CHEMBIOCHEM

Supporting Information

Mechanism-Based Inhibition of Quinone Reductase 2 (NQO2): Selectivity for NQO2 over NQO1 and Structural Basis for Flavoprotein Inhibition

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SUPPORTING INFORMATION

Mechanism-based inhibition of quinone reductase 2 (NQO2). Selectivity for NQO2 over NQO1 and structural basis for inhibition

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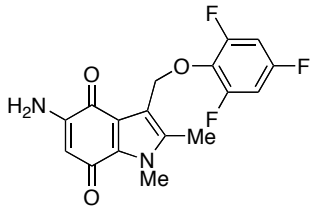
METHODS

Chemistry

General procedure for displacement by amino group: preparation of indolequinones 2

To a solution of the 5-methoxyindolequinone (0.05 mmol) in dry acetonitrile (1 mL) was added the corresponding amine (0.50 mmol). The mixture was stirred at room temperature for 12 days. The solvent and excess amine were removed under reduced pressure, and the residue was extracted with dichloromethane (3 × 20 mL), washed with water (3 × 20 mL), dried over Na_2SO_4, filtered, and the filtrate concentrated under reduced pressure.

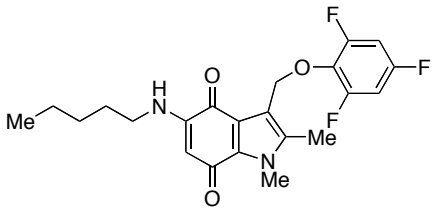
5-Amino-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2a



Ammonia gas was bubbled into a solution of 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b**(*1*) (100 mg, 0.27 mmol) at -78 °C in dry dichloromethane (2 mL), for 5 min. The reaction mixture was then warmed to room temperature

in a sealed tube and stirred for 2 days. The reaction mixture was extracted with dichloromethane (3 × 20 mL), washed with water (3 × 20 mL), dried with Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was purified by chromatography on alumina, eluting with 95% dichloromethane/methanol to give the *title compound* (17 mg, 18%) as a purple solid; mp 209 °C (decomp); (Found: M + H^+, 351.0955. $C_{17}H_{13}N_2O_3$ + H^+ requires 351.0951); λ_{max} (acetonitrile)/nm 239 (log ε 3.84), 310 (3.74); ν_{max} ($CHCl_3$)/cm^{-1} 3396, 3011, 1615, 1507, 1239, 1119, 1040; δ_H (400 MHz; d_6-DMSO) 7.17 (2 H, t, *J* 8.8, ArH), 5.20 (3 H, m, H-6 + CH_2), 3.84 (3 H, s, NMe), 2.18 (3 H, s, 2-Me); δ_C (125 MHz; d_6-DMSO) 178.3 (C), 178.2 (C), 156.8 (dt, *J* 241, 15, CF), 156.0 (ddd, *J* 246, 12, 8, CF), 150.2 (C), 137.3 (C), 131.4 (td, *J* 18, 5, C), 130.0 (C), 119.0 (C), 114.1 (C), 101.1 (ddd, *J* 27, 20, 8, CH), 98.0 (CH), 65.7 (CH_2), 31.3 (Me), 8.8 (Me); *m/z* (ESI) 723 (2 M + Na^+, 100%), 373 (MNa^+, 75%), 351 (MH^+, 10).

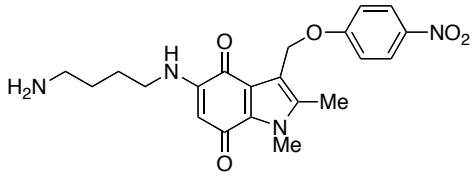
1,2-Dimethyl-5-pentylamino-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2b



According to the general procedure, 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b** (20 mg, 0.05 mmol) in acetonitrile (1 mL) was treated with pentylamine (58 μL, 0.50 mmol). Following workup, the mixture was purified by chromatography, eluting with 20-90% ethyl acetate/light petroleum and 2% triethylamine to give the *title compound* (22 mg, 95%) as a purple solid; mp 148-150 °C; (Found: M + H^+, 421.1725. $C_{22}H_{23}F_3N_2O_3$ + H^+ requires 421.1734); λ_{max} (acetonitrile)/nm 314 (log ε 3.93), 345 (3.47), 510 (3.15); ν_{max} ($CHCl_3$)/cm^{-1} 3378, 2933, 1604, 1507, 1461; δ_H (400 MHz; $CDCl_3$) 6.66 (2 H, t, *J*

8.0, ArH), 5.81 (1 H, t, *J* 8.0, NH), 5.28 (2 H, s, CH_2), 5.13 (1 H, s, H-6), 3.93 (3 H, s, NMe), 3.09 (2 H, q, *J* 6.9, NCH_2), 2.28 (3 H, s, 2-Me), 1.65 (2 H, m, CH_2), 1.37 (4 H, m, CH_2), 0.93 (3 H, t, *J* 6.5, Me); δ_C (125 MHz; $CDCl_3$) 179.0 (C), 178.7 (C), 157.3 (dt, *J* 245, 15, CF), 156.6 (ddd, *J* 247, 11, 7, CF), 148.2 (C), 136.8 (C), 131.8 (td, *J* 15, 5, C), 131.2 (C), 119.5 (C), 115.1 (C), 100.6 (ddd, *J* 27, 20, 7, CH), 96.4 (CH), 66.2 (CH_2), 42.7 (CH_2), 32.4 (Me), 29.2 (CH_2), 27.9 (CH_2), 22.3 (CH_2), 13.9 (Me), 9.3 (Me); *m/z* (ESI) 863 ($2M + Na^+$, 100%), 443 (MNa^+, 90), 421 (MH^+, 83).

