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The role of whole genome sequencing in characterizing the mechanism of action of anti-tuberculosis compounds: demonstrated with para-amino salicylic acid and its analogue

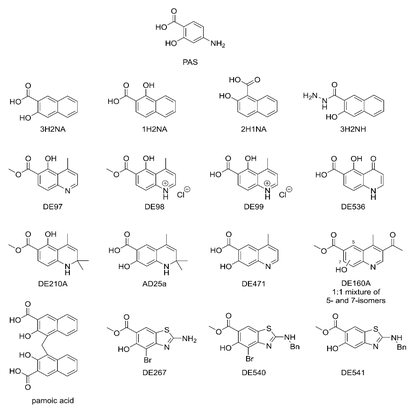
Satta G[1,2], Witney AA[3], Ere D[4], Disney A[4], Begum N[1], Canseco JO[1], Ratledge C[4], Boa AN[4], McHugh TD[1].

Background

Para-aminosalicylic acid (PAS) was one of the first antibiotics to be used against tuberculosis (TB) and it is still one of the last remaining drugs available to treat extensively drug-resistant (XDR) disease (**1, 2**). Despite being on the market for decades, the mechanism of action of PAS is not completely understood yet (**3**). Sixteen new compounds against *Mycobacterium tuberculosis* were created in the laboratory as salicylate analogues (based on their chemical structures) and their antimycobacterial activity had never been tested before. The main aim of this project was to test the activity of these new analogues and to understand their mechanism of action (including PAS).

Methods

The compounds (whose chemical structure is shown below) were tested using three different methods (spot culture, resazurin and BACTEC/MGIT system) (**4, 5, 6**). Additionally, resistant mutants were created against PAS and the most promising analogue; whole genome sequencing (WGS) was performed to understand their mechanism of action.



Results: susceptibility testing

1. Five PAS analogues (DE471, 1H2NA, 2H1NA, 3H1NA and AD25a) inhibited the growth of *M. tuberculosis* using the spot culture method (**Table 1**).
2. The five active compounds on the spot culture were further tested with the resazurin method for the determination of their critical concentration (**Table 2**). One compound in particular, AD25a, showed the lowest critical concentration (0.04 µg/ml) among the analogues.
3. The compound AD25a was selected for further testing using the BACTEC/MGIT method (**Table 3**).

Compound	Maximum concentration used (µg/ml)	Growth/No growth of *M. tuberculosis*
DE97	27	Growth
DE98	27.3	Growth
DE99	24	Growth
DE471	28.6	No growth
DE160A	32.6	Growth
DE210A	41	Growth
DE267(2)	39.6	Growth
DE540	26.6	Growth
DE536(2)	35.3	Growth
DE541	25	Growth
1H2NA	74	No growth
2H1NA	53.3	No growth
3H2NA	53	No growth
3H2NH	36	Growth
PAMOIC ACID	37.6	Growth
AD25a	10.8	No growth
PAS control	26.9	No Growth

Table 1: Spot culture screening results. Five compounds inhibited the growth of *M. tuberculosis* H37Rv. The active compounds (DE471, 1H2NA, 2H1NA, 3H1NA and AD25a) are highlighted in grey.

Compound	Critical concentration
AD25a	0.04 µg/ml
DE471	1.79 µg/ml
3H2NA	26.5 µg/ml
1H2NA	74 µg/ml
2H1NA	53.3 µg/ml

Table 2: Resazurin assay results. The five compounds potentially active against *M. tuberculosis* were further tested with the resazurin assay. AD25a (highlighted in grey) showed the lowest critical concentration among the compounds tested.

	Growth Units and different concentrations of AD25a						
Time (hours)	GC	1.54 µg/ml	0.77 µg/ml	0.39 µg/ml	0.19 µg/ml	0.1 µg/ml	0.05 µg/ml
0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0
40	0	0	0	0	0	0	1
47	0	0	0	0	0	1	5
136	8	0	0	0	>100	>100	>100
142	29	0	0	0	>100	>100	>100
159	156	0	0	0	>100	>100	>100
168	251	0	0	0	>100	>100	>100
174	**400**	**0**	**0**	**1**	**>100**	**>100**	**>100**

Table 3:BACTEC/MGIT testing results. The table above shows the growth units of H37Rv over time (in hours) at different concentrations of AD25a (from 1.54 µg/ml to 0.05 µg/ml). When the growth control (GC) reaches 400 growth units, any concentrations with a growth unit less than 100 is considered as active against *M. tuberculosis* (susceptible). If the growth units are more than 100, the concentration tested is considered as non-active (resistant). The lowest active concentration was 0.39 µg/ml (circled in yellow).

Results: mechanism of action

The WGS analysis of PAS- and AD25a-resistant mutants identified a total of 40 single nucleotide polymorphisms (SNPs) in the PAS resistant mutants and 28 SNPs in the AD25a resistant mutants (when compared to the reference strain H37Rv). The SNPs identified in the PAS and AD25a resistant mutants did not overlap. The genes *rrs*, *rrl* and *folC* were mostly involved in the PAS resistant mutants.

