PZA Mechanism of Action: Insights from Transcriptional Profiling

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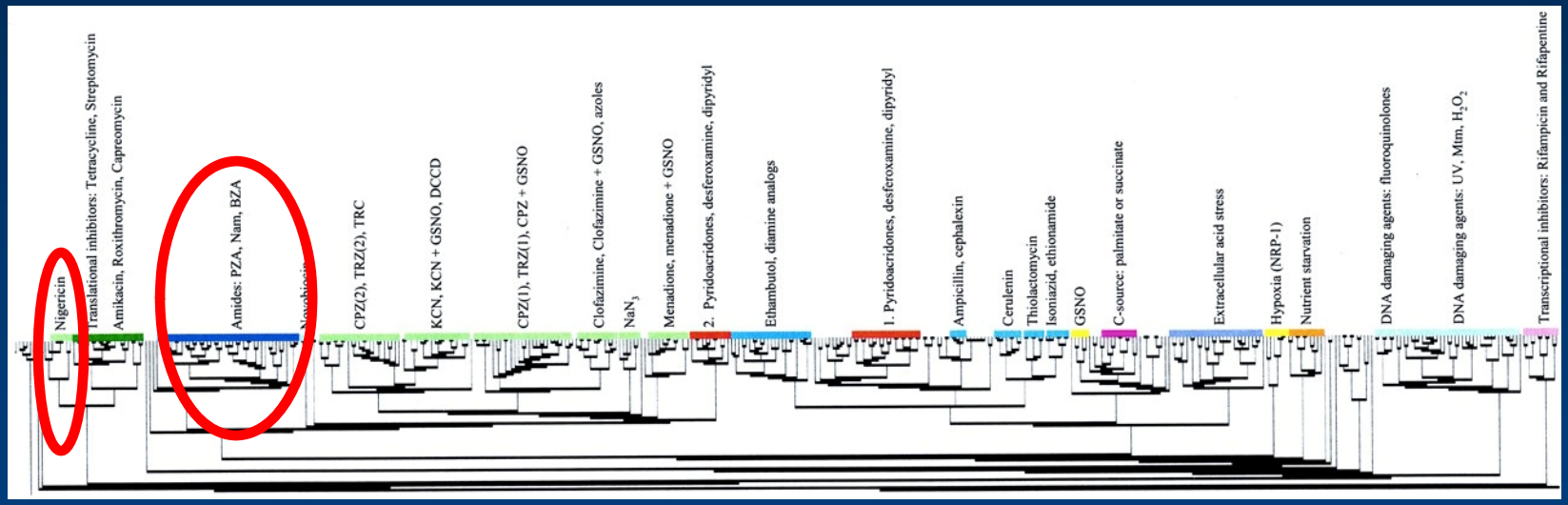
Suggested mechanisms of action for PZA

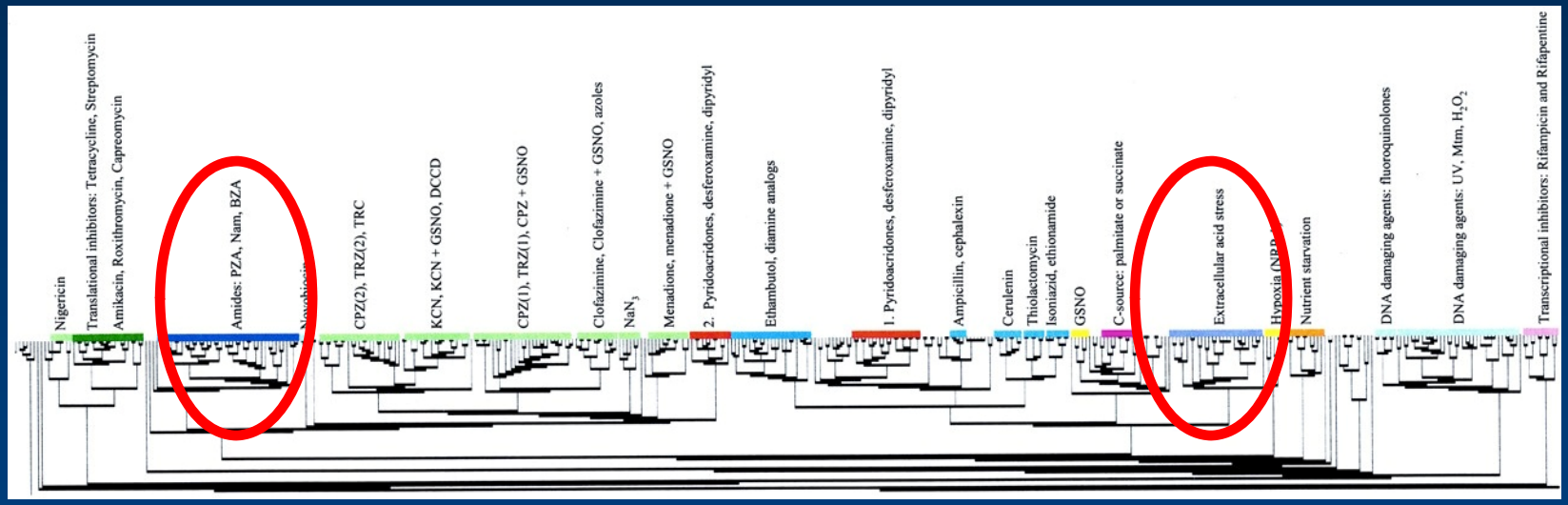
- Disruption of membrane function
- Acidification of cytoplasm
- Inhibition of FAS I
- Inhibition of trans-translation
- Interference with NAD metabolism

The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism: novel insights into drug mechanisms of action.
J Biol Chem. 2004 Sep 17;279(38):40174-84. **Boshoff HI, Myers TG, Copp BR, McNeil MR, Wilson MA, Barry CE 3rd**

Disruption of membrane function



Acidification of cytoplasm



Inhibition of FAS I

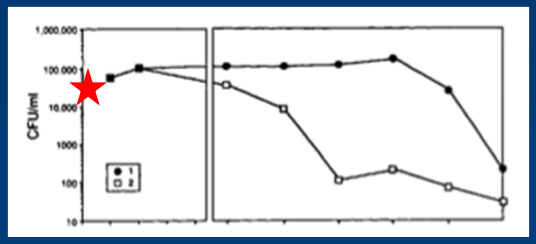
Inhibition of trans-translation

- What would be the impact of inhibition of trans-translation on transcriptional profiles?
- In the absence of gene expression data on this, we will leave the relevance of this potential mechanism to be weighed by others based on other data types

Interference with NAD metabolism

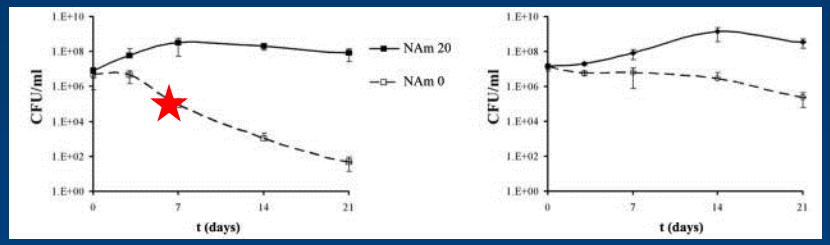
- Is there a data set that will allow this comparison?
- Yes, but has substantial differences from the Boshoff et al. 2004 data set

