UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Pharmaceutical Sciences

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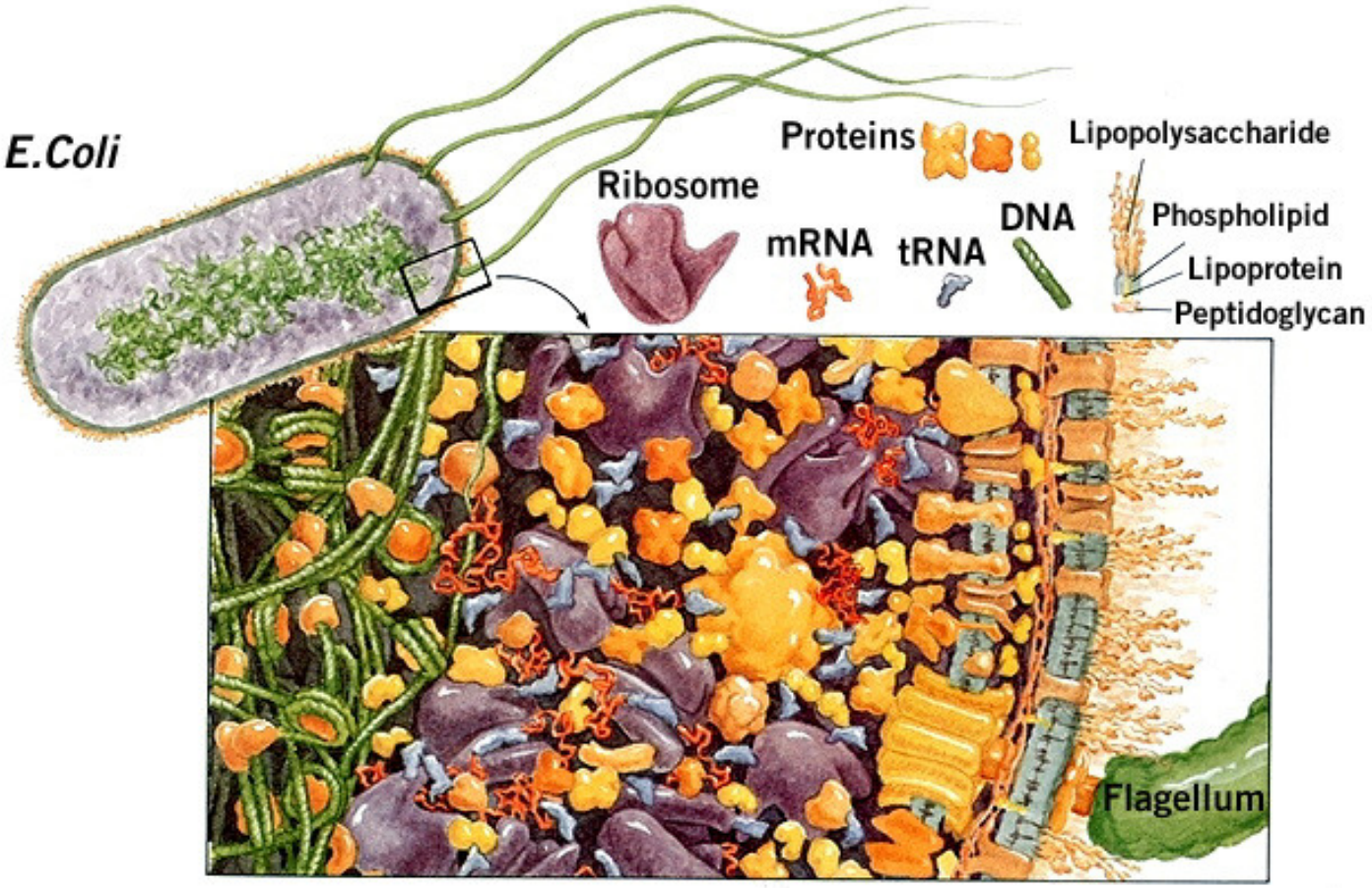
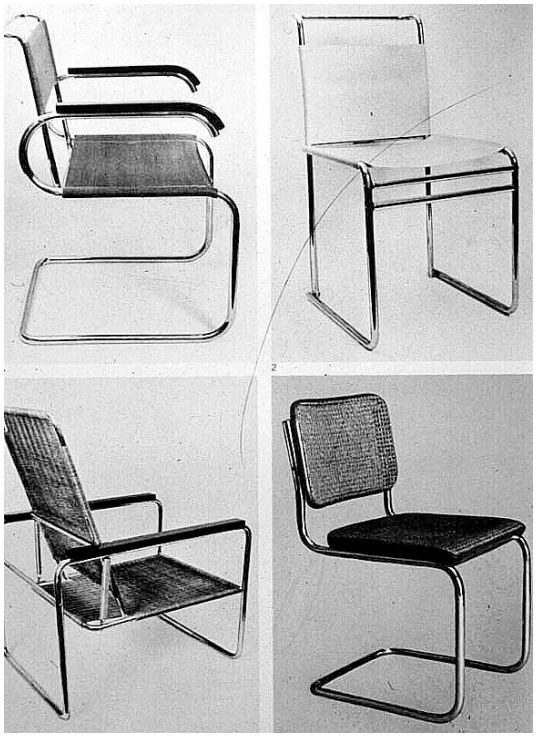
Lecture Topics

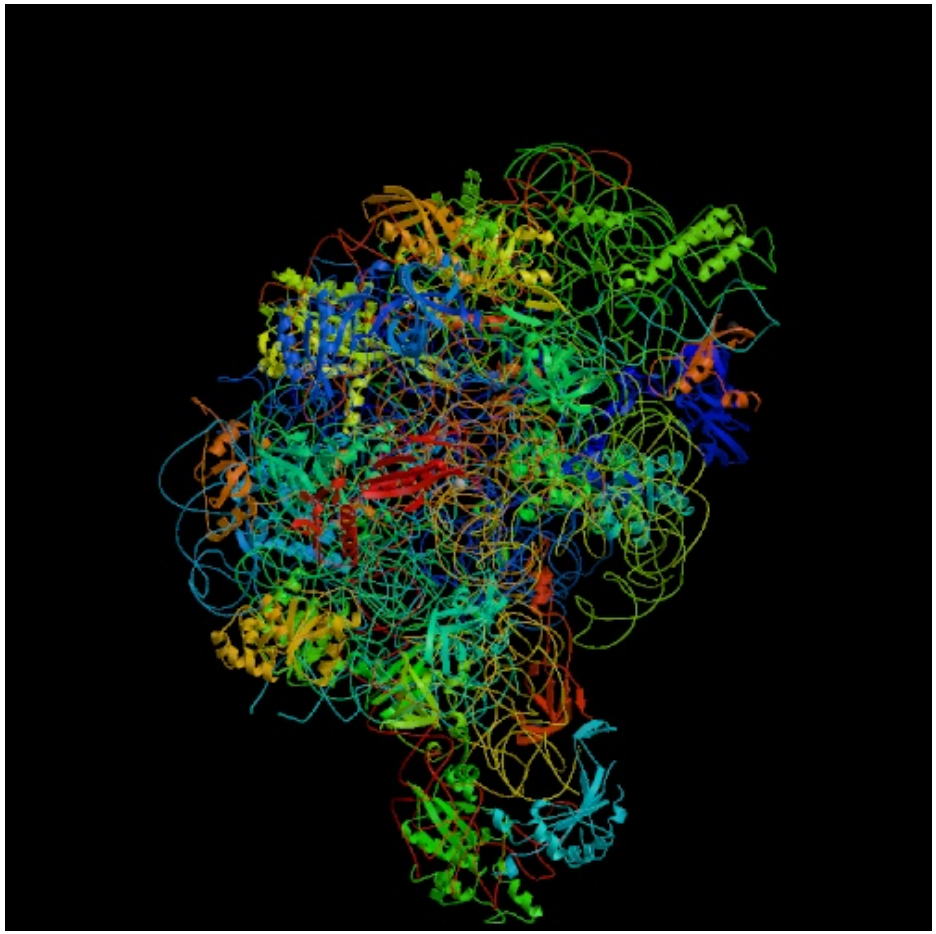
- **Structural biology: understanding biology**
- **Structural Biology: understanding disease**
- **Structural biology: medicines discovery**
- **Structural biology: diagnosis**
- **Structural biology: medicines safety**

Structural Biology





Atomic resolution Structural Biology



Venkatraman Ramakrishnan, Thomas A. Steitz, Ada E. Yonath, Nobel Laureates, 2009

Atomic resolution structural biology
Protein data base repository (www.rcsb.org)

