A rationale approach for drug targeting with special reference to *Leishmania* cytosolic SIR2

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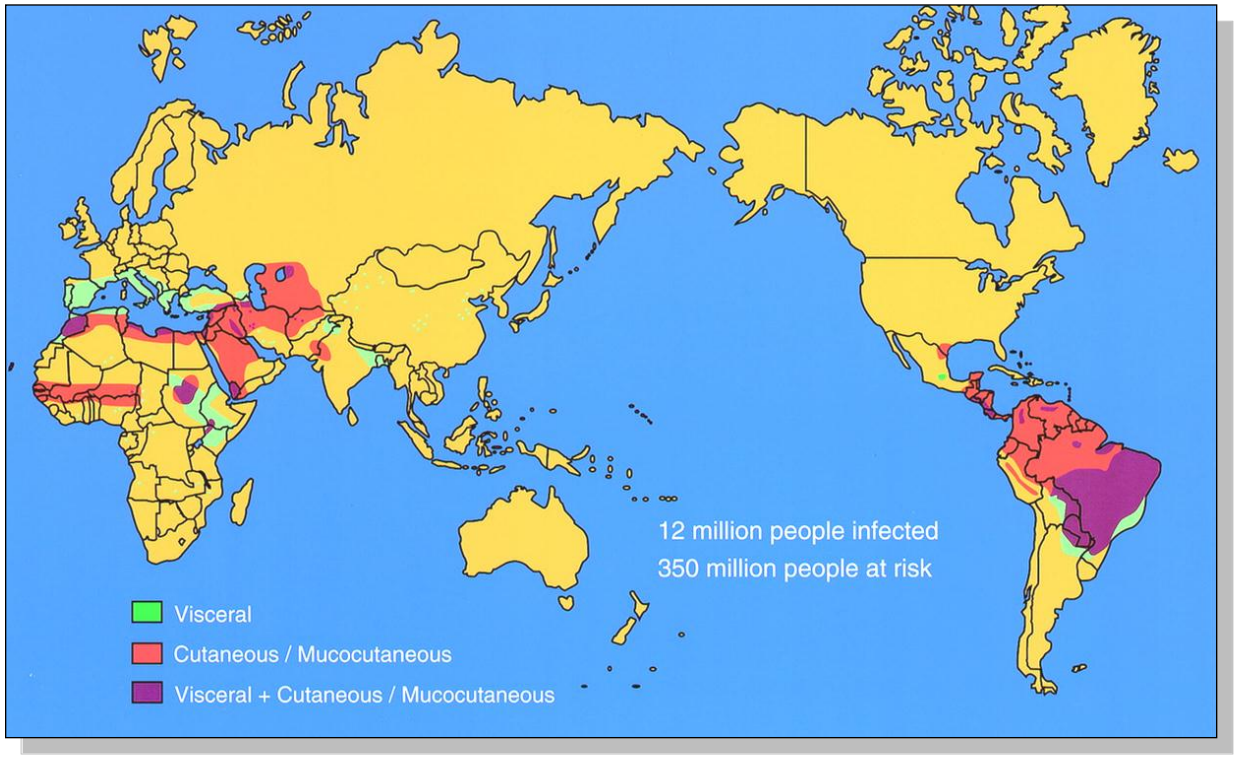
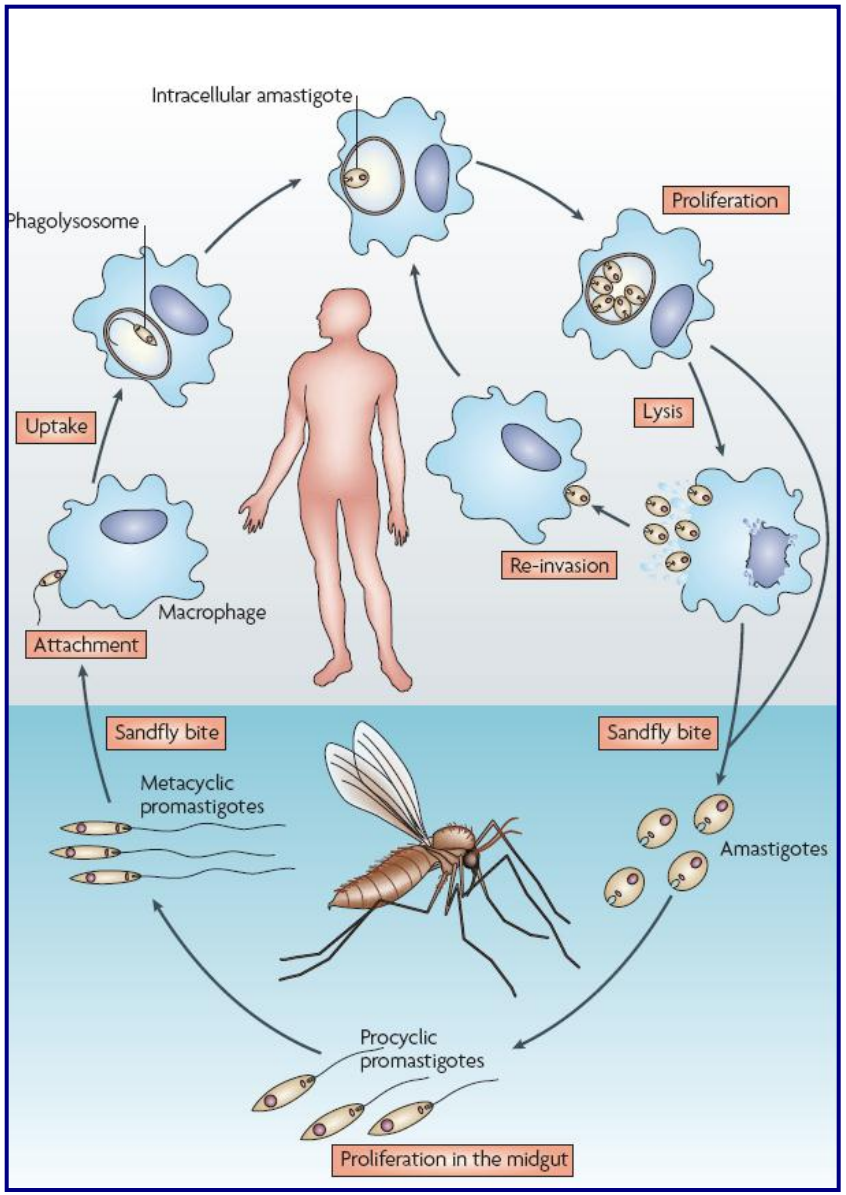
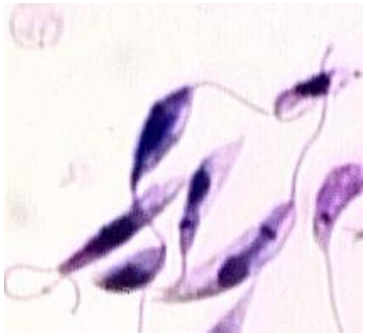
Leishmaniasis

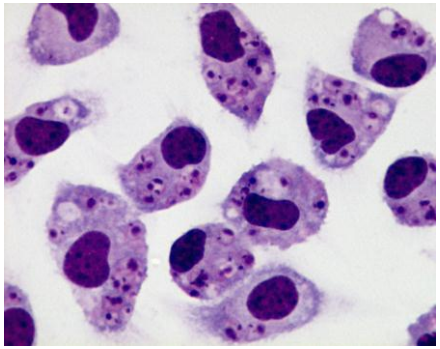


- 12 millions infected individuals
- 350 millions at risk
- Annual incidence: 2 millions

Leishmania life cycle







VL treatment

First-line drugs

Pentavalent antimonials (sodium stibogluconate-Pentostam; meglumine antimoniate- Glucantime**)**

Second-line drugs

Amphotericin B
- AmBisome (liposomes)

→ First-line
Europe; US

Other drugs

Miltefosine

Paromomycin

Limited efficacy

- Resistances
- Side effects
- HIV/ *Leishmania*
- Medical Supervision
- Cost

Disease Control

↓

Treatment

↓

No human vaccine available

Neglected diseases: drug discovery

- Combination of commercially available drugs
- Discovery of new applications for existent drugs
- Discovery of new molecules

- ✓Screening of compounds library
- ✓Target based drug discovery

Identification of *Leishmania major* gene encoding a protein belonging to the silent information regulatory proteins (SIR2) family

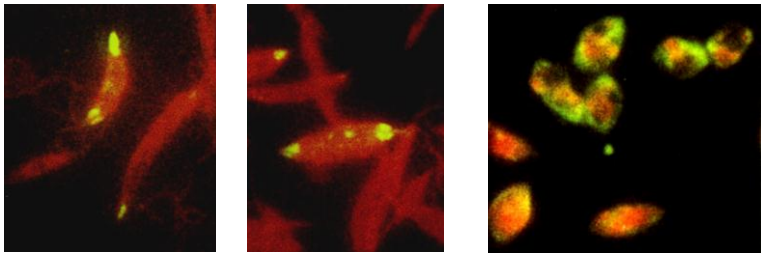
Screening of *Leishmania major* cDNA library

LmSIR2 : sharing 40% sequence identity to the yeast ySIR2

(Yahiaoui *et al*., *Gene*, 1996)

(Zemzoumi *et al*., *Bio. Cell*, 1998)

- ✓ Identification of SIR2 homologues in other *Leishmania* species (*L. amazonensis, L. infantum, L. amazonensis*)
- ✓ Cytoplasmic localization

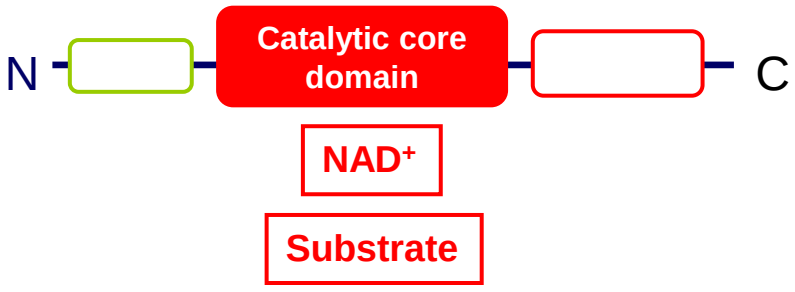


Promastigotes *L. major* Amastigotes *L. major*

SIR2 family

Humans

- SIRT1 nuclear
- SIRT2 cytoplasmic
- SIRT3 mitochondrial
- SIRT4 mitochondrial
- SIRT5 mitochondrial
- SIRT6 nuclear
- SIRT7 nuclear



Protozoa

Leishmania: SIR2RP1 (cytoplasmic)
- SIR2RP2 and RP3 (mitochondria)

T. brucei: SIR2RP1- SIR2RP3
nuclear

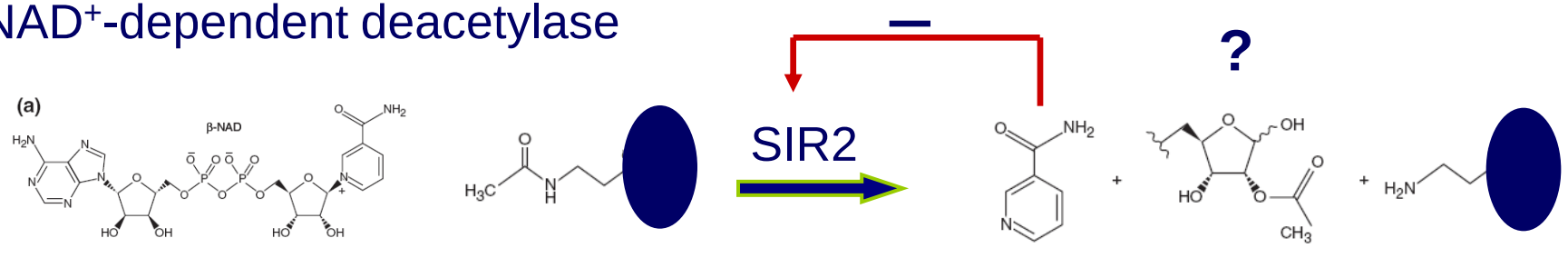
Plasmodium: PfSIR2
nuclear

Subcellular localization
Nucleus
Cytosol
Mitochondria

Substrate
H3 and H4
Tubulin
MyoD
p53

SIR2 enzymatic properties

NAD+-dependent deacetylase



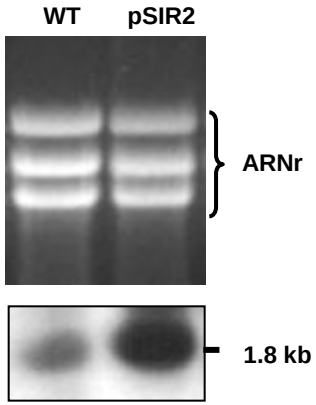
Adapted from North BJ, 2004

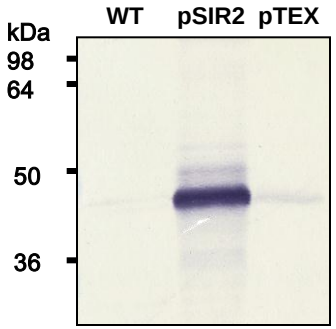
ADP-ribosyltransferase

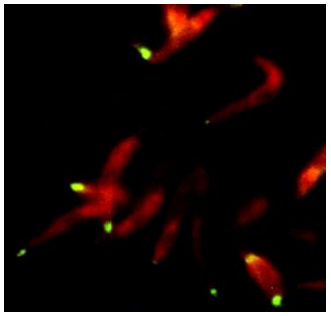
Adapted from Kowiesky TM, 2008

Overexpression of *LmSIR2* gene and parasite phenotypic characterization

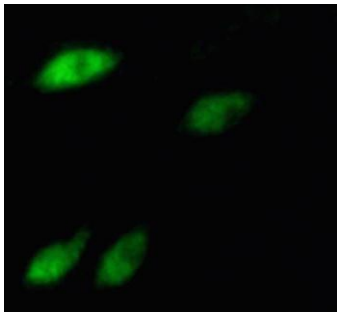
- pTEX plasmid: regulatory 5' and 3'UTR of T. *cruzi* glyceraldehyde phosphate dehydrogenis encoding gene *(GAPDH)* carrying *LmSIR2 gene*





