Effects of PZA and PZA analogs

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Acknowledgements

Halimah Sayahi

Beatriz Bolivar

Acknowledgements

Jason Seeley

Kaitlin Peck

Outline and Introduction

- *Hypothesis*
  - Pyrazinamide, a nicotinamide analog, may have more than one effect on either the pathogen *or the host*.
- *Pyrazinamide in anti-tuberculous therapy*
  - Nicotinamide analog as an anti-tuberculous drug in 1952
  - *Efficacy established directly in murine model*
  - Clinical utility in combination with other anti-tuberculous agents to decrease the duration of therapy
- *Pyrazinamide analogs*
  - Pyrazinoate esters
  - 5-Substitution
- *Pyrazinamide as an anti-leishmanial agent*
  - *Superior activity in murine model*

Outline (contd)

- *Inhibition of M. tuberculosis FAS I*
  - Spectrophotometric assay
  - Radiometric assay
  - Saturation Transfer Difference (STD) NMR studies
    - The binding epitope
    - NADPH binding
    - Binding of pyrazinamide analogs
- *Binding of PZA and PZA analogs to human sirtuins SIRT1, 2, 3, 5, and 6*
    - STD NMR experiments
    - Docking studies
    - Spectrophotometric assays
- *Summary*
- *Acknowledgments*

Antimycobacterial Activity of Pyrazinoate Esters

Table 1. Minimum Inhibitory Concentrations (MIC)[a] of Pyrazinoic Acids or Amides against Various Mycobacteria

	M. avium complex		*M. kansasii*	*M. tuberculosis*		
pyrazinoic acid (amide)	101[b]	ATCC 49601	S[c]	ATCC 35801	ATCC 27294	ATCC 35828[d]
pyrazinoic acid	>1024	>1024	256	32	32	32
pyrazinamide	>2048	>2048	2048	32	16	2048
5-chloropyrazinoic acid	1024	>1024	64	64	64	64
5-methylpyrazinoic acid	1024	>1024	1024	64	64	>64

[a] MIC is the minimum inhibitory concentration (μg/mL). [b] Clinical isolate from Lowell Young, Kuzell Institute for Arthritis and Infectious Disease. [c] Clinical isolate Veterans Administration Medical Center, Syracuse, NY. [d] Resistant to PZA.

Table 3. Minimum Inhibitory Concentrations (MIC) of Esters of 5-Chloropyrazinoic Acid against Various Mycobacteria[a]

		M. avium complex		*M. kansasii*	*M. tuberculosis*		
compd	5-chloropyrazinoate	101	ATCC 49601	S	ATCC 35801	ATCC 27294	ATCC 35828
10	methyl	256	64	2	8	4	16
11	ethyl	128	128	2	2	1	>16
12	*n*-propyl	16	16	0.25	≤0.03	0.06	0.25
13	*n*-butyl	64	128	≤1	0.5	0.25	0.5
14	*n*-pentyl	32	32	≤0.03	0.25	0.06	0.12
15	*n*-hexyl	32	16	0.015	0.125	0.125	0.5
16	*n*-heptyl	8	16	≤0.03	≤0.03	0.06	≤0.03
17	*n*-octyl	16	16	≤0.03	≤0.03	0.06	≤0.03
18	*n*-nonyl	32	16	≤0.125	1	≤0.03	≤0.03
19	*n*-decyl	32	16	0.5	0.25	0.5	0.5

Cynamon, M.H. ; Gimi, R.; Sharpe, C.A.; Bergmann, K.E.; Gyenes, F.; and Welch, J.T. "Pyrazinoic Acid Esters with Broad Spectrum *In vitro* Antimycobacterial Activity, " *J. Med. Chem.*. **1995**, *38*, 3902-3907

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5-chloropyrazinoic acid	1024	>1024	64	64	64	64
5-methylpyrazinoic acid	1024	>1024	1024	64	64	>64

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5-methylpyrazinoic acid	1024	>1024	1024	64	64	>64

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TABLE 1. MICs of pyrazinamide analogs for various mycobacteria

Organism	MIC (μg/ml) of:			
	PZA	5-Cl PZA	PA	5-Cl PA
M. tuberculosis strain				
ATCC 27294	64	16	32	128
ATCC 35801	32	16	32	64
ATCC 35828	>2,048	32	32	256
VA 205	>2,048	32	64	256
BDDIS 20	>2,048	32	64	256
DHMH 4319	2,048	8	16	128
CDC-BP-98	2,048	16	32	128
M. bovis strain				
ATCC 35720	>2,048	8	32	128
ATCC 27289	>2,048	8	64	256
Nontuberculous mycobacteria				
M. kansasii S	2,048	64	256	64
M. smegmatis 19420	>2,048	32	>2,048	512
M. fortuitum 49403	>2,048	32	>2,048	256
M. avium 49601	>2,048	32	>2,048	>1,024

R. J. Speirs, J.T. Welch and M. H. Cynamon, "In Vitro Antimycobacterial Activity of 5-Chloropyrazinamide," *Antimicrob. Agents Chemother.*, **1998**, *42*, 462-463

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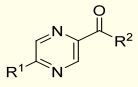
A Comparison of IC_{50} Values for PZA Analogs



Inhibitor	R^1	R^2	IC_{50}[a] (μM)
PZA	H	NH_2	8930
5-Cl-PZA	Cl	NH_2	151
n-Pr-POE	H	O-*n*-Pr	1722
t-Bu-POE	H	O-*t*-Bu	1359
n-Pr-5-Cl-POE	Cl	O-*n*-Pr	5.9
t-Bu-5-Cl-POE	Cl	O-*t*-Bu	15.1
n-Pr-5-F-POE	F	O-*n*-Pr	1.5
t-Bu-5-F-POE	F	O-*t*-Bu	4.3
n-Pr-5-Cl-PZA	Cl	N-*n*-Pr	8.1
t-Bu-5-Cl-PZA	Cl	N-*t*-Bu	8.7

[a]IC_{50} values obtained at 100 μM NADPH. Results obtained from spectrometric assays using purified FASI (3 μg/mL).

S. C. Ngo, O. Zimhony, W. J. Chung, H. Sayahi, W. R. Jacobs, Jr. and J. T. Welch. *Antimicrobial Agents Chemother*. **2007,** 51(7), 2430-2435

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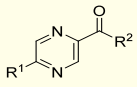

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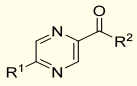

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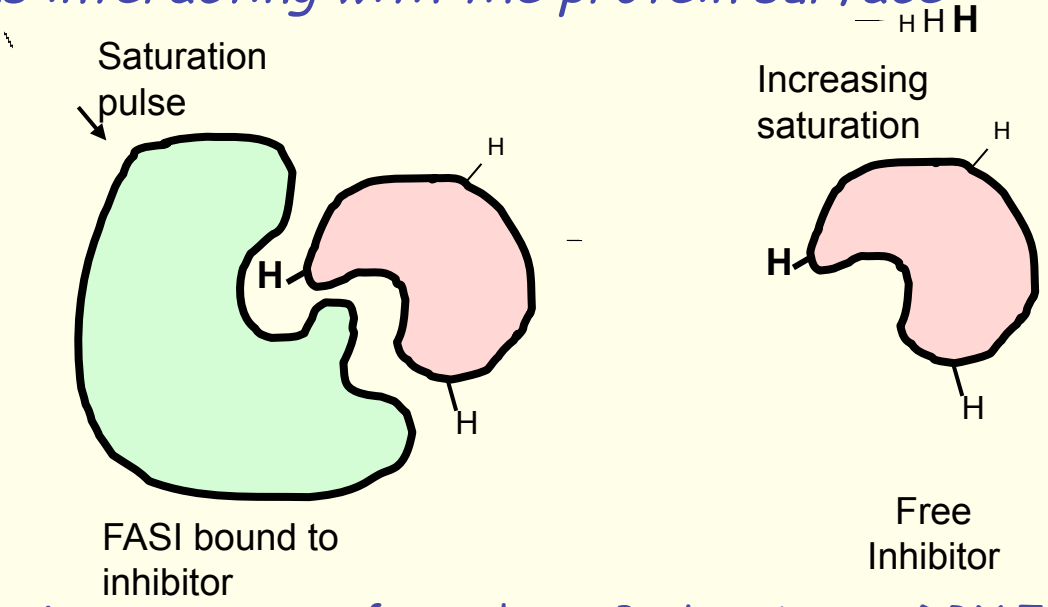
Saturation transfer difference (STD) NMR

- *Intermolecular transfer of magnetization from a protein to a bound ligand*[1,2]
  - Protein is irradiated with a selective saturation pulse
  - Spin saturation of the protein is transferred to the bound ligand
  - Ligand protons in close contact with the protein have a higher degree of saturation

1. Mayer, M.; Meyer. *J. Am. Chem. Soc*. **2001**, *123*, 6108-6117.
2. Wang, Y; Dingjiang, L; Daniel, W. *Magn. Reson. Chem*. **2004**, 42, 485-489.

Saturation transfer difference (STD) NMR

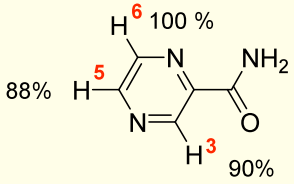
- *Cartoon of a inhibitor bound to FAS I showing different protons interacting with the protein surface*

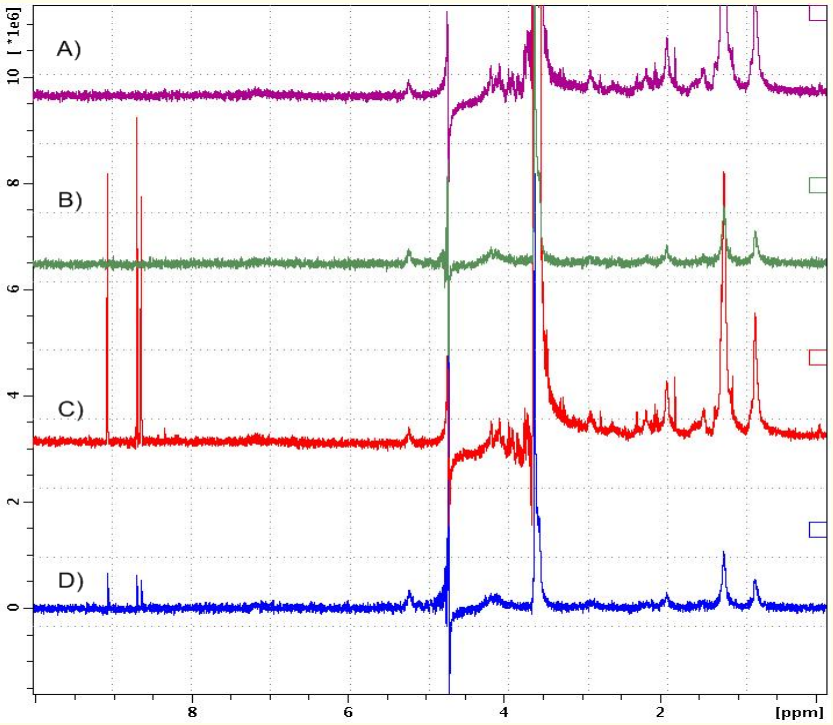


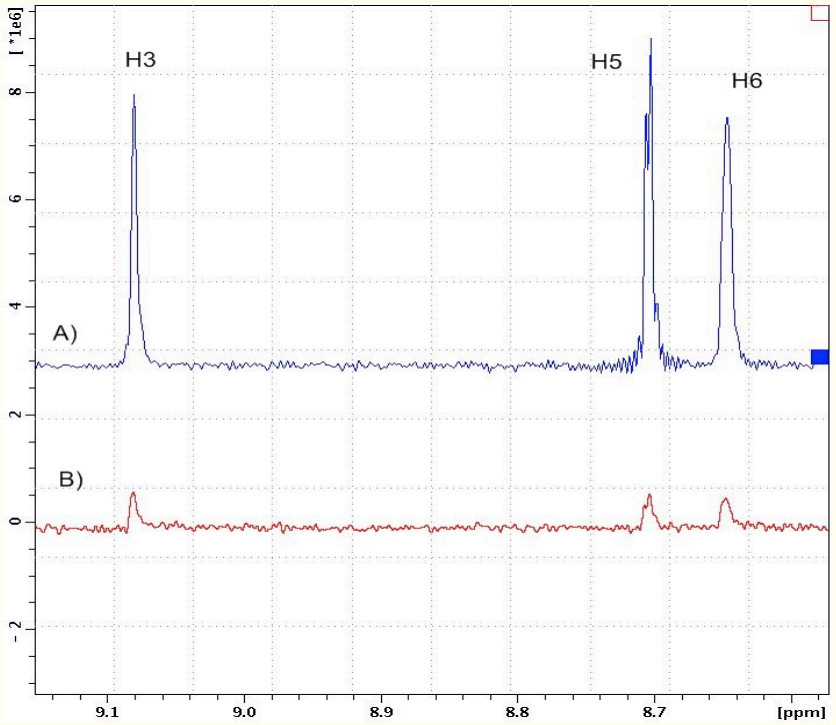
- Experiments were performed on a Bruker Avance DRX 700-MHz spectrometer equipped with a 5-mm inverse triple-resonance probe head at room temperature.
- NMR samples were prepared in 500 µL D20 containing 100 mM NaCl and 10 mM KPB buffer at 7.2 pH.
- The pulse sequence was a modification of the pulse sequence in Mayer.[1]

