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Ίδρυμα Ευγενίδου
ΑΘΗΝΑ
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6ο ΠΑΝΕΛΛΗΝΙΟ ΣΥΝΕΔΡΙΟ ΕΛΛΗΝΙΚΗΣ ΕΤΑΙΡΕΙΑΣ ΒΙΟΧΗΜΕΙΑΣ ΚΑΙ ΜΟΡΙΑΚΗΣ ΒΙΟΛΟΓΙΑΣ

Biochemical characterization & subcellular localization of the Tyrosine and Phosphoinositide DSP, *Ld*TyrPIP_22, a potential drug target from *Leishmania donovani*

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Group of intracellular parasitism
Department of Microbiology

Ελληνικό Ινστιτούτο Παστέρ
Institut Pasteur Hellenique

Kinetoplastida

Trypanosomatidae

The parasite *Leishmania* spp

Developmental forms and hosts

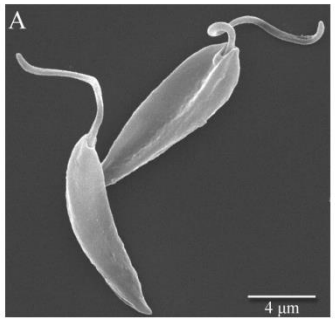
sandfly-insect vector

Lutzomyia

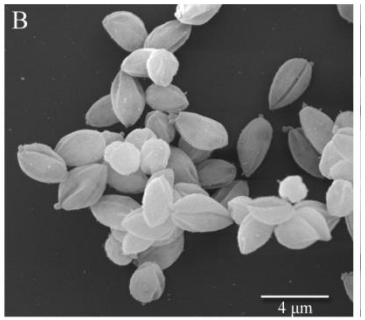
Phlebotomus

2 developmental forms

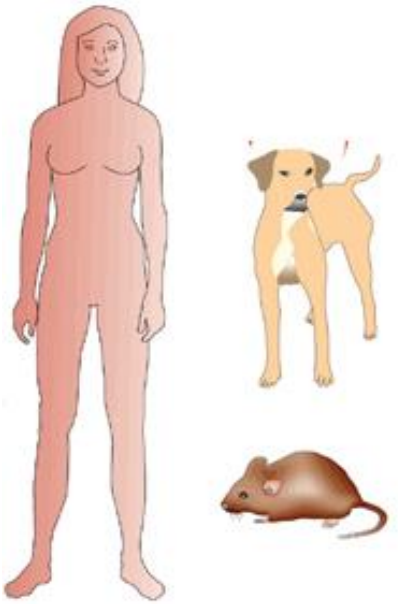
promastigote



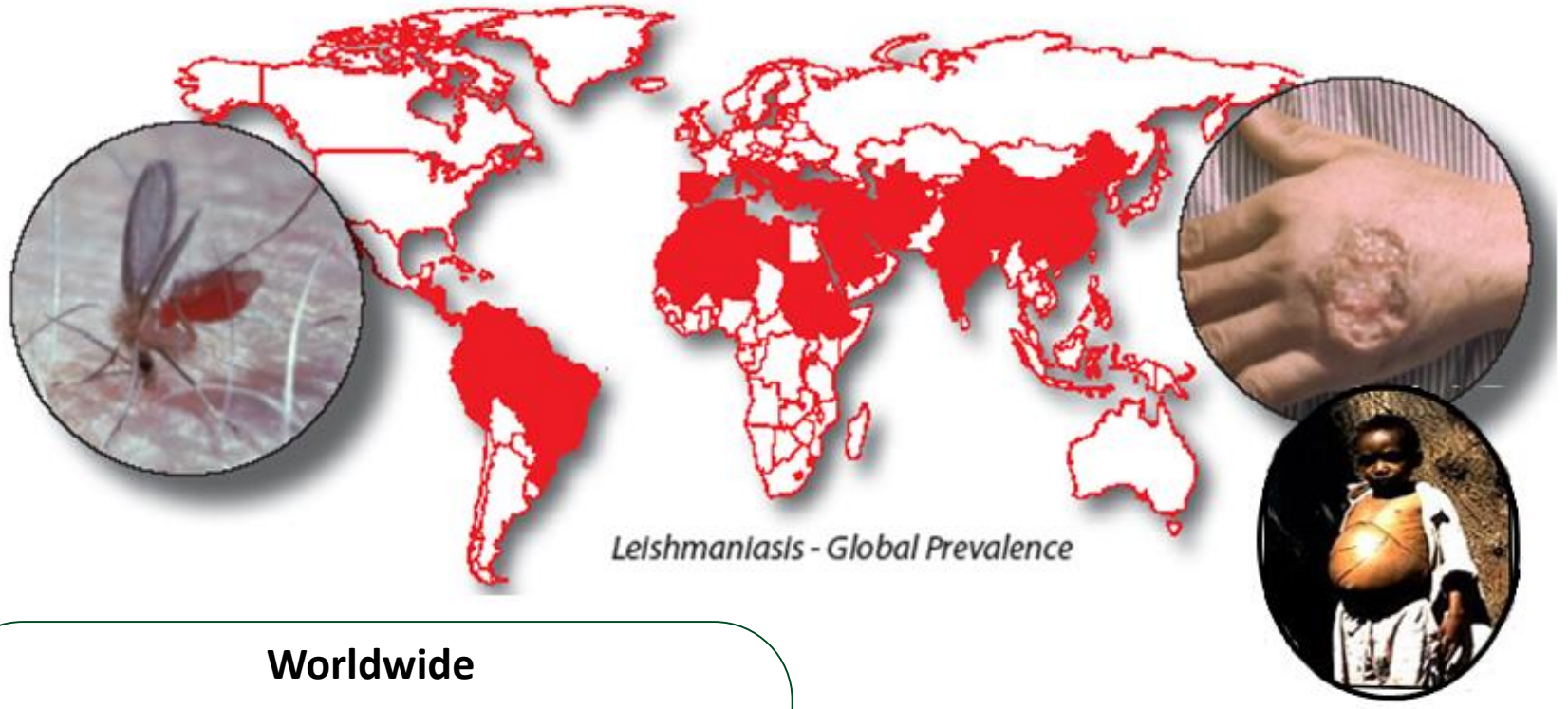
amastigote



mammalian-host



Leishmaniases: a neglected disease



Worldwide

- 98 endemic countries
- 12.000.000 infected people
- 1.300.000 new cases per year
- 20-30.000 deaths per year

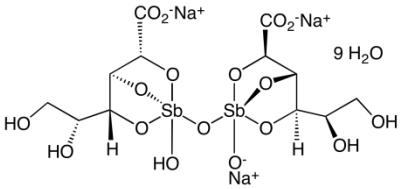
Mediterranean region

- **700** new cases per year

Clinical forms

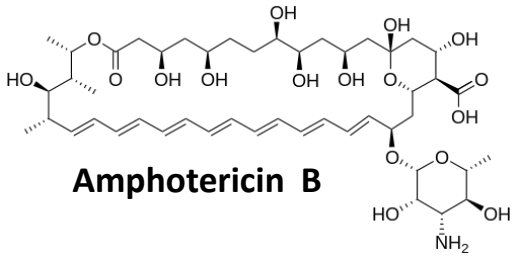
- Visceral Leishmaniasis (**VL**)
- Cutaneus Leishmaniasis (**CL**)
- Mucocutaneus Leishmaniaisis (**MCL**)