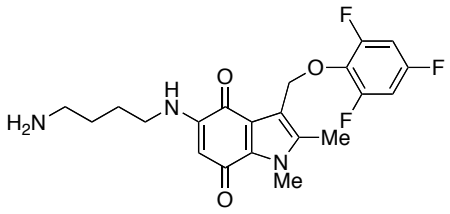
5-(4-Aminobutyl)amino-1,2-dimethyl-3-(4-nitrophenoxy)methylindole-4,7-dione 2c



According to the general procedure, 5-methoxy-1,2-dimethyl-3-(4-nitrophenoxy)methylindole-4,7-dione **1a**(*2*) (30 mg, 0.08 mmol) in acetonitrile (1 mL) was treated with 1,4-diaminobutane (85 µL, 0.84 mmol). Following workup, the residue was purified by chromatography using alumina, eluting with 95% dichloromethane/methanol to give the *title compound* (11 mg, 30%) as a purple solid; mp 155-157 °C; (Found: $M + H^+$, 413.1816. $C_{21}H_{24}N_4O_5 + H^+$ requires 413.1819); λ_{max} (acetonitrile)/nm 241 (log ε 4.15), 311 (4.19); ν_{max} ($CHCl_3$)/cm^{-1} 3379, 2996, 1596, 1504, 1462, 1343; δ_H (400 MHz; $CDCl_3$) 8.20 (2 H, d, *J* 8.0, ArH), 7.06 (2 H, d, *J* 8.0, ArH), 6.04 (1 H, bs, NH), 5.34 (2 H, s, CH_2), 5.16 (1 H, s, H-6), 3.94 (3 H, s, NMe), 3.14 (2 H, ~q, *J* 6.6, CH_2), 2.76 (2 H, t, *J* 6.6, CH_2), 2.29 (3 H, s, 2-Me), 1.73 (2 H, quin, *J* 7.3, CH_2), 1.55 (2 H, quin, *J* 7.3, CH_2); δ_C (100 MHz; $CDCl_3$) 179.0 (C), 178.8 (C), 163.8 (C), 148.1 (C), 141.5 (C), 136.2 (C), 131.4 (C), 126.0 (CH), 119.3 (C), 114.8 (CH), 114.6 (C), 96.7 (C), 61.3 (CH_2), 42.7 (CH_2), 41.6

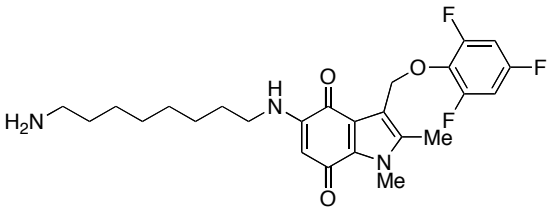
(CH_2), 32.4 (Me), 30.9 (CH_2), 25.6 (CH_2), 9.8 (Me); *m/z* (ESI) 435 (MNa^+, 21%), 413 (MH^+, 61), 274 (100).

5-(4-Aminobutyl)amino-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2d



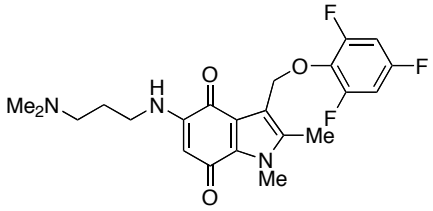
According to the general procedure, 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b** (20 mg, 0.05 mmol) in acetonitrile (1 mL) was treated with 1,4-diaminobutane (55 μL, 0.5 mmol). Following workup, the residue was purified by chromatography on alumina, eluting with 95% dichloromethane/methanol to give the *title compound* (12 mg, 52%) as a purple solid; mp 141-143 °C; (Found: M + H^+, 422.1689. $C_{21}H_{22}F_3N_3O_3$ + H^+ requires 422.1686); λ_{max} (acetonitrile)/nm 244 (log ε 4.02), 312 (3.85), 349 (3.54); ν_{max} ($CHCl_3$)/cm^{-1} 3379, 2948, 1602, 1507, 1462; δ_H (400 MHz; $CDCl_3$) 6.66 (2 H, t, *J* 8.0, ArH), 5.99 (1 H, bs, NH), 5.27 (2 H, s, CH_2), 5.12 (1 H, s, H-6), 3.92 (3 H, s, NMe), 3.13 (2 H, ~q, *J* 6.6, CH_2), 2.75 (2 H, t, *J* 6.6, CH_2), 2.27 (3 H, s, 2-Me), 1.71 (2 H, quin, *J* 7.2, CH_2), 1.53 (2 H, quin, *J* 7.2, CH_2); δ_C (125 MHz; $CDCl_3$) 179.0 (C), 178.7 (C), 157.3 (dt, *J* 244, 15, CF), 156.6 (ddd, *J* 248, 11, 8, CF), 148.2 (C), 136.8 (C), 131.8 (td, *J* 15, 5, C), 131.2 (C), 119.5 (C), 100.6 (ddd, *J* 26, 20, 6, CH), 96.5 (CH), 66.2 (CH_2), 53.5 (C), 42.6 (CH_2), 41.7 (CH_2), 32.4 (Me), 31.0 (CH_2), 25.6 (CH_2), 9.3 (Me); *m/z* (ESI) 444 (MNa^+, 30%), 422 (MH^+, 64), 274 (100).

5-(8-Aminooctyl)amino-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2e



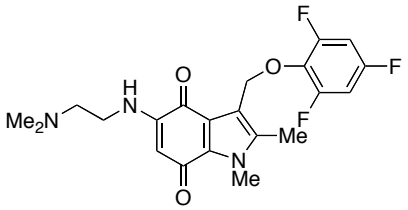
According to the general procedure, 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b** (30 mg, 0.08 mmol) in acetonitrile (1 mL) was treated with 1,8-diamino-octane (118 mg, 0.82 mmol). Following workup, the crude product was purified by chromatography, on alumina, eluting with 95% dichloromethane/methanol to give the *title compound* (14 mg, 35%) as a purple solid; mp 156-158 °C; (Found: M + H^+, 478.2303. $C_{25}H_{30}F_3N_3O_3$ + H^+ requires 478.2312); λ_{max} (acetonitrile)/nm 245 (log ε 4.03), 313 (3.85), 347 (3.52); ν_{max} ($CHCl_3$)/cm^{-1} 3378, 3011, 2934, 1603, 1507, 1462, 1239; δ_H (400 MHz; $CDCl_3$) 6.65 (2 H, t, *J* 8.2, ArH), 5.81 (1 H, bs, NH), 5.26 (2 H, s, CH_2), 5.12 (1 H, s, H-6), 3.92 (3 H, s, NMe), 3.08 (2 H, ~q, *J* 6.8, CH_2), 2.69 (2 H, t, *J* 6.8, CH_2), 2.27 (3 H, s, 2-Me), 1.64 (2 H, m, CH_2), 1.45 (2 H, m, CH_2), 1.32 (8 H, m, CH_2); δ_C (100 MHz; $CDCl_3$) 178.9 (C), 178.7 (C), 157.3 (dt, *J* 244, 14, CF), 156.6 (ddd, *J* 248, 14, 8, CF), 148.2 (C), 136.8 (C), 131.8 (td, *J* 15, 5, C), 131.2 (C), 119.6 (C), 115.2 (C), 100.6 (ddd, *J* 27, 24, 8, CH), 96.4 (CH), 66.2 (CH_2), 42.7 (CH_2), 42.1 (CH_2), 33.6 (CH_2), 32.3 (CH_2), 29.3 (Me), 29.2 (CH_2), 28.2 (CH_2), 26.9 (CH_2), 26.8 (Me), 9.3 (CH_2); *m/z* (ESI) 500 (MNa^+, 14%), 478 (MH^+, 19), 330 (100).

