Dichloro-naphthoquinone as a non-classical inhibitor of the mycobacterial carbonic anhydrase Rv3588c

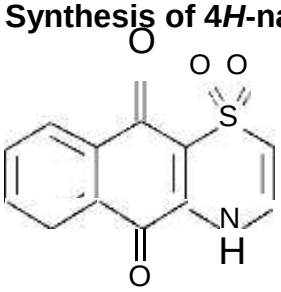
Supplementary Materials and Methods

2,3-Dichloronaphthoquinone (**1**) and 2-methoxynaphthoquinone (**4**) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) as Catalogue #D67200 and Catalogue #189162, respectively.

Synthesis of 3,4-dihydro-2*H*-naphtho[2,3-*b*][1,4]thiazine-5,10-dione 1,1-dioxide (7)

Compound **7** was synthesised using a previously reported procedure.[1,2]

Synthesis of 4*H*-naphtho[2,3-*b*][1,4]thiazine-5,10-dione 1,1-dioxide (8)

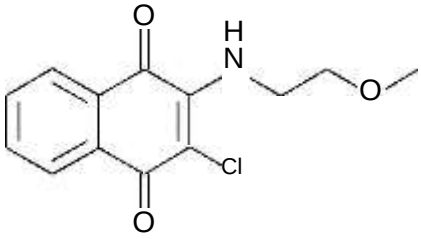


To a solution of **7** (8.0 mg, 0.03 mmol) in MeOH (5 mL) was added 40% KOH aqueous solution (5 mL).[3] The open reaction mixture was stirred at room temperature for 4 days, then pre-adsorbed onto Alltech C18-bonded silica (~1 g, 40-60 m) and packed into an Alltech stainless steel guard cartridge (10 mm 30 mm) as a semi-dried material. The cartridge was subsequently attached to a ThermoElectron Betasil C18-bonded silica HPLC column (5 m, 143 Å, 21.2 mm 150 mm) and isocratic conditions of 90% H2O (0.1% TFA)/10% MeOH (0.1% TFA) were employed for the first 10 min, then a linear gradient to MeOH (0.1% TFA) was run over 40 min, followed by isocratic conditions of MeOH (0.1% TFA) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 1 min) were collected by time from the start of the HPLC run. The desired product, compound **8**, eluted at 29-39 min (5.1 mg, 63%). **^{1}H NMR** (DMSO-*d*6, 500 MHz): δ 11.28 (NH, *br s*), 8.08 (1H, *dd*, *J* = 7.5, 1.5 Hz), 8.06 (1H, *dd*, *J* = 7.5, 1.5 Hz), 7.95 (1H, *ddd*, *J* = 7.5, 7.5, 1.5 Hz), 7.87 (1H, *ddd*, *J* = 7.5, 7.5, 1.5 Hz), 7.13 (1H, *d*, *J* = 9.0 Hz), 6.55 (1H, *d*, *J* = 9.0 Hz); **^{13}C NMR** (DMSO-*d*6, 125 MHz): δ 178.4 (C), 177.9 (C), 140.4 (C), 135.5 (CH), 133.9 (CH), 131.7 (C), 130.5 (CH), 130.1 (C), 126.6 (CH), 125.9 (CH), 115.8 (C), 111.8 (CH); **(+)-LRESIMS** *m/z* 262 (100) $[M+H]^+$.

Synthesis of amine derivatives of 2,3-dichloro-1,4-naphthoquinone

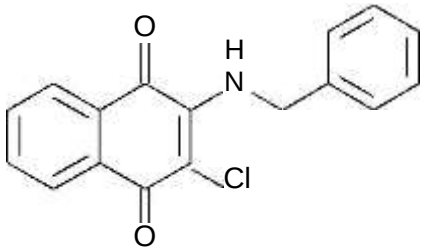
The library of N-alkylated derivatives (**10**-**15**) was synthesised using a previously reported procedure.[4]

2-Chloro-3-[(2-methoxyethyl)amino]naphthalene-1,4-dione (**10**)[5]