Gene	Function	PAS2 1st	PAS2 2nd	PAS4 1st	PAS4 2nd
		SNP Position			
Rv0486	Glycosyltransferase			576252	
Rv1072	Conserved membrane protein		1196380		
Rv1204c	Conserved protein				1347266
rrs	16s RNA gene		1472240		
rrs	16s RNA gene		1472242		1472242
rrs	16s RNA gene		1472251		1472251
rrs	16s RNA gene		1472252		1472252
rrs	16s RNA gene		1472253		1472253
rrs	16s RNA gene		1472256		1472256
rrs	16s RNA gene		1472259		1472259
rrs	16s RNA gene			1472616	
rrs	16s RNA gene	1473062			
rrs	16s RNA gene	1473068			
rrl	23s RNA gene		1475816		
rrl	23s RNA gene		1475817		
rrl	23s RNA gene		1476126		
rrl	23s RNA gene		1476131		
rrl	23s RNA gene		1476141		
rrl	23s RNA gene		1476153		
rrl	23s RNA gene		1476154		
rrl	23s RNA gene		1476164		
rrl	23s RNA gene		1476165		
rrl	23s RNA gene			1476455	
rrl	23s RNA gene			1476456	
Rv1431	Conserved membrane protein		1669561		
nadB	L-aspartate oxidase		1796003		
Rv2215	E2 component of pyruvate	2482556			
ahpD	Alkyl hydroperoxide reductase		2727108	2727108	
FolC	Folylpolyglutamate synthase				2747141
Rv2095	Conserved hypothetical protein		3012106		
cysA3	Thiosulfate sulfurtransferase		3484226		
Rv3202c	Possible ATP-dependent DNA helicase			3578723	
Rv3218	Conserved protein		3594639	3594639	
Rv3218	Conserved protein	3594791			
Rv3232c	Polyphosphate kinase		3609025		
RV3505	Acetyl-CoA dehydrogenase	3924194			
Rv3785	Hyphotetical protein				4232224
Rv3894c	Type VII secretion system		4378811		

Table 4: PAS-resistant mutants. The table shows the genes involved in the PAS-resistant mutants, with the respective function and SNP position in the genome. Only non-synonymous SNPs were considered. The genes *rrs*, *rrl* and *folC* are highlighted in grey and circled in yellow. All these SNPs are different from the SNPs involving the AD25a resistant mutants.

Gene	Function	AD25a 1	AD25a 2	AD25a 3	AD25a 4	AD25a 5	AD25a 6
		SNP Position					
Rv0197	Possible oxido-reductase	234494					
Rv0279c	PE family		338167				
Rv0388c	PPE family	467508					
Rv0532	PE family		623425				
Rv0532	PE family		623428				
Rv0532	PE family	623472	623472	623472	623472	623472	623472
Rv0578c	PE family	672491	672491				
Rv0746	PE family	836426	836426				
Rv0746	PE family	836454	836454	836454	836454		
Rv0746	PE family	836538	836538	836538	836538	836538	836538
Rv0747	PE family		839123	839123			
Rv0747	PE family		839129	839129			839129
Rv0747	PE family		840338				
Rv0747	PE family		840340				
Rv0833	PE family		845275				
Rv0834c	PE family		929943				
PE_PGRS25	PE family	1572865					
PE_PGRS25	PE family		1573326				
PE_PGRS25	PE family		1573335				
PE_PGRS31	PE family		2001220				
Rv2015c	Conserved hypothetical protein		2262896				
PE_PGRS43	PE family		2805256				
Rv2540	Chorismate synthase		2863654				
Rv3347	PPE family			3745738			
Rv3515	PE family					3948347	
PE_PGRS61	PE family	4094140					
Rv3655c	Hypothetical protein	4095000					

Table 5: AD25a-resistant mutants. The table shows the genes involved with the respective function and SNP position in the genome. Only non-synonymous SNPs were considered. The majority of SNPs involve PE family (proline-glutamic acid) with unknow function. All these SNPs are different from the SNPs involving the PAS resistant mutants.

Conclusions

The complete difference in the mutation profiles suggests that AD25a has a mechanism of action different to that of PAS, despite AD25a being synthesized as a salicylate analogue. WGS analysis of PAS resistant mutants has also provided some interesting results. In particular, all our PAS mutants showed mutations in the *rrs* and *rrl* genes (16S and 23S RNA genes, respectively). These mutations should affect the ribosomes and the overall synthesis of proteins. This highlights a new potential mechanism of resistance for PAS that has never been observed before.

References

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1. University College London
2. Imperial College Healthcare NHS Trust
3. St George's University of London
4. University of Hull

Contact email: Giovanni.satta@nhs.net