5-(3-Dimethylamino)propylamino-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2f



According to the general procedure, 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b** (20 mg, 0.05 mmol) in acetonitrile (1 mL) was treated with 3-dimethylamino-1-propylamine (63 μL, 0.5 mmol). Following workup, the residue was purified by chromatography, eluting with 20-90% ethyl acetate/light petroleum and 2% triethylamine to give the *title compound* (23 mg, 95%) as a purple solid; mp 90-91 °C; (Found: M + H^+, 436.1857. $C_{22}H_{24}F_3N_4O_5$ + H^+ requires 436.1843); λ_{max} (acetonitrile)/nm 312 (log ε 4.06), 347 (3.76), 518 (3.26); ν_{max} ($CHCl_3$)/cm^{-1} 3377, 1604, 1507, 1461, 1119; δ_H (400 MHz; $CDCl_3$) 6.65 (3 H, m, NH + ArH), 5.28 (2 H, s, CH_2), 5.13 (1 H, s, H-6), 3.93 (3 H, s, NMe), 3.18 (2 H, ~q, *J* 6.6, CH_2), 2.39 (2 H, t, *J* 6.6, CH_2), 2.27 (3 H, s, 2-Me), 2.26 (6 H, s, NMe_2), 1.80 (2 H, quin, *J* 6.6, CH_2); δ_C (125 MHz; $CDCl_3$) 179.0 (C), 178.7 (C), 157.3 (dt, *J* 244, 14, CF), 156.6 (ddd, *J* 250, 10, 7, CF), 148.6 (C), 136.7 (C), 131.9 (td, *J* 12, 5, C), 131.2 (C), 119.6 (C), 115.1 (C), 100.6 (ddd, *J* 27, 20, 5, CH), 96.3 (CH), 66.3 (CH_2), 57.6 (CH_2), 45.4 (Me), 41.8 (CH_2), 32.3 (Me), 25.5 (CH_2), 9.3 (Me); *m/z* (ESI) 458 (MNa^+, 3%), 436 (MH^+, 100).

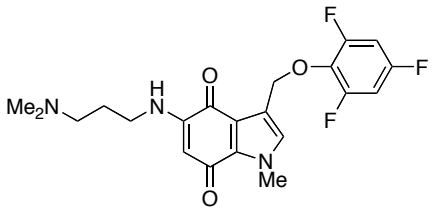
5-(2-Dimethylamino)ethylamino-1,2-dimethyl-3-(2,4,6-trifluorophenoxymethyl)indole-4,7-dione 2g



According to the general procedure, 5-methoxy-1,2-dimethyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione **1b** (30 mg, 0.08 mmol) in acetonitrile (1 mL) was treated with *N*,*N*-dimethylethylenediamine (88 μL, 0.81 mmol). Following workup, the residue was purified by chromatography, eluting with 20-90% ethyl acetate/light petroleum and 2%

triethylamine to give the *title compound* (30 mg, 88%) as a purple solid; mp 129-131 °C; (Found: M + H^+, 422.1682. $C_{21}H_{22}F_3N_3O_3$ + H^+ requires 422.1686); λ_{max} (acetonitrile)/nm 311 (log ε 4.03); ν_{max} ($CHCl_3$)/cm^{-1} 3369, 2934, 2360, 1604, 1507, 1460, 1119; δ_H (400 MHz; $CDCl_3$) 6.65 (2 H, t, *J* 8.3, ArH), 6.34 (1 H, bs, NH), 5.28 (2 H, s, CH_2), 5.11 (1 H, s, H-6), 3.92 (3 H, s, NMe), 3.13 (2 H, ~q, *J* 5.5, CH_2), 2.57 (2 H, t, *J* 5.5, CH_2), 2.27 (9 H, s, 2-Me, NMe_2); δ_C (100 MHz; $CDCl_3$) 178.9 (C), 178.6 (C), 157.3 (dt, *J* 244, 14, CF), 156.6 (ddd, *J* 248, 15, 8, CF), 148.3 (C), 136.8 (C), 131.9 (td, *J* 15, 5, C), 131.1 (C), 119.6 (C), 115.2 (C), 100.6 (ddd, *J* 27, 24, 8, CH), 96.7 (CH), 66.3 (CH_2), 56.5 (CH_2), 45.1 (Me), 39.8 (CH_2), 32.3 (Me), 9.3 (Me); *m/z* (ESI) 444 (MNa^+, 36%), 422 (MH^+, 100).

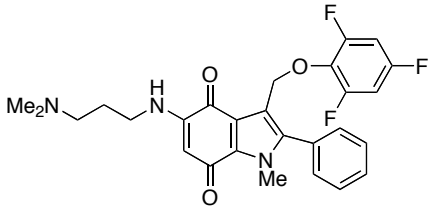
5-(3-Dimethylamino)propylamino-1-methyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione 2h



According to the general procedure, 5-methoxy-1-methyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione(*3*) (24 mg, 0.07 mmol) in acetonitrile (1 mL) was treated with 3-dimethylamino-1-propylamine (88 μL, 0.69 mmol). Following workup, the crude product was purified by chromatography, eluting with 20-90% ethyl acetate/light petroleum and triethylamine to give the *title compound* (17 mg, 58%) as a purple solid; mp 101-103 °C; (Found: M + H^+, 422.1723. $C_{21}H_{22}F_3N_3O_3$ + H^+ requires 422.1686); λ_{max} (acetonitrile)/nm 306 (log ε 3.93); ν_{max} ($CHCl_3$)/cm^{-1} 3378, 3011, 1596, 1508, 1239; δ_H (400 MHz; $CDCl_3$) 6.87 (1 H, bs, NH), 6.80 (1 H, s, H-2), 6.67 (2 H, t, *J* 8.4, ArH), 5.28 (2 H, s, CH_2), 5.17 (1 H, s, H-6), 3.96 (3