Therapeutic approaches for Leishmaniases

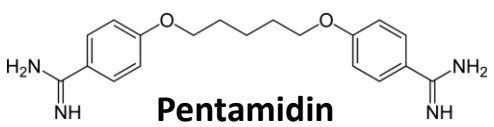


Pentavalent antimonials

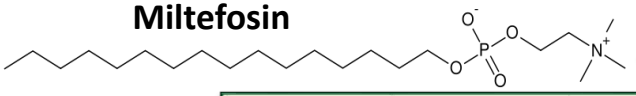
cardiotoxicity
Long term iv administration



Amphotericin B



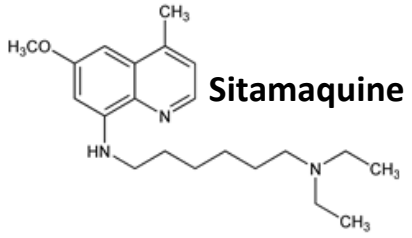
Pentamidin



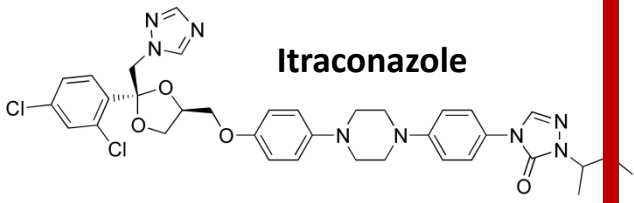
Miltefosin

Cardiovascular symptoms

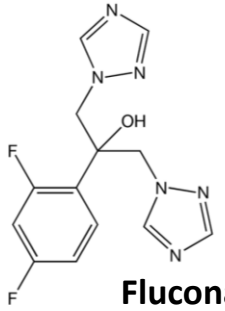
toxicity



Sitamaquine



Itraconazole



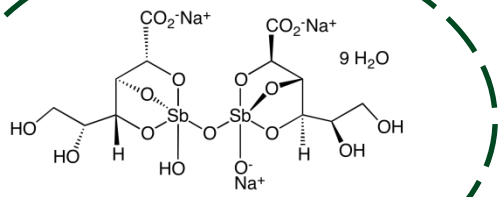
Fluconazole

- side effects
- high cost
- hospital treatment
- resistant strains

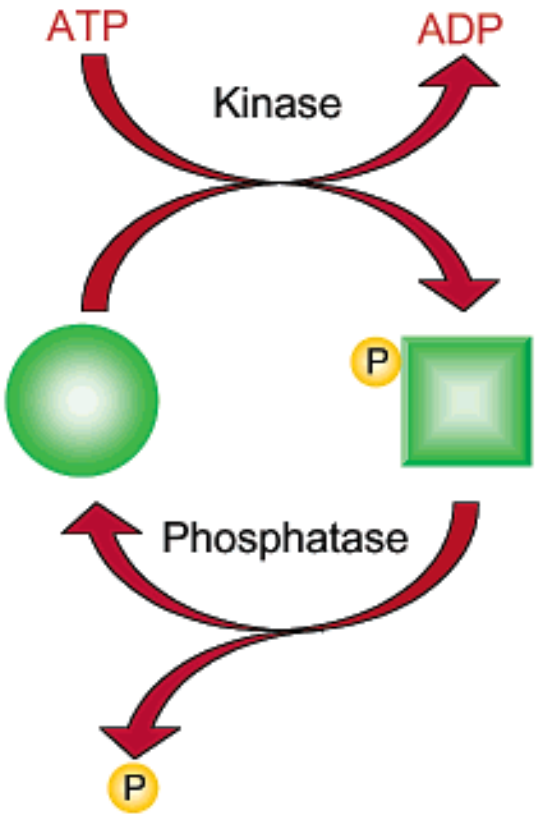
Necessity for development new, non-toxic drugs and vaccines

Ashford, Int. J. Parasitol., 2000

Phosphorylation /Dephosphorylation



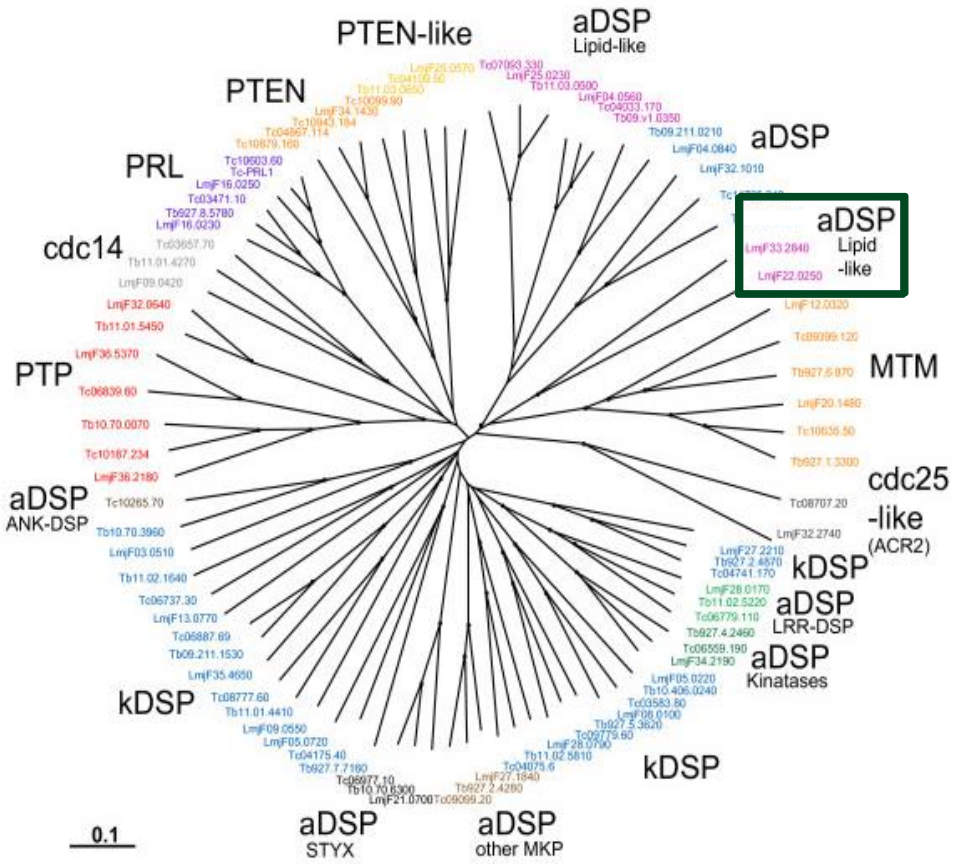
Pentavalent antimonials



Many intracellular pathogens use kinases and phosphatases to block or modify host cell functions in order to secure their survival in them, which leads to disease.

