Supplemental Material

Ethylene bis-imidazoles are highly potent and selective activators for isozymes VA and VII of carbonic anhydrases, with potential nootropic effect

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Summary:

Materials and Methods

Materials: The following materials were used as received: bromine, NH_3/MeOH 7N solution (Acros/Fisher Scientific, Pittsburgh, PA), 2,5-hexanedione (Alfa Aesar/VWR International, West Chester, PA), benzonitrile, propionitrile, methyl amidine hydrochloride (TCI America, Portland, OR), amidine acetate (Sigma Aldrich, St Louis, MO). Other solvents (HPLC quality), salts and acids were purchased from Fisher Scientific (Pittsburgh, PA), EMD (Gibbstown, NJ), and VWR International (West Chester, PA).

Techniques: The purity and the structure identity of the intermediary and final products were assessed by a combination of techniques that included thin-layer chromatography (TLC), HPLC-MS, ^{1}H-, ^{13}C- and ^{19}F-NMR, and high resolution mass spectrometry (HR-MS).

TLC was carried out on SiO_2-precoated aluminum plates (silica gel with F254 indicator; layer thickness 200 μm; pore size 60 Å, from Sigma-Aldrich.

The melting points and were determined via Thermolyne heating stage microscope (Dubuque, IA), equipped with an Olympus 5X objective, at heating/cooling rate of ~ 4 ^{o}C/min and were uncorrected.

The purity of compounds was assessed via LC-MS using an Agilent 1200 HPLC-DAD-MS system equipped with a G1315A DAD and a 6130 Quadrupole MS via a ZORBAX SB-C18 column eluted with H_2O (0.1% HCOOH)/MeCN (0.1% HCOOH) 95/5 to 0/100 linear gradient.

NMR spectra were recorded at ≈300 K with a Bruker Avance III 400 Plus spectrometer equipped with a 5 mm indirect detection probe, operating at 400 MHz for ^{1}H-NMR, at 100 MHz for ^{13}C-NMR, and at 376 MHz for ^{19}F-NMR. Chemical shifts are

reported as δ values, using tetramethylsilane (TMS) as the internal standard for proton spectra and the solvent resonance for carbon spectra. Assignments were made based on chemical shifts, signal intensity, COSY, HMQC, and HMBC sequences. For ^{1}H NMR, data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, sep = septet, m = multiplet), coupling constants *J* (Hz) and integration.

High resolution mass spectrometry (HR-MS) was performed on a LTQ Orbitrap XL hybrid ion trap-orbitrap mass spectrometer (Thermo Scientific, Waltham, MA).

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1,6-dibromo-2,5-hexanedione 15.

The compound was prepared via bromination of 2,5-hexanedione **14**, according to the procedure adapted from Meziere et al. [1]. The reaction workup was modified as follows: after precipitation with ether, the precipitate was discarded and the filtrate, which contains the useful product, was passed through a thin silica column, when most of the impurities remained trapped on the silica layer. The resulting clear solution was concentrated in vacuum to give a pale yellow precipitate which was further dissolved in DCM and precipitated with ether/hexanes to give white crystals of **14**. mp 108.0 – 109.0 ^{o}C; Lit. mp 112.0-113.0 ^{o}C [1]. ^{1}H NMR (400 MHz, $CDCl_3$, *δ, ppm*): 3.96 (2H, s, 1-CH_2(Br)CO), 2.99 (2H, s, 3-CH_2CO).

Amidines 17c-e were not commercially available and were prepared from the corresponding nitriles **16c-e** accordingly to the procedure of Barker and collaborators [2]. Thus, in a typical preparation procedure, nitrile (**16c-e**) (1 equiv.) was dissolved in a mixture of diethyl ether and anhydrous methanol (20 mL). Dry HCl gas, was subsequently bubbled through the

solution for 2 h at 0°C. The resulting reaction mixture was cooled and stored in the freezer (-20 °C) for 48 h. Evaporation of the solvent gave the corresponding imidate hydrochloride which was further washed with diethyl ether. The imidate hydrochloride was treated with NH_3/MeOH solution 7N (5 equiv.) and the resulting reaction mixture was stirred overnight at room temperature. Solvent removal under reduced pressure gave the corresponding amidine hydrochlorides (**17c-e**), which were used in the condensation step.