1. Mayer, M.; Meyer. *J. Am. Chem. Soc*. **2001**, *123*, 6108-6117.

STD NMR results: PZA binds to *Mtb* FASI



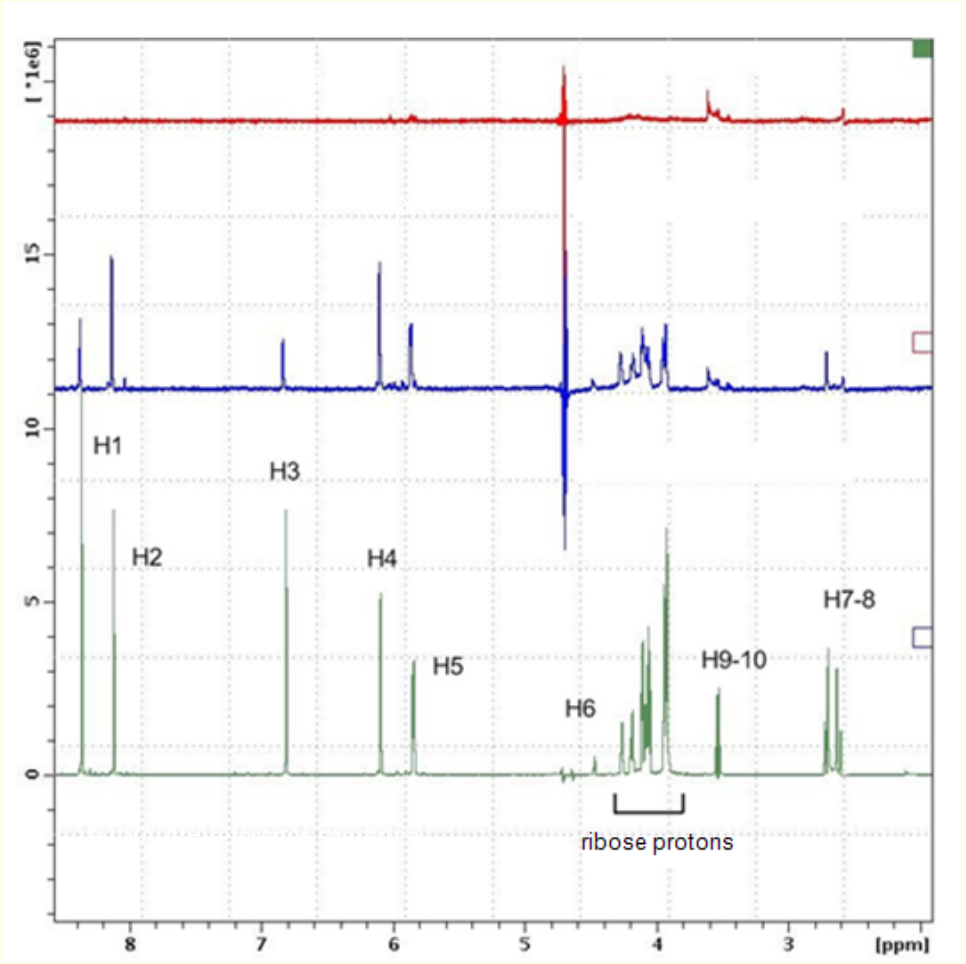


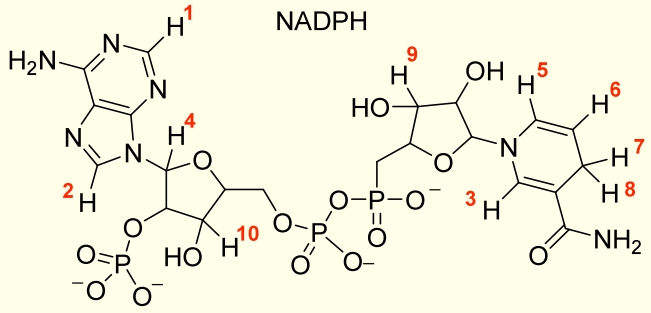


A) NMR spectrum of *Mtb* FAS I with $T1\rho$ filter
B) STD spectrum of FAS I
C) NMR spectrum of FASI (0.2 μM) and PZA (500 μM)
D) STD spectrum corresponding to C

A) Reference NMR spectrum of FAS I and PZA
B) STD spectrum of the mixture in A

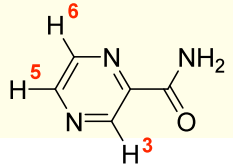
STD NMR of NADPH

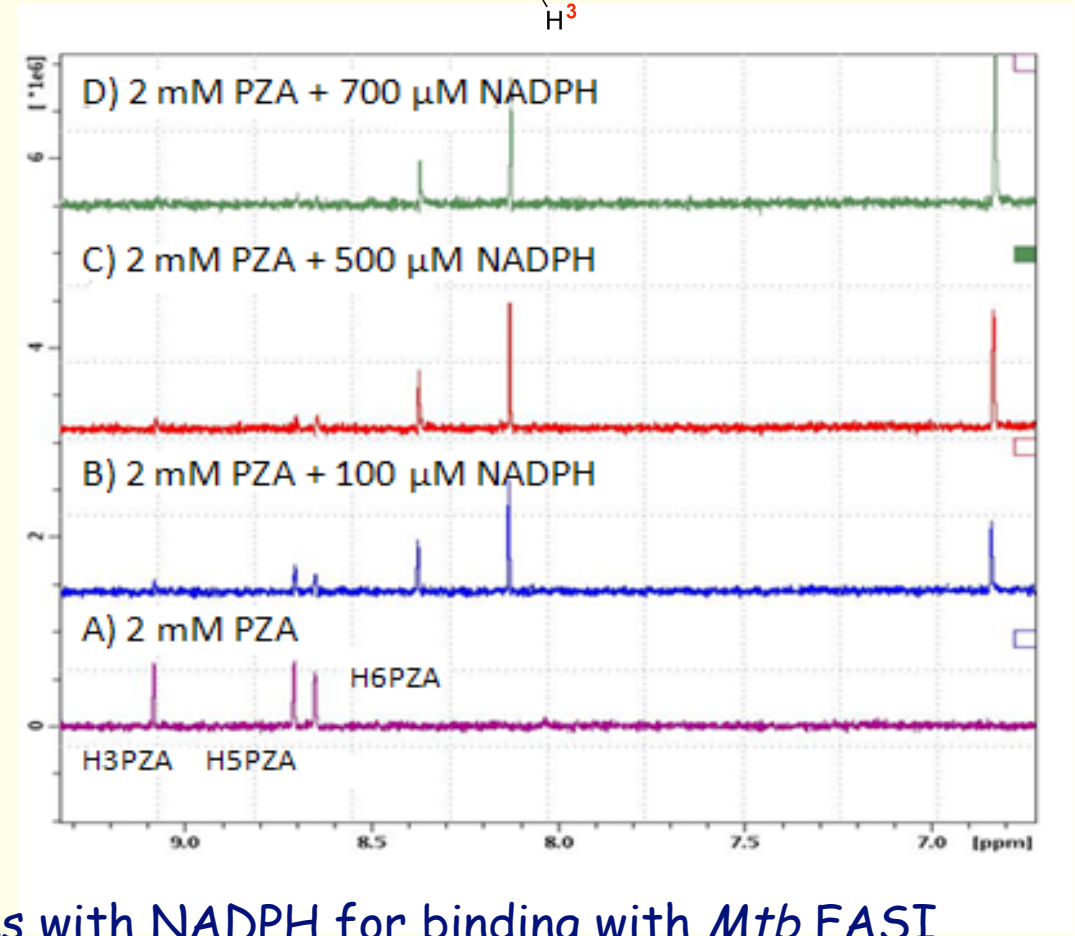




NADPH resonance	STD signal saturation (%)
H1	30
H2	52
H3	32
H5	92
H6	13
H7-H8	34

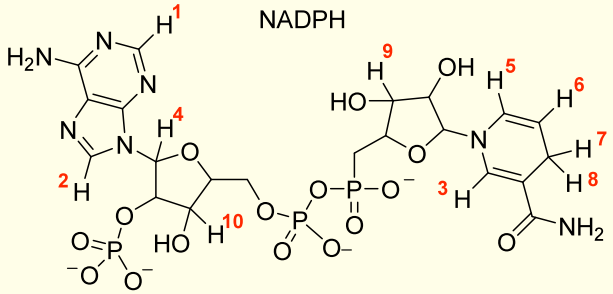
PZA and NADPH bind competitively to FASI

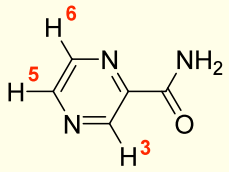


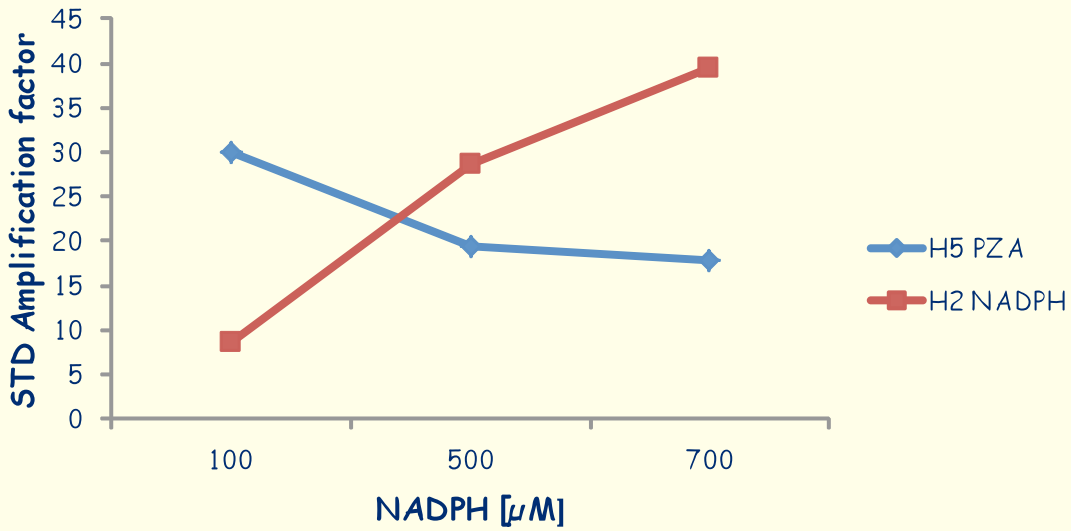


PZA competes with NADPH for binding with *Mtb* FASI.
STD signal of PZA resonances decrease upon increasing concentration of NADPH.

