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Synthesis and synergistic antimycobacterial screening of chlorpromazine and its metabolites

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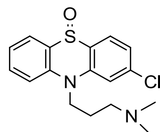
Experimental

General

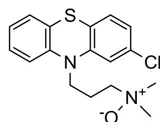
All the chemicals and solvents including 7-hydroxychlorpromazine **(M2)** were purchased from Sigma Aldrich (SA) or Merck (SA). 25-Desacetylrifampicin was purchased from American Custom Chemicals Corporation (San Diego, USA). Reactions were monitored by thin layer chromatography (TLC) using Merck TLC silica gel 60 F_{254} aluminium-backed pre-coated plates and were visualized by ultraviolet light at 254 nm. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Varian Unity XR400 MHz (^{1}H at 400 MHz, ^{13}C at 100 MHz), or a Bruker Ultrashield 400 Plus spectrometer (^{1}H at 400 MHz, ^{13}C at 101 MHz). Chemical shifts for ^{1}H and ^{13}C NMR were reported using tetramethylsilane (TMS) as the internal standard. NMR data is presented as chemical shift in ppm on the δ scale relative to $^{\delta}$TMS = 0, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet), coupling constant (*J*/ Hz). High Performance Liquid Chromatography (HPLC) for purity checks and preparative scale isolations was done using a Waters HPLC 2727 instrument with a Waters 2424 PDA detector and a Waters X-bridge C18 column (19mm x 150mm x 5µM, analytical or 19mm x 250mm x 5µM, preparative). 10mM Ammonium acetate in water and 10mM ammonium acetate in methanol were used as eluents. An Agilent 1200 Rapid Resolution HPLC system coupled to an AB SCIEX 4000 QTRAP® mass spectrophotometer was used for LC/MS analysis. A Kinetex® C_{18} HPLC column (150mm x 2.1mm, packed with 2.6µM fused core particles) was used for all LC-MS analysis using 5mM ammnonium formate in water and acetonitrile as mobile phase.

CPZ sulfoxide (M1)



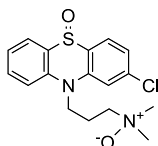
The target compound (0.2g, 10%) was obtained as a side product in the reaction that led to the formation of chlorpromazine-*N*-oxide. On thin layer chromatography (normal phase silica gel) and column chromatography, it eluted after the *N*-oxide. The structure was confirmed using NMR and LC/MS data (Fig 8). R_f 0.3 (15% MeOH-DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (m, 1H, Ar*H*), 7.89 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.63 (m, 1H, Ar*H*), 7.54 (m, 2H, Ar*H*), 7.30 (m, 1H, Ar*H*), 7.24 (dd, *J* = 8.2 and 1.8 Hz, 1H, Ar*H*), 4.42 (m, 2H, C*H*$_2$N), 2.75 (t, *J* = 7.2 Hz, 2H, C*H*$_2$N(CH_3)$_2$), 2.46 (s, 6H, N(C*H*$_3$)$_2$), 2.25 (m, 2H, CH_2C*H*$_2$$CH_2$); ^{13}C NMR (100M Hz, $CDCl_3$) δ 133.1, 132.4, 131.2, 122.7, 122.3, 116.3, 55.5, 45.4, 44.2 and 23.9; ESI: *m/z* 335 $[M+H]^+$.

Chlorpromazine-*N*-oxide (M3)



m-Chloroperbenzoic acid (1.5g, 0.0084 mol) was added portion wise at 0 °C to a solution of CPZ (2g, 0.0056 mol) in dichloromethane (50 mL) and 4N NaOH solution (400 µl) and the reaction mixture was stirred for 4h at 0 °C. The solvent was evaporated *in vacuo* before setting the pH of reaction at 10 using 4N NaOH solution and extracted with dichloromethane (3 × 15 mL). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification through column chromatography afforded target compound as oil (0.78g, 40%); R_f 0.35 (15% MeOH-DCM); 1H NMR (400 MHz, $CDCl_3$) δ 7.17 (m, 2H, Ar*H*), 7.04 (d, *J* = 8.20 Hz, 1H, Ar*H*), 6.92 (m, 4H, Ar*H*), 4.02 (t, *J* = 6.30 Hz, 2H, C*H*$_2$N), 3.34 (t, *J* = 7.52 Hz, 2H, C*H*$_2$N(CH_3)$_2$), 3.05 (s, 6H, N(C*H*$_3$)$_2$), 2.36 (m, 2H, CH_2C*H*$_2$$CH_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 128.2, 127.8, 127.7, 123.5, 122.9, 116.3, 68.4, 58.8, 44.9 and 21.3; ESI: *m/z* 335 $[M+H]^+$.