PZA^{50} 7H12 pH 5.0



day 2 3 4 5 9 16 24 30 37 45

M. bovis ΔnadABC *M. tuberculosis ΔnadABC*

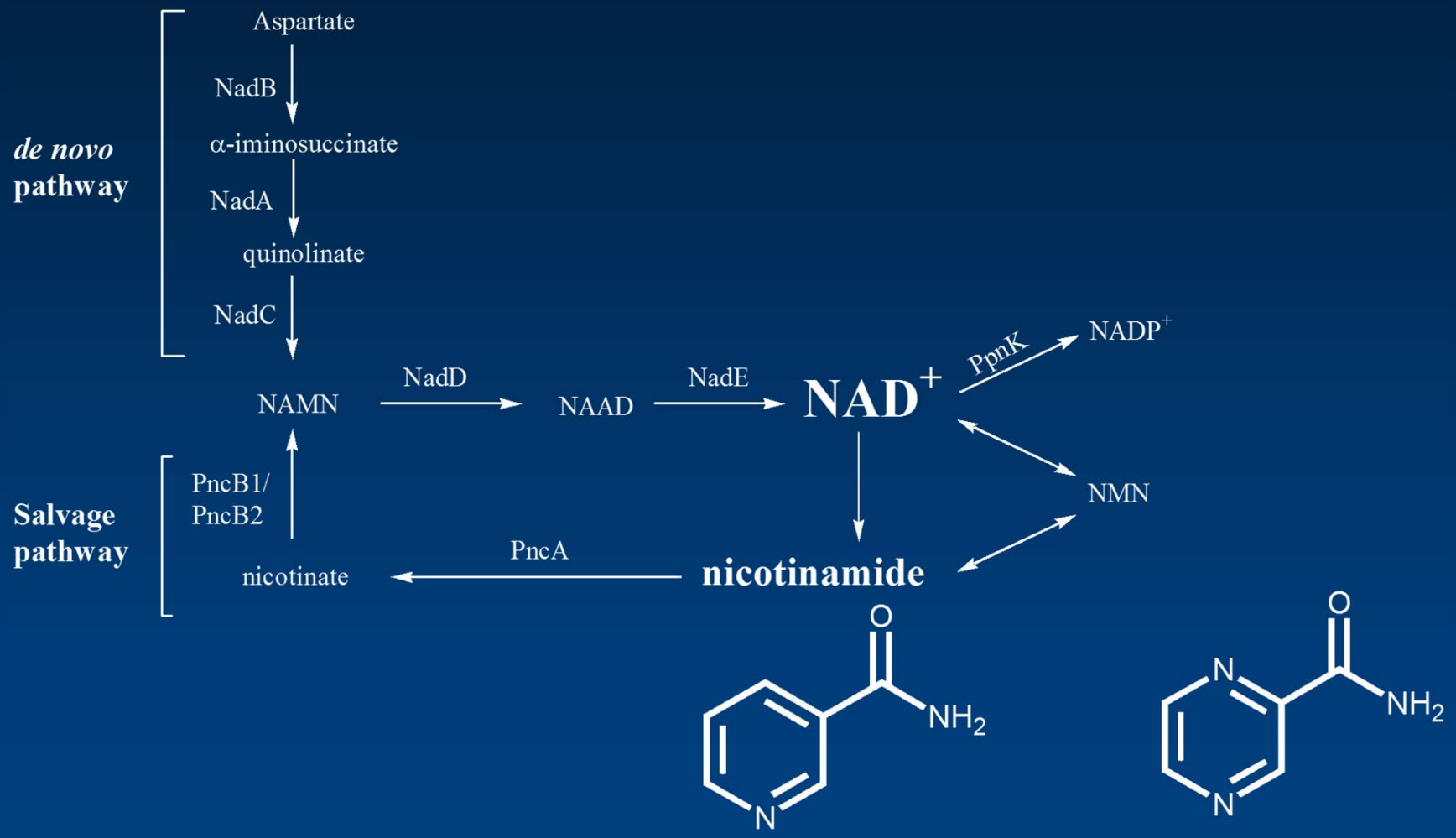


★= time point of transcriptional profile

Pyrazinamide sterilizing activity in vitro against semidormant Mycobacterium tuberculosis bacterial populations.
Am Rev Respir Dis. 1992 May;145(5):1223-5. Heifets L, Lindholm-Levy P.

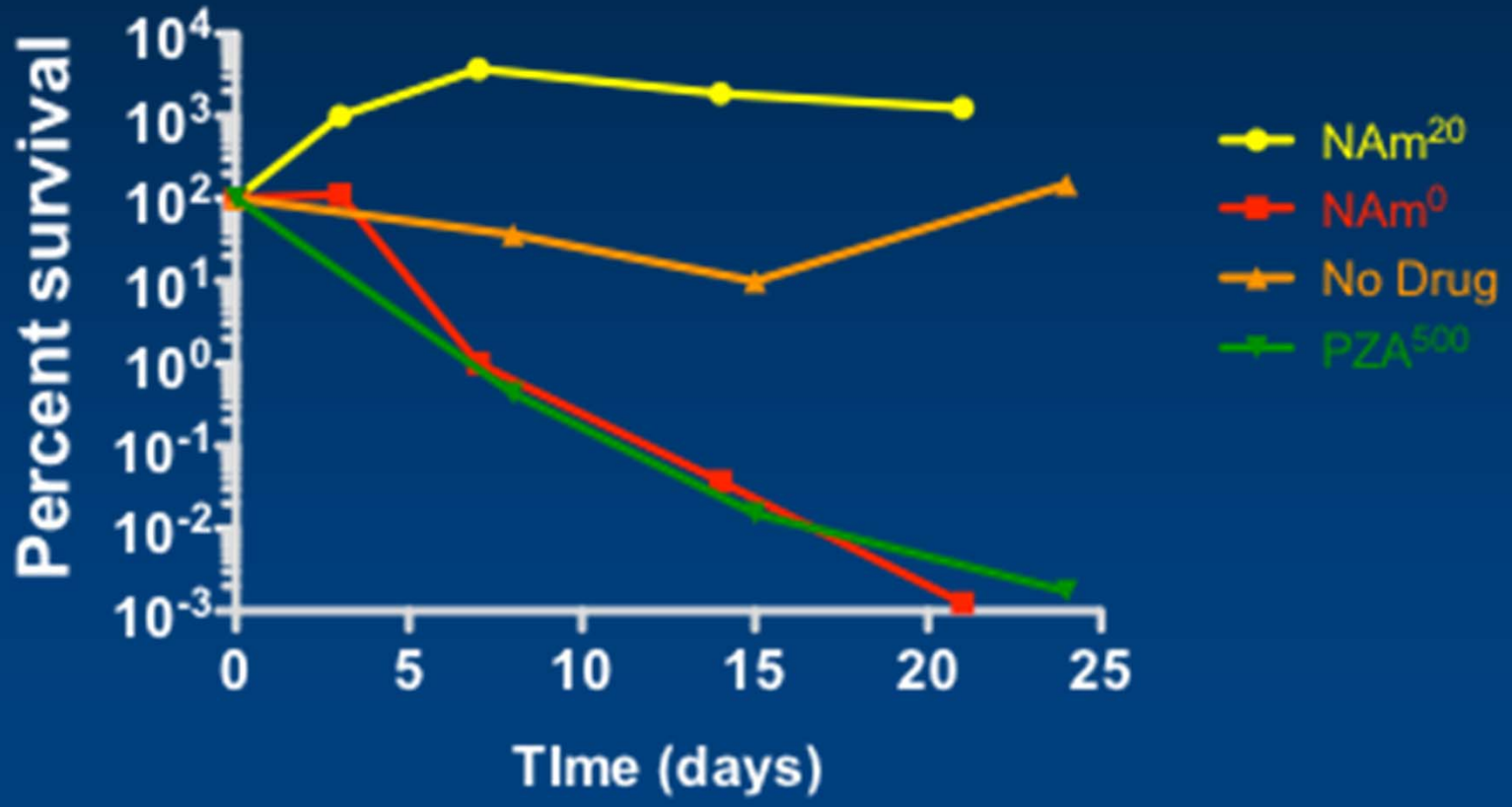
NAD+ auxotrophy is bacteriocidal for the tubercle bacilli.
Mol Microbiol. 2010 Apr;76(2):365-77. Epub 2010 Feb 28. Vilchèze C, Weinrick B, Wong KW, Chen B, Jacobs WR Jr.

NAD^+ biosynthetic pathway



PZA

PZA treatment and NAm starvation kill with similar kinetics



Substantial intersection of PZA 16hr w/ *M. bovis* NAD starvation

Intersection includes heat shock, protease, antioxidant, aerobic, electron transport, transcription/translation, transport (mmpLs), and lipid anabolism genes

So, proximal PZA mode of action is consistent with disrupted NAD metabolism, what's the target?