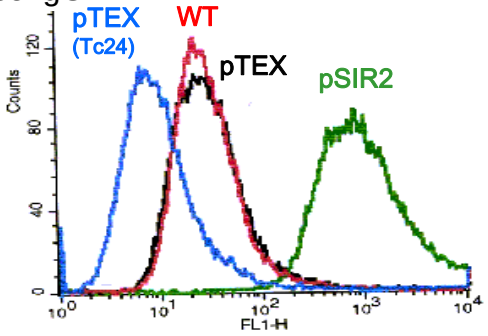


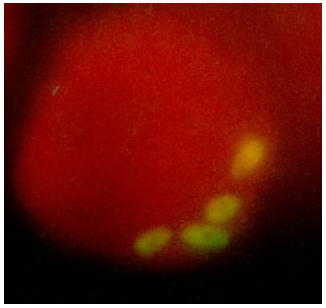
WT *L. infantum*

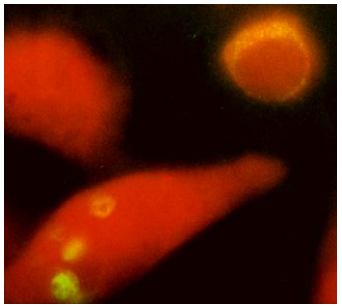


promastigotes pSIR2

Parasites + mAb anti-LmSIR2 + FITC-labeled rabbit Ig anti-mouse IgG





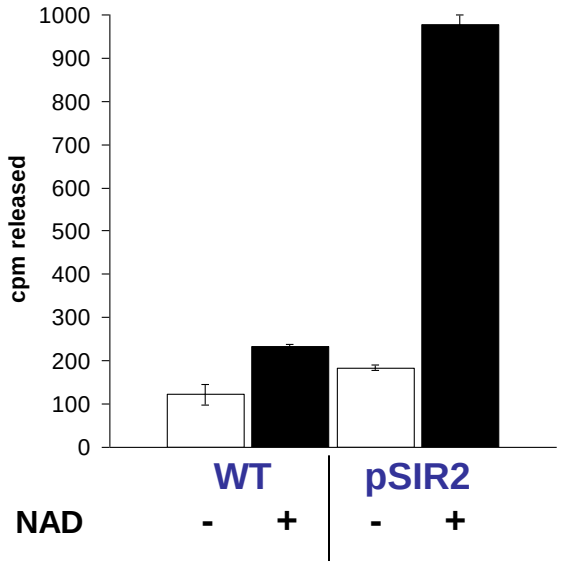


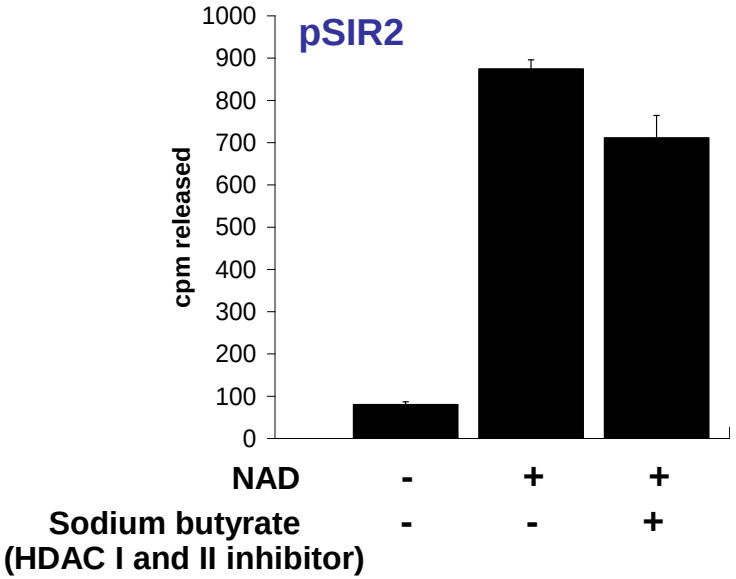
pSIR2 intracellular amastigotes (THP-1)

6 times increase of LmSIR2 protein synthesis

NAD+-dependant deacetylase activity in pSIR2 extracts

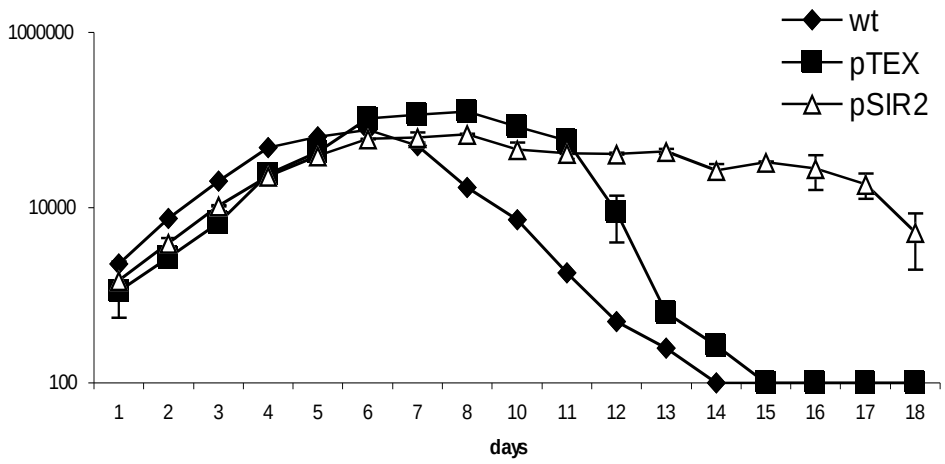
•[^{3}H]-labeled histone peptide incubated with parasite extracts with or without NAD





Growth of axenic amastigotes which overexpress LmSIR2

Normal culture conditions (MAA20 : pH 5, 37°C)



Overexpression of LmSIR2 increased survival of stationary phase axenic amastigotes

Vergnes et al., Gene, 2002, 296: 139-150

L. infantum (*LiSIR2*) gene sequencing and functional characterization

Methodology :

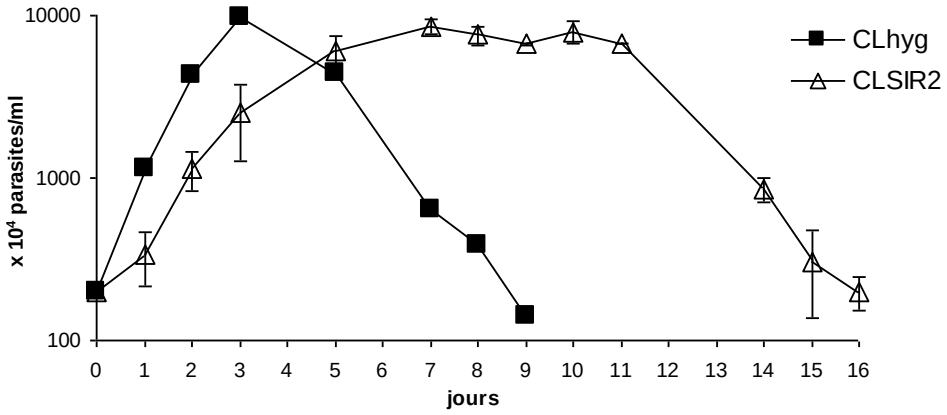
- Screening of a genomic *L. infantum* (CLhyg) library using LmSIR2 labeled cDNA as a probe

-Isolation of a genomic *Hind* III fragment of 5.9kb and sequencing :

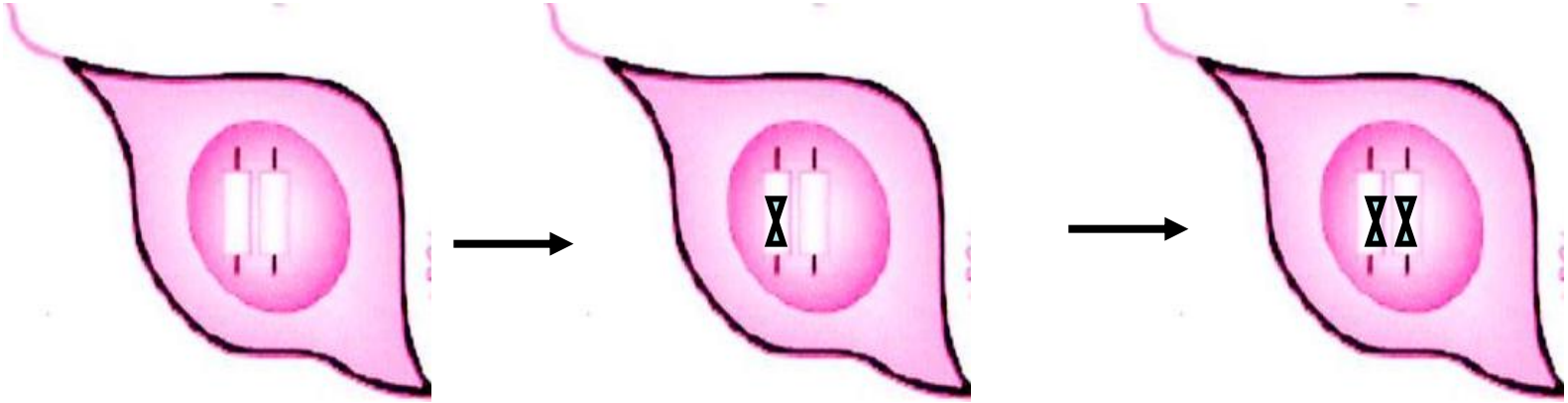
« LiSIR2 » (373aa) : 93% identity to LmSIR2

- Transfection of WT L. infantum promastigotes with either the empty cosmid (CLhyg) or the cosmid carrying the *LiSIR2* gene (CLSIR2)

Under normal culture conditions (MAA20 : pH 5, 37°C)



Reverse genetic approaches



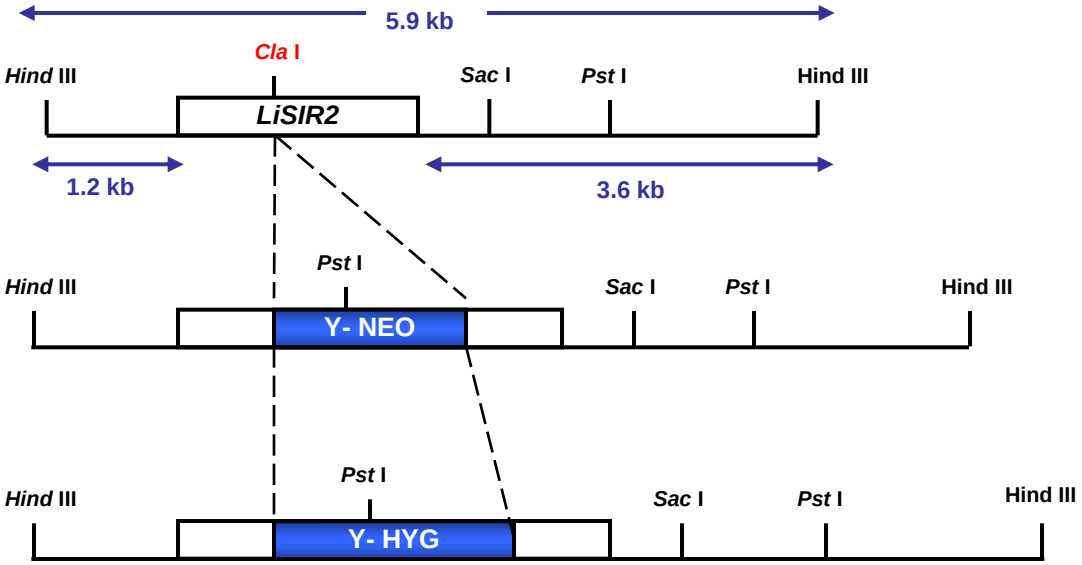
-Defined DNA construct carrying drug resistance gene (selection)
-Transfection
-Homologous recombination
-Mutant parasites
-Phenotype studies

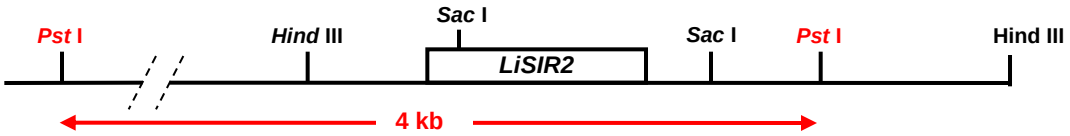
LiSIR2 gene inactivation by homologous recombination « Knock out »