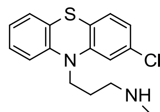
Chlorpromazine-*N*-*S*-dioxide (M4)



Chlorpromazine sulfoxide **(M1)** (0.4g, 1.1 mol) was oxidized using *m*-Chloroperbenzoic acid (0.3g, 1.7 mol) in a similar

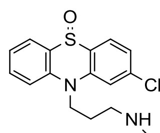
manner as described for Chlorpromazine-*N*-oxide **(M3)** yielding chlorpromazine-*N*-*S*-dioxide **(M4)** as white crystalline solid (0.24g, 65%); R_f 0.2 (20% MeOH-DCM); M. pt. 105-107^{0}C; 1H NMR (400MHz, $CDCl_3$) δ 7.88 (m, 2H, Ar*H*), 7.60 (d, *J* = 8.20 Hz, 1H, Ar*H*), 7.28 (m, 4H, Ar*H*), 4.56 (m, 2H, CH_2N), 3.30 (t, *J* = 7.6 Hz, 2H, CH_2N(CH_3)$_2$), 3.01 (s, 6H, N(CH_3)$_2$), 2.25 (m, 2H, CH_2C$H_2$$CH_2$); ^{13}C NMR (100MHz, $CDCl_3$) δ 133.1, 131.9, 130.7, 122.9, 122.6, 117.2, 67.0, 59.2, 44.3 and 21.8; ESI: *m/z* 351 $[M+H]^+$.

Nor-chlorpromazine (M5)



Solution of Chlorpromazine-*N*-oxide **(M3)** (0.6g 0.0018 mol) in methanol (10 mL) was cooled to 0 °C followed by an addition of a solution of ferrous sulphate (1g, 3.59 mol) in methanol (1 mL). The reaction was stirred at 0 °C for 3h. Solvent was removed under reduced pressure to obtain reddish brown solid, which was dissolved in 0.1M EDTA solution (1.86g in 50 mL H_2O) at pH 10 adjusted using NH_3 solutions. Extraction was carried out using dichloromethane (3 × 15 mL). Organic extracts were dried over anhydrous $MgSO_4$ and removed under reduced pressure. Purification through column chromatography afforded products as oil (65mg, 11%); R_f 0.3 (15% MeOH-DCM); ^{1}H NMR (400MHz, $CDCl_3$) δ 7.20 (m, 2H, Ar*H*), 7.09 (m, 1H, Ar*H*), 6.96 (m, 4H, Ar*H*), 4.03 (t, *J* = 6.5 Hz, 2H, CH_2N), 2.95 (t, *J* = 7.1 Hz, 2H, CH_2NCH_3), 2.51 (s, 3H, NCH_3), 2.21 (m, 2H, CH_2C$H_2$$CH_2$)]; ^{13}C NMR (100MHz, $CDCl_3$) δ 128.2, 127.8, 127.7, 123.4, 122.8, 116.1, 47.9, 44.9, 34.1 and 24.6; ESI: *m/z* 305 $[M+H]^+$.

Nor-chlorpromazine sulfoxide (M6)



m-Chloroperbenzoic acid (0.124g, 0.0007 mmol) was added portion wise at 0 °C to a solution of nor-chlorpromazine **(M5)** (0.2g, 0.0005 mmol) in dichloromethane (50 mL) and 4N NaOH solution (400 µl) and the reaction mixture was stirred for 4h at 0 °C. The solvent was evaporated *in vacuo* before setting the pH of reaction at 10 using 4N NaOH solution and extracted with dichloromethane (3 × 15 mL). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification through column chromatography afforded target compound as oil (46.7mg, 30%); R_f 0.2 (80% MeOH-DCM); ^{1}H NMR (400MHz, $CDCl_3$) δ 7.86 (m, 2H, Ar*H*), 7.57 (m, 1H, Ar*H*), 7.25 (m, 4H, Ar*H*), 4.35 (m, 2H, CH_2N), 2.79 (t, *J* = 6.8 Hz, 2H, CH_2NCH_3), 2.48 (s, 3H, NCH_3), 2.11 (m, 2H, CH_2C$H_2$$CH_2$)]; ^{13}CNMR (100MHz, $CDCl_3$) 133.2, 132.2, 131.0, 122.8, 122.4, 116.6, 47.3, 45.2, 34.6 and 24.7; ESI: *m/z* 321 $[M+H]^+$.