Hypothesis:

PZA activity requires sensitizing the cell - something up or down at low pH – it's not the pH that's important, it's the induction / repression (may happen in vivo at higher pH)

Rationale:

Low extracellular pH enhances POA accumulation but does not lower cytoplasmic pH, could the target be repressed at low pH, making it more vulnerable?

Low pH may sensitize *M. tb* to perturbations to NAD(P) metabolism

pH 5.5 up	
protease	heat shock
antioxidant	pckA
gltA	*mez*
cydA	*pdhA*
nadB	*citE*
pncA	*asd*
mmpL	*ndh*

pH 5.5	down		
transcription	translation	lipid	anabolism
pntAB	*emb*	*panD*	*ribG*
fum	*mmpL*	*fts*	*rpsA*
mdh	*proC*	*fix*	*glcB*
atp	*adhB*	*ilv*	*panB*
nuo	*zwf*	*pca*	*mbt*
acpS	*inhA*	*mtr*	*ndk*

Annotated pH-regulated genes have a preponderance of genes that code for NAD(P) requiring or synthesizing enzymes

Mycobacterium tuberculosis invasion of macrophages: linking bacterial gene expression to environmental cues. Cell Host Microbe. 2007 Nov 15;2(5):352-64. **Rohde KH, Abramovitch RB, Russell DG.**

Conclusions

- PZA mode of action is consistent with disruption of NAD metabolism
- Low pH may sensitize *M. tb* to perturbations of NAD(P) metabolism
- Overexpression of low pH downregulated essential genes that require NAD(P) may increase PZA resistance and reveal target/s

Use of Structural Analogs to Find the PZA Target(s)

Demystifying Pyrazinamide

September 4-5, 2012

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Oren Zimhony Kaplan Medical Center Hebrew University Israel
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Jiaoyu Deng Wuhan Institute of Virology, Chinese Academy of Sciences
Jeff Cox UCSF SF
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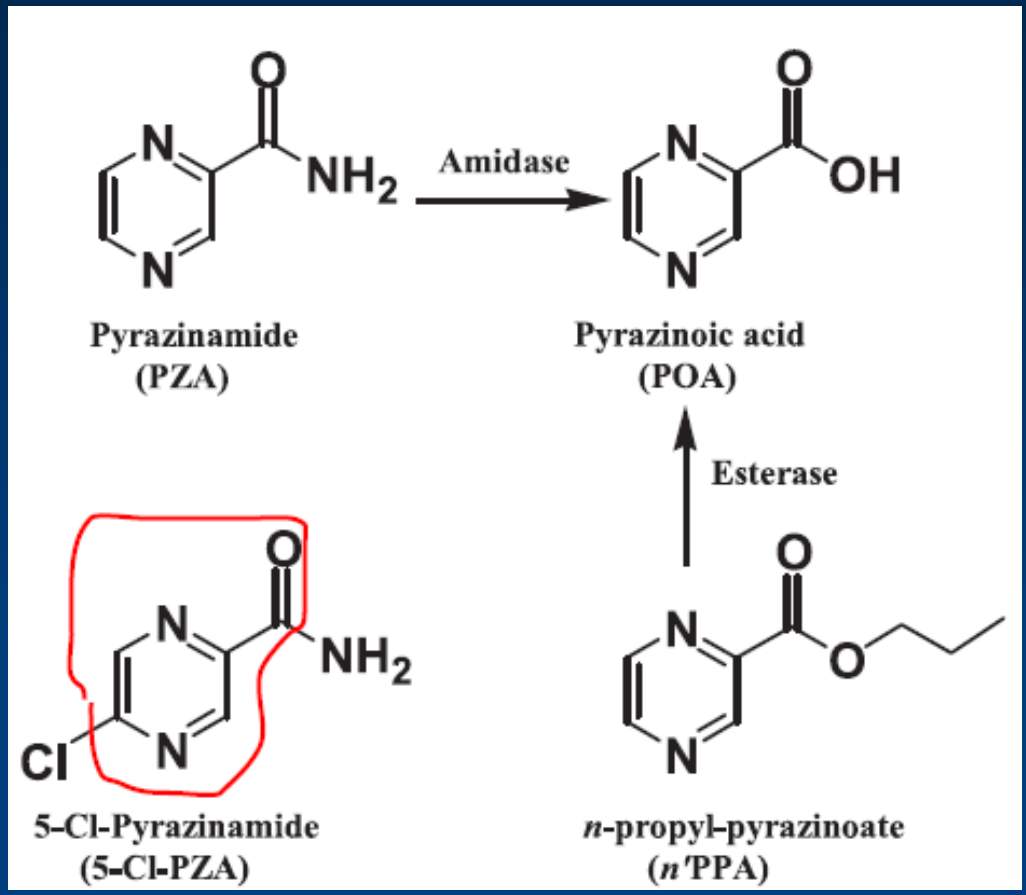
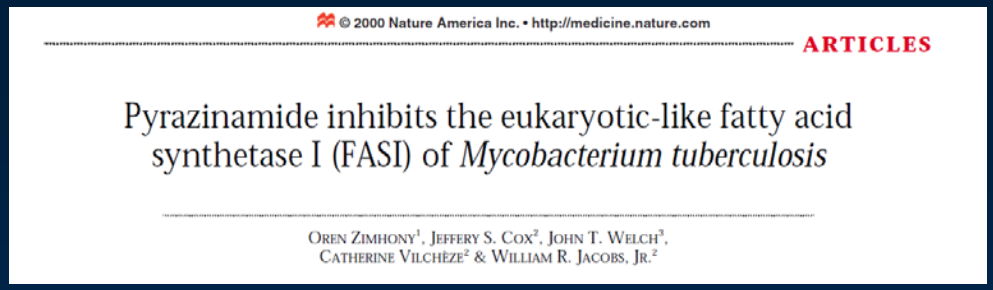
The minimal structure of pyrazine ring with an acyl group confers anti-mycobacterial activity due to a common target





Pyrazinamide inhibits the eukaryotic-like fatty acid synthetase I (FASI) of *Mycobacterium tuberculosis*

Oren Zimhony[1], Jeffery S. Cox[2], John T. Welch[3], Catherine Vilchèze[2] & William R. Jacobs, Jr.[2]

Hypothesis: PZA inhibits *Mtb* FAS I

Evidence that FAS I is the target of PZA and POA:

- Overexpression of *fas1* confers resistance to the analog 5-Cl-PZA in *M. smegmatis*
- PZA / POA and 5-Cl-PZA inhibit C16:0 synthesis in replicating bacilli in correlation with anti-mycobacterial activity
- 5-Cl-PZA, POA inhibit FAS I in cell free assay

Problems

- Controversy whether POA inhibits FAS I (H Boshoff et al.)
- No ability to overexpress *fas1* in *Mtb*.
- No POA^R or 5-Cl-PZA^R mutants in tubercle bacilli
- Inoculum effect makes it difficult to correlate FAS I inhibition with bacteriocidal effect.