NADPH displaces PZA



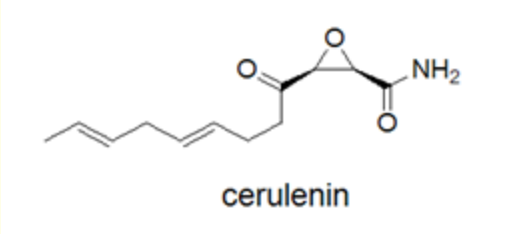




PZA K_D value of 70-123 μM based on a K_I value of 41 μM for NADPH

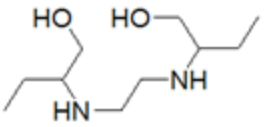
Control experiments

Positive control: cerulenin

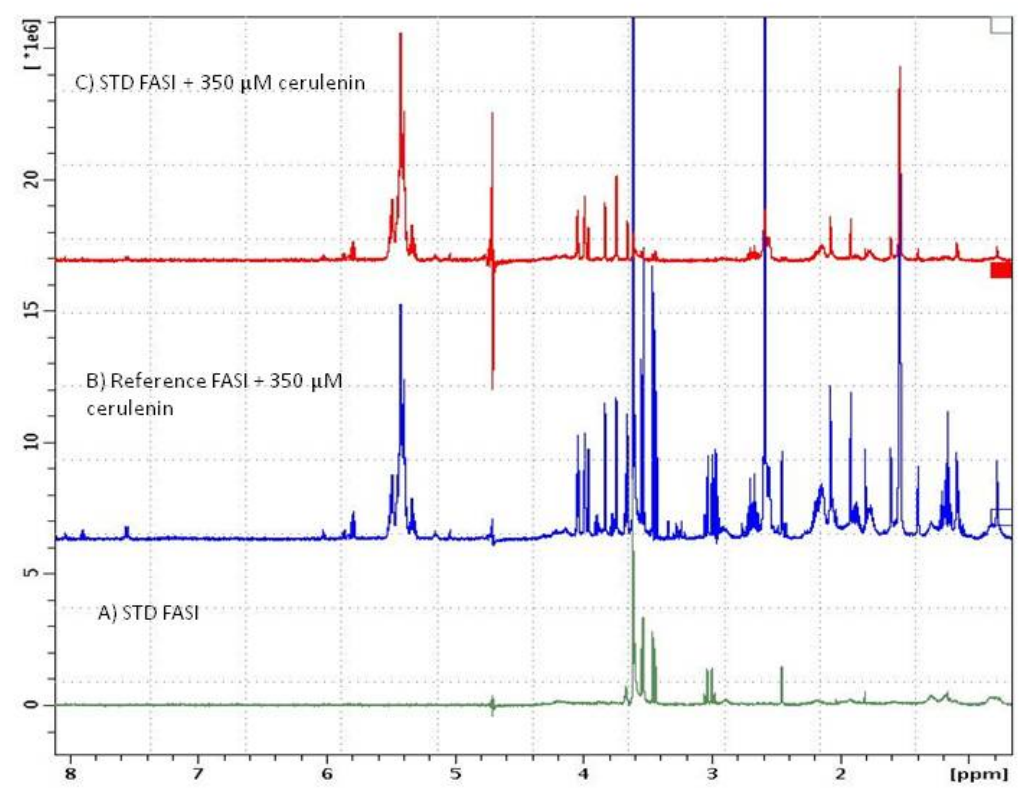


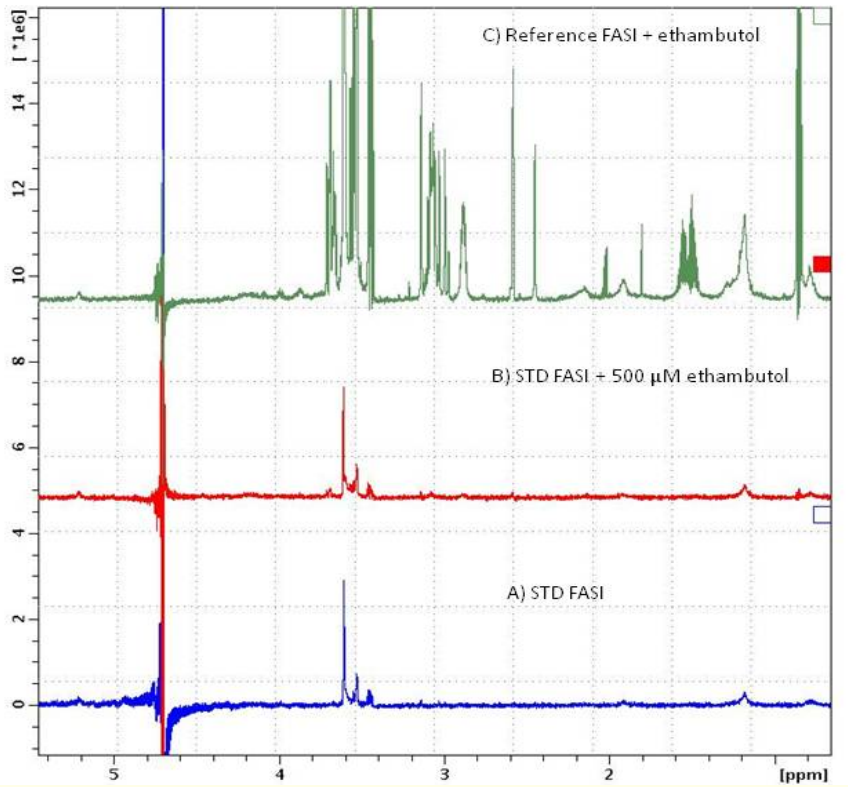
cerulenin

Negative control: ethambutol



ethambutol





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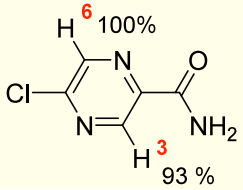
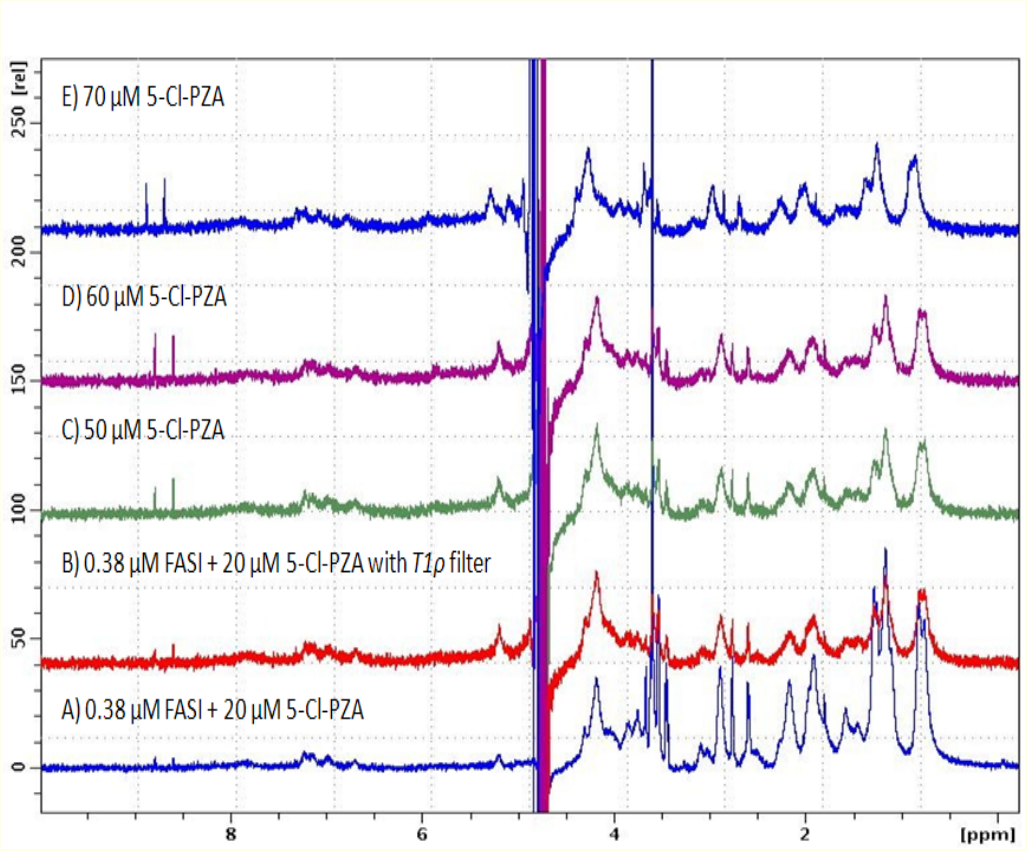
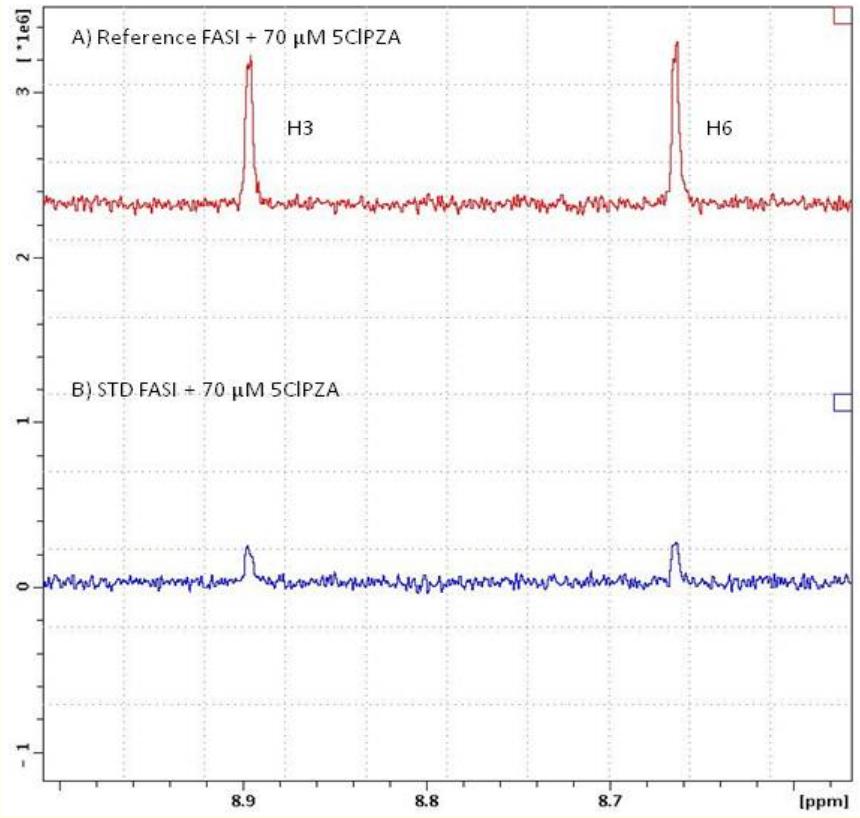
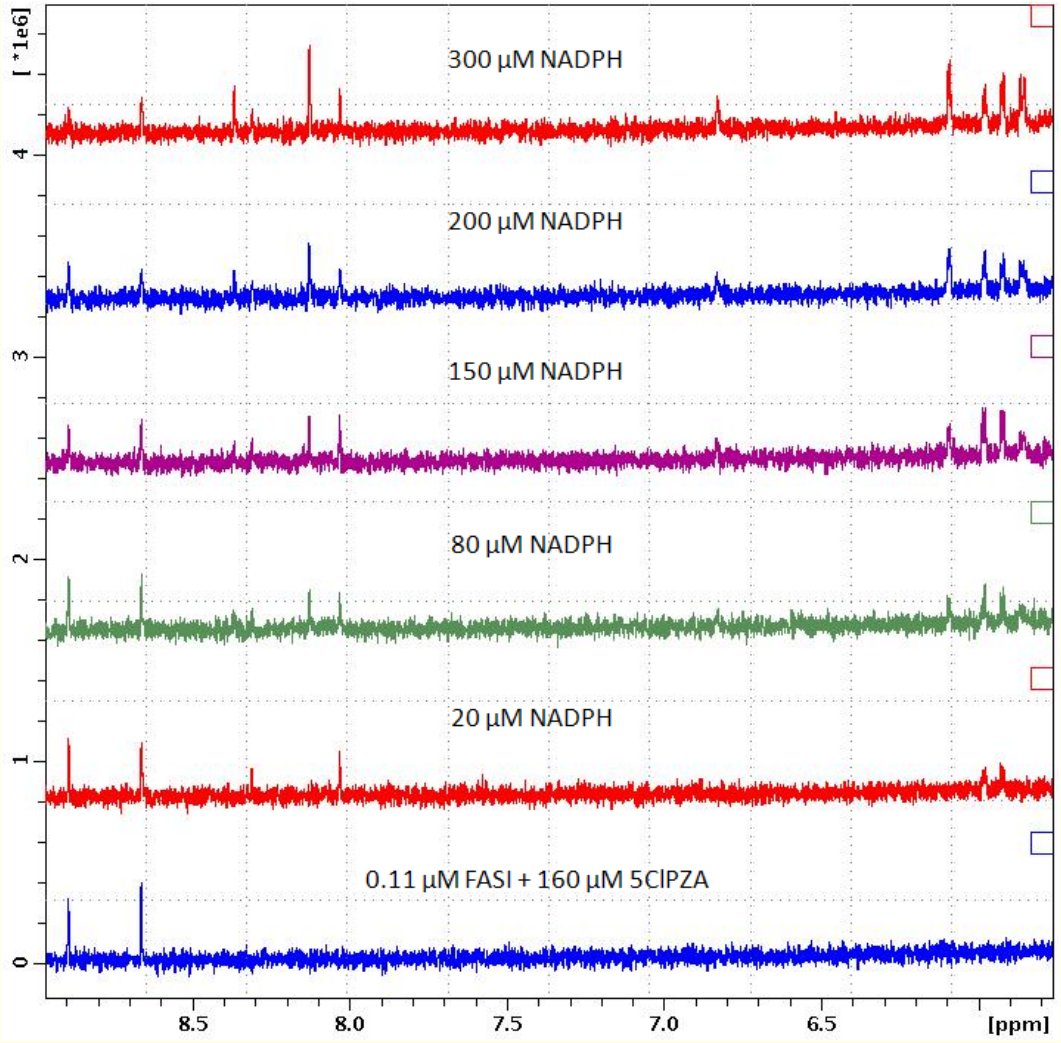




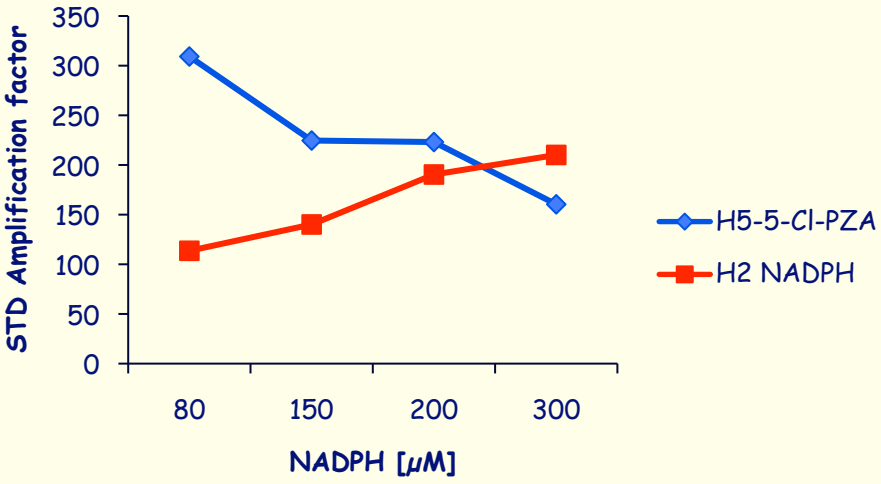


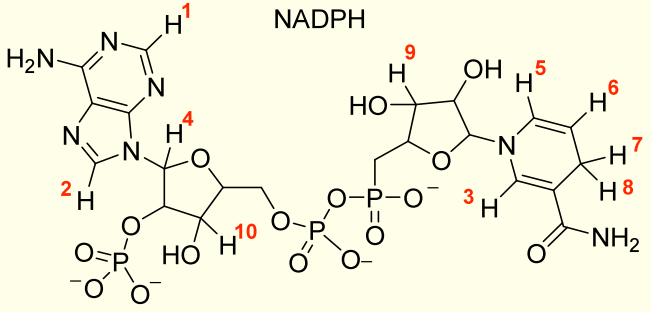
5-Cl-PZA and NADPH compete for FASI



Increasing concentrations of NADPH were added to a sample containing 5-Cl-PZA (160 µM). The intensity of the peaks corresponding to 5-Cl-PZA decrease at increasing NADPH concentrations.

Increasing NADPH displaces 5-Cl-PZA from FASI



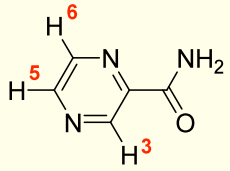


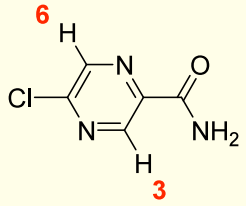
NADPH resonance	Saturation degree %
H1	*15*
H2	*38*
H3	*25*
H4	*72*
H6	*13*
H7-8	*26*
H9-10	*13*

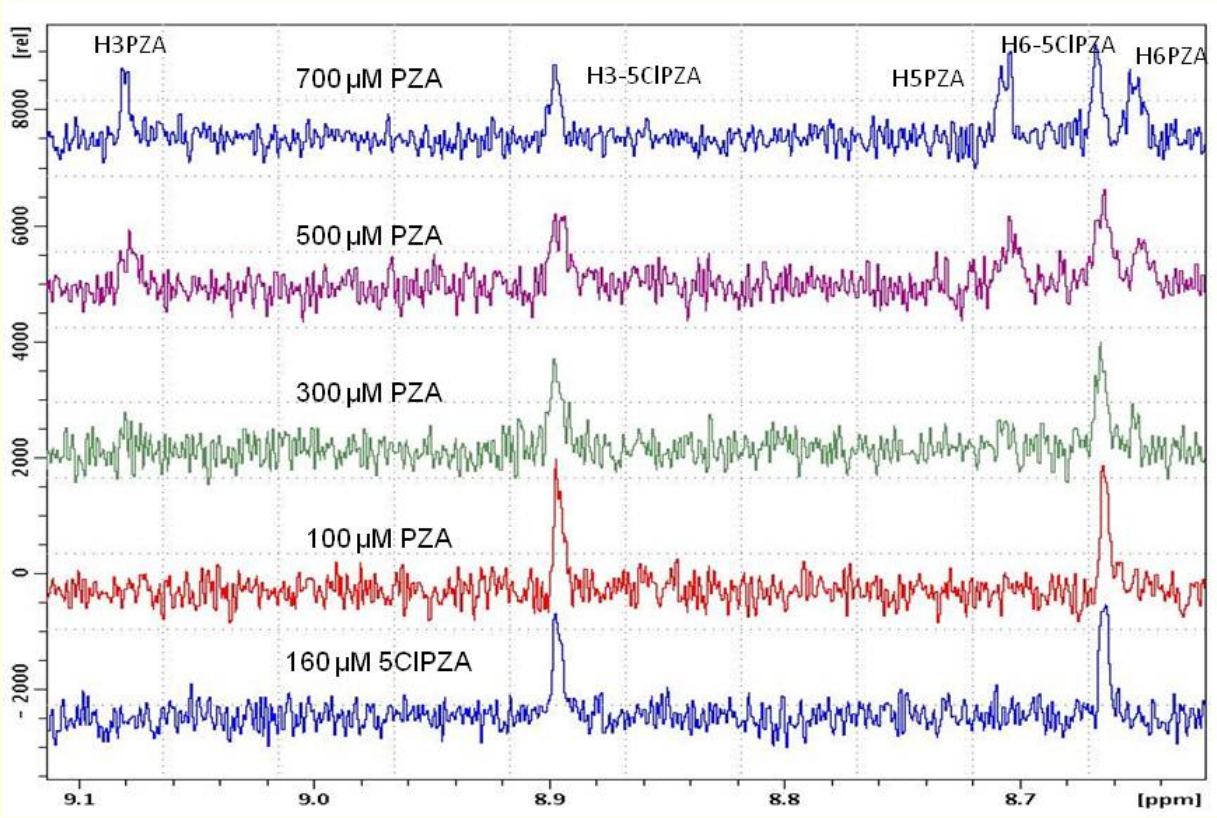
STD factor of 5-Cl-PZA decreases (160 μM) as the concentration of NADPH increases.

K_D of 10.2-40.7 μM based on a K_I value of 41 μM for NADPH.