DSPs and ALPs in the *Leishmania* phosphatome

Phylogenetic tree of trypanosomatid phosphatases



ALPs from bacteria or lower eukaryotes

No human homologues

- ✓ **MptpB** (***Mycobacterium tuberculosis***)
- ✓ **LipA** (***Listeria monocytogenes***)

Brenchley et al., BMC Genomics, 2007; van Ooijen et al., Molecular Plant, 2008

***Ld*TyrPIP_22**

Leishmania donovani

Tyrosine and **P**hospho**I**nositide dual specificity **P**hosphatase located in chromosome **22**

Bioinformatics analysis

ClustalW multiple sequence alignment

Leishmania spp	identity
L. donovani (LDBPK_220120)	>99%
L. infantum (LinJ_22_0120)	>99%
L. major (LmjF_22_0250)	>95%
L. mexicana (LmxM_22_0250)	>93%
L. tarentolae (LtaP22.0250)	>85%
L. braziliensis (LbrM.22.0240)	>57%

NP_465325)

Kotopouli A., et al, P63, HSBNB (EEBMB) congress, Crete, 2012

*Ld*TyrPIP_22: molecular tools of study

A

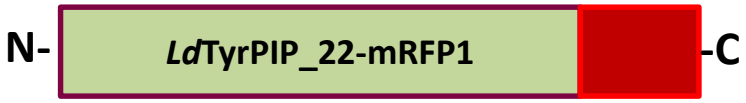
pTriEx1.1

bacteria & mammalian cells

- Purification/ enzyme kinetics
- Biochemical assays
- pAb production

B

pLexsy-sat

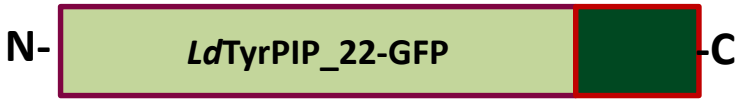


Leishmania

- Subcellular localization (IF/ Biochemistry)