***In vitro* metabolite generation**

Human Liver Microsomes (HLM) and Rat Liver Microsomes (RLM)

Chlorpromazine (10 µM) was incubated at 37 °C with a solution of human (pooled human mixed gender, Xenotech) and rat(male rat IGS, Xenotech) liver microsomes and NADPH (1 mM) in phosphate buffer (100 mM, pH 7.4) containing magnesium chloride (5 mM) for 1 hour while shaking. An equal volume of ice cold acetonitrile was added to stop the reaction and to precipitate the proteins. After centrifuging the mixture at 14000 rpm for 30 minutes the supernatant was transferred to a HPLC vial. Control samples with no NADPH, no microsomes and a T0 sample were also included and processed in a similar way to the samples.

Structural characterization of metabolites

An agilent 1200 Rapid Resolution HPLC system coupled to an AB SCIEX 4000 QTRAP® mass spectrophotometer was used for LC/MS analysis. A kinetex C_{18} HPLC column (150mm x 2.1mm, packed with 2.6µM fused core particles) was used for all analysis with a mobile phase flow rate of 0.4ml/min and a column temperature of 40°C. The mobile phase used in all experiments consisted of 5mM ammnonium formate buffer. Mobile phase A was mainly aqueous consisting of 5% acetonitrile at pH 3 (pH adjusted using fomic acid) while mobile phase B consisted of 95% acetonitrile. Identification of the metabolites formed by all the systems was carried out using IDA-EMS experiments. Hybrid triple quadrupole-linear ion trap (QqQ_{LIT}) system (MS/MS) was used for comprehensive study of fragmentation mechanisms.
The retention times and fragmentation patterns of the synthesized metabolites were compared to those of the microsomal metabolites to confirm their identity. Extracted ion chromatograms of the human and rat microsomal incubations as well as the identities confirmed using the synthesized metabolites are show in figure S1 and S2 below.

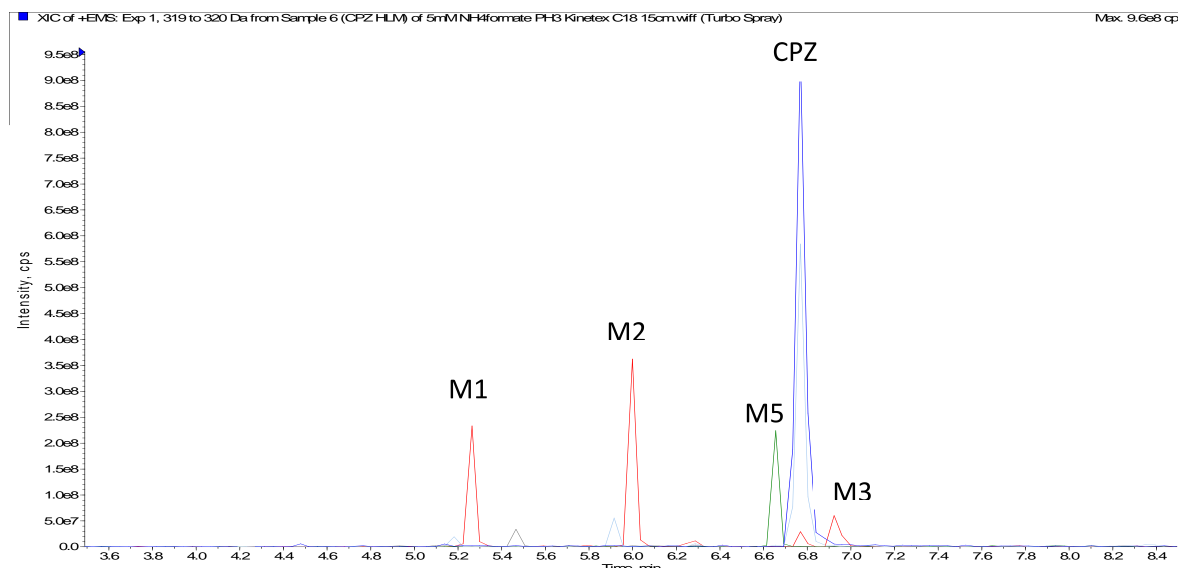


Figure S1: Extracted ion chromatogram of chlorpromazine and its metabolites from a human liver microsomal incubation of chlorpromazine.

