopropan-2-yl]amino]-4-methylpentanoic acid is the commercially available isomer (EMD Millipore).

2.5.1. Automated-Flash Chromatography—Flash column purification was performed on a Teledyne Isco Combiflash Rf200i by using the column containing ResiSep C18 Gold 40grams and the following conditions: Mobilephase A: 0.1% TFA in water; Mobilephase B: 0.1% TFA in acetonitrile. Gradient: 0%B to 75%B in 0 to 60 min.

2.5.2. LC-MS—High resolution mass spectrometry with LC was performed on a Waters Synapt G2Si instrument with Aquity HPLC by using Acquity BEH C18 (100 × 2.1)mm 1.7micron column and the following conditions: Mobilephase A: 0.1%TFA in water; Mobilephase B: 0.1%TFA in acetonitrile. Gradient: 0%B to 75%B in 0 to 60 min (Fig 1).

2.6. Data analysis

Concentration-inhibition curves were analyzed by nonlinear regression by using GraphPad prism (Prism software), and maximum inhibition ($I_{\max}$) and pIC_{50} values were obtained, which indicate efficacy and potency, respectively. Where applicable, goodness of fit for the nonlinear regression ranged from 0.88 to 0.97.

For the determination of the K_m and $V_{\max}$ hydrolysis rate data was fitted to a nonlinear regression for Michaelis-Menten kinetics (GraphPad Prism). Based on the published literature that showed evidence for mixed-type enzyme inhibition by both MLN4760 and DX600 (Huang et al., 2003; Jiang et al., 2014; Pedersen et al., 2011), hydrolysis rate values were fitted to a mixed model of enzyme inhibition with nonlinear regression using GraphPad Prism for the determination of dissociation constant, K_i . Where applicable, data was tested for statistical significance by using two-sample t-test. Data sets were considered significantly different if a 'P' value of <0.05 was observed.

3. Results

3.1. Determination of optimal substrate concentration for enzyme assays

The optimal substrate concentration for enzyme assays was determined by evaluating $V_{\max}$ and K_m of the substrates over a range of concentrations. As shown in Fig 2, ACE substrate showed highest $V_{\max}$ or K_m at a concentration of 50 μ M, and a higher concentration (75 μ M) either decreased (recombinant human ACE (rhACE) or human MNCs (huMNCs)) or unchanged (mouse MNCs (MuMNCs)) $V_{\max}$ (n=4) (Fig 2A-2C, Table 1). Along similar lines, ACE2 substrate showed highest $V_{\max}$ or K_m at 50 μ M concentration, and a higher concentration either decreased or unchanged either $V_{\max}$ or K_m (n=4) (Fig 2D-2F, Table 1). Therefore substrates were used at a concentration of 50 μ M for subsequent assays.

3.2. Substrate selectivity of ACE and ACE2 assays

In the light of the report (Huang et al., 2003; Wysocki et al., 2006) that used the substrate, 7-Mca-YVADAPK(Dnp), for the assays of both ACE and ACE2 activities, we questioned if it would be a reliable approach in our experimental setting. In rhACE2, MLN-4760 racemic mixture and its isomers (10 μ M) effectively quenched cleavage of the 7-Mca-YVADAPK(Dnp), whereas captopril was ineffective, indicating ACE2-selectivity of the substrate (n=6, Fig 3A). Instead, when the assay was carried out with the substrate Mca-RPPGFSAFK-Dnp, increase in fluorescence was not observed (n=6, Fig 3B), indicating the substrate-specificity of the assay. Then, we examined rhACE activity kinetics with ACE selective substrate. ACE activity was completely blocked by captopril, which confirmed the ACE-specificity of the substrate (n=6, Fig 3C). Surprisingly, we observed 43±4% inhibition of rhACE activity by MLN-4760, either racemate or isomers (10 μ M) (Figure 3C). No

activity was detected (n=6, Fig 3D), when ACE was assayed by using the substrate 7-Mca-YVADAPK(Dnp), further conforming ACE2-specificity of this substrate.