C

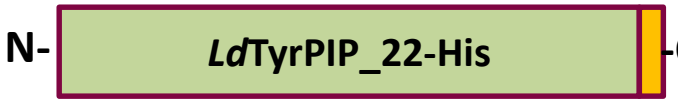
pEGFP-N3

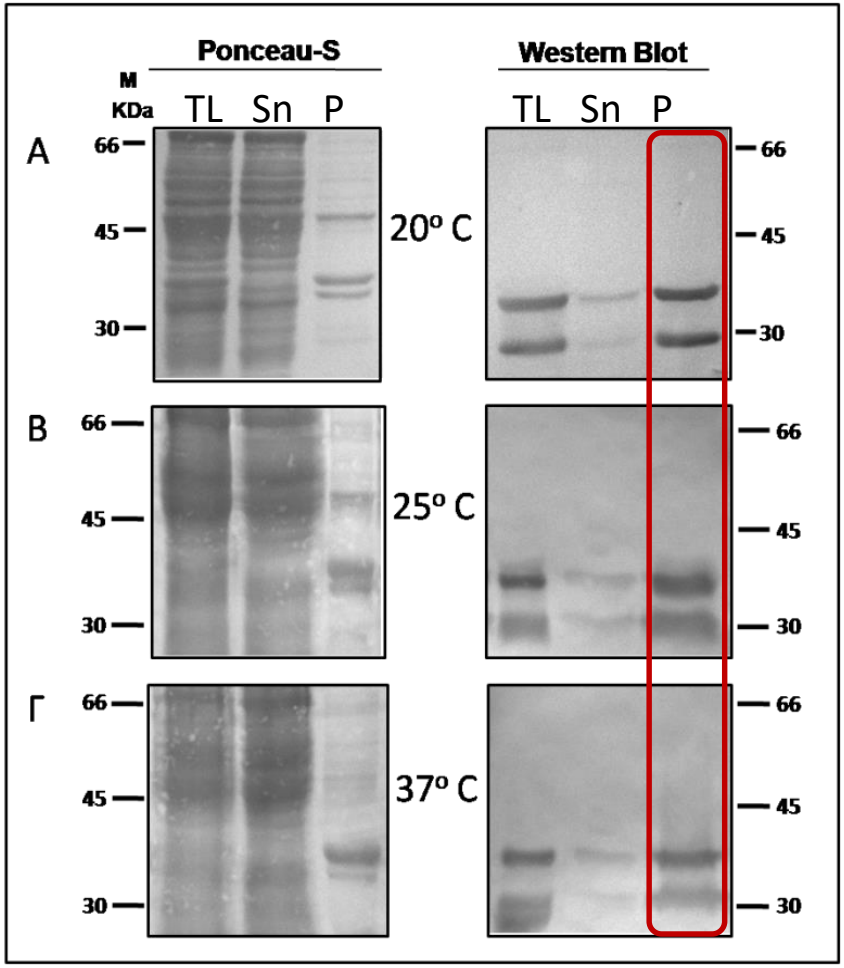


mammalian cells

- Microscopy

r*Ld*TyrPIP22-His expression from *E. coli*

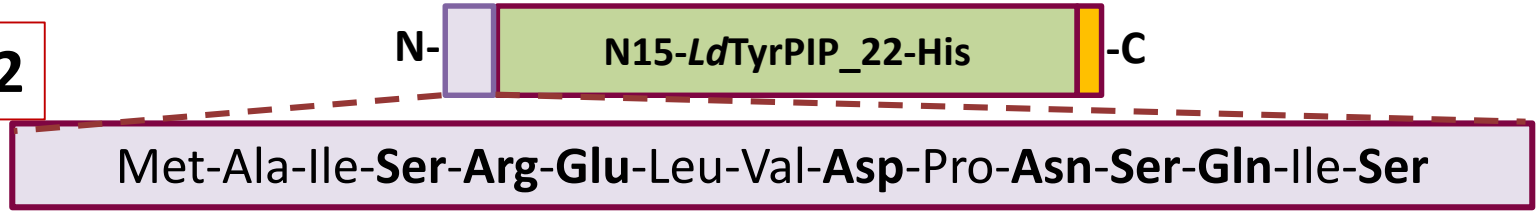


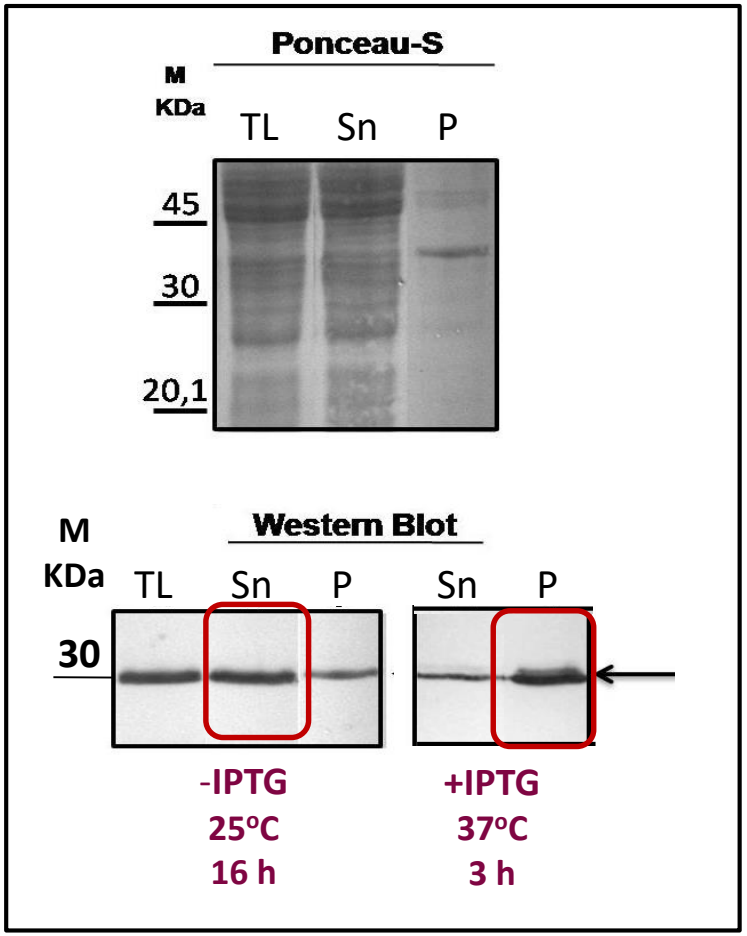


anti-His

r*Ld*TyrPIP22-His expression from *E. coli*

A2

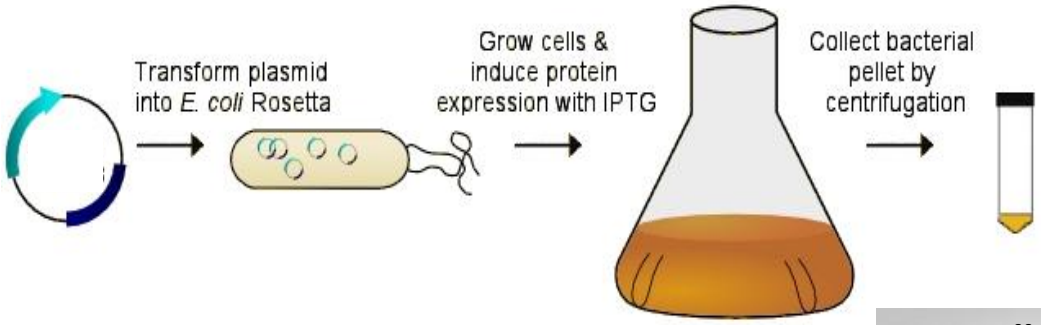


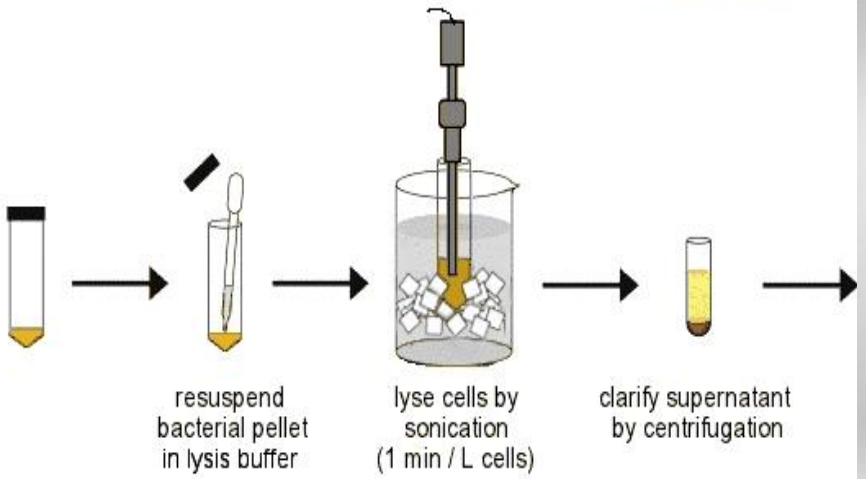


anti-His

rN15-*Ld*TyrPIP_22-His purification from *E. coli* lysates

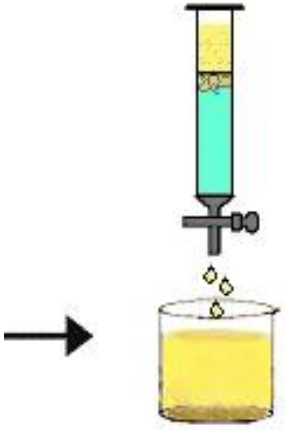
IPTG (0,5mM) induction at 20°C for 20 h





FPLC (ÄKTA)-Ni^{2+} column

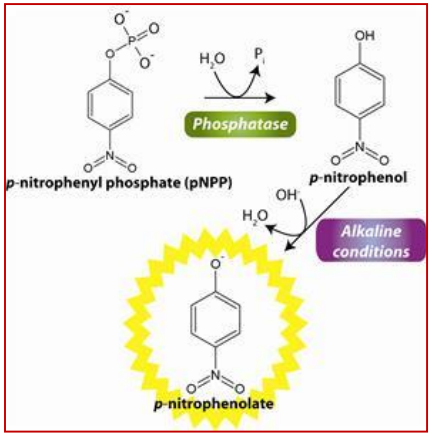
Talon (Co^{2+}) column



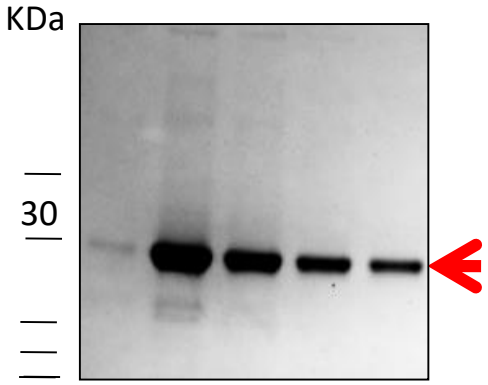
rN15-*Ld*TyrPIP_22 catalytic activity

EMBL

- 25ºC
- pNPP: 10mM
- enzyme: 2 µM

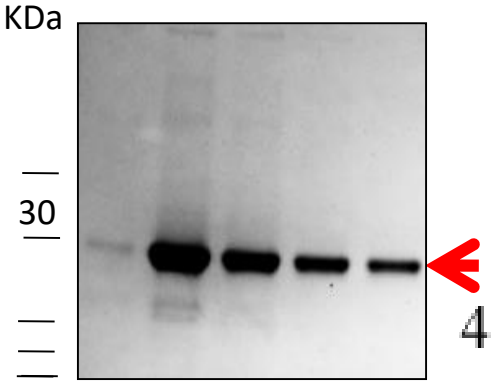


SDS-PAGE