Ethyl amidine hydrochloride **(17c)**. Isolated 1.87 g; yield 22 %; mp 135.0 – 136.0 °C; Lit. mp 128.0 -130.0 °C [2]. **^{1}H NMR** (D_2O, 400 MHz) $_H$ 2.42 – 2.40 (2H, m, 2-C*H*$_2$CH$_3$), 1.15 (3H, m, 3-*CH*$_3$CH$_2$). **^{13}C NMR** (D_2O, 100 MHz) $_C$ 172.6 (1-*C*(NH$_2$)NH$_2$Cl), 25.7 (2-*C*H$_2$CH$_3$), 10.1 *(3-C*H$_3$CH$_2$).

Isopropyl amidine hydrochloride **(17d)**. Isolated 1.75 g; yield 30 %; mp 161.0 -162.0 °C; Lit. mp [3] 161.0 °C ^{1}H NMR (D_2O, 400 MHz) $_H$ 2.63 (1H sep, *J* = 7.0 Hz, 2-*CH*C(NH$_2$)NH$_2$Cl), 1.14 (6H, d, *J* = 7.0 Hz, 3-(C*H*$_3$)$_2$CHC(NH$_2$)NH$_2$Cl). ^{13}C NMR (D_2O, 100 MHz,) $_C$ 175.9 (1-*C*(NH$_2$)NH$_2$Cl), 32.2(2-*C*H(CH$_3$)$_2$), 18.5 (3-(*C*H$_3$)$_2$CH).

Phenyl amidine hydrochloride **(17e)**. Isolated 1.38g; yield (25 %); mp 177.0 – 178.0 °C. Lit mp 171 °C [4]. ^{1}H NMR (DMSO, 400 MHz) $_H$ 9.46 (4H, s, 1-C(N*H*$_2$)N*H*$_2$Cl), 7.93 – 7.55 (m, 5H –C$_6$*H*$_5$). ^{13}C NMR (DMSO, 100 MHz) $_C$ 165.81(1-*C*(NH$_2$)N*H*$_2$Cl), 133.7 (–*C*$_6$H$_5$), 128.9 (–*C*$_6$H$_5$), 128.1(–*C*$_6$H$_5$), 127.9 (–*C*$_6$H$_5$).

General procedure for the synthesis of compounds 13a-e (adapted from Leschke et al. [5])

Amidine hydrochloride **17** (0.190g, 2.00mmol) was dissolved in 5 mL NH_3/MeOH (7N). The resulting reaction mixture was stirred at rt for 10 min in a CEM microwave vial. Then 1,6-dibromo-2,5-hexanedione **2** (0.272g, 1.00mmol) was added and the vial was capped. The reaction mixture was subsequently subjected to microwave irradiation (40 min, 200W, 120^{o}C) in a CEM Discover microwave reactor equipped with an auto sampler. The reaction was monitored by TLC and LC-MS and if not completed, it was irradiated for an additional 40 min under same reaction conditions. The crude product was purified on a Teledyne ISCO Combiflash® R_f (direct phase) using DCM/MeOH gradients. Fractions containing the useful product were combined and were subsequently purified by reverse phase liquid chromatography using H_2O (0.1% TFA)/ACN (0.1% TFA) 97/3 gradient to 0/100 over 18 min, a Phenomenex Gemini® 5 µm C18 LC column (110 Å, 150 x 30 mm) and a Gilson GX-281 preparative LC system. Pure fractions collected (assessed via LC-MS) were grouped and evaporated to dryness to yield the final products as orange-brown powders (as trifluoroacetates). Final purity was confirmed via LC-MS and was found to exceed 96% in all cases.

1,2-Bis(1H-imidazol-4-yl)ethane (13a) brown crystals, yield 40 %, mp 200-202 ^{o}C; Lit. mp 200.0-201.0 ^{o}C. HPLC purity 99%, **^{1}H NMR** (D_2O, 400 MHz) $_{H}$ 8.42 (2H, d, *J* = 1.2 Hz, 2-C*H*), 7.04 (2H, d, *J* = 1.2 Hz, 4-*CH*), 2.98 (2H, s, 6-CH_2). **^{13}C NMR** (D_2O, 100 MHz) $_{C}$ 133.3 (2-C), 131.7 (4-C), 116.0 (5-C), 23.2 (6-*C*H_2). ***Trifluoroacetate salt:*** brown crystals, HPLC purity 99%, R_t = 0.17 min. **^{1}H NMR** (D_2O, 400 MHz) $_{H}$ 8.42 (2H, d, *J* = 1.2 Hz, 2-

C*H*), 7.04 (2H, d, *J* = 1.2 Hz, 4-C*H*), 2.96 (4H, s, 6-CH_2). **^{13}C NMR** (D_2O, 100 MHz) $_C$ 162.3 (1C, q, *J* = 36.2 Hz 1"-*C*(O)CF_3), 133.1(2-*C*), 131.4 (4-*C*), 116.0 (q, *J* = 290.8 Hz, 2"-*C*F_3), 115.9 (5-*C*), 23.0 (6-*C*H_2). **^{19}F NMR** (D_2O, 376 MHz) $_F$ -75.80 (2"-CF_3). **HRMS** (ESI) Calculated for $C_8H_{10}N_4$ 163.0979, found 163.0982 (MH^+).