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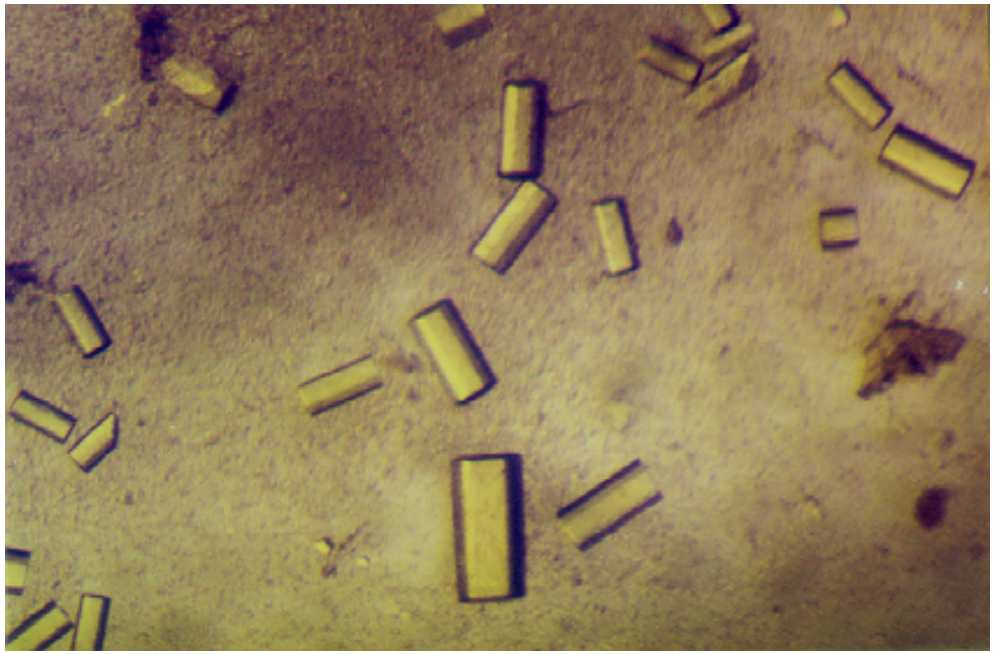
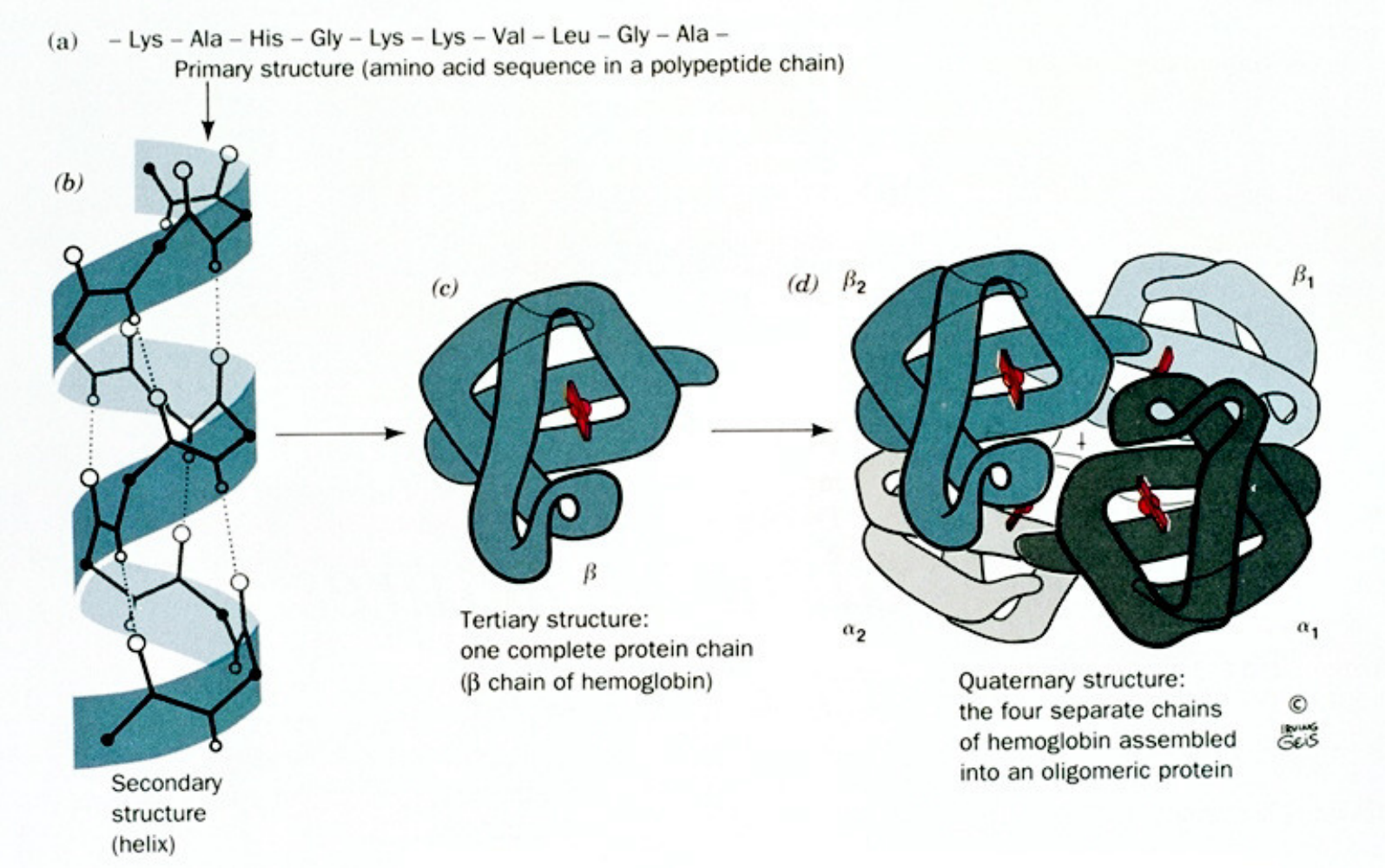


No matter which technique always remember

Experimentally determined MODEL

(data ≠ information ≠ knowledge ≠ wisdom)

Structure function relationship



Drug discovery and development

- **The long and complex process to make a medicine available to Patients**

Drug discovery pipeline

- **Hit compounds (or lead compounds)**
- **Hit/lead optimization**
- **Preclinical development**
- **Clinical trials (phases I-IV)**
- **Market**

Drug discovery

- **MUCH more to consider than BINDING**

Drug discovery
medicine-target interaction

- **Target(s) – Medicine interactions**
- **Molecular target(s) – Medicine interactions**
- **Binding: energetic and kinetic aspects**
- **Molecular Recognition (selectivity)**
- **Stability (affinity - potency) (reversible vs irreversible)**
- **Kinetics (potency) (on and off binding)**

Drug discovery
hit/lead identification

- **In vitro screenings of chemical libraries (synthetic, focused, natural compounds) by using the recombinant targets**

- **Potency and selectivity on target**
- **Permeability, preliminary pharmacological properties**

Drug discovery hit/lead identification

- **Ex-vivo (in vivo, e.g. bacterium) screenings of chemical libraries (synthetic, focused, natural compounds), phenotype screening**

- **Potency and selectivity on target**
- **Permeability, preliminary pharmacological properties**

Drug discovery hit/lead identification

- **In silico structure-based screenings**
- **Rational structure-based drug design**

- **Potency and selectivity on target**
- **Permeability, preliminary pharmacological properties**

Drug discovery

- **Medicines**
- **Chemicals (ligand-protein or ligand-RNA or ligand-DNA interactions)**
- **Biologicals (mainly but not only protein-protein interactions)**

Biological Medicines

- **Therapeutic proteins**
- **Gene therapies**
- **Cell therapies (autologous – heterologous)**
- **Gene modified cell therapies**

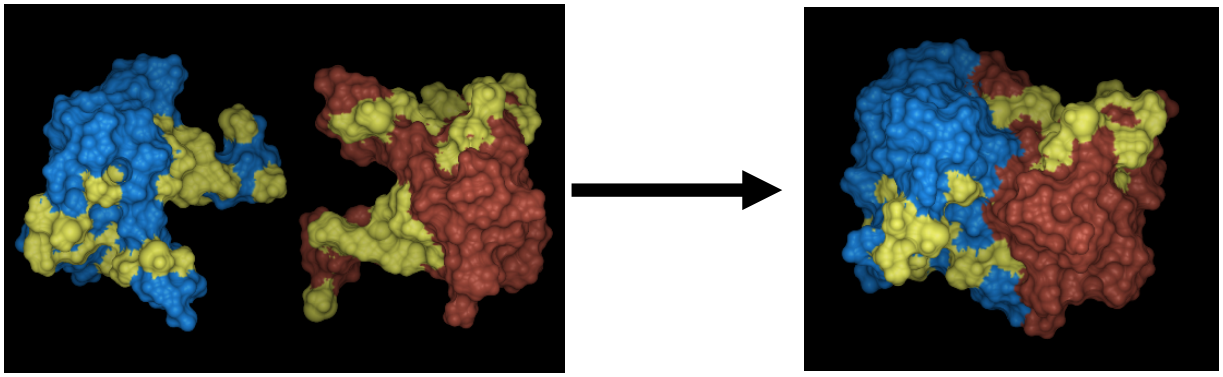
Biological Medicines
Therapeutic proteins

- **Insuline(s)**
- **Enzymes**
- **Monoclonal antibodies (*trastuzumab*)**
- **Receptors**
- **Fusion proteins (*tagraxofusp*, first in the market by the end of 2019, blastic cancer)**
- **Cytokines (interleukin)**
- **Etc**