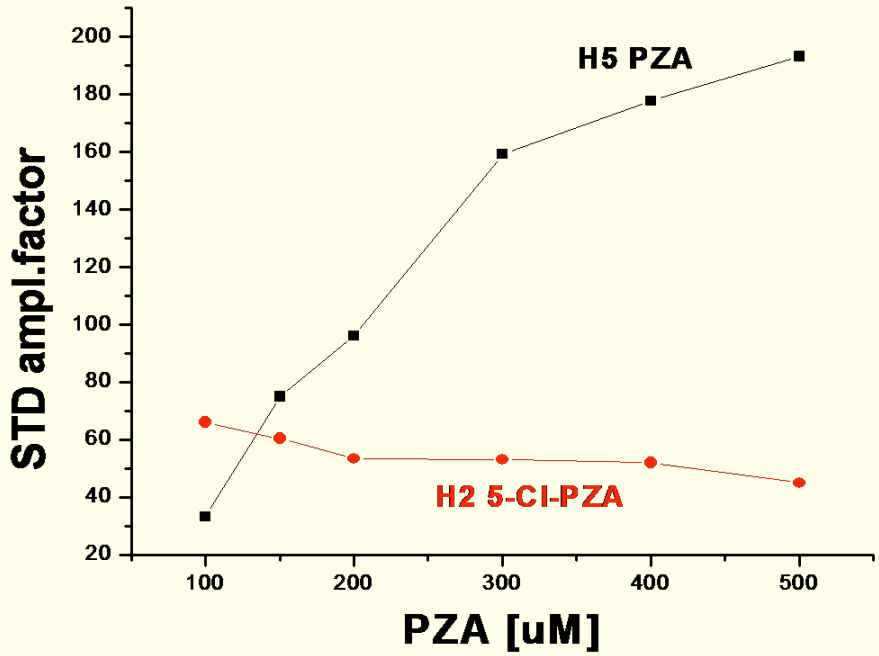
PZA can displace 5-Cl-PZA from FASI





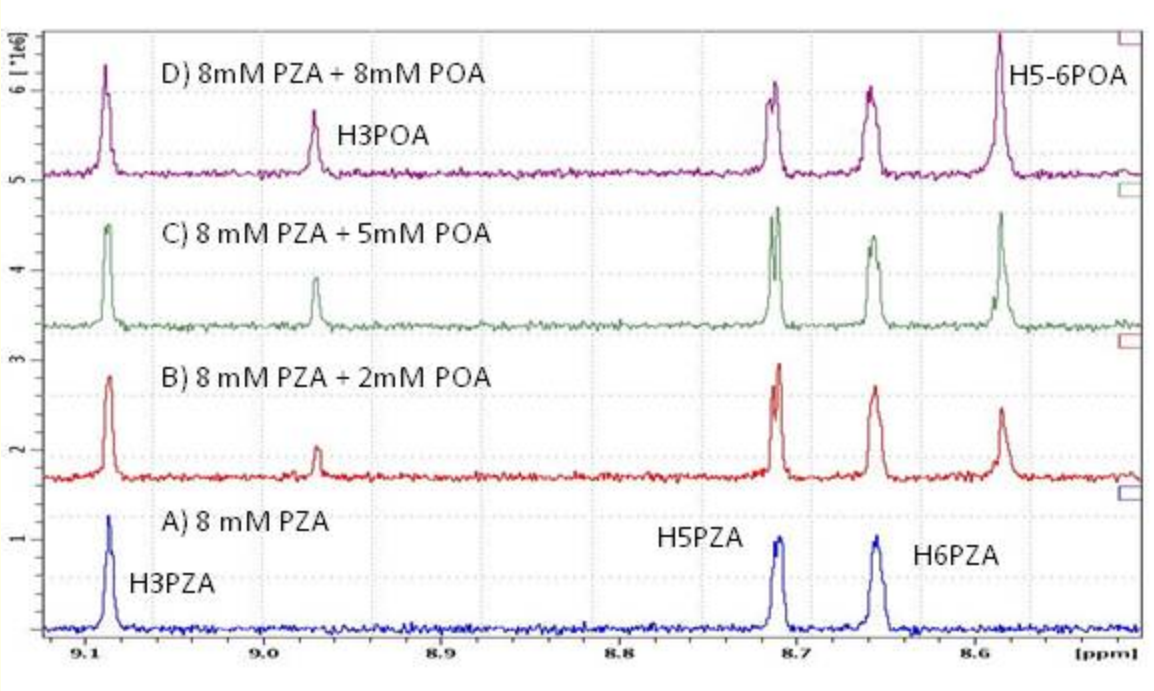


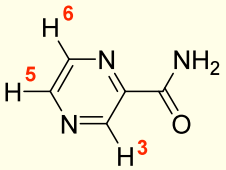
PZA displaces 5-Cl-PZA from FASI

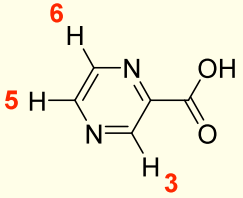


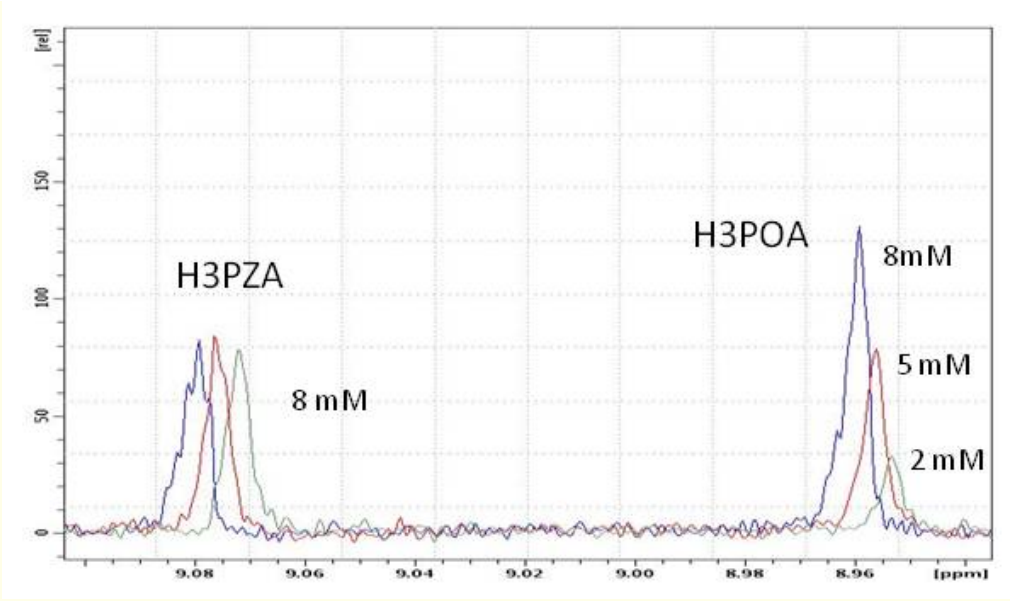
K_I of 492 μM for PZA based on a known K_D value of 57 μM for 5-Cl-PZA

PZA and POA do not compete for FAS I

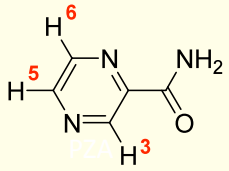


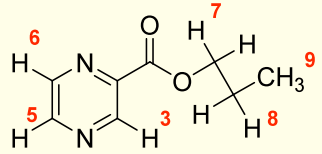




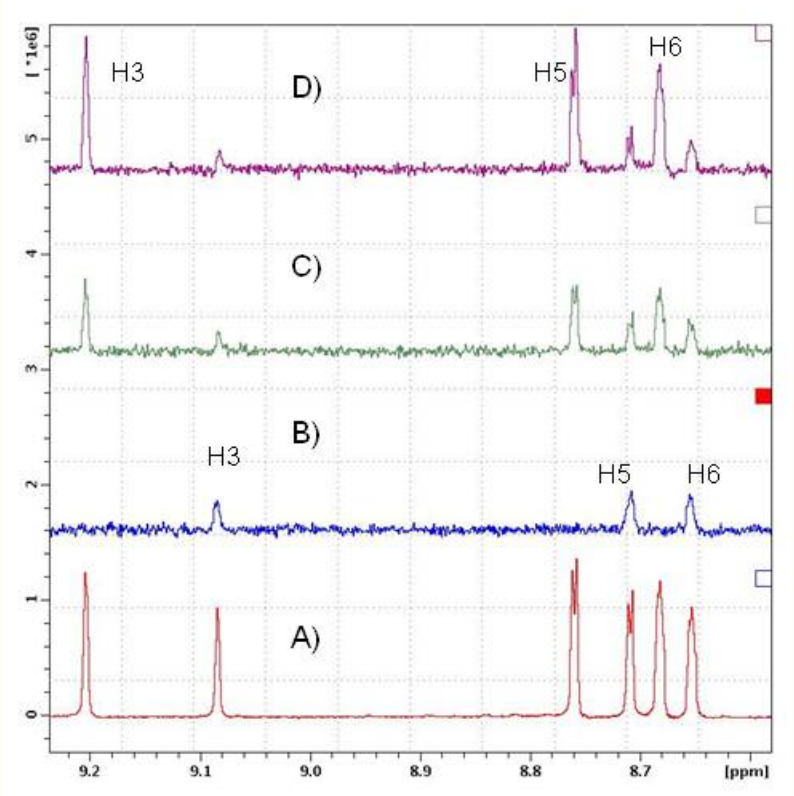


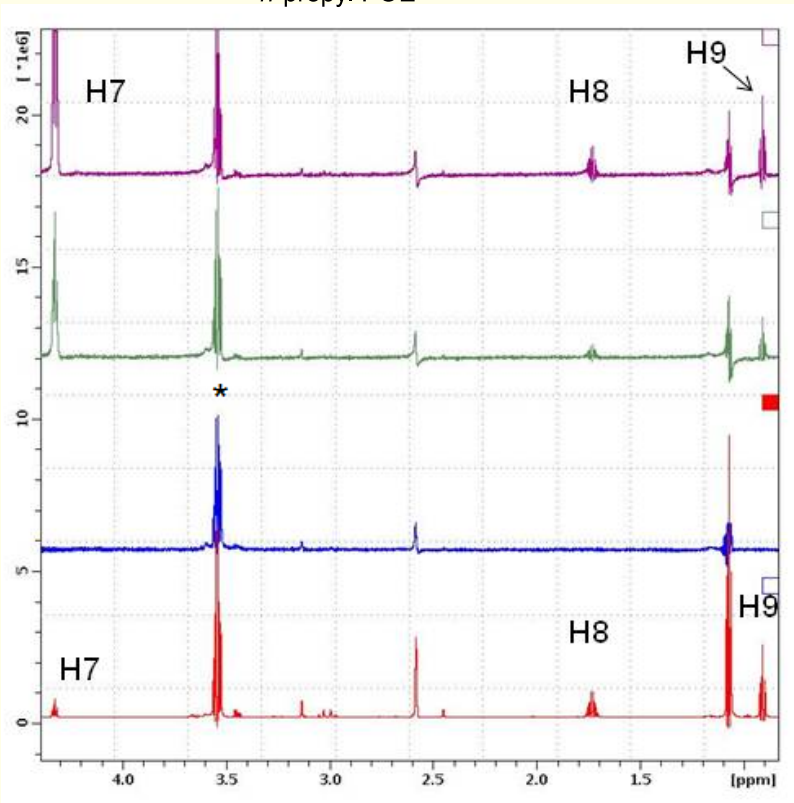
PZA and *n*-propyl-POE





n-propyl POE

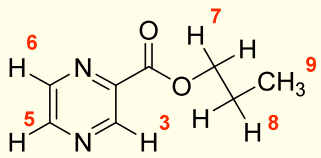




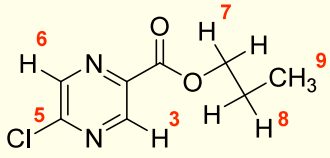
A) Reference spectrum of 2 mM PZA, 2 mM *n*-propyl-POE and *Mtb* FASI
B) STD spectrum of *Mtb* FASI and 2 mM PZA
C) STD spectrum of FASI, 2mM PZA and 2 mM *n*-propyl-POE
D) STD spectrum of FASI 2 mM PZA and 4 mM *n*-propyl-POE

* Indicate impurities in the prep that are not efficiently substracted in the STD spectrum.