Leishmania : diploïd, mitosis : 2 steps for inactivation of both alleles

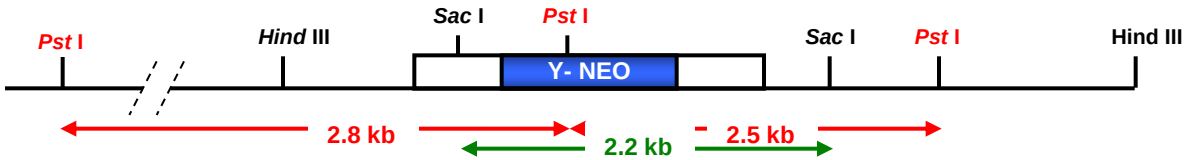
Methodology : gene disruption

- *Hind* III genomic fragment of 5.9 kb carrying the *LiSIR2* gene
- Y-NEO and Y-HYG integration into the LiSIR2 catalytic domain (*Cla* I)
- Plasmid DNA construct was digested using Hind III for transfection purposes
- Drug selection and cloning of parasites

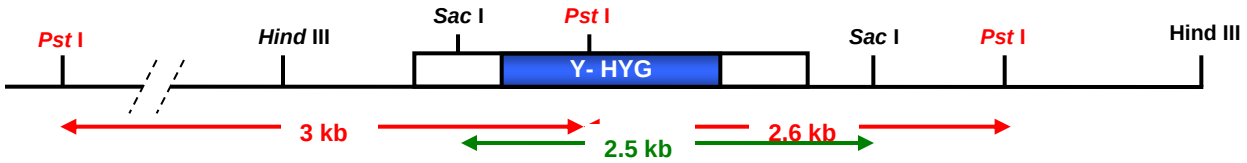




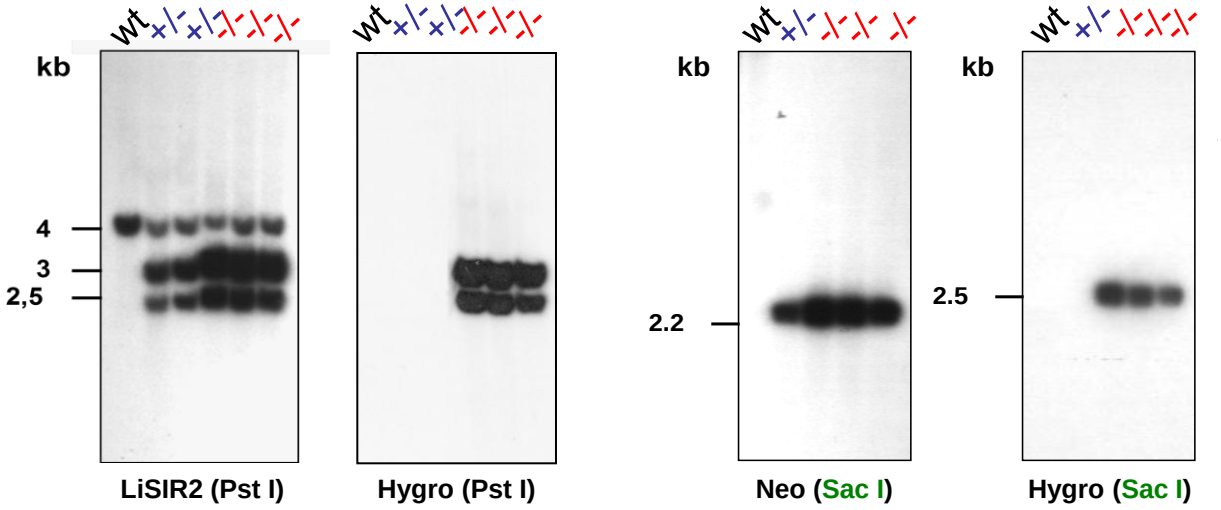
***WT LiSIR2* Locus**



***LiSIR2::neo* Locus**



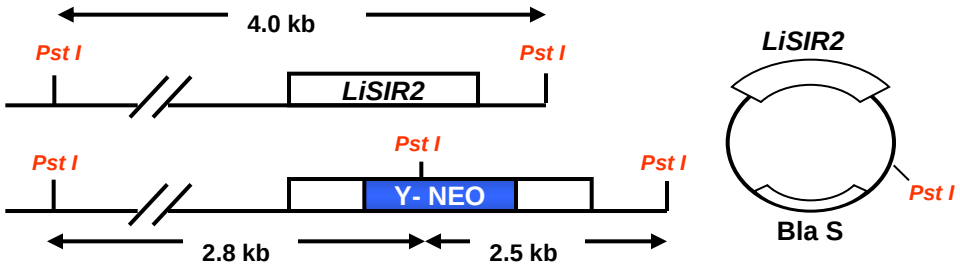
***LiSIR2::hyg* Locus**



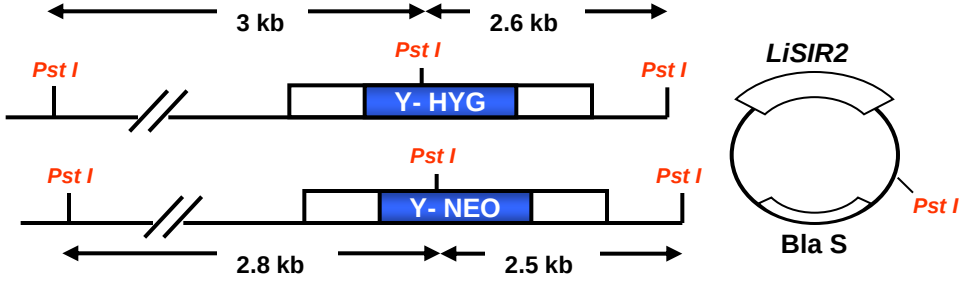
- Production of single mutant parasites LiSIR2+/-

- No possibility to get viable double mutants LiSIR2-/- (neo/hyg or hyg/neo)

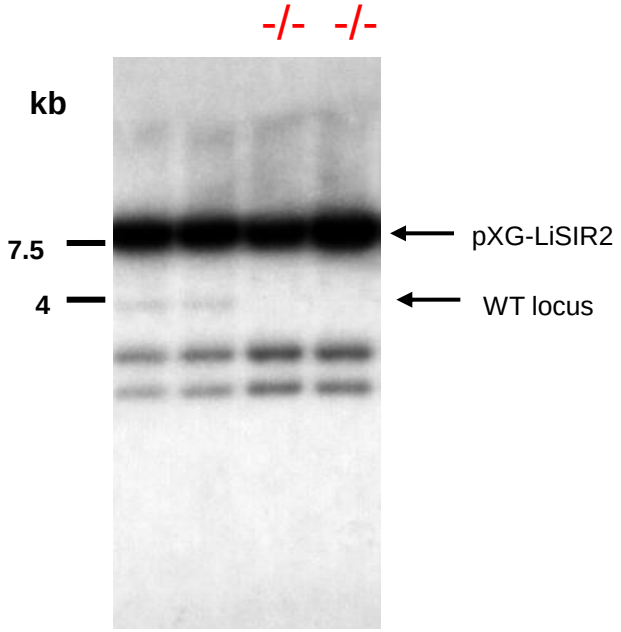
Inactivation of both *LiSIR2* alleles required episomal rescue



LiSIR2/LiSIR2::neo [pXG-LiSIR2]



LiSIR2::neo/LiSIR2::hyg[pXG-LiSIR2]



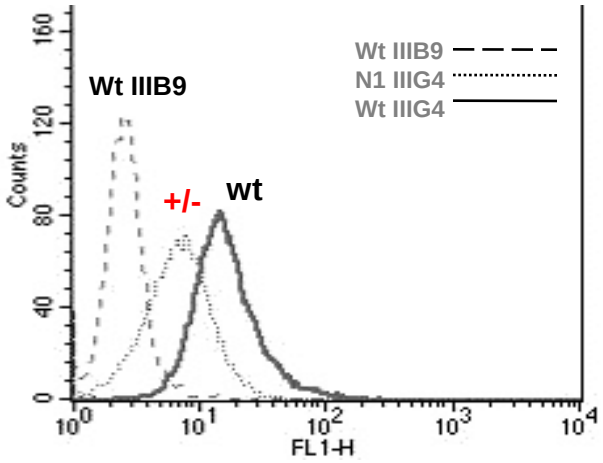
Pst I digestion, *LiSIR2 probe hybridization*

LiSIR2 : essential for parasite survival

Vergnes et al., 2005, 363: 85-96

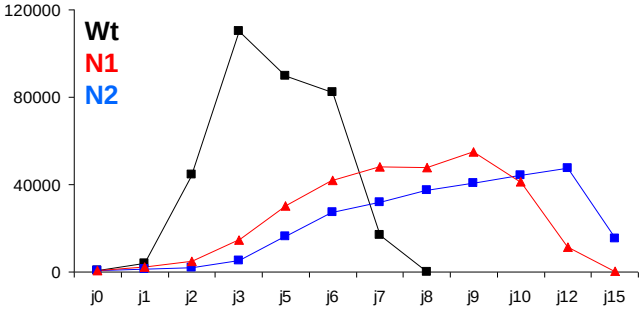
Growth phenotype of LiSIR2+/- *in vitro*

N1, N2 : LiSIR2/LiSIR2::neo

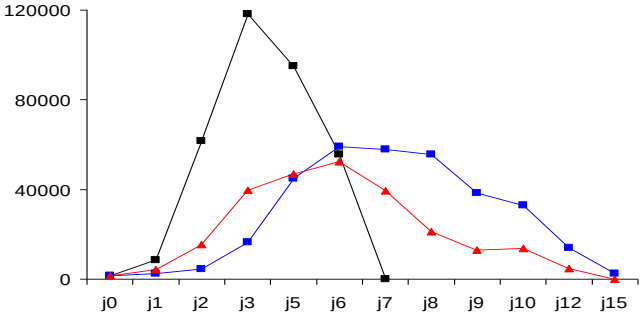


amastigotes

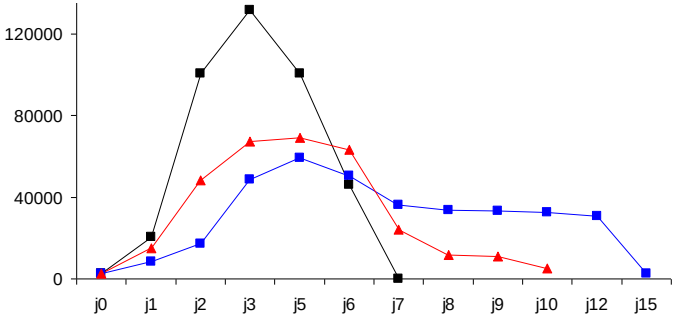
10^6 p/ml



2.10^6 p/ml



4.10^6 p/ml

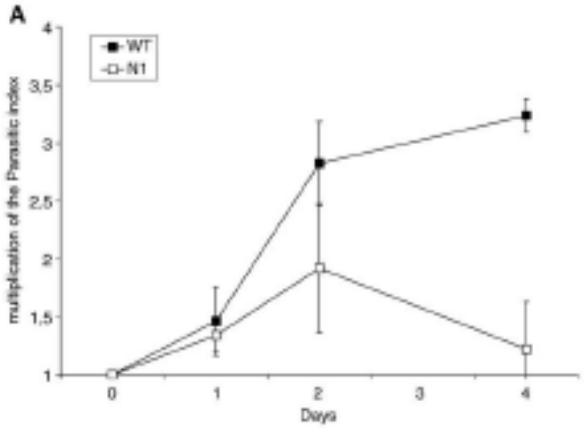


Inactivation of one *LiSIR2* allele alters the *in vitro* growth capacity of axenic amastigotes

