Supporting Information

Improved potency of indole-based NorA efflux pump inhibitors: from serendipity towards rational design and development.

Federica Buonerba,[§‡] Susan Lepri,[§‡] Laura Goracci,[§] Bryan D. Schindler, [ζ]Susan M. Seo, [ζ] Glenn W. Kaatz,[¥ ζ*] and Gabriele Cruciani.[§*]

[§]Department of Chemistry, Biology and Biotechnology, University of Perugia, 06123 Perugia, Italy.
[ζ]The John D. Dingell Department of Veterans Affairs Medical Center, Detroit, MI 48201, USA
[¥]Department of Internal Medicine, Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, MI 48201, USA

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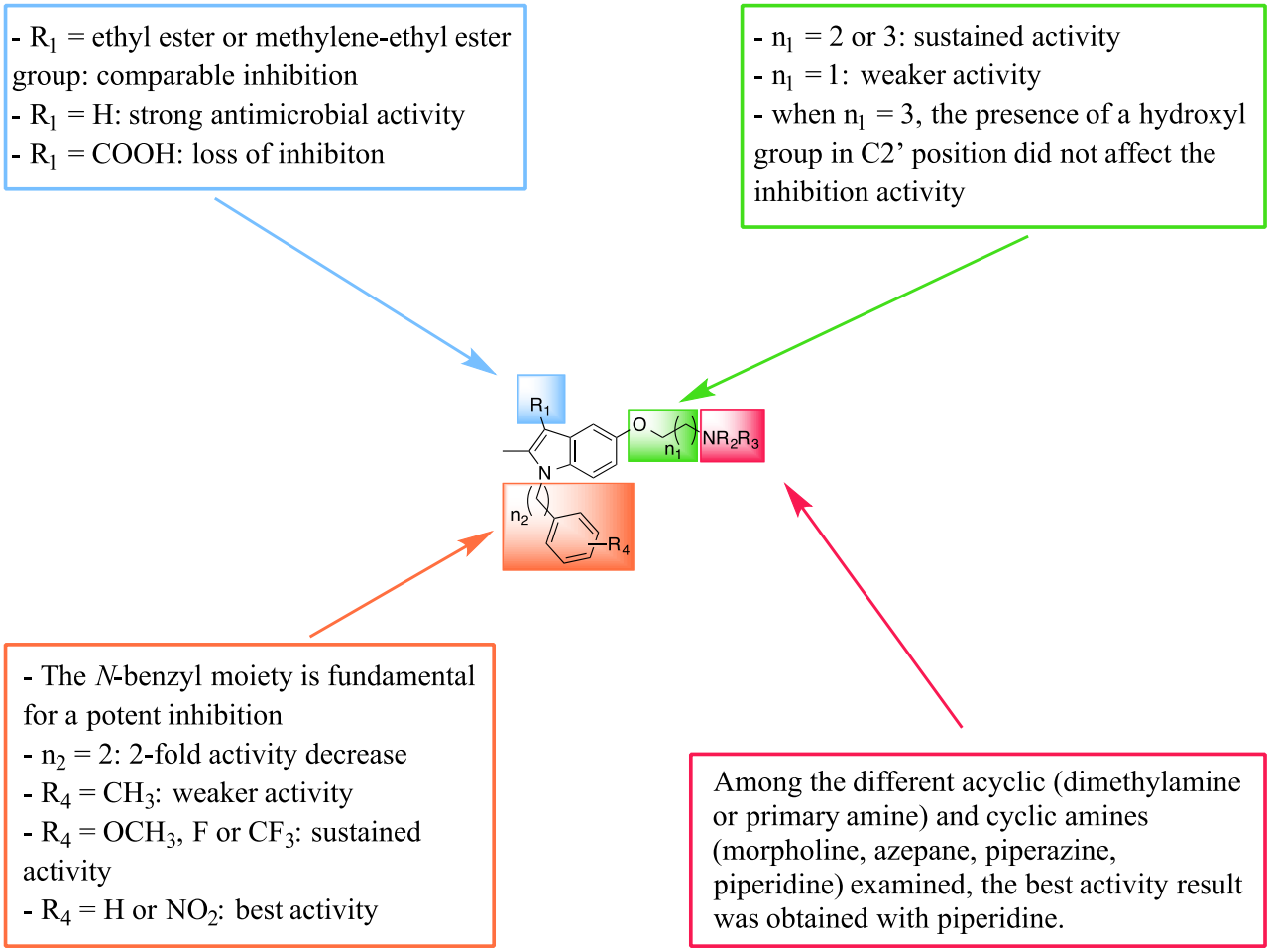

Figure S1. Previous SAR study for N-benzyl indole compounds as a novel class of NorA EPIs.[1]

EXPERIMENTAL SECTION

Biology

Tested compounds

Compounds **4** and **5** were acquired from SPECS. Purities of all tested compounds (including synthesized and acquired ones) were ≥98% pure as determined by UHPLC: on Agilent Technologies 1290; column, Phenomenex AERIS Peptide 1.2 mm × 100 mm (1.7 μm); flow rate, 0.8 mL/min; acquisition time, 20 min; DAD 190-650 nm; oven temperature, 45 °C; gradient of acetonitrile in water containing 0.1% of formic acid (0-100% in 20 minutes).