POA inhibits fatty acid synthesis in replicating tubercle bacilli

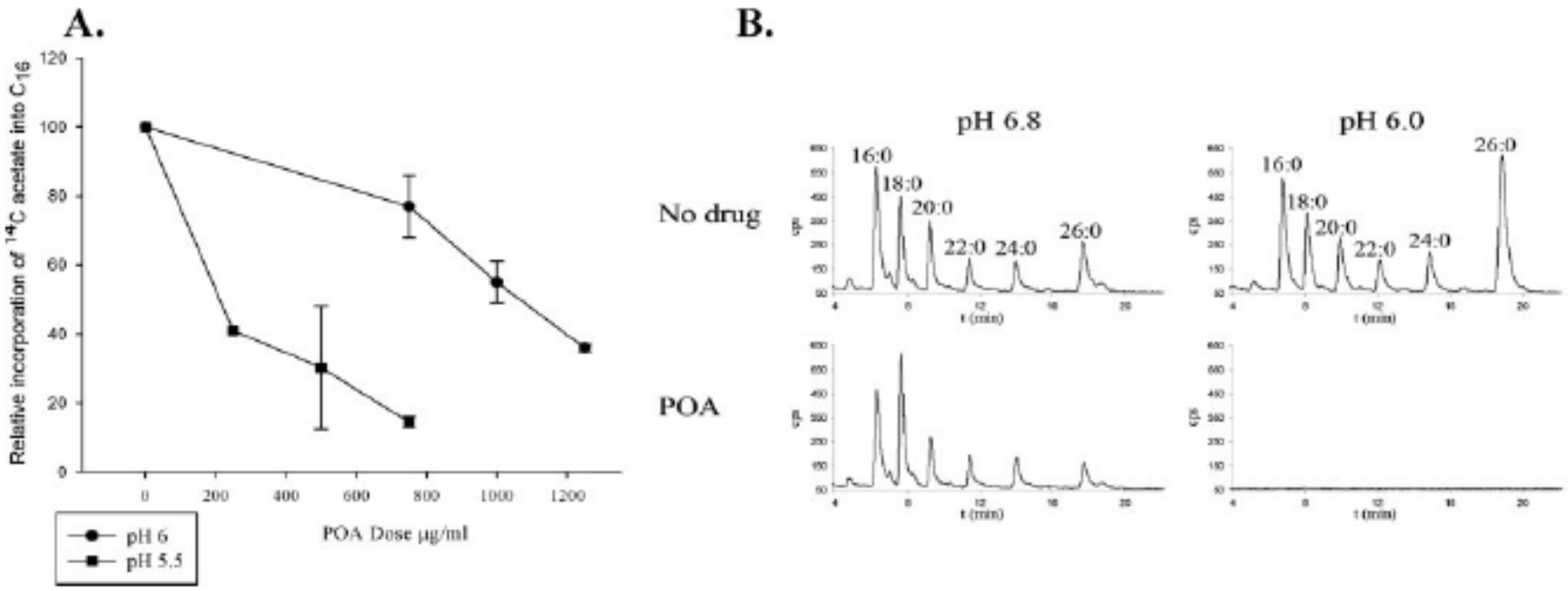
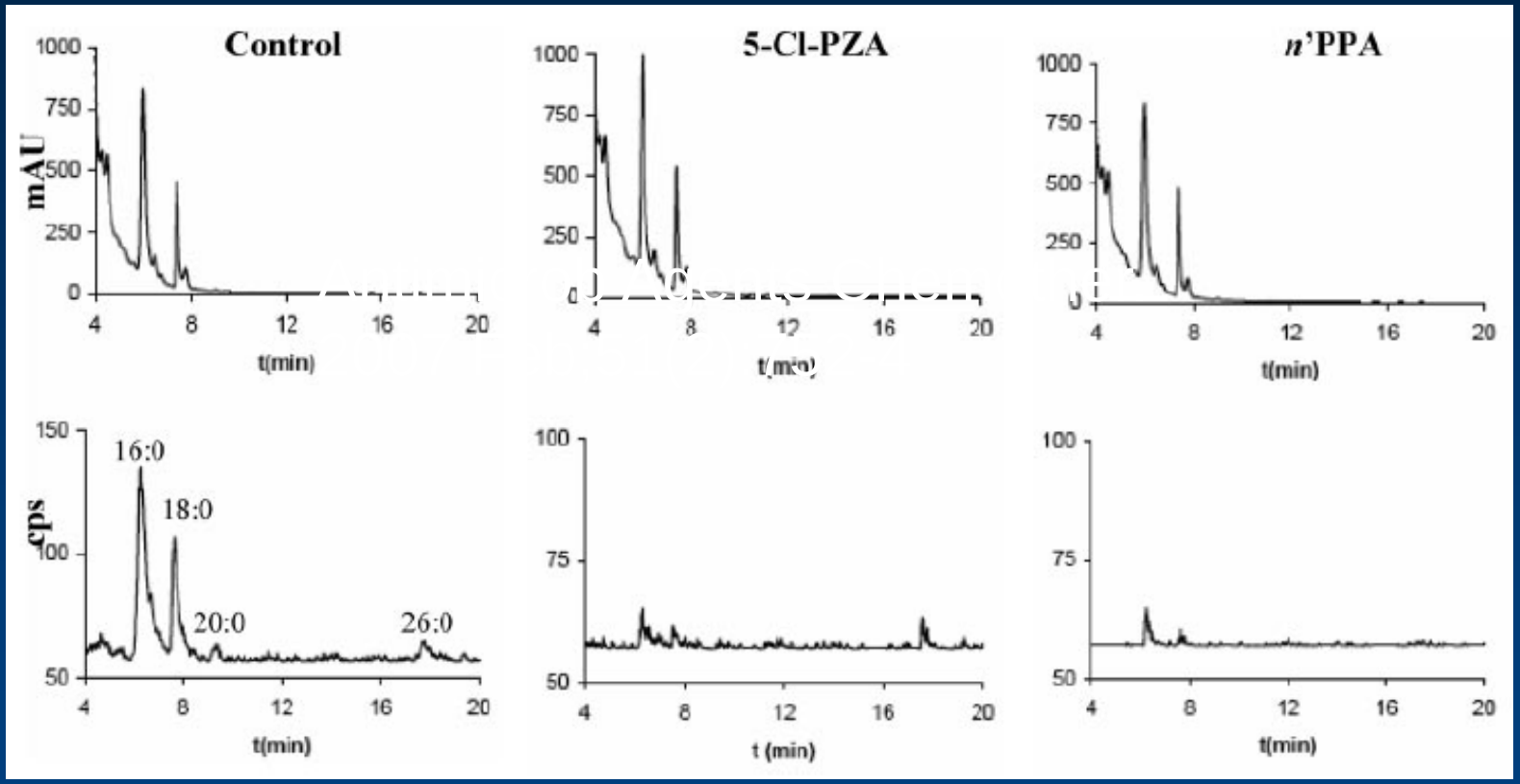


FIG. 3. (A) Quantitative analysis of 1-^{14}C-labeled C_{16} (*y* axis) from *M. tuberculosis* bacilli treated with different concentrations of POA (*x* axis) at pH 5.5 or 6. (B) HPLC chromatogram of extracted 1-^{14}C-labeled fatty acids from *M. bovis* BCG bacilli treated with POA (1,500 µg/ml) at either pH 6 or 6.8.

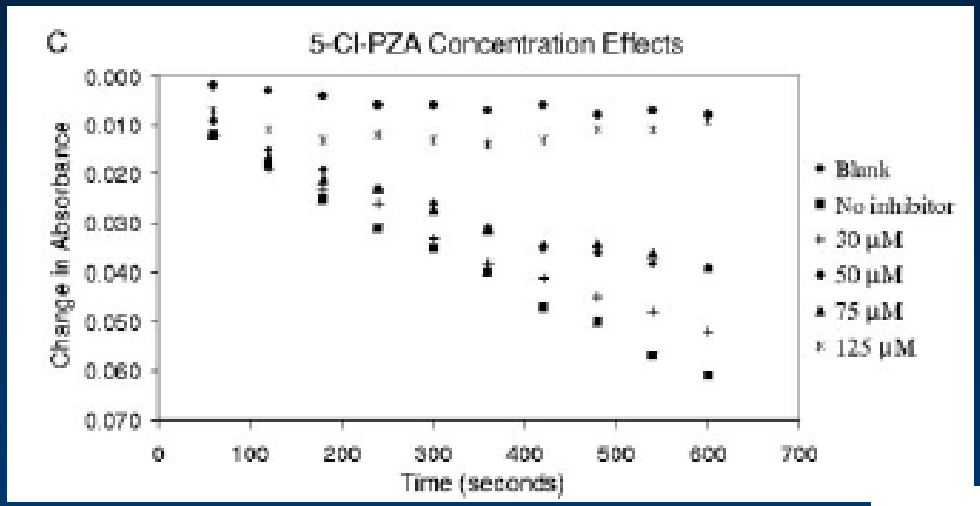
Zimhony O et al Antimicrob Agents Chemother. 2007 Feb;51:752-4