Since rhACE activity was significantly inhibited by MLN-4760, we sought to determine the selectivity of these inhibitors for rhACE2 over rhACE. Concentration-dependent inhibition of rhACE or rhACE2 activities was observed with all three inhibitors. Maximum inhibition ($I_{\max}$) of rhACE2 by racemic mixture, isomer A and isomer B were $94\pm2\%$, $93\pm1\%$ and $80\pm3\%$, respectively (Fig 3E-G). Isomer B is less selective than the racemate or the isomer A for rhACE2 versus rhACE ($P<0.001$, n=6). Efficacy of isomer B was significantly lower than that of racemate or the isomer A ($P<0.001$, n=6). $I_{\max}$ values of racemic mixture, isomer A and isomer B at rhACE were found to be $48\pm4\%$, $49\pm5\%$ and $46\pm1\%$, respectively. Inhibitory potencies (pIC_{50}) at ACE2 were 8.5 ± 0.1 , 8.9 ± 0.1 and 8.01 ± 0.1 , respectively, and at ACE were 4.4 ± 0.2 , 4.4 ± 0.3 and 5.0 ± 0.1 , respectively, suggesting 600-10,000-fold selectivity (Fig 3E-G).

3.2. ACE vs ACE2 selectivity of MLN-4760 in human MNCs and CD34⁺ cells

Then we sought to determine the selectivity of MLN-4760 at wild type (WT) human ACE2 over ACE. In human MNCs, racemic mixture and isomer A showed only 3-fold selectivity for ACE2 over ACE (Fig 4A and 4B), whereas isomer B has 20-fold selectivity for ACE2 (Fig 4C). $I_{\max}$ values of the three inhibitors at ACE were 22 ± 2 , 20 ± 2 and $34\pm1\%$, respectively, and at ACE2 was, 34 ± 2 , 35 ± 1 , and $63\pm2\%$, respectively. Isomer B is more efficacious than the racemate ($P<0.003$, n=6) or the isomer A ($P<0.004$, n=6). Total ACE activity as determined by captopril-inhibitable enzyme activity in human MNCs was found to be $49\pm3\%$.

Then the selectivity of MLN isomer B, which is more potent over racemic mixture or the isomer A, was evaluated in human CD34⁺ cells. Isomer B showed 63-fold selectivity for ACE2 over ACE with an $I_{\max}$ of $38\pm4\%$ at ACE2 and $19\pm2\%$ at ACE (n=6) (Fig 4D). The observed pIC_{50} was 6.5 ± 0.2 and 4.9 ± 1 for ACE2 and ACE, respectively. Total ACE activity in human CD34⁺ cells was found to be $32\pm2\%$, which was significantly lower than the ACE2 activity ($P<0.04$, n=6).

3.3. ACE vs ACE2 selectivity of MLN-4760 in murine heart and bone marrow cells

Then we sought to determine the selectivity of MLN-4760 at WT murine ACE2 over ACE. In murine heart, the selectivity of isomer B is 100-fold for ACE2 over ACE, and racemic mixture and isomer A were not selective (n=6) (Fig 5A-5C). Inhibitory potencies (pIC_{50}) of racemic mixture, isomer A and isomer B at ACE2 were 4.7 ± 0.1 , 5.1 ± 0.1 and 6.6 ± 0.1 (n=6), respectively, and at ACE were 4.4 ± 0.1 , 4.8 ± 0.3 and 4.2 ± 0.1 (n=6), respectively. $I_{\max}$ values of these three inhibitors at ACE2 were, 40 ± 3 , 26 ± 2 , and $54\pm4\%$ (n=6), respectively, and at ACE was 26 ± 2 , 14 ± 3 and $20\pm3\%$, respectively ($P<0.05$, n=6, for isomer B at ACE2). In MNCs, similar profile was observed with the isomer B being more selective (228-fold) at ACE2 over ACE. Inhibitory potencies (pIC_{50}) of racemic mixture, isomer A and isomer B at ACE2 were 6.9 ± 0.1 , 7.1 ± 0.2 and 7.1 ± 0.1 , respectively (n=6), and at ACE were 6.2 ± 0.1 , 6.6 ± 0.2 and 4.7 ± 0.2 (n=6), respectively. $I_{\max}$ of the three inhibitors at ACE2 was, 35 ± 1 , 32 ± 4 , and $43\pm1\%$ (n=6), respectively, and at ACE were 26 ± 2 , 20 ± 1 and $22\pm1\%$ (n=6),

respectively (Fig 5D-5F). Total ACE activity in murine heart and MNCs and Lin⁻ cells was found to be 67±5% and 36±2% (n=6), respectively.

Then the selectivity of isomer B was evaluated in murine Lin⁻ cells. Isomer B showed 25±3% inhibition while its effect on ACE was less than 10±2% (Fig 5G). Inhibitory potency (pIC₅₀) at ACE2 was 5.0±0.2. Total ACE activity in murine Lin⁻ cells was found to be 19±4%, which was significantly lower than the ACE2 activity detected by the isomer B of MLN-4760 (P<0.01, n=5).