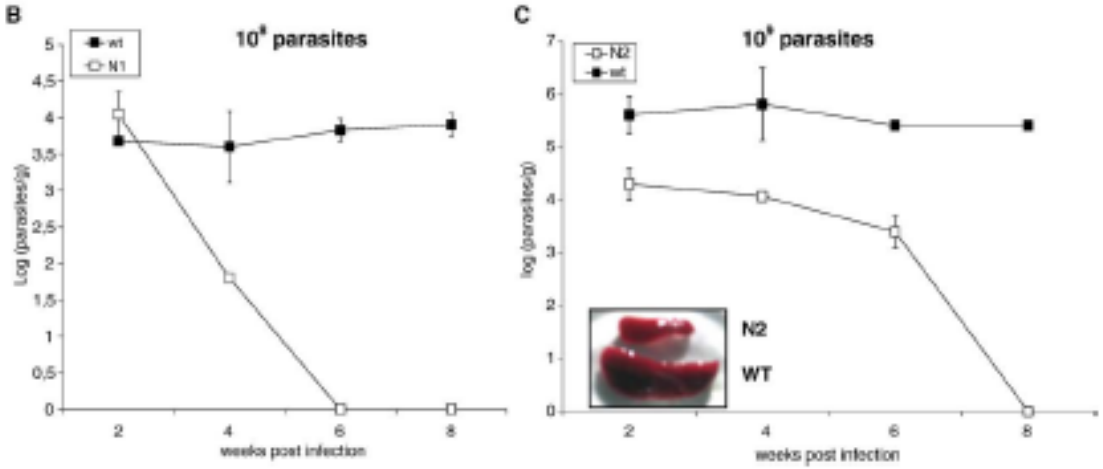
Target identification and validation

Intracellular amastigotes

In vitro



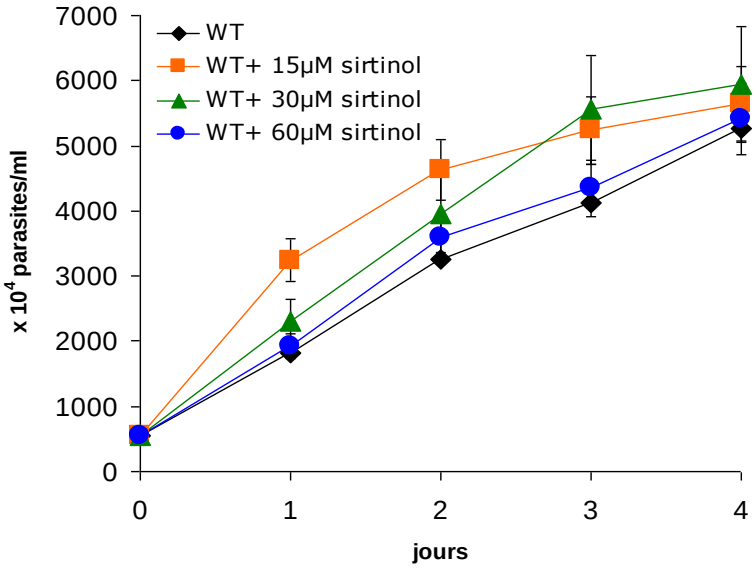
In vivo



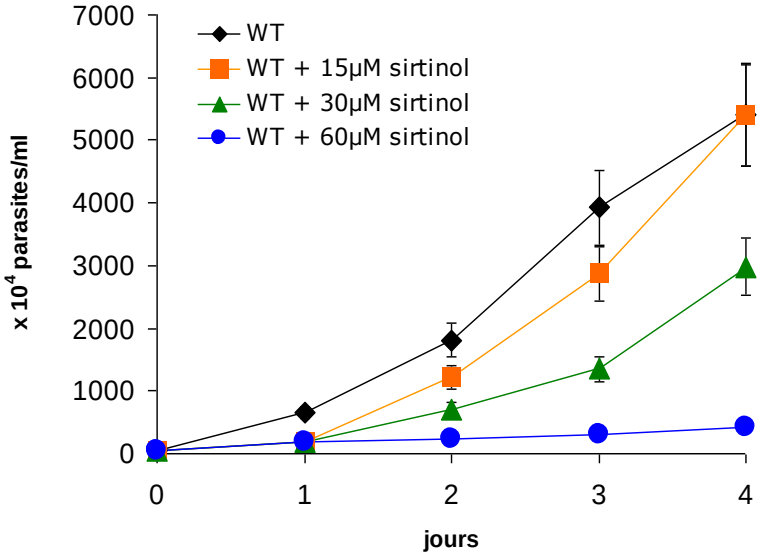
Parasite growth inhibitory activity of sirtinol

sirtinol : « SIR Two Inhibitor NaphtOL » (Grozinger et al., *J Biol Chem*, 2001) (inhibition of ySIR2 and SIRT2 *in vitro*)

promastigotes



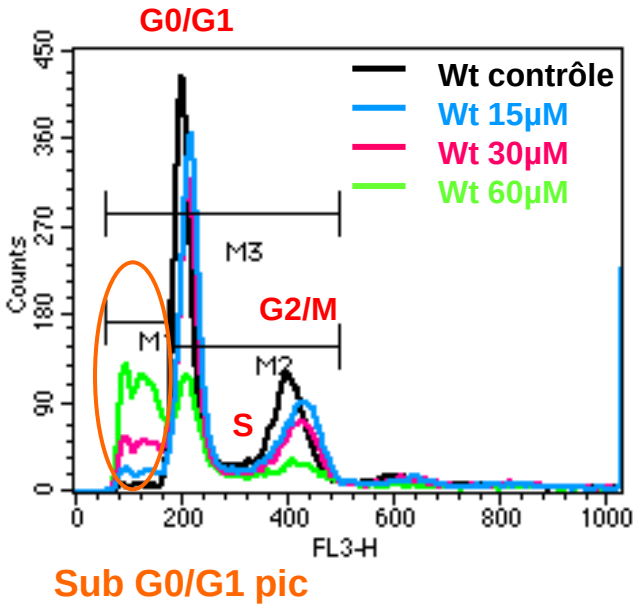
amastigotes



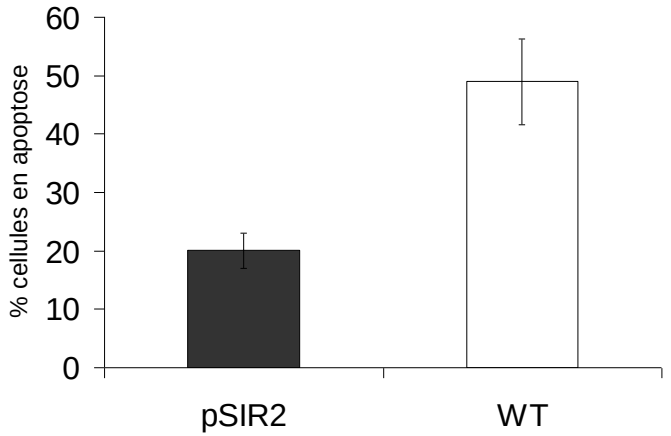
Sirtinol inhibited the *in vitro* proliferation of amastigotes in a dose-dependant manner

Leishmanicidal activity of Sirtinol toward amastigotes

Activity of sirtinol on amastigote cell cycle after 4 days of culture



Percent cells at sub G0/G1 after 4 days of culture in the presence of 60µM sirtinol

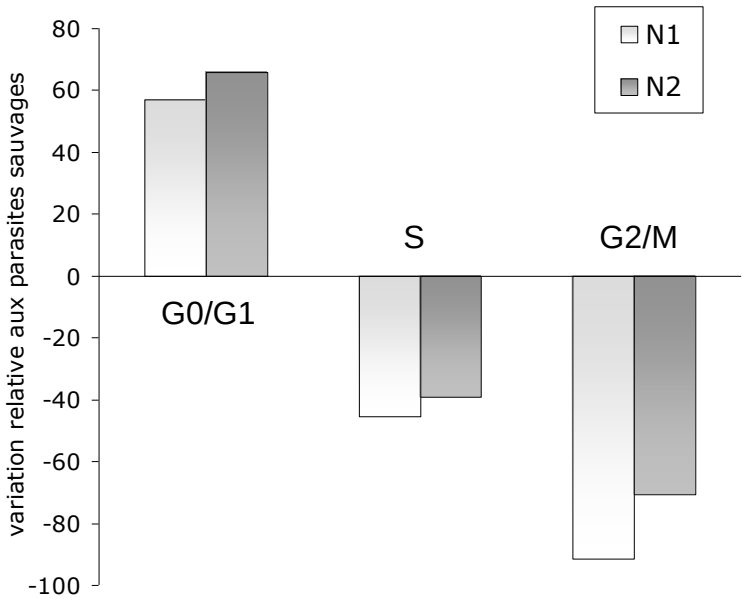


Sirtinol induced the appearance of a sub G0/G1 pic which revealed the presence of DNA fragmentation, reminiscent of the apoptotic phenotype.

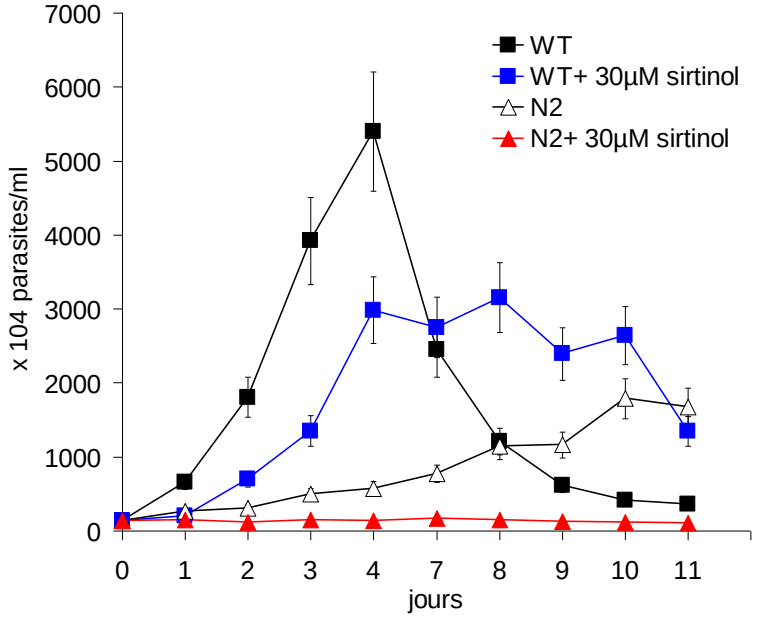
A certain degree of protection against apoptosis (30%) could be seen in the case of amastigotes which overexpress LiSIR2

Growth phenotype of LiSIR2+/- *in vitro*

Cell cycle analysis at 4 days of culture N1, N2 : LiSIR2/LiSIR2::neo



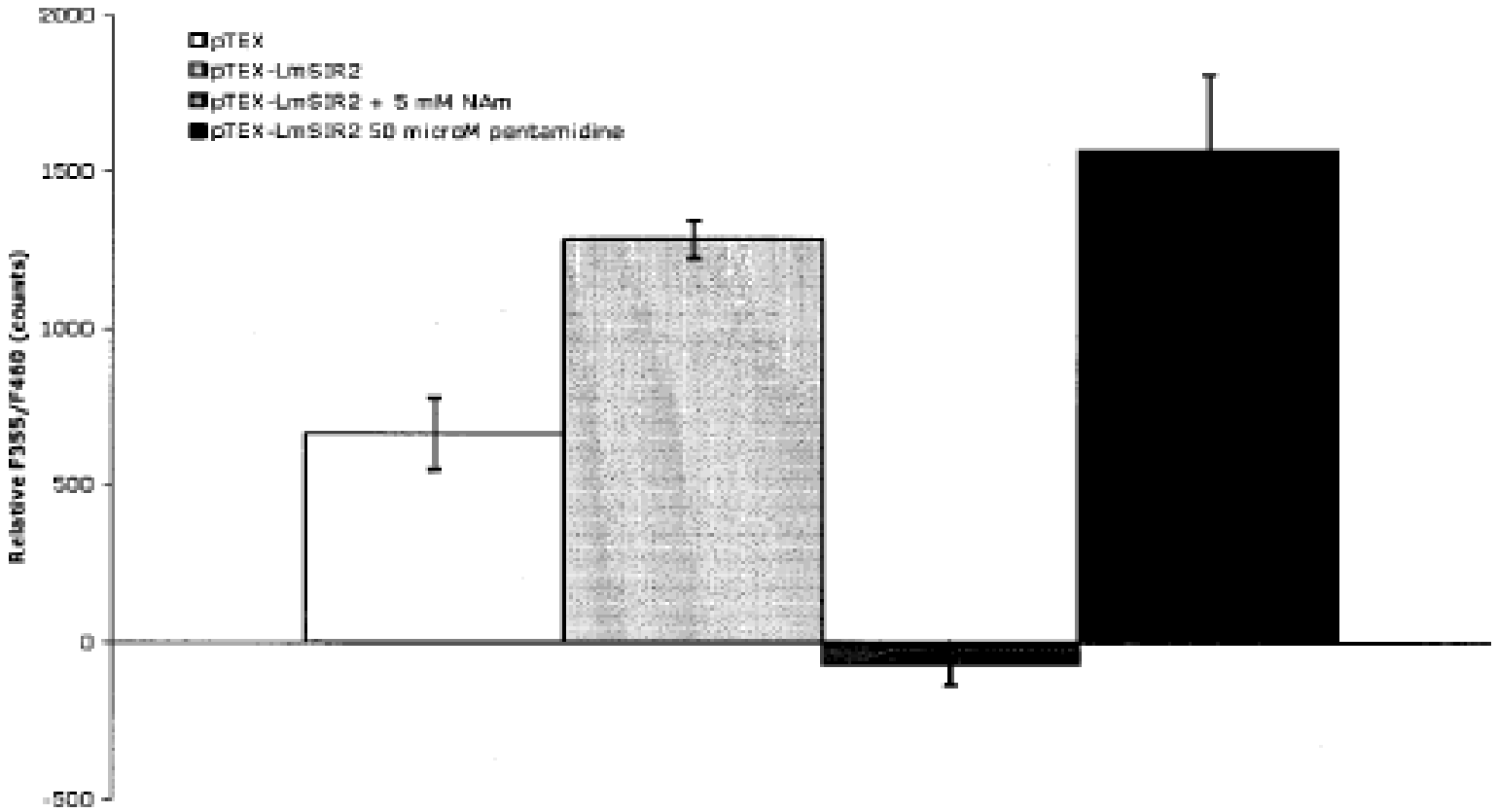
Effect of sirtinol on the growth of LiSIR2+/- clones



The mutants are blocked at G0/G1 of the cell cycle

Inhibition of the remaining LiSIR2 protein by sirtinol was detrimental for the growth of single mutant amastigotes

Vergnes et al., Acta Trop., 2005, 94, 107-115



Nicotinamide a physiological inhibitor of SIR2 enzyme, at 5 mM concentration almost completely blocked the NAD-dependant deacetylase activity present in the total extracts from parasites which overexpress LmSIR2