H, s, NMe), 3.20 (2 H, ~q, *J* 6.5, CH_2), 2.42 (2 H, t, *J* 6.5, CH_2), 2.27 (6 H, s, NMe_2), 1.81 (2 H, quin, *J* 6.5, CH_2); δ_C (125 MHz; $CDCl_3$) 178.8 (C), 178.4 (C), 157.2 (dt, *J* 244, 14, CF), 156.2 (ddd, *J* 248, 11, 8, CF), 149.2 (C), 132.3 (C), 132.2 (td, *J* 17, 5, C), 127.4 (CH), 119.6 (C), 119.3 (C), 100.6 (ddd, *J* 27, 20, 7, CH), 96.4 (CH), 68.5 (CH_2), 57.7 (CH_2), 45.3 (Me), 42.0 (CH_2), 33.3 (Me), 25.3 (CH_2); *m/z* (ESI) 444 (MNa^+, 2%), 422 (MH^+, 100).

5-(3-Dimethylamino)propylamino-1-methyl-2-phenyl-3-(2,4,6-trifluorophenoxy-methyl)indole-4,7-dione 2i



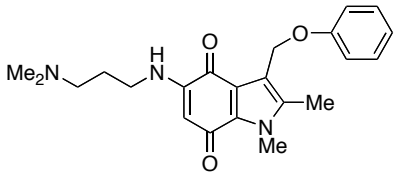
(a) Thionyl chloride (0.65 mL, 8.9 mmol) was added at 0 °C to a solution of 3-hydroxymethyl-5-methoxy-1-methyl-2-phenylindole-4,7-dione(*2*) (50 mg, 0.2 mmol) in dry dichloromethane (2 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was used directly into the next step.

To the crude 3-chloromethyl-5-methoxy-1-methyl-2-phenylindole-4,7-dione in DMF (2 mL) was added potassium carbonate (65 mg, 0.5 mmol) and 2,4,6-trifluorophenol (186 mg, 0.45 mmol) at room temperature. The mixture was stirred for 12 h at room temperature. Water was added and the reaction mixture was extracted with ethyl acetate (3 × 30 mL). The organic layer was washed with water (3 × 30 mL), and then dried with Na_2SO_4. The solvent was removed under reduced pressure. The crude product was purified by chromatography, eluting with 10-50% ethyl acetate/light petroleum to give *5-methoxy-1-methyl-2-phenyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione* (57 mg, 79% over the two steps) as a red solid; mp

159-161 °C; (Found: M + Na^+, 450.0933. $C_{23}H_{16}F_3NO_4$ + Na^+ requires 450.0924); λ_{max} (acetonitrile)/nm 268 (log ε 4.29), 337 (3.47); ν_{max} ($CHCl_3$)/cm^{-1} 3011, 1639, 1603, 1508, 1454, 1175; δ_H (300 MHz; $CDCl_3$) 7.51 (3 H, m, ArH), 7.44 (2 H, m, ArH), 6.60 (2 H, t, *J* 9.0, ArH), 5.71 (1 H, s, 6-H), 5.12 (2 H, s, CH_2), 3.85 (3 H, s, Me), 3.84 (3 H, s, Me); δ_C (125 MHz; $CDCl_3$) 179.2 (C), 177.5 (C), 160.0 (C), 157.3 (dt, *J* 244, 14, CF), 156.5 (ddd, *J* 249, 11, 7, CF), 142.9 (C), 131.9 (td, *J* 15, 5, C), 130.5 (CH), 129.6 (C), 129.5 (CH), 128.7 (CH), 128.1 (C), 121.9 (C), 117.3 (C), 106.9 (CH), 100.5 (ddd, *J* 26, 20, 7, CH), 65.8 (CH_2), 56.5 (Me), 34.2 (Me); *m/z* (ESI) 450 (MNa^+, 100%).

(b) According to the general procedure, the above 5-methoxy-1-methyl-2-phenyl-3-(2,4,6-trifluorophenoxy)methylindole-4,7-dione (25 mg, 0.07 mmol) in acetonitrile (1 mL) was treated with 3-dimethylamino-1-propylamine (78 μL, 0.70 mmol). Following workup, the crude product was purified by chromatography on alumina, eluting with 95% dichloromethane/methanol to give the *title compound* **xx** (28 mg, 95%) as a purple sticky oil; (Found: M + H^+, 498.1998. $C_{27}H_{26}F_3N_3O_3$ + H^+ requires 498.1999); λ_{max} (acetonitrile)/nm 266 (log ε 3.85), 313 (3.63); ν_{max} ($CHCl_3$)/cm^{-1} 3370, 3011, 2359, 1598, 1508, 1454; δ_H (400 MHz; $CDCl_3$) 7.46 (3 H, m, ArH), 7.36 (2 H, m, ArH), 6.75 (1 H, bs, NH), 6.58 (2 H, t, *J* 8.3, ArH), 5.23 (1 H, s, 6-H), 5.10 (2 H, s, CH_2), 3.84 (3 H, s, NMe), 3.22 (2 H, ~q, *J* 6.3, CH_2), 2.41 (2 H, t, *J* 6.3, CH_2), 2.27 (6 H, s, NMe_2), 1.82 (2 H, quin, *J* 6.3, CH_2); δ_C (125 MHz; $CDCl_3$) 179.0 (C), 178.5 (C), 157.2 (dt, *J* 244, 14, CF), 156.5 (ddd, *J* 250, 12, 9, CF), 148.8 (C), 141.1 (C), 132.0 (C), 131.9 (td, *J* 12, 6, C), 130.6 (CH), 129.1 (CH), 128.6 (CH), 128.5 (C), 119.9 (C), 116.3 (C), 100.4 (ddd, *J* 26, 20, 6, CH), 96.7 (CH), 66.2 (CH_2), 57.6 (CH_2), 45.4 (Me), 41.9 (CH_2), 34.1 (Me), 25.5 (CH_2); *m/z* (ESI) 498 (MH^+, 100%).