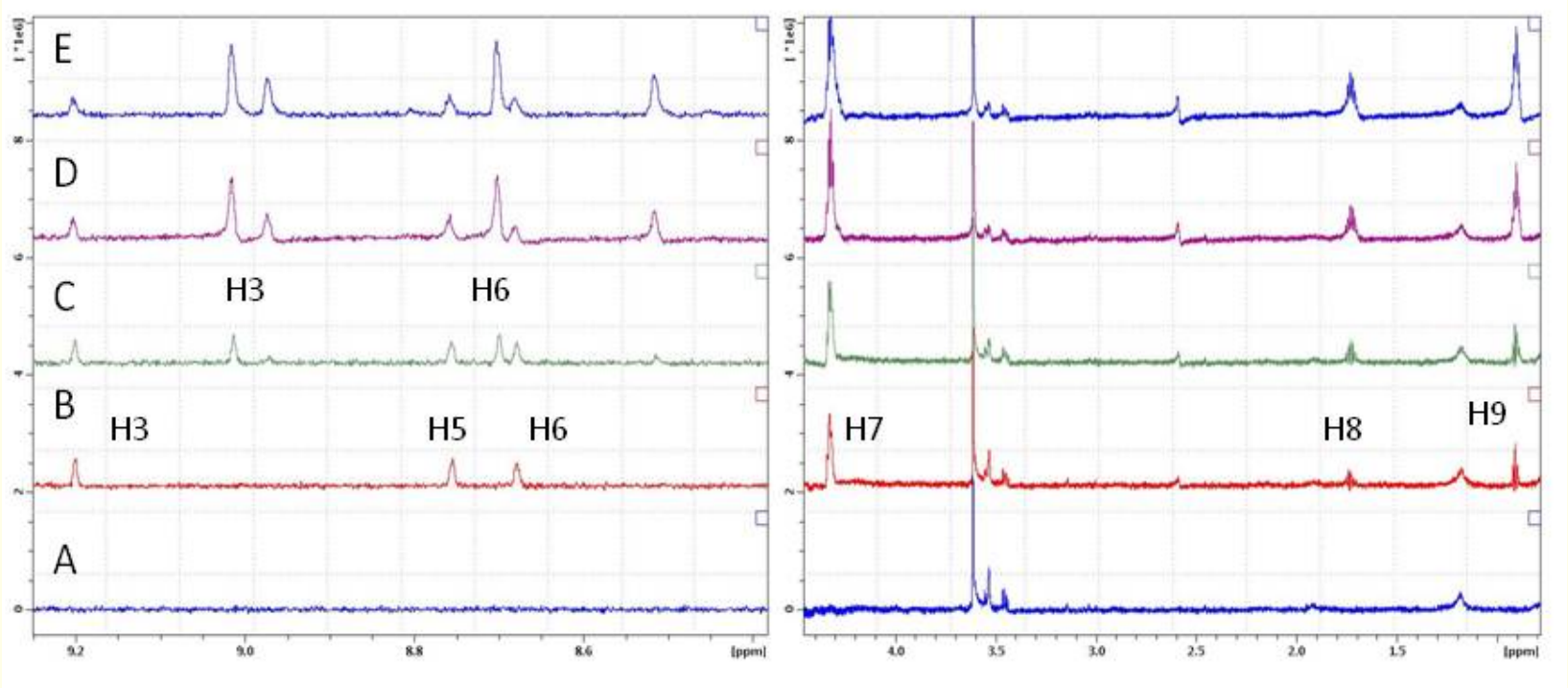
n-propyl-POE and *n*-propyl-5-Cl-POE



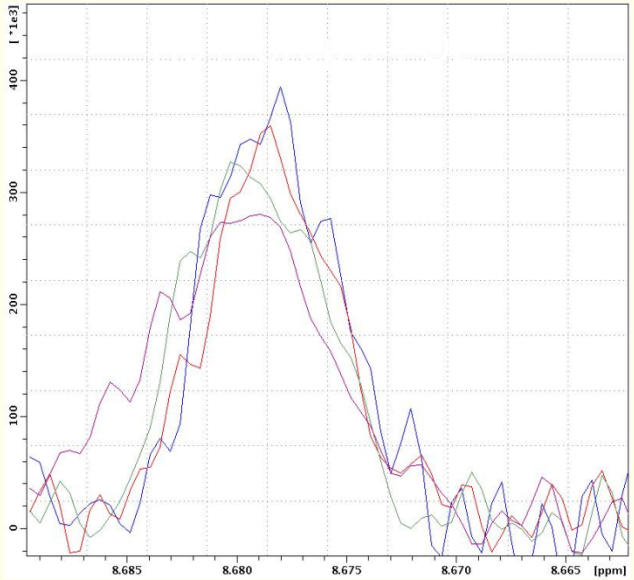
n-propyl POE

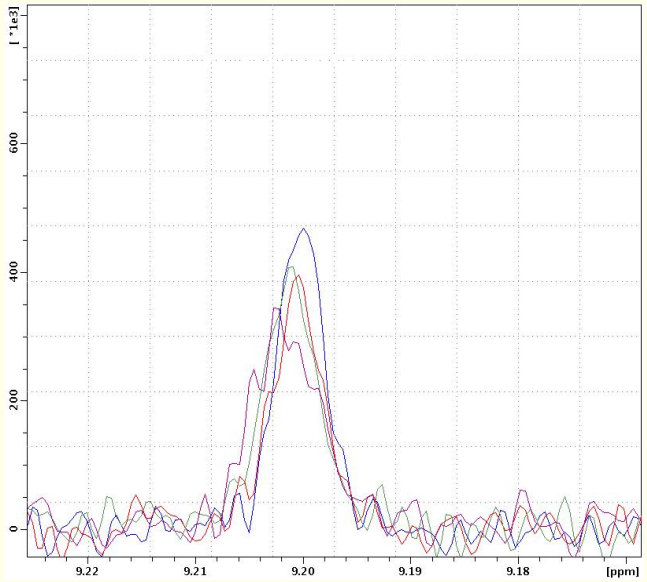


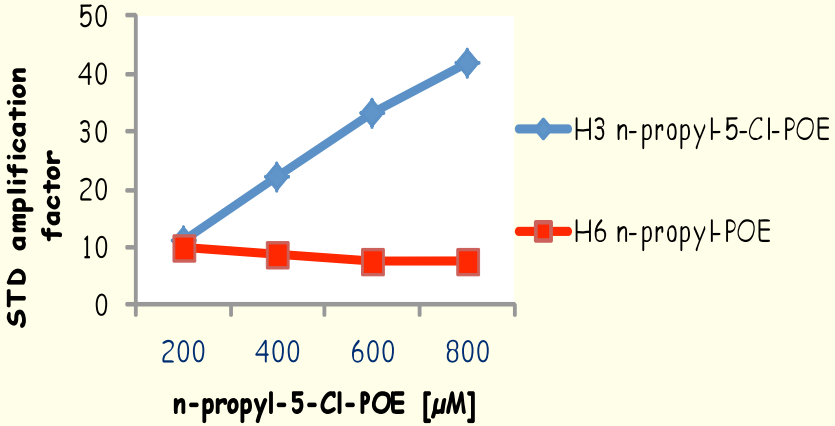
n-propyl 5-Cl-POE

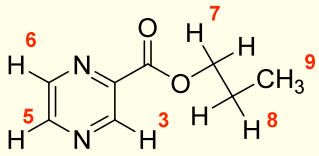


n-Propyl-POE and *n*-propyl-5-Cl-POE

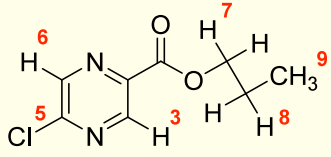






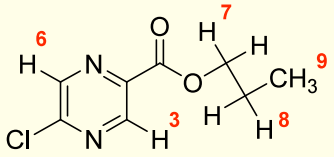


n-propyl POE

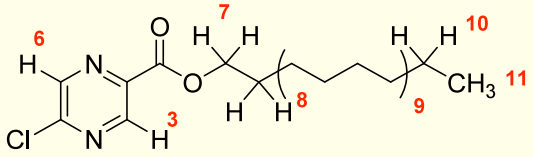


n-propyl 5-Cl-POE

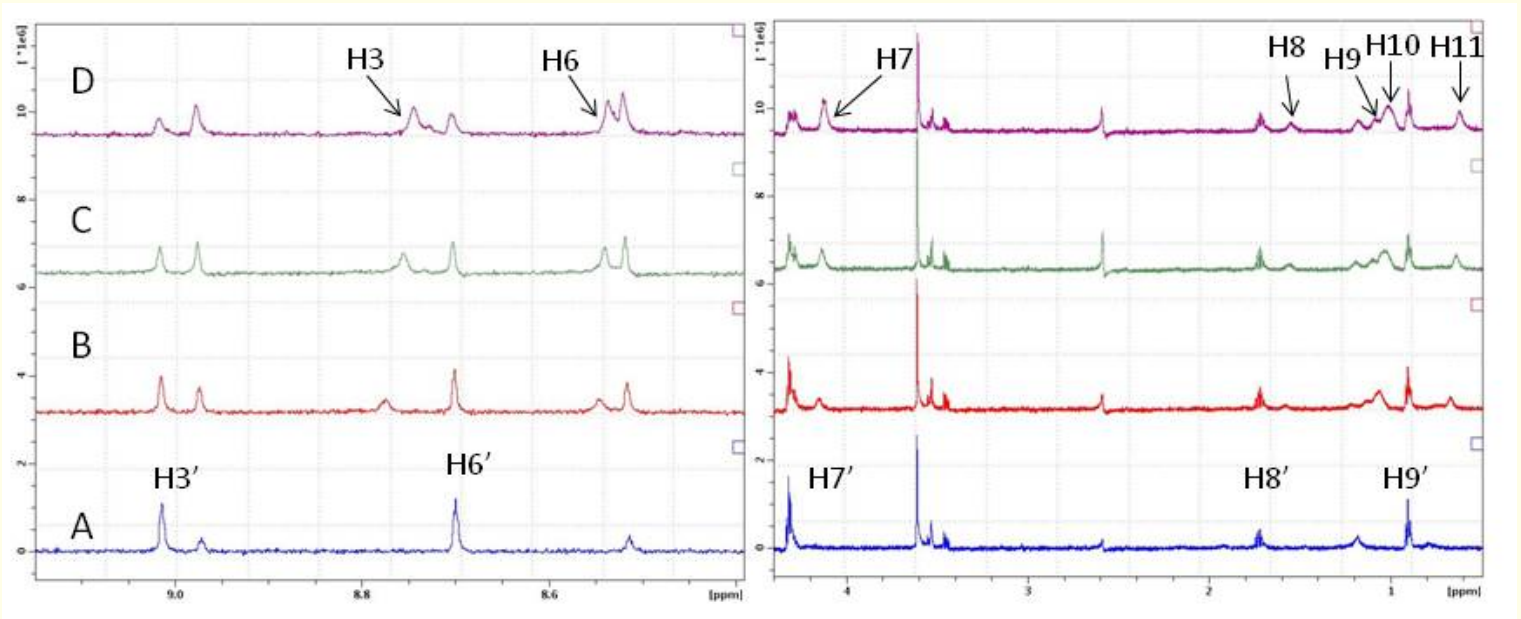
n-Propyl 5-Cl-POE and *n*-octyl 5-Cl-POE



n-propyl 5-Cl-POE



n-octyl 5-Cl-POE



A) STD spectrum of FAS I and 600 μM *n*-propyl-5-Cl-POE.
B-D) STD spectra of FAS I and 600 μM *n*-propyl-5-Cl-POE with 200, 400 and 600 μM *n*-octyl-5-Cl-POE.

Dissociation Constants

- *NADPH displaces PZA and binds to FAS I in a competitive fashion.*
- *Based on STD competition titration, 5-Cl PZA binds to FAS I with dissociation binding constant K_D of 90 μM which is significantly lower than the PZA binding constant K_i of 250 μM.*
- *In agreement with spectrometric assays.*
- *NADPH binding constant K_i determination in progress*

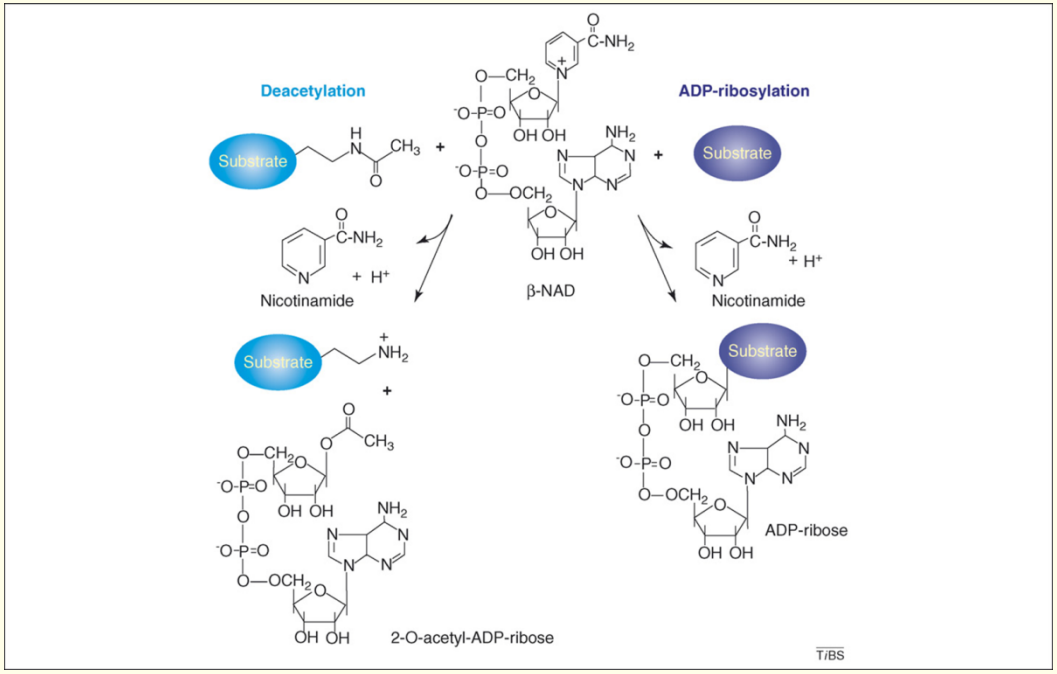
SIR 2 in *Leishmania infantum*

- *Identified in L. infantum by Ouaissi*[1]
  - Associated with remodeling of parasite
- *Disruption of SIR 2 lead to decreased virulence*
- *Explored chemical suppression*
  - Inhibition by nicotinamide was reported.
  - Prompted experiments with PZA[2] on anti-leishmanial activity

1. Tavares, J., A. Ouaissi, N. Santarem, D. Sereno, B. Vergnes, P. Sampaio, and A. Cordeiro-da-Silva. 2008. The Leishmania infantum cytosolic SIR2-related protein 1 (LiSIR2RP1) is an NAD+-dependent deacetylase and ADP-ribosyltransferase. Biochem. J. 415:377-386.
2. Mendez, S., R. Traslavina, M. Hinchman, L. Huang, P. Green, M. H. Cynamon, and J. T. Welch. 2009. The antituberculosis drug pyrazinamide affects the course of cutaneous leishmaniasis in vivo and increases activation of macrophages and dendritic cells. Antimicrobial Agents and Chemotherapy **53**:5114-5121.

Histone deacetylation and ADP-ribosylation

- *Sirtuins are a conserved class of trichostatin A-insensitive lysyl-deacetylases*
  - Catalyze the coupled lysine deacetylation with the formation of nicotinamide and *O*-acetyl-ADP-ribose from NAD^+.



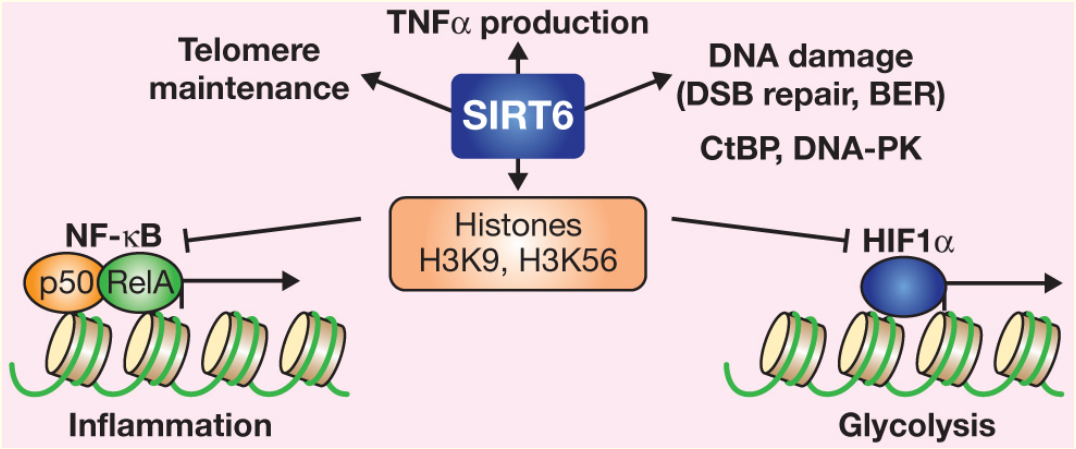
Westphal, C. H., M. A. Dipp, and L. Guarente. 2007. A therapeutic role for sirtuins in diseases of aging? Trends Biochem Sci 32:555-560

Sirtuins

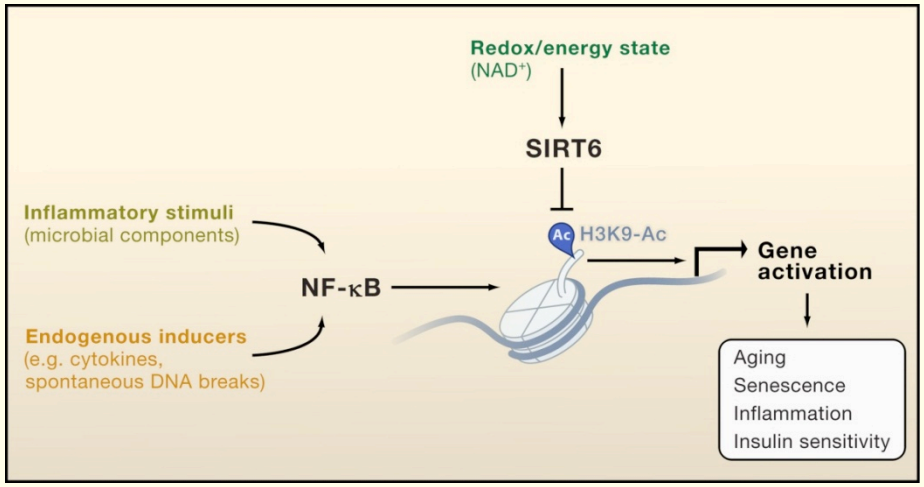
- *First discovered in yeast*
  - Silent information regulator 2 (SIR 2)
- *7 characteristic forms known*
  - SIRT1
    - Nuclear, mediates inflammation via NF-κB, initiates hypoxic stress response
  - SIRT2
    - Tubulin deacetylase, regulates cell cycle
  - SIRT3
    - Mitochondrial , regulates acetyl CoA synthetase
  - SIRT5
    - Mitochondrial, regulation of urea cycle
  - SIRT6
    - Nuclear, interacts with RelA, interferes with NF-κB signaling
  - SIRT7
    - Least studied, RNA polymerase I regulation

Imai, S, Armstrong, C.M., Kaeberlein, M., Guarente, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 464: 1313-19.