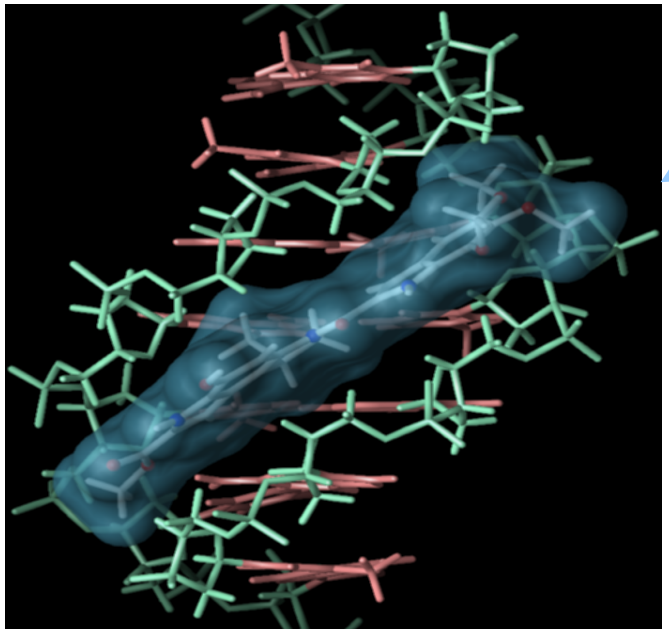
Drug discovery
Structural biology

- **Understanding interactions (description)**
- **Predicting and evaluating interactions**
- **Designing a small molecule**
- **Designing a protein or a multiple domains protein**

The reward: understanding-->control molecular interactions



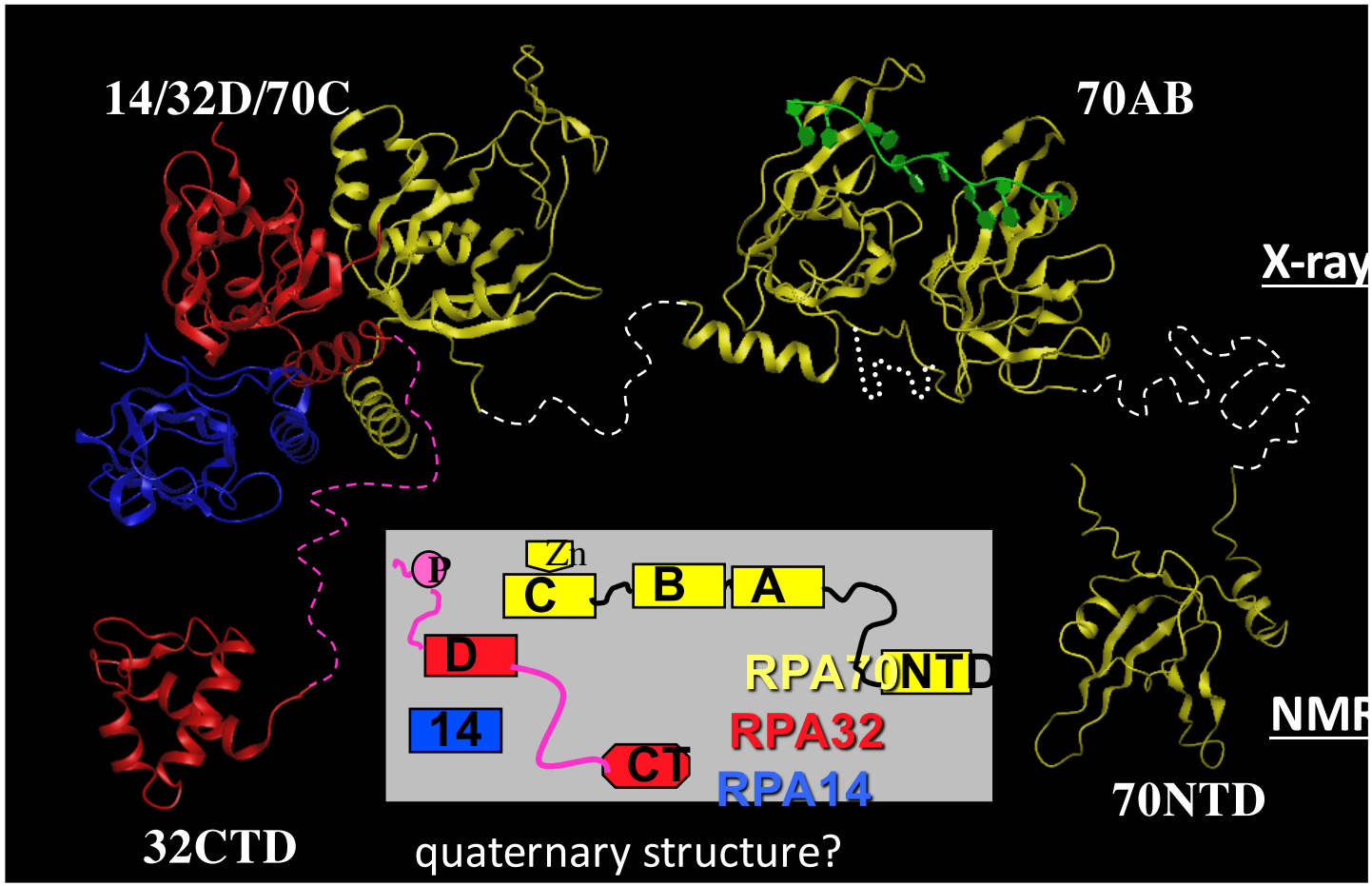
Protein-Protein



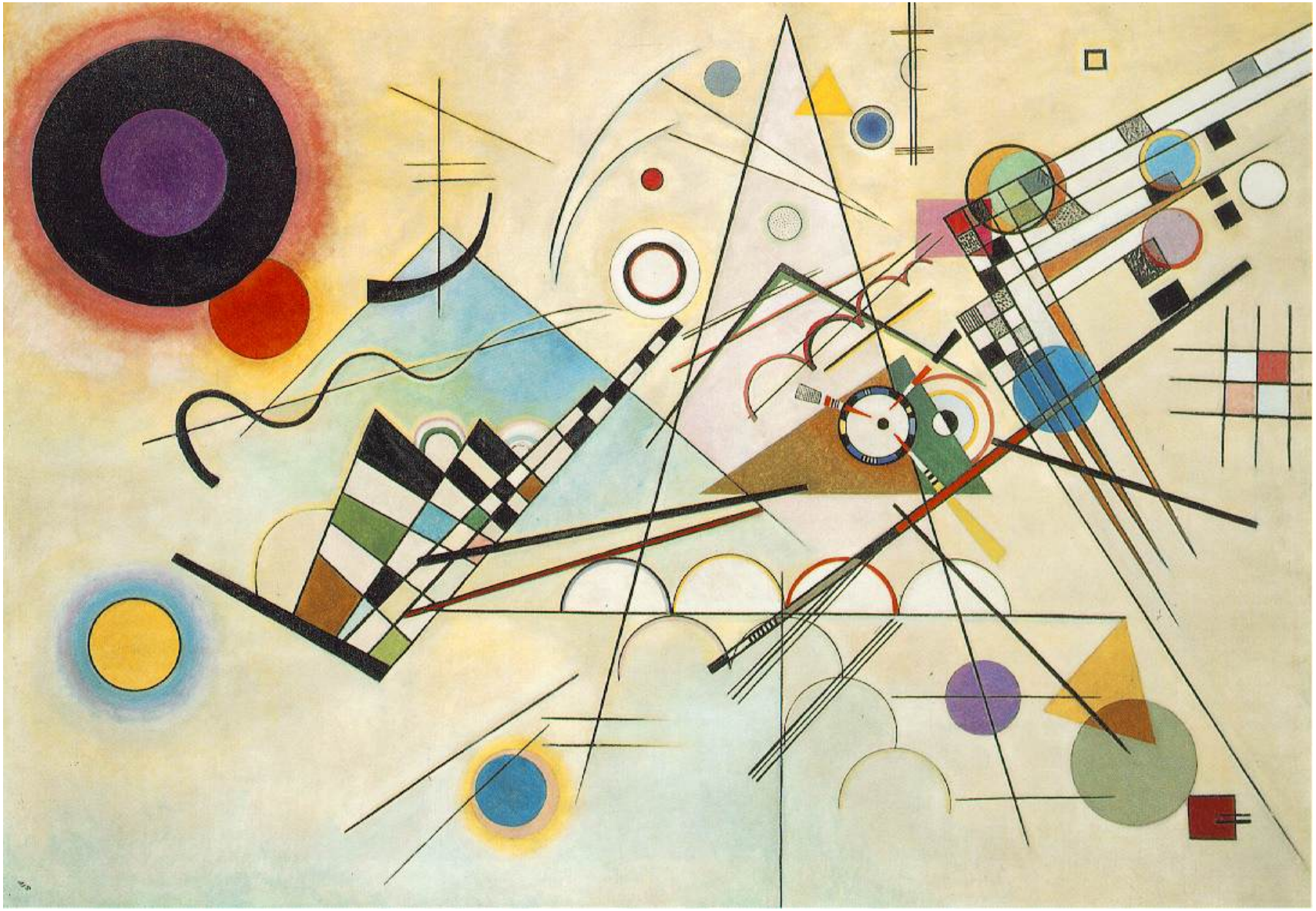
Drug-target interaction

duocarmycin

The reward: understanding-->control protein engineering



FUSION PROTEIN: *-fusp*



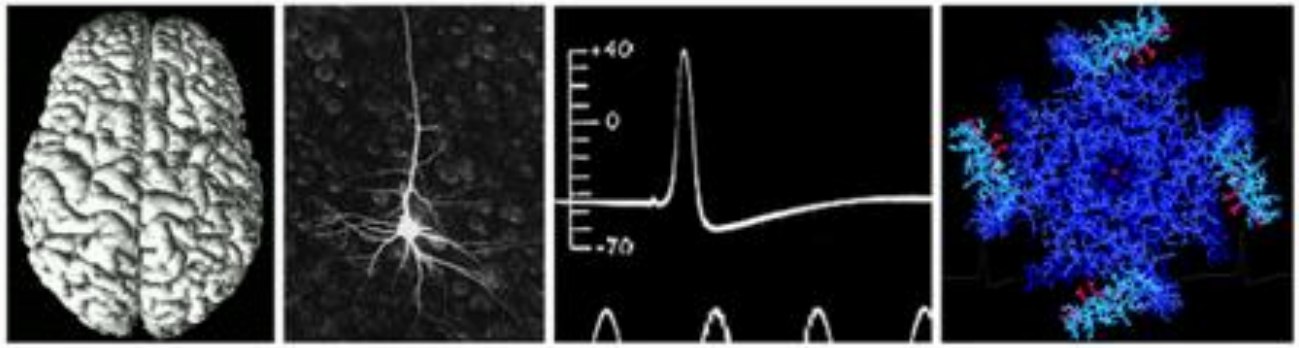
Sodium and Potassium: “almost” identical

“…sodium is almost the same as potassium (…..); the difference can be small, but they can lead to radically different consequences, like a railroad's switch point; the chemist's trade consists in good part in being aware of these differences, knowing them close up, and foreseeing their effects. And not only the chemist's trade.”