1,2-Bis(2-methyl-1H-imidazol-4-yl)ethane **(13b),** ***trifluoroacetate salt***, brown crystals, yield 30 %; MP. 180.0 (desc.) ^oC, HPLC purity 99%, R_t = 0.14 min. **^{1}H NMR** (D_2O, 400 MHz) $_H$ 6.91 (2H, s, 4-C*H*), 2.91 (4H, s, 6-CH_2), 2.46 (6H, s, 2'-CH_3). **^{13}C NMR** (D_2O, 100 MHz) $_C$ 163.0 (1C, q, *J* = 35.2 Hz, 1"-*C*(O)CF_3), 144.0 (2-*C*), 130.7 (4-C), 116.4(1C, q, *J* = 290.79 Hz, 2"-*C*F_3), 115.0 (5-C) 23.1(6-C), 10.6 (2'-C). **^{19}F NMR** (D_2O, 376 MHz), $_F$ -75.58 (2"-CF_3). **HRMS** (ESI) Calculated for $C_{10}H_{14}N_4$ 191.1292 found: 191.1293 (MH^+).

1,2-Bis(2-ethyl-1H-imidazol-4-yl)ethane **(13c). trifluoroacetate salt**, brown crystals, yield 53%. HPLC purity 96%, R_t = 0.18 min. **^{1}H NMR** (D_2O, 400 MHz) $_H$ 6.90 (2H s, 4-C*H*), 2.96 (4H s, 6-CH_2), 2.85 (4H q, *J* = 7.6 Hz, 2'-CH_2CH_3), 1.19 (6H t, *J* = 7.6 Hz, 3'-CH_3CH_2). **^{13}C NMR** (D_2O, 100 MHz) $_C$ 163.0 (1C, q, *J* = 35.2 Hz, 1"-*C*(O)CF_3), 148.8 (2-C), 130.8 (4-C), 116.3 (1C, q, *J* = 291.8 Hz, 2"-*C*F_3), 114.9 (5-C), 23.1(6-C), 19.0 (2'-C), 10.4 (3'-C). **^{19}F NMR** (D_2O, 376 MHz,) $_F$ -75.64 (2"-*C*F_3). **HRMS** (ESI) Calculated for $C_{12}H_{18}N_4$ 219.1605, found 219.1607 (MH^+).

1,2-Bis(2-isopropyl-1H-imidazol-4-yl)ethane **(13d)** ***trifluoroacetate salt***, yellow-brown crystals, yield 57 %. HPLC purity 98%, R_t = 0.21 min. ^{1}H NMR (D_2O, 400 MHz), *δ, ppm*, 6.85 (2H, s, 4-C*H*), 3.13 (2H, hep, *J* = 7.0 Hz, 6-CH_2), 2.86 (4H, s, 2'-C*H*$(CH_3)_2$), 1.20 (2H,

d, J = 7.0 Hz, -(CH_3)CH).^{13}C NMR (100 MHz, D_2O-d_2), δ, *ppm*, 162.8 (1C, q, J = 34.1 Hz, 1"-C(O)CF_3), 152.2 (2-C), 130.8 (4-C), 116.2 (1C, q, J = 291.7 Hz, 2"-CF_3), 114.7 (5-C), 26.3 (6-C), 23.1 (2'-C), 19.4 (3'-C). ^{19}F NMR (D_2O, 376 MHz,) $_F$ -75.62 (2"-CF_3). **HRMS** (ESI) Calculated for $C_{14}H_{22}N_4$ 247.1918, found 247.1922 (MH^+).

1,2-Bis(2-phenyl-1H-imidazol-4-yl)ethane **(13e) trifluoroacetate salt**, yellow crystals, yield 23%; MP. 265.0 – 266.0 ^{o}C. HPLC purity 96%, R_t = 0.24 min. ^{1}H NMR (DMSO, 400 MHz) $_H$ 8.00 – 7.97 (2H, m, -C_6H_5), 7.67 – 7.61 (6H, m, -C_6H_5), 7.59 (2H, s, -C_6H_5), 3.13 (4H, s, 6-CH_2). ^{13}C NMR (DMSO, 100 MHz) $_C$ 157.8 (1C, q, J = 34.2 Hz, 1"-C(O)CF_3), 149.5 (2-C), 143.3 (4-C), 131.3(-C_6H_5), 129.4(-C_6H_5), 126.2(-C_6H_5), 124.1(-C_6H_5), 117.0 (5-C), 116.3 (1C, q, J = 291.8 Hz, 2"-CF_3), 23.7 (6-C). ^{19}F NMR (DMSO, 376 MHz,) $_F$ -73.45 (2"-CF_3). **HRMS** (ESI) Calculated for $C_{20}H_{18}N_4$ $[M+1]^+$ 315.1575, found 315.1580 (MH^+).