5-(3-Dimethylamino)propylamino-1,2-dimethyl-3-phenoxymethylindole-4,7-dione 2j



According to the general procedure, 5-methoxy-1,2-dimethyl-3-phenoxymethylindole-4,7-dione **1c**(*4*)(10 mg, 0.03 mmol) in acetonitrile (1 mL) was treated with 3-dimethylamino-1-propylamine (40 μL, 0.32 mmol). Following workup, the crude product was purified by chromatography, eluting with 20-90% ethyl acetate/light petroleum and 2% triethylamine to give the *title compound* (7 mg, 56%) as a purple solid; mp 122-124 °C; (Found: M + H^+, 382.2135. $C_{22}H_{27}N_3O_3$ + H^+ requires 382.2125); λ_{max} (acetonitrile)/nm 311 (log ε 3.70); ν_{max} ($CHCl_3$)/cm^{-1} 3360, 3003, 1730, 1601, 1498, 1374, 1240; δ_H (400 MHz; $CDCl_3$) 7.27 (2 H, m, ArH), 7.02 (3 H, m, ArH), 6.70 (1 H, bs, NH), 5.25 (2 H, s, CH_2), 5.14 (1 H, s, H-6), 3.93 (3 H, s, NMe), 3.20 (2 H, q, *J* 6.6, CH_2), 2.43 (2 H, t, *J* 6.6, CH_2), 2.28 (6 H, s, NMe_2), 2.27 (3 H, s, 2-Me), 1.82 (2 H, quin, *J* 6.6, CH_2); δ_C (100 MHz; $CDCl_3$) 179.0 (C), 178.9 (C), 158.8 (C), 148.6 (C), 135.9 (C), 131.2 (C), 129.4 (CH), 120.8 (CH), 119.5 (C), 116.0 (C), 114.9 (CH), 96.4 (CH), 60.6 (CH_2), 57.6 (CH_2), 45.3 (Me), 41.8 (CH_2), 32.3 (Me), 25.4 (CH_2), 9.7 (Me); *m/z* (ESI) 404 (MNa^+, 2%), 382 (MH^+, 100).

Biochemical Assays

Recombinant human NQO1 was purified from *E coli* using Cibacron-blue affinity chromatography as previously described.(*5*) Recombinant human NQO2 was purchased from Sigma (St. Louis, MO). The inhibition of recombinant human NQO1 and NQO2 by indolequinones was measured independently. Briefly, indolequinones (dissolved in DMSO) were added to reaction buffer; 50 mM potassium phosphate buffer, pH 7.4 containing 125 mM NaCl, 5

μM FAD, 1 mg/mL BSA and 2 μg/mL NQO1 or NQO2 in the absence and presence of either NADH (NQO1) or NRH (NQO2) at 30 °C. To determine 100% activity remaining reactions were carried out in the presence of DMSO. After 5 min, an aliquot of reaction buffer was removed and diluted 100-fold (NQO1) or 50-fold (NQO2) in stop buffer; 50 mM potassium phosphate, pH 7.4 containing 250 mM sucrose, 5 μM FAD and 0.2% (v/v) Tween-20. To determine NQO1 activity, 200 μM NADH and 40 μM DCPIP were added to stop buffer (1 mL final volume) and a linear decrease in absorbance was measured spectrophotometrically at 600 nm. To determine NQO2 activity 200 μM NRH, 10 μM menadione and 0.25 mg/mL MTT were added to stop buffer (1 mL final volume) and a linear increase in absorbance was measured spectrophotometrically at 550 nm.

Protein Crystallography

Human NQO2 was expressed and purified as previously described.(*6*) Freshly purified and non-frozen NQO2 was used for all crystallization experiments.

Co-crystals of the NQO2 - indolequinone **2c** complex were grown using the vapour diffusion sitting drop method. A volume of 2 μl of protein solution, at an approximate concentration of 30 mg/mL, was mixed with an equal volume of reservoir solution (1.60 M ammonium sulfate, 100 mM Na-HEPES pH 7, 12 μM FAD, 1 mM dithiothreitol, 200 μM **2c**). Pale pink rod shaped crystals grew in about a week. Prior to the diffraction experiments, the crystals were briefly soaked in a cryoprotectant solution (1.7 M ammonium sulfate and 1.2 M sodium malonate) and then flash-frozen and stored in liquid nitrogen.

X-ray diffraction data was collected at the European Synchrotron Radiation Facility (ESRF) beamline ID23-1 at 100 K, using an ADSC Q315R CCD detector. The data processing

and scaling was carried out using Mosflm(*7*) and Scala,(*8*) using the CCP4 program suite.(*9, 10*) Initial phases for the complex structure were obtained with Phaser(*11*) using the human NQO2 structure (PDB ID 1QR2(*12*)) as the starting model. Visualization of electron density maps, and model fitting and rebuilding was carried out using Coot.(*13*) Refinement was carried out using Refmac,(*14*) using positional and B-factor restrained refinement. Details of the data collection and processing statistics are given in Table 1.

The inhibitor structure was the last component of the model to be fitted into the density maps. Whilst the presence of the planar central indolequinone ring structure of the inhibitor was clear in the initial maps, the position of the two conformationally flexible substituents was more difficult to ascertain. Four alternative orientations of the ligand were therefore modeled and refined, and inspection of the associated maps (both $F_o - F_c$ and $2F_o - F_c$) allowed a preferred orientation to be discerned. In addition, the positioning of this preferred orientation of the inhibitor allowed a hydrogen bond interaction between Asp117 (main chain carbonyl oxygen) and the primary amine of the aminobutylamino substituent.

The B factors for the nitrophenoxy side chain of the inhibitor (67 to 94 $Å^2$) were higher than those for the indolequinone moiety (52 to 64 $Å^2$) and the aminobutylamino side chain (53 to 57 $Å^2$), probably reflecting its high conformational flexibility. This observation is of note since it indicates uncertainty in the refined position of these atoms. The availability of higher resolution data would be of value to reinforce the orientation of binding proposed here, including possible conformations of the nitrophenoxy side chain.

Figures were drawn using the software Pymol(*15*) and Sybyl.(*16*) The atomic coordinates and structure factors have been deposited in the Protein Data Bank (3O73).

