

## Effects of interactions of antibacterial drugs with each other and with 6-mercaptopurine on *in vitro* growth of *Mycobacterium avium* subspecies *paratuberculosis*

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Received 9 July 2009; returned 18 August 2009; revised 24 August 2009; accepted 24 August 2009

**Objectives:** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been targeted for treatment with clarithromycin and rifamycin derivatives in numerous cases of Crohn's disease (CD). 6-Mercaptopurine and its pro-drug azathioprine are widely used as immunomodulators in the treatment of CD and have recently been shown to have anti-MAP activity *in vitro*. The objectives of the study were to evaluate the *in vitro* effects on MAP of (i) 6-mercaptopurine when combined with each of eight conventional antibacterial agents with *in vitro* anti-MAP activity and (ii) antibacterial combinations consisting of two drugs (clarithromycin combined with amikacin, rifampicin, ciprofloxacin or ethambutol) and three drugs (clarithromycin, rifabutin and clofazimine).

**Methods:** The drug interaction effects on nine human isolates of MAP were determined by the checkerboard method adapted for the BACTEC™ MGIT™ 960 culture system and by calculation of the fractional inhibitory concentration index (FICI) for drug combinations.

**Results:** Synergism ( $FICI \leq 0.5$ ) was observed between 6-mercaptopurine and azithromycin (seven isolates), clarithromycin, rifampicin, rifabutin (four isolates each) and ethambutol (two isolates). 6-Mercaptopurine was not antagonistic with any of the antibacterial agents tested. Among the combinations of two and three antibacterials tested, the clarithromycin/rifampicin combination was synergistic against four isolates, while all other combinations showed no interaction.

**Conclusions:** This *in vitro* study suggests that 6-mercaptopurine may be synergistic with macrolides and rifamycin derivatives against MAP. The activity of clarithromycin against MAP seems to be enhanced by rifampicin.

Keywords: drug combination, FIC, immunomodulator, antibiotics, susceptibility, paratuberculosis, Crohn's disease

### Introduction

Treatment of mycobacterial diseases requires a combination of drugs to limit antimicrobial drug resistance during long-term therapy and to deal with the innate susceptibility differences among mycobacteria in different physiological states, e.g. dormant *in vivo*.<sup>1</sup> *In vitro* interaction studies on antimicrobial agents are necessary to select synergistic and avoid antagonistic antimicrobial combinations.

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic inflammatory bowel disease affecting a broad range of animals including primates.<sup>2</sup> It is also the most investigated potential causative agent of

Crohn's disease (CD), a chronic, debilitating inflammatory bowel disease of humans.<sup>3,4</sup> Owing to the strong MAP-CD association,<sup>5,6</sup> potential anti-MAP agents (macrolides, rifamycin derivatives and clofazimine) were used in clinical trials and case studies with promising outcomes in most reports,<sup>7-12</sup> but not in all.<sup>13,14</sup>

Earlier *in vitro* studies have shown that macrolides, especially clarithromycin, are highly effective against MAP.<sup>15,16</sup> This observation is consistent with antimicrobial susceptibility data for *M. avium* complex (MAC).<sup>17,18</sup> Presently, the recommended treatment and prophylactic regimens for disease due to MAC are combinations of two or three drugs: a macrolide (clarithromycin or azithromycin) in combination with rifampicin or rifabutin and/or ethambutol.<sup>19</sup> For CD, a combination of antimicrobials

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## Effect of drug combinations on *M. avium* subspecies *paratuberculosis*

along with immunosuppressive agent(s) was recommended as preferable to using antibiotics alone.<sup>20</sup>

Azathioprine is an immunomodulator that induces and maintains remission in CD.<sup>21</sup> Azathioprine and its derivative 6-mercaptopurine are eventually metabolized *in vivo* forming thioguanine nucleotides (TGNs), which induce cytotoxicity and immunosuppression.<sup>22</sup> Interestingly, 6-mercaptopurine was recently shown to have anti-MAP activity *in vitro*.<sup>23,24</sup> The objectives of this work were to evaluate the actions of clarithromycin or 6-mercaptopurine with other anti-MAP agents and with each other on the *in vitro* growth of MAP.

