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Graphical abstract

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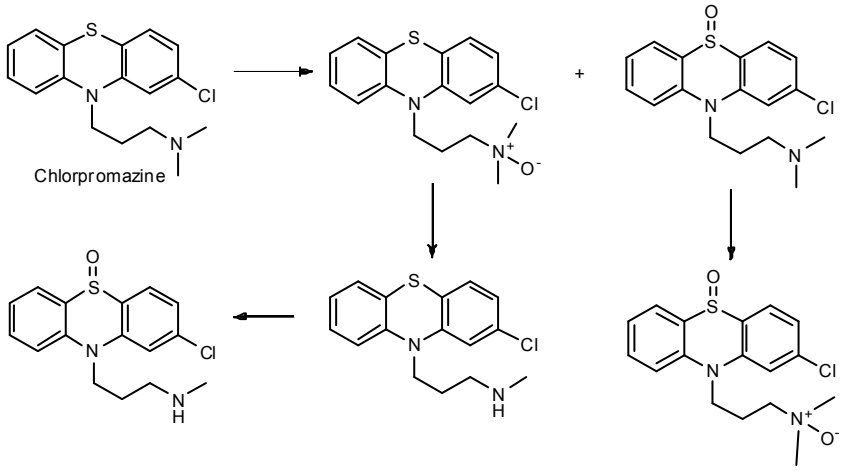

Chlorpromazine (CPZ) metabolites naturally generated *in vivo* were synthesized via a non-classical Polonovskii reaction. CPZ and the synthesized metabolites exhibited clear synergy when tested in combination with a number of antituberculosis drugs suggesting that these could be potential partners that could be used for anti-TB drug development.

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CONCISE ARTICLE

Synthesis and synergistic antimycobacterial screening of chlorpromazine and its metabolites

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Abstract

The antimycobacterial activities of chlorpromazine and its metabolites were evaluated alone and in combination with antitubercular drugs. Although associated with limited antimycobacterial activity when tested individually, chlorpromazine and its metabolites exhibited clear synergy when tested in combination with a number of aminoglycosides as well as the active metabolite of rifampicin, 25-desaceteylrifampicin. The combination of chlorpromazine and spectinomycin was associated with the greatest synergy, yielding a fractional inhibitory concentration index (FICI) of 0.31. Synergistic interactions were also observed for combinations of 7-hydroxychlorpromazine or nor-chlorpromazine with kanamycin, streptomycin, spectinomycin and 25-desacetylrifampicin (FICI 0.19 -0.5).

Introduction

Every year, approximately 9 million people develop active tuberculosis (TB), 30% of which reflect co-infection with HIV.[1–3] However, the number of new TB drugs that make it into the market is very low which is of great concern for a disease whose global incidence remains elevated, resulting in almost 1.4 million deaths per annum.[4] This problem is further compounded by the continued emergence of drug resistance, which severely limits the utility of existing drugs. Clinically, anti-TB drugs are administered in combination to maximize their efficacy and prevent resistance. However, most antimycobacterial drug discovery efforts are based on screening of single agents. An alternative and potentially more relevant strategy[5–8] is the use of synergistic screening (so-called "checkerboard assays"[6,9,10]) to investigate the activity of two agents in combination. Synergy is defined where the biological activity of a combination of two drugs against a given microorganism is greater than the sum of the individual activities of each member of that combination.[11] Practically, this is determined through the calculation of the fractional inhibitory concentration index (FICI).[6,10] A key goal of any drug discovery programme, is to synthesise metabolically stable analogues of a lead compound, and this applies equally to antimycobacterial drugs. However, it has been recognized that pharmacologically active metabolites have in some instances been successfully developed as drugs, which often possess superior physicochemical, pharmacodynamics and pharmacokinetic properties compared to the parent drugs.[12] Accordingly, we have become interested in studying the relative contribution of metabolites to antimycobacterial activity.

Phenothiazines have been used for many years in the clinical management of psychosis. However, they have also been reported to have *in vitro* antimycobacterial activity specifically inhibiting NADH:menaquinone oxidoreductase which is responsible for aerobic respiration.[13] Studies have demonstrated that thioridazine, a phenothiazine, has activity in mice and against multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis*.[14–20] Thioridazine in combination with several antibiotics causes synergy. The phenothiazine inhibits protein synthesis necessary for bacteria cell wall leading to death of the mycobacteria.[21] Chlorpromazine (CPZ), another phenothiazine, was selected as a proof-of-concept for this study. Previously, CPZ was reported to exhibit a 4-fold reduction of efflux pump activity in *M. avium*.[18] In another study, CPZ in combination with some anti-TB drugs was shown to exhibit synergism.[22] This makes CPZ a potential partner for combination studies with anti-TB drugs. The fast replicating non-pathogen *M. smegmatis* was used as a mycobacterial model in this study. This is consistent with other reports which have successfully applied *M. smegmatis* for the preliminary identification of hit compounds[23] as well as promising drug combinations.[6]