Sereno et al., Antimicrob. Agents Chemother., 2005, 49, 808-812

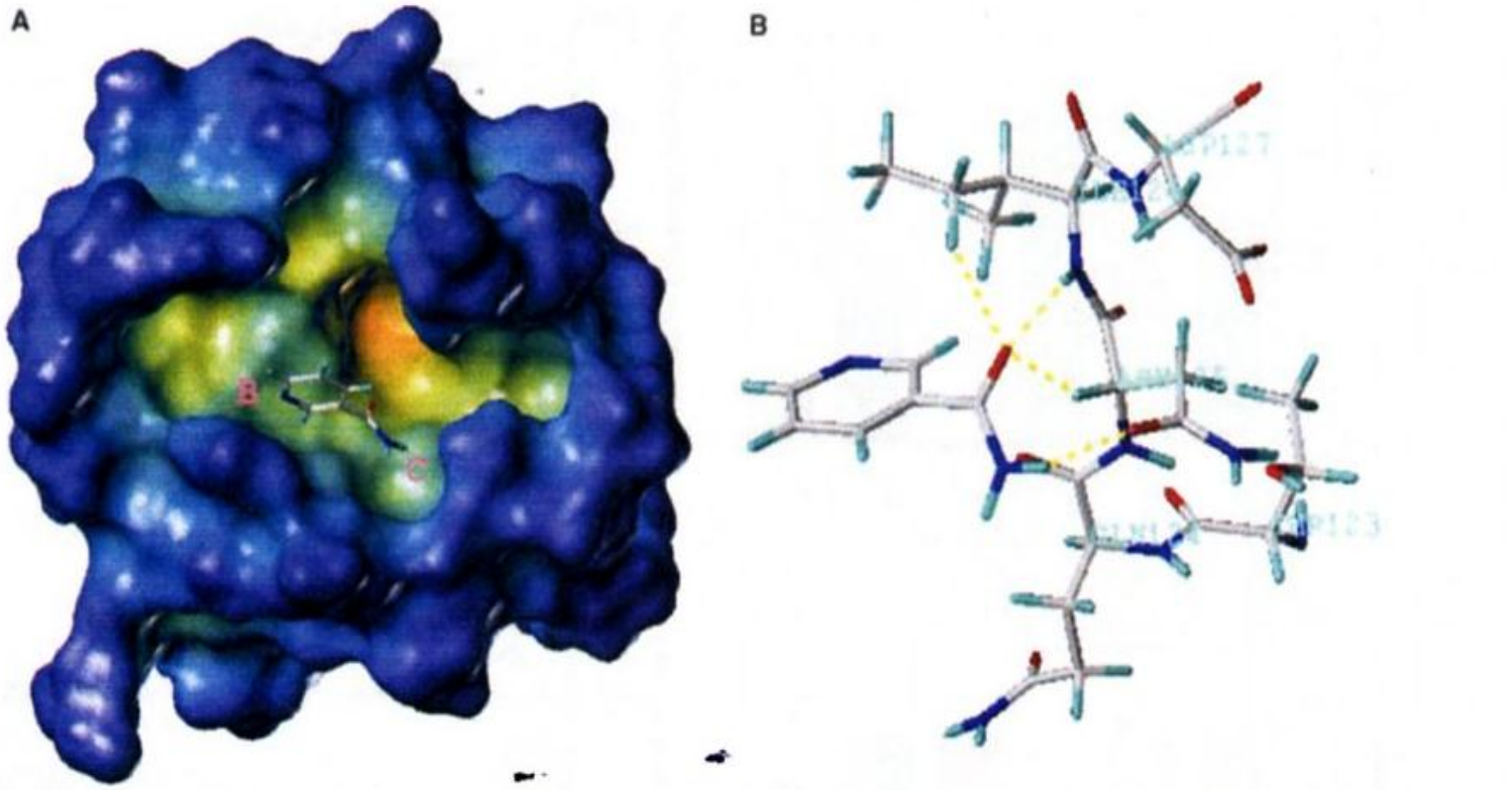


Figure 2: LmSir2 model. (A) MEPS surface showing docking of nicotinamide. (B) Interaction of nicotinamide with active side residues.

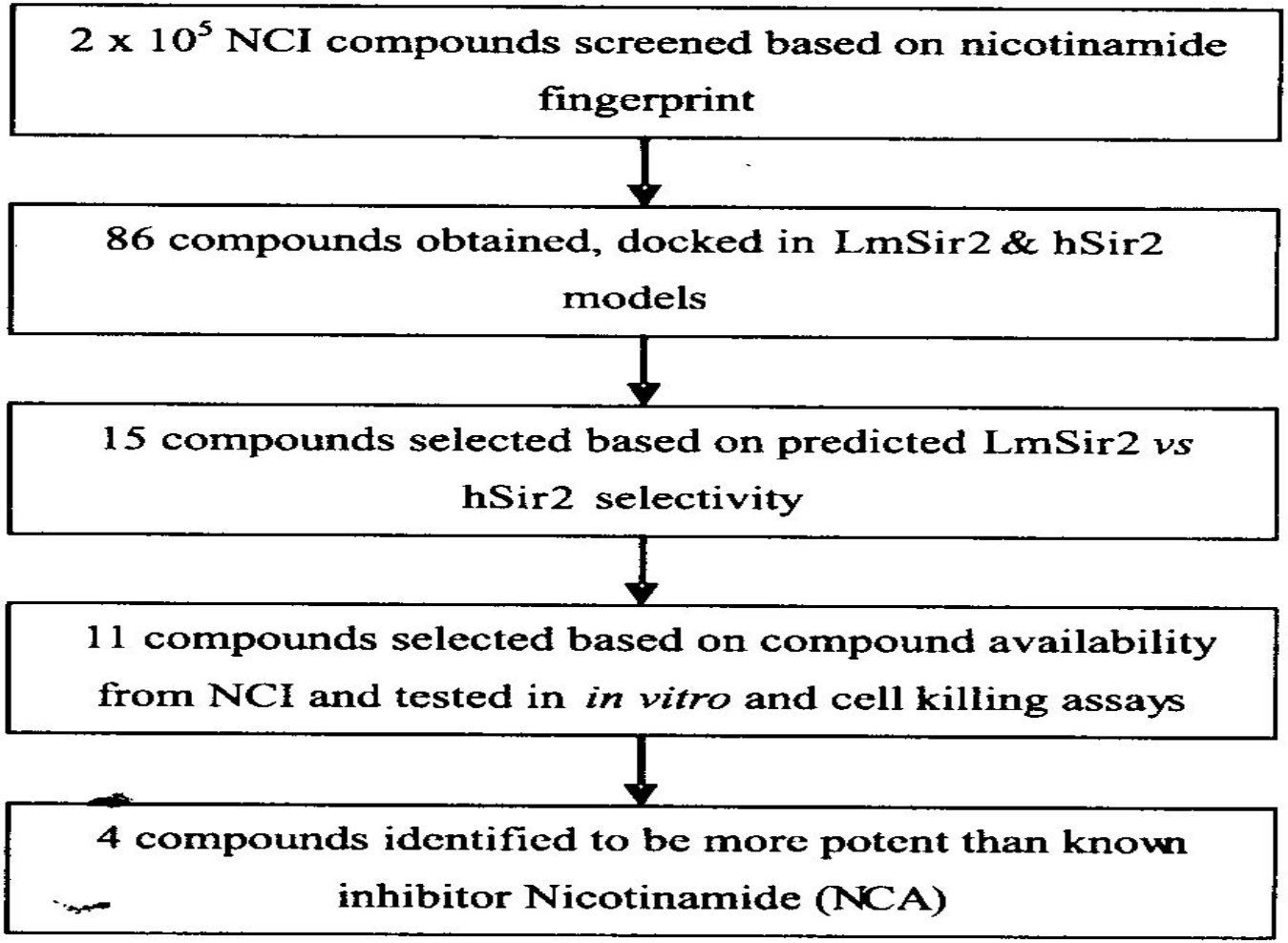


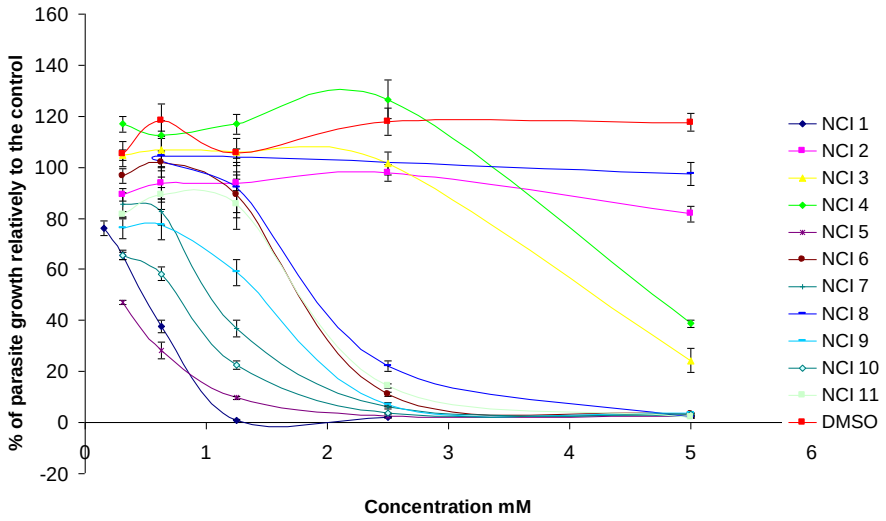
Figure 1: Flow diagram of screening protocol used in the study.

National Cancer Institute database

National Cancer Institute

Compound library screening : Computational analysis

1: 2-aminonicotinohydrazide
2:_methyl 6-methylnicotinate
3::N'-acetylnicotinohydrazide
4:6-(dimethylamino)nicotinamide
5:_6-(benzyloxy)nicotinamide
6: 2-hydroxy-6 methylnicotinamide
7: N-(2-fluorophenyl)nicotinamide
8: 2-amino-5-cyano-6-(methylthio)nicotinamide
9:3-methoxy-N-methyl-2-oxo-1(2H)-10:pyridinecarboxamide
10:2-(2-ethyl-1,3-thiazol-4 yl)acetamide
11:3,5-pyridinedicarboxamide



L. infantum amastigotes expressing luciferase incubated with different compounds
The viability was evaluated by the luciferase assay

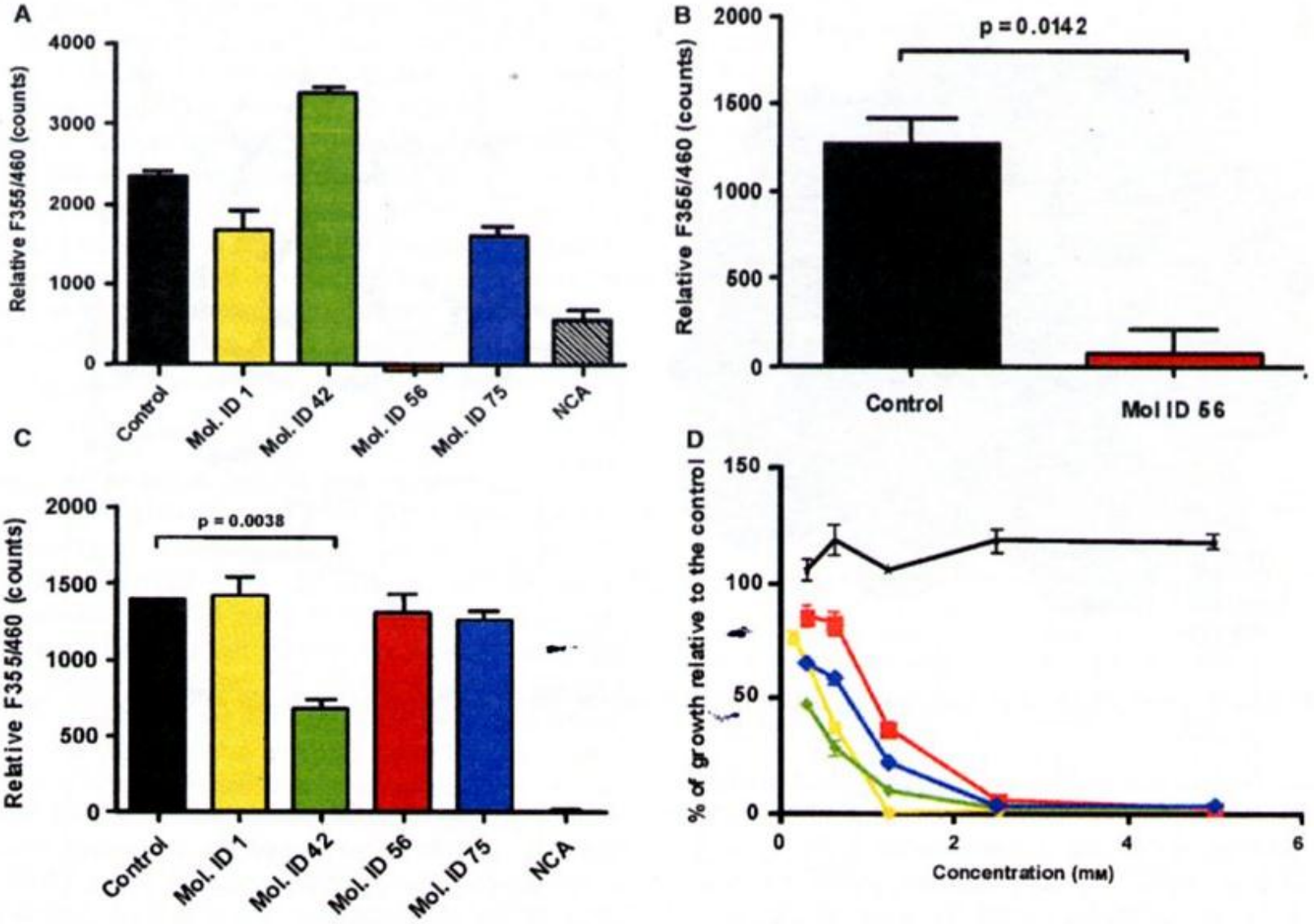
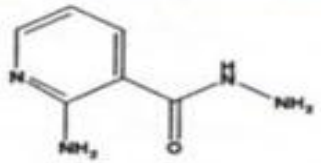
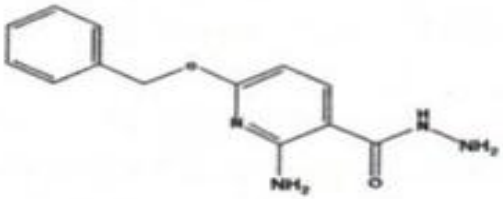
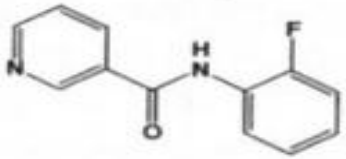
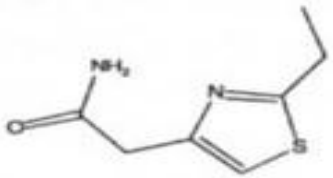
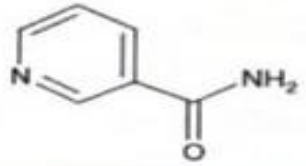