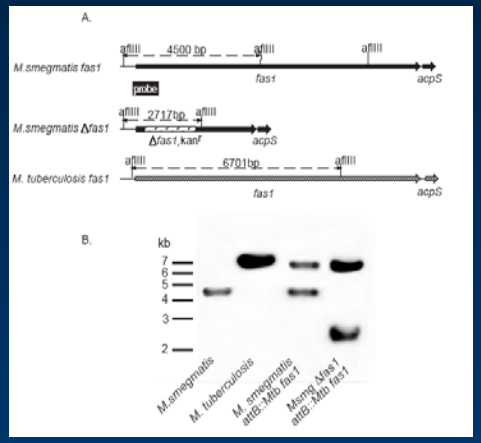
A Pyrazinoate Ester, *n*'PPA inhibits fatty acid synthesis like 5-Cl-PZA in *M. tuberculosis*



Zimhony O et al Antimicrob Agents Chemother. 2007 Feb;51:752-4

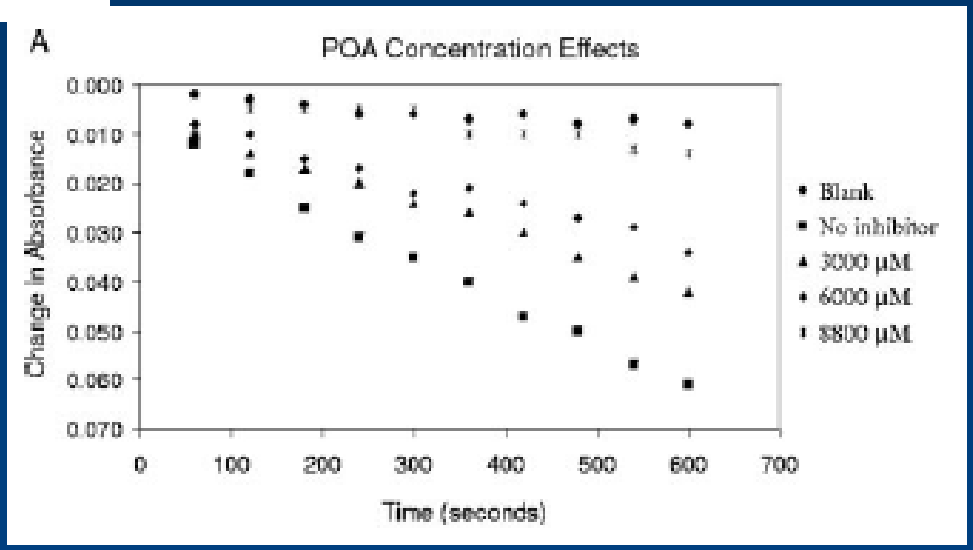
M. tuberculosis FAS I inhibition in cell free system using NADPH oxidation



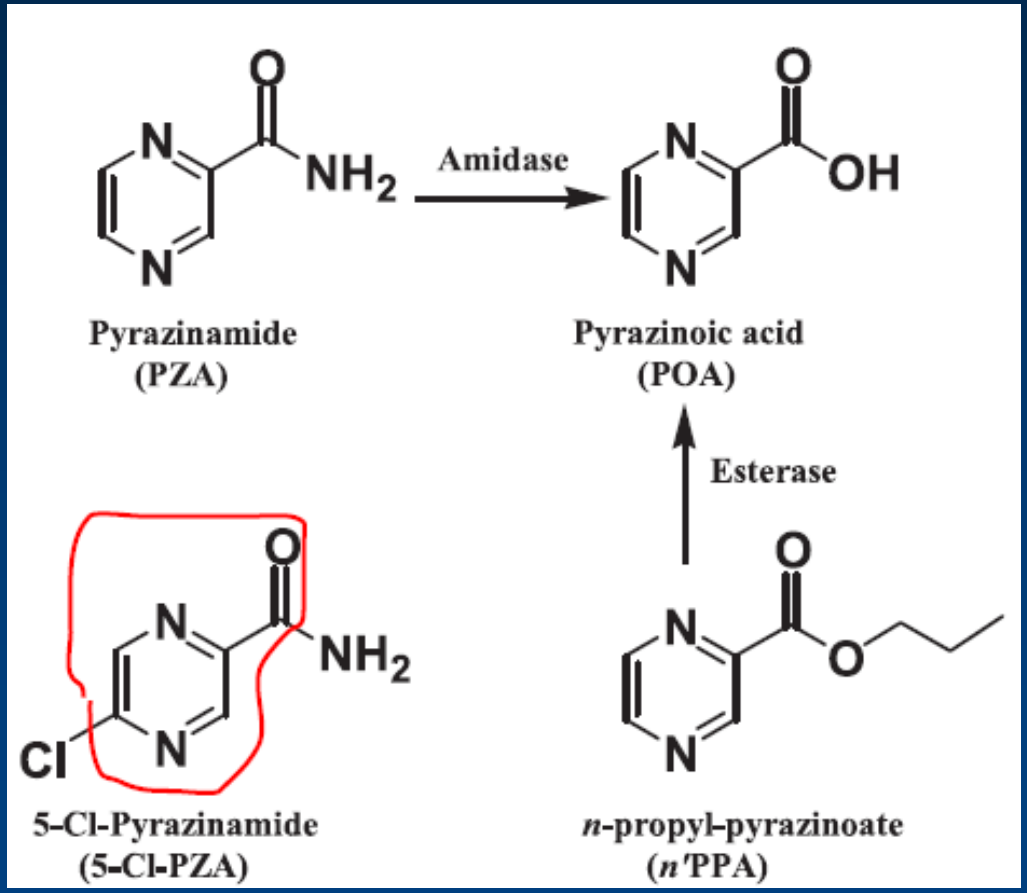


Effect of increasing inhibitor concentration on NADPH oxidation

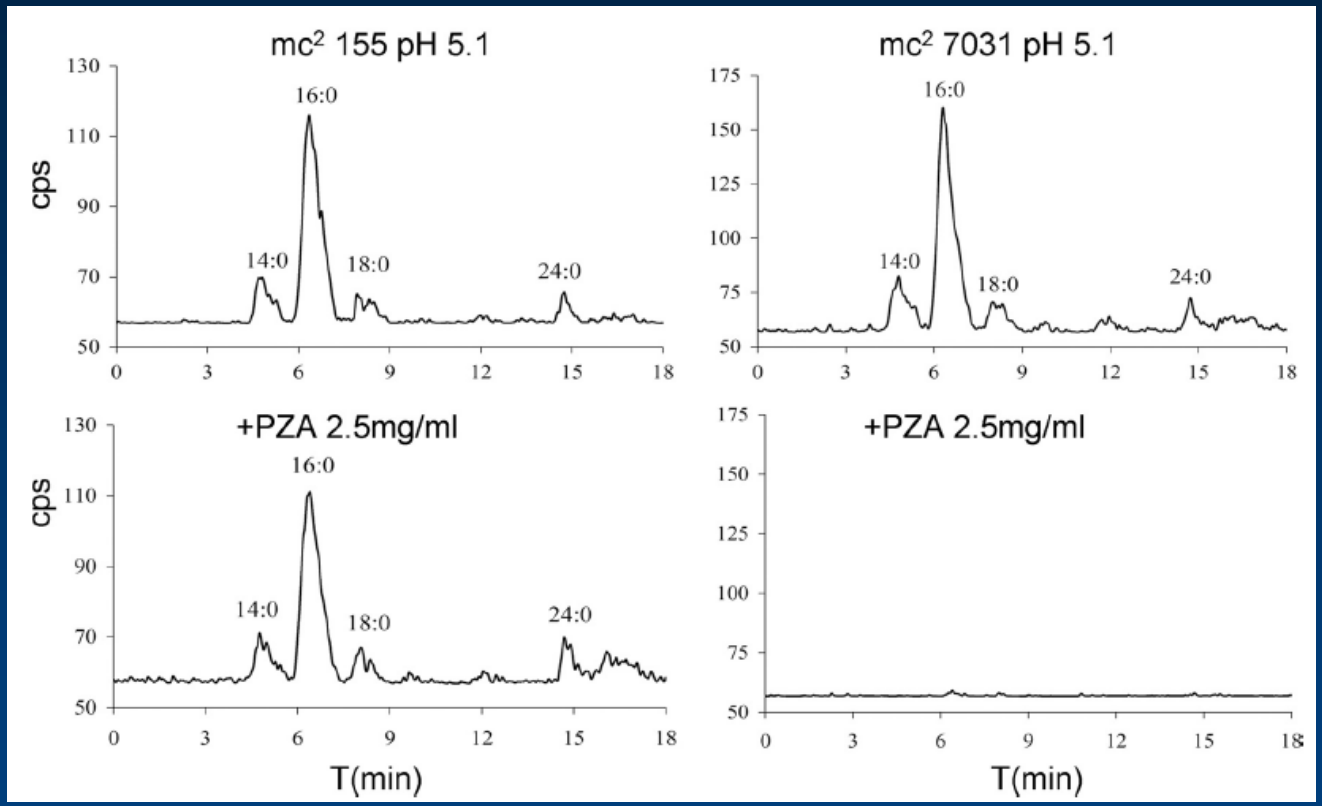
Ngo S.C et al Antimicrob Agents Chemother. 2007 Jul;51:2430-5