3.4. ACE versus ACE2 selectivity of DX600

Then the selectivity of a peptide inhibitor of ACE2, DX600, was determined in ACE and ACE2 assays. DX600 inhibited rhACE2 activity by 47±3% with a pIC₅₀ of 8.0±0.1 (n=6) (Fig 6A) while only 14±3% of rhACE activity was inhibited at the concentration of 30µM (concentrations higher than 30µM were not used in this study). In human MNCs, 42±4% inhibition with a pIC₅₀ of 6.5±0.1 at ACE2, whereas only 15±2% of ACE activity was inhibited by 10µM DX600 (Fig 6B). ACE2 activity uncovered by DX600 was significantly lower than that revealed by the isomer B of MLN-4760 (P<0.004, n=6). In human CD34⁺ cells the observed I_{max} at ACE2 was 30±4% with a pIC₅₀ of 6.8±0.2, whereas at ACE the I_{max} was 10±2% with a pIC₅₀ of 5.5±0.6 (n=6) (Fig 6C). ACE2 activity uncovered was significantly lower than that shown by the isomer B of MLN-4760 (P<0.04, n=6).

In murine heart, DX600 inhibited ACE2 with an I_{max} of 45±5 % and a pIC₅₀ of 6.7±0.1, while ACE was inhibited by 15±2 % with a pIC₅₀ of 5.8±0.3 (n=5) (Fig 6D). In murine MNCs, 33±1 % inhibition with a pIC₅₀ of 6.4±0.1 at ACE2, whereas only 15±2% of ACE activity was inhibited with a pIC₅₀ of 5.4±0.4 (Fig 6E). In murine Lin⁻ cells the observed I_{max} at ACE2 was 23±2% with a pIC₅₀ of 5.8±0.4, whereas at ACE the I_{max} was only 7±1% (Fig 6F). ACE2 activity uncovered by DX600 was significantly lower than that revealed by the isomer B of MLN-4760 in murine heart (P<0.04, n=6), MNCs (P<0.03, n=6) and Lin⁻ cells (P<0.05, n=5).

Finally, we have determined K_i values of the antagonists in different samples for comparison with IC₅₀ values obtained in the above studies. Table 2 shows K_i and IC₅₀ values for isomer B of MLN4760 and DX600 in the recombinant enzymes, and human and mouse MNCs (n=4 for all samples). K_i values are agreeable with IC₅₀ values that were obtained in the study. Affinity of the isomer B of MLN 4760 is in the following order, recombinant human >human > murine, and similar order of affinity was observed with DX600 (Table 2).

4. Discussion

This study reports important pharmacological clarifications in regards to the efficacy and selectivity of ACE2 inhibitors, MLN-4760 and DX600, and provides for the first time quantitative estimates of ACE and ACE2 activities in human and murine bone marrow-derived mononuclear cells and stem/progenitor cells. Furthermore our study shows that the use of recombinant enzymes alone may not provide reliable activity measurements. Selectivity of MLN-4760 or DX600 is concentration-dependent. MLN4760 is more selective

for ACE2 than DX600 up to the concentration of 10 μ M in human and murine bone marrow cells, and in murine heart. Study mainly evaluated the potency of inhibitors, IC₅₀ that was further confirmed by determining Ki. It is important to note that the rate of substrate hydrolysis differed among recombinant human, and native human and murine ACE2s. This further suggests that the inhibitor potencies are required to be optimized in the tissue or cell-type of interest for a reliable interpretation of experimental outcomes. It is important to note that in vitro studies cannot be directly extrapolated to an in vivo setting however in vitro or ex vivo cell- or tissue-based assays are powerful tools for reliable estimates of antagonist selectivity and potencies as it is completely free from several physiological influences that would occur in an in vivo setting. In vitro studies provide a strong basis for the selection of an antagonist for an in vivo experimental design.