## Materials and methods

### MAP strains

Nine human (CD cases) isolates of MAP were tested. They included five clinical strains (UCF3, UCF4, UCF5, UCF7 and UCF8) kindly donated by Saleh Naser (University of Central Florida, Orlando, FL, USA) and four ATCC strains (43015, 43544, 43545 and 49164). All the isolates were verified as MAP by assay for insertion sequence (IS)900 by PCR,<sup>25</sup> as well as by *in vitro* growth characteristics. The isolates were grown in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD, USA) supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) and 2 mg/L mycobactin J (Allied Monitor, Fayette, MO, USA). Eight-week-old cultures were harvested and resuspended in fresh medium containing glycerol (final concentration of 20%, v/v). The bacterial suspension was declumped by vortexing in screw-capped glass test tubes containing 3 mm glass beads. The suspension was then allowed to stand for 30 min. The supernatant of MAP obtained by this method was found to contain bacilli evenly spread out as single cells on a Ziehl–Neelsen-stained smear. The harvested supernatant was stored as aliquots at  $-80^{\circ}\text{C}$ .

### Preparation of inocula for *in vitro* drug susceptibility testing

Frozen stock cultures were thawed and added to 4 mL of PBS in screw-capped test tubes containing 3 mm glass beads and declumped once again as described above. The resulting supernatant was harvested and its turbidity adjusted using PBS to an optical density at 600 nm ( $\text{OD}_{600}$ ) of  $\sim 0.13$  using a spectrophotometer (Biomate 3, Thermo Fisher Scientific, Waltham, MA, USA) to match a 0.5 McFarland turbidity standard. MGIT<sup>TM</sup> ParaTB medium tubes (Becton Dickinson) were inoculated with 0.1 mL of the MAP suspension (drug testing and growth control tubes) or a 100-fold dilution of the suspension (1:100 growth control). The organism was added to the culture medium 24 h prior to adding drugs in order to permit the bacilli to adjust to the growth conditions and prepare for log-phase growth. Tubes of MGIT<sup>TM</sup> ParaTB medium so inoculated were found to contain  $1 \times 10^5$ – $5 \times 10^5$  cfu/mL prior to adding drugs. Minor but consistent differences in growth rates were observed between strains as reflected in the time to detection (TTD) values in the MGIT system (discussed below).

### Drugs

Azithromycin, clarithromycin, amikacin, ciprofloxacin, ethambutol, clofazimine and 6-mercaptopurine were purchased from Sigma-Aldrich (St Louis, MO, USA). Rifampicin and rifabutin were purchased from USP (Rockville, MD, USA). Stock solutions of drugs were prepared using the most appropriate solvent: water (amikacin and ethambutol); 0.1 N sodium hydroxide (ciprofloxacin); DMSO (6-mercaptopurine);

methanol (rifampicin and rifabutin); ethanol (azithromycin and clarithromycin); or methanol acidified by trace amounts of glacial acetic acid (clofazimine). Drug stock solutions were filter sterilized, if required, and stored at  $-80^{\circ}\text{C}$  for up to 2 months, except for 6-mercaptopurine, which was freshly made each time it was used. Prior to testing, each drug was freshly diluted in sterile deionized water.

### *In vitro* drug interaction testing

The method described for mycobacteria was adapted for the BACTEC<sup>TM</sup> MGIT<sup>TM</sup>960 system (Becton Dickinson).<sup>26</sup> Briefly, each drug in a combination (two-drug and three-drug combinations) was tested at their respective MICs and at up to five doubling dilutions (sub-MICs) for the strain being tested. Anticipating one doubling dilution difference from previously determined MICs, two more sets of combinations each containing one of the drugs at 2× the MIC and doubling dilutions thereof were tested. MICs of each individual drug were re-determined in the same experiment in which drug combinations were tested. All drug dilutions as either individual drugs or combinations were added to the culture medium in a total volume of 0.1 mL.

Tubes were incubated in the MGIT<sup>TM</sup>960 instrument and growth was recorded as a TTD value. The TTDs for the strains ranged from 2.5 to 4.4 days (average  $3.56 \pm 0.69$ ) in control tubes and from 7 to 12.7 days (average  $9.87 \pm 1.86$ ) in 1% control tubes. The MIC of individual drugs was defined as the lowest drug concentration required to suppress MAP growth resulting in a TTD greater than that of the 1% growth control. The MIC of a drug in a combination ( $\text{MIC}_{\text{comb}}$ ) was defined as the lowest concentration of that drug in the mixture that resulted in a TTD greater than the 1% growth control.