Ethidium bromide efflux inhibition

All compounds were evaluated for their ability to inhibit efflux of EtBr by SA-1199B, a well-characterized strain that overexpresses *norA*.[2] The efflux inhibition assay employed a real-time microtiter-based fluorometric approach exactly as described previously.[1] Efflux activity of SA-1199B was expressed as percent fluorescence decrease over a 5-min time course. Efflux inhibition by test compounds was determined using the equation [efflux in the absence]-[efflux in the presence of test compound]/[efflux in absence of test compound] × 100, giving the percent efflux inhibition observed. If a 50 μM concentration of test compound achieved ≥ 80% efflux inhibition, a series of concentrations were tested to quantify potency by determining the 50% inhibitory concentration (IC_{50}).

Intrinsic antimicrobial activity (MIC)

In addition to IC_{50} determinations, compounds with ≥80% efflux inhibition were evaluated for intrinsic antimicrobial activity by determining minimal inhibitory concentrations (MIC) employing a microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines.[3] The antimicrobial activity of selected compounds having less than 80% efflux inhibition were also determined. Compounds having an MIC value greater than 100 μg/mL were considered antimicrobially inactive. In these instances, efflux inhibitory activity was considered to be unrelated to any direct antimicrobial effect on the test strain.

Fractional Inhibitory Concentration (FIC) Index

Checkerboard combination plate assays were performed with EtBr and each EPI candidate using SA-1199B strain. Concentrations of 0-100 µg/mL of each compound and 0-80 µg/mL of ciprofloxacin were utilized and the Fractional Inhibitory Concentration (FIC) Index was calculated by finding the lowest concentration of each EPI candidate causing at least a 4-fold reduction in the EtBr MIC. FIC Index = $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of EPI and EtBr alone, respectively, and C_A and C_B are the concentrations of the compounds in combination in wells at which the EtBr MIC is reduced at least 4-fold. Interpretation of FIC Index values: ≤ 0.5, synergy; > 0.5 - ≤ 4.0, indifference; > 4.0, antagonism.

Statistical analysis

Data for efflux inhibition and resultant IC_{50} values are expressed as mean ± SD from three independent experiments. Statistical comparisons were performed using Student's t-test. Differences were considered statistically significant when $p < 0.05$.

performed using Student's t-test. Differences were considered statistically significant when $p < 0.002$.

Chemistry

Compound characterization

Diethyl 5,5'-((azanediylbis(2-hydroxypropane-3,1-diyl))bis(oxy))bis(1-benzyl-2-methyl-1*H*-indole-3-carboxylate) (3). Mp 147 – 149 °C; ^{1}H NMR ($CDCl_3$) δ 7.70 (s, 2H), 7.33 – 7.18 (m, 6H), 7.09 (d, *J* = 8.9 Hz, 2H), 7.02 – 6.88 (m, 4H), 6.83 (dd, *J* = 8.9, 2.5 Hz, 2H), 5.29 (s, 4H), 4.39 (q, *J* = 7.1 Hz, 4H), 4.17 (bs, 2H), 4.12 – 4.03 (m, 4H), 3.04 – 2.82 (m, 4H), 2.69 (s, 6H), 2.62 – 2.45 (bm, 3H), 1.44 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 166.1 (2C), 154.7 (2C), 145.3 (2C), 136.3 (2C), 131.7 (2C), 128.9 (4C), 127.7 (2C), 127.6 (2C), 125.9 (4C), 112.3 (2C), 110.3 (2C), 105.1 (2C), 104.4 (2C), 71.1 (2C), 68.8 (2C), 59.5 (2C), 52.1, 52.0, 46.7 (2C), 14.7 (2C), 12.1 (2C); HRMS: calcd for $C_{44}H_{49}N_3O_8$ 748.3598 [$M+H^+$], found 748.3614 [$M+H^+$].