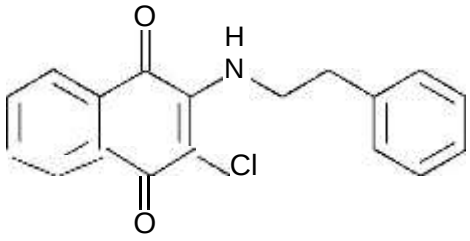
2-Methoxyethylamine (0.4 mL, 6.5 mmol. 14.8 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 µmol) in 5 mL ethanol. The reaction was stirred at room temperature for 4.5 hours then dried under nitrogen. The resulting crystals were redissolved in DCM and absorbed on to silica, dried, and purified by silica SPE cartridge using a 10% stepwise gradient from *n*-hexane to EtOAc (10 mL elutions). Fractions 3-5 (80-60% *n*-hexane) contained the desired compound. The fractions were combined and dried first under nitrogen and then under vacuum to give **10** (102.9 mg, 88%) as a bright red crystalline solid.
^{1}H NMR (methanol-d_4, 500 MHz): δ 8.05 (2H, m), 7.78 (1H, m), 7.70 (1H, m), 4.04 (2H, t, J = 5.5 Hz), 3.64 (2H, t, J = 5.5 Hz), 3.39 (3H, s); **(+)-LRESIMS** *m/z* 266 (100), 268 (30) $[M+H]^+$.

2-Chloro-3-(benzylamino)-naphthalene-1,4-dione (**11**)[5]



Benzylamine (0.46 mL, 4.4 mmol, 10 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 µmol, 1 eq.) in 5 mL ethanol. The reaction was stirred at room temperature for 3 hours and dried under nitrogen. The crude product was absorbed onto silica overnight and subjected to silica SPE cartridge chromatography using a 10% stepwise gradient from *n*-hexane to 50% *n*-hexane-50% EtOAc (10 mL elutions). Fractions 3-5 (80-60% *n*-hexane) were combined and dried under nitrogen then under vacuum. The resulting red solid was subjected to HPLC using a ThermoElectron C18 Betasil 5 µm 143 Å (21.2 × 150 mm) column at a flow rate of 9 mL/min and isocratic conditions of 30% MeOH (0.1% TFA) – 70% H2O (0.1% TFA) for 10 minutes, followed by a linear gradient to 100% MeOH (0.1% TFA) over 40 minutes, then isocratic 100% MeOH (0.1% TFA) for 10 minutes. Sixty fractions (60 x 1 min) were collected from the start of the HPLC run. Fractions 42-44 and 46 yielded 35.6 mg of pure compound **11** (27.3%). The fractions were combined, dried under nitrogen and then under vacuum.
^{1}H NMR (methanol-d_4, 500 MHz): δ 8.05 (2H, m), 7.77 (1H, ddd, J = 7.6, 7.6, 1.3 Hz), 7.69 (1H, ddd, J = 7.6, 7.6, 1.3 Hz), 7.37-7.21 (5H, m), 5.05 (2H, s); **(+)-LRESIMS** *m/z* 298 (100), 300 (30) $[M+H]^+$.

2-Chloro-3-(phenethylamino)naphthalene-1,4-dione (**12**)[6]

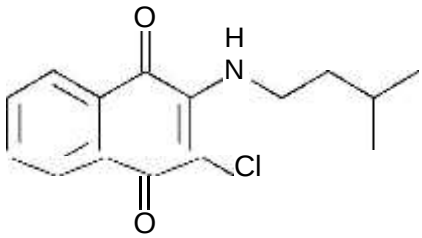


Phenethylamine (0.51 mL, 4.4 mmol, 10 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 µmol, 1 eq.) in 5 mL ethanol. The reaction was stirred for 3 hours at room temperature then dried under nitrogen. The crude product was absorbed onto silica and subjected to silica SPE cartridge chromatography using a 10% stepwise gradient from *n*-hexane to 50% *n*-hexane-50% EtOAc (10 mL elutions). Fractions 3-5 (80-60% *n*-hexane) were combined and