Primo Levi, The Periodic Table

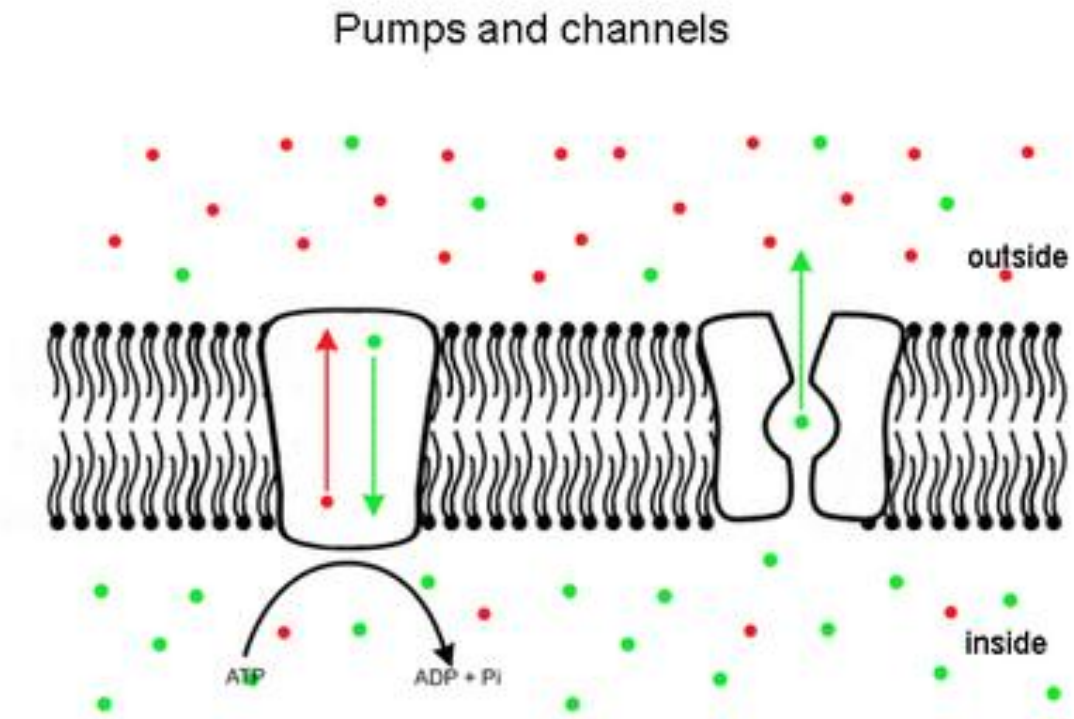
Ion channels

Ion channels are nature's electrical impulse generators

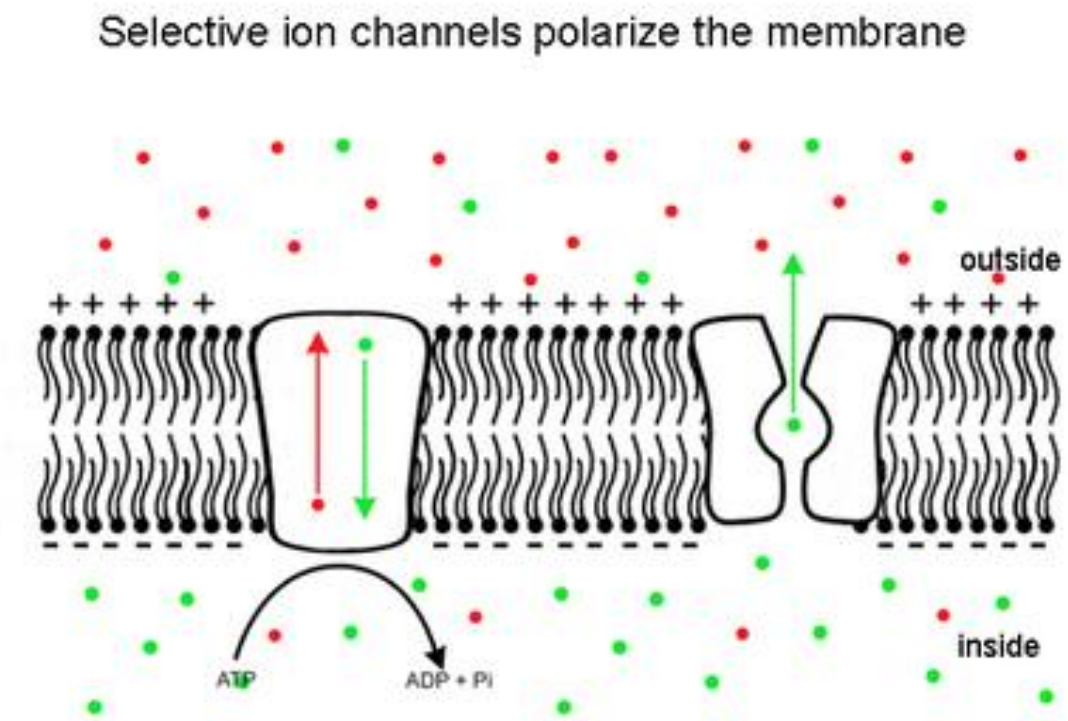


Roderick MacKinnon (2003 Nobel laureate)