SIRT6 Activity



Nakagawa, T., Guarente, L. 2011. Sirtuins at a glance. J. Cell Sci. 124: 833-8



Ghosh, S., M.S. Hayden 2008. New regulators of NF-κB in inflammation. Nature Rev. Immun. 8: 837-48

M. tuberculosis SIRT 2 and SIRT 6

- *Zhang and coworkers cloned and overexpressed RV1151c (putative SIRT2) from M. tuberculosis*[1]
  - No inhibition by PZA
- *Sequence comparision with SIRT 6*

```
1    MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTASGIPDFRGPHGV   70  Q8N6T7   SIRT6_HUMAN
1    MRVAVLSGAGISAESG---VPTFRD-----------------DKNGLWARFDPYELSSTQGWLRNPERV   49  P66813   NPD_MYCTU

71   WTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLRFLVSQNVDGLHVRSGFPRDKLAELHGNMFVEE  140  Q8N6T7   SIRT6_HUMAN
50   W-----GWYLWRHYLVANVEPNDGHRAIAAWQDHAEVS-VITQNVDDLHERAGSG--AVHHLHGSLFEFR  111  P66813   NPD_MYCTU

141  CAKCKTQYVRDTVVGTMGLKATGRLCTVAKARGLRACRGELRDTILDWEDSLPDRDLALADEASRNADLS  210  Q8N6T7   SIRT6_HUMAN
112  CARCGVPYT--------DALPEMPEPAIEVEPPVCDCGGLIRPDIVWFGEPLPEEPWRSAVEATGSADVM  173  P66813   NPD_MYCTU

211  ITLGTSLQIRPSGNLPLATKRRGGRLVIVNLQPTKHDRHADLRIHGYVDEVMTRLMKHLGLEIPAWDGPR  280  Q8N6T7   SIRT6_HUMAN
174  VVVGTSAIVYPAAGLPDLALARGTAVIEVNPEPTPLSGSATISIRESASQALPGLLERL---------PA  234  P66813   NPD_MYCTU

281  VLERALPPLPRPPTPKLEPKEESPTRINGSIPAGPKQEPCAQHNGSEPASPKRERPTSPAPHRPPKRVKA  350  Q8N6T7   SIRT6_HUMAN
235  LLK-------------------------------------------------------------------  237  P66813   NPD_MYCTU

351  KAVPS  355  Q8N6T7   SIRT6_HUMAN
238  -----  237  P66813   NPD_MYCTU
```

J. Gu; Deng, J.-Y; Li, R.; Wei, H.; Zhang, Z; Zhou, Y.; Zhang, Y.; Zhang, X.-E. 2009 Cloning and characterization of NAD-dependent Protein deacetylase (Rv1151c) from M. tuberculosis, Biochem. (Moscow) 74: 743-8

Human and murine SIRT 6

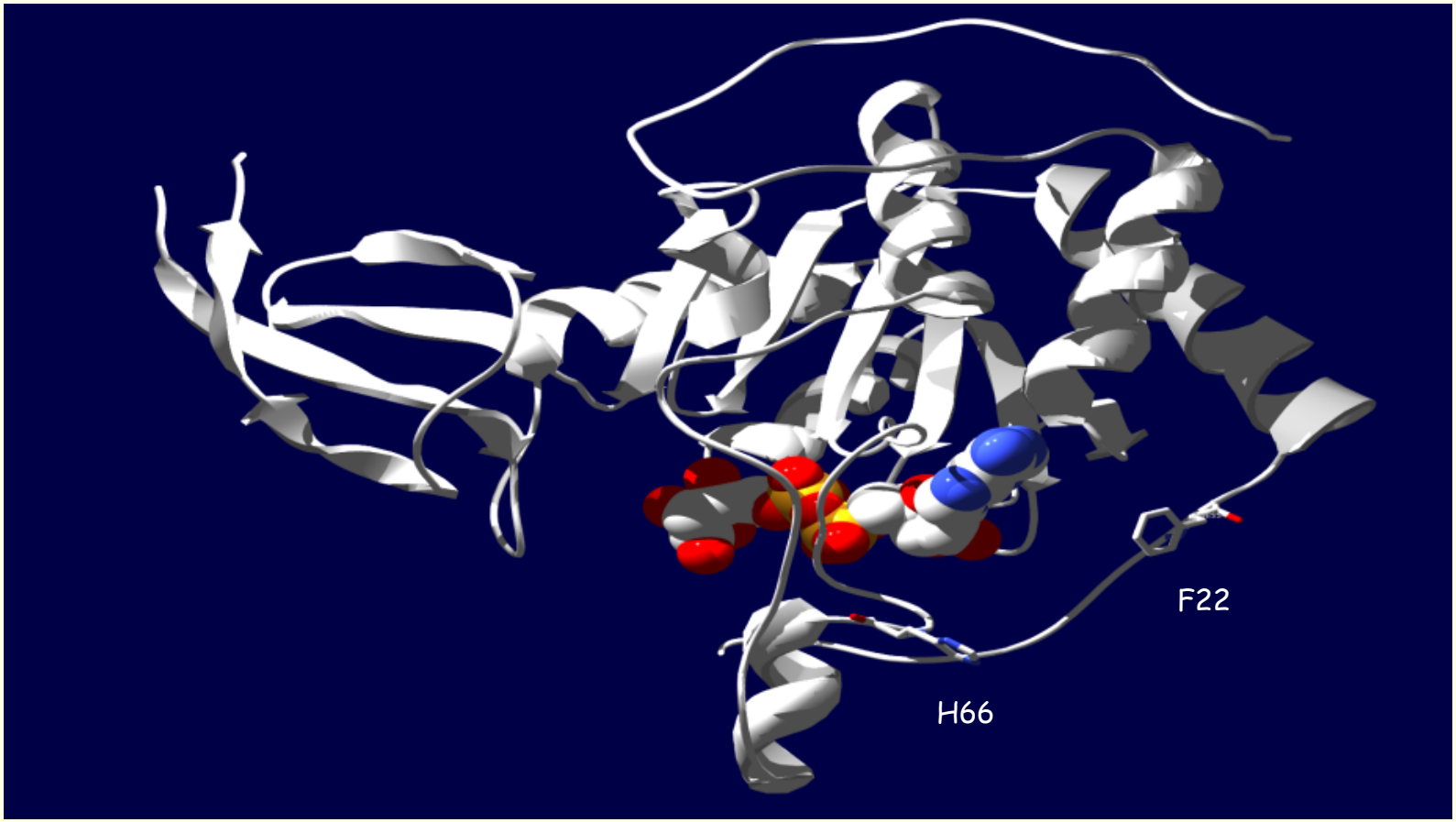
- *Conserved across species.*

```
1    MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTASGIPDFRGPHGV   70  Q8N6T7   SIRT6_HUMAN
1    MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLMWQSSSVVFHTGAGISTASGIPDFRGPHGV   70  P59941   SIRT6_MOUSE
     ****************************************:****************************

71   WTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLRFLVSQNVDGLHVRSGFPRDKLAELHGNMFVEE  140  Q8N6T7   SIRT6_HUMAN
71   WTMEERGLAPKFDTTFENARPSKTHMALVQLERMGFLSFLVSQNVDGLHVRSGFPRDKLAELHGNMFVEE  140  P59941   SIRT6_MOUSE
     *****************.***::**********:*:* ********************************

141  CAKCKTQYVRDTVVGTMGLKATGRLCTVAKARGLRACRGELRDTILDWEDSLPDRDLALADEASRNADLS  210  Q8N6T7   SIRT6_HUMAN
141  CPKCKTQYVRDTVVGTMGLKATGRLCTVAKTRGLRACRGELRDTILDWEDSLPDRDLMLADEASRTADLS  210  P59941   SIRT6_MOUSE
     *.****************************:************************ *******.****

211  ITLGTSLQIRPSGNLPLATKRRGGRLVIVNLQPTKHDRHADLRIHGYVDEVMTRLMKHLGLEIPAWDGPR  280  Q8N6T7   SIRT6_HUMAN
211  VTLGTSLQIRPSGNLPLATKRRGGRLVIVNLQPTKHDRQADLRIHGYVDEVMCRLMKHLGLEIPAWDGPC  280  P59941   SIRT6_MOUSE
     :************************************:************* ***************

281  VLERALPPLPRPPTPKLEPKEESPTRINGSIPAGPKQEPCAQHNGSEPASPKRERPTSPAPHRPPKRVKA  350  Q8N6T7   SIRT6_HUMAN
281  VLDKALPPLPRPVALKAEP----PVHLNGAVHVSYKS-----------------KPNSPILHRPPKRVKT  329  P59941   SIRT6_MOUSE
     **::******** : * **    *.::**:: .. *.                 :*.**  ********:

351  KAVPS  355  Q8N6T7   SIRT6_HUMAN
330  EAAPS  334  P59941   SIRT6_MOUSE
     :*.**
```

Comparison SIRT 1, 2, 5, and 6

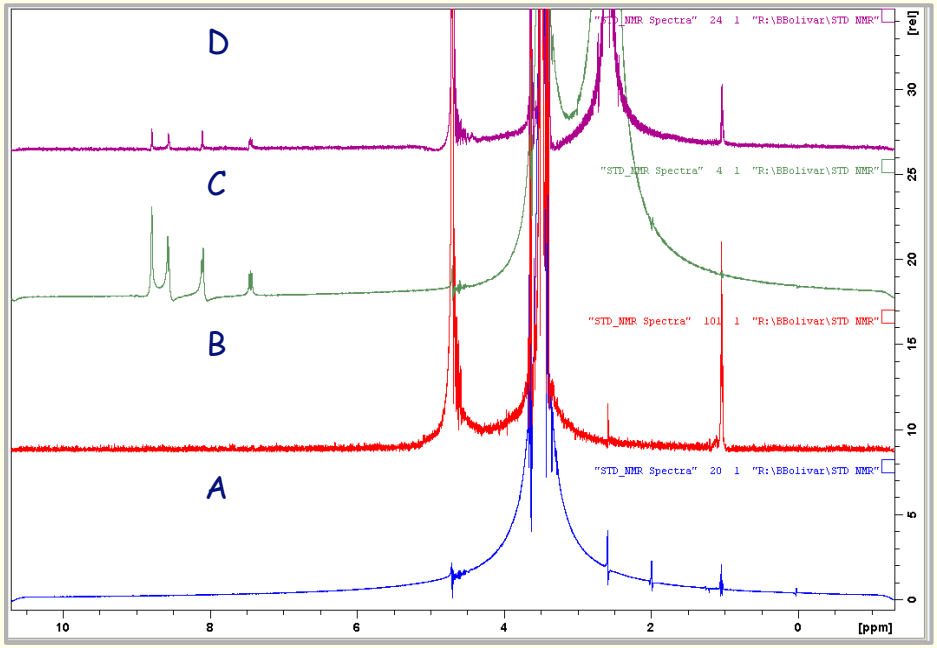
- *Little homology across sirtuins*

ADP-ribose bound to human SIRT6 subunit a[1]

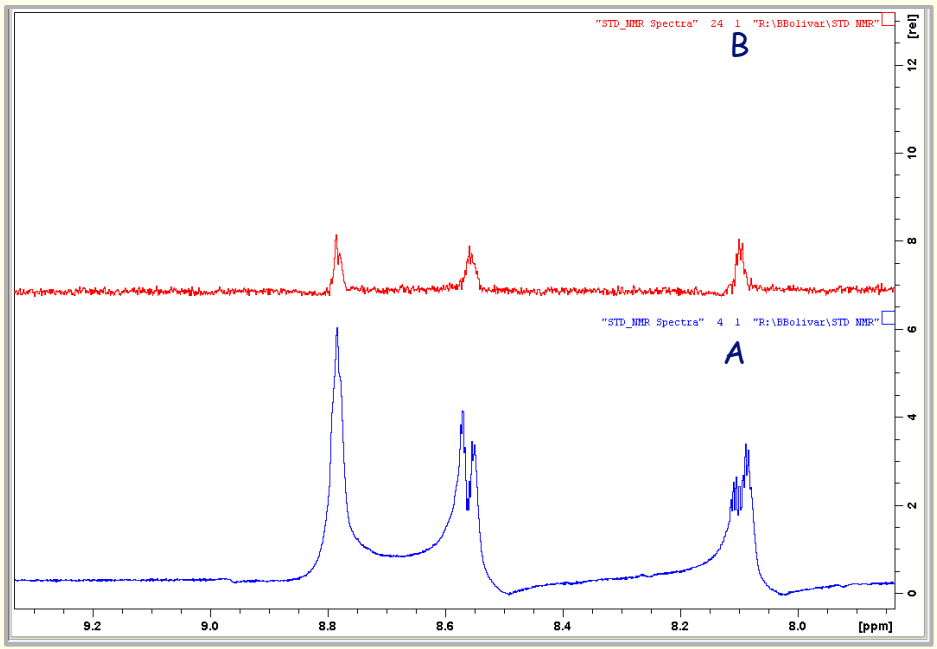


Pan, P. W., J. L. Feldman, M. K. Devries, A. Dong, A. M. Edwards, and J. M. Denu. 2011. Structure and Biochemical Functions of SIRT6. J Biol Chem **286**:14575-14587.

