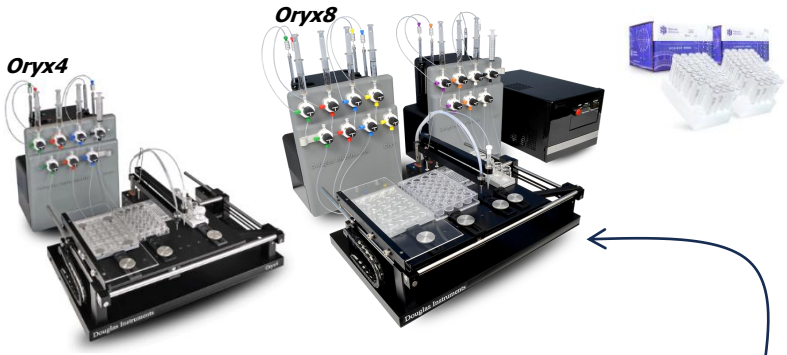
TRYPANOTHIONE REDUCTASE: ONE TARGET, DIFFERENT APPROACHES FOR THE DEVELOPMENT OF A BROAD-SPECTRUM TRYPANOCIDAL DRUG

Andrea Ilari (IBPM-CNR)

IBPM CNR

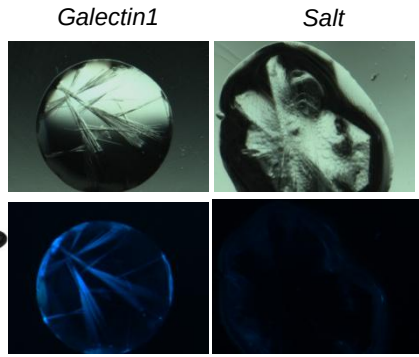
National Research Council of Italy

The Biocrystal Facility at CNR-IBPM Roma



Crystallization and optimization

Crystallization valutation



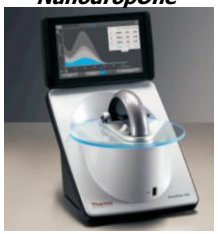
Structure determination of proteins and proteins in complex with lead compound by X-ray crystallography for SBDD

Liquid handler

Sample preparation

Jasco spectrophotmeter

NanodropOne



Synchrotron beamtime

Crystal harvest and shipping

The Biocrystal Facility at CNR-IBPM Roma

High performance platform for Surface plasmon resonance (SPR)

APPLICATIONS:

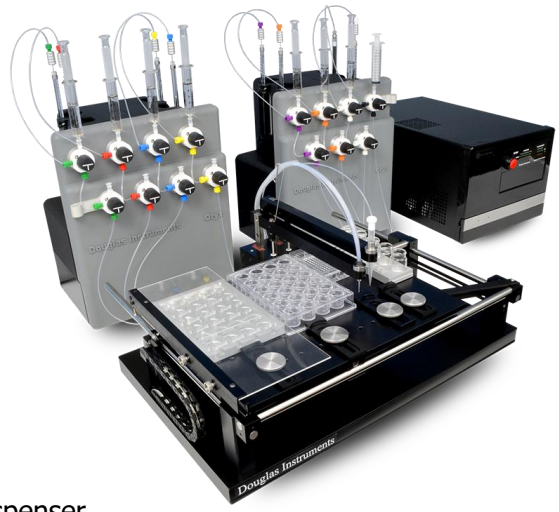
- High-quality binding kinetics and affinity data for the rapid characterization of biomolecular interactions.
- high-throughput Hits Identification for DRUG DISCOVERY

Mass photometry

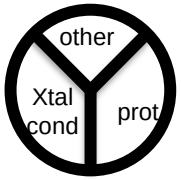
APPLICATIONS:

- Interaction between macromolecules
- Sample characterization for protein crystallization
- Study of protein oligomerization processes
- Macromolecular assemblies determination

Crystallization



LCP dispenser

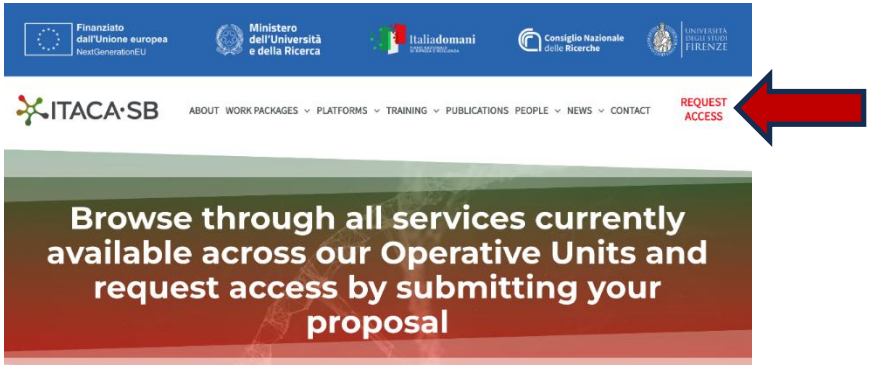


Up until 7 different channels

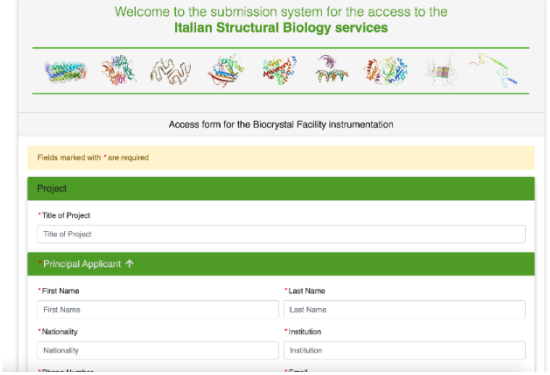
Oryx4 & Oryx8

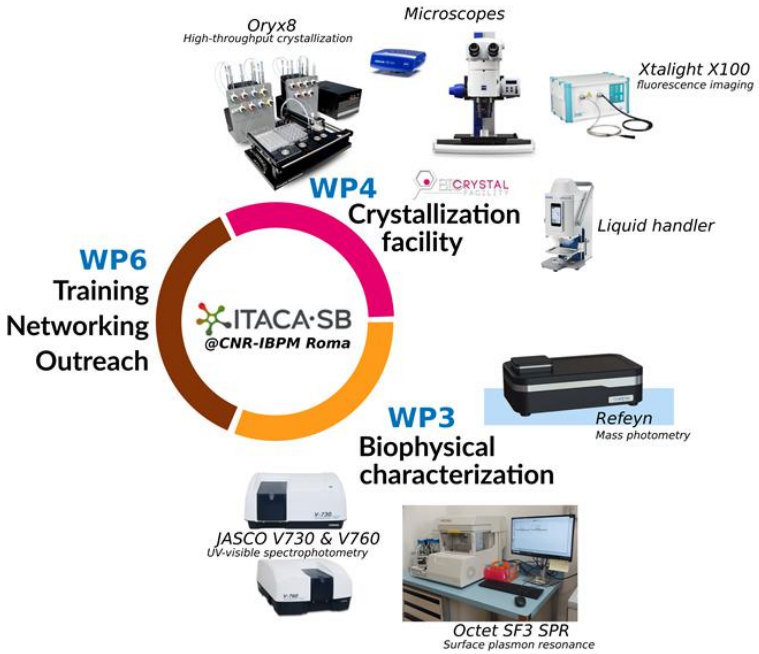
- “Soluble” proteins
- Membrane proteins using the lipidic cubic phase
- Vapor diffusion
- Under-oil microbatch
- Optimization

BIOCRYSTAL FACILITY IS PART OF ITACA.SB

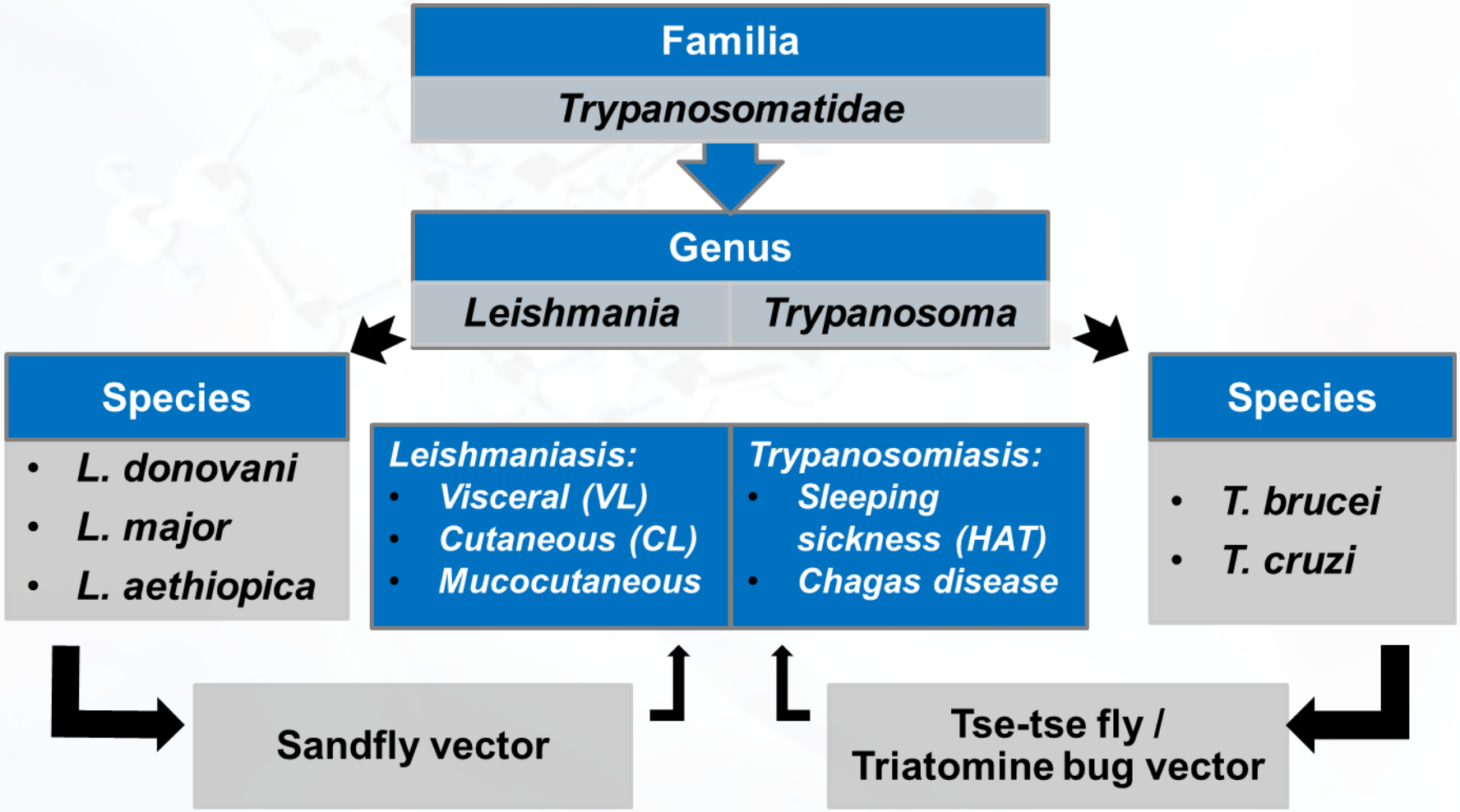


https://www.itaca-sb.it/



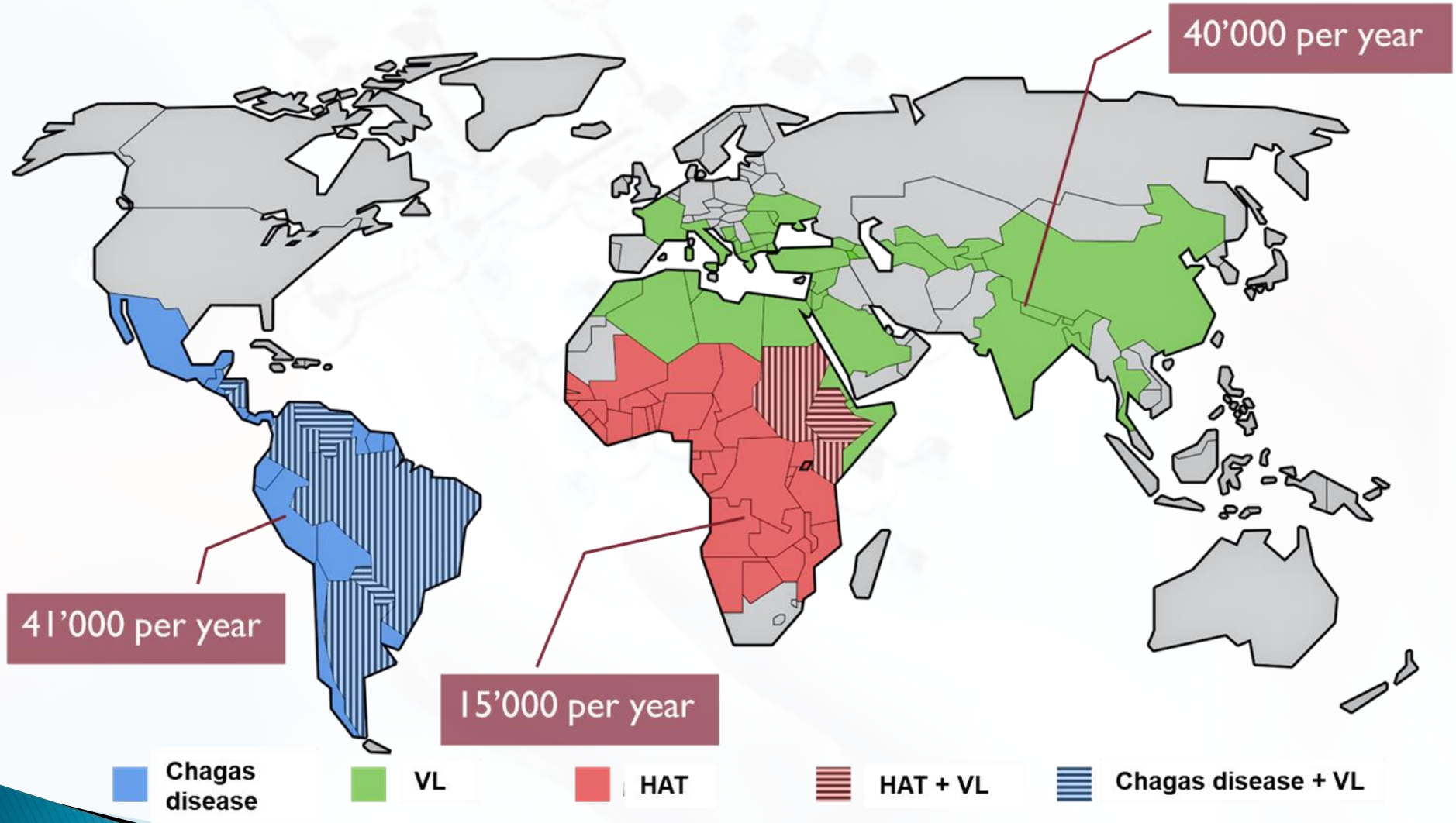


Trypanosomatid caused diseases



Areas of Active Transmission of Diseases Due to Trypanosomatids.

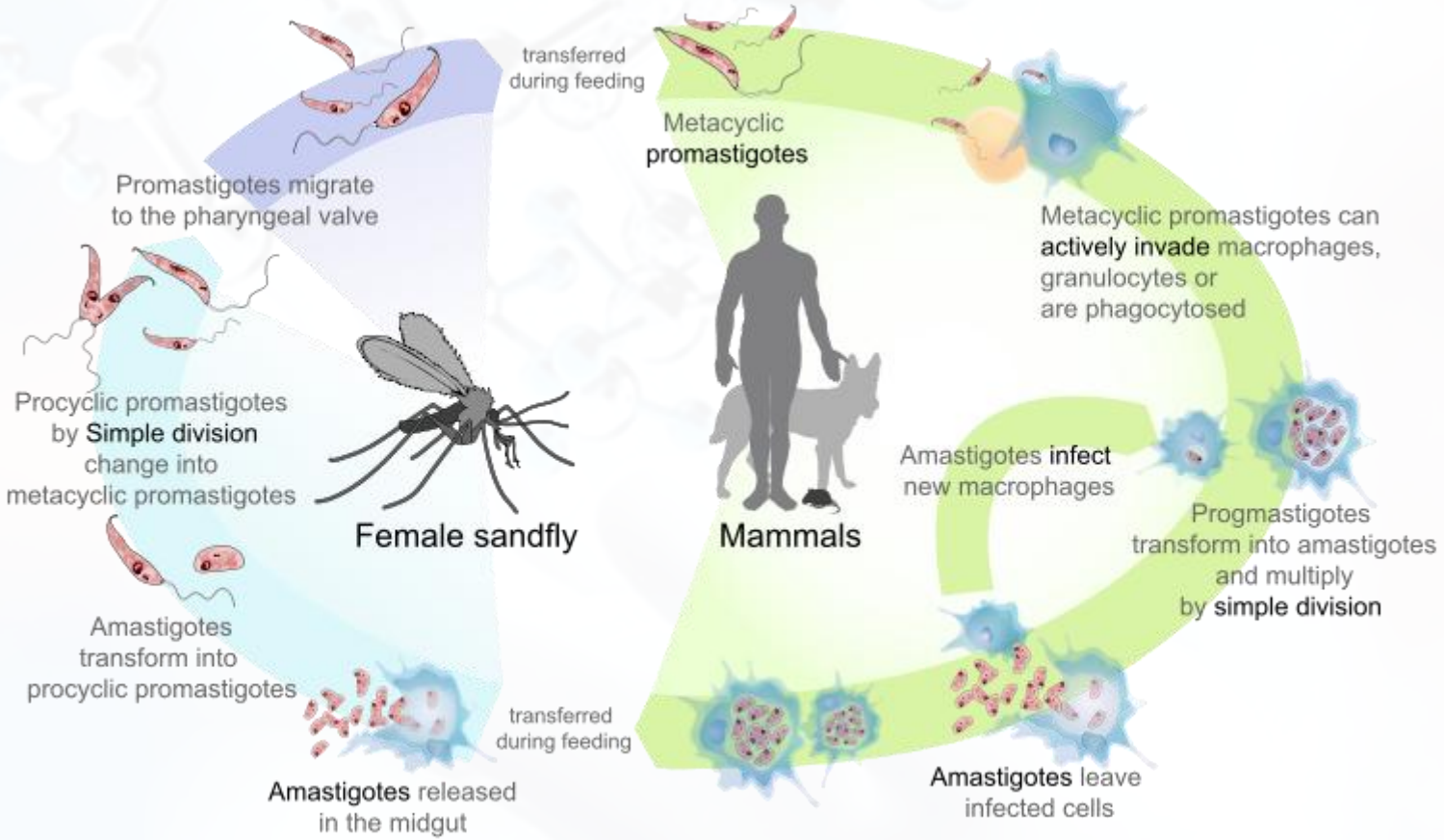
20 millions of people affected and 100000 deaths every year



Berthier et Trends in parasitology 2016

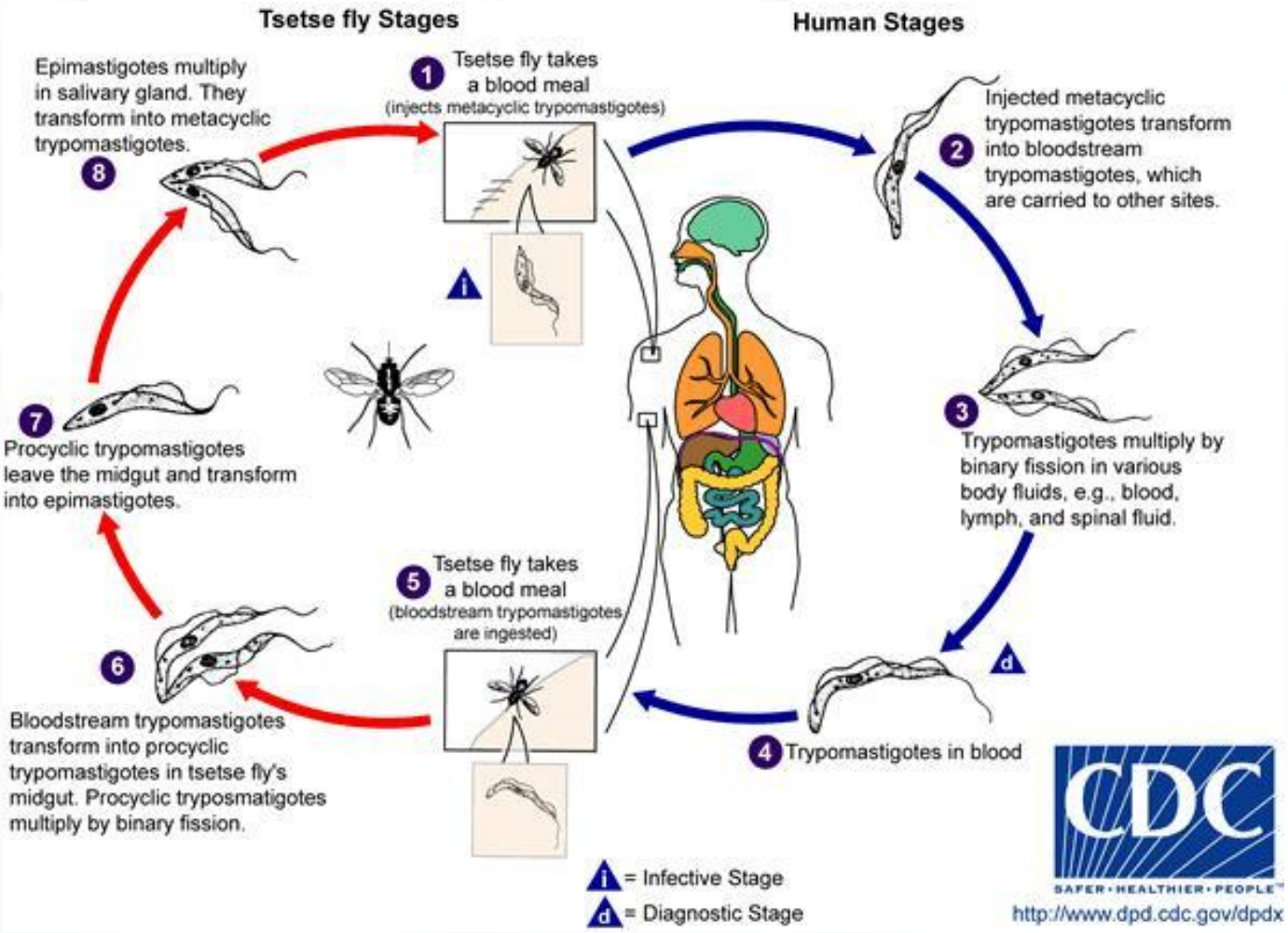
Leishmaniasis is a neglected disease

2 million of new cases occur annually, 60000 deaths/year (a rate surpassed only by malaria)

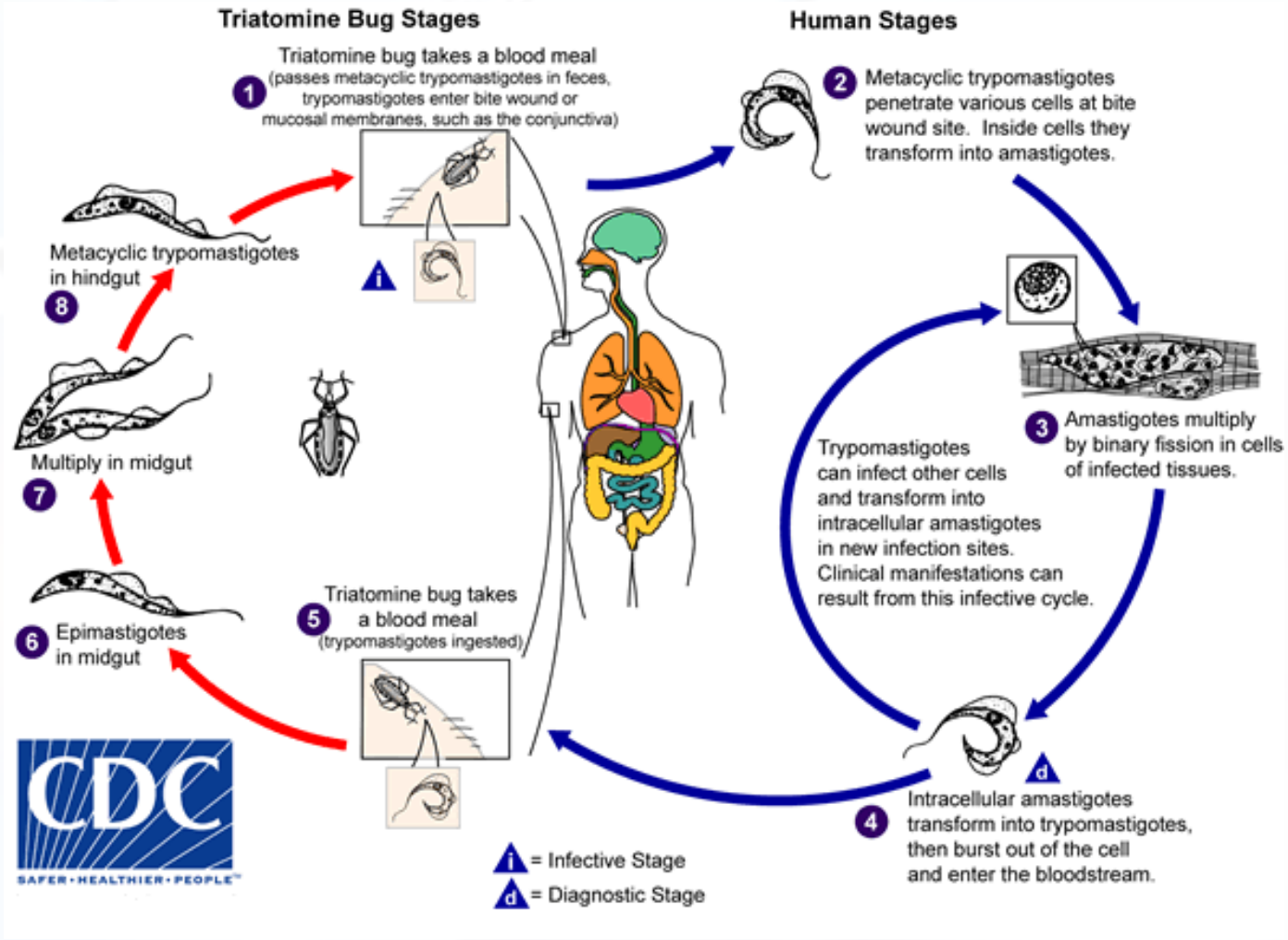


http://www.who.int/leishmaniasis/

African trypanosoma cycle

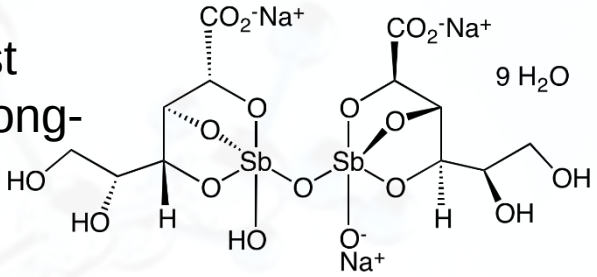


American trypanosoma cycle

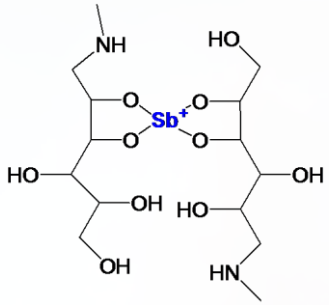


Drug therapy against Leishmaniasis

pentavalent antimonials: the most used drugs for over 70 years are, long-term treatments, high toxicity, drug resistance.