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)propyl)piperazin-1-yl)propoxy)-1-benzyl-2-methylindole-3-carboxylate (7). Mp 103 – 104 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 2.5 Hz, 1H), 7.58 – 7.48 (m, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.35 – 7.19 (m, 6H), 7.10 (d, *J* = 8.8 Hz, 1H), 6.99 (dt, *J* = 18.3, 7.2 Hz, 4H), 6.81 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.31 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 4.09 (t, *J* = 6.3 Hz, 2H), 4.01 (t, *J* = 6.2 Hz, 2H), 2.70 (s, 3H), 2.62 – 2.34 (m, 12H), 2.00 (p, *J* = 6.7 Hz, 2H), 1.90 (p, *J* = 6.5 Hz, 2H), 1.45 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 155.8, 155.0, 145.0, 138.5, 136.3, 131.3, 130.9, 130.7, 129.5 (2C), 128.9 (2C), 128.5, 127.7 (2C), 127.6, 127.5, 126.7, 125.8 (2C), 120.8, 112.5, 112.4, 110.1, 104.7, 104.2, 66.9, 66.7, 59.3, 55.4, 55.2, 53.2 (2C), 53.1 (2C), 46.6, 26.9, 26.6, 14.6, 12.0; HRMS calcd for $C_{41}H_{47}N_3O_4$ 646.3645 $[M+H]^+$, found 646.3644 $[M+H]^+$.

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)ethyl)piperazin-1-yl)ethoxy)-1-benzyl-2-methylindole-3-carboxylate (8). Mp 87 – 88 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 2.3 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.36 (t, *J* = 7.7 Hz, 2H), 7.32 – 7.15 (m, 6H), 7.06 (d, *J* = 8.8 Hz, 1H), 7.00 (t, *J* = 7.7 Hz, 1H), 6.97 – 6.88 (m, 3H), 6.81 (d, *J* = 9.0 Hz, 1H), 5.22 (s, 2H), 4.39 (q, *J* = 7.1 Hz, 2H), 4.17 (t, *J* = 5.8 Hz, 2H), 4.06 (t, *J* = 5.8 Hz, 2H), 2.82 (t, *J* = 5.8 Hz, 2H), 2.71 (t, *J* = 5.7 Hz, 2H), 2.66 (s, 3H), 2.64 – 2.46 (m, 8H), 1.43 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 155.7, 154.8, 145.1, 138.5, 136.3, 131.4, 131.1, 130.8, 129.6 (2C), 128.9 (2C), 128.5, 127.8 (2C), 127.6, 127.5, 126.8, 125.8 (2C), 121.1, 112.7, 112.5, 110.2, 104.9, 104.3, 66.8, 66.4, 59.4, 57.4, 57.0, 53.6 (2C), 53.5 (2C), 46.5, 14.7, 12.0; HRMS calcd for $C_{39}H_{43}N_3O_4$ 618.3332 $[M+H]^+$, found 618.3337 $[M+H]^+$.

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)propyl)piperazin-1-yl)ethoxy)-1-benzyl-2-methylindole-3-carboxylate (9). Mp 102 – 103 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (d, *J* = 2.5 Hz, 1H), 7.58 – 7.48 (m, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.35 – 7.20 (m, 6H), 7.11 (d, *J* = 8.8 Hz, 1H), 6.99 (td, *J* = 18.3, 7.4 Hz, 4H), 6.83 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.32 (s, 2H), 4.41 (q, *J* = 7.1 Hz, 2H), 4.19 (t, *J* = 5.8 Hz, 2H), 4.01 (t, *J* = 6.1 Hz, 2H), 2.85 (t, *J* = 5.8 Hz, 2H), 2.70 (s, 3H), 2.63 (bs, 4H), 2.56 – 2.35 (m, 6H), 1.90 (p, *J* = 6.5 Hz, 2H), 1.45 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$)

δ 166.1, 155.8, 154.9, 145.2, 138.5, 136.4, 131.5, 131.0, 130.8, 129.6 (2C), 128.9 (2C), 128.5, 127.8 (2C), 127.6, 127.5, 126.8, 125.9 (2C), 120.9, 112.6, 112.5, 110.2, 104.9, 104.4, 66.7, 66.4, 59.4, 57.4, 55.3, 53.5 (2C), 53.1 (2C), 46.6, 26.6, 14.7, 12.1; HRMS calcd for $C_{40}H_{45}N_3O_4$ 632.3488 $[M+H]^+$, found 632.3481 $[M+H]^+$.