The minimal structure of pyrazine ring with an acyl group confers anti-mycobacterial activity and FAS I inhibition



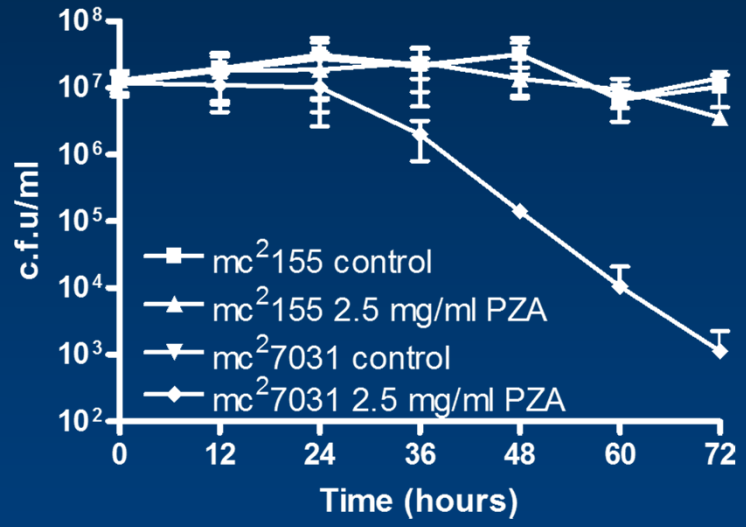
Susceptibility to PZA in PZA susceptible strain of *M. smegmatis* translates into inhibition of FA synthesis



HPLC analysis of C16:0 to C26:0 fatty acids from PZA-treated *M.smegmatis* mc²155 and mc²7031, treated with 2.5 mg/ml PZA for 2 h, and then pulsed with [1-^{14}C]acetate for an additional 2 h

Baughn AD Antimicrob Agents Chemother. 2010 Dec;54:5323-8

Inhibition of FAS I in PZA susceptible *M. smegmatis* correlates with bactericidal effect



Binding of PZA and POA to *Mtb* FAS I through STD NMR studies

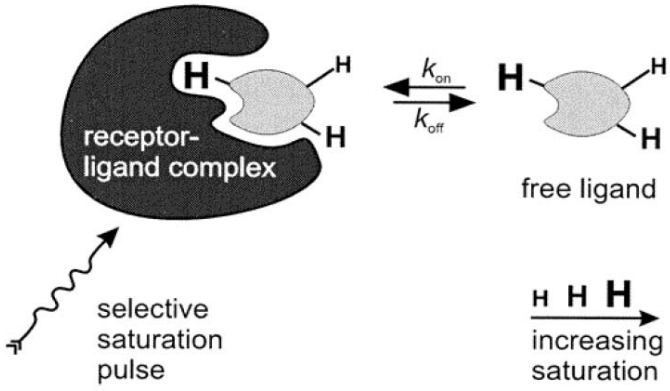
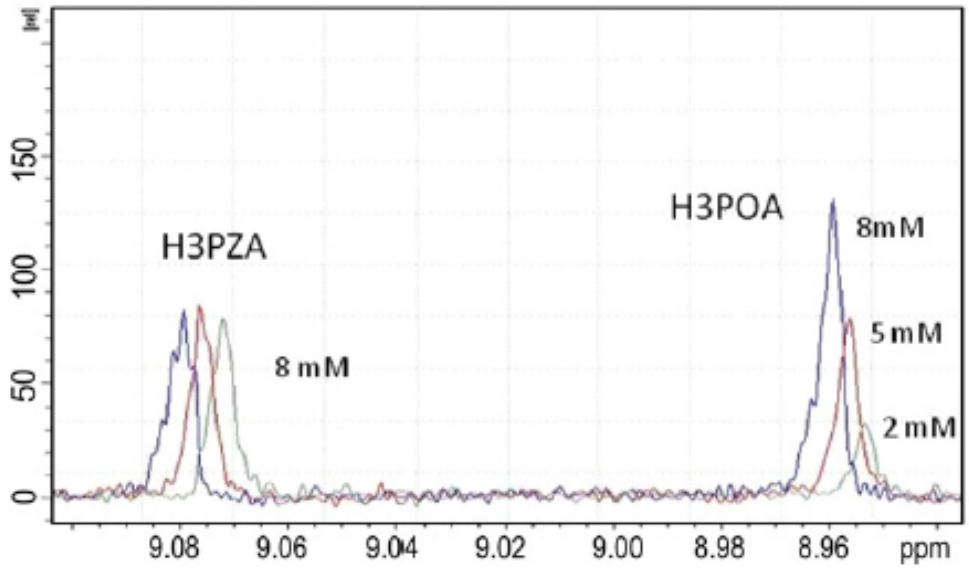


Figure 8. Cartoon depicting STD-NMR experiment.[135]



Sayahi H. et al
Bioorganic and Medicinal Chemistry letters 2011 Aug 15;21:4804-7

A unifying model/proposal for PZA activity

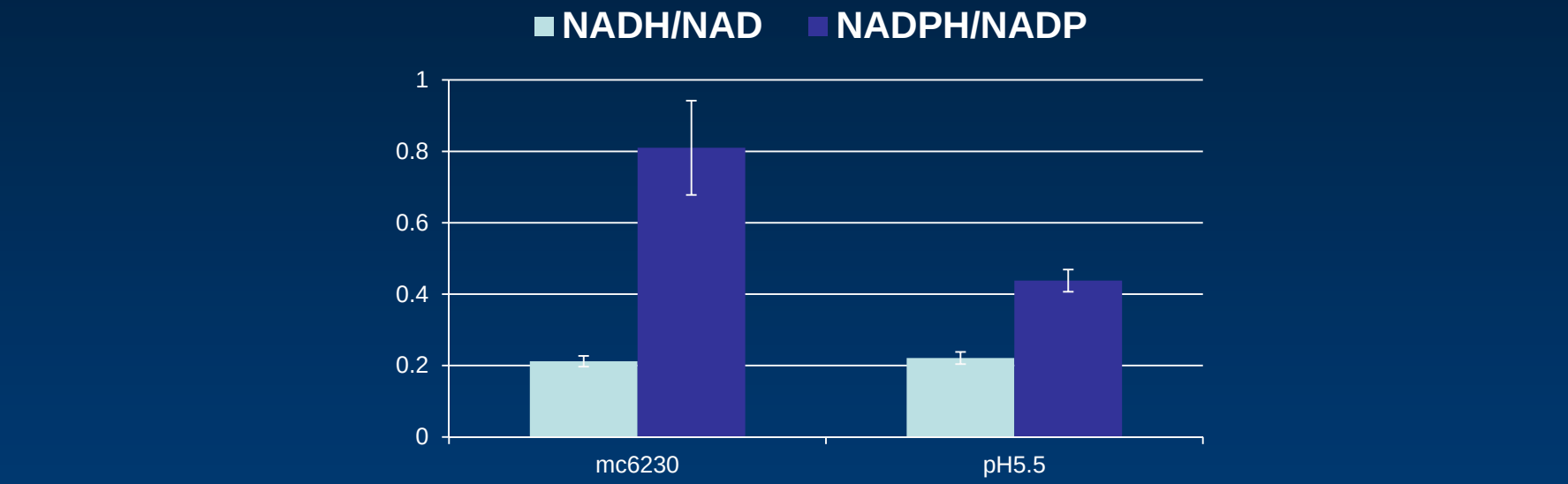
- PZA/POA affects replicating bacilli.
- PZA particularly affects replicating bacilli that reside within extracellular anoxic environment.
- This particular condition is associated with "huge accumulation" (Zhang Y et al J. Bacteriol 1999 181, 2044-9) of POA.
- POA activity then results in complete fatty acid synthesis inhibition leading to cell death.