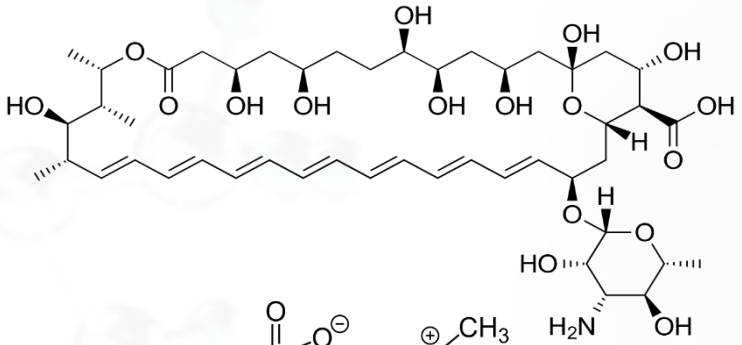


Sodium stibogluconate

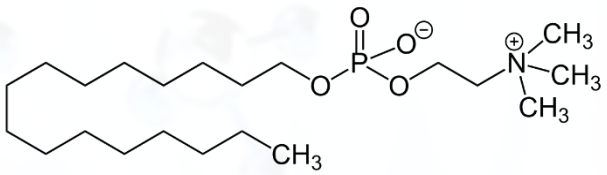


Meglumine antimoniate

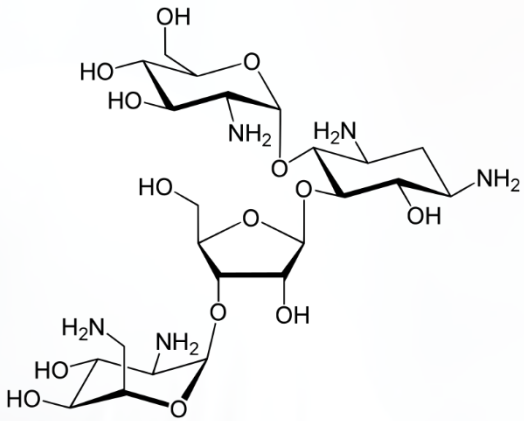
Amphotericin B: a polyene antifungal drug, associates with ergosterol, forming a transmembrane channel leading to monovalent ion leak;
AmBisome: liposomal formulation.
High cost



Miltefosine: (2-(hexadecoxy-oxido-phosphoryl)oxyethyl-trimethyl-azanium): developed as antineoplastic.
High toxicity



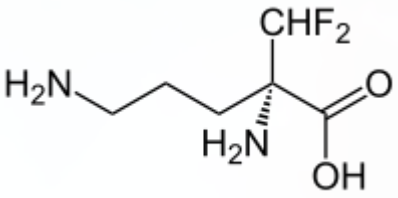
Paromomycin: an aminoglycoside antibiotic, inhibits protein synthesis by binding to 16S ribosomal RNA. Drug resitance



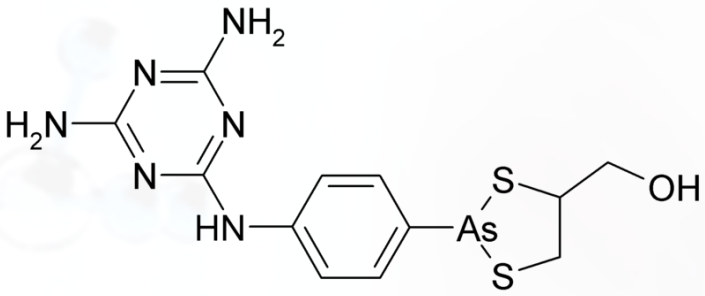
Drug therapy against Trypanosomiases

Drugs for HAT treatment

Eflornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (**ODC**). Eflornithine is effective only for West African sleeping sickness (caused by *T. brucei gambiense*); it has no effect on East African sleeping sickness (caused by *T. brucei rhodesiense*).

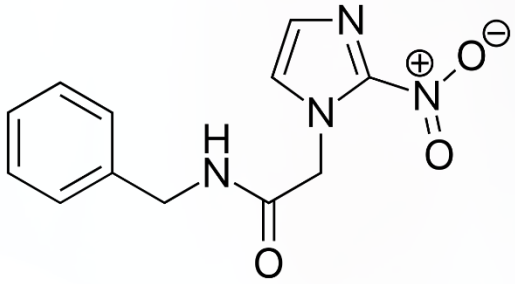


Melarsoprol is a very toxic, arsenic-based drug against all Trypanosomiases. It inhibits the **pyruvate kinase** and other **thiol proteins (also TR)** High toxicity**.**

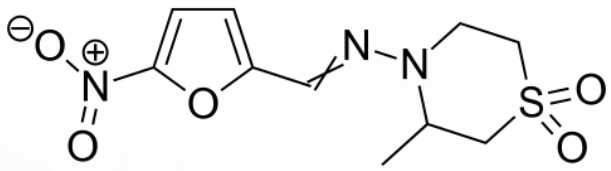


Drugs for Chagas Disease treatment

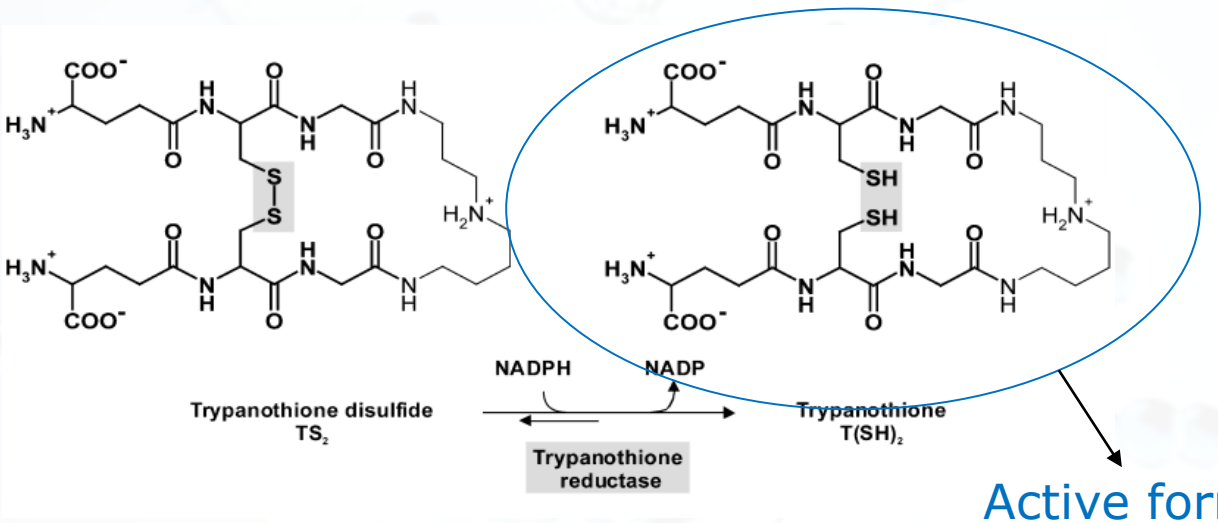
Benznidazole. covalent modification of biomolecules, due to the generation of ROS from reduction of the nitro group. High toxicity, poor efficacy in chronic phase.



Nifurtimox, the second line treatment, is prescribed in cases where benznidazole is not well tolerated. Its mode of action, again, relates to the reduction of the nitro group, leading to the formation of ROS, High toxicity



Trypanothione, a glutathione-spermidine conjugate protects parasites from oxidative damage



Alan Fairlamb

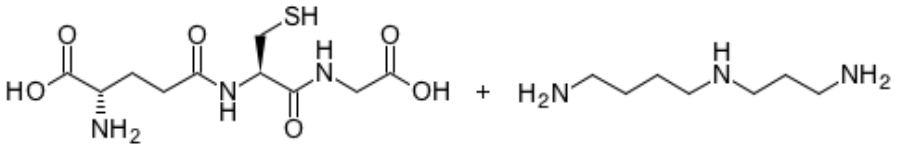
Trypanothione is a polyamine dithiol active in its reduced form $T(SH)_2$ and protects parasites from oxidative damage.

It is used by the **enzymes tryparedoxin/tryparedoxin peroxidase** to reduce the hydrogen peroxide produced by macrophages during the infection.

It is **essential** for the **parasite survival and virulence** and it is absent in mammalian cells.

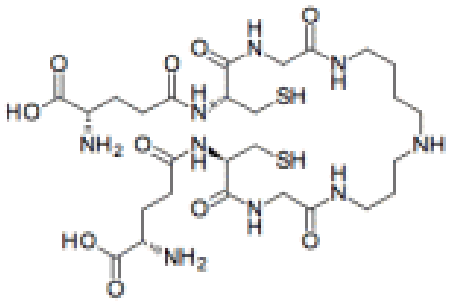
Trypanothione metabolism

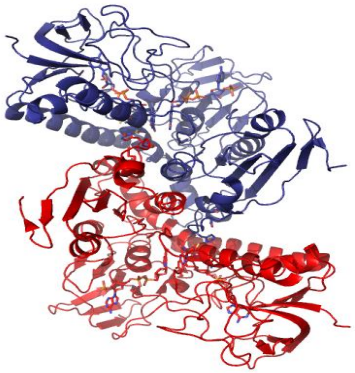
2 Glutathione + Spermidine



Trypanothione synthetase

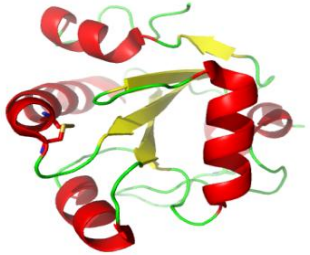
Trypanothione



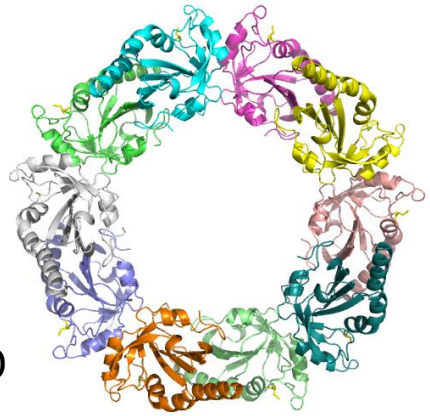


$T[S]_2$ → Trypanothione reductase → $T[SH]_2$

The enzymes of the trypanothione metabolism are good drug targets because are essential and unique



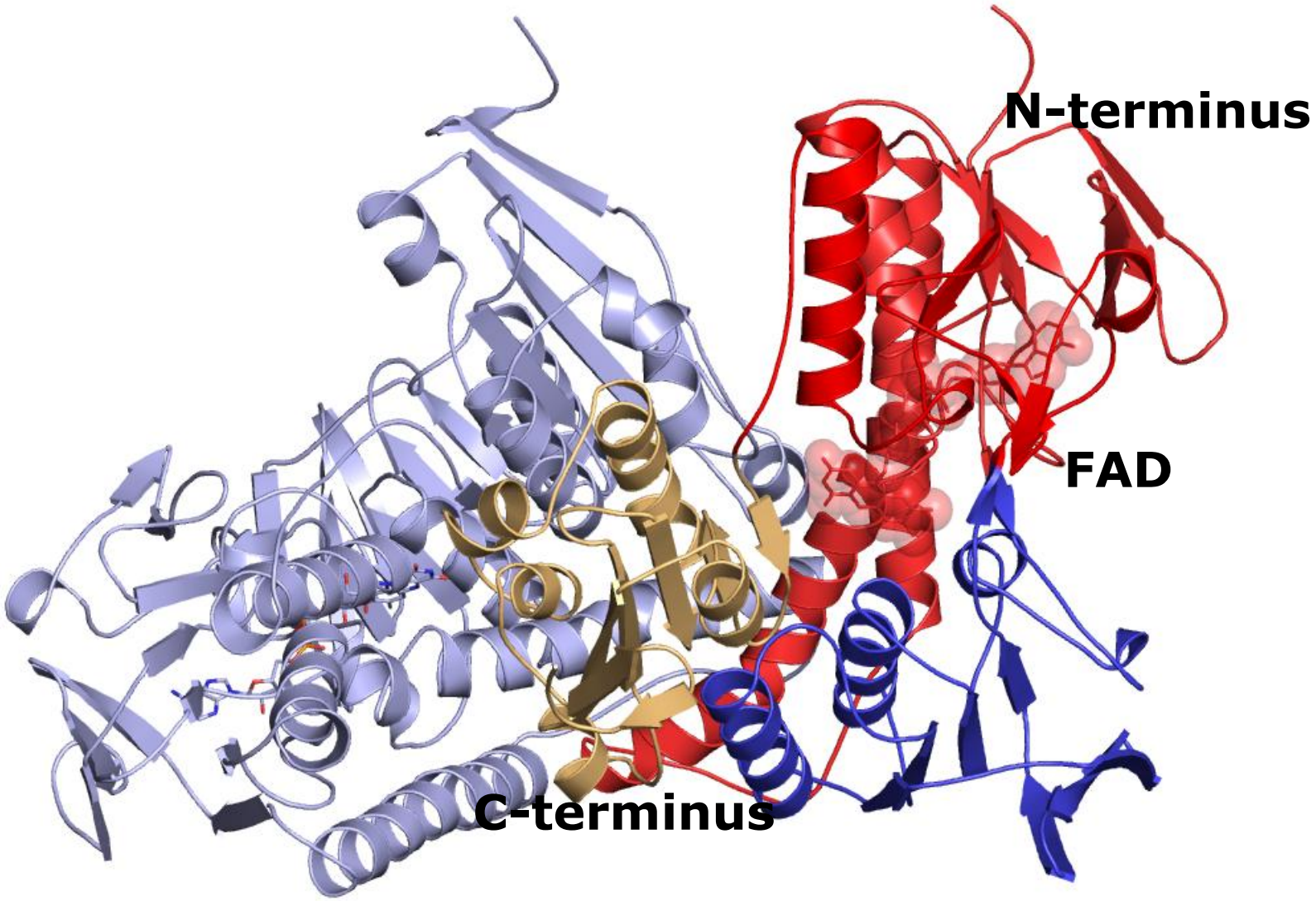
$TXN[SH]_2$ → Tryparedoxin → $TXN[S]_2$



ROOH → Tryparedoxin peroxidase → $ROH + H_2O$

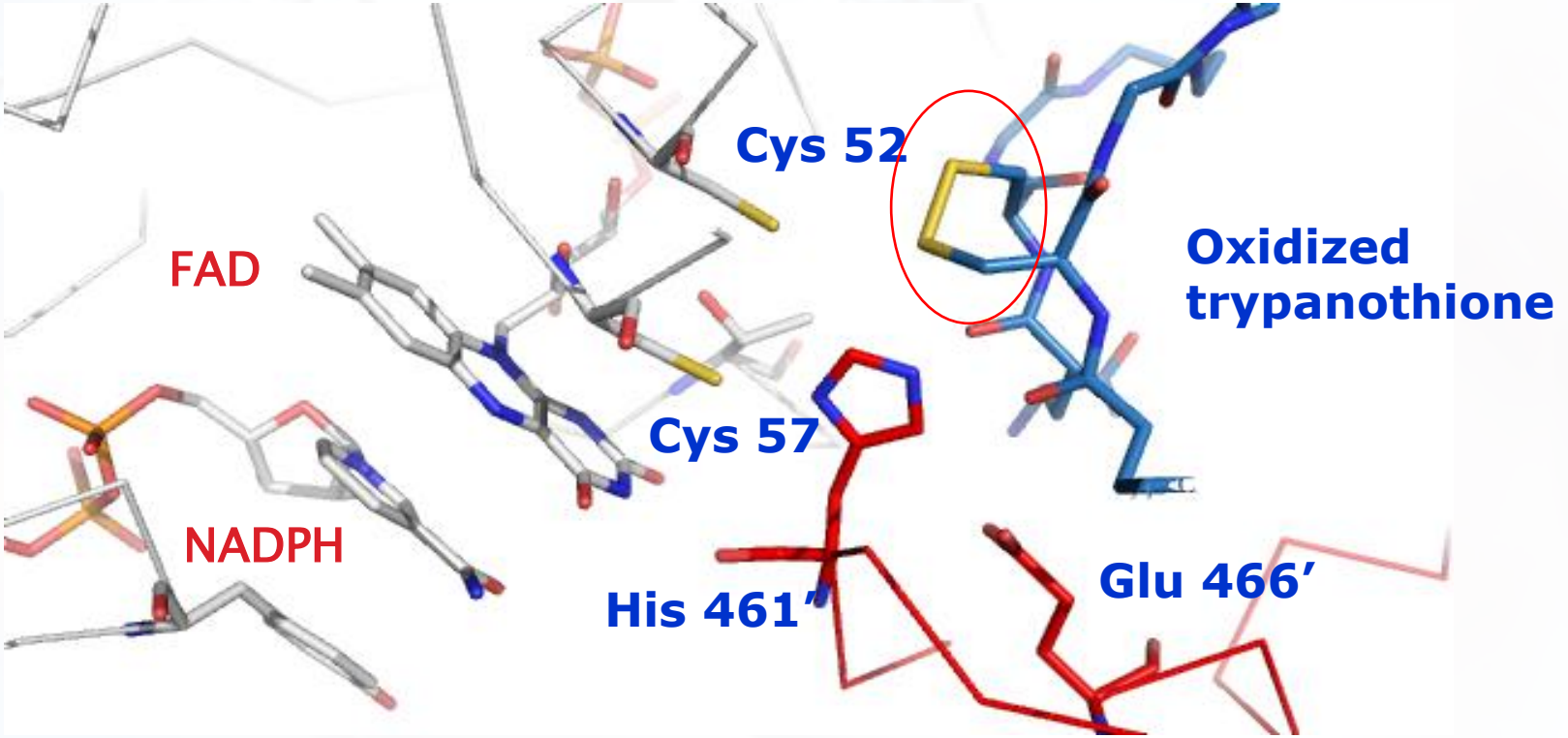
Colotti, Ilari, 2011. Polyamine metabolism in Leishmania: from arginine to trypanothione, Amino Acids

TR is a homodimer. Each monomer is formed by three domains



The residues involved in the TS_2 reduction are: Cys52, Cys57, His461' and Glu466'

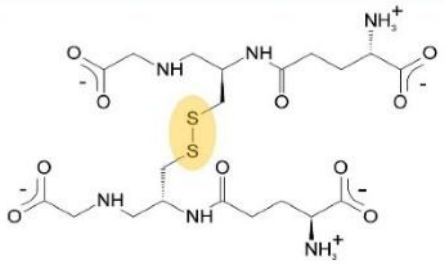
$$NADPH + TS_2 + H^+ \rightarrow NADP^+ + T(SH)_2$$



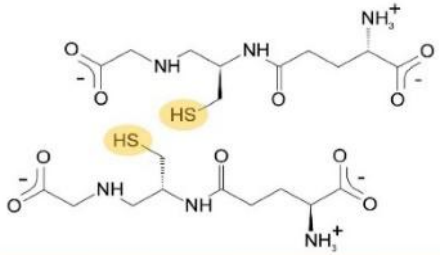
One of the protein cysteines, Cys52, **is activated** similarly to cysteine proteases by the **His461'-Glu466'** pair and reacts with TS_2 to produce a mixed disulfide followed by nucleophile attack of the second protein cysteine (Cys57) on Cys52

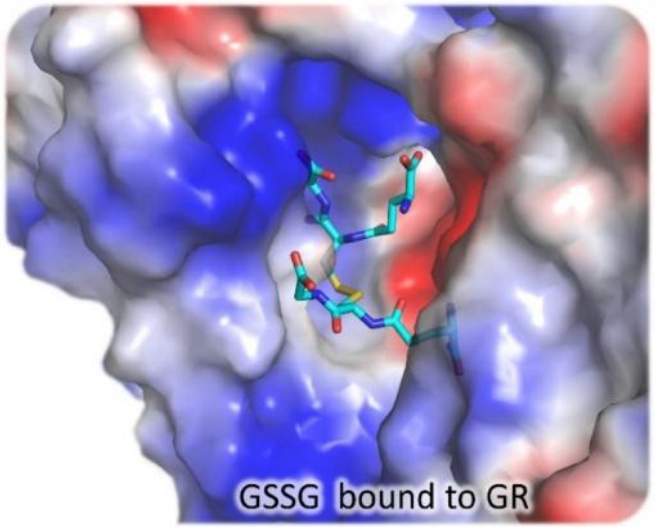
Leishmania TR vs human GR

Oxidized glutathione **GSSG**

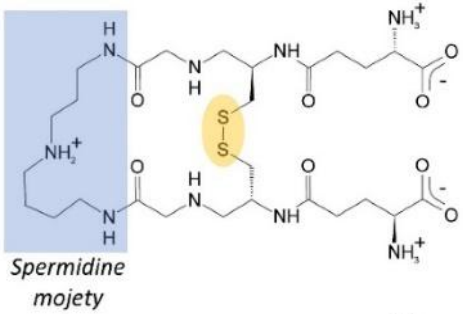


Reduced glutathione **GSH**

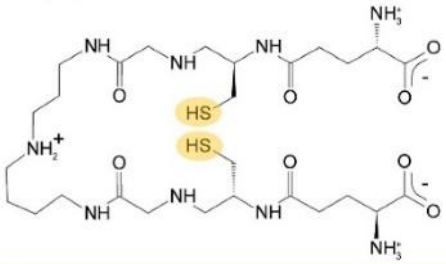


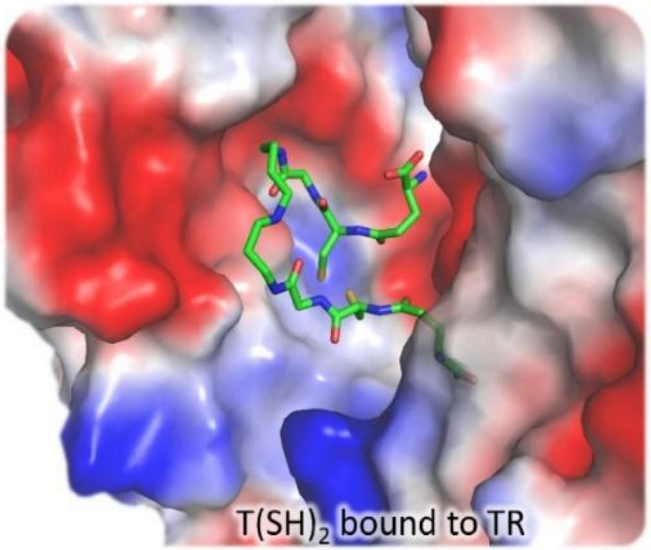


Oxidized trypanothione **TS_2**



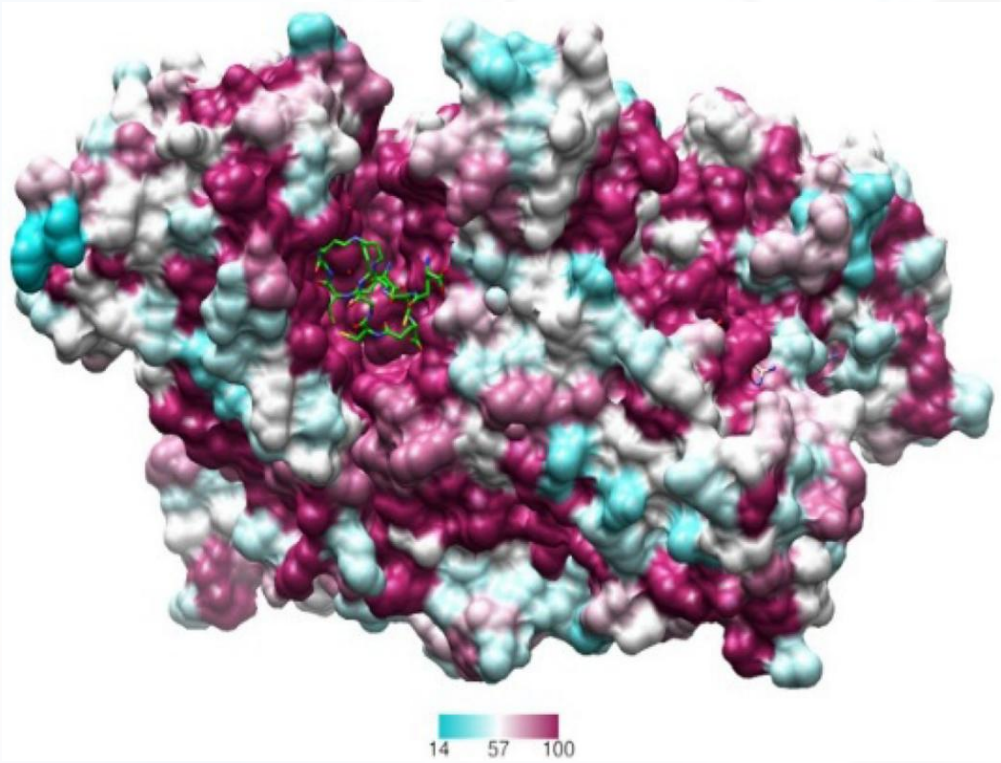
Reduced trypanothione **$T(SH)_2$**





The residues lining the active sites are identical among TRs

Violet indicates identical residues



The dimer of TR from T. brucei (PDB: 2wow) is colored according to the percentage of residues identity with respect to other TR **(Battista et al. Molecules 2020)**

TRs from all Trypanosomatidae share at least
67% of primary sequence,

with >82% identity among Leishmania spp. and >80% among Trypanosoma spp.