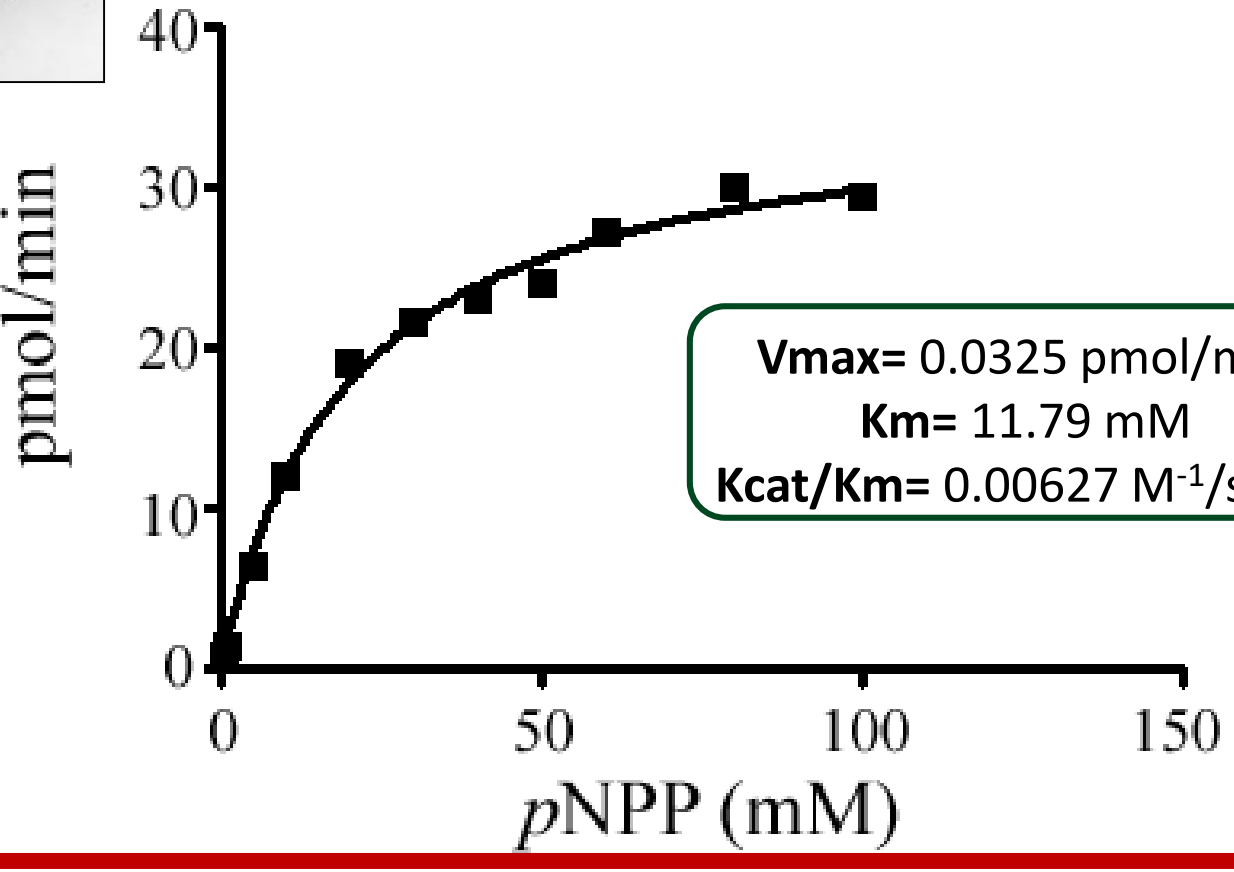
Optimum pH= 6

rN15-*Ld*TyrPIP_22 catalytic activity



SDS-PAGE

- 25°C
- enzyme: 2µM
- Substrate: pNPP



Vmax= 0.0325 pmol/min
Km= 11.79 mM
Kcat/Km= 0.00627 M^{-1}/sec^{-1}

rN15-*Ld*TyrPIP_22-His substrate specificity

EnzcheK kit

pTyr peptides (1 mM)	
IEDNE**pY**TARQG	?
DEEDT**pY**TMPST	No
PEGHE**pY**p**Y**RVRE	No
KKKK**pY**PKKK	No
LGSRA**pY**PHFCA	Yes
STEPQ**pY**QPGEN	Yes
DPSDN**pY**AEPID	Yes
EEEDI**pY**EVLPD	Yes
DNEDV**pY**TFKTP	Yes
IEDP**pY**p**Y**GNDSD	Yes
QENPI**pY**KSPIN	Yes
D**pY**p**Y**R	Yes

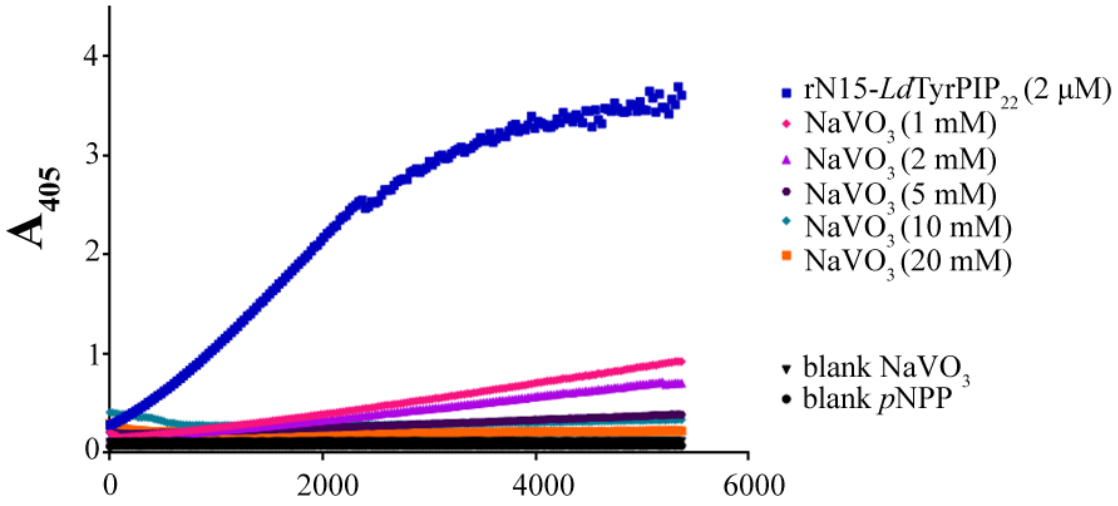
pH 7

Phosphoinositides (PIs) (100 µM)	
PI3P	Yes
PI4P	Yes
PI5P	No
$PI(4,5)P_2$	No
$PI(3,4)P_2$	No
$PI(3,5)P_2$	No
$PI(3,4,5)P_3$	No
pSer/Thr peptides (60 µM)	
Ac-IQAAA**pSpT**P	No
Ac-I**pS**PPPTANL	No
Biotin-QGRDKYK**pT**LRQIRQG	No