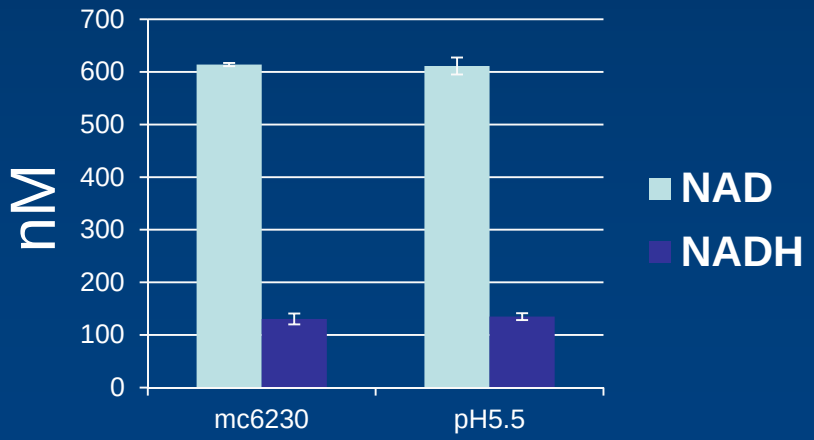
Zimhony O et al Dissecting " mechanism of action" into conditions for susceptibility and to target identification through pyrazinamide analogs. Review in preparation

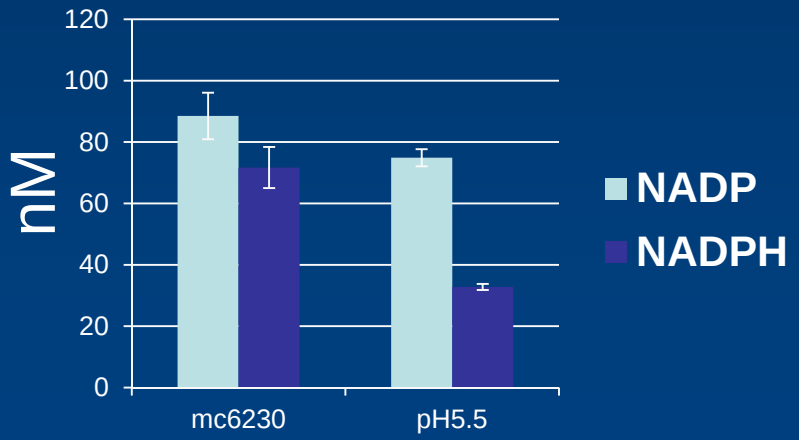
Further studies to test how FAS I inhibition correlates with the death of *M. tuberculosis*

- Isolate POA resistant mutant of *Mtb*
- Generate conditionally inactivated *fas 1*
- Test the effect of *fas 1* domain overexpression on resistance
- PZA Microarrays using 5-Cl-PZA and POA (later time points)
- Metabolomic analyses
- Measure accumulation of POA in *Mtb* bacilli in vivo.

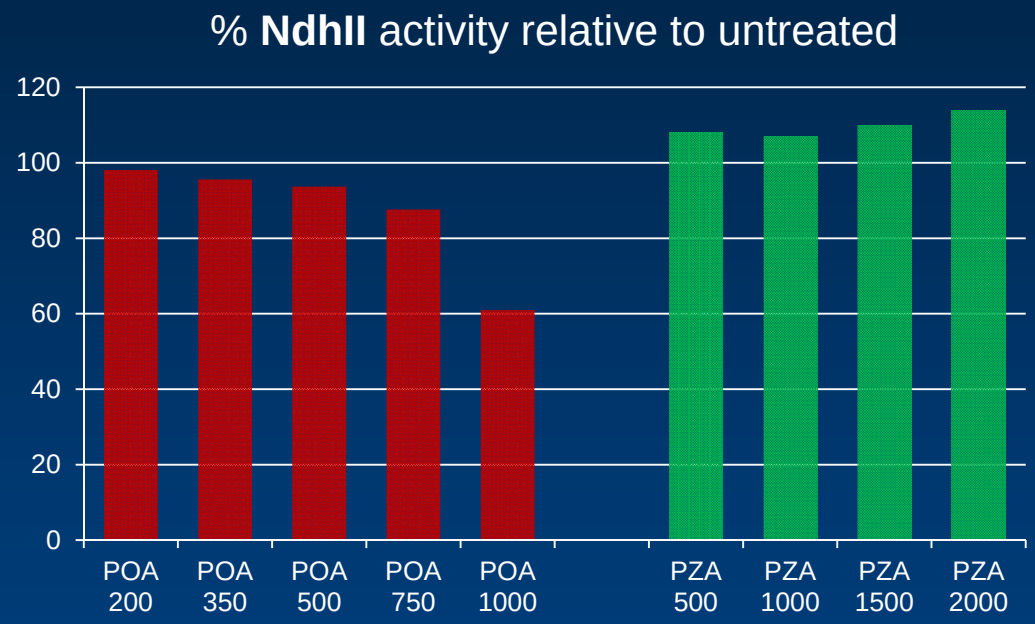
Cofactor concentrations in mc²6230 grown at normal pH (6.8) vs pH 5.5