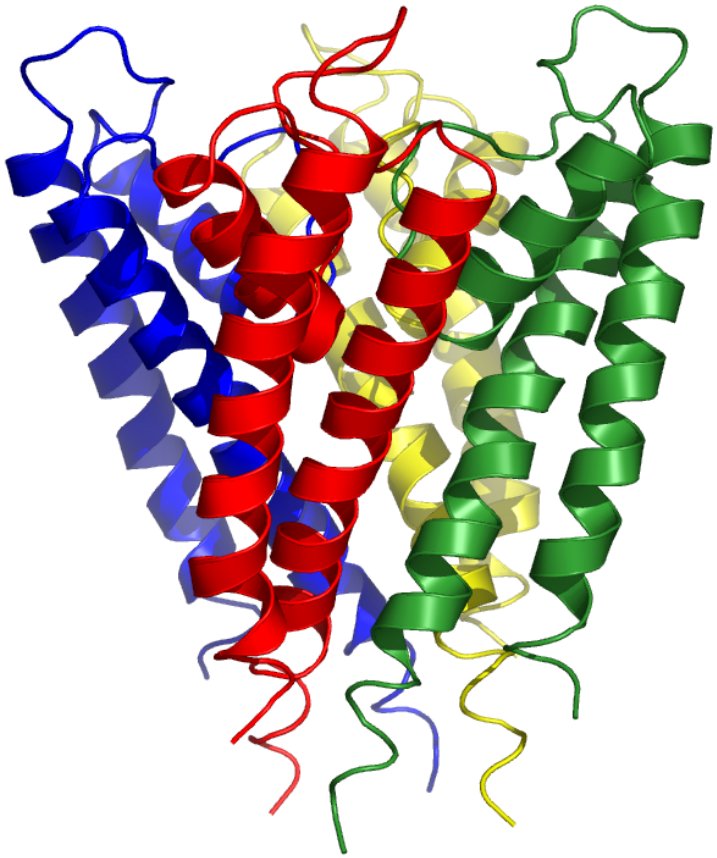
Pumps and ion channels

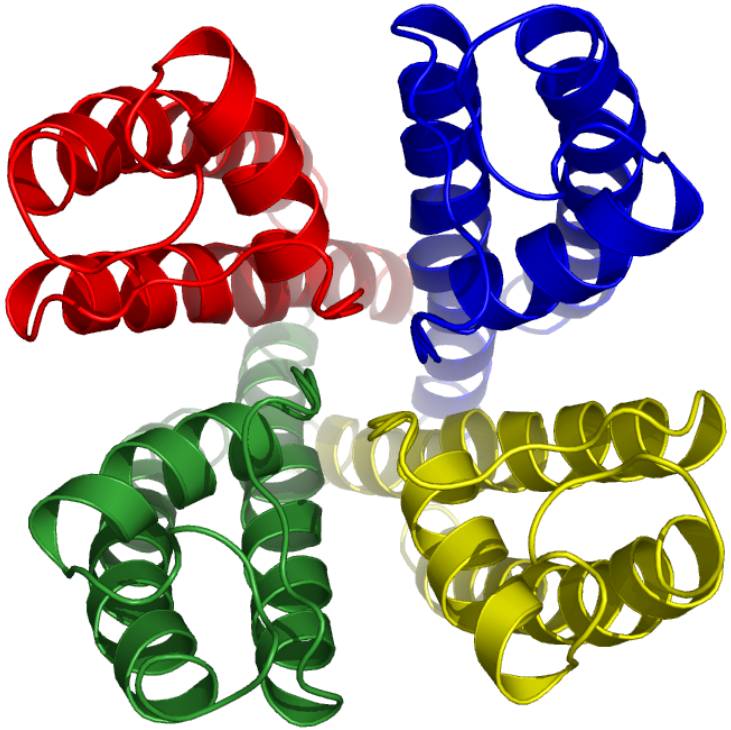


Selectivity and directionality

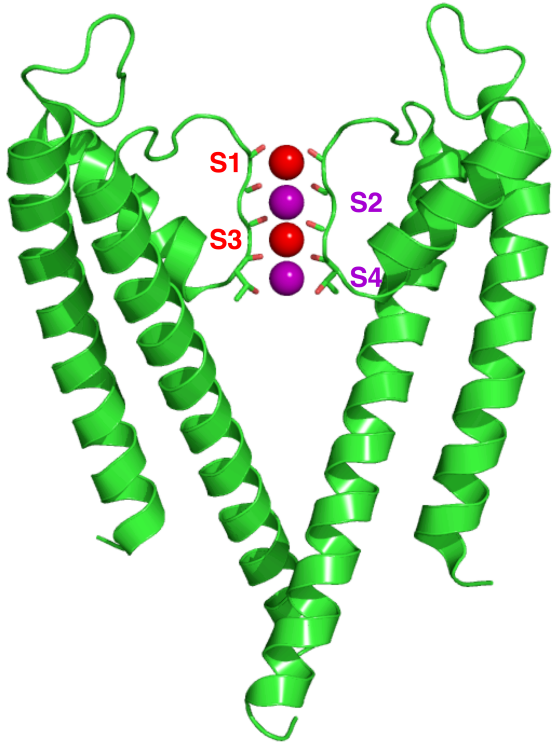


Potassium channel crystal structure

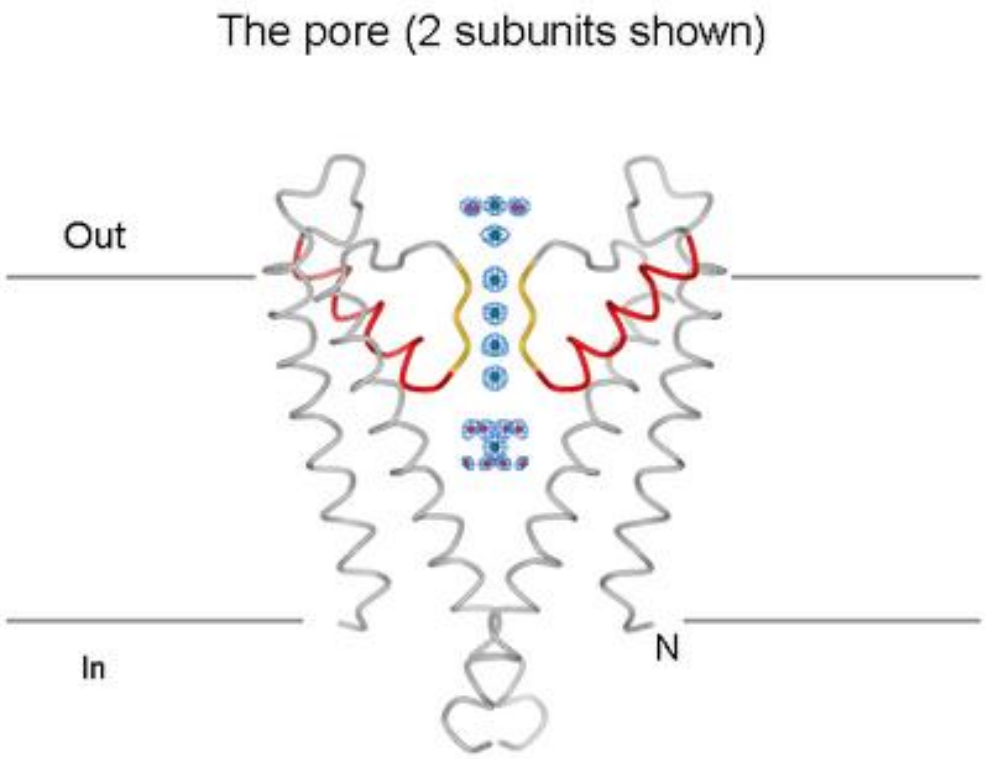




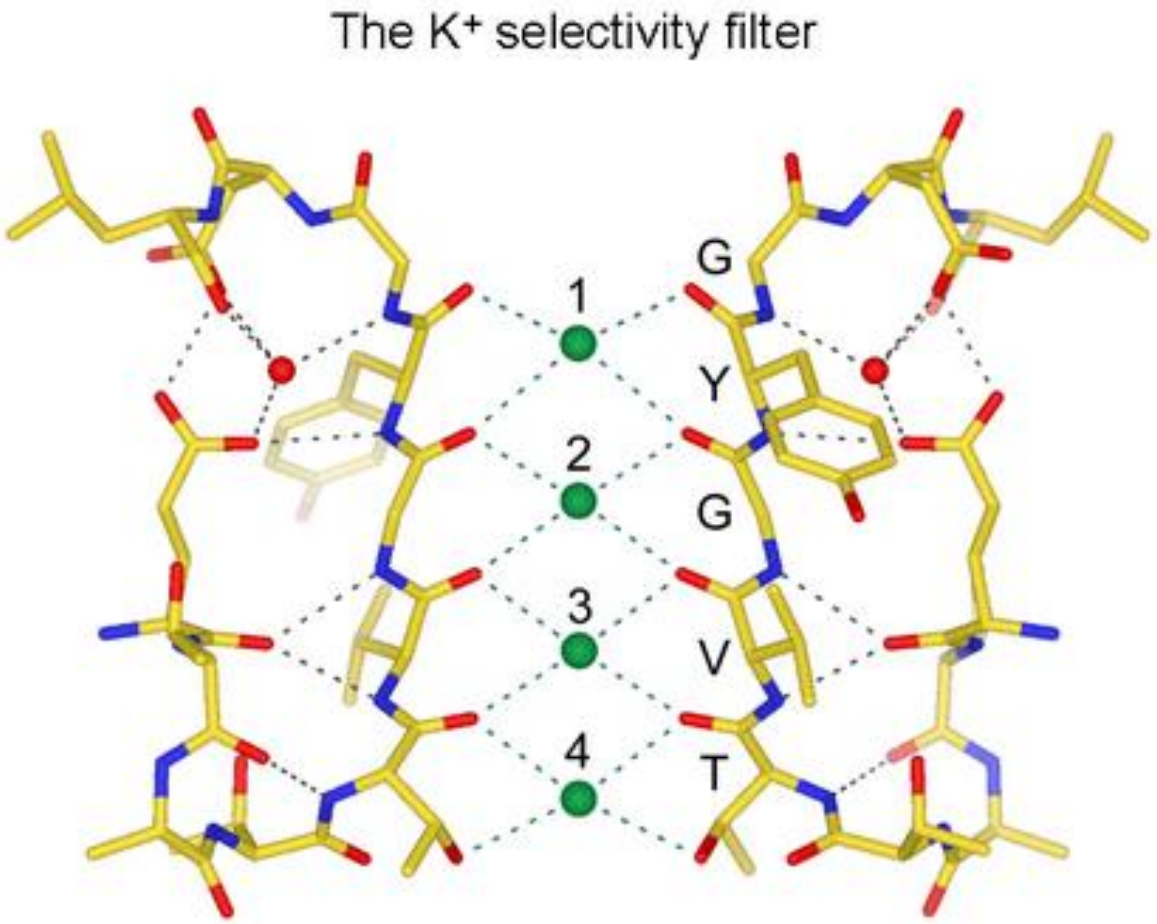
A funnel assembly



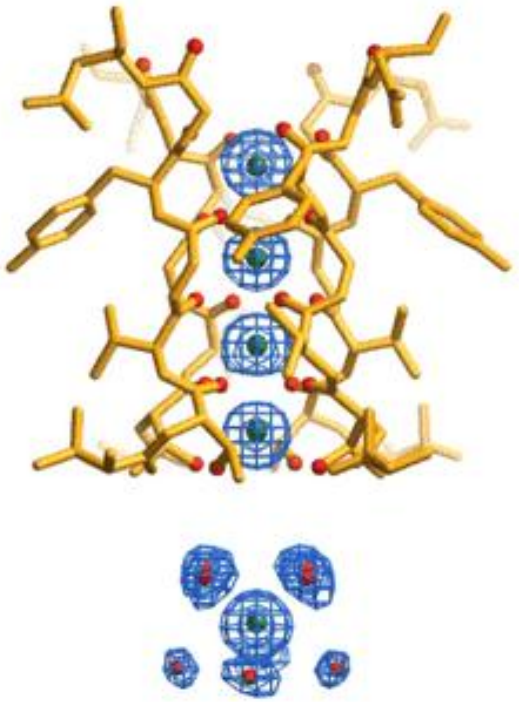
The selectivity pore

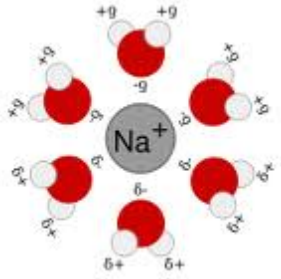


The selectivity pore