Similarity reaches 100% for residues shaping both substrates' binding sites,

1. A good TR inhibitor can be used to find a broad spectrum anti-trypanocidal drug
2. We have used both TbTR and LiTR for Our structural studies

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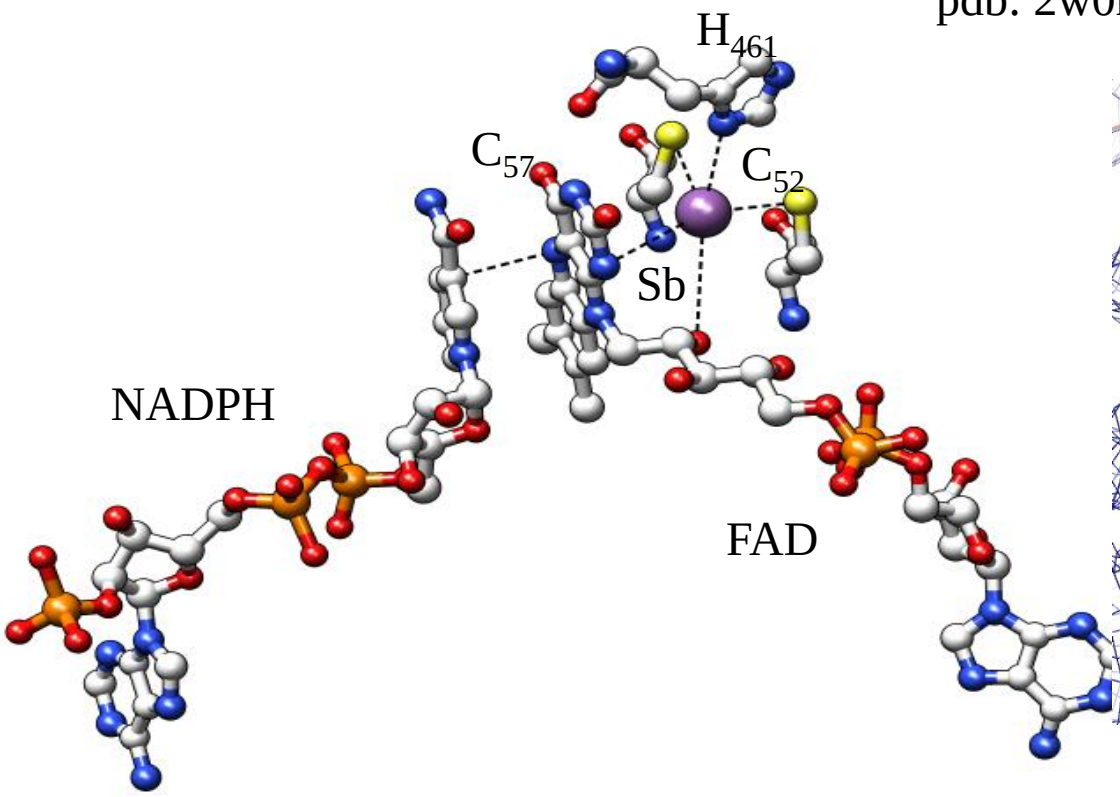
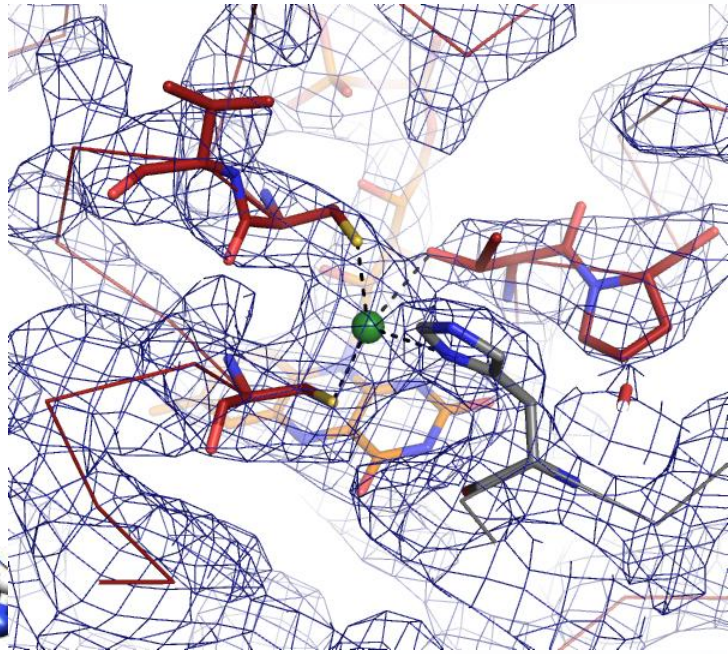
Antimonial drugs inhibits TR with high efficiency

The measured K_i for Sb(III) is 1.5 ± 0.4 μM indicating that Sb(III) is a very effective inhibitor of the enzyme.

pdb: 2w0h





Baiocco P, Colotti G, Franceschini S, Ilari A. Molecular basis of antimony treatment in leishmaniasis. J. Med. Chem. (2009) 52(8):2603-12.

Different approaches to find new lead compounds targeting TR

1. Drug Repositioning or repurposing involves the investigation of existing drugs for new therapeutic purposes.This approach would allow pharmaceutical companies to save money reducing the number of required clinical trial

2. Structure-based drug discovery. This method exploit the knowledge of the protein structure and of its complex with inhibitor to design new drugs

3. High throughput screening. Using robotics, data processing/control software, liquid handling devices, and sensitive detectors, high-throughput screening allows a researcher to quickly conduct hundred of thousands of chemical, genetic, or pharmacological tests.

4. Fragment-Based Drug Discovery (FBDD). FBDD is a powerful method to develop potent small-molecule compounds starting from fragments binding weakly to crystallized targets

5. PROTAC: Proteolisis Targeting Chimeras.
Degradation of target protein through UPS

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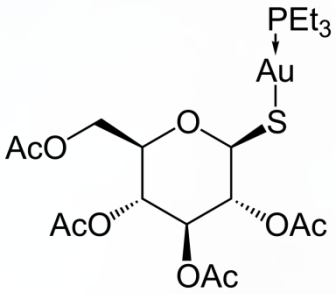
Drug Repositioning: Auranofin bind to and inhibits TR

cytotoxicity

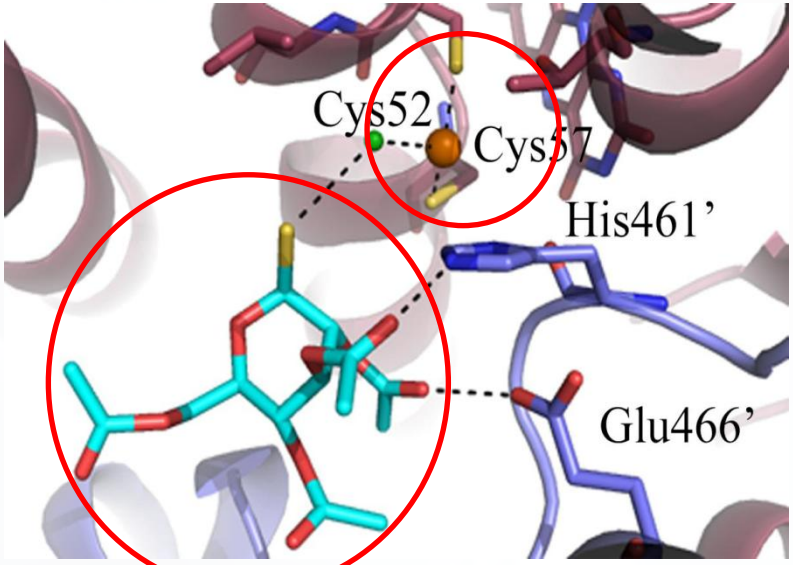
Compounds	K_i (nM)	IC_{50}(μM)
Au (III)		
$AuCl_3$	160±5	10.1
$[(bipy)Au(OH)_2][PF_6]$	40±15	8.8
Auoxo1	68±20	22.8
$Au(Pbi)Cl_2$	90±20	6.6
$K[Au(Sac)_4]$	200±70	14.9
[Au(NNO)Cl]	25000±8000	6.1
$[Au(pyox^{iPr})Cl_2][PF_6]$	75±20	1.4
$[Au(pyox^{Bn})Cl_2][PF_6]$	185±40	5.05
Au(III)+Au(I)		
$[Cl_2Au^{III}(Pbi)Au^{I}(PPh_3)][PF_6]$	**22±11**	**0.6**
Au(I)		
Auranofin	155±35	0.5
$K[Au(Sac)_2]$	140±30	52.7
[(TPA)Au(Sac)]	50±18	8.5

Auranofin is able To inhibit promastigote with an IC_{50}=9.68 μM

Auranofin is by the World Health Organization as an antirheumatic agent (brand name Ridaura).



Co-crystallization with TR: **Au** binds to the residues involved in trypanothione reduction, andthe **3,4,5-triacetyloxy-6-(acetyloxymethyl)oxane-2-thiolate** competes with the binding of trypanothione



Ilari, Baiocco, Messori, Fiorillo, Gramiccia, Di Muccio, Colotti; Amino Acids (2012), 42:803-811.

Structure-based drug discovery: we start to screen of GSK LeishBox compounds as TR inhibitors

GlaxoSmithKline whole-cell HTS against L. donovani, T. cruzi and T. brucei vs. human cells (THP1-derived macrophages, HepG2), allow
to screen a library of 1.8 million compounds:

Leish-Box: 192 compounds vs. L. donovani
Chagas-Box: 222 compounds vs. T. cruzi
HAT-Box: 192 compounds vs. T. brucei

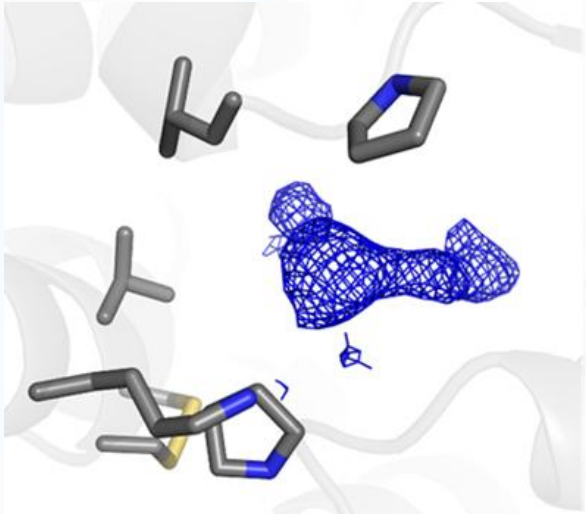
Ilari A, Fiorillo A, Colotti G. et al. Toward a Drug Against All Kinetoplastids: From LeishBox to Specific and Potent Trypanothione Reductase Inhibitors. Mol Pharm. 2018 Aug 6;15(8):3069-3078.

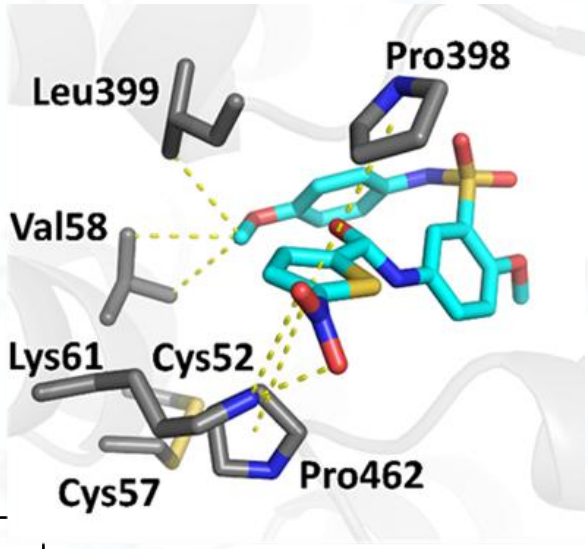
	TR Inhibition IC_{50}	GR Inhibition IC_{50}
A1/7	**0,52 ± 0,14 µM**	**No inhibition**
F1/7	**5,58 ± 0,86 µM**	**No inhibition**
C5/7	**0,22 ± 0,05 µM**	**3,2 µM**
B10/7	1,96 ± 0,30 µM	3,7 µM
C10/7	**0,19 ± 0,08 µM**	**No inhibition**
G1/9	2,24 ± 0,52 µM	>25 µM
G2/9	5,96 ± 0,84 µM	>25 µM

The compounds with best selectivity index are 4:

- **have similar structures;**
- **also inhibit T.brucei and T.cruzi;**
- **A1/7 is the only compound in common in all 3 GSK boxes**

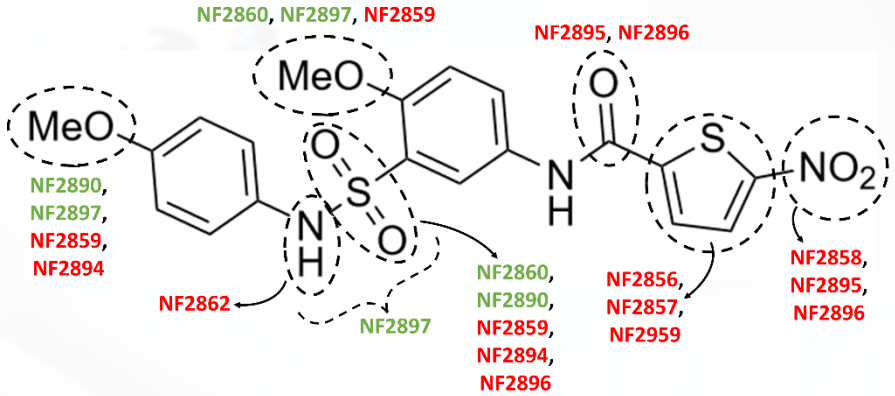
Structure-based drug discovery: we start from the X-ray structure of TR from *T. brucei* in complex with A1/7

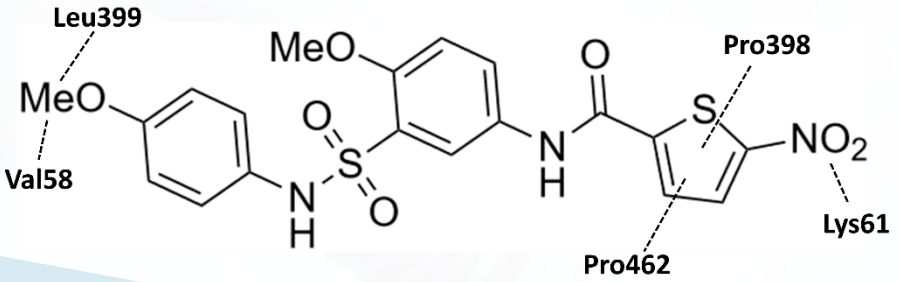




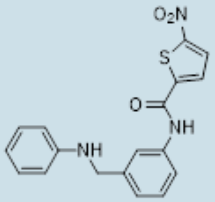
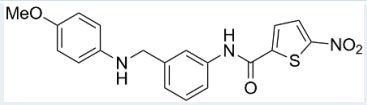
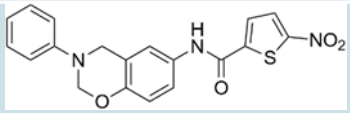
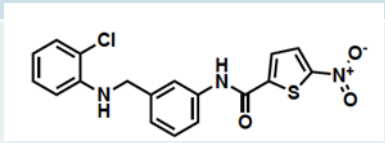
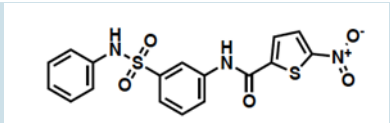
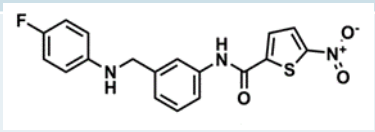
Based on the structure of the complex we design new and more potent inhibitors

Fo – Fc map contoured at 2.5 σ
A1/7 bound in the TS_2 cavity



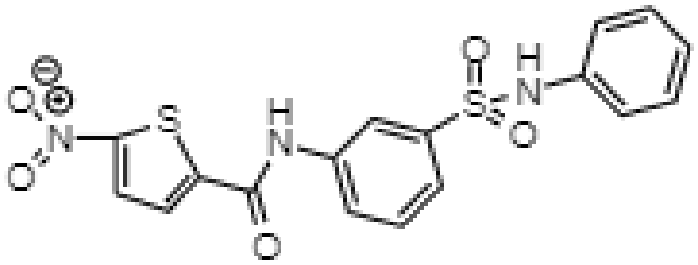


Structure-based drug discovery: compounds inhibiting TR in the nanomolar range.

Compound	Formula	pIC50	IC50 (μM)
NF2860		6.63±0.39	0.24
NF2890		6.44±0.32	0.36
NF2897		6.72±0.57	0.19
NF2954		6.61±0.15	0.25
NF2975		7.01±0.56	0.10
NF2955		6.72±0.67	0.19

Structure-based drug discovery: Conclusion

We succeed in finding
A TR inhibitor in the nanomolar
Range (IC_{50} (NF2925)=100 nM)



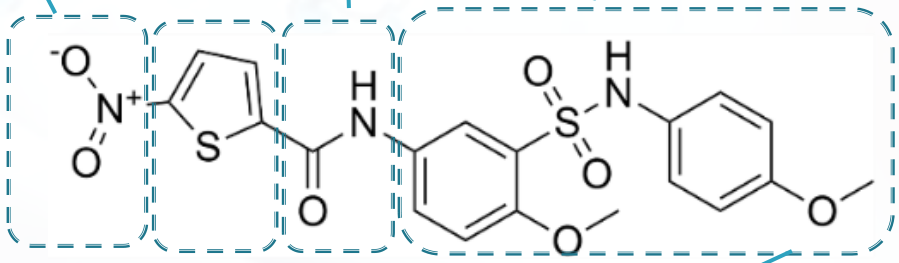
NF2975

Carboxamide
moiety
Not very important

Nitro group
Essential

Sulfamoyl amino group important

A1/7

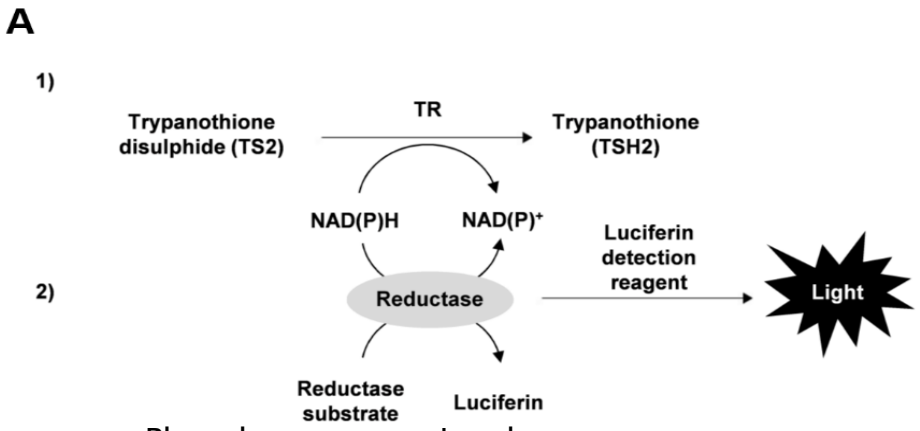


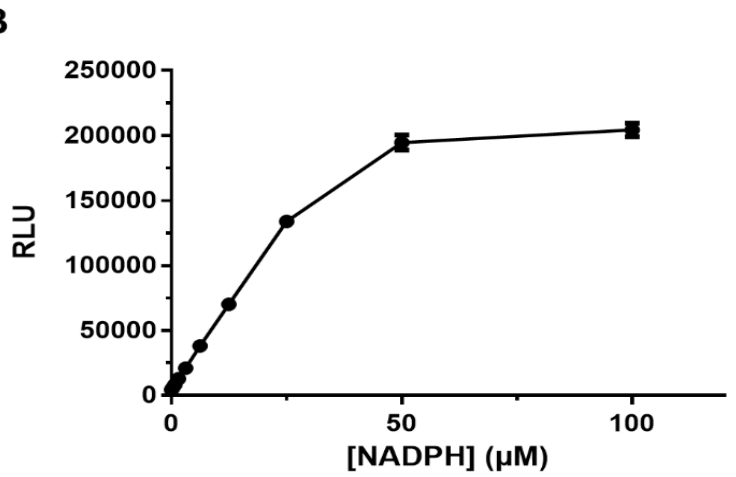
Thiophene group
Very important

Methoxy groups decreases
the affinity of the compound and
Should be removed

High Throughput Screening on TR

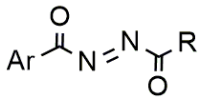
HTS was performed a collection of approximately **120,000 small molecules** through the CNCCS public-private consortium (www.cnccs.it). **A new homogeneous bioluminescent assays was set up in which the residual NADPH after reduction of TS_2 is detected by a luciferine/luciferase based system.**

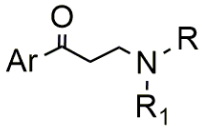




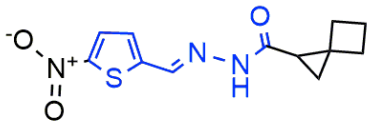
- Phosphorescence signal
- At concentrations below 50 μM the signal is linear