Table 1: Crystallographic data and refinement statistics

Crystallographic data statistics	
Beamline	ESRF ID23-1
Wavelength (Å)	0.9762
Data Processing	
Space group	$P2_12_12_1$
Unit cell dimensions (Å)	a=57.08, b=82.05, c=106.34
Total no. of observations*	125867 (18749)
Unique reflections*	28053 (4230)
Resolution range (Å)*	35.45–2.0 (2.11–2.00)
R_{sym} (%)*	10.6 (67.9)
R_{meas}(%)*	0.119(0.769)
R_{pim}(%)*	0.053(0.349)
Mean I / σI*	7.5 (2.0)
Completeness (%)*	82.3 (86.3)
Multiplicity*	4.5 (4.4)
Model Refinement	
Resolution range (Å)*	35.45–2.0 (2.05–2.00)
Free R value test set (%)	5.0
R_{work} / R_{free} (%)*	22.0 (30.5) / 28.8 (31)
No. of non-hydrogen protein atoms	3648
No. of ligand atoms	168
No. of water atoms	62
Average overall B, (Å^2)	36.80
rms deviation bond lengths (Å)	0.021
rms deviation bond angles (°)	1.92

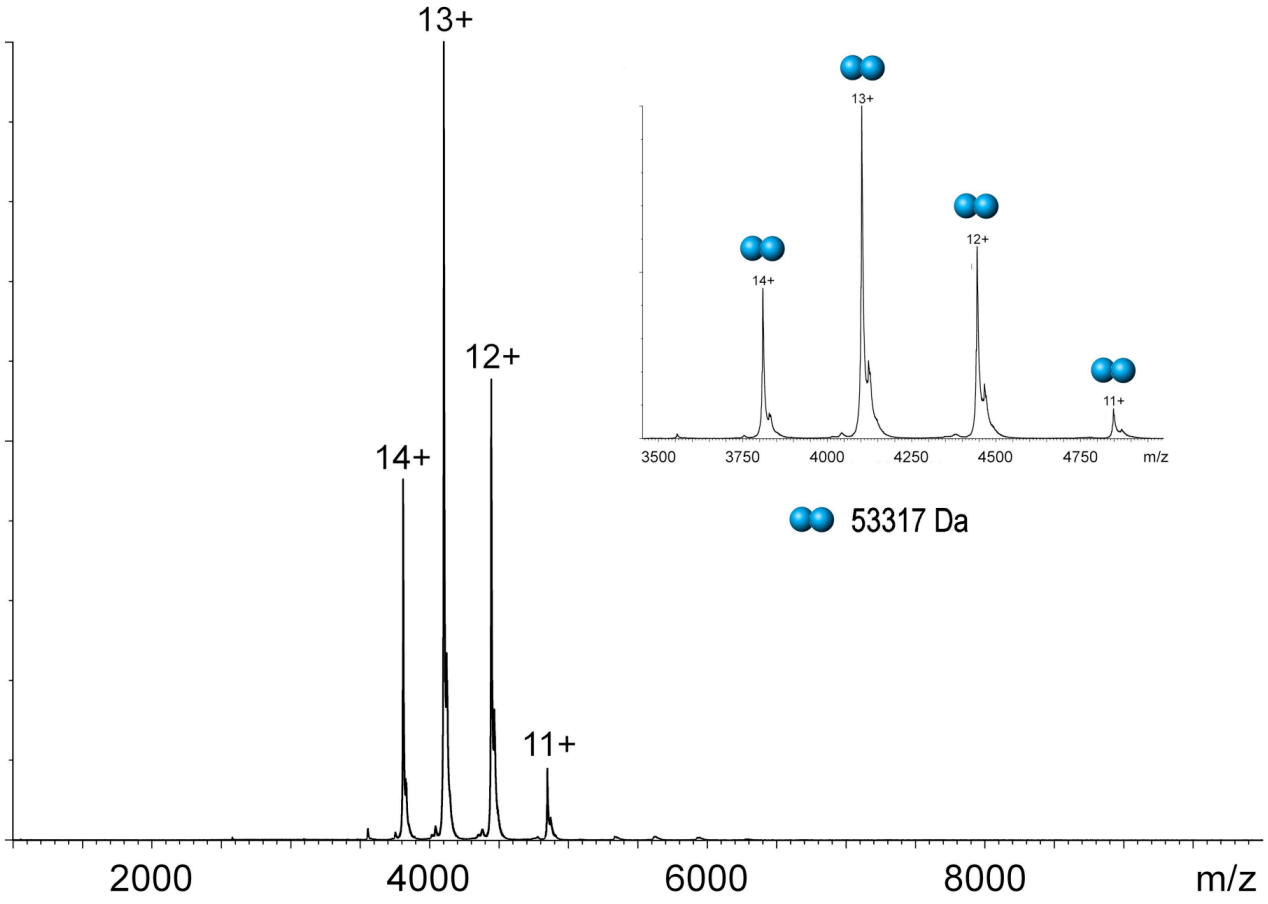
Data and statistics for highest resolution shell are given in parentheses

These X-ray diffraction experiments were performed on the ID23-1 beamline at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. We are grateful to Dr Gordon Leonard at ESRF for providing assistance in using beamline ID23-1.