dried under nitrogen then under vacuum. The resulting red solid was subjected to HPLC using a ThermoElectron C18 Betasil 5 µm 143 Å (21.2 × 150 mm) column at a flow rate of 9 mL/min and isocratic conditions of 30% MeOH (0.1% TFA) – 70% H_2O (0.1% TFA) for 10 minutes, followed by a linear gradient to 100% MeOH (0.1% TFA) over 40 minutes, then isocratic 100% MeOH (0.1% TFA) for 10 minutes. Sixty fractions (60 x 1 min) were collected from the start of the HPLC run. Fractions 44-48 were combined and dried under nitrogen then under vacuum to give compound **12** (66.4 mg, 48.4%).

^{1}H NMR (methanol-d_4, 500 MHz): δ 8.04 (1H, app. d, J = 7.6 Hz), 8.00 (1H, app. d, J = 7.6 Hz), 7.77 (1H, ddd, J = 7.6, 7.6, 1.3 Hz), 7.68 (1H, ddd, J = 7.6, 7.6 1.3 Hz), 7.29-7.15 (5H, m), 4.07 (2H, t, J = 7.4 Hz,), 2.98 (2H, t, J = 7.4 Hz); **(+)-LRESIMS** *m/z* 312 (100), 314 (30) $[M+H]^+$.

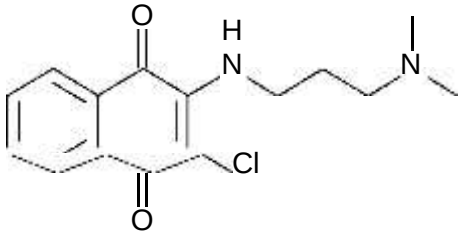
2-Chloro-3-(isopentylamino)naphthalene-1,4-dione (**13**)[7]



Isopentylamine (0.29 mL, 4.4 mmol, 10 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 µmol, 1 eq.) in 5 mL ethanol. The solution was stirred for 2 hours 45 minutes at room temperature, the reaction remained translucent bright red throughout the reaction with a small amount of precipitate. The reaction mixture was dried under nitrogen, redissolved in DCM and preabsorbed on to silica overnight. The crude product was purified by silica SPE cartridge chromatography using a 10% stepwise gradient from *n*-hexane to 50% *n*-hexane-50% EtOAc (10 mL elutions). Fractions 2-3 (90-80% *n*-hexane) were combined and dried under nitrogen then under vacuum to give **13** (88.3 mg, 72.3%).

^{1}H NMR (methanol-d_4, 500 MHz): δ 8.04 (1H, app. d, J = 7.8 Hz), 8.02 (1H, app. d, J = 7.8 Hz), 7.77 (1H, ddd, J = 7.8, 7.8, 1.1 Hz), 7.68 (1H, ddd, J = 7.8, 7.8, 1.1 Hz), 3.85 (2H, t, J = 7.6 Hz), 1.70 (1H, app. nonet, J = 6.6 Hz), 1.60 (2H, m), 0.98 (6H, d, J = 6.6 Hz); **(+)-LRESIMS** *m/z* 278 (100), 280 (30) $[M+H]^+$.

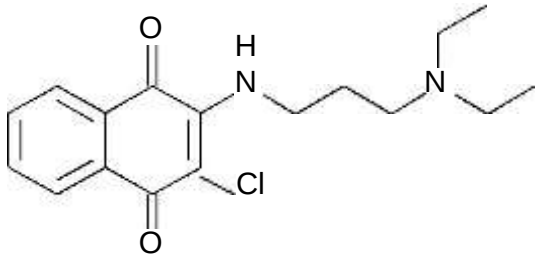
2-Chloro-3-{[3-(dimethylamino)propyl]amino}naphthalene-1,4-dione (**14**)[7]



3-(Dimethylamino)propylamine (0.47 mL, 4.4 mmol, 10 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 µmol, 1 eq.) in 3 mL ethanol. The reaction was stirred for 4 hours at room temperature then dried under nitrogen to give a viscous, dark red oil. The oil was resuspended in DCM and methanol, absorbed onto silica (30-40 micron) overnight and subjected to silica SPE cartridge chromatography using a 10% stepwise gradient from *n*-hexane to 50% *n*-hexane-50% EtOAc (10 mL elutions). Two drops of triethylamine were added to each elution volume before being run through the column. Fractions 2-4 (90-70% *n*-hexane) were combined and dried under nitrogen then under vacuum to give **14** (40.6 mg, 31.6% yield).