Substructure

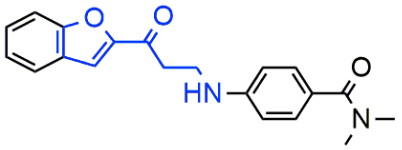




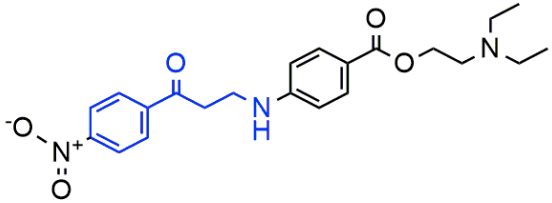
Examples



Compound 1
IC_{50}
1764.9 ± 371.2 nM



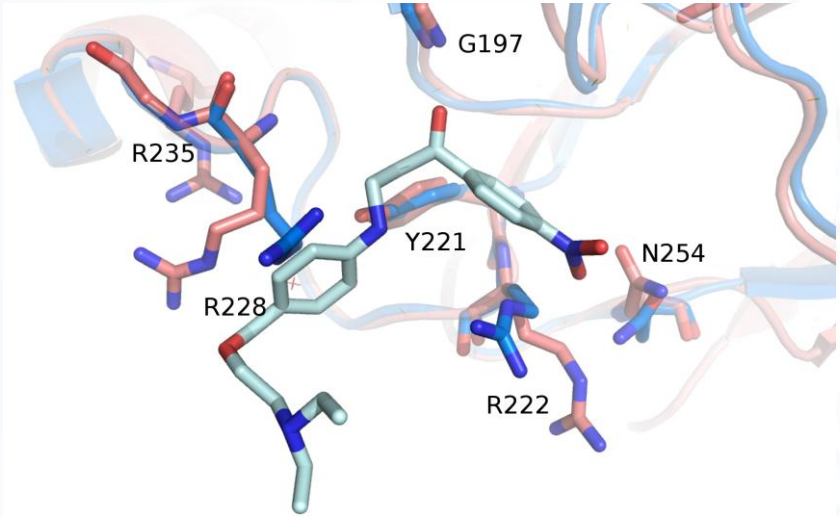
Compound 2
IC_{50}
13279.6 ± 2691.3 nM



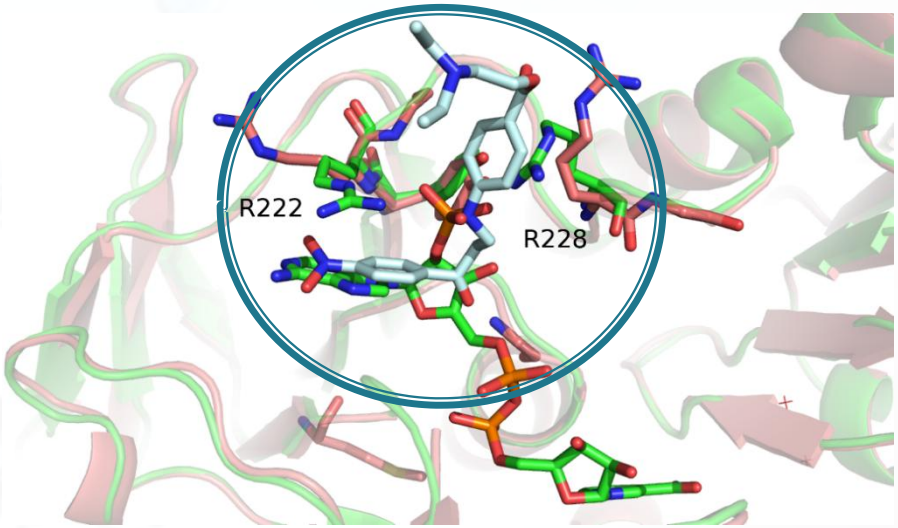
Compound 3
IC_{50}
7513.2 ± 2532.2 nM

Compound 3 binds to NADPH binding site

Compound **3**-TR complex crystal structure: in the NADPH binding site



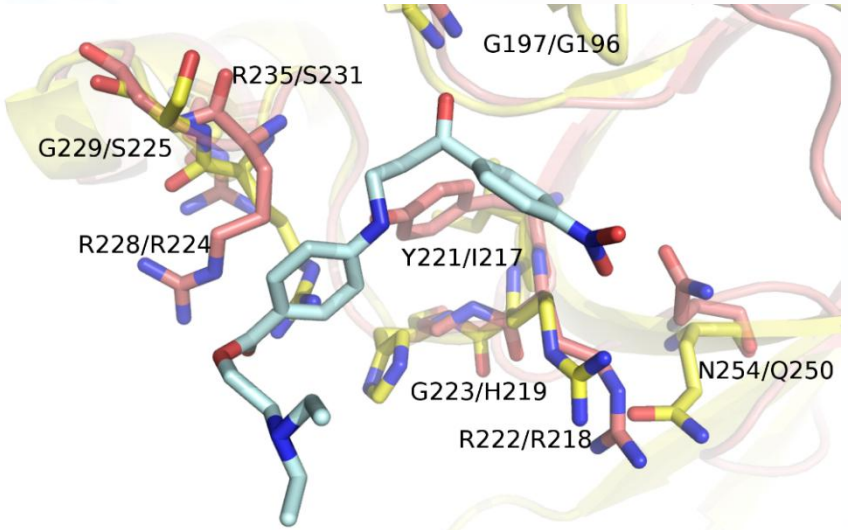
C3-TR complex (magenta) vs. apo TR (blue) (C3 is colored cyan)



C**3**-TR complex (C3 is colored cyan) vs. TR in complex with NADPH (NADPH is colored green)

The site is unique, not present in GR

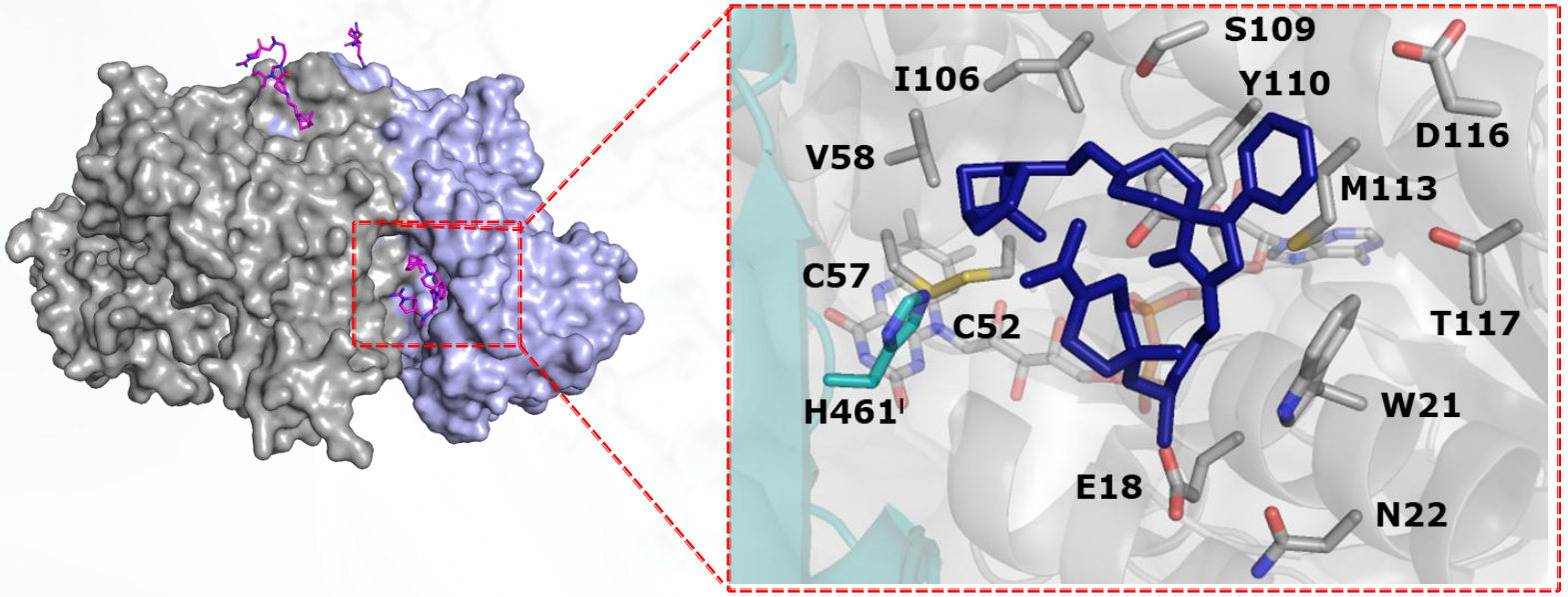
C**3**-TR complex (magenta) vs. apo GR (yellow) (C3 is colored cyan) . Y221, G229,R235,G223 are not conserved in GR

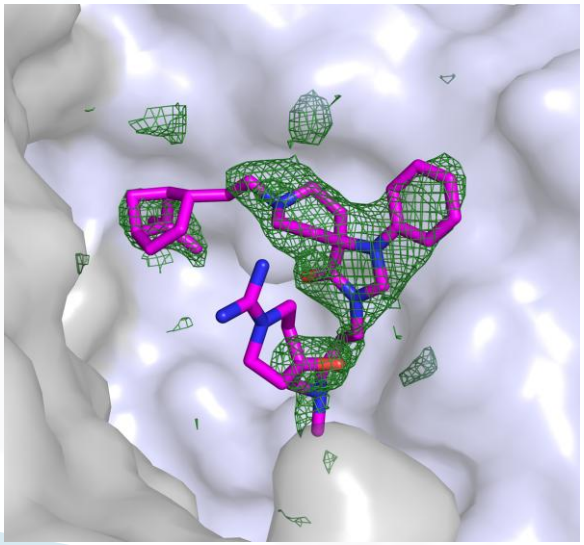


Turcano L,…,Fiorillo A, Harper S, Bresciani A, Colotti G, Ilari A. **PLoS Negl Trop Dis. 2018 26;12(11):e0006969**

Compound 1 binds to Trypanothione binding site

Compound 1 from the HTS on TbTR (3097 compounds already active on *Trypanosoma brucei*) IC50 of 3.5 ± 2.2 µM



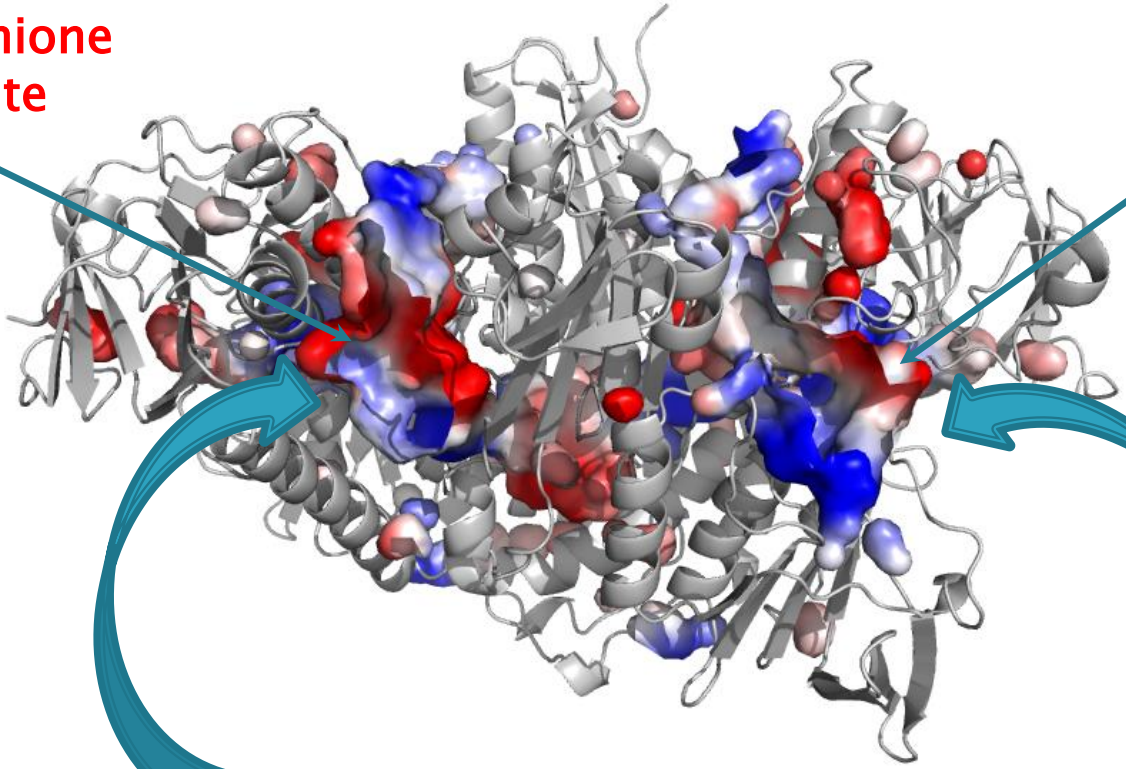


Turcano L, Battista T, De Haro ET, Missineo A, Alli C, Paonessa G, Colotti G,Harper S, Fiorillo A, Ilari A, Bresciani A. Spiro-containing derivatives showantiparasitic activity against Trypanosoma brucei through inhibition of thetrypanothione reductase enzyme**. PLoS Negl Trop Dis. 2020 May 21;14(5):e0008339.**

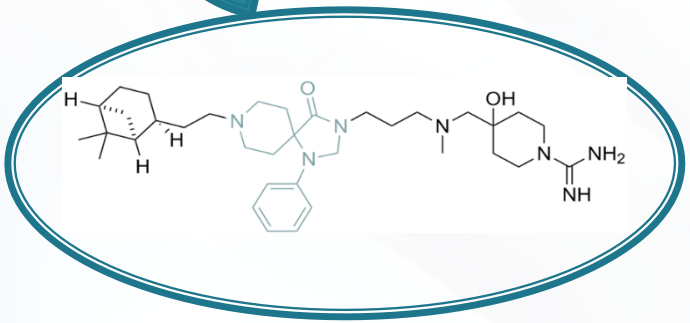
High Throughput Screening on TR: conclusion

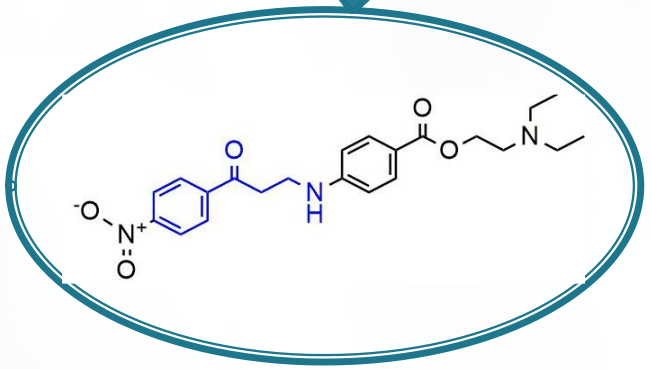
Trypanothione Binding site

NADPH cavity



Compound 1:
IC50 = 3.5 μM





Compound 3: IC50 = 7.5 μM

IBPM CNR

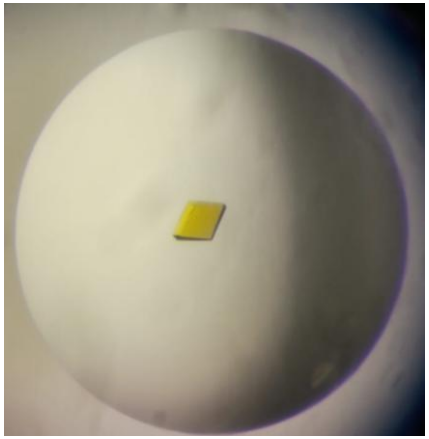
Fragment-based screening is now well-established as a powerful approach to early drug ("lead") discovery.

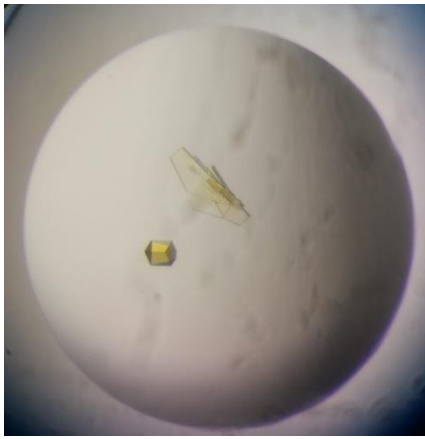
Screening (semi)automatizzato
di piccoli composti (max 2-300 uma) *in cristallo*

Consente di identificare:
- Nuovi leads
- Nuovi siti di legame

Il target deve avere un buon
«comportamento cristallografico»

TR di *Trypanosoma brucei* è un target ideale:
- Cristallizzazione riproducibile
- Diffrange a 1.6-2Å
- Non richiede crioprotezione
- Tollera bene DMSO

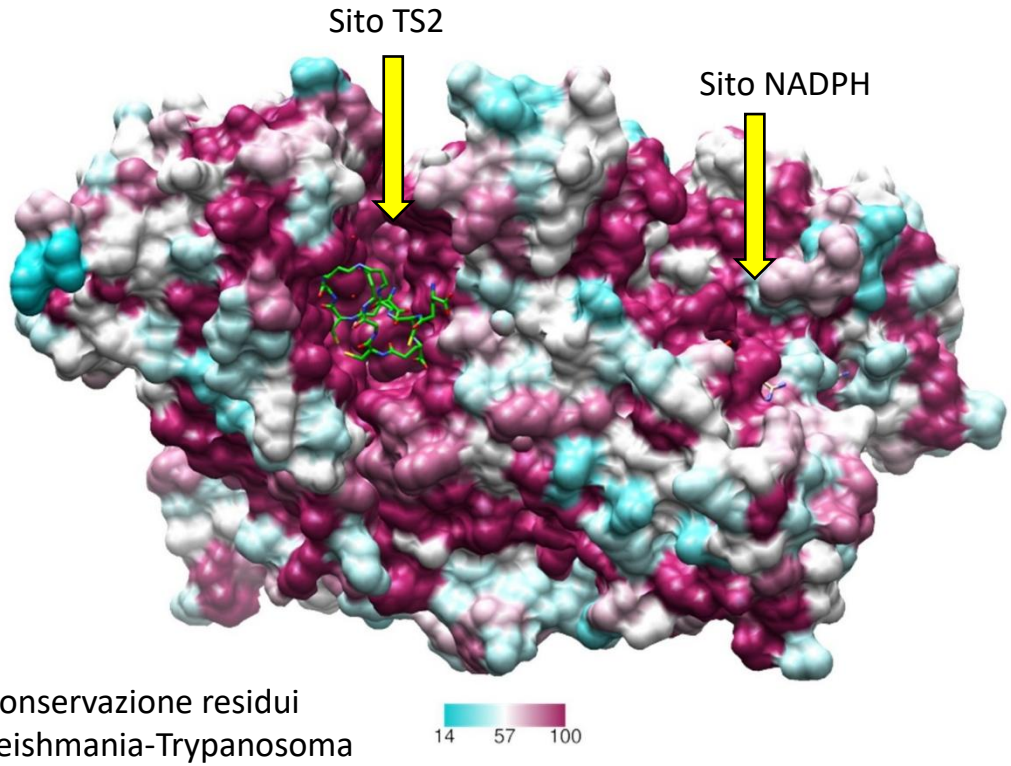




Library testata: **DSiP** ("*Diamond-SGC-iNEXT Poised library*")

design principle is to allow rapid, cheap follow-up synthesis to provide quick SAR data.
Poised fragments contain at least one functional group which can be synthesised using a robust, well-characterised reaction. Reactions include amide couplings, Suzuki-type aryl-aryl couplings and reductive aminations.

The library is aligned with the availability of compounds in Enamine REAL Database. It is possible for anybody to order a copy by contacting Enamine directly and mention "DSI-poised" and Diamond/XChem.



Conservazione residui
Leishmania-Trypanosoma

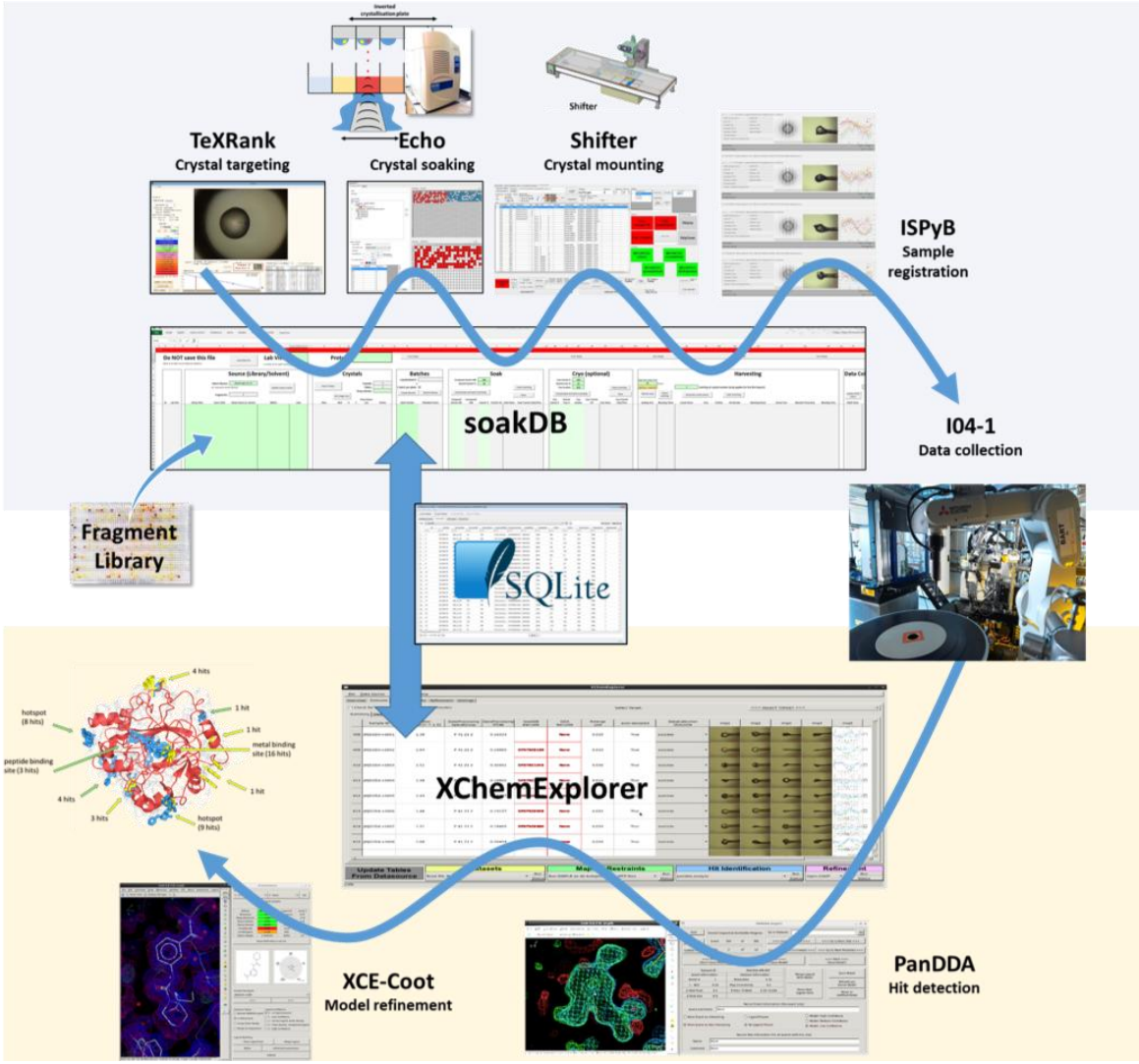
I siti identificati in trypanosoma potrebbero essere conservati o meno in Leishmania.

XCHEM

Diamond Fragment Screening

Fragment-based screening is now well-established as a powerful approach to early drug ("lead") discovery.

- ✓ Cristallizzazione automatizzata
- ✓ Composti aggiunti tramite ultrasuoni
- ✓ Supporto software per gestione e analisi dati
- ✓ Analisi PanDDA per identificazione di hit



PANDDA Processing Output

Summary of Processing of Datasets

Dataset Summary

Interesting (69%)

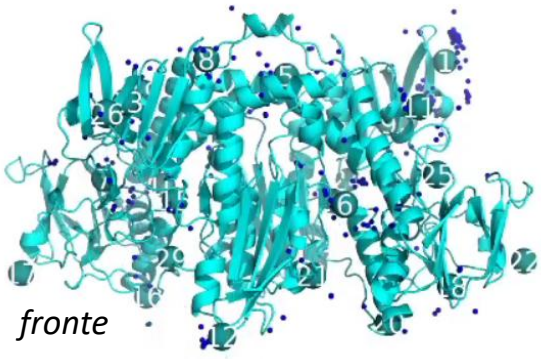
Analysed: 356

Interesting: 249

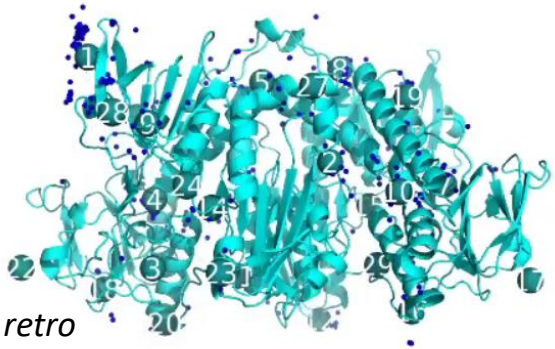
- ✓ Testati più di 300 composti
- ✓ Nel 69% dei dataset si è osservato un ‘evento’

  (legame, var. conform., ….)
- ✓ Gli ‘eventi’ vengono clusterizzati in base al sito.