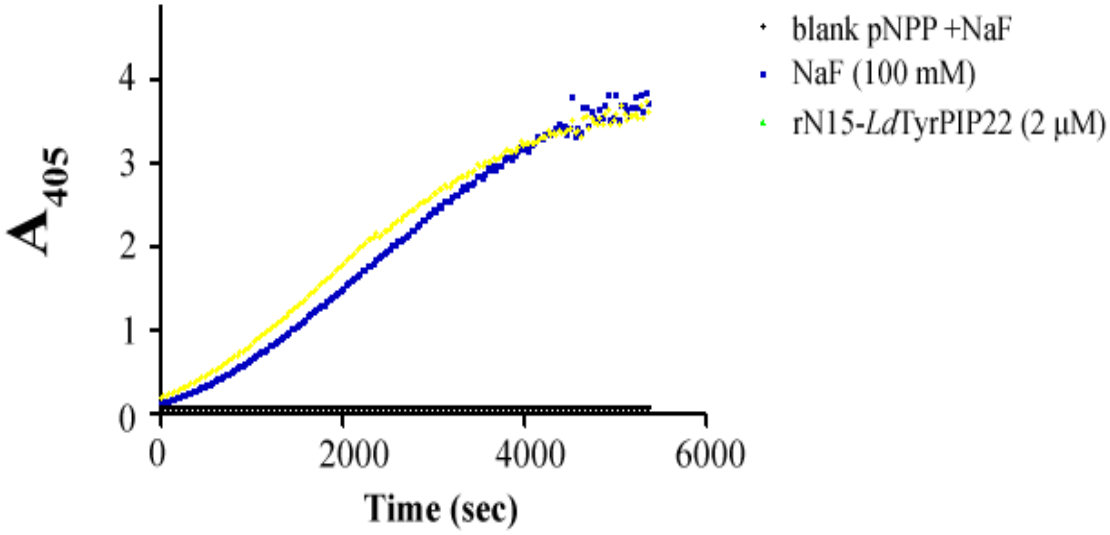
rN15-*Ld*TyrPIP_22-His inhibition assay

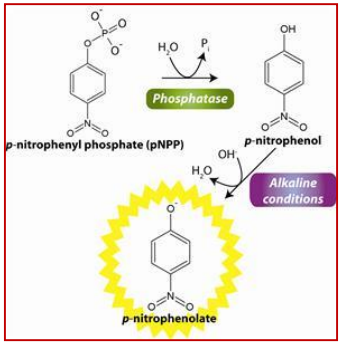
- 25°C
- Enzyme: 2µM
- PNPP : 10 mM

$NaVO_3$

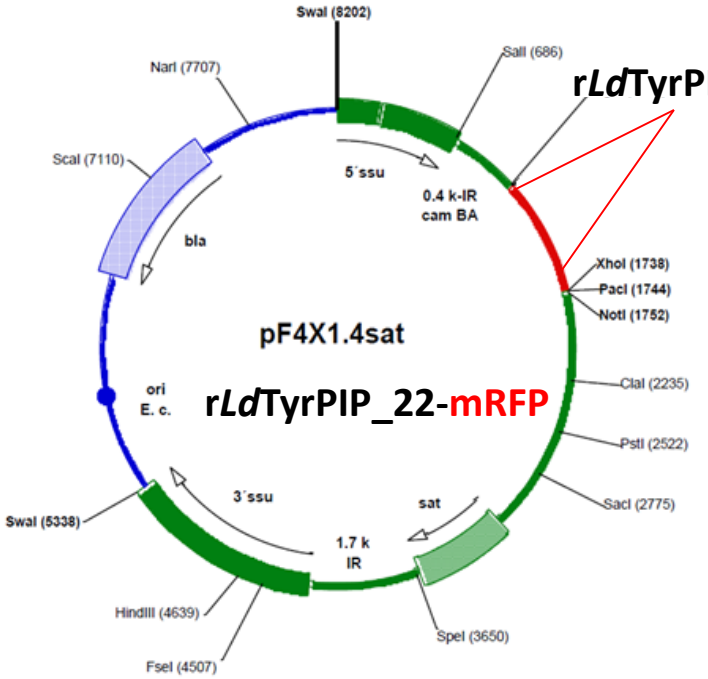


NaF



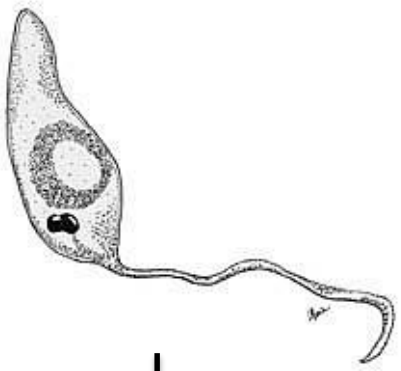


Generation of trangenic *L. donovani*-r*Ld*TyrPIP_22-mRFP

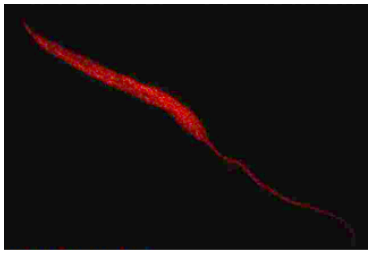


r*Ld*TyrPIP22-mRFP

electroporation

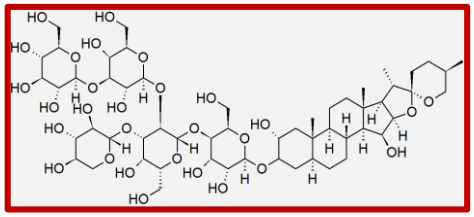


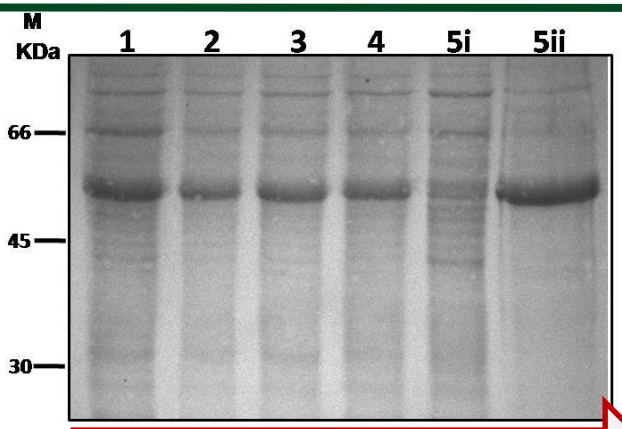
Selection with antibiotic Nourseothricin



*Ld*TyrPIP_22 subcellular localization in *L. donovani*-r*Ld*TyrPIP_22-mRFP

Digitonin fractionation



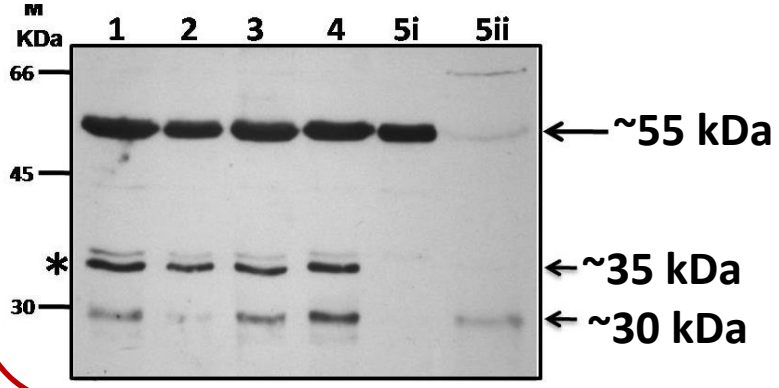


digitonin (24 ng/mL-12 µg/mL)