STD NMR results: NAM binding to SIRT6

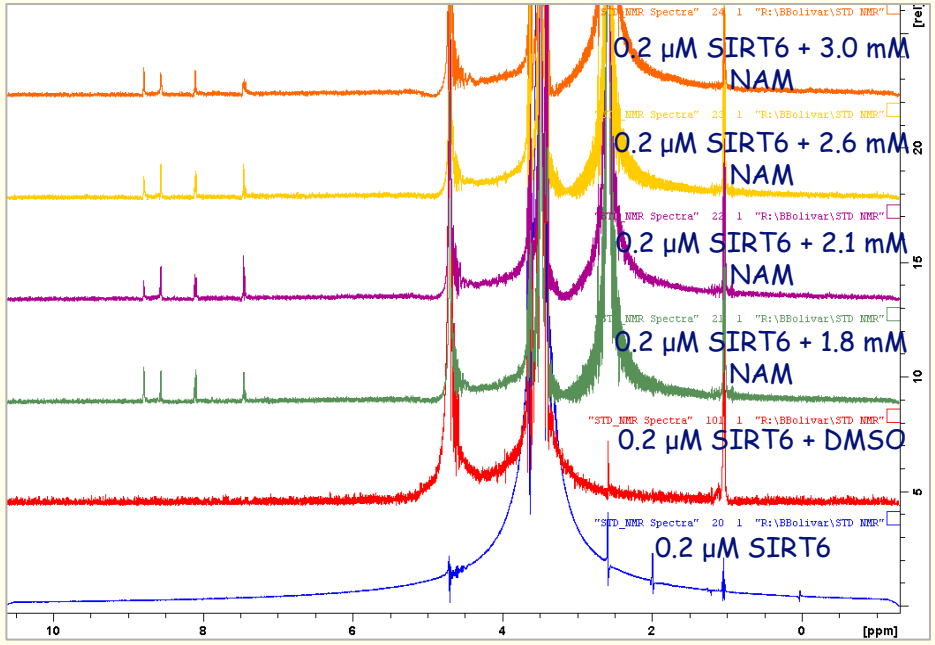


A. Reference Spectrum SIRT6
B. STD Spectrum SIRT6 and DMSO (solvent)
C. Reference Spectrum SIRT6 and NAM
D. STD Spectrum SIRT6 and NAM

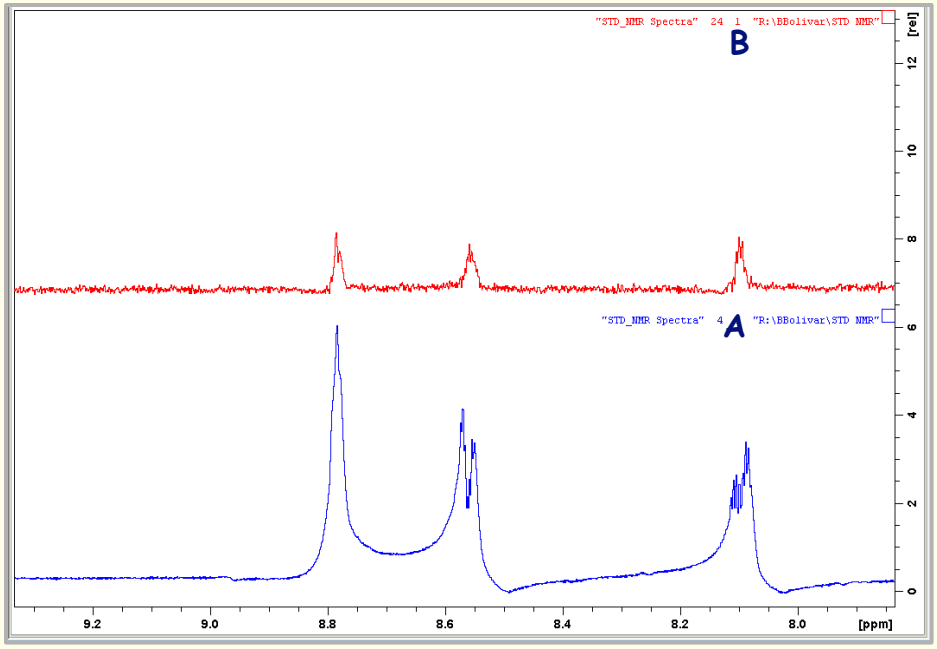


A. Reference Spectrum SIRT6 and NAM
B. STD Spectrum SIRT6 and NAM

STD NMR Results: NAM-as SIRT6 Ligand

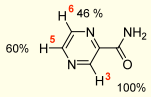


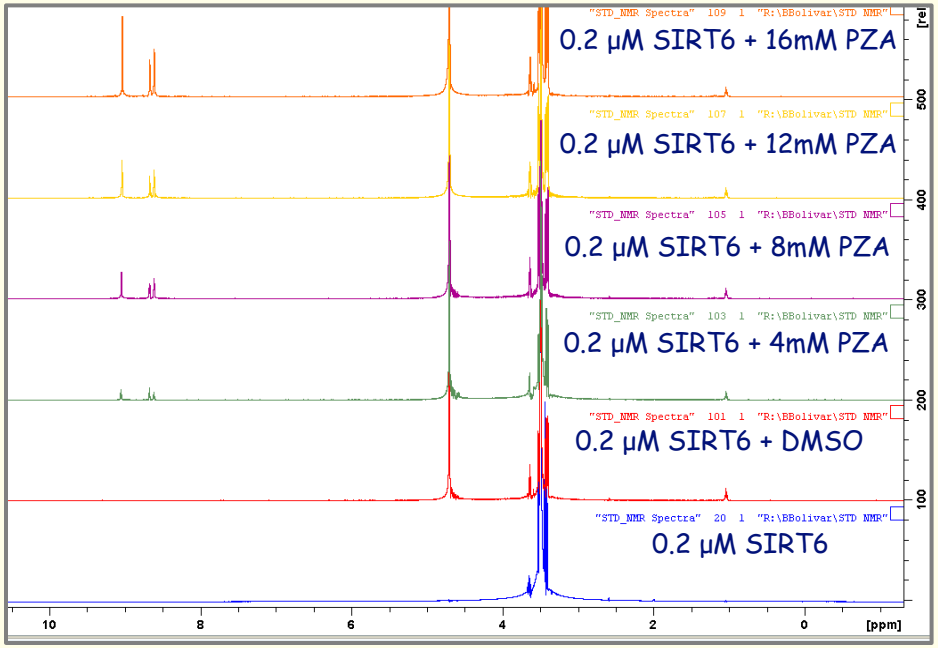
STD Spectra SIRT6 and NAM



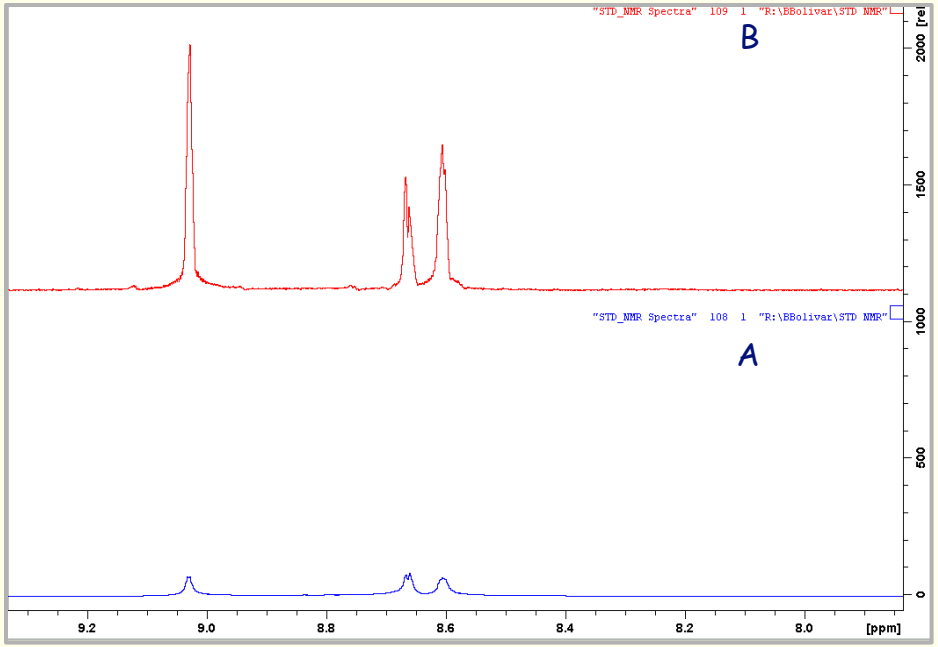
A. Reference Spectrum SIRT6 and NAM
B. STD Spectra SIRT6 and NAM

STD NMR results: PZA binding to SIRT6



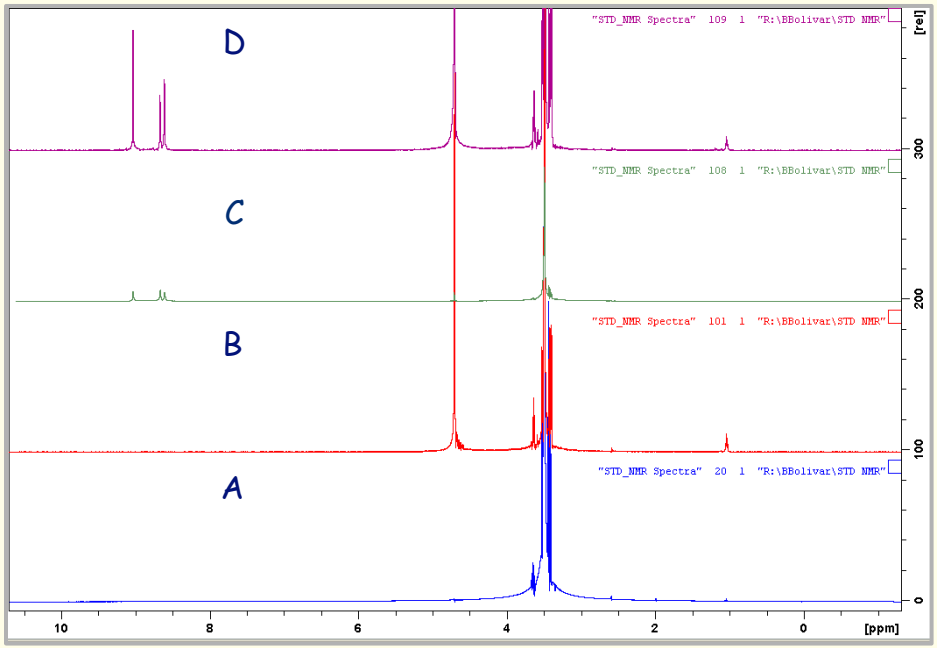


STD Spectra SIRT6 and PZA

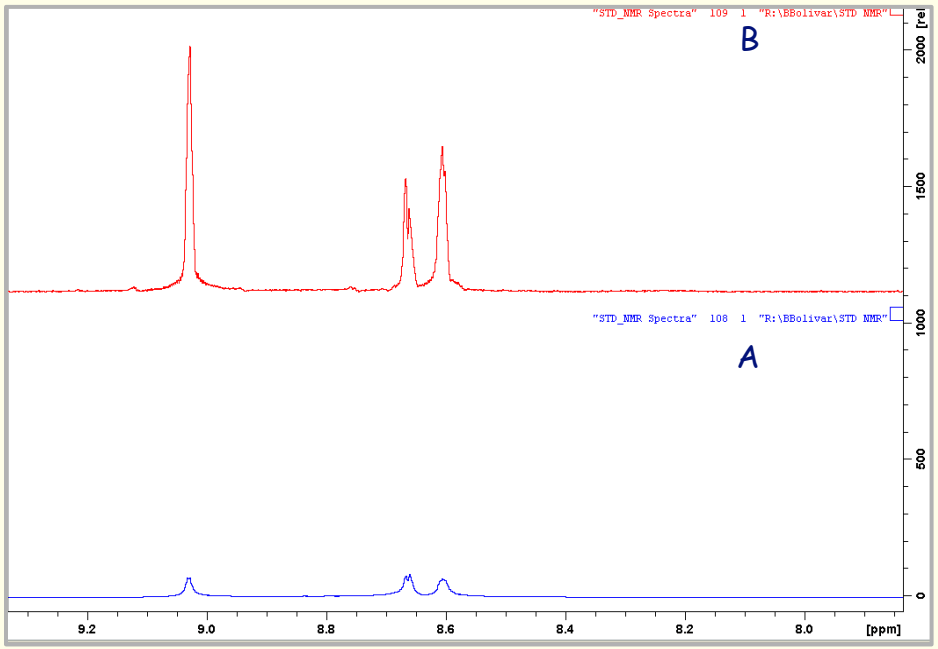


A. Reference Spectrum SIRT6 and PZA
B. STD Spectrum SIRT6 and PZA

STD NMR results: PZA binding to SIRT6

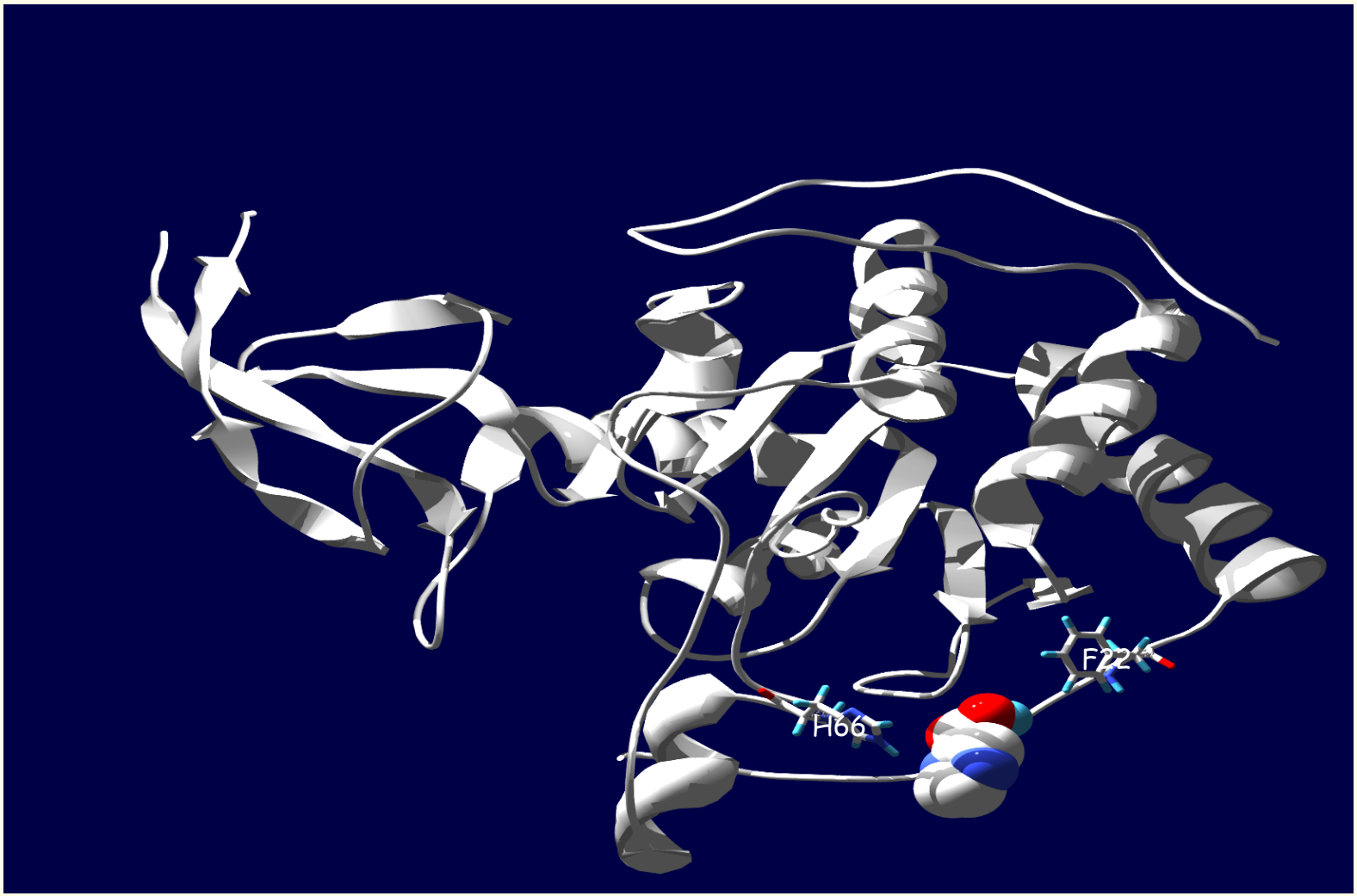


A. Reference Spectrum SIRT6
B. STD Spectrum SIRT6 and DMSO (solvent)
C. Reference Spectrum SIRT6 and PZA
D. STD Spectrum SIRT6 and PZA