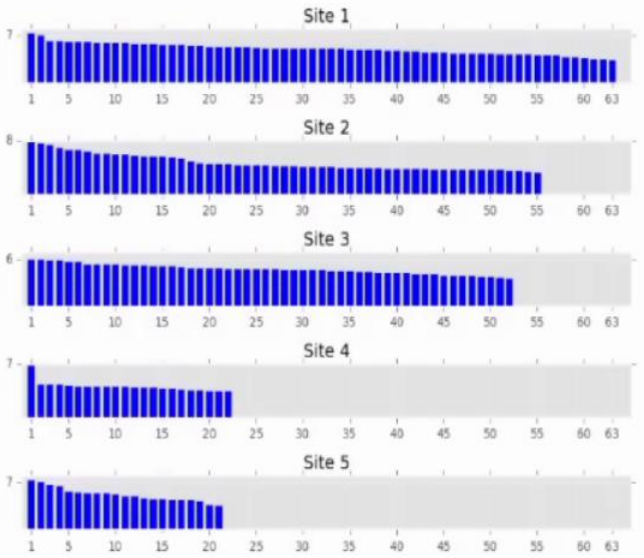
L’analisi preliminare ha identificato **33 siti.**



fronte



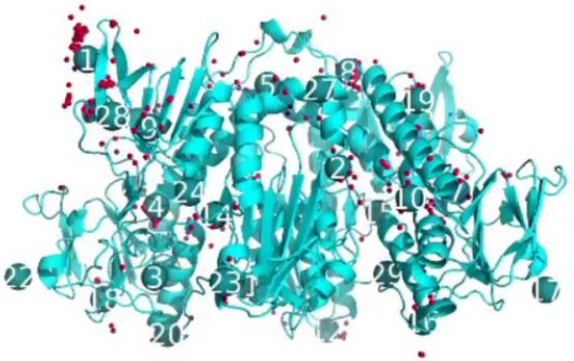
retro



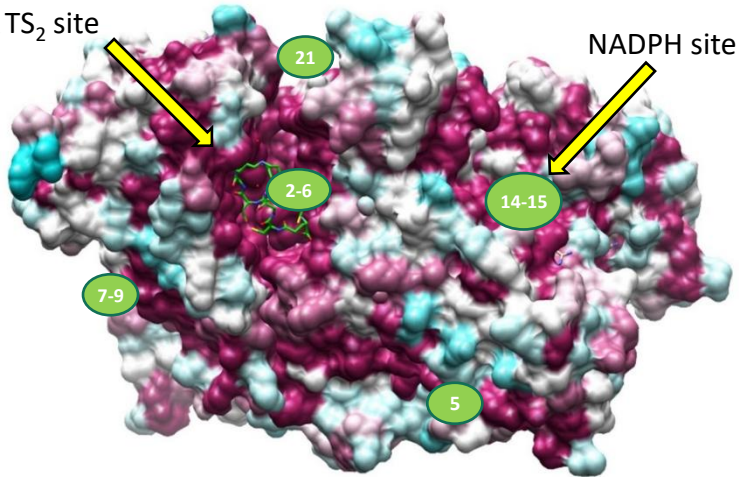
data analysis with XChem Explorer (XCE)

357 datasets analysed with PanDDA
249 classified as interesting

480 events clustered in **33 sites.**



Visual inspection



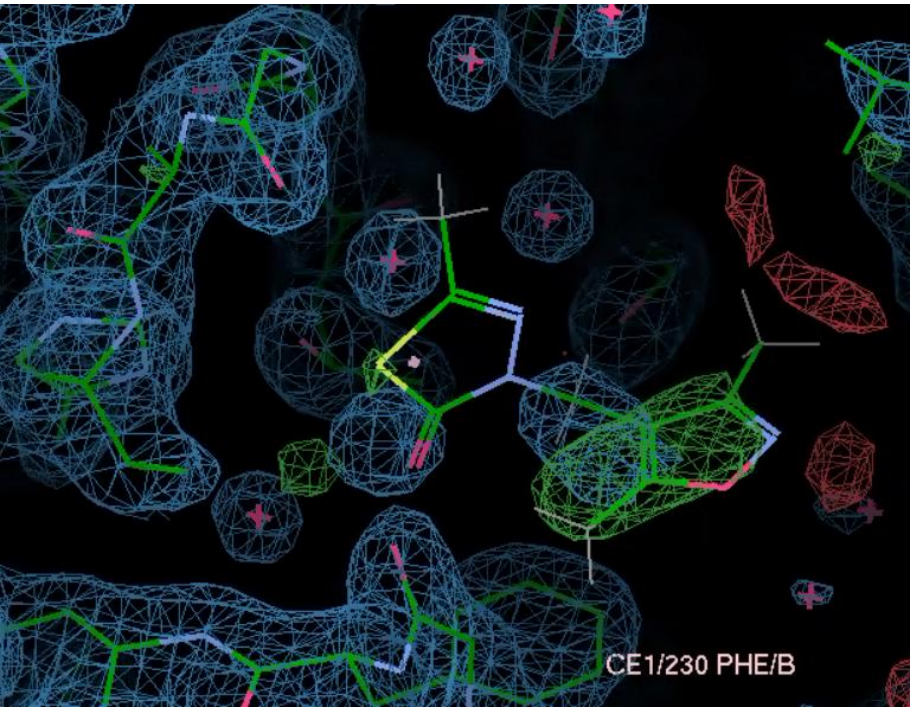
21 'true' binding events in **8 sites**

- 12 fragment hits (res. 1.6-2.0 Å)
- 5 independent binding sites

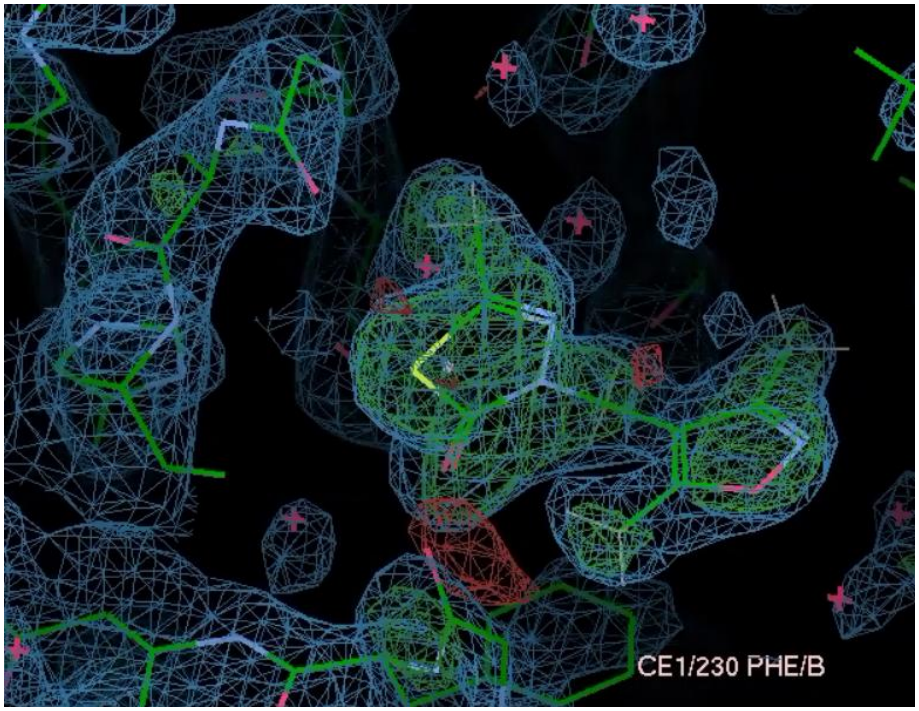
Binding site	n. of ligs
2-6 (TS_2)	5
14-15 (NADPH)	4
5	2
7-9	1
21	1

Effetto analisi PanDDA

Sito NADPH (sample 90)



Mappa classica

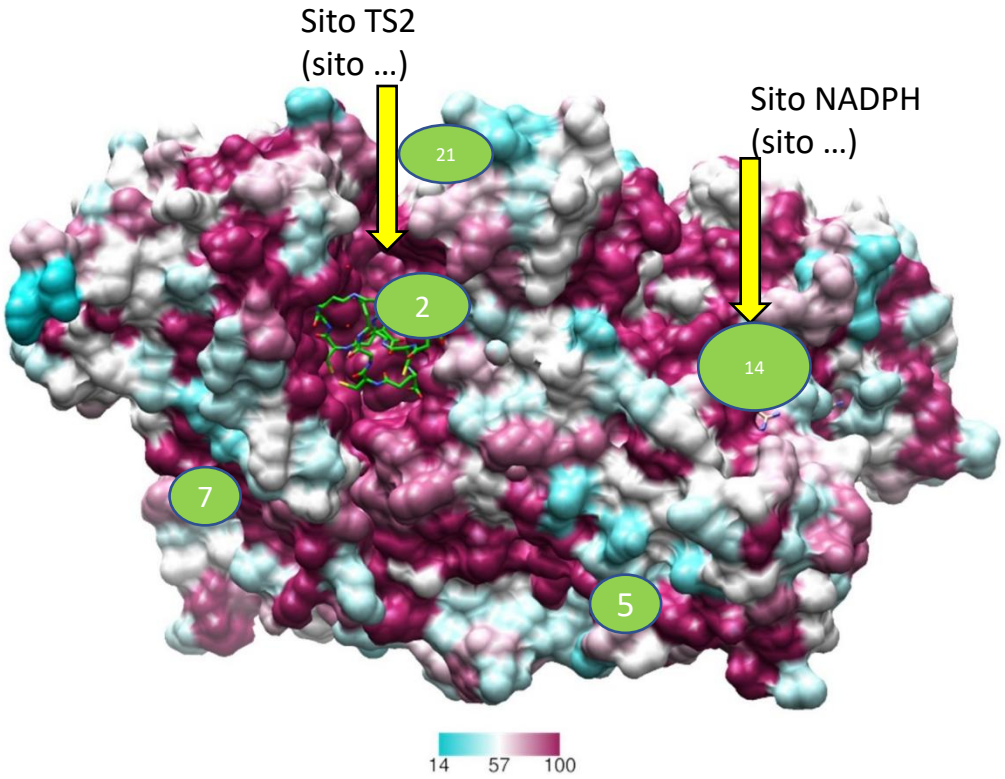


Analisi PanDDA

21 eventi di binding

5 siti reali

12 ligandi (risoluzione 1.6-1.97Å)

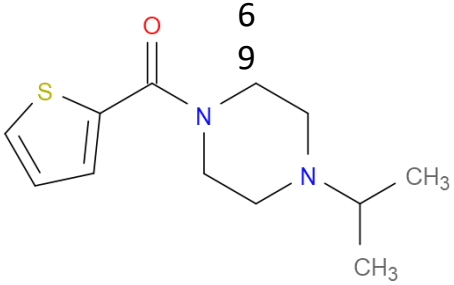


Sito	ligandi	Sample	pdb
2-6 (TS2)	5	69, 71, 109, 221, 371	
5 (IRBM3)	2	60, 90	
7-9	1	94	
14-15 (NADPH)	4	64, 68, 90, 117	
21	1	296	

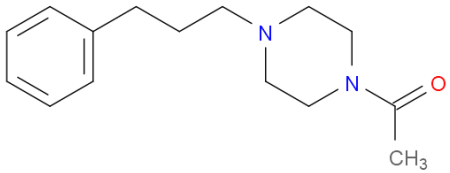
Sito 2-6
(TS2)

Porzione che punta verso l'esterno

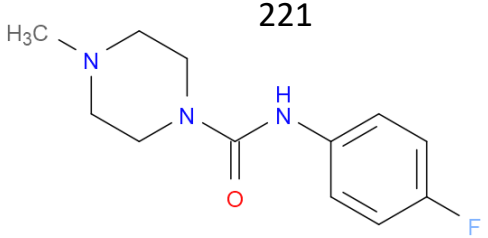
6
9

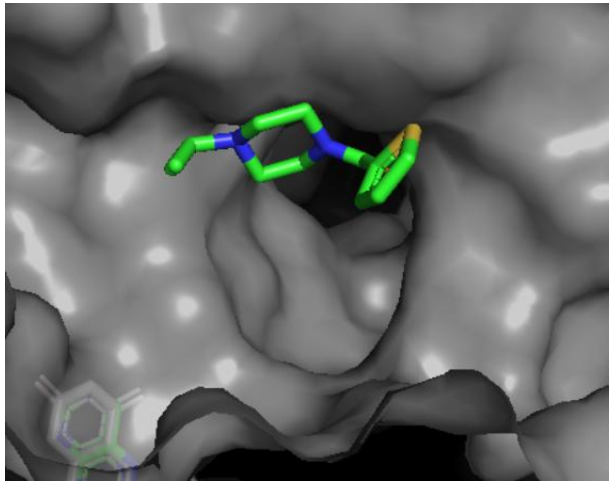


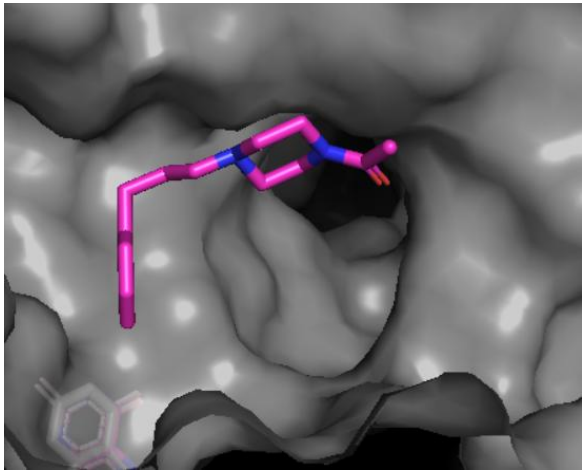
109

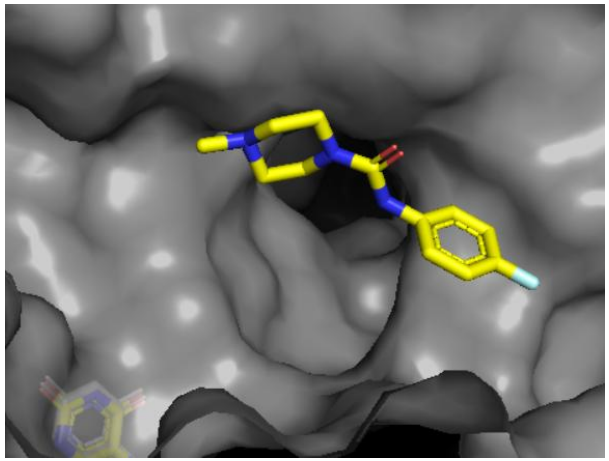


221



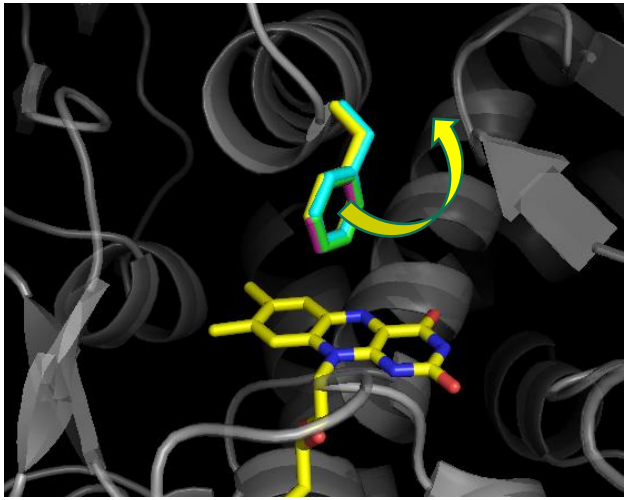




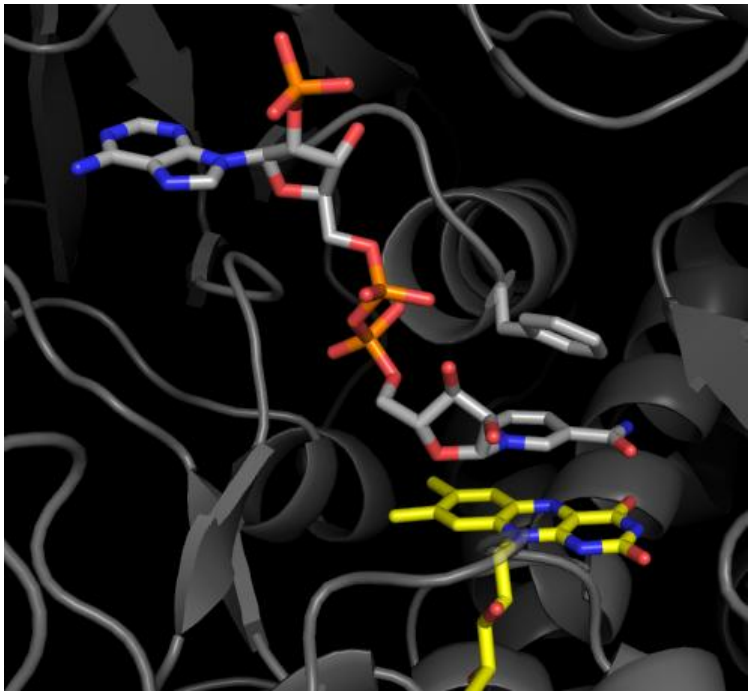


Frags in proximity of NADPH-site: the doorstop-pocket

'door opening'
needed for NADPH binding in NAD-FAD reductases



Apo or frag-bound TbTR



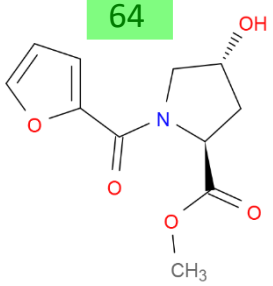
NADPH-bound TbTR

Silvestri et al., ACS Chem Biol, 2018 (SmTGR: Thioredoxin-glutathione Reductase from *Schistosoma Mansoni*)

Frags in proximity of NADPH-site: the doorstop-pocket

Binding site	n. of ligs
14-15 (NADPH)	4

64



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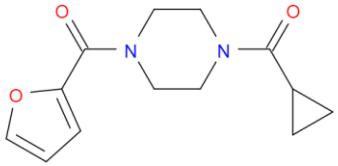

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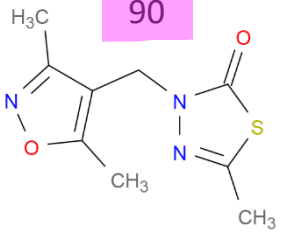

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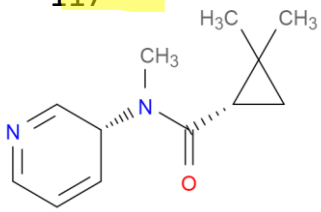
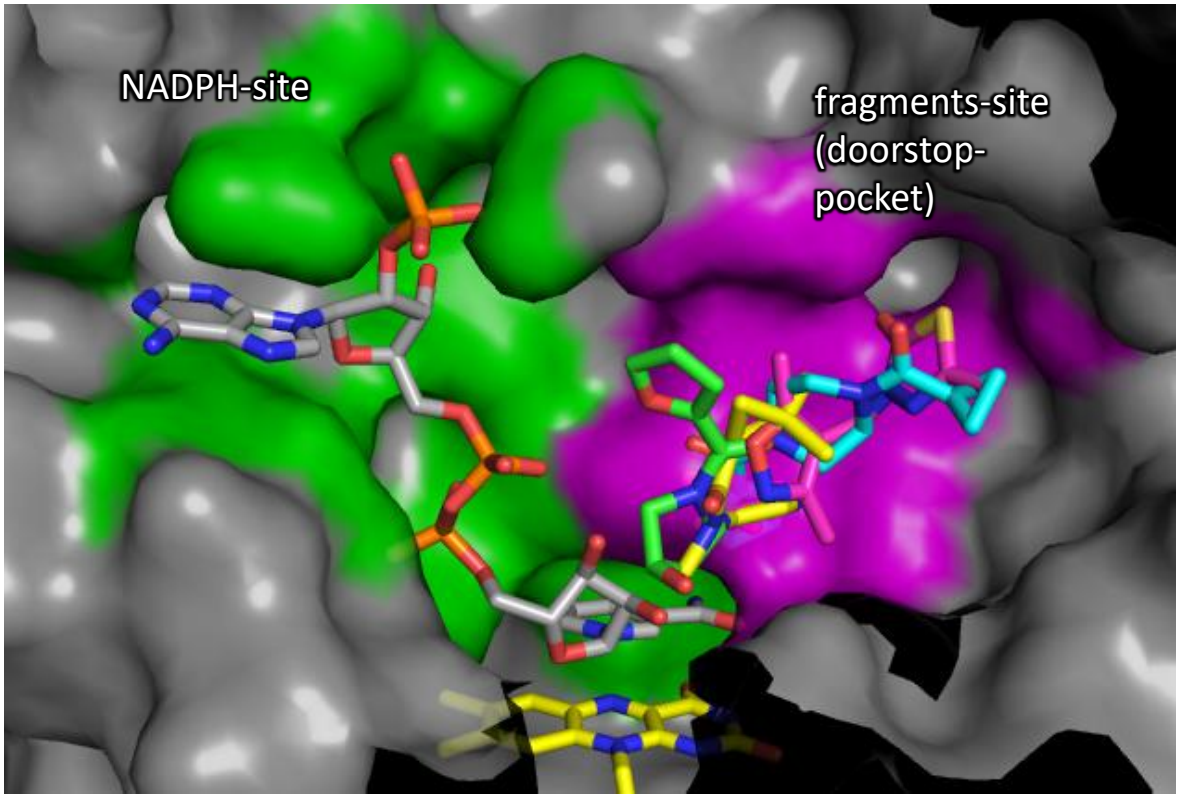
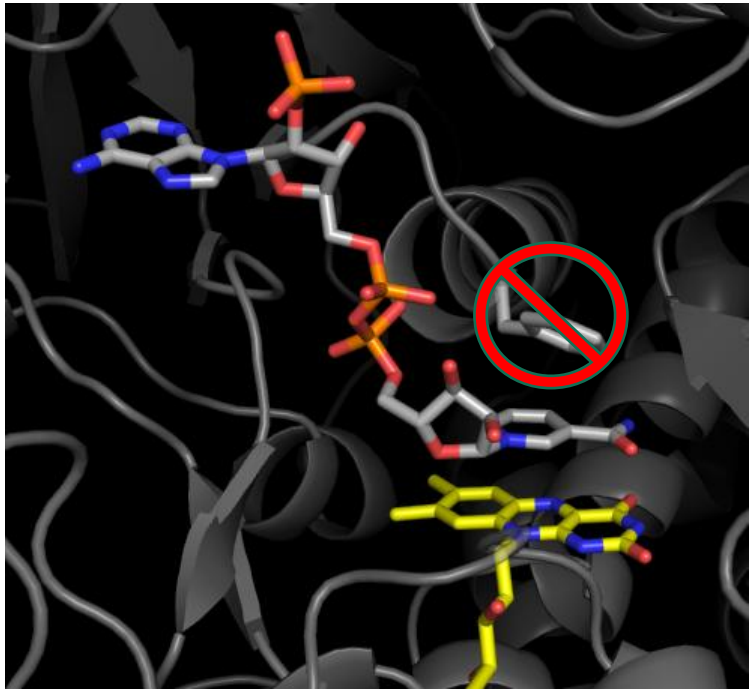




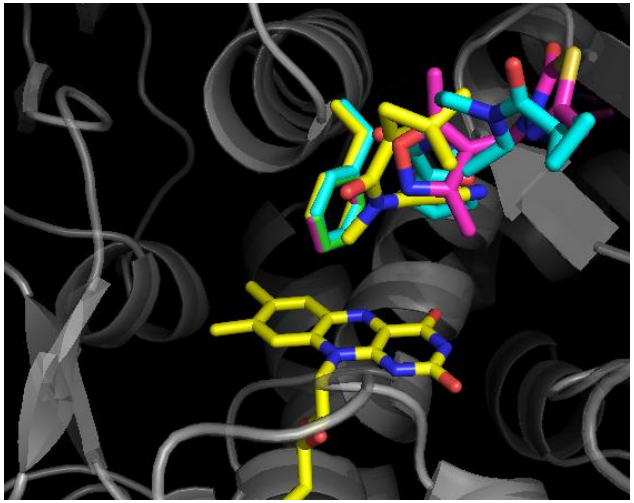
We are interested in developing inhibitors starting from the identified fragments