^{1}H NMR (methanol-d_4, 500 MHz): δ 8.02 (1H, *dd*, J = 7.8, 1.1 Hz), 7.99 (1H, *dd*, J = 7.8, 1.1 Hz), 7.75 (1H, *ddd*, J = 7.8, 7.8, 1.1 Hz), 7.66 (1H, *ddd*, J = 7.8, 7.8, 1.1 Hz), 3.87 (2H, t, J = 7.0 Hz), 2.44 (2H, t, J = 7.0 Hz), 2.26 (6H, s), 1.87 (2H, p, J = 7.0 Hz); **(+)-LRESIMS** *m/z* 293 (100), 295 (30) $[M+H]^+$.

2-Chloro-3-{[3-(diethylamino)propyl]amino}naphthalene-1,4-dione (**15**)[7]



3-(Diethylamino)propylamine (0.47 mL, 4.4 mmol, 10 eq.) was added to a solution of 2,3-dichloro-1,4-naphthoquinone (**1**) (100 mg, 440 μmol, 1 eq.) in 3 mL ethanol. The reaction was stirred for 4 hours at room temperature then dried under nitrogen to give a viscous, dark red oil. The oil was resuspended in DCM and methanol, absorbed onto silica overnight and subjected to silica SPE cartridge chromatography using a 10% stepwise gradient from *n*-hexane to 50% *n*-hexane-50% EtOAc (10 mL elutions). Two drops of triethylamine were added to each elution volume before being run through the column. Fractions 2-3 (90-80% *n*-hexane) were combined and dried under nitrogen then under vacuum to give **15** (108 mg, 77%).

^{1}H NMR (methanol-d_4, 500 MHz): δ 8.04 (1H, app. *d*, J = 7.8 Hz), 8.01 (1H, app. *d*, J = 7.8 Hz), 7.76 (1H, *ddd*, J = 7.8, 7.8, 0.8 Hz), 7.68 (1H, *ddd*, J = 7.8, 7.8, 0.8 Hz), 3.89 (2H, t, J = 6.9 Hz), 2.59 (6H, m), 1.85 (2H, p, J = 6.9), 1.06 (6H, m); **(+)-LRESIMS** *m/z* 321 (100), 323 (30) $[M+H]^+$.

Protein expression and purification

Mycobacterial carbonic anhydrases

Recombinant Rv1284 and N-terminally His-tagged Rv3588c, as well as recombinant human carbonic anhydrase II were overexpressed in bacterial culture and purified as described previously.[8] Briefly, expression plasmids were transformed into *Escherichia coli* BL21-AI and liquid overnight cultures (1 L) were used to seed the production cultures in a total of 8 L of LB^+ medium.[9] Following induction, cells were incubated for 4 h at 37°C, then harvested and lysed by multiple freeze-thaw cycles and the resulting suspension was subjected to sonication. After separation of soluble contents by centrifugation, the target mycobacterial protein was purified by a series of chromatographic steps (non-tagged protein: anion exchange with QA52 resin at pH 8.0 and cation exchange with SP-Sepharose at pH 4.5; His-tagged protein: anion exchange with QA52 resin at pH 8.0 and immobilised metal ion affinity with Ni-NTA resin at pH 8.0). The final protein samples were dialysed against 100 mM NaCl, 20 mM HEPES (pH 7.5) and concentrated using Millipore Amicon Ultra-15 ultrafiltration cartridges (Merck Millipore, Bayswater, VIC, Australia).