The fractional inhibitory concentration (FIC) of each drug in a mixture was calculated as  $\text{MIC}_{\text{comb}}/\text{MIC}$ . Drug interaction was determined based on the FIC index (FICI) for the specific drug combination, which is the sum of FICs.

$$\text{FICI} = (\text{MIC}_{\text{drug A comb}}/\text{MIC}_{\text{drug A alone}}) + (\text{MIC}_{\text{drug B comb}}/\text{MIC}_{\text{drug B alone}})$$

For two-drug combinations, drug interactions were considered synergistic if the FICI was  $\leq 0.5$  and antagonistic if the FICI was

**Table 1.** Effects of 6-mercaptopurine in combination with other anti-MAP agents on the *in vitro* growth of nine MAP isolates

Antibiotic combined with 6-MP	No. of isolates against which the combination showed:			FICI (range)
	synergy	no interaction	antagonism	
AZM	7	2	0	0.28–0.62
CLR	4	5	0	0.37–1.0
AMK	0	9	0	0.62–2.25
CIP	0	9	0	0.62–1.25
RIF	4	5	0	0.37–1.0
RFB	4	5	0	0.37–1.5
EMB	2	7	0	0.19–1.5
CLF	0	9	0	0.75–1.5

6-MP, 6-mercaptopurine; AZM, azithromycin; CLR, clarithromycin; AMK, amikacin; CIP, ciprofloxacin; RIF, rifampicin; RFB, rifabutin; EMB, ethambutol; CLF, clofazimine.

**Table 2.** MIC and FICI data of individual MAP strains for two-drug combinations containing 6-mercaptopurine

Strain	6-MP+CIP					6-MP+AZM					6-MP+CLR					6-MP+AMK				
	6-MP		CIP		FICI	6-MP		AZM		FICI	6-MP		CLR		FICI	6-MP		AMK		FICI
	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	
UCF3	4	2	1	0.5	1	4	1	4	1	0.5	4	1	1	0.25	0.5	4	1	6.25	3.12	0.75
UCF4	4	2	1	0.25	0.75	4	1	0.5	0.125	0.5	4	1	0.12	0.03	0.5	4	1	1.56	3.12	2.25
UCF5	2	0.5	0.5	0.25	0.75	2	0.25	1	0.5	0.62	2	0.25	0.12	0.06	0.62	2	0.125	1.56	1.56	1
UCF7	1	0.5	0.5	0.25	1	1	0.25	2	0.5	0.5	1	0.5	0.12	0.06	1	1	0.06	1.56	1.56	1
UCF8	2	1	0.25	0.12	1	2	0.5	0.5	0.12	0.5	2	1	0.12	0.03	0.75	2	0.25	0.78	0.39	0.62
ATCC 43015	2	0.5	0.5	0.5	1.25	4	0.5	0.5	0.25	0.62	4	0.25	0.12	0.06	0.56	2	0.5	3.12	1.56	0.75
ATCC 43544	64	32	2	0.5	0.75	64	16	1	0.25	0.5	64	16	0.25	0.03	0.37	64	4	3.12	3.12	1
ATCC 43545	64	8	4	2	0.62	64	2	2	0.5	0.28	64	8	0.12	0.03	0.37	64	4	1.56	1.56	1
ATCC 49164	128	8	1	1	1	128	16	2	0.5	0.37	128	2	0.25	0.125	0.52	128	32	3.12	1.56	0.75
Strain	6-MP+RIF					6-MP+RFB					6-MP+EMB					6-MP+CLF				
	6-MP		RIF		FICI	6-MP		RFB		FICI	6-MP		EMB		FICI	6-MP		CLF		FICI
	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>		MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	
UCF3	4	2	6	1.5	0.75	4	1	2	0.5	0.5	4	2	5	1.25	0.75	4	4	0.62	0.31	1.5
UCF4	4	1	0.38	0.09	0.5	4	2	0.5	0.125	0.75	4	0.5	2.5	1.25	0.62	4	2	1.25	0.31	0.75
UCF5	2	1	0.75	0.09	0.62	2	1	0.25	0.06	0.75	2	0.5	5	2.5	0.75	2	2	0.31	0.15	1.5
UCF7	1	0.5	1.5	0.75	1	1	0.5	0.5	0.25	1	1	1	5	2.5	1.5	1	1	0.31	0.15	1.5
UCF8	2	1	0.19	0.09	1	2	2	0.12	0.06	1.5	2	1	2.5	0.62	0.75	2	1	0.31	0.15	1
ATCC 43015	4	0.25	0.06	0.06	1	1	0.5	0.12	0.06	1	1	0.25	5	2.5	0.75	1	0.5	0.62	0.31	1
ATCC 43544	64	16	0.75	0.09	0.37	64	16	0.25	0.03	0.37	64	4	10	1.25	0.19	64	32	0.62	0.15	0.75
ATCC 43545	64	16	1.5	0.38	0.5	64	16	0.5	0.12	0.5	64	8	5	1.25	0.37	64	16	1.25	0.62	0.75
ATCC 49164	128	16	6	1.5	0.37	128	16	4	1	0.37	128	16	5	2.5	0.62	128	32	0.62	0.62	1.25

6-MP, 6-mercaptopurine; CIP, ciprofloxacin; AZM, azithromycin; CLR, clarithromycin; AMK, amikacin; RIF, rifampicin; RFB, rifabutin; EMB, ethambutol; CLF, clofazimine. Grey shading indicates synergy (FICI ≤ 0.5).