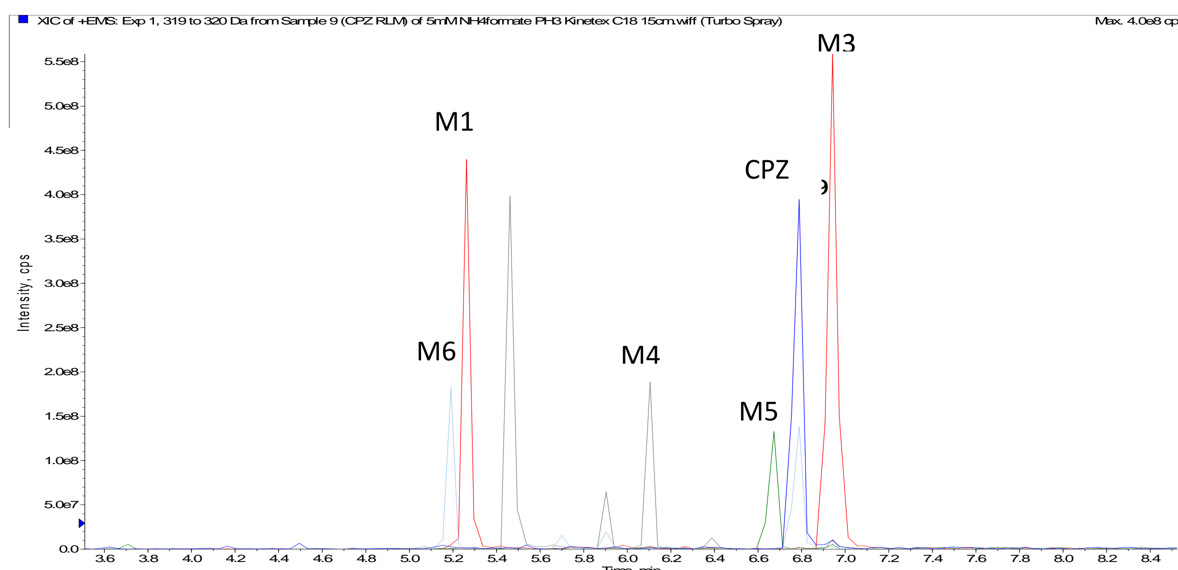


Figure S2: Extracted ion chromatogram of chlorpromazine and its metabolites from a rat liver microsomal incubation of chlorpromazine.

Antimycobacterial screening

Broth microdilution assay/mycobacterial alamar blue assay (MABA).

Testing for antimycobacterial activity was done using microdilution alamar blue assay (MABA) as described.[27] Briefly, a pre-culture of *M. smegmatis* (mc^2155) was prepared from a glycerol stock and grown for 16h followed by a subculture grown to an OD_{600} 0.6-0.8 (exponential phase) in filter-sterilized 7H9 media supplemented with 10% OADC, 0.2% glycerol and 0.25% Tween 80 (20% in H_2O). The media was added to 96 microtitre well plates followed by addition of the drugs which were then serially diluted. Finally the culture (diluted x 1000) was added to the wells. The controls included media and the solvent used to dissolve the drugs at a concentration corresponding to that of the working solutions of the drugs. Incubation was done at 37 °C with no shaking for 48h then resazurin dye was added to the plates. Further incubation was carried out for 24h in the same conditions.

Synergistic drug combinations for TB therapy-high-throughput screen

A two dimensional array of serial dilutions of two test compounds was prepared in 96 well plates and a mycobacterium culture was added to the wells (section above). Incubations were carried out under the same conditions as described (section above). Relative MIC concentrations in wells representing various ratios of the two compounds was then used for calculations to determine whether paired combinations exert inhibitory effects that are more than the sum of their effects alone (synergy). Cell viability was then determined and the fractional inhibitory concentration index (FICI) values which correspond to either compound (A or B) were defined as[6,24]:

MIC of A (combination)/MIC of A (singly) + MIC of B (combination)/MIC of B (singly)