Enzyme activities of ACE and ACE2 enzymes, zinc-dependent carboxy peptidases, have been reported in several tissues and body fluids derived from rat and mice (Lindsey et al., 1987; Pedersen et al., 2011; Tikellis et al., 2008; Wysocki et al., 2006). Activity assays were always based on the propensity to cleave a surrogate substrates. Few studies have indeed used one substrate, which we found in the current study ACE2-specific, for determining both ACE and ACE2 activities that were further differentiated by using selective antagonists (Huang et al., 2003; Wysocki et al., 2006). In our experimental conditions, we observed that ACE and ACE2 assays required enzyme-specific substrates, and we conclude that the use of enzyme-selective substrates may be crucial for obtaining reliable estimates of enzyme activities. Our study is in agreement with the report by Tikellis et al (2008) that used a three amino acid-long substrates that are ACE- or ACE2-specific, and further confirmed the activities by using selective inhibitors.

4.1. ACE versus ACE2 selectivity of MLN4760

MLN-4760, racemate and isomers B, showed almost similar inhibition of rhACE2 with the isomer B being less efficacious by 20%. Interestingly, all three molecules showed significant inhibition of rhACE. Racemate and the isomer A were relatively more selective than the isomer B for rhACE2 compared to rhACE. The selectivity profiles were found to be different in human and mouse wild type cell/tissue lysates compared to that observed in recombinant enzymes. In human MNCs, the isomer B of MLN-4760 is more (28-fold) selective for ACE2 over ACE, and uncovered more activity of ACE2 as well as ACE compared to either the racemate or the isomer A, which showed poor or no selectivity for ACE2. In murine heart and MNCs, isomer B showed 100- and 228-fold selectivity for ACE2, while the others were not selective with lower efficacy. Higher efficacy of the isomer B at ACE2 paralleled with its higher efficacy at ACE in all samples tested however the ACE activity detected by the isomer B is very lower than the total ACE detected by captopril. Interestingly, rhACE could be significantly inhibited by MLN-4760 at a concentration of <10 μ M, which was not observed in assays with cell/tissue lysates. At this concentration, the isomer B appears to be highly selective for ACE2 with minimal or no inhibition of ACE in both human and murine samples tested in the current study.

4.2. Relative selectivity and efficacy of MLN-4760 and DX600

DX600-mediated inhibition of either rhACE or rhACE2 was relatively lower than that produced by MLN-4760 molecules. Our study suggested that DX600 is relatively more selective for ACE2 however less efficacious compared to MLN-4760. In human MNCs, ACE2 activity uncovered by DX600 was significantly lower than that observed with the isomer B (42 vs 63%), and similar difference was observed in their efficacies of ACE inhibition. Along similar lines, DX600-inhibitable ACE2 or ACE were lower than that demonstrated by the isomer B in murine heart and MNCs. Collectively, these findings imply that DX600 is less efficacious than MLN-4760 in inhibiting ACE2 as well as ACE in human and murine cell/tissue lysates. It is important to note that at 10 μ M concentration, both isomer B of MLN-4760 and DX600 show their respective maximum inhibition of ACE2 with minimum inhibition of ACE.

4.3. ACE and ACE2 activities in human and murine stem progenitor cells

This study for the first time reports the quantitative enzyme activities of ACEs in BMSPCs. The expression of active ACE is lower than ACE2 in human CD34⁺ cells or murine Lin⁻ cells. ACE2 activity uncovered by isomer B of MLN-4760 is 3-4 fold higher than ACE activity. Therefore ACE2 appears to be the predominant isoform expressed in these primitive cells which at least in part contribute for the cardiovascular reparative and endothelial regenerative functions of these cells. Evidence now has been accumulating in support of the cardiovascular protective functions of ACE2/Ang-(1-7)/Mas axis, which involves stimulation of the reparative functions of BMSPCs (Jarajapu et al 2012). Experimental studies in human diabetic CD34⁺ cells, derived from patients with diabetes or pulmonary hypertension, and in mouse or rat models of diabetes, pulmonary arterial hypertension and heart failure (Jarajapu et al., 2013; Shenoy et al., 2013; Singh, 2014; Wang et al., 2010). Therefore, stimulation of ACE2/Ang-(1-7)/Mas receptor axis, either by activation or overexpression of ACE2 or treatment with Ang-(1-7), would be a promising approach for enhancing the cardiovascular reparative outcomes of cell-based therapies. It is important to note that the human CD34⁺ cells although derived from bone marrow, were obtained from peripheral blood. Bone marrow environment is relatively hypoxic while the circulating cells are exposed to different gradients of oxygen in the arterial and venous circulation. Hypoxia is known to modulate the reparative functions and the expression of angiogenic genes in BMPSCs (Bradley et al., 1978; Jarajapu et al., 2014; Smith et al., 1986) however the effect of hypoxia on the expression of ACE and ACE2 is not known. It is likely that the functional enzyme levels observed in the circulating CD34⁺ cells are not reflective of the levels in bone marrow-resident cells.