CA enzyme assay. The CA catalysed CO_2 hydration activity of the eight human isozymes was determined using an Applied Photophysics stopped-flow instrument [6]. The aqueous solutions used contained 0.2 mM indicator (phenol red), together with 10 mM Hepes (pH 7.5) as buffer and 0.1 M Na_2SO_4 for maintaining constant ionic strength. The absorbance maximum for the indicator (557 nm) was used in all experiments. The CO_2 hydration reaction catalysed by CA isozymes was followed for a period of 10 s at 25°C. In order to determine the kinetic parameters and activation constants for the compounds tested, the CO_2 concentrations were varied from 1.7 to 17 mM. At least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity for each activator.

The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates.

Stock solutions of activators (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. In order to allow the formation of the E-A complex, the activator and enzyme solutions were preincubated together for 15 min in the case of standard assay at room temperature, or for prolonged periods of 24-72 h, at 4 °C, prior to the assay.

The activation constant (K_A), which is defined similarly with the inhibition constant K_I, can be obtained by considering the classical Michaelis-Menten equation (equation *1*, which has been fitted by non-linear least squares using PRISM 3) [7, 8]:

$$v = v_{max} /\{1 + K_M /[S] (1 + [A]_f/K_A)\} \qquad (1)$$

where $[A]_f$ is the free concentration of activator.

If one works at substrate concentrations considerably lower than K_M ($[S] << K_M$) then the obtained competitive steady-state equation for determining the activation constant is given by equation *2*, which takes into account that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$) [7,8]:

$$v = v_0 \,.\, K_A /\{K_A + ([A]_t - 0.5\, \{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t.[E]t)^{1/2}\}\} \qquad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator [7-9].

The initial ionization state of the activator (free base or bis-trifluoroacetate salt) does not affect the K_A, as exemplified for **13a** (Supplemental Table 1), proving that trifluoroacetate anion does not interfere with the activation process.

Supplemental Table 1. Activation profile of human CA isozymes (hCAs) with bis-imidazole **13a** in neutral form or as trifluoroacetate salt

No.	K_A* (μM)							
	hCA I	hCA II	hCA IV	hCA VA	hCA VII	hCA IX	hCA XII	hCA XIV
Histamine	2	125			37.5			
13a	16.4	68.5	1.25	0.021	0.015	9.51	8.63	13.9
13a·2TFA	16.1	71.0	1.22	0.020	0.013	9.28	8.71	14.1

We emphasize that K_M values in the presence and the absence of activators were the same for the various CA isozymes (within the limits of the experimental errors), as shown in Supplemental Table 2 for three representative isozymes. All values are mean from at least three determinations using the same stopped-flow, CO_2 hydrase method mentioned above. Standard errors were in the range of 5-10 % of the reported values (data not shown).

Supplemental Table 2: Activation of hCA isozymes I, II, III and IV, with L-histidine and compound **13e**, at 25°C, for the CO_2 hydration reaction.

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	$(k_{cat})_{L\text{-}His}$** (s^{-1})	K_M** (mM)	$(k_{cat})_{\mathbf{13e}}$*** (s^{-1})	K_M*** (mM)
hCA I[a]	2.0×10^5	4.0	13.4×10^5	4.1	8.2×10^5	4.0
hCA II[a]	1.4×10^6	9.3	4.3×10^6	9.3	2.9×10^6	9.2
hCA IV[b]	1.2×10^6	21.5	4.1×10^6	21.6	3.9×10^6	21.5

* Observed catalytic rate without activator. K_M – Michaelis constant of pure enzyme

** Observed catalytic rate in the presence of 10 μM activator (L-His). K_M**- Michaelis constant in the presence of 10 μM L-His

*** Observed catalytic rate in the presence of 10 μM activator **13e.** K_M***- Michaelis constant in the presence of 10 μM **13e**

[a] Human recombinant isozymes

[b] Truncated human recombinant isozyme lacking the first 20 amino acid residues which represent the signal peptide orienting the protein outside the cell

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