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)ethyl)piperazin-1-yl)propoxy)-1-benzyl-2-methylindole-3-carboxylate (10). Mp 96 – 98 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, *J* = 2.4 Hz, 1H), 7.57 – 7.51 (m, 2H), 7.38 (t, *J* = 7.6 Hz, 2H), 7.35 – 7.21 (m, 6H), 7.10 (d, *J* = 8.8 Hz, 1H), 7.03 (t, *J* = 7.5 Hz, 1H), 6.99 – 6.93 (m, 3H), 6.81 (dd, *J* = 8.9, 2.5 Hz, 1H), 5.31 (s, 2H), 4.91 – 4.52 (m, 4H), 4.40 (q, *J* = 7.1 Hz, 2H), 4.09 (q, *J* = 5.6 Hz, 4H), 2.72 (t, *J* = 5.8 Hz, 2H), 2.70 (s, 3H), 2.62 – 2.36 (m, 2H), 1.99 (p, *J* = 6.5 Hz, 2H), 1.77 (s, 4H), 1.45 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 155.6, 155.0, 145.0, 138.4, 136.3, 131.3, 131.1, 130.8, 129.6 (2C), 128.8 (2C), 128.5, 127.7 (2C), 127.6, 127.5, 126.7, 125.8 (2C), 121.0, 112.6, 112.4, 110.1, 104.7, 104.2, 66.8, 66.7, 59.4, 57.0, 55.3, 53.5 (2C), 53.1 (2C), 46.6, 26.9, 14.6, 12.0; HRMS calcd for $C_{40}H_{45}N_3O_4$ 632.3488 $[M+H]^+$, found 632.3486 $[M+H]^+$.

Ethyl 1-benzyl-5-(3-(4-(3-(4-fluorophenoxy)propyl)piperazin-1-yl)propoxy)-2-methyl-1*H*-indole-3-carboxylate (11). Mp 98 – 99 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 2.5 Hz, 1H), 7.35 – 7.22 (m, 3H), 7.13 (d, *J* = 8.9 Hz, 1H), 7.04 – 6.93 (m, 4H), 6.89 – 6.78 (m, 3H), 5.34 (s, 2H), 4.43 (q, *J* = 7.1 Hz, 2H), 4.12 (t, *J* = 6.3 Hz, 2H), 3.99 (t, *J* = 6.3 Hz, 2H), 2.72 (s, 2H), 2.65 – 2.47 (m, 12H), 2.10 – 1.93 (m, 4H), 1.48 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.2, 158.3, 155.5 (d, *J* = 87.1 Hz), 155.1, 145.1, 136.4, 131.4, 128.9 (2C), 127.6, 127.5, 125.9 (2C), 115.7 (d, *J* = 23.1 Hz, 2C), 115.4 (d, *J* = 7.9 Hz, 2C), 112.4, 110.2, 104.7, 104.3, 66.9, 66.9, 59.4, 55.4, 55.2 (2C), 53.3 (2C), 46.7, 27.0, 26.8, 14.7, 12.1; ^{19}F NMR (376 MHz, $CDCl_3$) δ -124.25 – -124.32 (m); HRMS: calcd for $C_{35}H_{42}FN_3O_4$ 588.3238 $[M+H]^+$, found 588.3232 $[M+H]^+$.

Ethyl 1-benzyl-5-(2-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)ethoxy)-2-methyl-1*H*-indole-3-carboxylate (12). Mp 70 – 71 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, *J* = 7.7 Hz, 2H), 7.70 (d, *J* = 2.5 Hz, 1H), 7.54 (t, *J* = 7.7 Hz, 2H), 7.50 – 7.43 (m, 1H), 7.34 – 7.19 (m,

4H), 7.14 – 7.06 (m, 2H), 7.00 – 6.90 (m, 3H), 6.81 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.29 (s, 2H), 4.38 (q, *J* = 7.1 Hz, 2H), 4.17 (t, *J* = 5.0 Hz, 2H), 3.88 (s, 2H), 3.76 (s, 3H), 3.05 (t, *J* = 5.0 Hz, 2H), 2.67 (s, 3H), 1.94 (bs, 1H, NH), 1.43 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.0, 160.0, 154.8, 150.3, 145.2, 141.3, 140.4, 136.3, 133.3, 131.5, 129.6 (2C), 129.1, 128.9 (2C), 127.6, 127.5, 125.8 (2C), 122.1 (2C), 121.2, 120.3, 112.6, 112.3, 110.2, 104.8, 104.3, 67.7, 59.4, 55.9, 53.2, 48.3, 46.6, 14.6, 12.0; HRMS calcd for $C_{36}H_{37}N_6O_5$ 633.2825 $[M+H]^+$, found 633.2819 $[M+H]^+$.

Ethyl 1-benzyl-5-(3-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (13). Mp 80 – 81 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.3 Hz, 2H), 7.69 (d, *J* = 2.5 Hz, 1H), 7.53 (t, *J* = 7.7 Hz, 2H), 7.49 – 7.42 (m, 1H), 7.23 (t, *J* = 8.3 Hz, 4H), 7.12 – 7.02 (m, 2H), 6.93 (d, *J* = 7.8 Hz, 3H), 6.78 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.27 (s, 2H), 4.37 (q, *J* = 7.1 Hz, 2H), 4.13 (t, *J* = 6.0 Hz, 2H), 3.81 (s, 2H), 3.71 (s, 3H), 2.86 (t, *J* = 6.8 Hz, 2H), 2.67 (s, 3H), 2.02 (p, *J* = 6.4 Hz, 2H), 1.78 (bs, 1H, NH), 1.42 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 160.0, 154.9, 150.2, 145.1, 141.2, 140.6, 136.3, 133.3, 131.4, 129.6 (2C), 129.1, 128.8 (2C), 127.6, 127.5, 125.8 (2C), 122.1 (2C), 121.1, 120.3, 112.6, 112.3, 110.2, 104.6, 104.2, 66.9, 59.3, 55.9, 53.6, 46.6, 46.6, 29.7, 14.6, 12.0; HRMS calcd for $C_{37}H_{38}N_6O_5$ 647.2982 $[M+H]^+$, found 647.2987 $[M+H]^+$.