## Effect of drug combinations on *M. avium* subspecies *paratuberculosis*

>4.0; there was considered to be no interaction if the FICI was >0.5 and ≤4.0.<sup>27</sup>

### Results and discussion

#### 6-Mercaptopurine–antibiotic combinations

It was recently shown that the immunosuppressive drugs azathioprine and its metabolite 6-mercaptopurine have anti-MAP activity.<sup>23,24</sup> These drugs could potentially affect the activity of antimicrobials when used together for treatment of CD. We assessed drug interactions *in vitro* by determining the FICI values of two-drug (6-mercaptopurine plus antimicrobial) combinations containing 6-mercaptopurine and one of the eight antibiotics that showed *in vitro* anti-MAP activity in our previous study.<sup>16</sup> MICs of 6-mercaptopurine for the MAP isolates used in the present study ranged from 1 to 128 mg/L. We have previously observed that the MBCs of 6-mercaptopurine for all the nine strains were >128 mg/L, the highest concentration tested. Even for a strain having an MIC of 1 mg/L, there was only a 0.4 log<sub>10</sub> reduction in cfu at 64 mg/L and there was no concentration-dependent killing. These data suggest that the drug is bacteriostatic to MAP. Antimicrobial drugs either showed synergy or no interaction with 6-mercaptopurine, and none of the combinations was antagonistic for MAP growth inhibition *in vitro* (Table 1). Azithromycin showed a synergistic interaction with 6-mercaptopurine for seven out of the nine MAP strains, while clarithromycin, rifampicin and rifabutin showed synergy with 6-mercaptopurine for four MAP strains each. Ethambutol was synergistic with 6-mercaptopurine for only two MAP strains. Anti-MAP activities of amikacin, ciprofloxacin and clofazimine were not affected by 6-mercaptopurine for any of the nine MAP isolates tested. Individual MICs and FICI values for all nine strains are shown in Table 2.

This is the first *in vitro* drug combination study on 6-mercaptopurine and antibiotics as anti-MAP agents and is relevant for the treatment of CD cases that are associated with MAP. In humans, azathioprine is rapidly converted into 6-mercaptopurine, which in turn is metabolized to TGNs—the active metabolites responsible for immunosuppression.<sup>22</sup> The low serum levels (<0.1 mg/L) and short half-lives (1–3 h) of 6-mercaptopurine are attributed to its rapid conversion into TGNs.<sup>28,29</sup> The concentrations of active metabolites and toxic

**Table 3.** Effect of antibiotic combinations containing clarithromycin on the *in vitro* growth of MAP isolates

Antibiotic combined with CLR	No. of isolates against which the combination showed:			FICI (range)
	synergy	no interaction	antagonism	
AMK	0	9	0	0.56–1.5
RIF	4	5	0	0.3–0.75
CIP	0	9	0	0.62–1.5
EMB	0	9	0	0.62–1.5
CLF and RFB	0	all three tested		0.75–1.25

CLR, clarithromycin; AMK, amikacin; RIF, rifampicin; CIP, ciprofloxacin; EMB, ethambutol; CLF, clofazimine. Nine isolates were tested except for the three-drug combination.