Because of the relevance of metabolites to the activity of known clinical compounds,[12] the synergistic combination screening was also performed with CPZ metabolites. CPZ is metabolized to chlorpromazine sulfoxide **(M1)**, 7-hydroxychlorpromazine **(M2)**, chlorpromazine-*N*-oxide **(M3)**, chlorpromazine-*N*-*S*-dioxide **(M4)**, nor-chlorpromazine **(M5)** and nor-chlorpromazine sulfoxide **(M6)** (Fig 1), among others.[24–26]

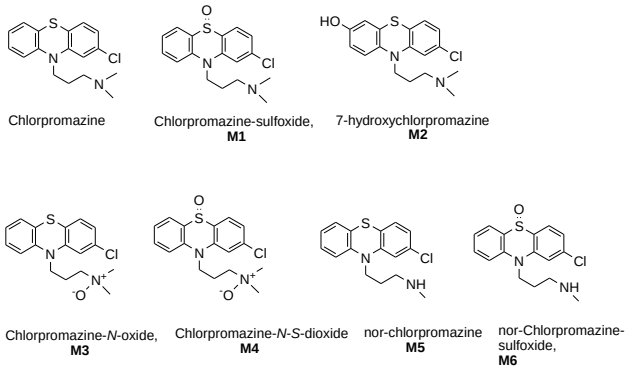
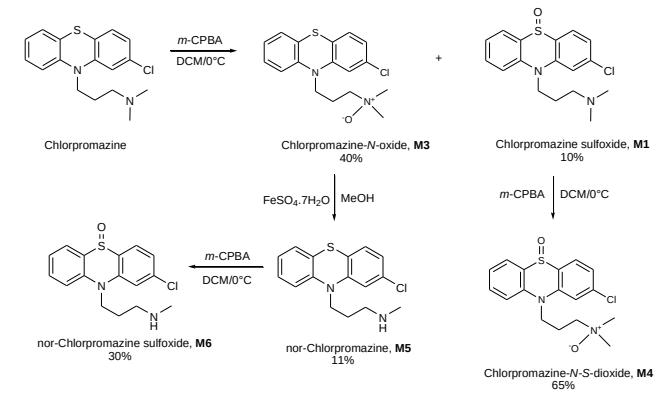


Fig 1. Chlorpromazine and its metabolites

Synthesis

CPZ- *N*-oxide **(M3),** CPZ sulfoxide **(M1),** nor-CPZ **(M5),** nor-CPZ sulfoxide **(M6)** and CPZ-*N*-*S*-dioxide **(M4)** were synthesized from CPZ via a non-classical Polonovski reaction (Scheme 1),[27] which involves oxidation of CPZ with *m*-chloroperbenzoic acid (*m*-CPBA) to afford *N*-oxide derivative **M3** as a major product and CPZ sulfoxide **(M1)** as a minor product. Subsequent demethylation of **M3** with ferrous sulphate in methanol yielded nor-CPZ **(M5)**. CPZ-*N*-*S*-dioxide **(M4)** was obtained by reacting **M1** with *m*-CPBA. Likewise, reaction of nor-CPZ **(M5)** with *m*-CPBA yielded nor-chlorpromazine sulfoxide **(M6)**. 7-hydroxyCPZ **(M2)** was purchased from Sigma Aldrich (SA).



Scheme 1: Synthesis of chlorpromazine metabolites

We confirmed the identities of the major CPZ metabolites by LC-MS analysis following exposure of CPZ to liver microsomes (See supplementary information).

For further investigation of the contribution of drug metabolites to the biological activity, we included 25-desacetylrifampicin in these experiments since it is the major active metabolite of rifampicin, a frontline TB drug.[28]

Results and discussion

Determination of MIC_{99} of Chlorpromazine and its metabolites

The minimum inhibitory concentrations (MIC_{99}) of CPZ and its metabolites are shown in table 1. The antimycobacterial activity of CPZ and its metabolites was generally low. Interestingly, the activity of CPZ metabolites, 7-hydroxyCPZ **(M2)** and nor-CPZ **(M5)** was comparable to that of chlorpromazine. CPZ-*N*-oxide **(M3)** has been reported to revert to CPZ in solution and this may contribute to some of its activity.[29] No antimycobacterial activity was observed for the other metabolites **(M1, M4, M6)** at the highest concentration tested.