Ethyl 1-benzyl-5-(4-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)butoxy)-2-methyl-1*H*-indole-3-carboxylate (14). Mp 96 – 98 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 – 7.74 (m, 2H), 7.62 (d, *J* = 2.5 Hz, 1H), 7.52 – 7.46 (m, 2H), 7.44 – 7.37 (m, 1H), 7.24 – 7.13 (m, 4H), 7.09 – 6.97 (m, 2H), 6.91 – 6.85 (m, 3H), 6.73 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.24 (s, 2H), 4.33 (q, *J* = 7.1 Hz, 2H), 4.00 (t, *J* = 6.3 Hz, 2H), 3.75 (s, 2H), 3.71 (s, 3H), 2.66 (t, *J* = 7.1 Hz, 2H), 2.62 (s, 3H), 1.81 (p, *J* = 6.5 Hz, 2H), 1.66 (p, *J* = 7.1 Hz, 2H), 1.46 (bs, 1H, NH), 1.37 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3, 160.2, 155.2, 150.4, 145.2, 141.4, 140.9, 136.5, 133.5, 131.5, 129.7 (2C), 129.3, 129.0 (2C), 127.7, 127.7, 126.0 (2C), 122.3 (2C), 121.3, 120.4, 112.8, 112.6, 110.3, 104.7, 104.4, 68.4, 59.5, 56.1, 53.8, 49.3, 46.8, 27.4, 26.9, 14.8, 12.2; HRMS: calcd for $C_{38}H_{40}N_6O_5$ 661.3138 $[M+H]^+$, found 661.3135 $[M+H]^+$.

Ethyl 1-benzyl-2-methyl-5-(3-(((1-phenyl-1*H*-tetrazol-5-yl)methyl)amino)propoxy)-1*H*-indole-3-carboxylate (15). Mp 79 – 80 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 2.3 Hz, 1H), 7.64 – 7.57 (m, 2H), 7.54 – 7.45 (m, 3H), 7.32 – 7.19 (m, 2H), 7.10 (d, *J* = 8.8 Hz, 1H), 6.96 (d, *J* = 6.9 Hz, 2H), 6.78 (d, *J* = 8.8 Hz, 1H), 5.31 (s, 2H), 4.39 (q, *J* = 7.2 Hz, 2H), 4.16 – 4.00 (m, 4H), 2.89 (t, *J* = 6.8 Hz, 2H), 2.70 (s, 3H), 1.97 (p, *J* = 6.4 Hz, 2H), 1.72 (bs, 1H, NH), 1.43 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 154.9, 153.5, 145.2, 136.4, 133.9, 131.4, 130.3, 129.8 (2C), 128.9 (2C), 127.7, 127.6, 125.9 (2C), 124.5 (2C), 112.3, 110.3, 104.7, 104.3, 66.3, 59.4, 46.7, 46.6, 42.2, 29.6, 14.7, 12.1; HRMS: calcd for $C_{30}H_{32}N_6O_3$ 525.2614 $[M+H]^+$, found 525.2615 $[M+H]^+$.

Ethyl 1-benzyl-5-(3-((4-fluoro-3-methoxybenzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (16). Colourless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 2.5 Hz, 1H), 7.35 – 7.25 (m, 3H), 7.13 (d, *J* = 8.8 Hz, 1H), 7.07 – 6.96 (m, 4H), 6.89 – 6.84 (m, 1H), 6.82 (dd, *J* = 8.9, 2.5 Hz, 1H), 5.35 (s, 2H), 4.43 (q, *J* = 7.1 Hz, 2H), 4.17 (t, *J* = 6.1 Hz, 2H), 3.87 (s, 3H), 3.80 (s, 2H), 2.88 (t, *J* = 6.8 Hz, 2H), 2.73 (s, 3H), 2.06 (p, *J* = 6.5 Hz, 2H), 1.67 (bs, 1H, NH), 1.47 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3, 155.1, 151.6 (d, *J* = 244.1 Hz), 147.6 (d, *J* = 10.8 Hz), 145.2, 136.9 (d, *J* = 3.7 Hz), 136.5, 131.5, 129.1 (2C), 127.8, 127.7, 126.0 (2C), 120.3 (d, *J* = 6.8 Hz), 115.7 (d, *J* = 18.3 Hz), 113.2 (d, *J* = 2.0 Hz), 112.5, 110.3, 104.8, 104.4, 67.1, 59.6, 56.3, 53.8, 46.8 (2C), 29.9, 14.8, 12.2; ^{19}F NMR (376 MHz, $CDCl_3$) δ -138.21 (ddd, *J* = 12.0, 8.2, 4.3 Hz); HRMS: calcd for $C_{30}H_{33}FN_2O_4$ 505.2503 $[M+H]^+$, found 505.2498 $[M+H]^+$.