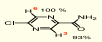


A. Reference Spectra SIRT6 and PZA
B. STD Spectra SIRT6 and PZA

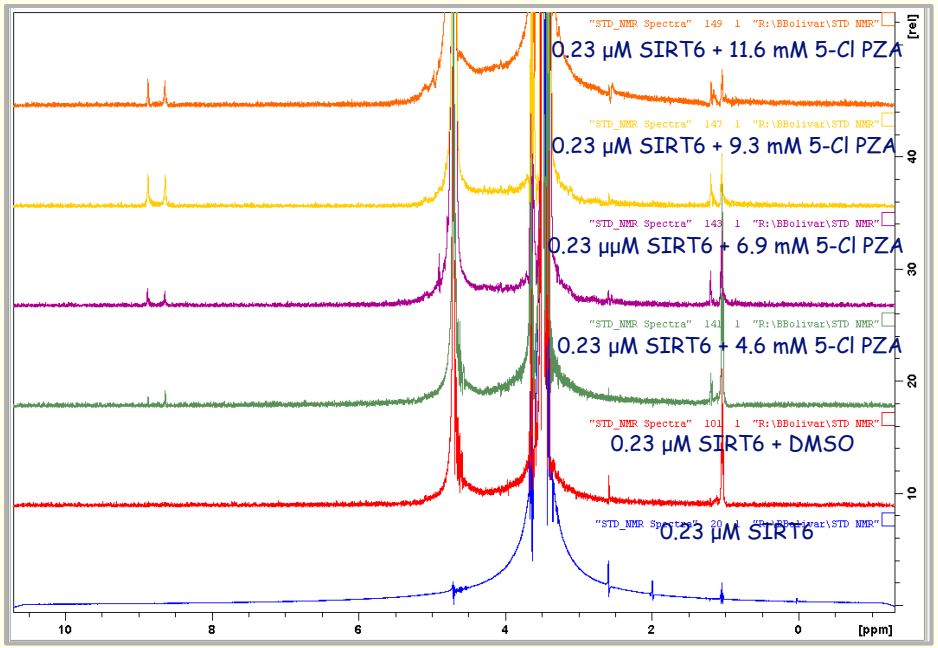
POA docked to human SIRT6 subunit a



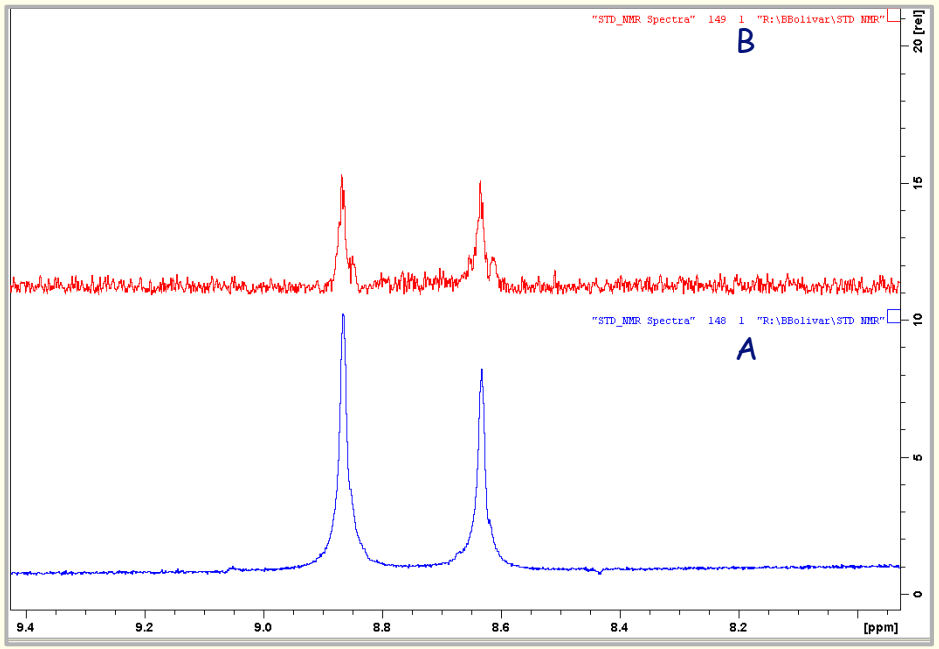
STD NMR results: 5-Cl PZA binding to SIRT6



To analyze the relative saturation degree for the individual protons, the largest signal in the STD spectrum, H6 in this case, was set to 100%. Hence, the relative degree of saturation for H3 93 % The relative degree of saturation of 5-Cl PZA protons suggests that all three protons are in intimate contact with the protein.

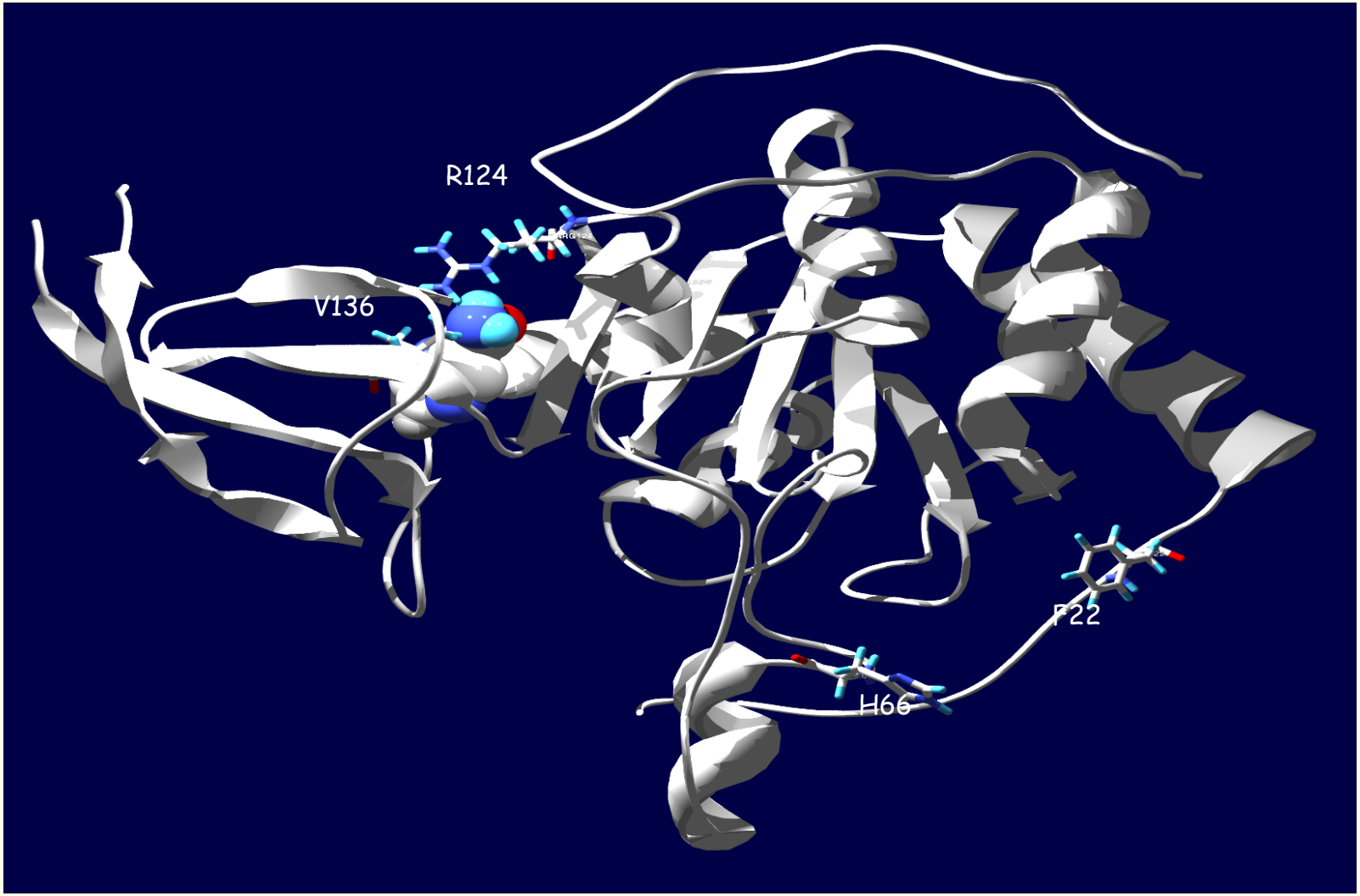


STD Spectra SIRT6 and 5-Cl PZA



A. NMR Reference Spectrum SIRT6 and PZA
B. STD Spectrum SIRT6 and PZA

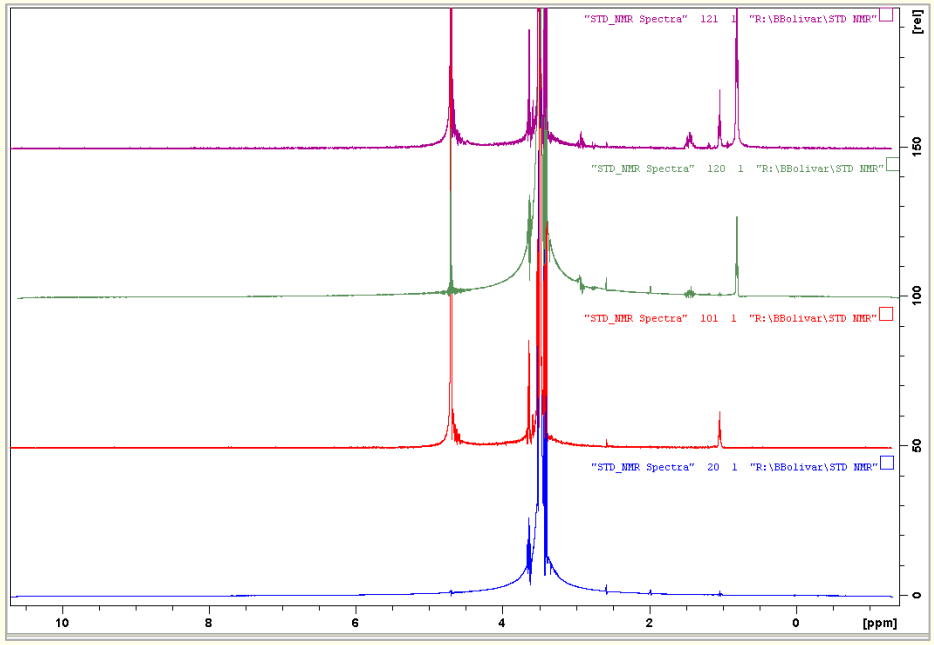
5-Cl PZA docked to human SIRT6 subunit a

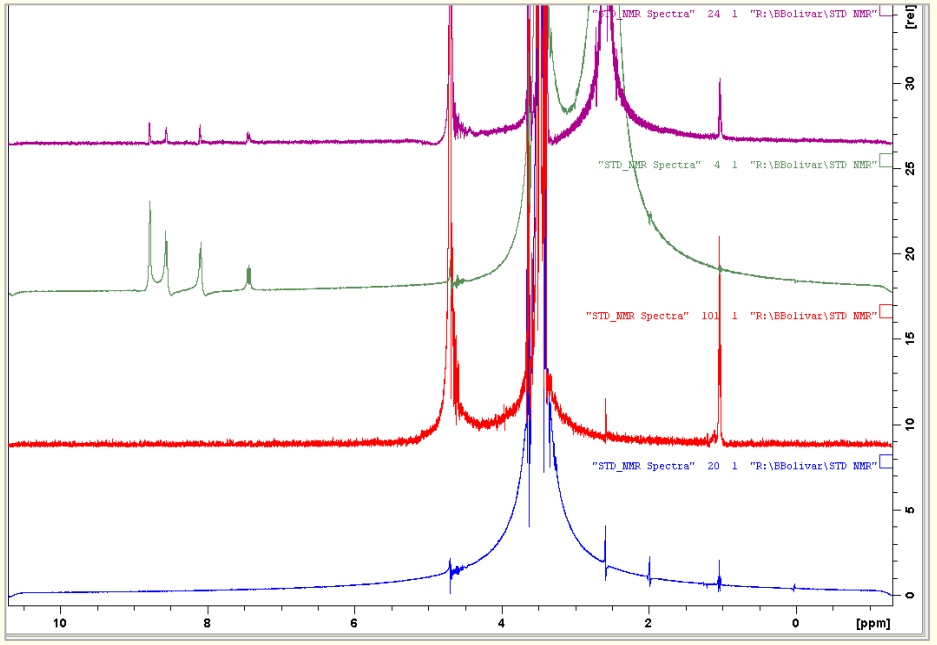


STD Control Experiments

Ethambutol

Nicotinamide





Negative Control

Positive Control

Summary

- *PZA analogs have up to 10^4 greater in vitro activity against Mtb*
- *5-Cl PZA and PZA have been shown to be competitive inhibitors of Mtb FAS I*
  - FAS I inhibitory activity correlates with whole cell inhibition of growth assays with Mtb, Msmeg et al.
- *PZA and NADPH bind Mtb FAS I competitively.*
- *POA and PZA do not bind Mtb FAS I competitively*
- *5-Cl PZA and PZA bind competitively by STD NMR methods*
- *Human SIRT6 binds PZA, NCA and 5-Cl PZA by STD NMR*
- *Human SIRT6 and SIRT1 inhibition by 5-Cl PZA measured in coupled enzyme assay*

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