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Fig 1. Chemical structures of stereoisomers A and B of MLN-4760 (**A** and **B**). Fig 1C depicts a representative of the separation of isomers by LC-MS.

Fig 2. Determination of optimal substrate concentration for ACE and ACE2 activity assays
Rate of hydrolysis was evaluated over a range of substrate concentrations in the presence of recombinant enzymes or human or mouse cell lysates with ACE- or ACE2-specific substrates. Shown were representatives of four repetitions (recombinant enzymes) or experiments carried out in four different samples (human or murine cells). (A) rhACE2 (B) human MNC ACE2 (C) murine MNC ACE2 (D) rhACE (E) human MNC ACE and (F) murine MNC ACE.

Fig 3. Enzyme specific substrates for ACE and ACE2 activity assays

ACE- and ACE2-specific substrates were completely hydrolyzed by (A) rhACE2 and (C) rhACE, respectively (n=6). (B) ACE-specific substrate was not hydrolyzed by rhACE2 (n=4), and along the similar lines, (D) ACE2-specific substrate was not hydrolyzed by rhACE (n=4). (E-G) ACE versus ACE2 selectivity of MLN-4760 in recombinant human enzymes (n=6). Racemic mixture and the isomers were denoted as MLN-4760-R, MLN-4760-A and MLN-4760-B.

Fig 4.

ACE versus ACE2 selectivity of MLN-4760, (A) racemic mixture, (B) isomer A and (C) isomer B, in human mononuclear cells (n=6). (D) ACE2 activity, and ACE versus ACE2 selectivity of the isomer B of MLN-4760 in human CD34⁺ cells (n=6). Racemic mixture and the isomers were denoted as MLN-4760-R, MLN-4760-A and MLN-4760-B.

Fig 5.

ACE versus ACE2 selectivity of MLN-4760, racemic mixture, and isomers A and B, in murine heart (A-C), and in murine mononuclear cells (D-F) (n=6). (G) ACE2 activity, and ACE versus ACE2 selectivity of the isomer B of MLN-4760 in murine Lin⁻ cells (n=5). Racemic mixture and the isomers were denoted as MLN-4760-R, MLN-4760-A and MLN-4760-B.

Fig 6.

ACE versus ACE2 selectivity of DX600 in (A) human recombinant enzymes (rhACE and rhACE2) (n=6), (B) human mononuclear cells (n=6), (C) human CD34⁺ cells (n=6), (D) murine heart (n=5), (E) murine mononuclear cells (n=5), and (F) murine Lin⁻ cells (n=5).

Table 1
Kinetic Parameters, $V_{\max}$ and K_m , for the substrates, MCA-RPGFSAFK-Dnp and 7-Mca-YVADAPK-Dnp, for ACE and ACE2, respectively

Enzyme	$V_{\max}$ (AFU/min/ μ g of protein)	K_m (μ M)
Recombinant Human ACE2	125 ± 13	23 ± 0.5
Human MNC ACE2	15 ± 4	57 ± 0.6
Murine MNC ACE2	22 ± 6	89 ± 1.9
Recombinant Human ACE	395 ± 70	57 ± 0.8
Human MNC ACE	53 ± 8	68 ± 1.7
Murine MNC ACE	57 ± 14	106 ± 2.7

A representative of time versus substrate hydrolysis curves for each sample shown above is provided in Fig 2. Assays were repeated four times in case of recombinant enzymes or carried out in four different samples in case of human and murine MNCs.

Table 2
Dissociation constant K_i and IC_{50} values of antagonists, isomer B of MLN4760 and DX600, in different samples

Enzyme	MLN4760 (Isomer B)		DX600	
	K_i (μ M)	IC_{50} (μ M)	K_i (μ M)	IC_{50} (μ M)
Recombinant Human ACE2	0.04 ± 0.005	0.01 ± 0.006	0.02 ± 0.008	0.01 ± 0.003
Human ACE2	0.2 ± 0.01	0.5 ± 0.03	0.2 ± 0.01	0.3 ± 0.05
Murine ACE2	2.1 ± 0.2	7.1 ± 0.63	1.2 ± 0.9	0.4 ± 0.07

K_i values were determined as described in the text. Assays were repeated four times in case of recombinant ACE2 or carried out in four different samples in case of human and murine cells. IC_{50} values were obtained from current study as described in the results section.