Ethyl 1-benzyl-5-(2-hydroxy-3-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (17). Mp 110 – 112 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.83 (m, 2H), 7.71 (d, *J* = 2.5 Hz, 1H), 7.59 – 7.53 (m, 2H), 7.51 – 7.44 (m, 1H), 7.32 – 7.20 (m, 4H), 7.10 (d, *J* = 8.9 Hz, 1H), 7.07 (d, *J* = 1.9 Hz, 1H), 6.99 – 6.91 (m, 3H), 6.83 (dd, *J* = 8.8, 2.5 Hz, 1H), 5.29 (s, 2H), 4.39 (q, *J* = 7.1 Hz, 2H), 4.20 – 4.04 (m, 3H), 3.85 (d, *J* = 2.5 Hz, 2H), 3.77 (s, 3H), 2.91 (dd, *J* = 12.1, 3.9 Hz, 1H), 2.84 (dd, *J* = 12.1, 7.5 Hz, 1H), 2.68 (s, 3H), 1.44 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 160.1, 154.7, 150.3, 145.3, 141.4, 140.4, 136.3, 133.3, 131.6, 129.6 (2C), 129.2, 128.9 (2C), 127.7, 127.6, 125.9 (2C),

122.1 (2C), 121.3, 120.4, 112.7, 112.3, 110.3, 105.0, 104.3, 71.2, 68.8, 59.5, 56.0, 53.5, 51.5, 46.6, 14.7, 12.1; HRMS: calcd for $C_{37}H_{38}N_6O_6$ 663.2931 $[M+H]^+$, found 663.2928 $[M+H]^+$.

2-(2-Bromoethoxy)-1,1'-biphenyl (18a). Mp 79 – 80°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.59 (m, 2H), 7.49 – 7.30 (m, 5H), 7.15 – 7.05 (m, 1H), 6.97 (d, *J* = 8.1 Hz, 1H), 4.25 (t, *J* = 6.2 Hz, 2H), 3.54 (t, *J* = 6.2 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.0, 138.1, 131.3, 131.1, 129.7 (2C), 128.6, 128.0 (2C), 127.0, 121.9, 113.2, 68.5, 29.3. HRMS: calcd for $C_{14}H_{13}{}^{79}BrO$ 277.0228 $[M + H]^+$, found 277.0238 $[M + H]^+$.

2-(3-Bromopropoxy)-1,1'-biphenyl (18b). Mp 129 – 131 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, *J* = 7.8 Hz, 2H), 7.45 (t, *J* = 7.6 Hz, 2H), 7.37 (t, *J* = 7.9 Hz, 3H), 7.14 – 7.02 (m, 2H), 4.15 (t, *J* = 5.7 Hz, 2H), 3.51 (t, *J* = 6.4 Hz, 2H), 2.26 (p, *J* = 5.9 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.5, 138.5, 131.2, 131.0, 129.5 (2C), 128.7, 127.9 (2C), 126.9, 121.3, 112.7, 65.7, 32.3, 30.4; HRMS: calcd for $C_{15}H_{15}{}^{79}BrO$ 290.0306 $[M + H]^+$, found 291.0365 $[M + H]^+$.

1-(2-[(1,1'-Biphenyl)-2-yloxy)ethyl]piperazine (19a). Mp 75-77 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 – 7.50 (m, 2H), 7.45 – 7.35 (m, 1H), 7.35 – 7.23 (m, 4H), 7.03 (td, *J* = 7.5, 1.1 Hz, 1H), 7.00 – 6.94 (m, 1H), 4.09 (t, *J* = 5.7 Hz, 2H), 2.84 (t, *J* = 5.0 Hz, 4H), 2.70 (t, *J* = 5.7 Hz, 2H), 2.42 (bs, 4H), 1.53 (bs, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.7, 138.4, 131.1, 130.8, 129.6 (2C), 128.5, 127.7 (2C), 126.7, 121.0, 112.6, 66.7, 57.6, 54.9 (2C), 46.1 (2C); HRMS: calcd $C_{18}H_{22}N_2O$ 283.1810 $[M+H]^+$, found 283.1822 $[M+H]^+$.