Frags in proximity of NADPH-site: the doorstop-pocket



NADPH-bound TbTR

'door opening'
needed for NADPH binding in NAD-FAD reductases



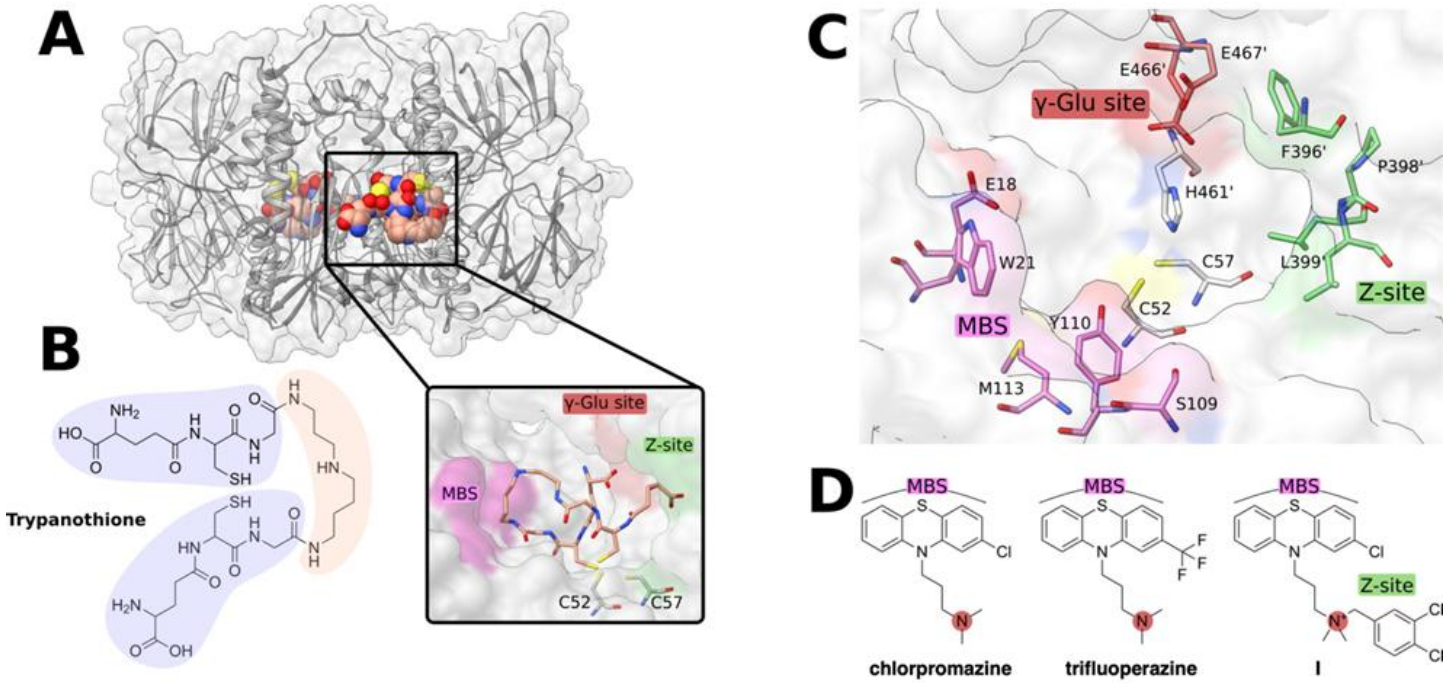
Apo or frag-bound TbTR

doorstop

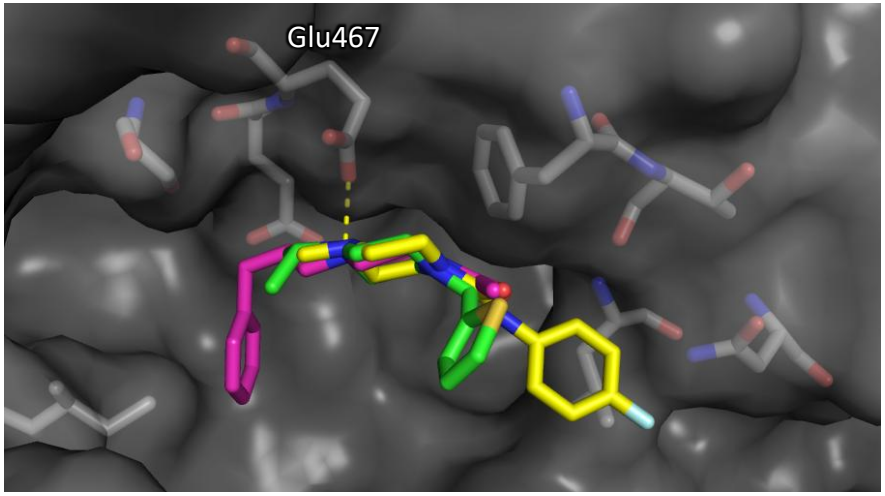
- Ligand-binding at the doorstop pocket hampers the shift of aromatic residue hampering NADPH binding

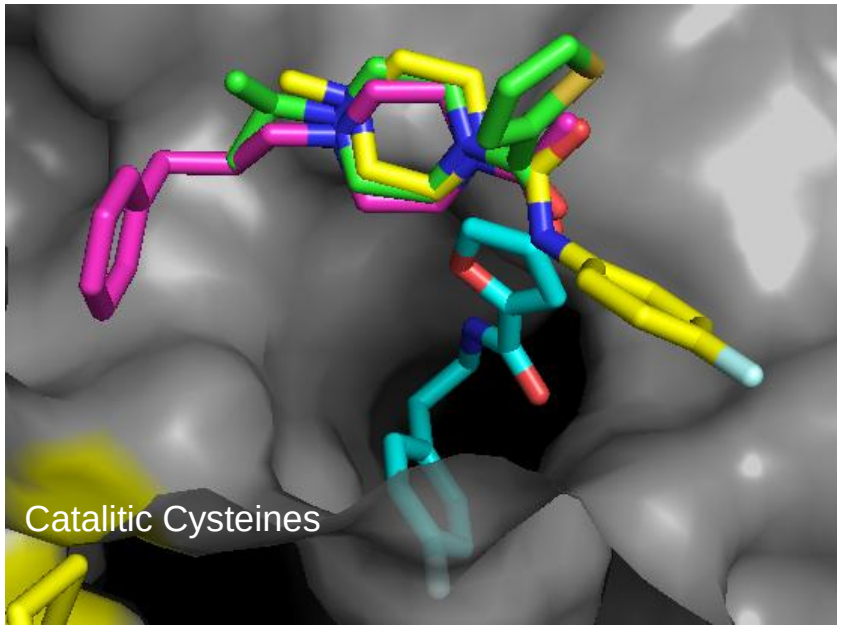
TR inhibition

Z-site in the typanothione binding site



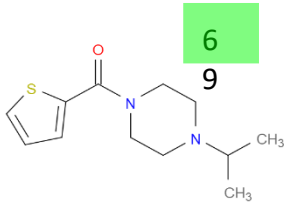
Frags at the TS_2 cavity (Z-site)

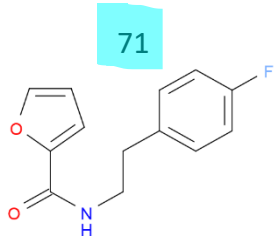


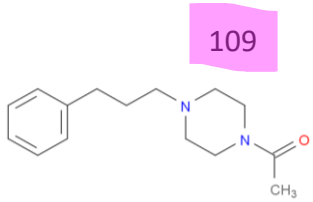


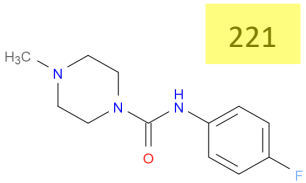
Piperazine ring

- *Electrostatic interaction with Glu467*
- *Shape complementarity*

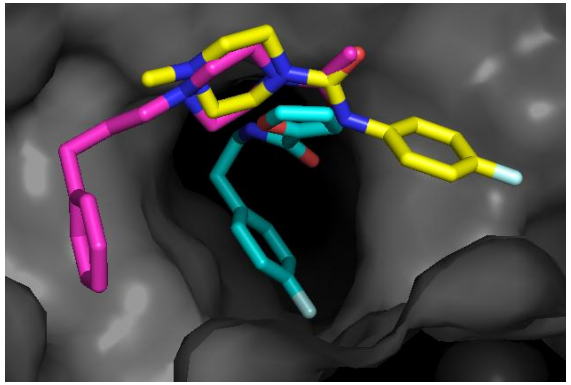




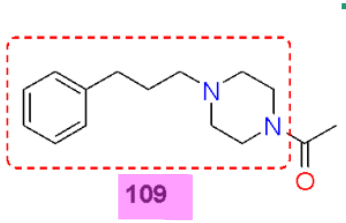




Design of fragment hybrids



3 selected fragments



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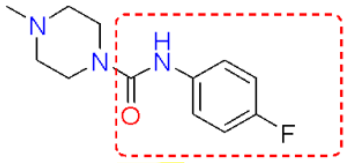

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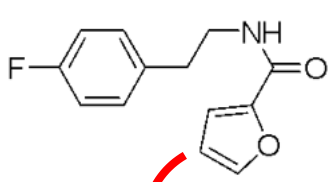
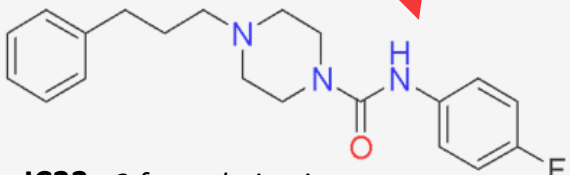
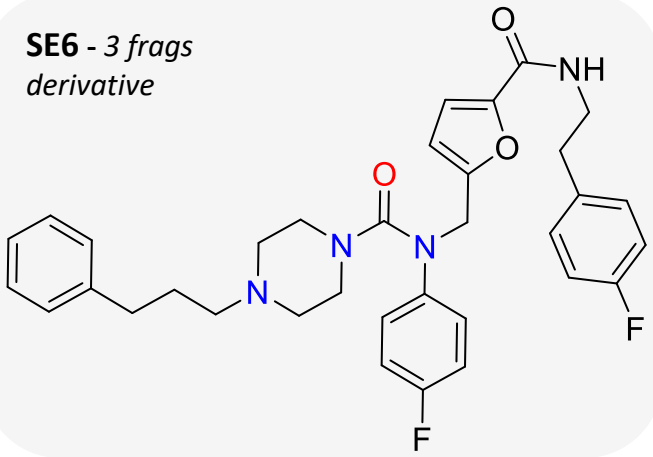




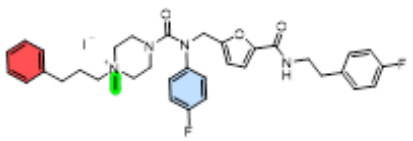
JC33 - *2 frags derivative*

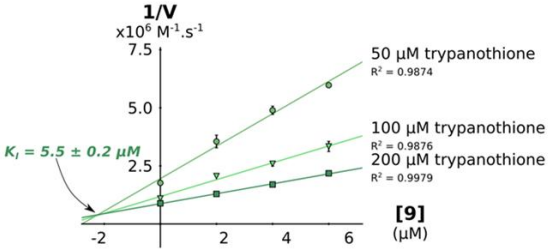
SE6 - *3 frags derivative*



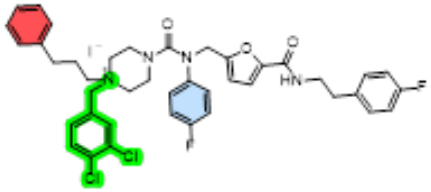
Three compounds with high inhibitory capacity

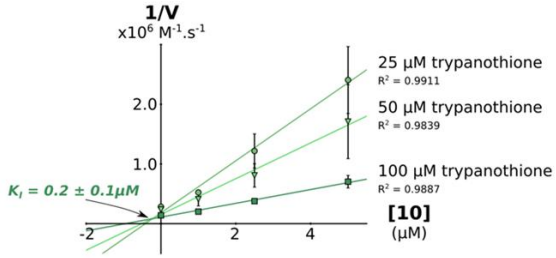
9 AC7



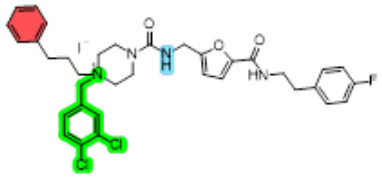


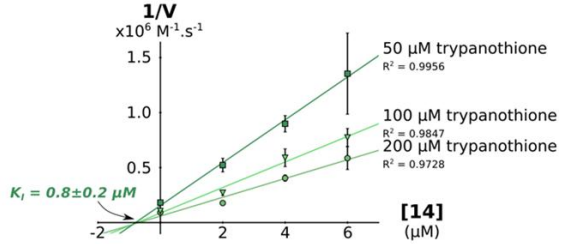
10 SE13



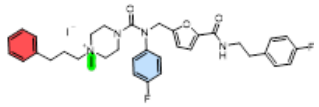


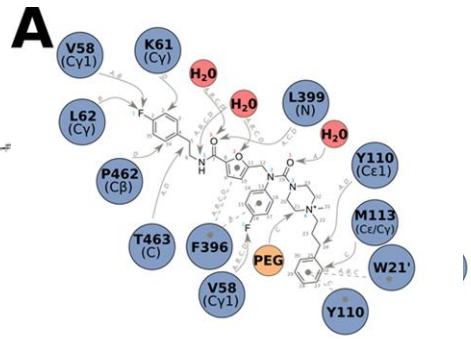
14 ADF01

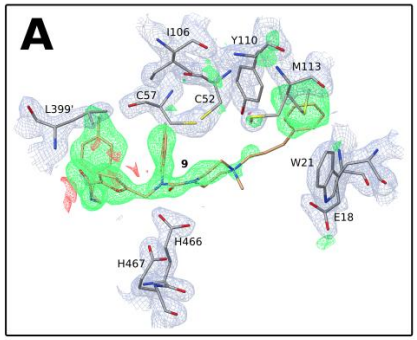




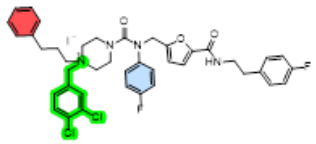
9 AC7

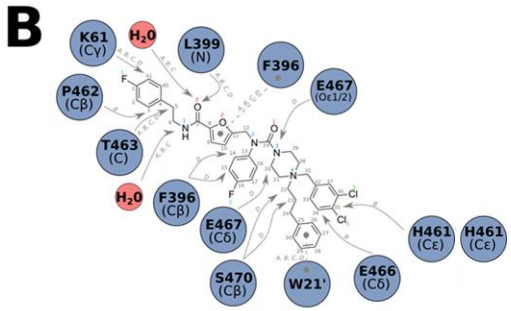


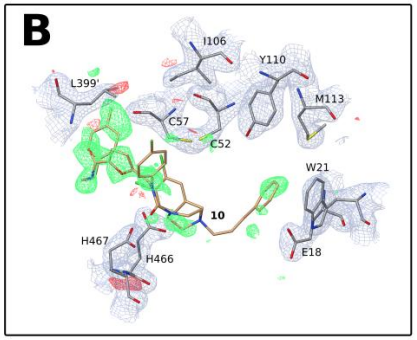




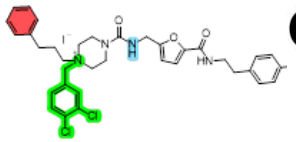
10 SE13

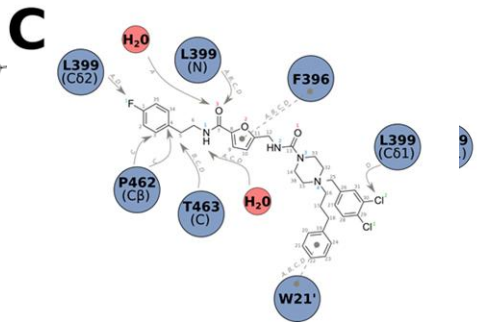


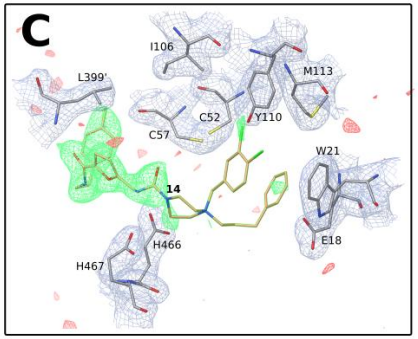


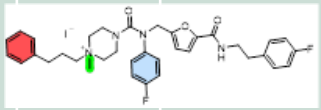
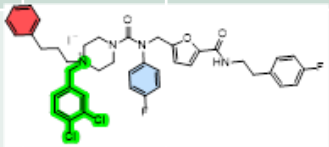
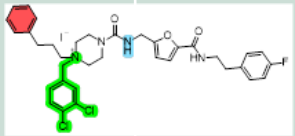


14 ADF01



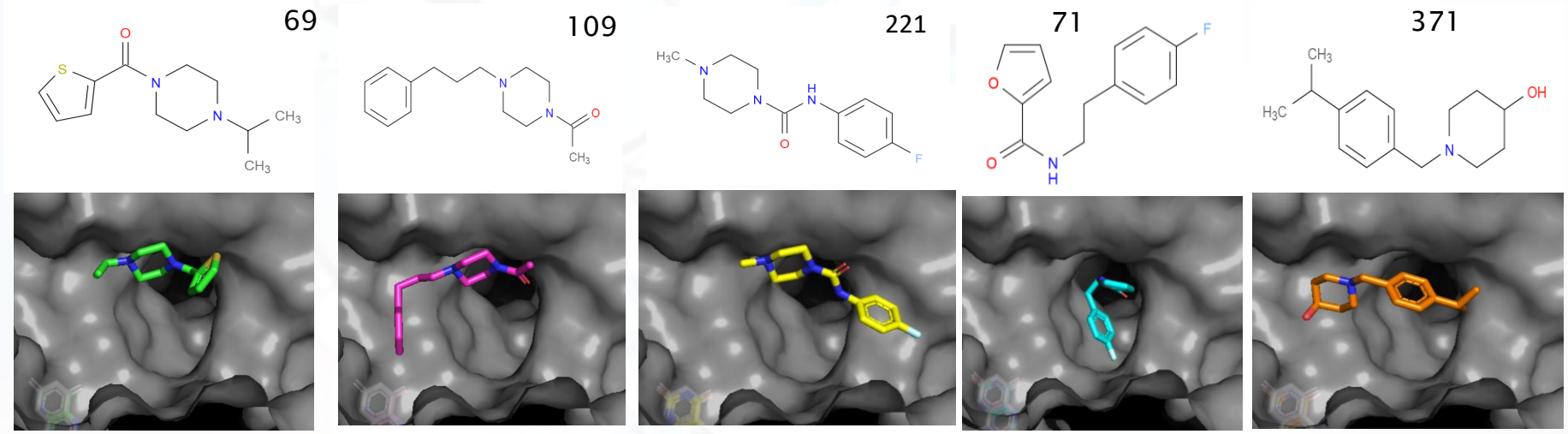


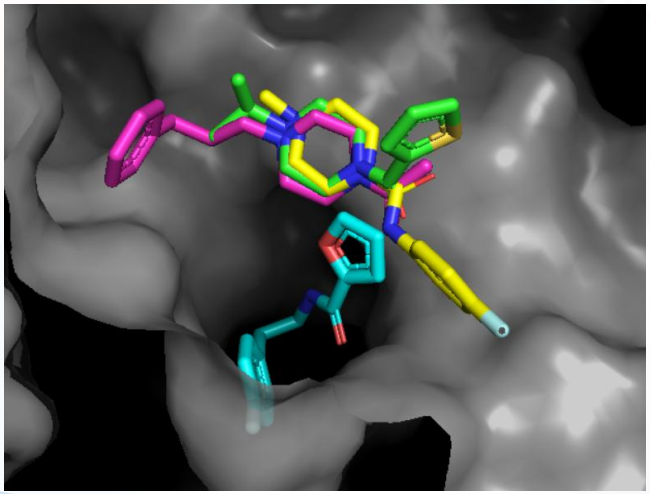


Compound			IC_{50} *Li*TR (µM)	IC50 hGR (µM)	SI
9 AC7			20.5 ± 2.0	62.4 ±12.4	3.0
10 SE13			1.31 ± 0.07	2.3 ± 0.1	1.7
14 ADF01			2.35 ± 0.21	3.7 ± 0.3	1.6

Compound	Axenic Amastigote EC50 ± SE (µM) (95%CI)	macrophage CC50 ± SE (µM) (95%CI)	Intra-Macrophage Amastigote EC50 ± SE (µM) (95%CI)	SI
9 AC7	10.43 ± 1.1 (8.3-13.2)	29.9 ± 4.2 (22.5-40.1)	15.32 ± 2.3 (11.34 - 20.71)	2.8
10 SE13	11.0 ± 1.9 (7.633-15.89)	12.5 ± 0.8 (11.1-14.3)	n.t.	1.1
14 ADF01	8.98 ± 0.4 (8.2-9.9)	12.7 ± 0.9 (11.1-14.7)	40% of reduction at 12.5 µM	1.4

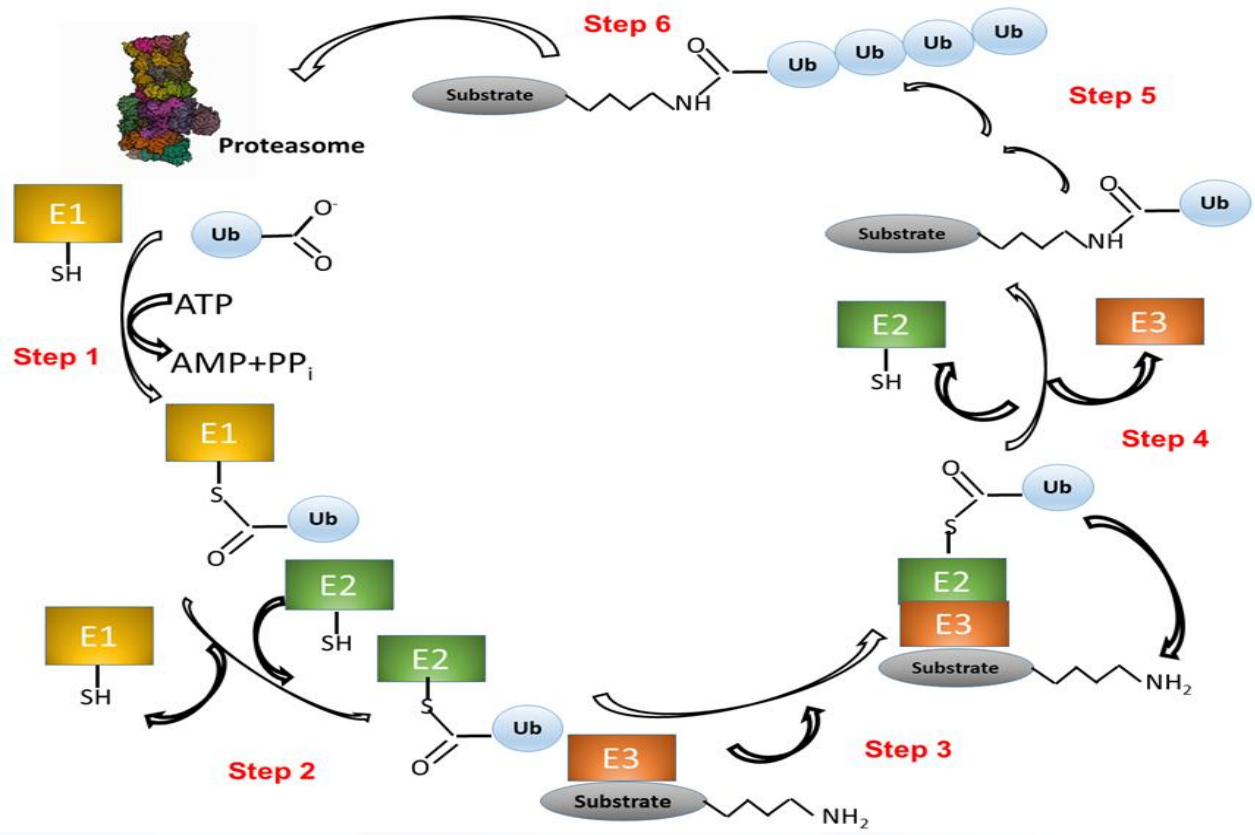
Fragment-Based Drug Discovery: Ligands at the TS_2 binding site





These ligands are cose and/or Superimposed in the trypanothione binding sites and can be used to synthesize new and more effective lead compounds