A
Relative F355/460 (counts)
4000
3000
2000
1000
0
Control
Mol. ID 1
Mol. ID 42
Mol. ID 56
Mol. ID 75
NCA
B
Relative F355/460 (counts)
2000
1500
1000
500
0
p = 0.0142
Control
Mol ID 56
C
Relative F355/460 (counts)
2000
1500
1000
500
0
p = 0.0038
Control
Mol. ID 1
Mol. ID 42
Mol. ID 56
Mol. ID 75
NCA
D
% of growth relative to the control
150
100
50
0
0
2
4
6
Concentration (mM)

Table 1: Docking scores of and amastigote growth inhibition by the four active compounds and NCA

Mol ID	Structure	FlexX Score		IC_{50} (mM)
		LmSir2	hSir2	
1		−23.7	−15.1	0.49 ± 0.009
42		−16.1	−16.9	0.28 ± 0.036
56		−13.4	−9.6	1.49 ± 0.021
75		−4.2	−8.6	0.82 ± 0.079
NCA		−10.9	−11.0	5.5 ± 0.05

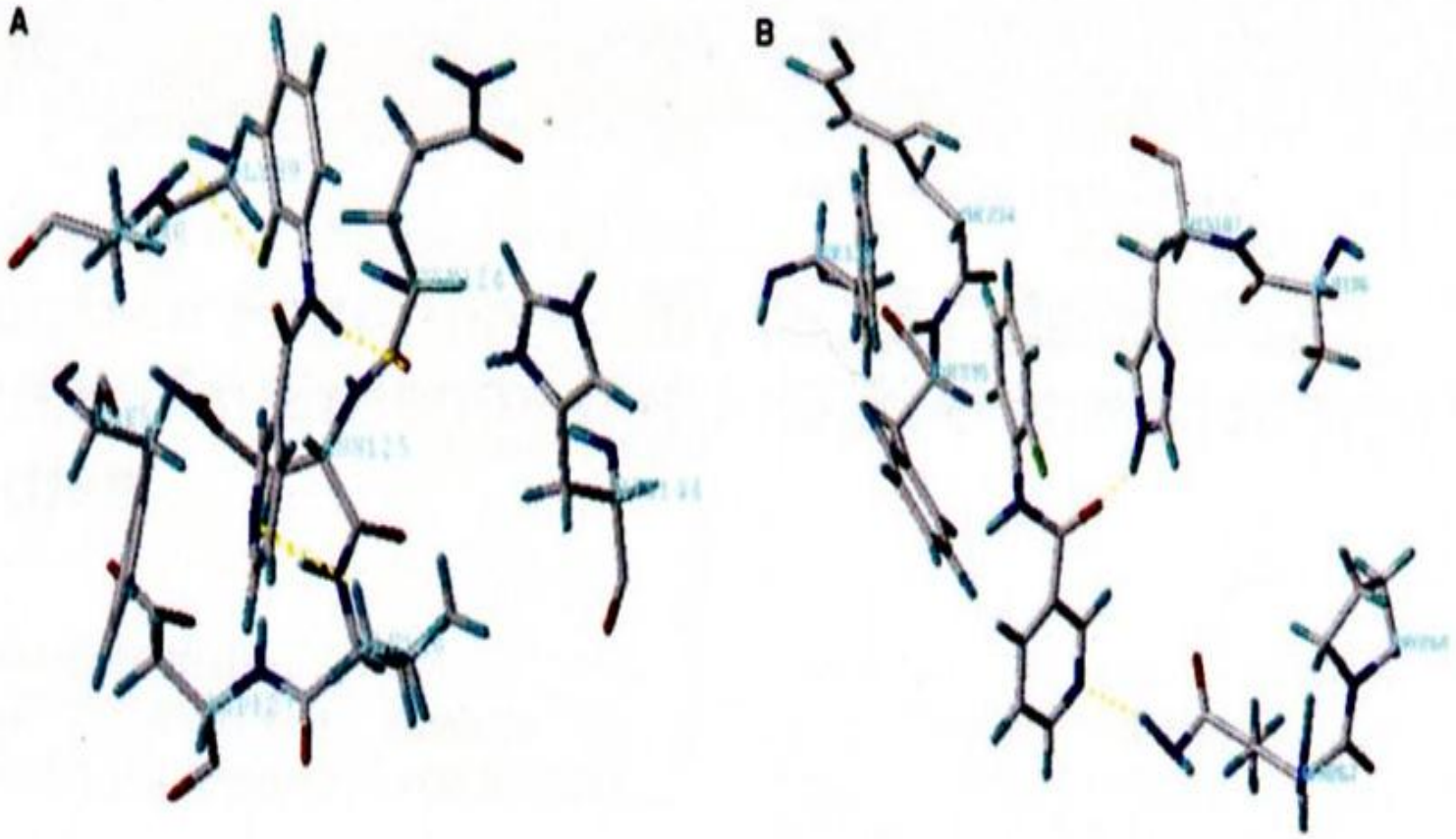
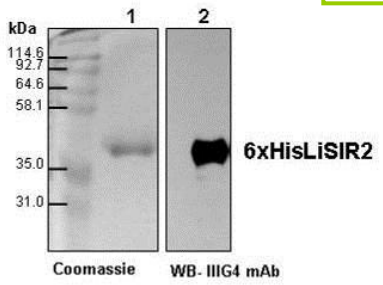


Figure 5: Compound **56** selectively docked in *Leishmania* sirtuin (A) as compared to human sirtuin (B).

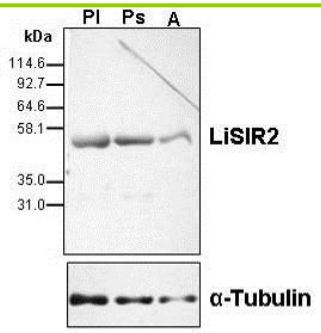
Kadam et al., Chem Biol Drug Des. 2008, 71: 501-6.

LiSIR2RP1 is a NAD+ dependent deacetylase

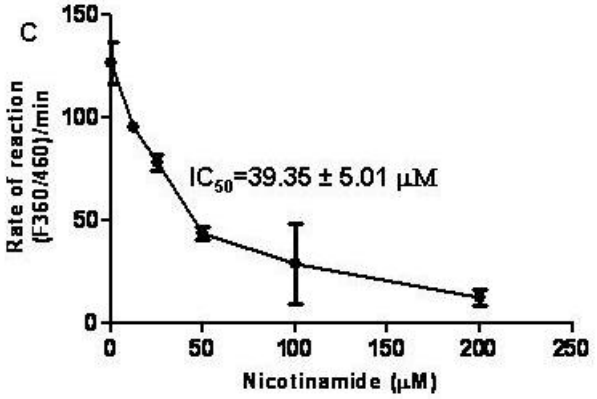
r*Li*SIR2RP1



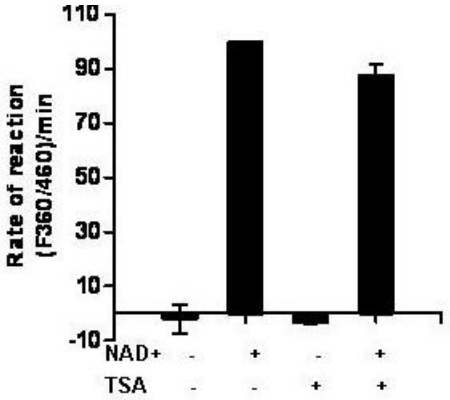
Protein expression in *Leishmania extracts*