1-(3-[(1,1'-Biphenyl)-2-yloxy)propyl]piperazine (19b). Mp 166 – 167 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (t, *J* = 6.9 Hz, 2H), 7.31 (d, *J* = 7.6 Hz, 2H), 7.28 – 7.17 (m, 3H), 6.95 (td, *J* = 7.5, 2.6 Hz, 1H), 6.90 (dd, *J* = 8.3, 3.5 Hz, 1H), 5.68 (s, 1H), 3.92 (q, *J* = 6.1 Hz, 2H), 3.00 (t, *J* = 4.9 Hz, 2H), 2.55 – 2.45 (m, 3H), 2.39 (q, *J* = 8.2, 7.6 Hz, 3H), 1.92 – 1.72 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.8, 138.6, 131.0, 130.8, 129.6 (2C), 128.6, 127.8 (2C), 126.9, 121.1, 112.8, 66.3, 54.8, 53.1, 50.7 (2C), 44.1 (2C); HRMS: calcd for $C_{19}H_{24}N_2O$ 297.1967 $[M+H]^+$, found 297.1950 $[M+H]^+$.

3-Methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzaldehyde (21). *t*-BuOK (3 g, 26.6 mmol) was added to a solution of vanillin (3.4 g, 22.1 mmol) in dry DMF (50 ml) under nitrogen atmosphere.

Upon complete dissolution of the base, 5-chloro-1-phenyltetrazole (4 g, 22.1 mmol) was added and the mixture was stirred at room temperature for 18 h. The reaction mixture was poured into ice water and the formed precipitate was filtered off under vacuum, washed with H_2O and dried under vacuum obtaining 4.8 g of a white solid (73% yield): mp 126 – 128 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.01 (s, 1H), 7.91 – 7.83 (m, 2H), 7.66 – 7.51 (m, 6H), 3.90 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.6, 159.2, 151.1, 146.7, 135.7, 133.0, 129.7 (2C), 129.4, 124.8, 122.1 (2C), 121.9, 111.5, 56.3; HRMS: calcd $C_{15}H_{12}N_4O_3$ 297.0988 $[M+H]^+$, found 297.0983 $[M+H]^+$.

(3-Methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)phenyl)methanamine (22). Mp 97 – 98 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 – 7.71 (m, 2H), 7.45 – 7.33 (m, 3H), 7.16 – 7.14 (m, 1H), 6.95 (s, 1H), 6.82 – 6.80 (m, 1H), 3.73 (s, 2H), 3.65 (s, 3H), 2.25 (bs, 2H, NH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.1, 150.3, 143.0, 141.1, 133.2, 129.7 (2C), 129.3, 122.1 (2C), 121.3, 119,4, 112.0, 56.0, 46.0; HRMS: calcd $C_{15}H_{15}N_5O_2$ 298.1304 $[M + H]^+$, found 298.1297 $[M + H]^+$.

1-(3-Chloropropoxy)-4-fluorobenzene. 1-Bromo-3-chloropropane (176 mmol) was added to a stirred mixture of 4-fluorophenol (59 mmol) and K_2CO_3 (8.1 g, 59 mmol) in ethanol (100 ml) and refluxed for 6 h. After cooling, precipitated salt was filtered off and the filtrate was concentrated under reduced pressure. The residue was diluted with H_2O and extracted with AcOEt. The combined AcOEt layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated under vacuum and purified by column chromatography on silica gel (eluent PE/AcOEt 95:5) to give a pale yellow oil (1.2 g , 71% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (t, *J* = 8.7 Hz, 2H), 6.86 – 6.78 (m, 2H), 4.04 (t, *J* = 5.9 Hz, 2H), 3.71 (t, *J* = 6.2 Hz, 2H), 2.19 (p, *J* = 6.1 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.5, 155.4 (d, *J* = 129.0 Hz), 115.7 (d, *J* = 23.1 Hz, 2C), 115.5 (d, *J* = 8.0 Hz, 2C), 64.9, 41.4, 32.2; ^{19}F NMR (376 MHz, DMSO-d_6) δ -124.25; HRMS: calcd $C_9H_{10}ClFO$ 189.0482 $[M+H]^+$, found 189.0486 $[M+H]^+$.

Chloroacetanilide.[4] A solution of chloroacetyl chloride (3.1 ml, 38.6 mmol) in dry DMF (10 ml) was added dropwise to a solution of aniline (2.9 ml, 32 mmol) and Et_3N (4.4 ml, 32 mmol) in DMF (25 ml) cooled at 0 °C. The reaction mixture was stirred overnight under nitrogen atmosphere; the

mixture was poured into ice water and the formed precipitate was filtered off and dried to obtain a white solid (5.1 g, 94% yield): mp 86 – 88 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (s, 1H), 7.65 – 7.50 (m, 2H), 7.45 – 7.31 (m, 2H), 7.16 (ddt, *J* = 7.7, 7.0, 1.2 Hz, 1H), 4.17 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.9, 136.7, 129.1 (2C), 125.3, 120.2 (2C), 42.9; HRMS: calcd C_8H_8ClNO 170.0373 $[M+H]^+$, found 170.0379 $[M+H]^+$.