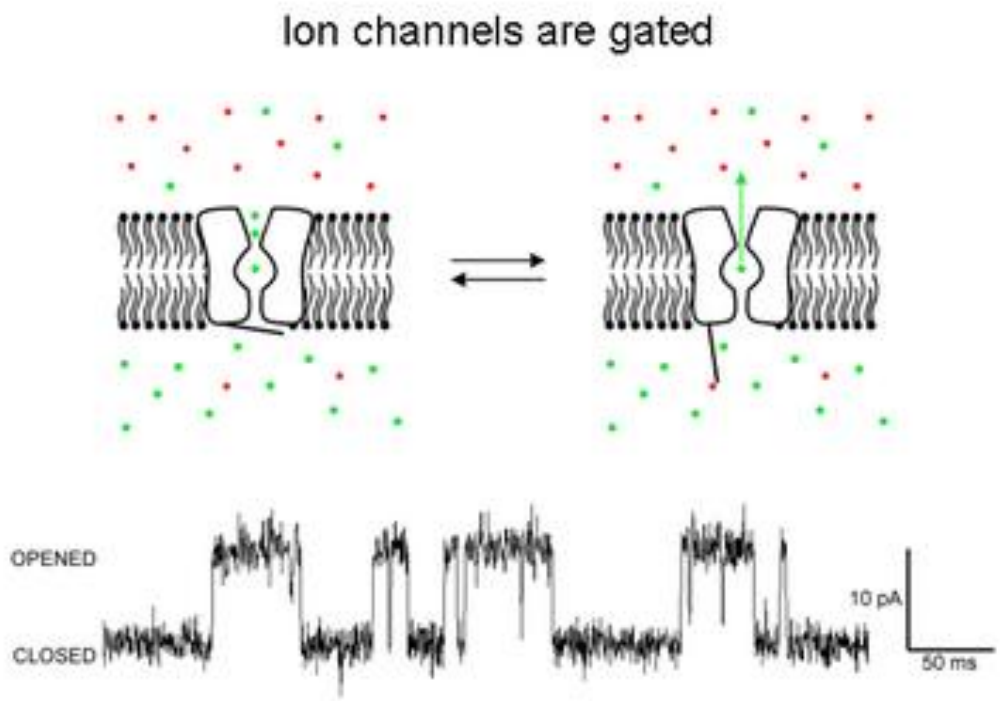


The selectivity pore



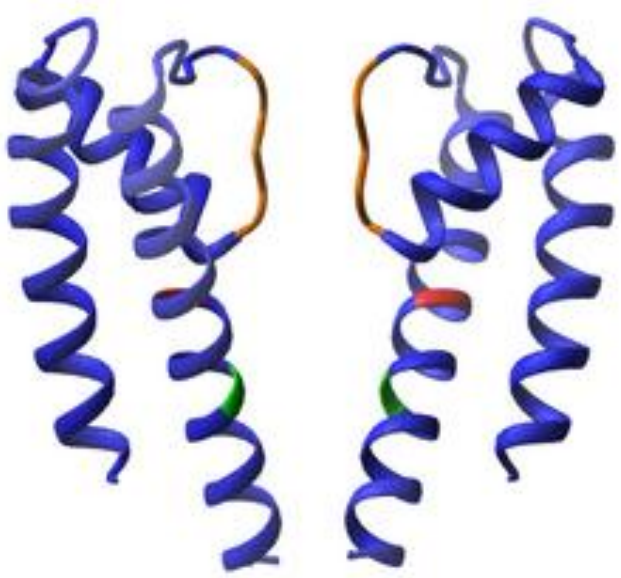


A gating mechanism



A gating mechanism

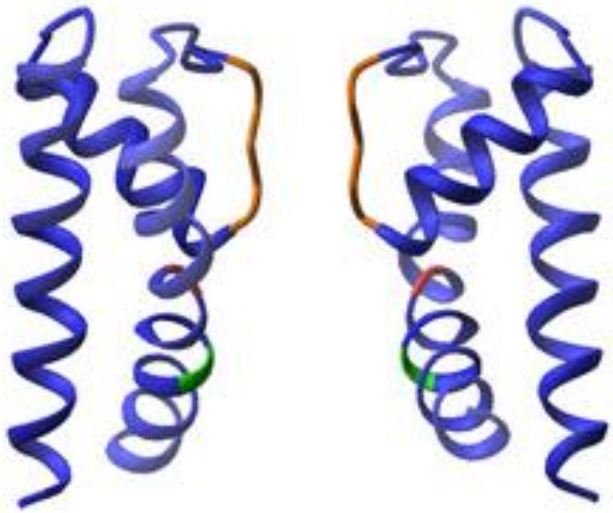
Mechanics of pore gating



(KcsA: closed)

A gating mechanism

Mechanics of pore gating



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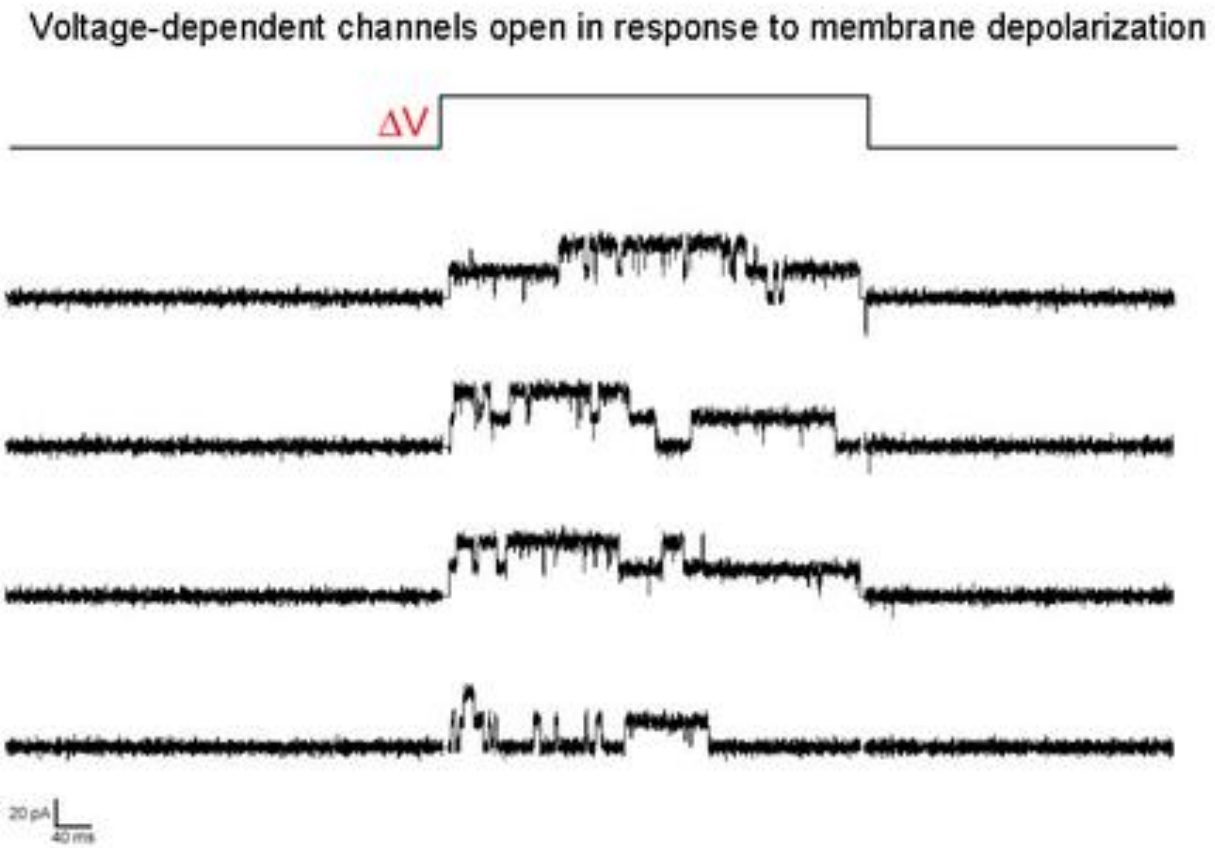
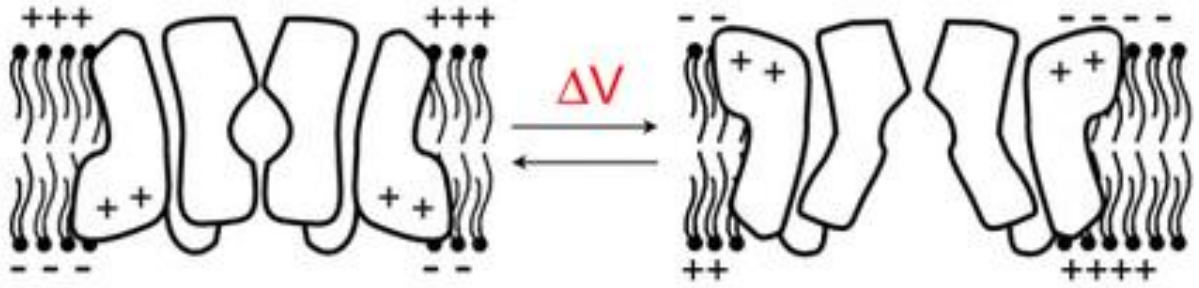
A gating mechanism voltage dependent