Table 1: MIC_{99} of Chlorpromazine and its metabolites

Compound	MIC_{99} (μM)
CPZ	117.26
CPZ sulfoxide (**M1**)	>1990.89
7-hydroxyCPZ (**M2**)	124.44
CPZ-*N*-oxide (**M3**)	995.43
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10
nor-CPZ (**M5**)	136.70
nor-CPZ sulfoxide (**M6**)	>2077.89

Synergistic/Matrix screening

Table 2 and 3 summarize the MIC_{99} of the individual compounds, the lowest MIC_{99} achieved in the various combinations of CPZ and its metabolites with known anti-TB drugs, and the fractional inhibitory concentration indices (FICI). Synergy is assigned where the FICI $\leq$ 0.5; a FICI $\geq$ 4 is considered an antagonistic interaction, while any value falling in between is indicates no interaction.[7] Generally, combinations of CPZ with known anti-TB drugs exhibited improved activity against *M. smegmatis*.

A combination of CPZ with spectinomycin exhibited a synergistic effect with a FICI of 0.31. Combinations of 7-hydroxyCPZ **(M2)** resulted in synergistic effects with kanamycin and spectinomycin (FICI 0.50 and 0.19 respectively).

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Table 2: MIC_{99} & FICI of CPZ and its active metabolites in combination with anti-TB drugs, in *M. smegmatis*

Compound	MIC_{99} (μM) Singly	MIC_{99} (μM) combination	FICI	Compound	MIC_{99} (μM) Singly	MIC_{99} (μM) combination	FICI
Rifampicin	2.53	0.32	0.63	Spectinomycin	84.12	5.25	0.19
CPZ	117.27	58.62		7-hydroxyCPZ (**M2**)	124.44	15.56	
Rifampicin	1.26	0.63	0.50	Chlorpromazine	117.27	29.32	0.75
25-Desacetylrifampicin	2.66	1.33		7-hydroxyCPZ (**M2**)	124.44	62.21	
25-Desacetylrifampicin	5.33	1.33	0.50	Rifampicin	1.26	0.63	1.00
CPZ	117.27	29.32		CPZ-*N*-oxide (**M3**)	995.43	497.73	
Ethambutol	0.76	NC	-	25-Desacetylrifampicin	2.66	0.67	0.75
CPZ	117.27			CPZ-*N*-oxide (**M3**)	995.43	497.73	
Kanamycin	7.16	3.57	1.00	Ethambutol	0.76	IAE	-
CPZ	117.27	58.62		CPZ-*N*-oxide (**M3**)	995.43		
Streptomycin	0.07	0.02	0.79	Kanamycin	1.79	0.89	1.00
CPZ	58.62	29.32		CPZ-*N*-oxide (**M3**)	995.43	497.73	
Spectinomycin	84.12	5.25	0.31	Streptomycin	0.29	0.07	0.74
CPZ	117.27	29.32		CPZ-*N*-oxide(**M3**)	995.43	497.73	
TMC207	0.05	0.01	0.70	Spectinomycin	168.22	84.12	0.63
CPZ	117.27	58.62		CPZ-*N*-oxide (**M3**)	995.43	124.44	
Nalidixic acid	1435.2	358.81	-	CPZ	117.27	58.62	0.75
CPZ	117.27	58.62		CPZ-*N*-oxide (**M3**)	995.43	248.85	
Ciprofloxacin	0.63	0.30	0.73	Rifampicin	1.26	0.32	0.50
CPZ	117.27	29.32		Nor-CPZ (**M5**)	136.70	34.18	
Levofloxacin	0.58	NC	-	25-Desacetylrifampicin	2.66	0.67	0.50
CPZ	117.27			Nor-CPZ (**M5**)	136.70	34.18	
Rifampicin	2.53	0.32	0.63	Ethambutol	1.51	IAE	-
7-hydroxyCPZ(**M2**)	62.21	31.12		Nor-CPZ (**M5**)	63.33		
25-Desacetylrifampicin	1.33	0.33	0.75	Kanamycin	3.57	0.89	0.50
7-hydroxyCPZ (**M2**)	124.44	62.21		Nor-CPZ (**M5**)	136.70	34.18	
Ethambutol	0.76	IAE	-	Streptomycin	0.29	0.07	0.49
7-hydroxyCPZ (**M2**)	124.44			Nor-CPZ (**M5**)	136.70	34.18	
Kanamycin	3.57	0.89	0.50	Spectinomycin	84.12	5.25	0.31
7-hydroxyCPZ (**M2**)	124.44	31.12		Nor-CPZ (**M5**)	136.70	34.18	
Streptomycin	0.07	0.02	0.79	CPZ	117.27	29.32	0.75
7-hydroxyCPZ (**M2**)	124.44	62.21		Nor-CPZ (**M5**)	136.70	68.33	

IAE – Inconsistent antagonistic effect; NC – No Change; Note: MIC_{99} of the two compounds in combination is less than the MIC_{99} of the individual compounds because in combination the compounds potentiate each other's activity