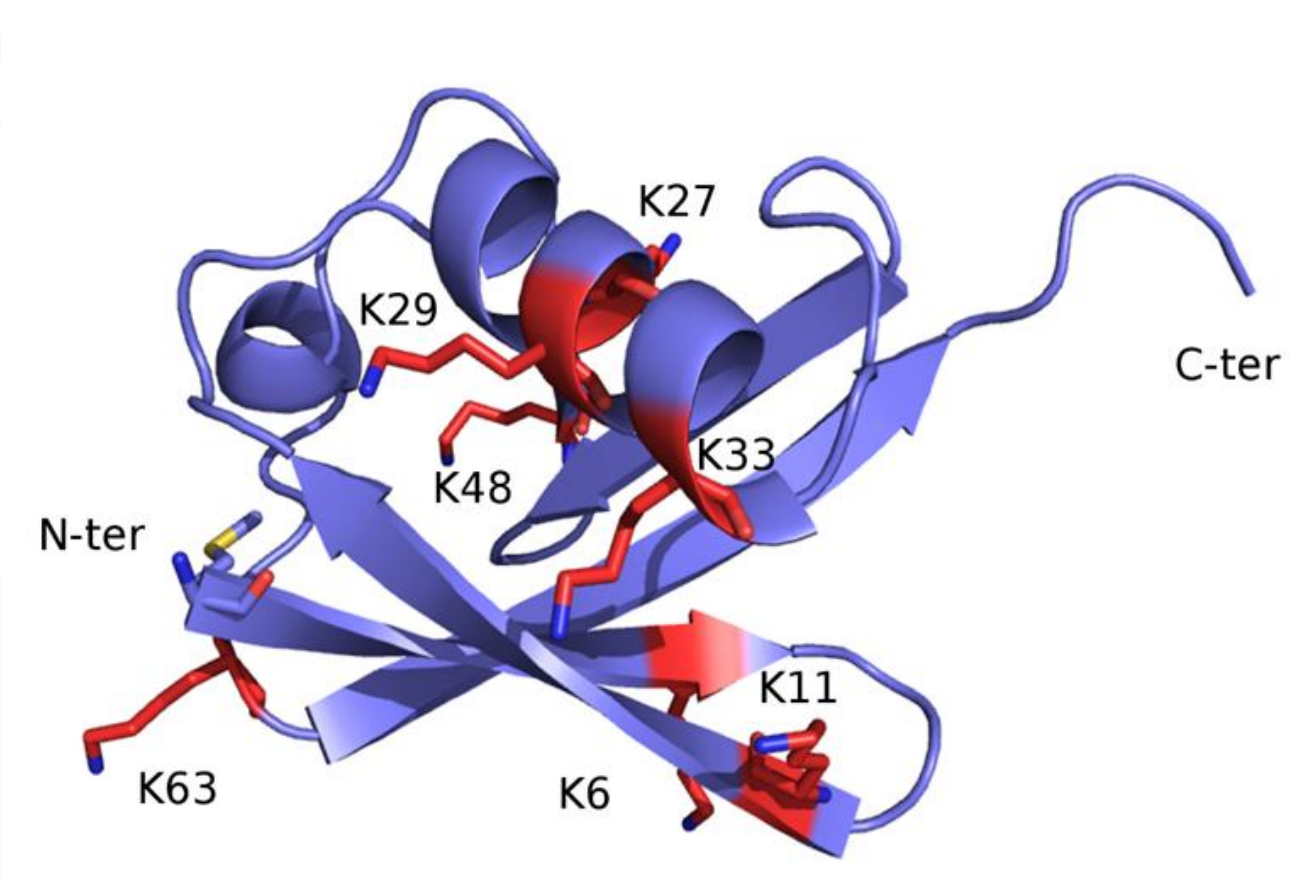
Innovative strategy: TR degradation through UPS



E1= ubiquitin activating enzyme
E2= ubiquitin conjugating enzyme
E3= E3 ligases

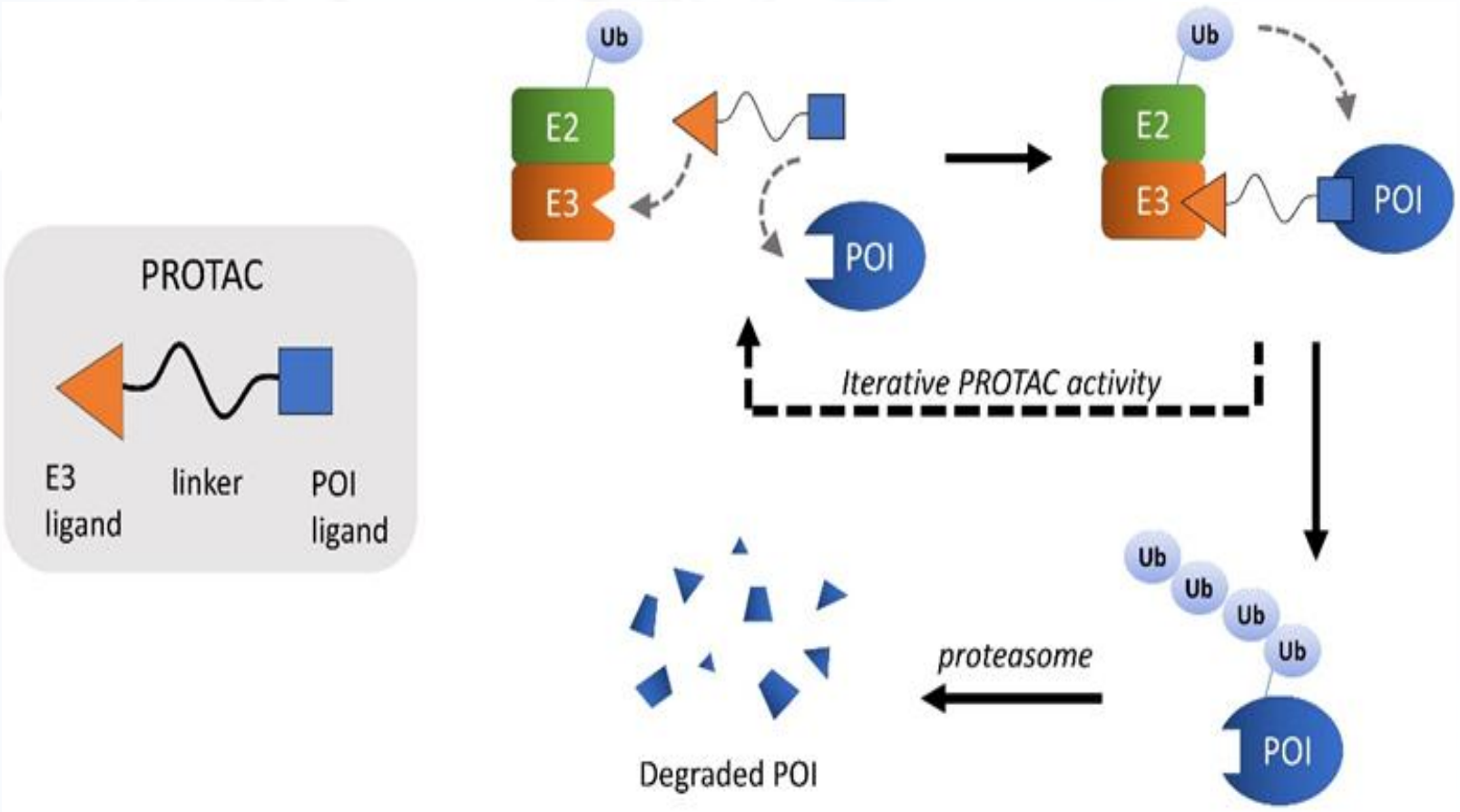
Polyubiquitination can interest one of the 7 ubiquitin lysines.

Ubiquitination through K48 determine the degradation of the target through UPS

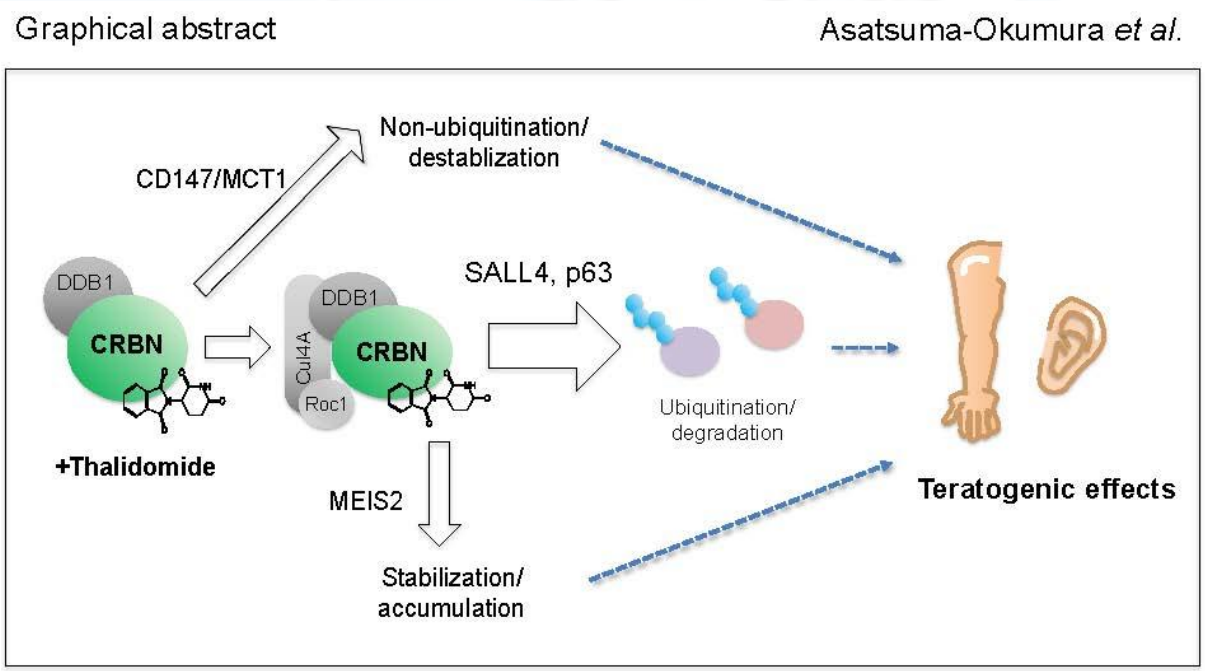


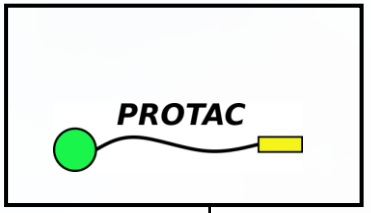
PROTAC: PROteolysis TArgeting Chimeras

POI: Protein of Interest

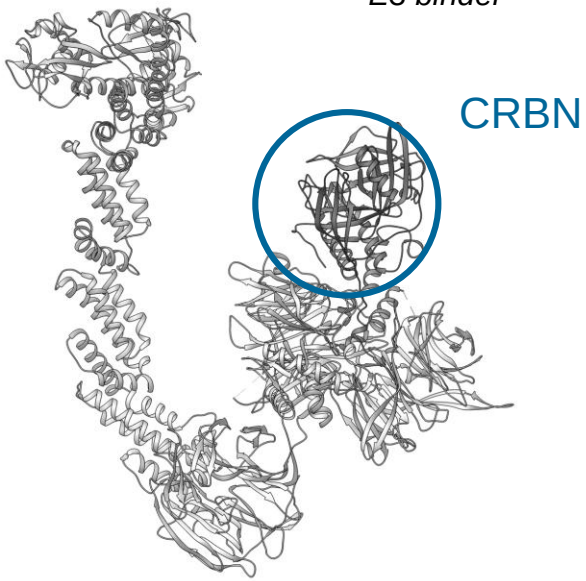


PROTACs design





E3 binder

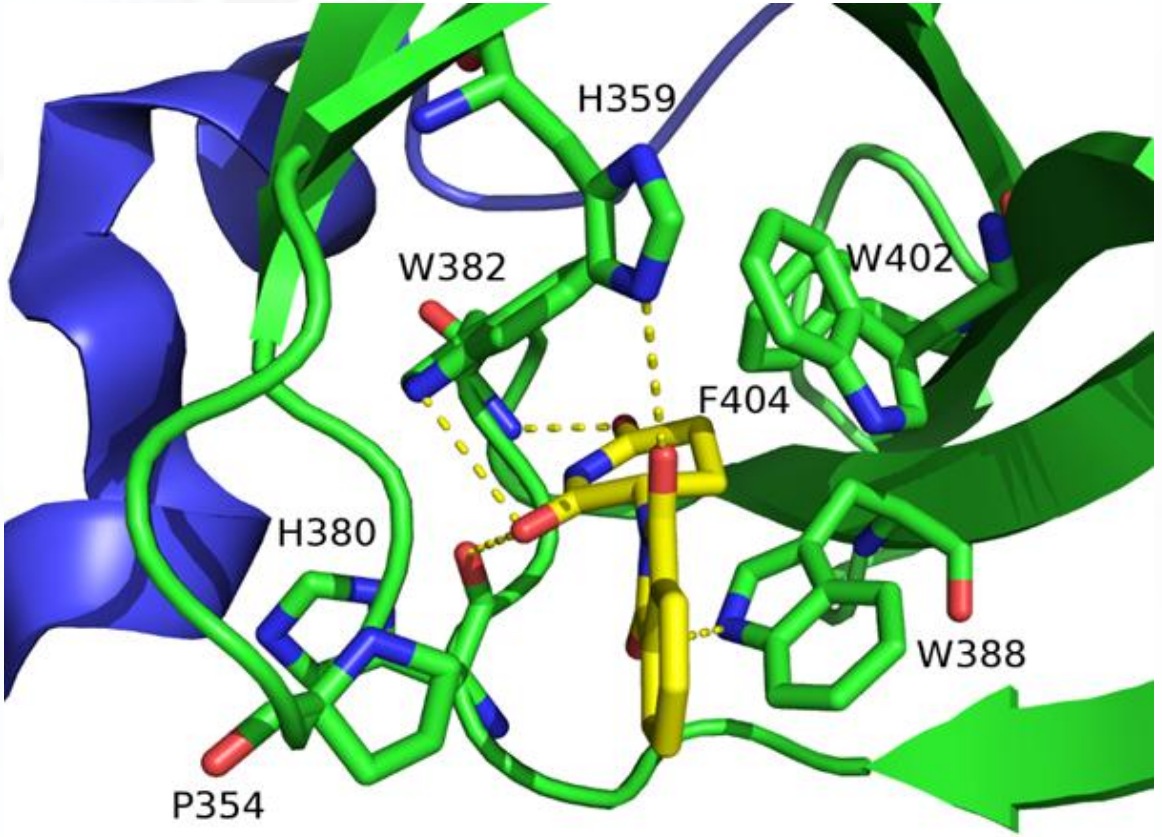


●**CRBN** (cereblon) → CRL4 = E3 ligase complex

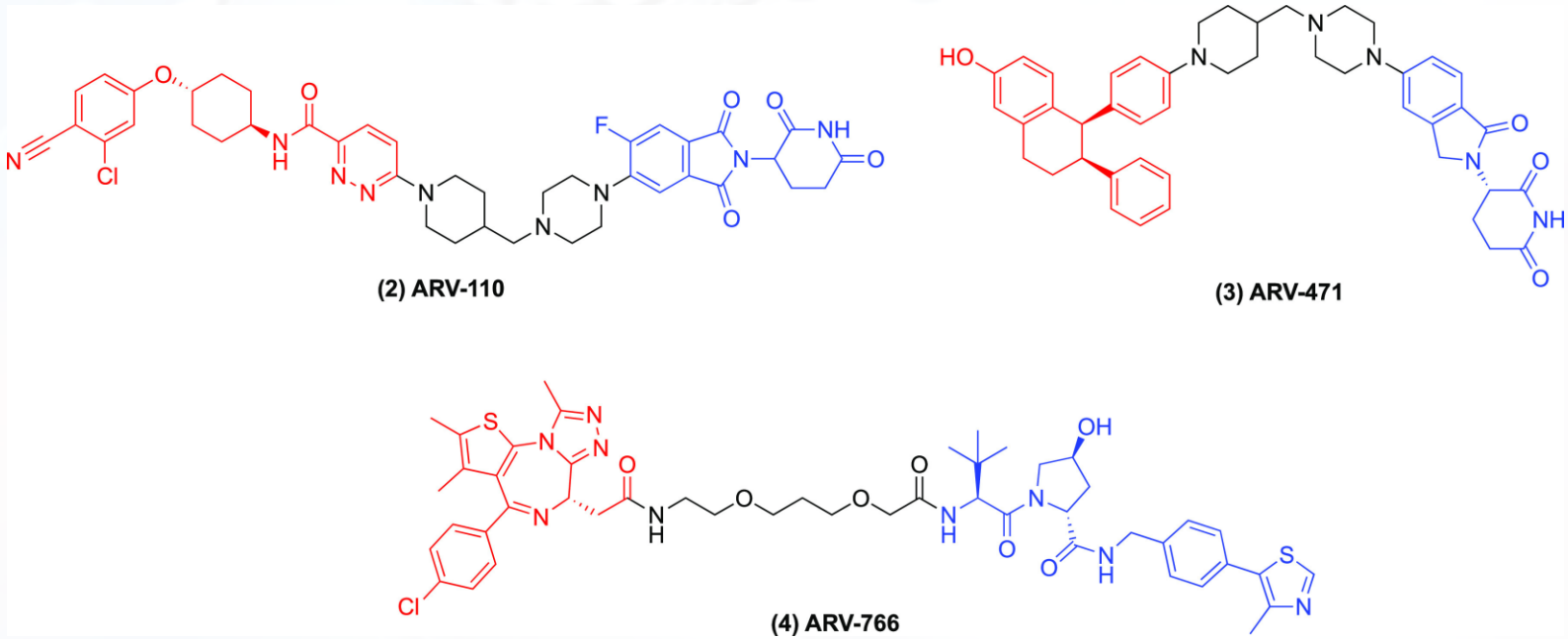
Fischer et al. (2014) Nature
Gu et al. (2018) Bioassays
Bricelj et al. (2021) Front Chem

X-ray structure of the complex between thalidomide and CRBN (PDB code: 4CI1)

Thalidomide and CRBN residues interacting with it are represented as sticks and coloured by atom type (N, blue; O, red; C, yellow and green for thalidomide and CRBN, respectively). Other residues are as ribbon and coloured green, in the thalidomide binding domain, and blue, in the rest of CRBN



PROTAC are already in clinical trials: promising strategy for cancer therapy



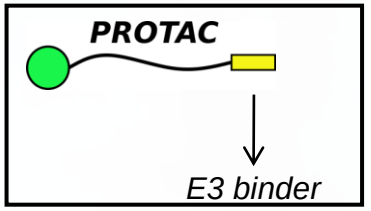
(2) ARV-110

(3) ARV-471

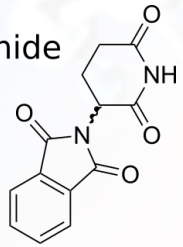
(4) ARV-766

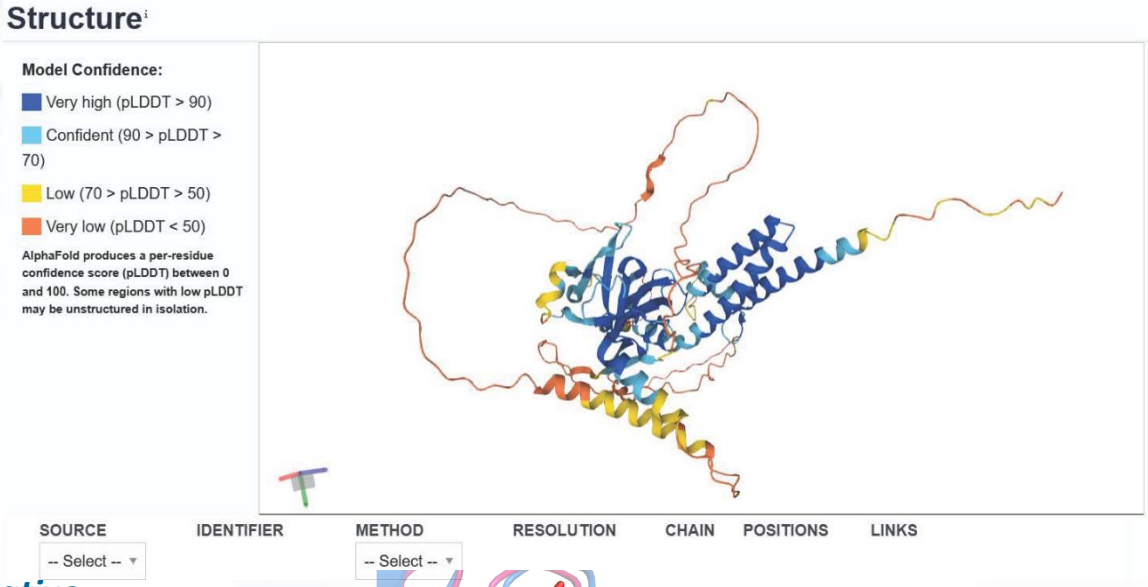
Biopharmaceutical company ARVINAS has three candidates in clinical trials for the treatment of prostate and breast cancers (ARV-110 (2),35 ARV-471 (3),36 and ARV-766 (

Looking for the E3-ligase in Leishmania



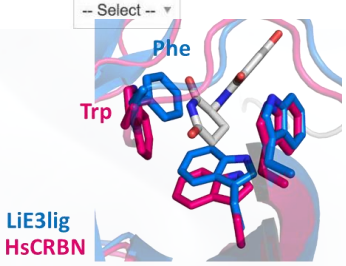
Thalidomide





A4HTX8 · A4HTX8_LEIIN

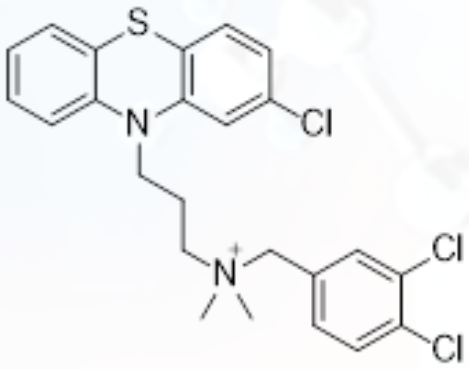
- Leishmania protein containing a ***putative Thalidomide Binding Domain***



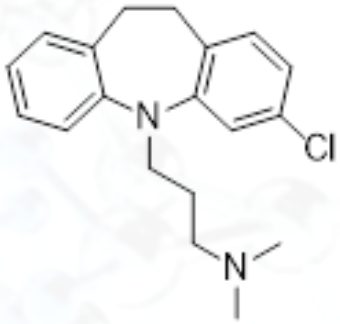
Too Flexible NO CRYSTALS !!!