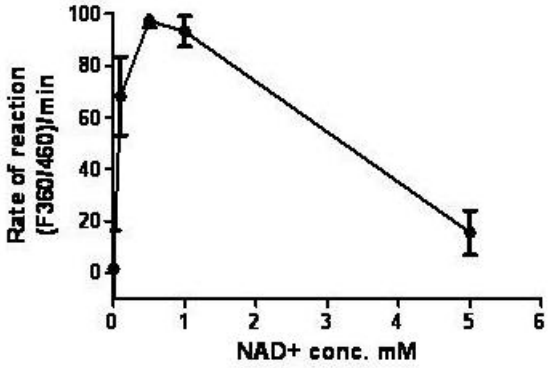
Nicotinamide effect

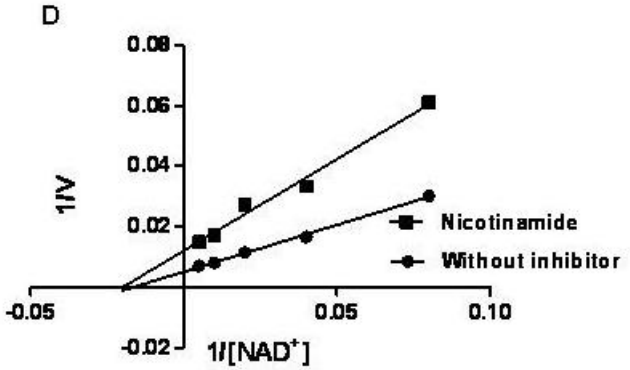


Deacetylase activity



[NAD+] effect



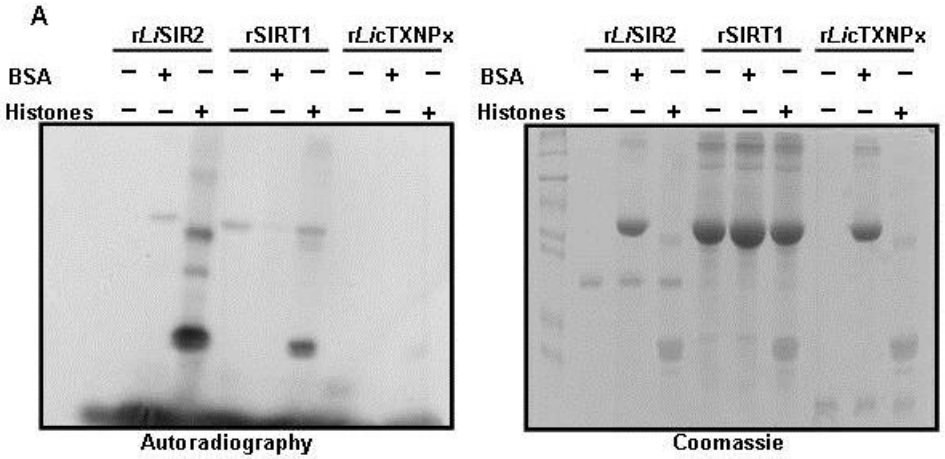


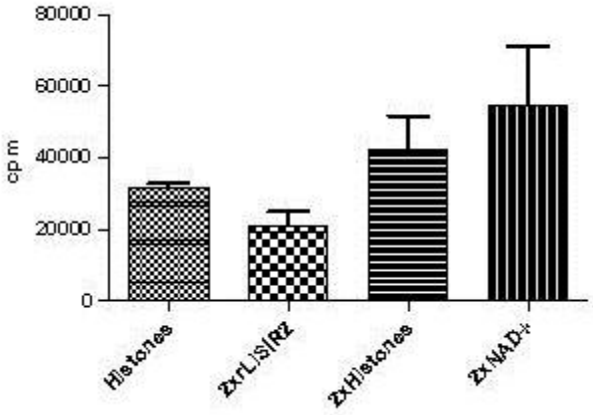
Class III NAD+ dependent deacetylase

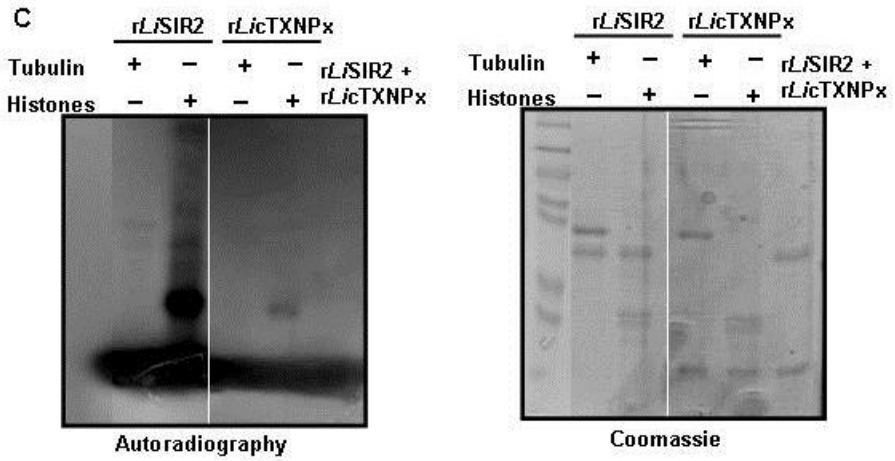
LiSIR2RP1 is inhibited by nicotinamide

LiSIR2RP1 expresses ADP-ribosyltransferase activity

ADP-ribosyltransferase activity



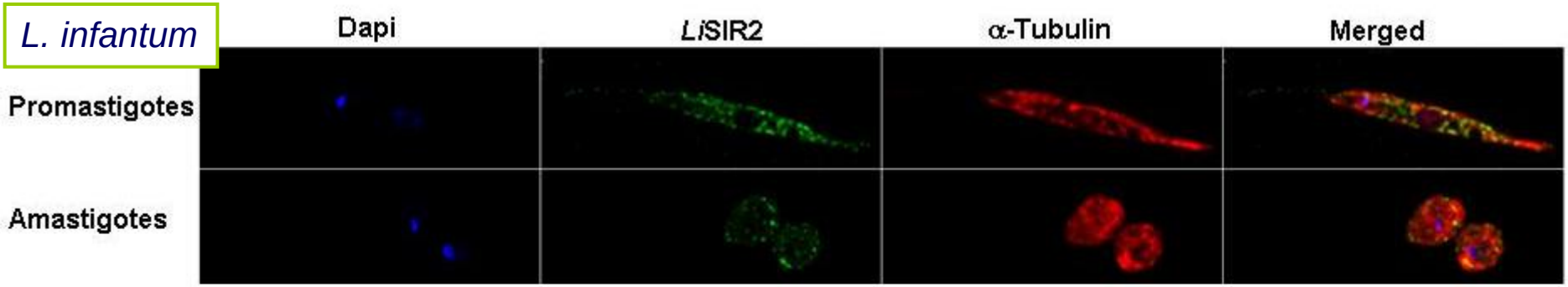




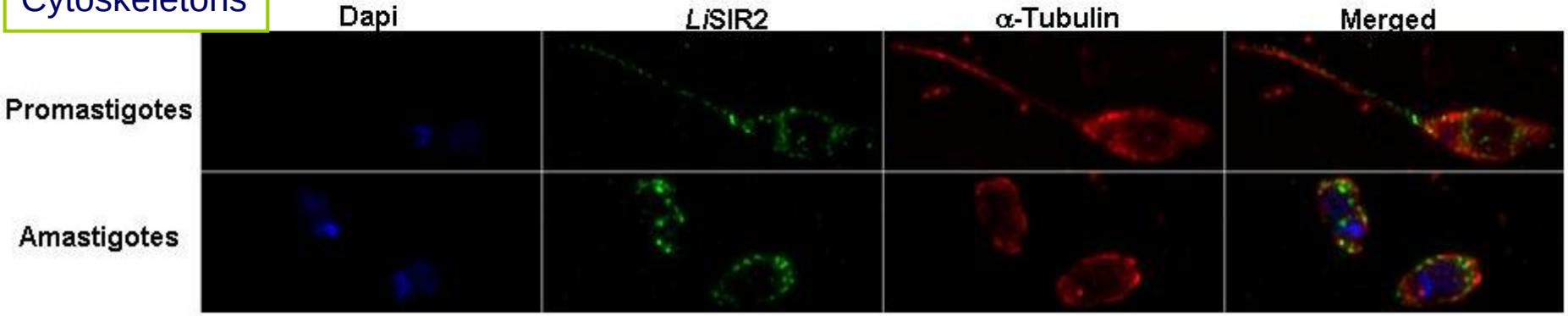
*Li*SIR2RP1
-is auto-ADP ribosylated in presence of histones and tubulin
- ADP-ribosylate histones, BSA and tubulin

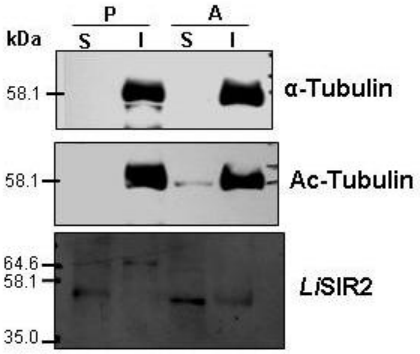
LiSIR2RP1 substrate identification

L. infantum



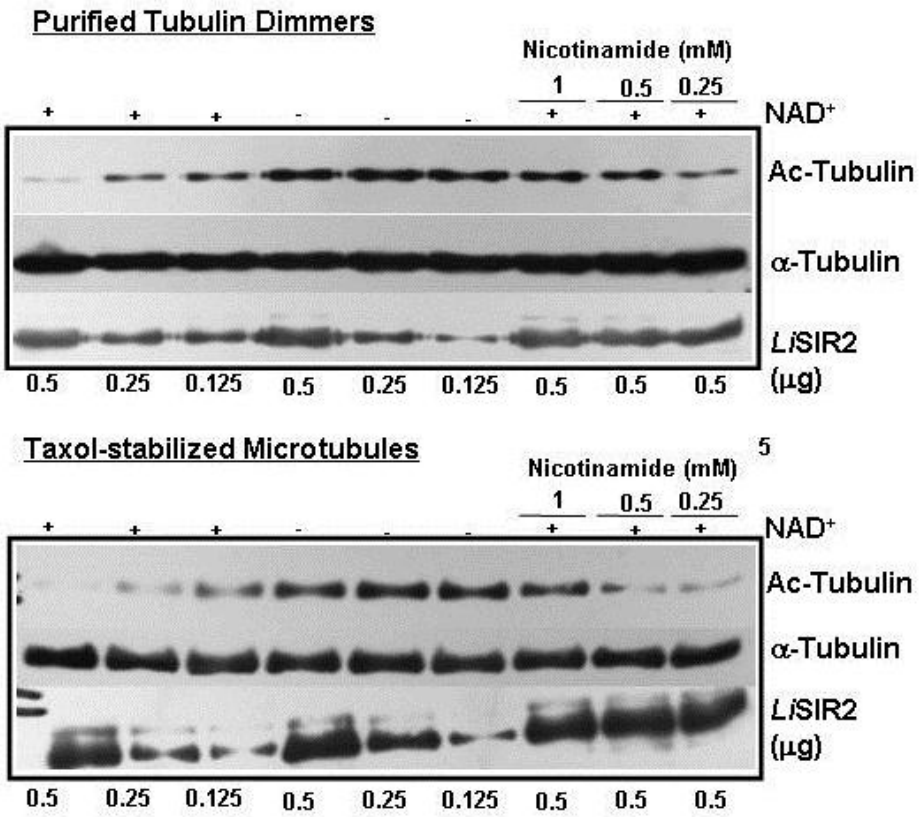
Cytoskeletons





*Li*SIR2RP1 is partially associated with microtubules

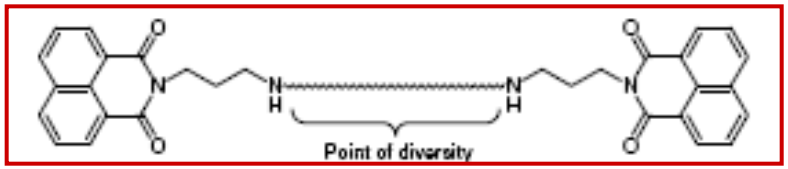
LiSIR2RP1 deacetylates α-tubulin



*Li*SIR2RP1 deacetylates tubulin either in dimers or taxol stabilized microtubules

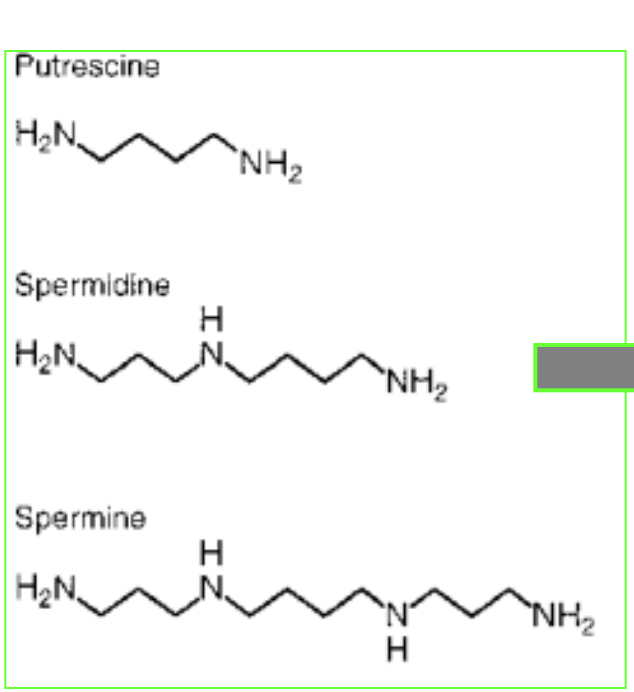
Tavares *et al.*, *Biochem. J.*, 2008, 415: 377-386

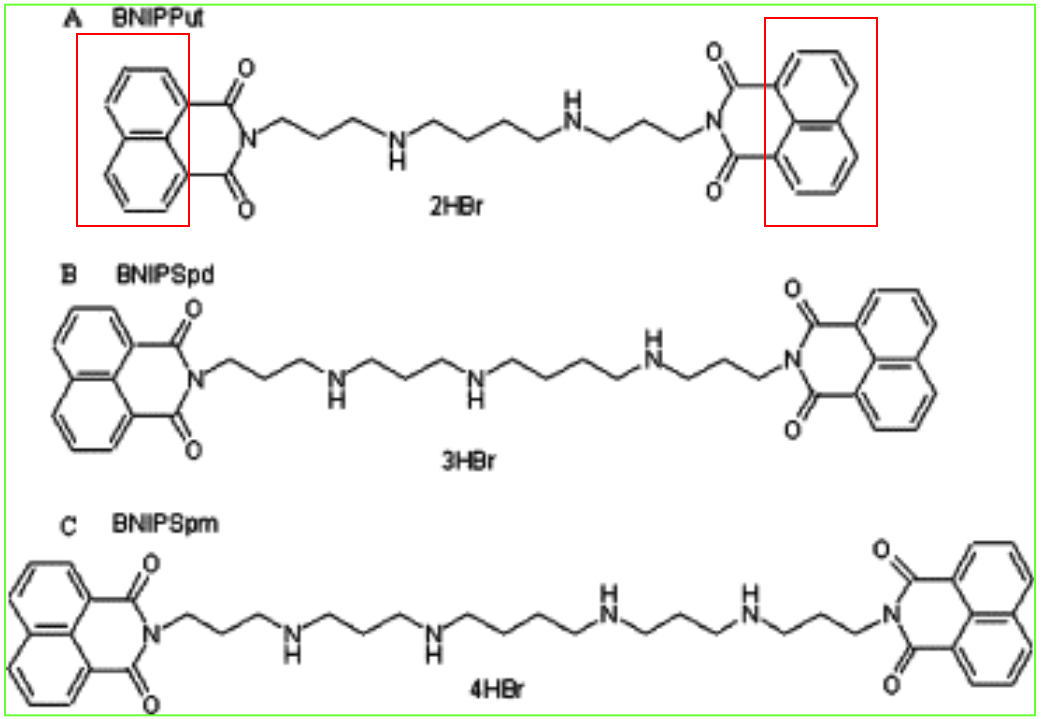
BNIPderivatives



??

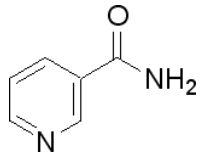
bisnaphthalimidopropyl (BNIP)





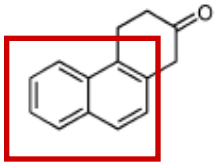
Tavares et al., Int. J. Parasitol. 2005, 35: 637-46

Search for target specific inhibitors

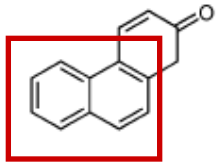


Nicotinamide

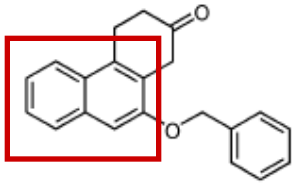
SIR2 inhibitors



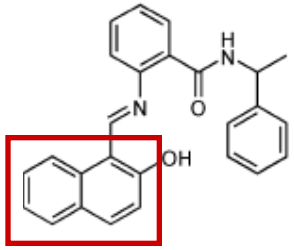
Splitomicin



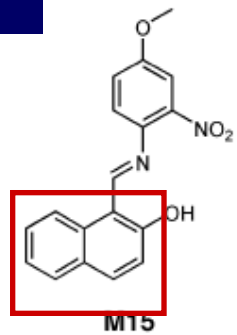
Dehydrosplitomicin



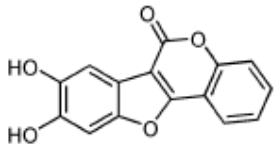
26



Sirtinol



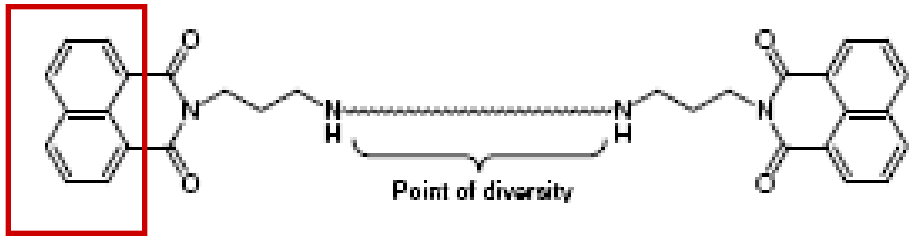
M15



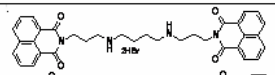
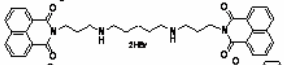
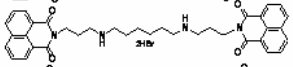
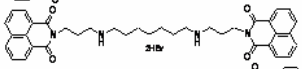
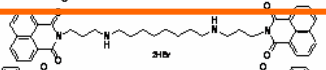
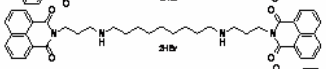
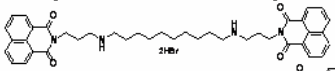
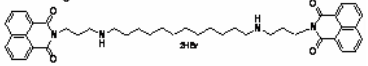
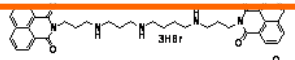
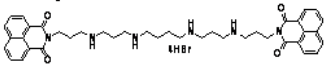
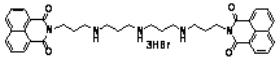
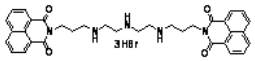
A3