Interestingly, nor-CPZ **(M5)** was able to augment the antimycobacterial activity of anti-TB drugs to a greater extent compared to CPZ and **M2.** Its synergistic effect was observed for combinations with rifampicin and its metabolite, 25-desacetylrifampicin, kanamycin, and streptomycin - all of which yielded a FICI value of 0.5, with the best interaction observed with spectinomycin (FICI 0.31). It is worth noting that even in combinations that exhibited a FICI > 0.5, a clear drop in the

MIC_{99} of several of the anti-TB drugs was observed. For instance, CPZ and its metabolites were able to cause a 4-8 fold drop in the MIC_{99} of rifampicin and its metabolite 25-desacetylrifampicin (table 2). As per the FICI definition, combinations that yield FICI > 0.5 but ≤ 4.0 indicate no interaction. However, for some of these (for example, CPZ plus rifampicin and CPZ-*N*-oxide plus streptomycin), the change in MIC_{99} of the known anti-TB drug suggests the potential to identify compounds and/or metabolites which can potentiate activity. Notably, for CPZ-*N*-oxide (**M3**) this effect is observed even where the metabolite itself is only very weakly active on its own. CPZ sulfoxide **(M1),** chlorpromazine-*N*-*S*-dioxide **(M4)** and nor-CPZ sulfoxide **(M6)** were all inactive hence FICI could not be calculated for the various combinations.

Table 3: MIC_{99} of inactive metabolites in combination with anti-TB drugs, in *M. smegmatis*

Drugs/Compound	MIC_{99} (μM) singly	MIC_{99} (μM) combination
Rifampicin	2.53	NC
CPZ sulfoxide (**M1**)	>1990.89	
Ethambutol	0.76	IAE
CPZ sulfoxide (**M1**)	>1990.89	
Kanamycin	1.79	0.89
CPZ sulfoxide (**M1**)	>1990.89	1990.89
Streptomycin	0.29	0.14
CPZ sulfoxide (**M1**)	>1990.89	62.21
Spectinomycin	168.22	10.52
CPZ sulfoxide (**M1**)	>1990.89	1990.89
Rifampicin	2.53	NC
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10	
Ethambutol	0.76	IAE
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10	
Kanamycin	1.76	0.89
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10	475.03
Streptomycin	0.29	0.14
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10	237.50
Spectinomycin	84.12	42.05
CPZ-*N*-*S*-dioxide (**M4**)	>1900.10	1900.10
Rifampicin	1.26	NC
nor-CPZ sulfoxide (**M6**)	>2077.89	
Ethambutol	0.76	IAE
nor-CPZ sulfoxide (**M6**)	>2077.89	
Kanamycin	1.79	NC
nor-CPZ sulfoxide (**M6**)	>2077.89	
Streptomycin	0.14	0.07
nor-CPZ sulfoxide (**M6**)	>2077.89	519.48
Spectinomycin	84.12	21.04
nor-CPZ sulfoxide (**M6**)	>2077.89	64.92

IAE – Inconsistent antagonistic effect; NC – No Change

Nevertheless, as seen in table 3, inactive metabolites were still able to augment the antimycobacterial activity of some of the anti-TB drugs used in this study. For example, CPZ sulfoxide **(M1)** and nor-CPZ sulfoxide **(M6)** decreased the MIC_{99} of spectinomycin 16-fold and 4-fold respectively. At least a 2-fold drop in MIC_{99} was observed for the other combinations.

Combinations of the parents (CPZ and rifampicin) with their metabolites yielded a FICI of ~1.00 which is expected of an additive interaction (table 2). Ethambutol with CPZ and its metabolites did not exhibit synergism but antagonism. This effect has been reported in previous studies.[30,22] The results clearly indicate that CPZ and its metabolites are able to increase *M. smegmatis* susceptibility to anti-TB drugs. Spectinomycin which hardly has any antimycobacterial activity exhibited the highest drop in MIC_{99}. Similar interactions were observed for spectinomycin and other drugs in a recent study by Ramón-García *et al*.[6] The basis for the propensity of spectinomycin to interact synergistically with a variety of different compound classes requires further investigation.

Aminoglycosides appeared to interact most with CPZ and its metabolites. It has been reported that aminoglycosides which are known to target ribosomes leading to inhibition of protein synthesis do tend to display synergistic effects when used in combination with other drugs such as cell wall synthesis inhibitors, which help to increase accumulation of the drug within the mycobacterial cell.[31] Elucidation of the molecular mechanism underlying those interactions that yielded FICI ≤ 0.5 would contribute significantly to the interpretation of these findings.

Conclusion

In conclusion, chlorpromazine and its metabolites can potentiate the activity of a number of anti-TB drugs. The similarity in activity of the metabolites to CPZ may offer alternate paths to the investigation of these agents as potential antimycobacterial drugs.

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Notes and references

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