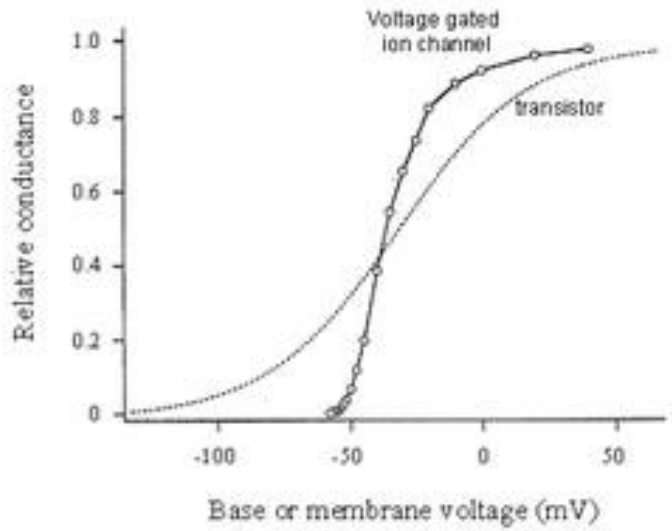


A gating mechanism voltage dependent



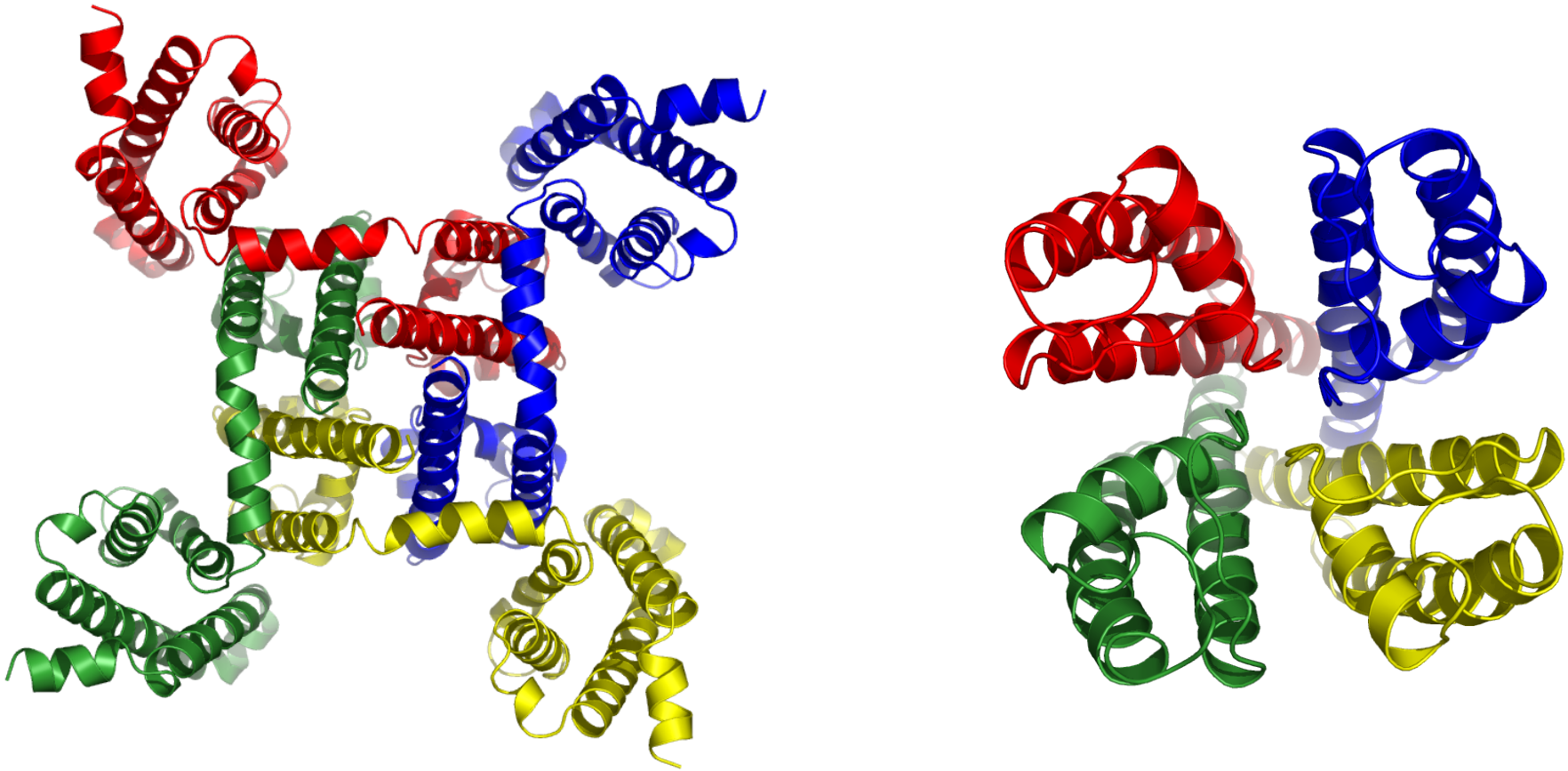
Nature electronic

Voltage-dependent channels are life's transistors

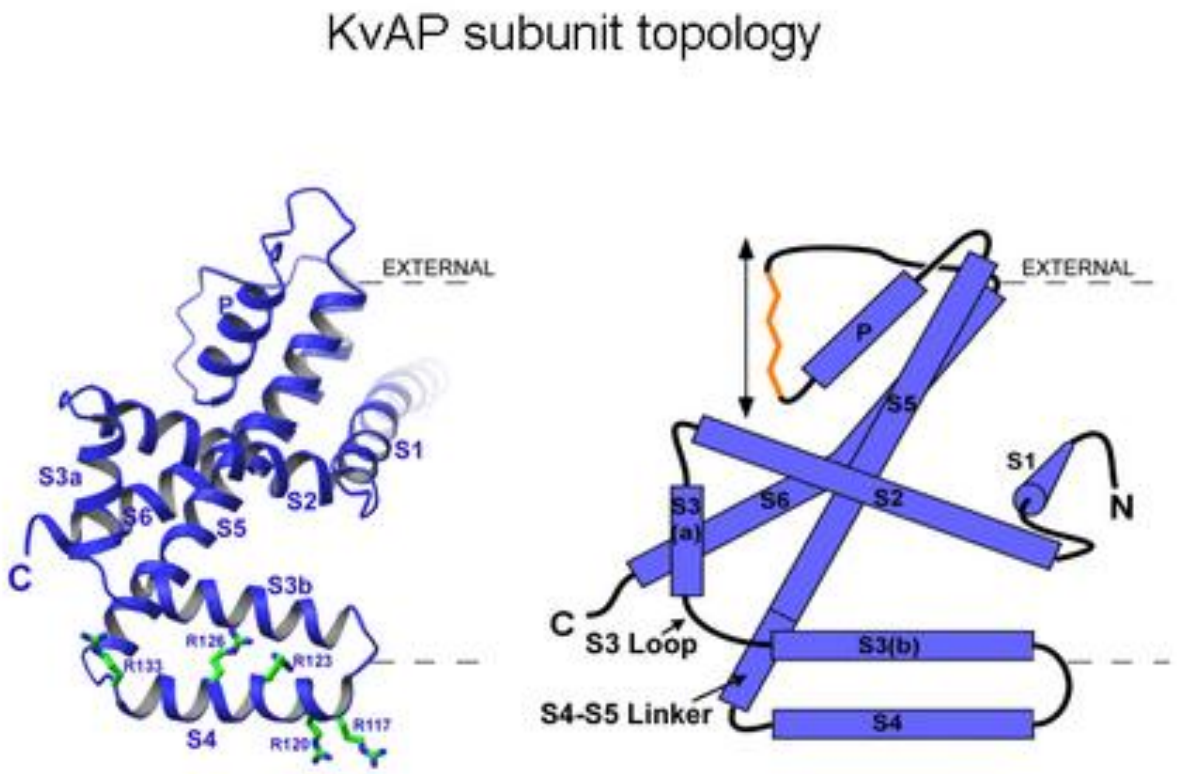


Sigworth, FJ (1994) Q Rev Biophys 27:1-40

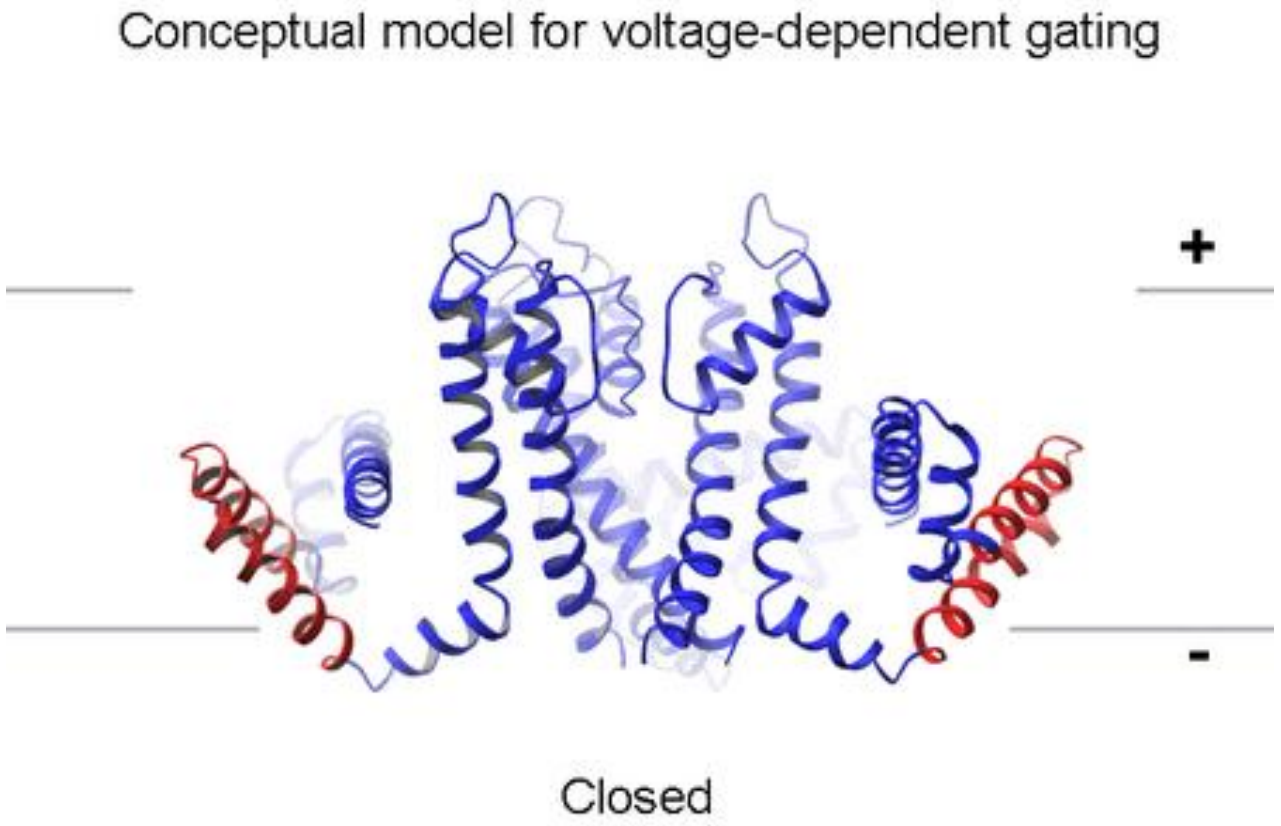
Voltage-dependent potassium channel crystal structure



Voltage-dependent potassium channel crystal structure

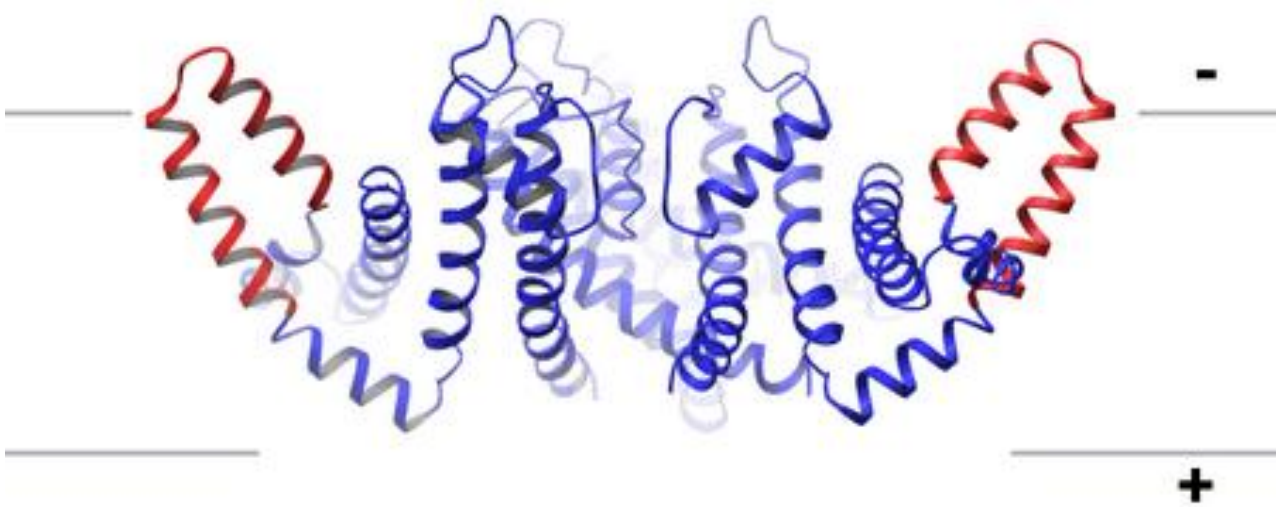


Structure-based gating mechanism



Structure-based gating mechanism

Conceptual model for voltage-dependent gating

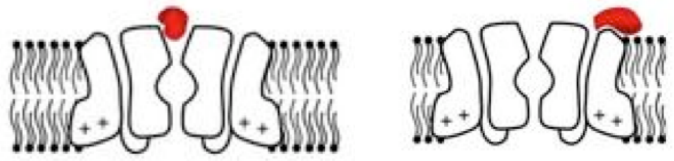