5-(Chloromethyl)-1-phenyl-1*H*-tetrazole. PCl_5 was added slowly to a solution of chloroacetanilide (4 g, 23.6 mmol) in dry benzene (26 ml) cooled to 0 °C. The mixture was stirred under nitrogen atmosphere at room temperature for 2 h, NaN_3 was added and after 30 min, H_2O was also added. The reaction mixture was refluxed for 5 h and, after cooling, was diluted with H_2O and extracted with CH_2Cl_2. The combined organic layers were washed with 5% NaOH aqueous solution and brine. After drying over anhydrous Na_2SO_4, the solvent was evaporated under reduce pressure and the residue was purified by column chromatography on Silica gel eluting with PET/AcOEt 7:3 to give a pale orange solid (2.7 g, 59% yield): mp 76 – 77 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 – 7.51 (m, 5H), 4.87 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 151.6, 133.0, 131.0, 130.1 (2C), 124.6 (2C), 31.3; HRMS: calcd $C_8H_7ClN_4$ 195.0437 $[M+H]^+$, found 195.0433 $[M+H]^+$.

Computational methods

A ligand-based virtual screening (LBVS) with FLAP[5,6] was performed to search for novel building blocks for NorA inhibitors. The SPECS database (SPECS_SC_20mg_total_Dec2009.sdf) containing 166839 compounds was used to perform a pre-filtering virtual screening run using compound **2** as a template, fixing the amino group as a HBD constraint. A total of 25 conformations for each SPECS compound were generated. Pre-filtering results were ranked according to the Glob-Sum similarity score and the 44 compounds showing a Glob-Sum> 0.2 were used to generate a FLAP database, in which each compound in different conformations and protonation states at pH 7.4 (evaluated using the MoKa algorithm)[7,8] is described in terms of GRID Molecular Interaction Fields (MIFs). A number of 50 conformations were used for database creation, and the GRID probes for hydrophobic (DRY),

hydrogen bond donor (N1) hydrogen bond acceptor (O) interactions and for shape (H) were used for MIFs generation. The template **2** was also described using the same settings, and was used in its protonated form, being the most abundant at pH 7.4. Thus, a second LBVS based on the GRID MIFs similarity was performed. Results were ranked by the Glob-Prod similarity score, and the FLAP poses for compounds with Glob-Prod >0.4 were visually inspected. Selection of the most promising building blocks was made according to Glob-Prod score, availability and cost.

Metabolism evaluation.

As previously reported,[1] substrates were incubated with human liver microsomes (HLM, 0.5 mg protein/ml) (BD Biosciences) according to the manufacturer's recommendations with minor modifications. Briefly, substrates at 10 µM final concentration were incubated in a shaking water bath for 5 minutes at 37 °C in 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 250 µl. The reactions were initiated by addition of 1 mM NADPH. After incubation for 0 and 30 minutes, 250 µl of cold acetonitrile (containing 0.6 mM labetalol as internal standard) was added to terminate the reaction. Proteins were precipitated by centrifugation at 12000 g for 5 min at 4 °C, and aliquots of supernatants were analyzed by HPLC-MS/MS. The LC/MS analyses were run on a Agilent 6540 UHD accurate mass Q-TOF LC/MSMS system (Agilent Technologies, Palo Alto, CA) governed by Agilent MassHunter software (B.05.00 version). The system consists of a binary pump, autosampler, thermostatic column compartment, DAD detector, source, and Q-TOF spectrometer. Chromatographic separation of the metabolites (Agilent 1290 UHPLC system) was performed with Aeris Peptide 1.7 JXB-C18, 100 x 2.1 mm (Phenomenex USA) at a constant temperature of 40 °C. The mobile phases consisted of A: H_2O/0.1% formic acid and B: acetonitrile/0.1% formic acid at the flow of 0.3 mL/min with the following gradient: Time 0 min, B 0%; Time 10 min, B 100%. The DAD Detector stored all the acquired spectra in the 10-640 nm range (2 nm spectrum step). The ion source was an Agilent Dual JetStream operating under positive ionization mode (4000 V), with nitrogen the desolvating gas (320 °C, 10 L/min, 35 psig). The fragmentor was set to 110 V, the skimmer to 65 V,

and the octrapole RF to 750 V. The spectrometric data were collected in All Ion mode in the 100-1000 mass range, with 3 scans/sec at Collision Energy of 0, 30, 40 V. The TOF operated at 2 GHz.

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