Fischer et al. (2014) Nature
Gu et al. (2018) Bioassays
Bricelj et al. (2021) Front Chem

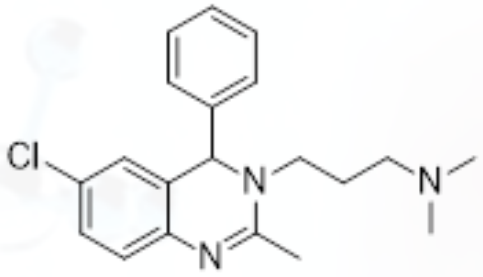
TR inhibitors



Ki= 0.12 uM (*Khan et al. 2000*)
IC50= 4 ug/mL



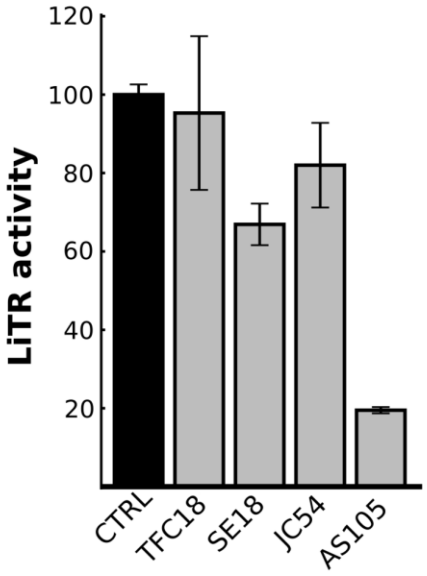
Ki= 6.5 uM (*Benson et al. 1992*)
IC50=15.45 uM



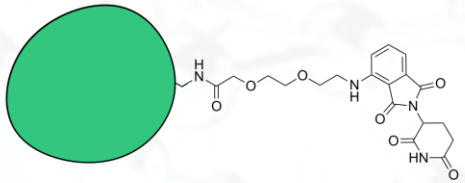
Ki= 1.0 uM (*Patterson et al. 2011*)
No data on Leishmania

Identification of TR binders

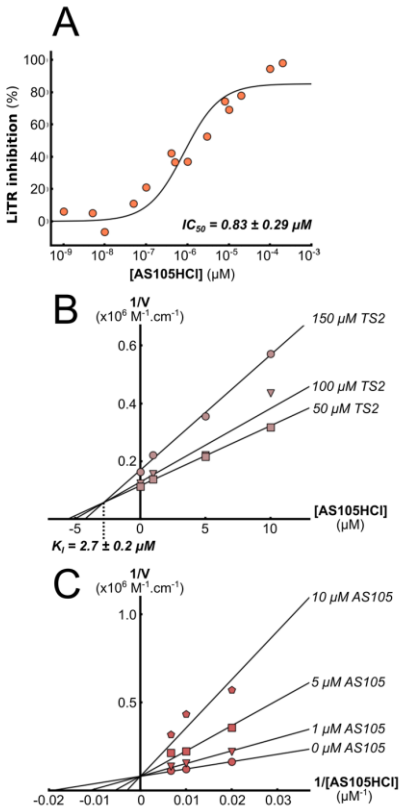
10 uM PROTAC, 100 uM NADPH, 150 uM trypanothione, 25°C, pH 7.4

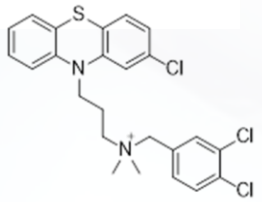


The best TR binder = **AS105**

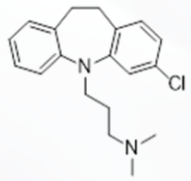


100 uM NADPH, 150 uM trypanothione, 25°C, pH 7.4

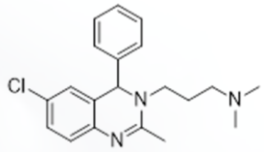




Ki= 0.12 uM (*Khan et al. 2000*)
IC50= 4 ug/mL

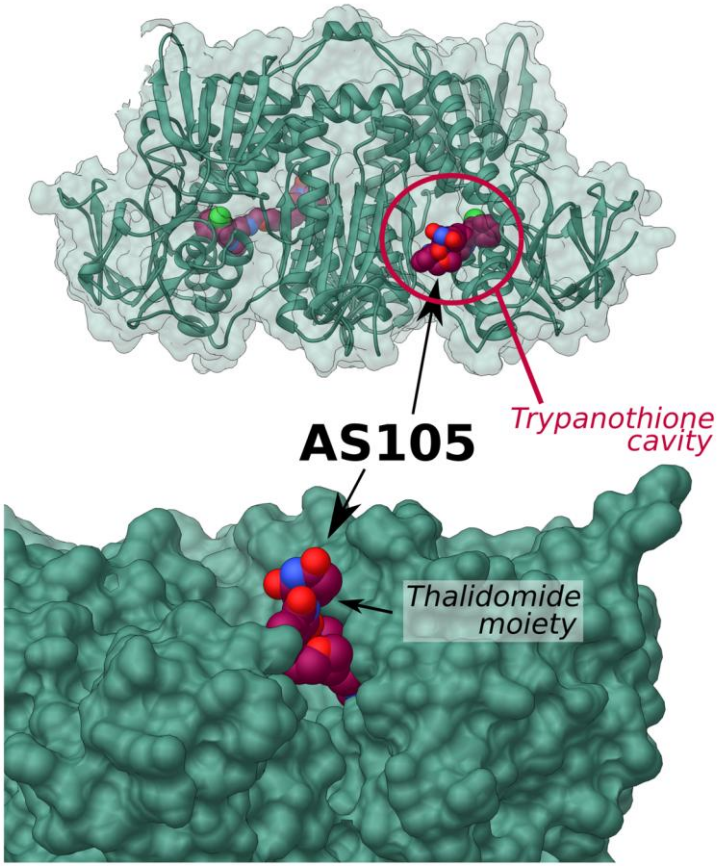


Ki= 6.5 uM (*Benson et al. 1992*)
IC50=15.45 uM



Ki= 1.0 uM (*Patterson et al. 2011*)
No data on Leishmania

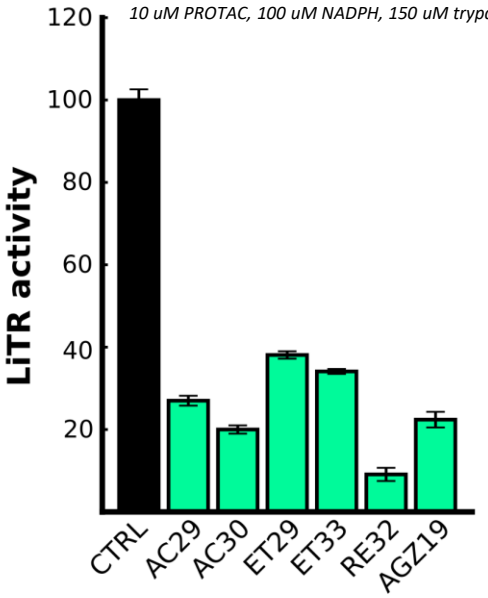
Crystal structure of TbTR bound to AS105



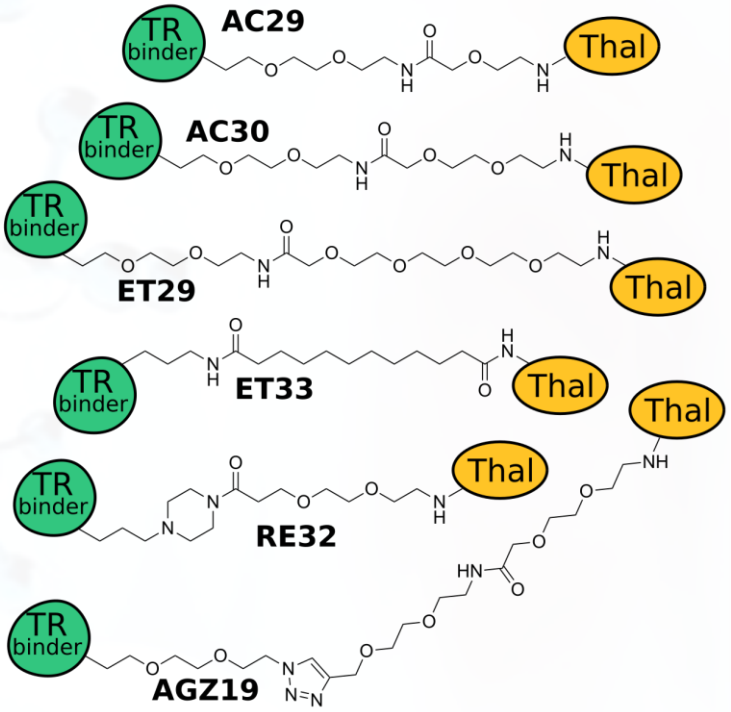
- **Crystallographic structure of TR from *Trypanosoma brucei* bound to AS105 at 2.1Å resolution**

➢ the linker seems **too short**

Linker optimization

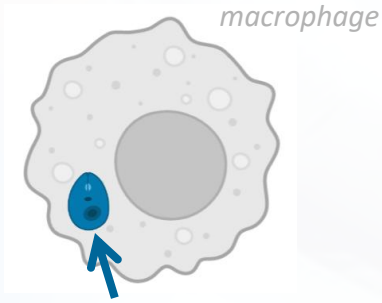


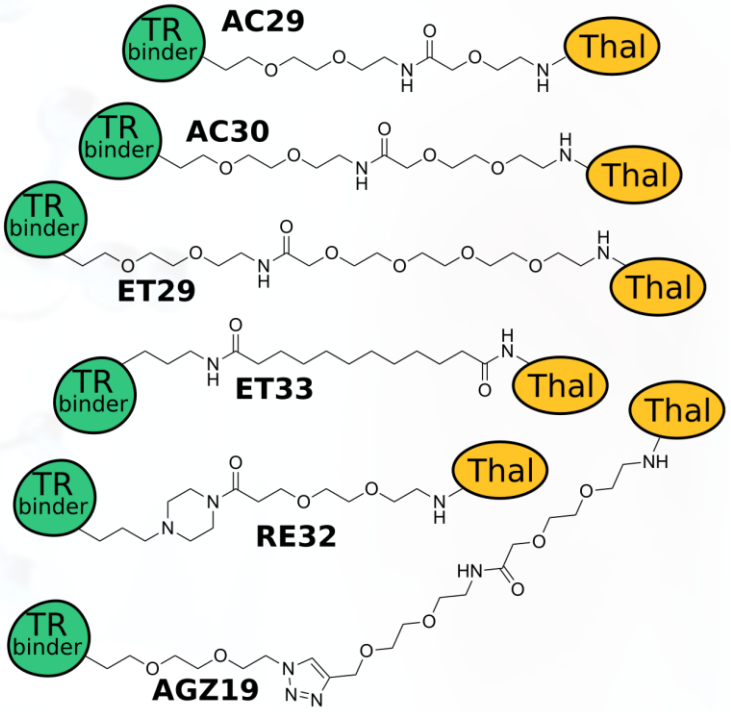
AC30=AP41



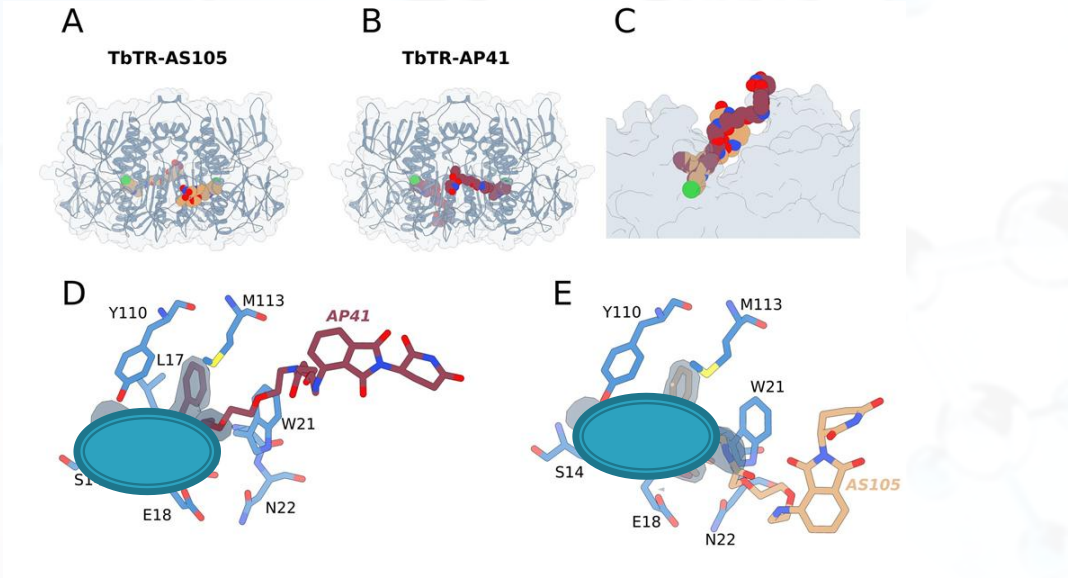
Linker optimization

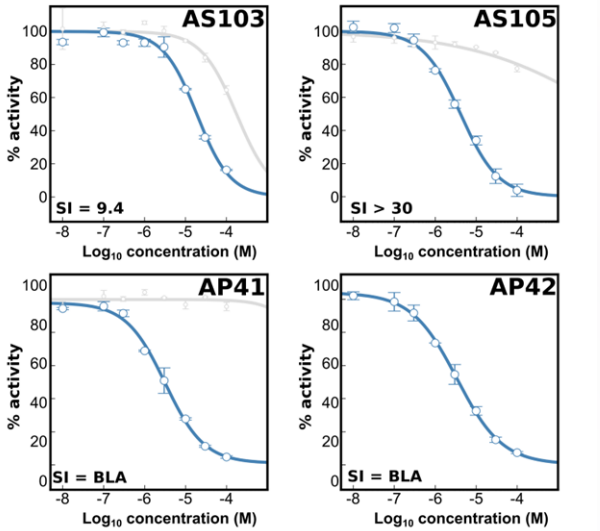
	Axenic amastigotes IC50 (µM)	Amastigotes intra macrophages % growth inhib	Cytotoxicity (macrophages) CC50 (µM)
AC29	8.3 ± 0.4	6.0 ± 1.7	25-50
AC30	9.86 ± 0.4	1.5 ± 0.7	25-30
ET29	20.6 ± 1.7	9% at 1 uM	20
ET33	23.5 ± 1.5	nd	nd
RE32	27.0 ± 2.7	high	< 2.5
AGZ19	22.4 ± 2.9	low	>50





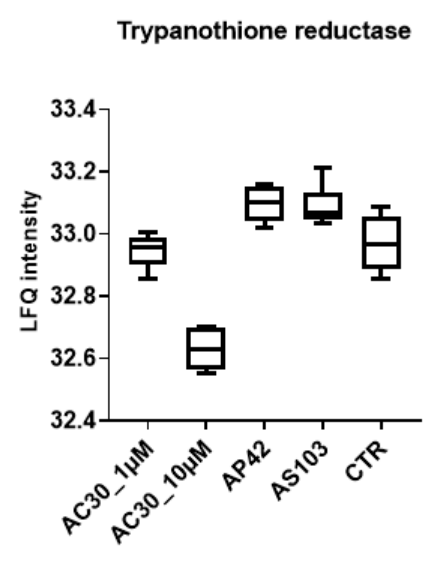
The AP41/AC30 compound binds TR thereby inhibiting its catalytic activity with high efficiency

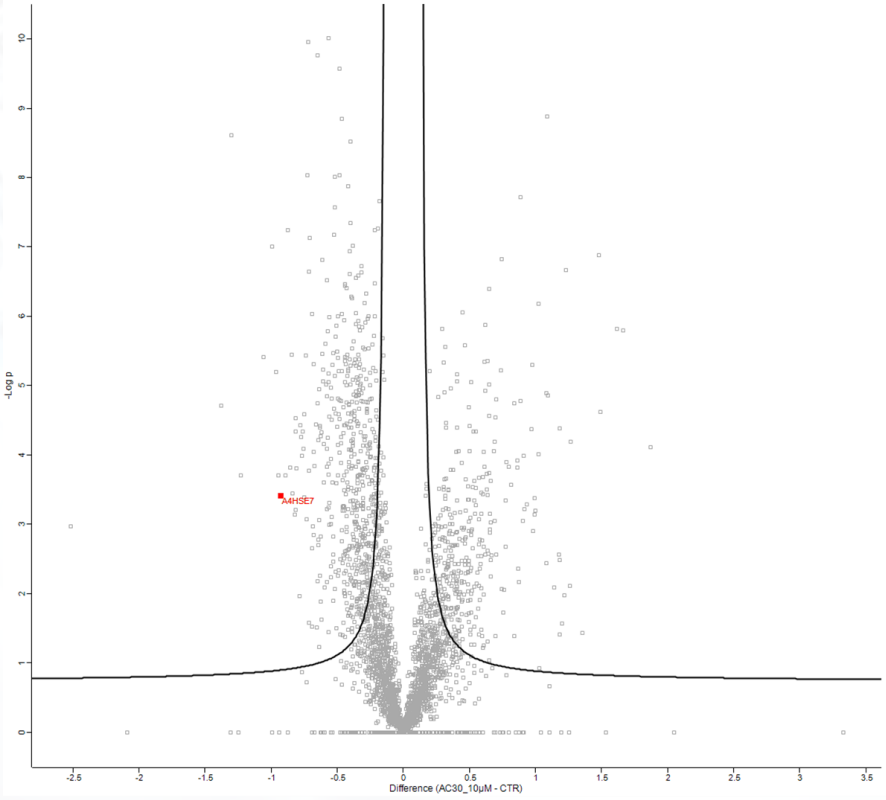




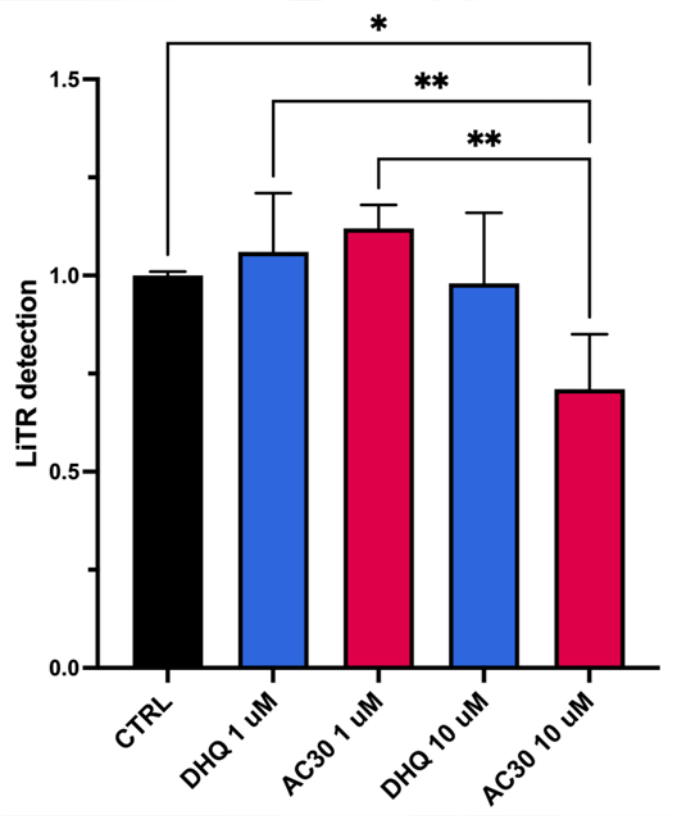
compound	LiTR IC50 (uM)	HsGR IC50 (uM)	SI
AS103 (DHQ)	15.7 +/- 0.2	~ 100 uM	9.4
AS105 (Short PROTAC)	4.0 +/- 0.6	>> 100 uM	>30
AP41 (Best PROTAC)	3.2 +/- 0.6	>> 100 uM	>30
AP42 (methylated PROTAC)	3.4 +/- 0.3	>> 100 uM	>30

Proteomic experiments: the compound AC30/AP41 decreases the concentration of TR in the cell





Western Blot analysis

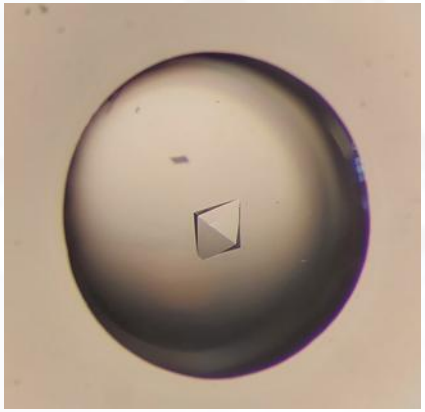


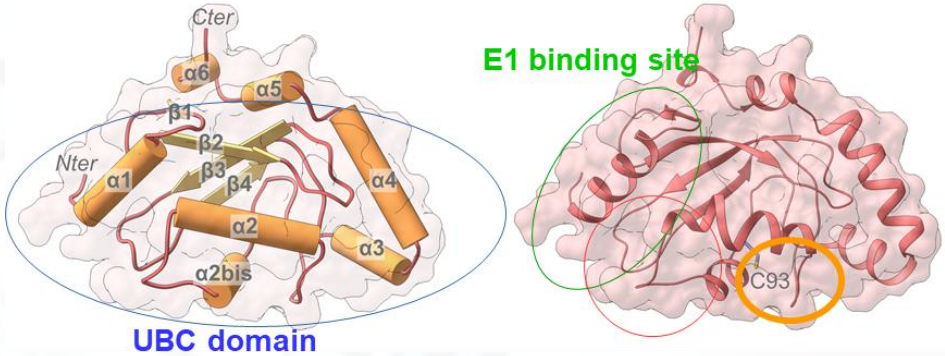
Western Blot to assess the degradation of LiTR

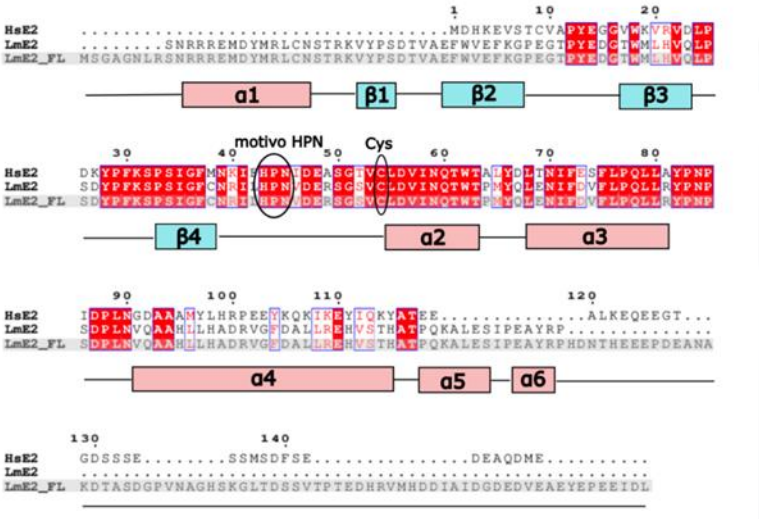
→ Done on axenic amastigotes harvested 6 hours after treatment
→ Axenic amastigote lysis
→ Total protein content measured by BCA assay
→ 10 ug of lysate/well
→ Housekeeper = alpha-tubulin

→ On the left, results obtained for a total of four independent experiments
→ ANOVA analysis
→ *p-value = 0,05
→ **pvalue = 0,01

LmUbC4 – Fragment screening



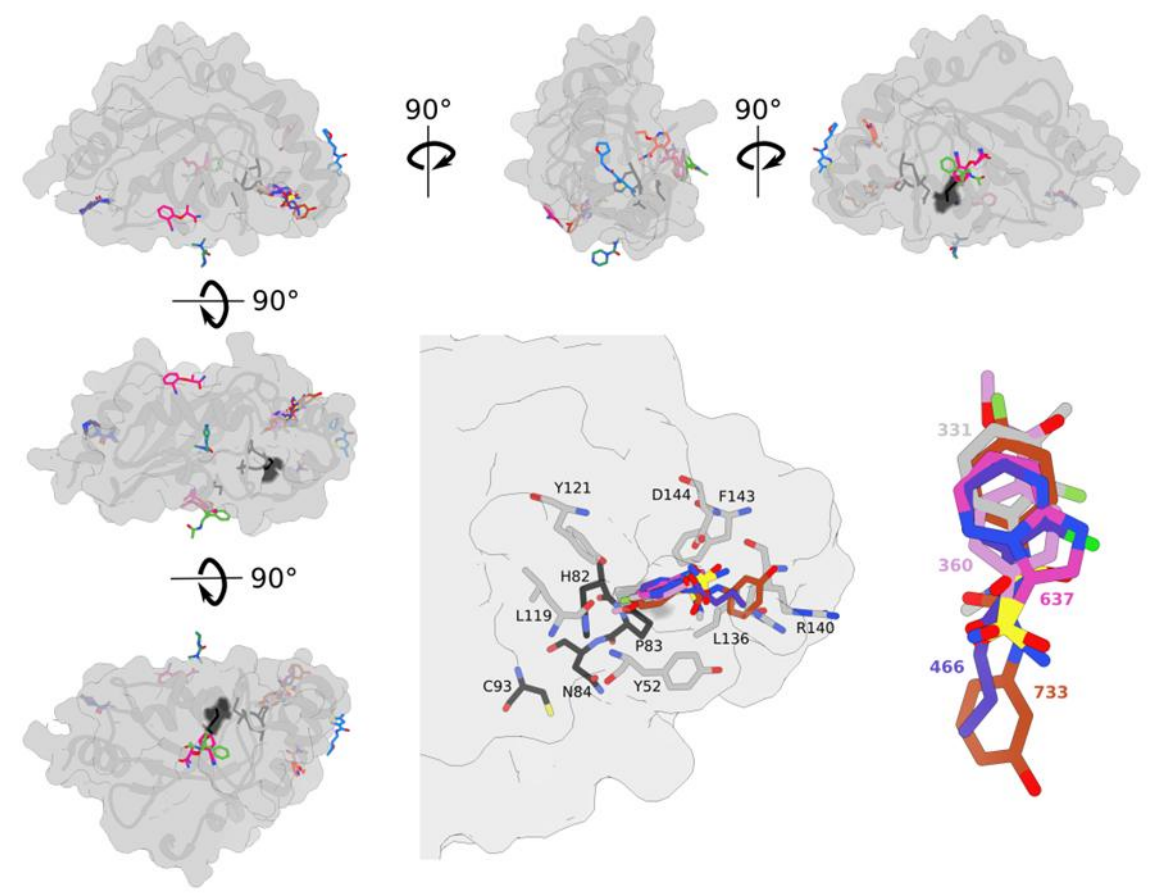




Structure of LmUbC4 determnined by X-ray crystallography

UBC domain = canonical E2 fold
Some human E2 → Cter extensions (intrinsically disordered) → functions
Catalytic Cys = Cys93
HPN motif conserved (10 residues prior Cys93)

LmUbC4 – Fragment screening



Framgent screening → identification of LmUbC4 binders

Identification of ligand binding in LmUbC4 cavities away from the catalytic Cys93.

5 main cavities:
One of them = "D144 cavity"
→ 5 fragments were identified
→ Away from Cys93
→ Only partially conserved (compared to the closest human homolog)