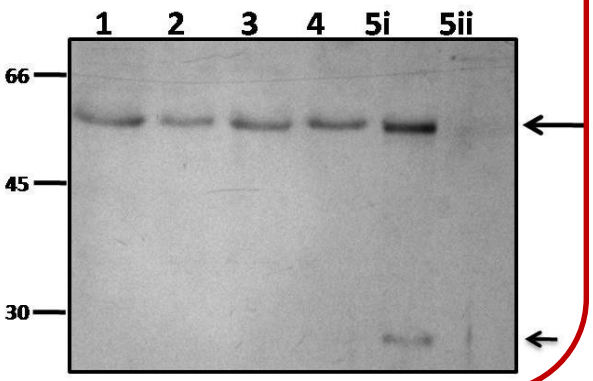
Membrane- cytoskeletical proteins

Western Blot

a-LdTyrPIP_22

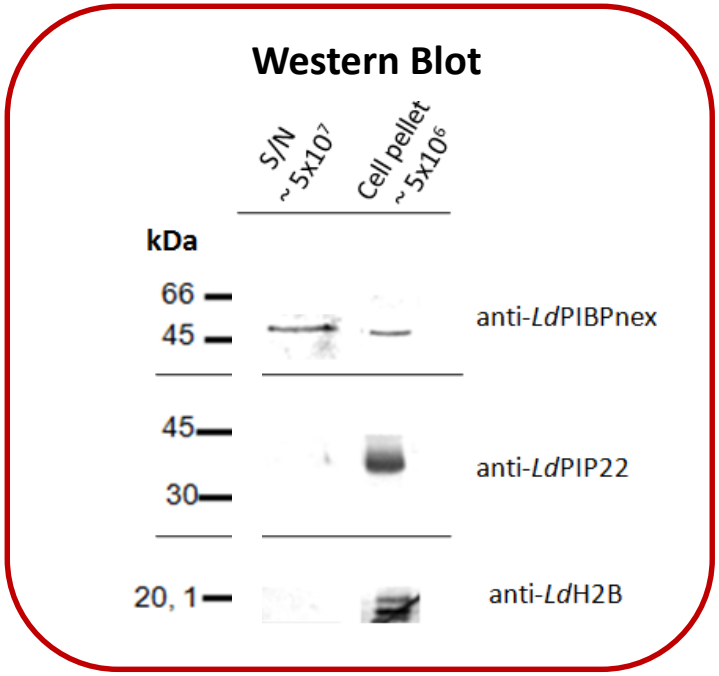


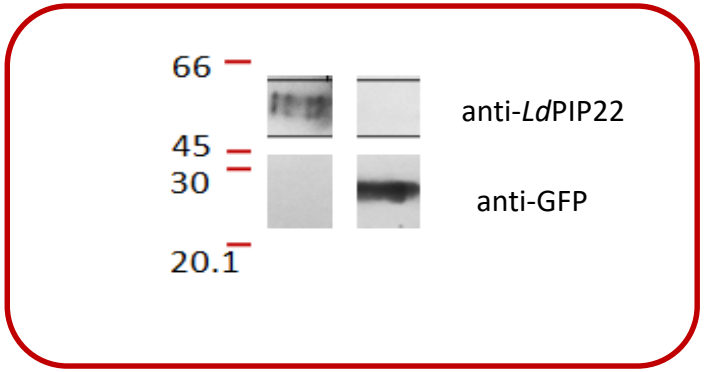
a-mRFP



*Ld*TyrPIP_22 subcellular localization in *L. donovani*-r*Ld*TyrPIP_22-mRFP

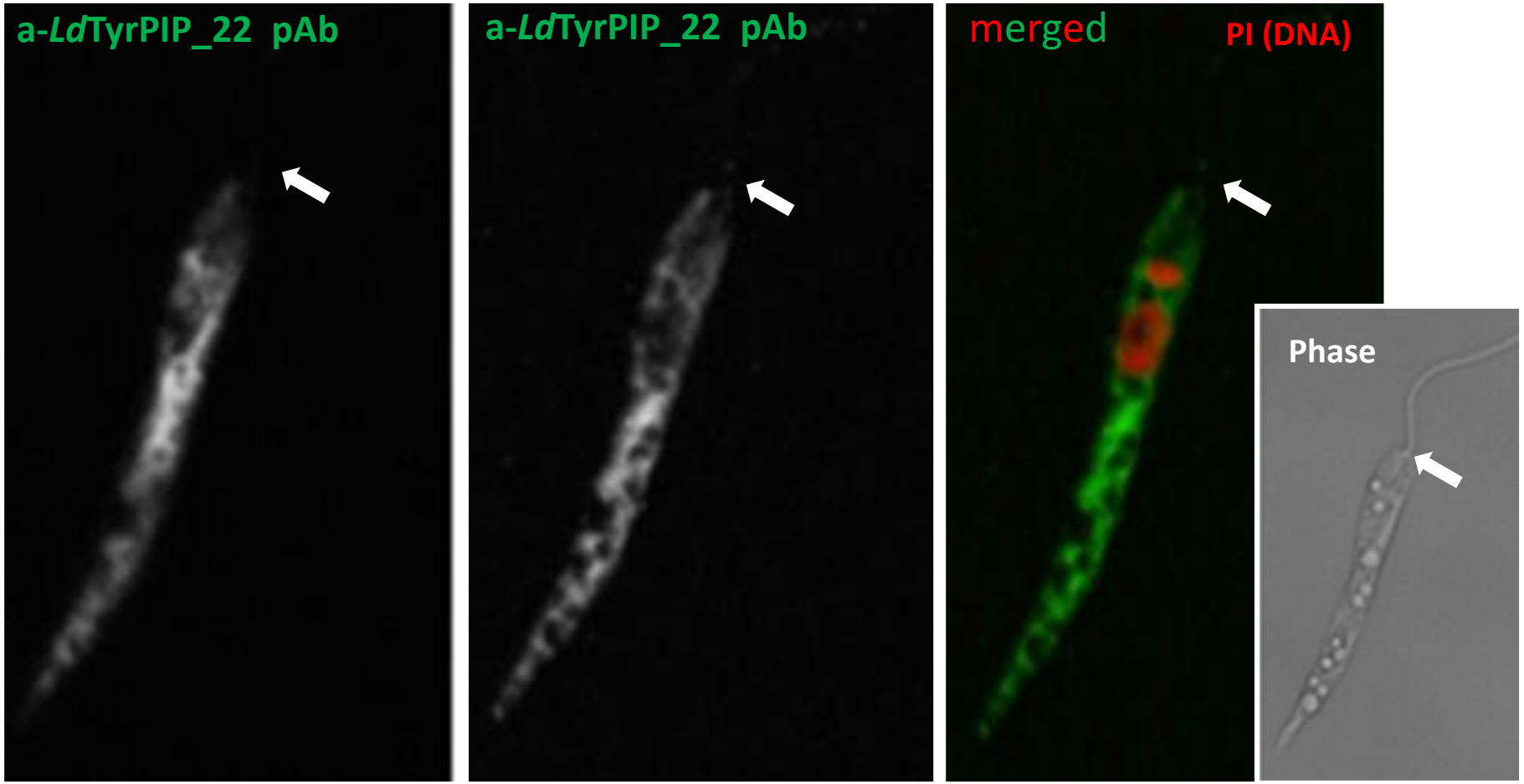
Secretion assay 25ºC





*Ld*TyrPIP_22 subcellular localization in *L. donovani*

Confocal microscopy



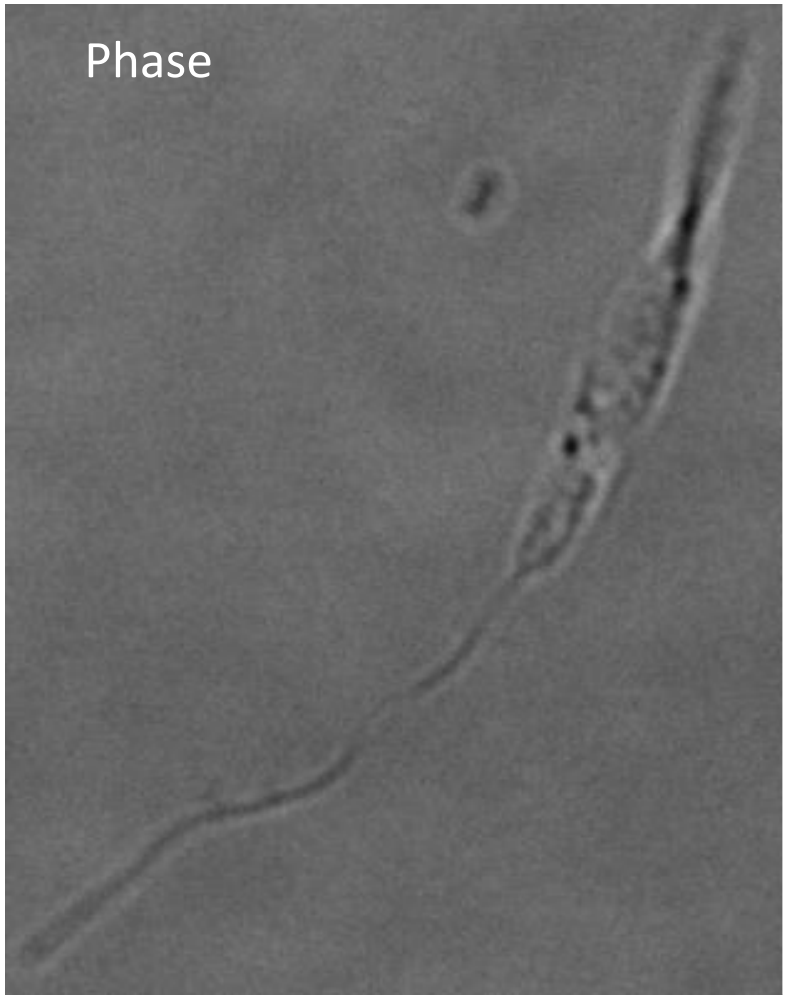
Native *Ld*TyrPIP_22 is localized mainly in the cell body and less in the flagelum, & in vesicle-like or filamental subcellular structures