➢The presence of a common structural moiety (naphthalene) in some molecules identified as Sirtuin inhibitors led us to re-evaluate the activity of bisnaphthalimidopropyl (BNIP) derivatives toward the enzyme activity and the parasite growth

BNIP derivatives



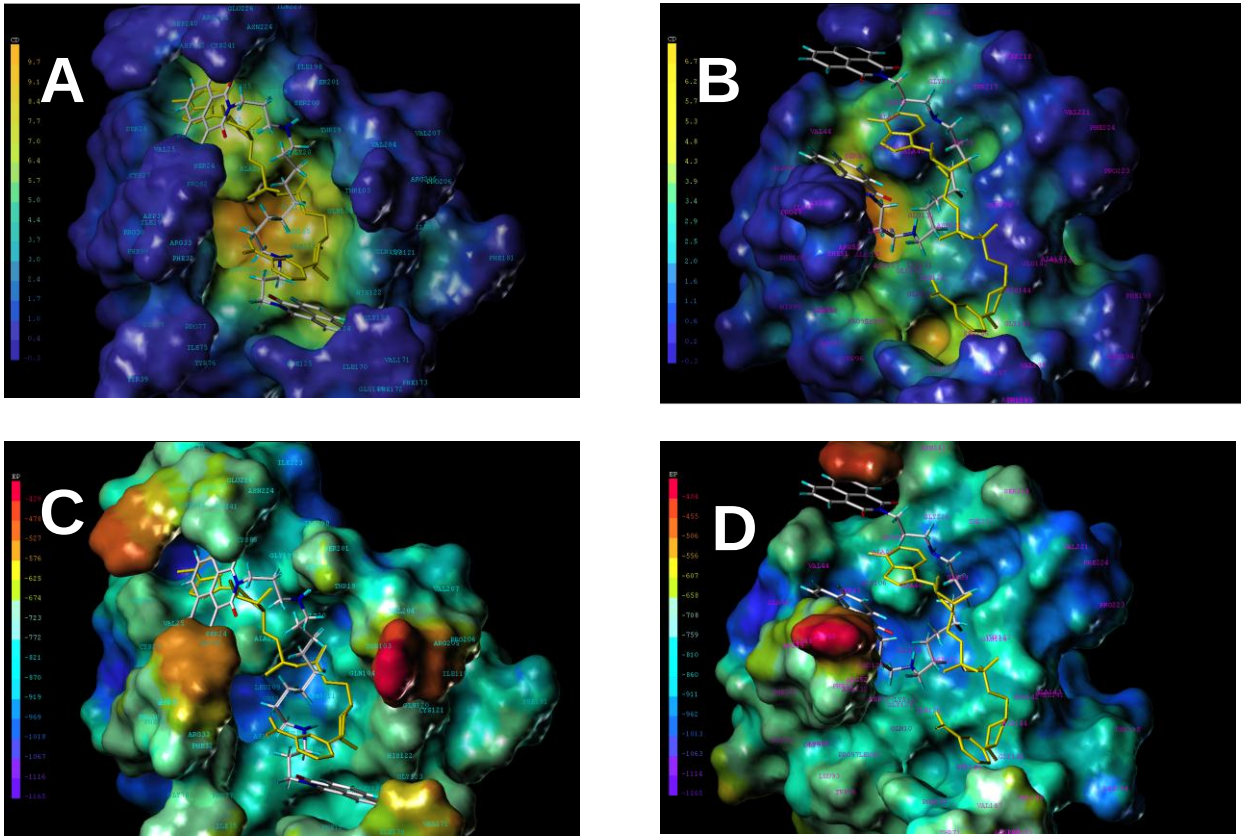
L. infantum SIR2RP1 (LiSIR2RP1) and human SIRT1 (hSIRT1) inhibitory activity of BNIP derivatives

Compound	Name	Structure	IC_{50} ± SD (μM)		Selectivity index (IC_{50}LiSIR2RP1/hSIRT1)
			LiSIR2RP1	hSIRT1	
1	BNIPDabut		35.0 ± 5.8	73.1 ± 14.9	2.1*
2	BNIPDapen		37.7 ± 6.9	82.2 ± 16.4	2.2 *
3	BNIPDahex		43.3 ± 9.5	93.5 ± 7.8	2.2**
4	BNIPDahep		52.7 ± 5.2	127.5 ± 31.9	2.4*
5	BNIPDaoct		9.2 ± 1.4	116.5 ± 23.3	12.7**
6	BNIPDanon		5.7 ± 0.2	97.4 ± 4.9	17.0***
7	BNIPDadec		11.2 ± 1.6	113.8 ± 22.7	10.2**
8	BNIPDadodec		10. 1± 1.2	94.7 ± 23.7	9.4**
9	BNIPSpd		17.9 ± 1.6	94.8 ± 23.7	5.3**
10	BNIPSpm		39.5 ± 6.5	102.2 ± 31.9	2.6**
11	BNIPDpta		32.8 ± 2.4	43.1 ± 9.3	1.3
12	BNIPDeta		54.7 ± 15.7	182.8 ± 22.2	3.3**

IC50 ± SD data are reported as the mean of at least three independent experiments.
*, p <0.05; **, p< 0.01; and ***, p<0.001 between the LiSIR2RP1 and the hSIRT1

The most active and selective inhibitor (parasite versus human enzyme) was BNIPDanon. Based on kinetic studies, its inhibitory activity is due to competition with NAD^+

Docking results of poly amines



A and C human hSIRT1, B and D LiSIR2RP1; A and B cavity depth, C and D electrostatic potential map of the protein molecule, NAD is yellow and other molecule is BNIPDanon.

BNIPDanon has six hydrogen bonding aas present in LiSIR2RP1 NAD binding cavity and docking score is -17.244.

BNIPDanon shows five hydrogen bonding with aas present in hSIRT1 NAD binding cavity and docking score is -15.889.

Other three molecules Dadodec, Dadec and Dadex are also docking in the same place with same pose in both the protein which basically validate the docking positions.

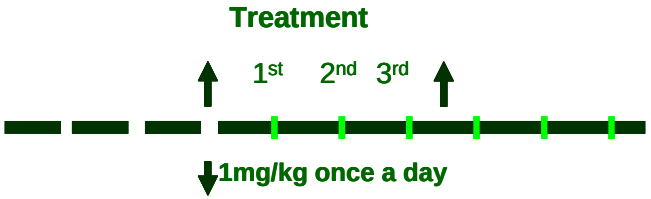
Tavares *et al., ChemMedChem*., 2010, 5: 140-7

Effect of BNIP derivatives on the intracellular development of *L. infantum* amastigotes

	Compound	Intracellular Amastigotes
1	BNIPDabut	4.53±0.54
2	BNIPDapen	1.26±0.18
3	BNIPDahex	3.46±0.48
4	BNIPDahep	1.12±0.0084
5	BNIPDaoct	2.43±0.19
6	BNIPDanon	6.03±0.67
7	BNIPDadec	1.02±0.41
8	BNIPDadodec	1.01±0.39
9	BNIPDpta	4.22±1.07
10	BNIPDeta	9.52±0.56

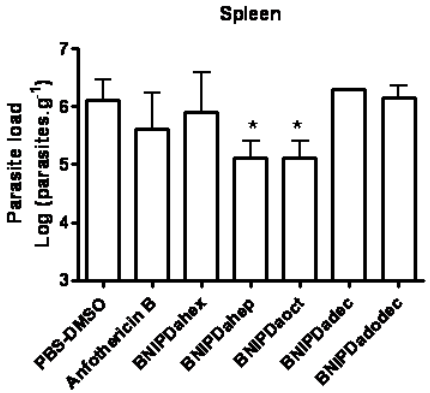
PMA differentiated THP-1 cells infected with amastigotes were incubated with a serial range of each drug concentrations during three days. The growth inhibitory effect of the drugs was determined by the luciferase assay and the IC_{50} calculated by linear regression analysis. Each experiment was performed in triplicate and the IC_{50} values represented correspond to the mean value obtained for at least three independent experiments.

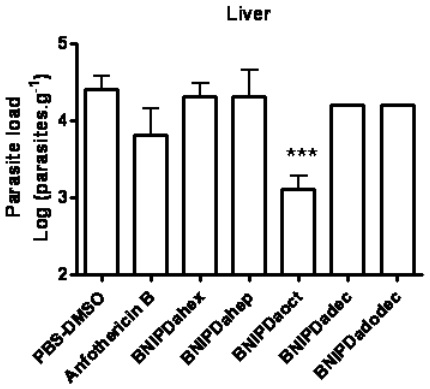
1x10 ^{8} *L. infantum* promastigotes IP



End

Parasite load evaluation





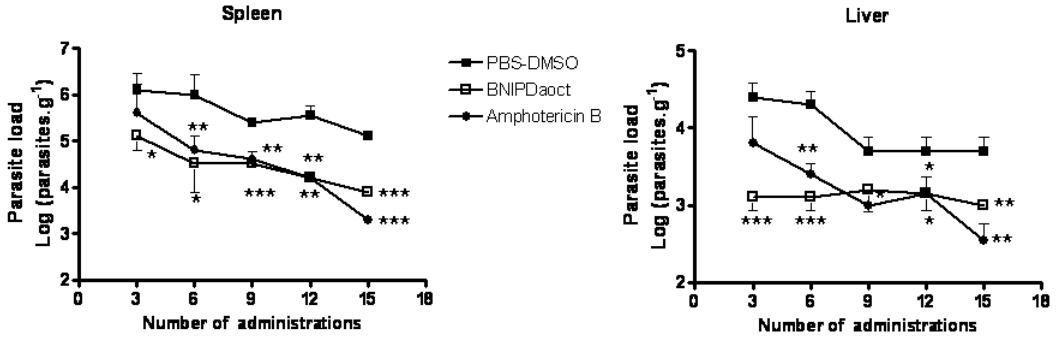


Table III. Hematologic changes after 6, 9, 12 or 15 days of consecutive administrations of 1mg/kg of each drug to *L. infantum* infected BALB/c mice.

Number Administrations	Treatment	White blood cell count ($10^3/mm^3$)	Red blood cell count ($10^6/mm^3$)	Hemoglobin concentration (g/dl)	Hematocrit	Lymphocyte count ($10^3/mm^3$)	Monocyte count ($10^3/mm^3$)	Granulocyte count ($10^3/mm^3$)
6	PBS-DMSO	4.37±0.76	8.60±0.45	14.2±0.7	42.4±1.6	3.23±0.57	0.70±0.20	0.43±0.15
	Amphotericin B	7.40±0.01*	9.10±0.032	14.5±0.7	45.6±0.2*	5.35±0.07*	1.30±0.0*	0.75±0.07
	BNIPDaoct	5.8±0.03*	8.32±0.35	13.8±0.5	41.3±2.1	4.30±0.26*	1.03±0.15	0.47±0.15
9	PBS-DMSO	3.20±0.57	8.42±0.70	13.7±1.3	41.9±4.7	2.25±0.49	0.50±0.14	0.45±0.070
	Amphotericin B	4.10±0.28	8.96±0.28	14.1±0.0	45.0±1.8	2.90±0.56	0.65±0.21	0.55±0.07
	BNIPDaoct	2.57±0.38	7.36±0.98	11.6±1.7	36.3±5.3	2.00±0.20	0.30±0.17	0.27±0.058
12	DMSO	4.38±1.30	9.46±0.42	14.5±0.7	46.9±2.2	3.33±0.49	0.60±0.18	0.45±0.060
	Amphotericin B	3.33±0.29	9.11 ±0.69	14.7±0.8	45.5±3.4	2.37±0.15	0.43±0.06	0.53±0.15
	BNIPDaoct	2.40±0.84	8.50±0.23	13.6±0.2	42.1±1.6*	1.55±0.49	0.50±0.28	0.35±0.071
15	DMSO	3.60±0.28	8.87±0.17	14.3±0.2	45.0±0.1	2.25±0.21	0.90±0.06	0.5±0.0
	Amphotericin B	6.03±1.09	9.25±0.26	15.0±0.3	48.0±1.5	4.17±0.60*	1.30±0.38	0.77±0.25
	BNIPDaoct	2.10±0.78	7.92±1.37	12.6±1.4	40.7±7.7	1.37±0.31*	0.40±0.30	0.33±0.23

Values represent the mean ± standard deviation of the means of 3 to 4 mice per group. *, $p<0.05$; ***, $p<0.001$ between the drug treated group and the control group.

Tavares *et al.*, unpublished

Summary

We believe that the investigations of parasite metabolic pathways may uncover new distinctive features and functional differences in target gene products that will be exploited for the design of selective means to interfere with their biological properties

Thank you