Human carbonic anhydrase II

Based on the mRNA sequence of human carbonic anhydrase 2 (gb:NM_000067), a codon-optimised synthetic gene was generated by GenScript (Piscataway, NJ, USA) and ligated into the vector pRSET_6c. The protein was expressed in *E. coli* BL21(DE3) cells following the general procedure as outlined above. The soluble cytosolic fraction after lysis was subjected to anion exchange chromatography using first QA52 and then Q-Sepharose resin.

Enzyme kinetics

The enzyme activity of carbonic anhydrases was evaluated using kinetic analysis of the CO2 hydration reaction with stopped-flow methodology following the general protocol by Khalifah. [10] The change of absorbance of *m*-cresol purple was monitored at a wavelength of 572 nm using a Bio-Logic SFM-100 MOS-LED stopped-flow instrument. All solutions were made with freshly filtered and degassed deionised water; CO2 was obtained from BOC (Brisbane, Qld, Australia) and other chemicals from Sigma-Aldrich (Castle Hill, NSW, Australia).

Compound stock solutions were prepared in DMSO or methanol at 60 mM concentration. For determination of relative activity, the sample buffer contained 100 mM Na2SO4, 25 mM TAPS (pH 8.5), 50 M *m*-cresol purple, 5 μM protein and 50 M compound. The substrate buffer consisted of CO2-saturated water. Reactions were initiated by mixing sample and substrate buffer at a 1:1 (v/v) ratio and were followed for the first 10 s, and at least five traces are analysed for each individual compound to determine the initial rates of the reaction.

For dose-response experiments, compounds were tested at five different concentrations ranging from 25 nM to 25 M and tested using CO2-saturated water as substrate, following the above protocol. Dose-response data were fitted using the software SDAR.[11]

Notes and references

1 S. Cao, B. T. Murphy, C. Foster, J. S. Lazo and D. G. I. Kingston, *Bioorg. Med. Chem.*, 2009, **17**, 2276–2281.

2 E. W. Chia, A. N. Pearce, M. V. Berridge, L. Larsen, N. B. Perry, C. E. Sansom, C. A. Godfrey, L. R. Hanton, G.-L. (Leon) Lu, M. Walton, W. A. Denny, V. L. Webb, B. R. Copp and J. L. Harper, *Bioorg. Med. Chem.*, 2008, **16**, 9432–9442.

3 R. H. Pouwer, S. M. Deydier, P. V. Le, B. D. Schwartz, N. C. Franken, R. A. Davis, M. J. Coster, S. A. Charman, M. D. Edstein, T. S. Skinner-Adams, K. T. Andrews, I. D. Jenkins and R. J. Quinn, *ACS Med. Chem. Lett.*, 2014, **5**, 178–182.

4 V. K. Singh, S. K. Verma, R. Kadu and S. M. Mobin, *RSC Adv.*, 2015, **5**, 43669–43686.

5 S.-H. S. Ho, M.-Y. Sim, W.-L. S. Yee, T. Yang, S.-P. J. Yuen and M.-L. Go, *Eur. J. Med. Chem.*, 2015, **104**, 42–56.

6 V. Prachayasittikul, R. Pingaew, A. Worachartcheewan, C. Nantasenamat, S. Prachayasittikul, S. Ruchirawat and V. Prachayasittikul, *Eur. J. Med. Chem.*, 2014, **84**, 247–263.

7 KR20140105413 (A), 2014.

8 N. von Gnielinski, L. Nienaber, L. Mason, S. Ellis, J. A. Triccas, R. A. Davis and A. Hofmann, *Med Chem Commun*, 2014, **5**, 1563–1566.

9 A. Hofmann, *Methods in Structural Chemistry*, Structural Chemistry Program, Griffith University, Brisbane, 3rd edn., 2016.

10 R. G. Khalifah, *J. Biol. Chem.*, 1971, **246**, 2561–2573.

11 S. Weeratunga, N.-J. Hu, A. Simon and A. Hofmann, *BMC Bioinformatics*, 2012, **13**, 201.