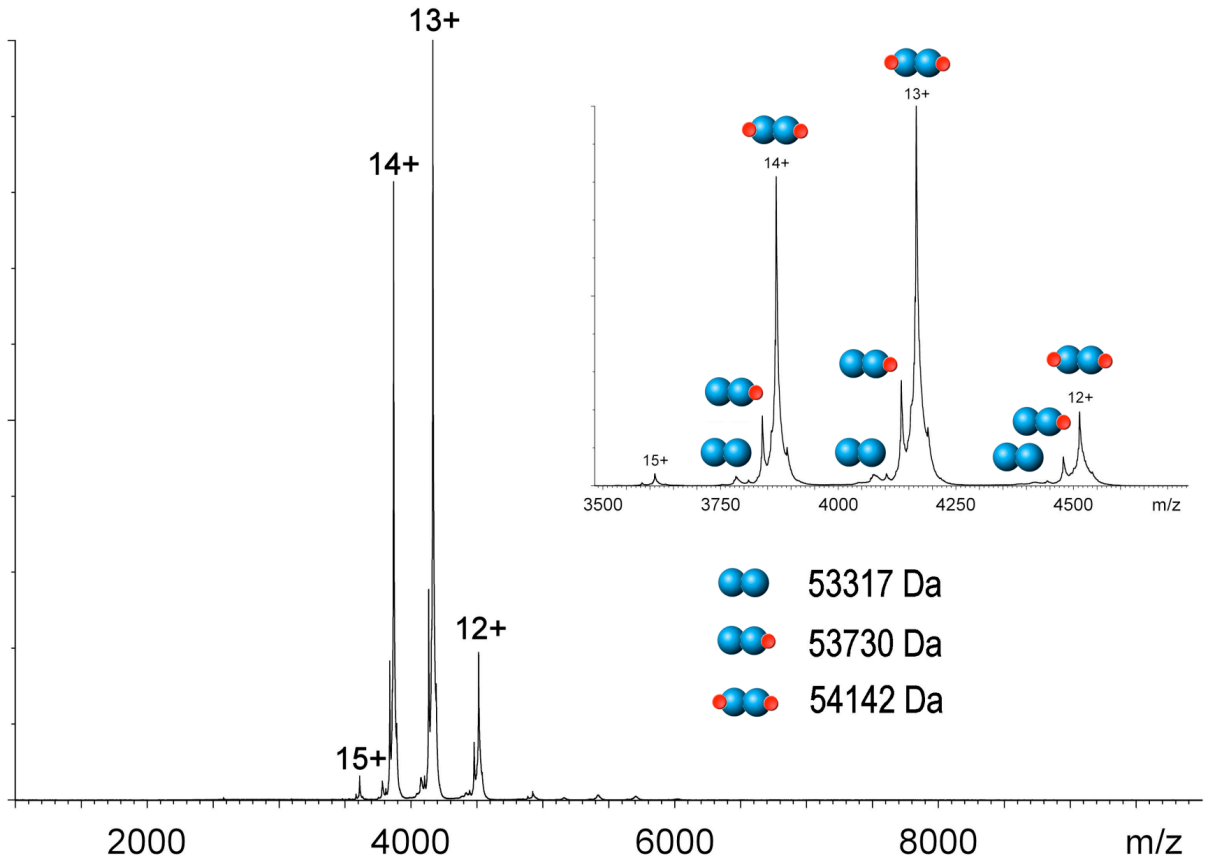
Mass Spectrometry

*Electrospray ionization-mass spectrometry on non-covalent NQO2:indolequinone **2c** complex*

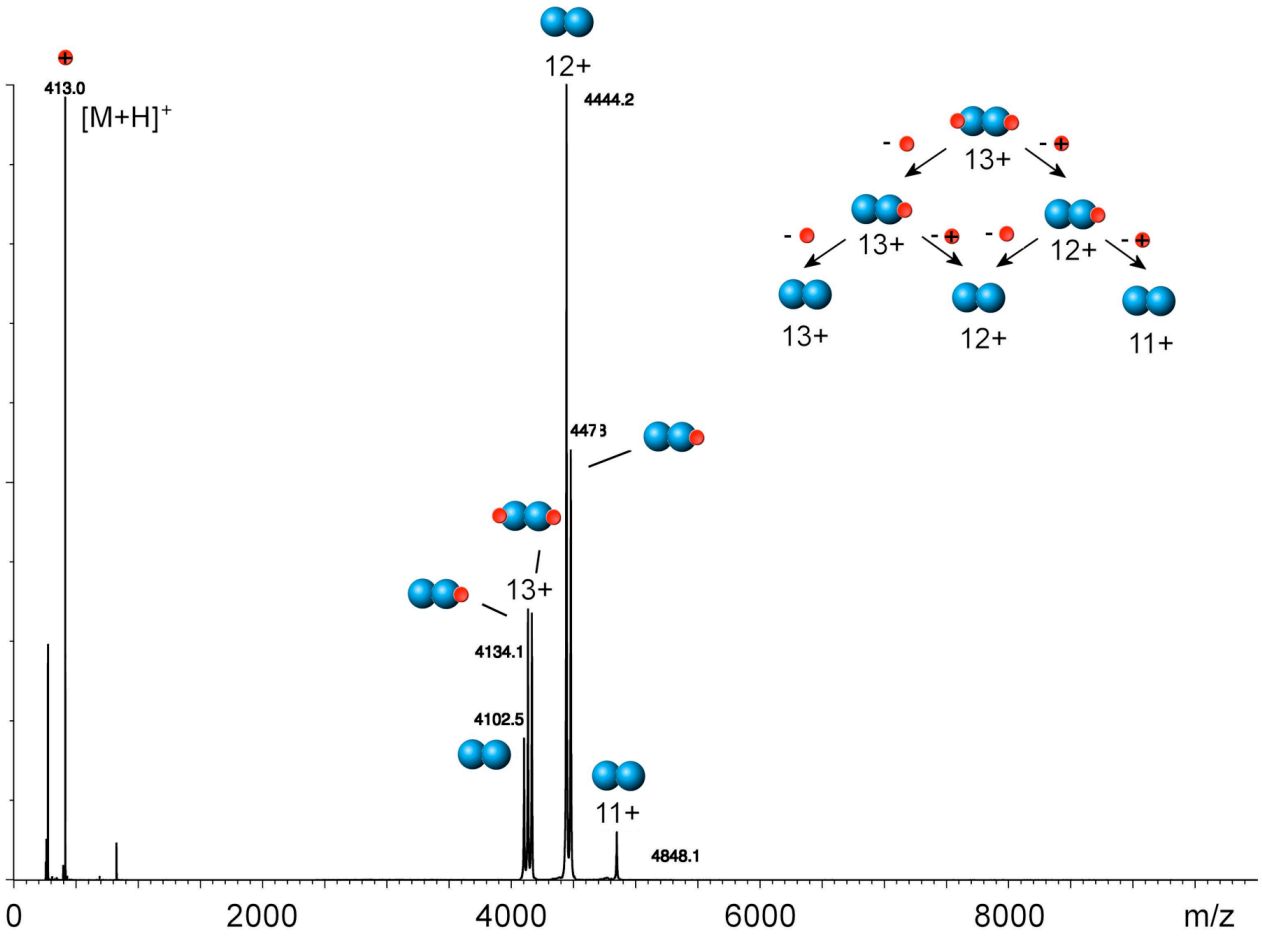
Electrospray ionization-mass spectrometry was performed on a Waters (Altrincham, UK) Synapt High Definition Mass Spectrometer (HDMS) - a hybrid quadrupole/ion mobility/orthogonal acceleration time of flight (oa-TOF) instrument. Samples were infused from borosilicate nanospray tips (Waters, thin wall design) using capillary voltages of 1.3-1.6 kV, with the source operating in positive ion mode. The sample cone was maintained at 30-40 V, with a backing pressure of 4.5 mbar providing collisional cooling in the intermediate vacuum region of the mass spectrometer. The trap T-wave collisional cell, containing argon gas held at a pressure of 2.5 $\times 10^{-2}$ mbar, was operated at 10 V for ion transmission and 30 V for MS/MS dissociation of NQO2:indole quinone complex ions. The oa-TOF-MS was scanned over the range *m/z* 500-10000 at a pressure of 1.8×10^{-6} mbar.