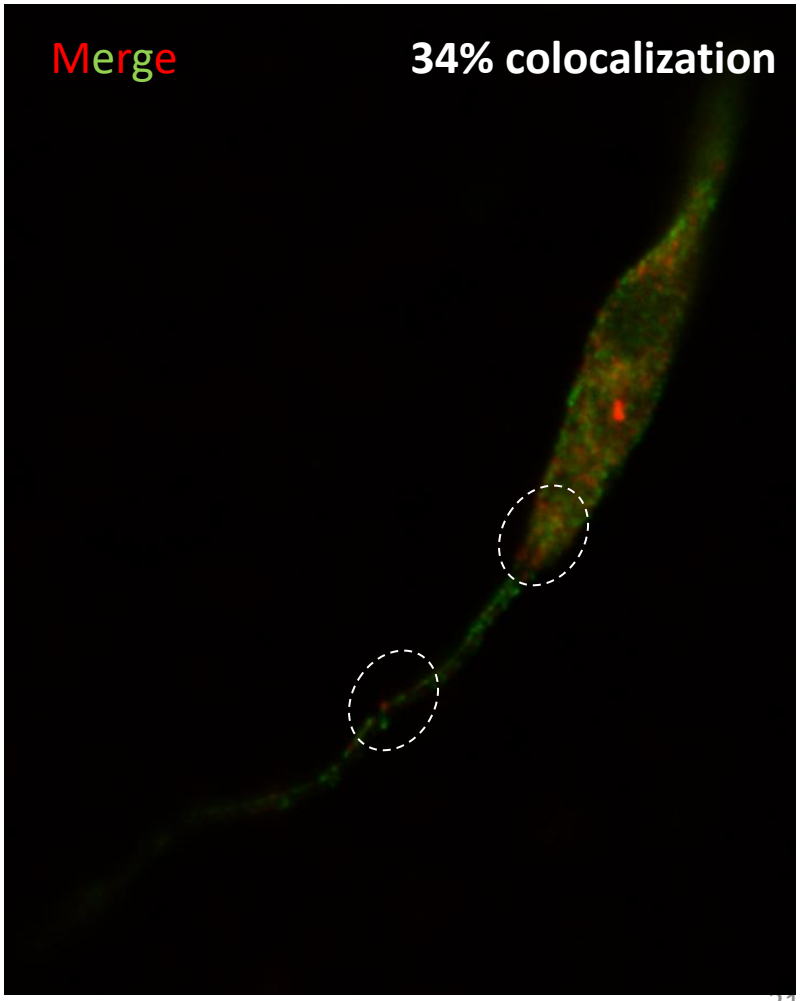
*Ld*TyrPIP_22 subcellular localization in *L. donovani* cells

EMBL

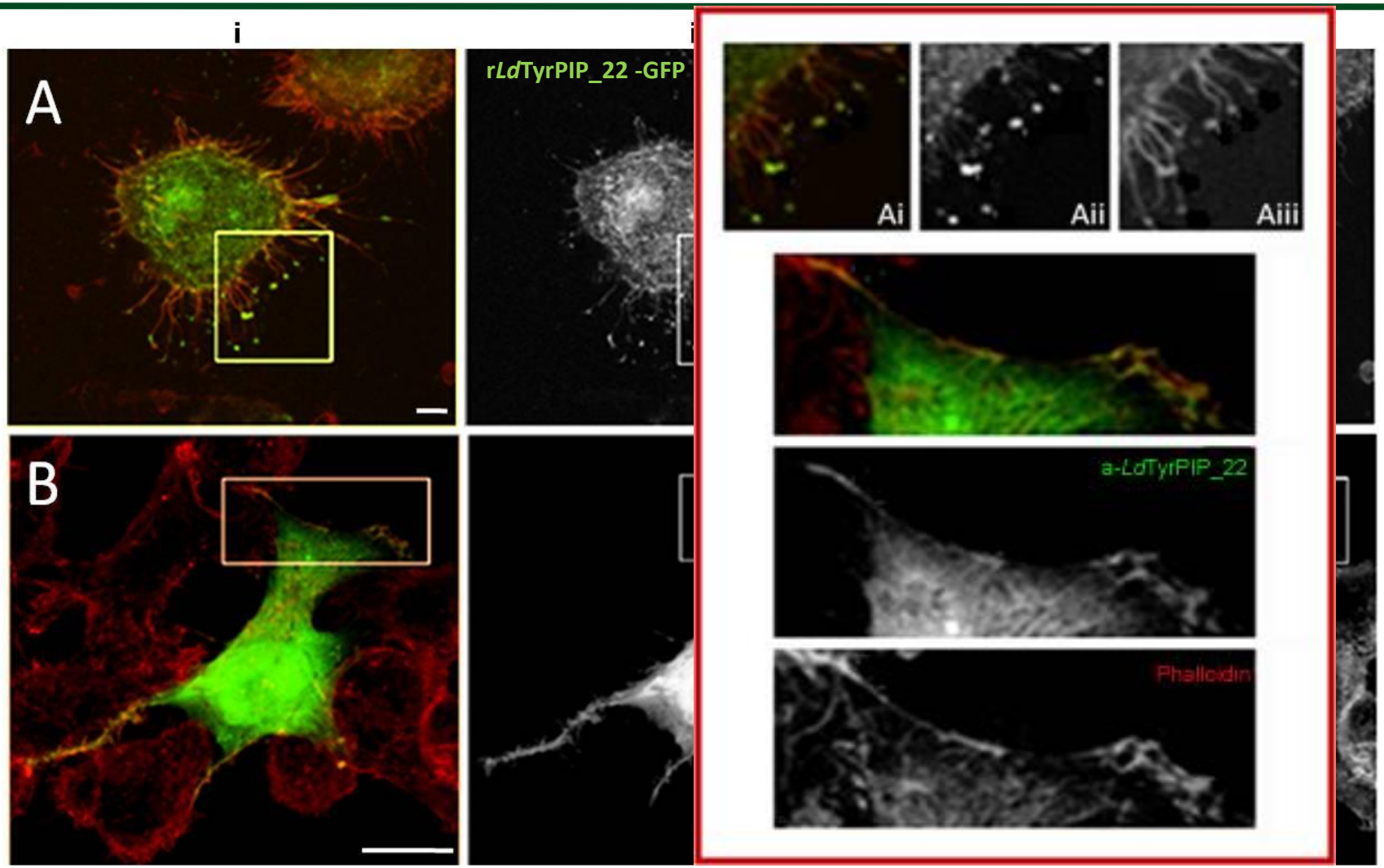
STED

super resolution





r*Ld*TyrPIP_22-GFP subcellular localization in HeLa cells



r*Ld*TyrPIP_22-GFP is localized on PM sites of high actin dynamics (lamellipodia & filopodia tips), enriched in PIs and PIPs that regulate membrane and actin dynamics

Conclusions- Future perspectives

- ***Ld*TyrPIP_22** exhibits dual specificity (DSP) activity (**pTyr & PIs)**
- In promastigote ***L. donovani* parasites *Ld*TyrPIP_22** is biochemically located in the soluble, membrane and cytoskeletal fraction. It could be also secreted.
- By **fluorescent microscopy** it localizes in localized mainly in the cell body and less in the flagelum, & in vesicle-like or filamental subcellular structures
- **r*Ld*TyrPIP_22-GFP**, transiently transfected in **HeLa cells**, is localized on PM sites of high actin and membrane dynamics (lamellipodia & filopodia tips), enriched in PIs and PIPs that regulate membrane and actin dynamics

- Study of the enzymatic properties of **r*Ld*TyrPIP_22-His** expressed in *Leishmania* cells, and possible role in virulence of *L. donovani in vitro* (infected macrophages) and *in vivo* (infected mice).
- Testing of phosphatase inhibitor molecules

Collaborations - Funding

Group of intracellular parasitism, HPI

Members:

Δρ. **Χαραλαμπία Μπολέτη**, group leader

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Interaction of r*Ld*TyrPIP_22 F-actin *in vitro*

In vitro **actin cosedimentation assay**

- Polymerization of actin in the presence of r*Ld*TyrPIP_22-8His