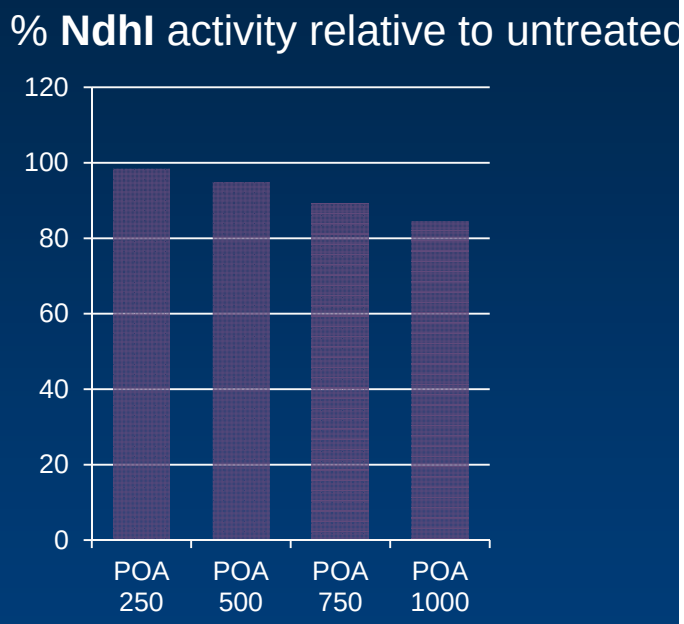






Effect of POA and PZA on Ndh type I and Ndh type II activities





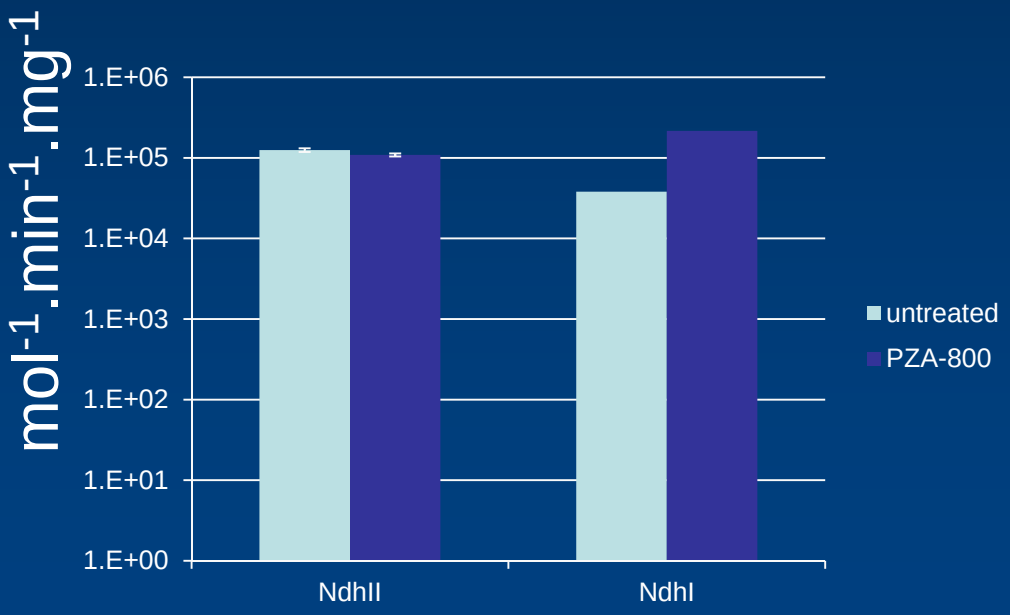
The membrane fraction was isolated from 100 ml $mc^2$6230 culture (OD =0.8) and resuspended in 0.4 ml MES buffer (pH 5.5).

10 μl of membrane fraction was treated with POA or PZA at rt for 20 min prior to measuring Ndh activity.

Concentrations of POA or PZA are in mg/l.

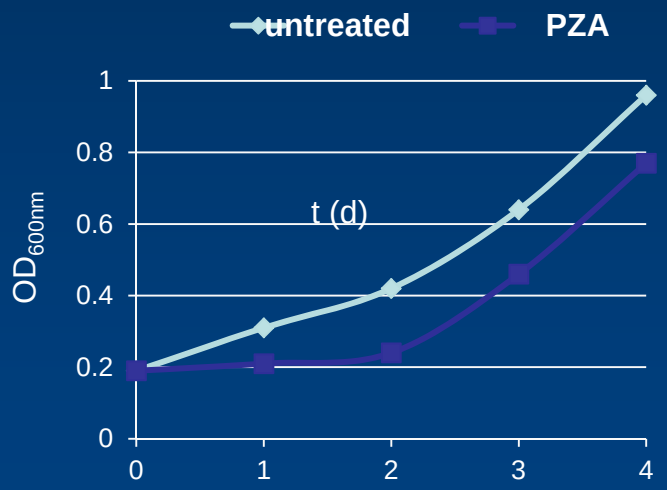
Effect of PZA on Ndh type I and Ndh type II activities

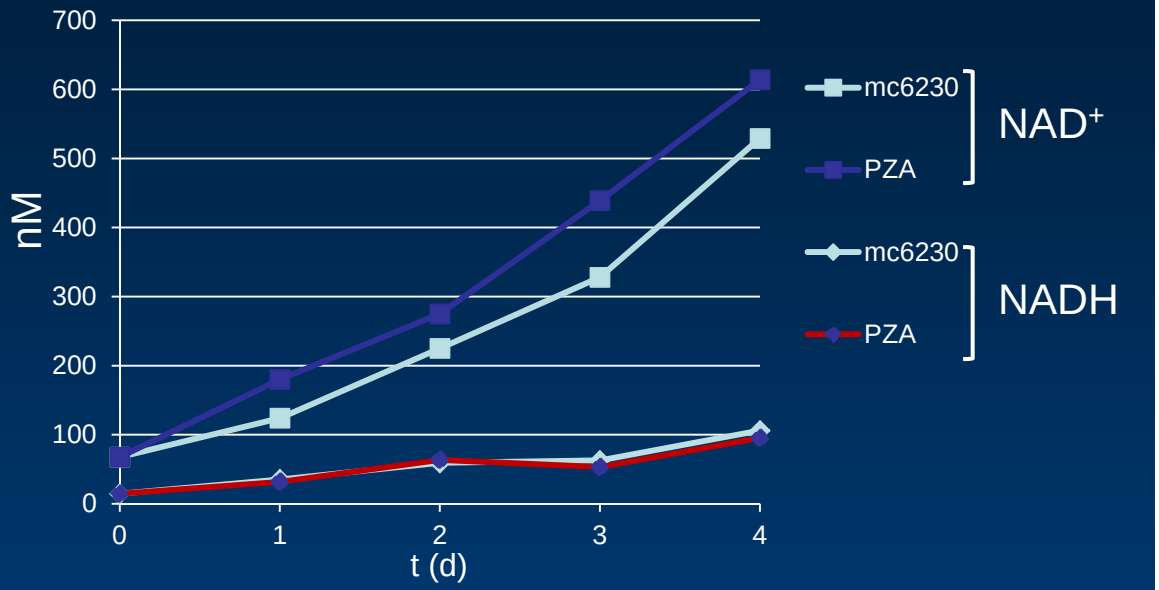
mc^{2}6230, grown in 7H9-OADC-gly-MES-tylo (pH 6.0), was treated with PZA (0.8 mg/ml) for 3 days prior to membrane fraction isolation and measurement of Ndh type I and type II activities.

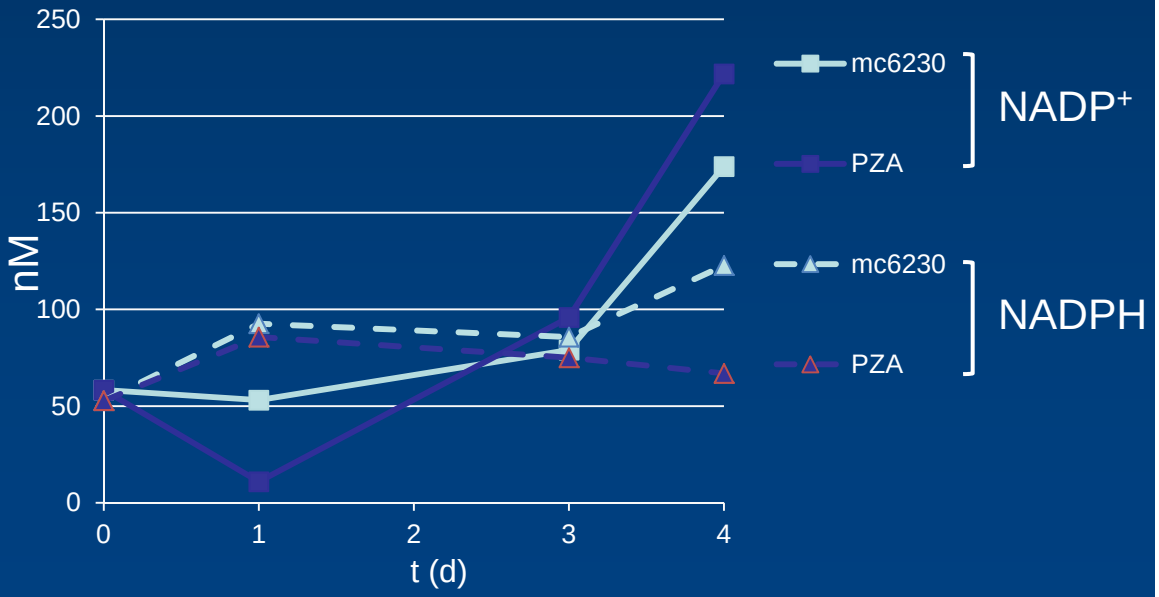


Nucleotide concentrations

Growth of mc²6230 in 7H9-OADC-MES (pH 6.0) treated or not with PZA (0.8 mg/ml)







Growth of H37Rv with PZA at pH 6.0