Nano-ESI-MS spectrum of NQO2 showing a dimeric protein of molecular mass 53317 Da.



Nano-ESI-MS spectrum of NQO2 (7 mM) in the presence of excess indolequinone **2c** showing signals at molecular mass 53730 and 54142 Da, corresponding to the non-covalent binding of one and two molecules of inhibitor (MW = 412) respectively.



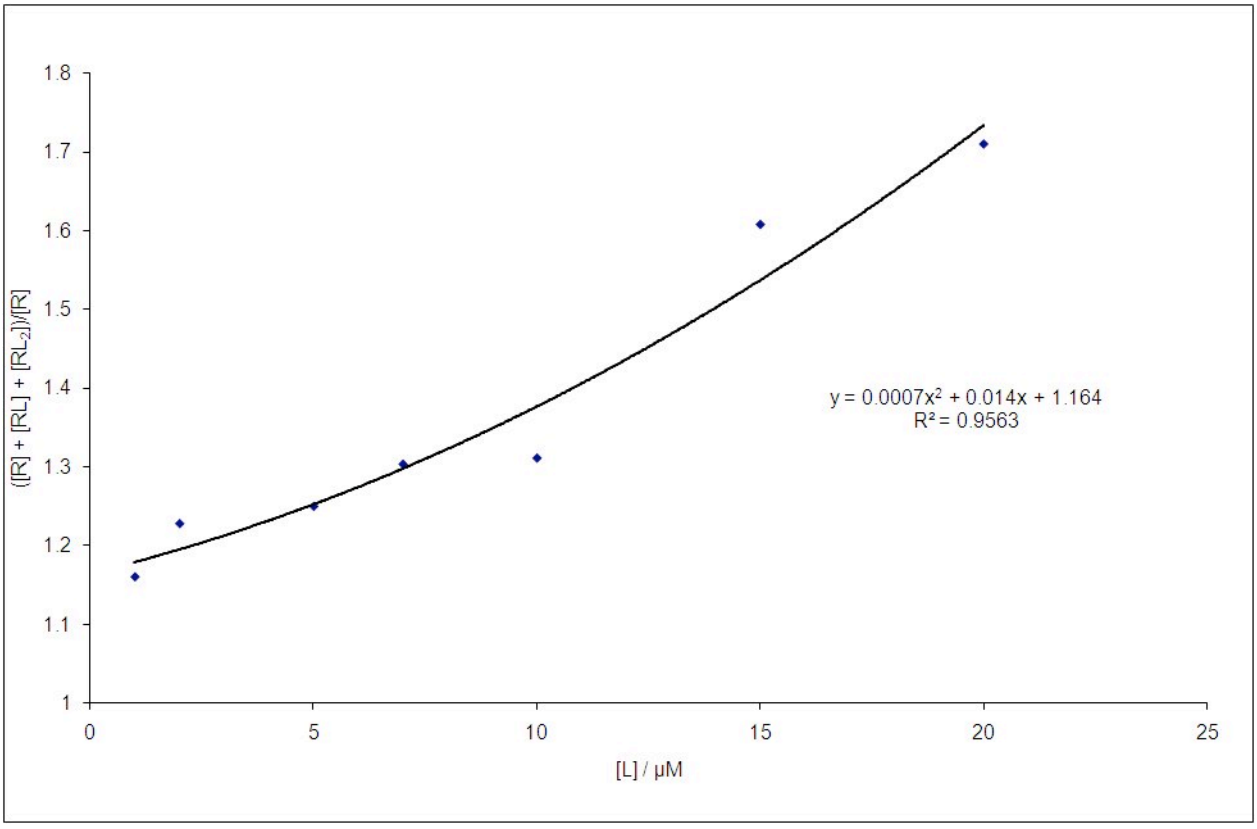
Nano-ESI-MS/MS spectrum of the $[M+13H]^{13+}$NQO2:indolequinone complex ion showing dissociative loss of the IQ inhibitor as a mixture of neutral and charged species.

Determination of apparent K_d

Apparent K_D values for the non-covalent binding of **2c** to NQO2 were estimated by titrating **2c** (1 to 20 μM) into a constant concentration of NQO2 (5 μM) and detecting ligand binding by ESI-MS (see mass spectrometry methods section). For a two-binding interaction it can be shown that:-

$$\frac{([R]+[RL]+[RL_2])}{[R]} = \frac{[L]^2}{K_{D1}K_{D2}} + \frac{[L]}{K_{D1}} + 1$$

Where (*17*) is the free protein concentration, [RL] the concentration of protein with one ligand bound, and [RL_2] the concentration of protein with two ligand molecules bound. The two dissociation constants K_{D1} and K_{D2} can be determined from the coefficients of a binomial plot of $([R] + [RL] + [RL_2])/[R]$ against [L]. The fit below gives a K_{d1} of 71 μM of and a K_{d2} of 19 μM.



Reducing incubation conditions for mass spectrometry

NQO2 (192 nM) was incubated with NRH (200 μM) and IQ (1 μM) in phosphate buffer (50 mM, pH 7.4) at room temperature for 30 min. Protein was then concentrated by centrifugation with 10,000 MWCO Viva Spin columns, and buffer exchanged into 25 mM ammonium acetate (25 mM, pH 7) by 5 concentration-dilution steps before nanoESI-MS analysis. Native MS analysis was conducted directly from the ammonium acetate solution. Denatured samples were prepared by dilution with 1 volume equivalent of methanol containing 0.2% formic acid.

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