**Table 4.** MIC and FICI data of individual MAP strains for two-drug combinations containing clarithromycin

Strain	CLR + CIP				CLR + AMK				CLR + RIF				CLR + EMB				
	CLR		CIP		CLR		AMK		CLR		RIF		CLR		EMB		
	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	MIC <sub>alone</sub>	MIC <sub>comb</sub>	
UCF3	0.25	0.25	0.5	0.25	0.25	0.25	1	1.13	0.25	0.03	1	0.25	0.37	0.25	0.06	2.5	1.25
UCF4	0.06	0.06	0.5	0.06	0.06	0.06	1	1.25	0.06	0.03	1	0.01	0.58	0.06	0.03	1.25	1.5
UCF5	0.12	0.06	0.5	0.06	0.12	0.06	4	0.25	0.12	0.03	0.06	0.03	0.75	0.12	0.03	2.5	1.25
UCF7	0.25	0.12	0.5	0.25	0.25	0.25	8	1.25	0.25	0.06	0.5	0.12	0.5	0.25	0.12	5	2.5
UCF8	0.06	0.03	0.25	0.06	0.06	0.06	0.5	1.5	0.06	0.01	0.06	0.01	0.3	0.06	0.03	1.25	0.62
ATCC 43015	0.12	0.06	0.5	0.12	0.12	0.06	4	0.75	0.12	0.03	0.06	0.01	0.42	0.12	0.03	2.5	1.25
ATCC 43544	0.06	0.01	0.25	0.06	0.06	0.03	1	1.5	0.06	0.01	0.06	0.03	0.67	0.06	0.03	2.5	1.25
ATCC 43545	0.25	0.12	1	0.25	0.25	0.25	2	1.25	0.25	0.03	0.5	0.25	0.62	0.25	0.06	2.5	1.25
ATCC 49164	0.5	0.25	2	0.5	0.5	0.25	4	1.5	0.5	0.06	1	0.5	0.62	0.5	0.06	5	2.5

CLR, clarithromycin; CIP, ciprofloxacin; AMK, amikacin; RIF, rifampicin; EMB, ethambutol. Grey shading indicates synergy (FICI ≤ 0.5).

metabolites of 6-mercaptopurine are known to vary among and within individuals due to the complexity of the metabolic pathways involved and genetic polymorphisms in the metabolizing enzymes.<sup>30</sup> TGN concentrations approaching 250 pmol/8×10<sup>8</sup> red blood cells are reported to be associated with disease remission.<sup>22</sup> Currently it is not known how 6-mercaptopurine inhibits the growth of MAP or whether the bacterium actively converts 6-mercaptopurine into TGNs. In this scenario, even though the *in vitro* data presented here are not sufficient to predict the clinical efficacy of synergistic 6-mercaptopurine–antibiotic combinations on MAP, it is encouraging to find that these combinations can be synergistic or neutral in their effects, but not antagonistic.

#### Drug combinations containing clarithromycin

Two-drug combinations, containing clarithromycin and one of the four anti-MAP agents amikacin, rifampicin, ciprofloxacin or ethambutol (each belonging to different antimicrobial drug classes), were tested against nine MAP isolates. In addition, a three-drug combination containing clarithromycin, rifabutin and clofazimine, a common combination used in CD trials targeting MAP, was tested against three MAP strains. Clarithromycin when combined with rifampicin showed either synergism (four isolates) or no effect (five isolates) on inhibition of MAP growth *in vitro* (Table 3). The other three drugs, amikacin, ciprofloxacin and ethambutol, did not show any interaction with clarithromycin for any of the nine MAP isolates tested. The three MAP isolates tested with the three-drug combination, clarithromycin, clofazimine and rifabutin, did not show any drug interaction. Individual MIC and FICI values for all nine strains for two-drug combinations containing clarithromycin are shown in Table 4.

Clarithromycin in our earlier *in vitro* study was highly active against MAP, with MICs ranging between 0.125 and 1.0 mg/L.<sup>16</sup> Similar observations have been made in the past by others.<sup>15</sup> The drug has also been tested as an anti-MAP agent in clinical trials against CD.<sup>12,14,20</sup> In the only prior report on the effect of drug combinations on *in vitro* growth of MAP, the combination of clarithromycin and ethambutol showed synergy for three of four MAP strains.<sup>15</sup> More drug interaction studies have been reported on *M. avium* isolates. Piersimoni *et al.*<sup>31</sup> demonstrated synergistic activity of clarithromycin with rifabutin, ethambutol and ciprofloxacin in 54%, 16% and 8% of 37 clinical *M. avium* isolates, respectively. In another study on *M. avium* clinical isolates, clarithromycin activity was enhanced by ethambutol and rifampicin in eight and three of nine strains tested, respectively.<sup>32</sup>

The study demonstrates the utility of a rapid (8–10 days) screening method for study of anti-MAP drug interactions in a research setting prior to *ex vivo* and *in vivo* experiments. This *in vitro* study also demonstrates, for the first time, potential synergistic anti-MAP effects of 6-mercaptopurine–antibacterial drug and clarithromycin–rifampicin drug combinations.

#### Acknowledgements

We thank Kelly Anklam for her valuable technical assistance.

#### Funding

This study was supported by funding from The Broad Medical Research Programme of the Broad Foundation.

#### Transparency declarations

M. T. C. was a paid consultant to Becton Dickinson. Other authors: none to declare.

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## Effect of drug combinations on *M. avium* subspecies *paratuberculosis*

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