Opened

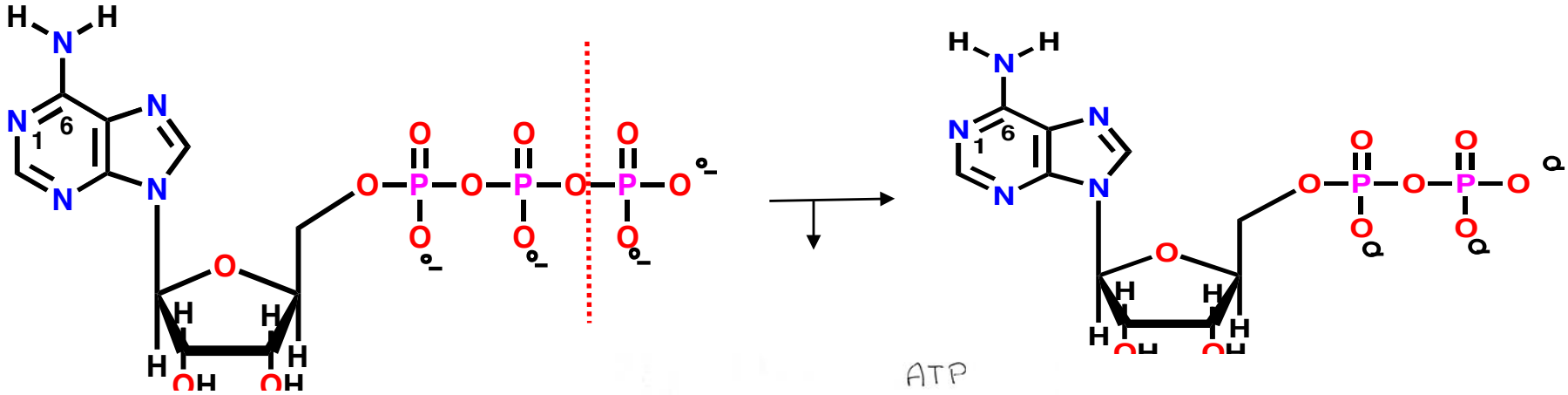
“Nothing in biology makes sense except in the light of Evolution” (T. Dobzhansky)

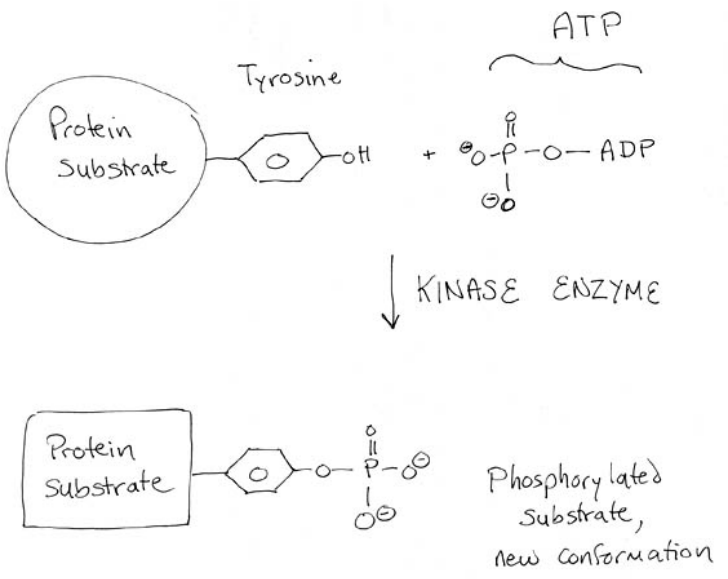
Many ion channels used in the nervous system are highly conserved across the tree of life and apparently arose for purposes other than electrical signal production.

Venoms contain inhibitors of ion channel function



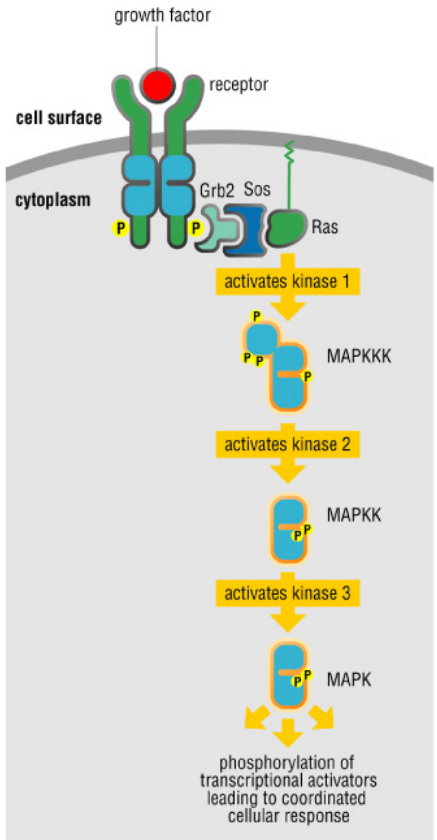
Protein kinases





Signal transduction cascade

From **Protein Structure and Function**
by Gregory A Petsko and Dagmar Ringe



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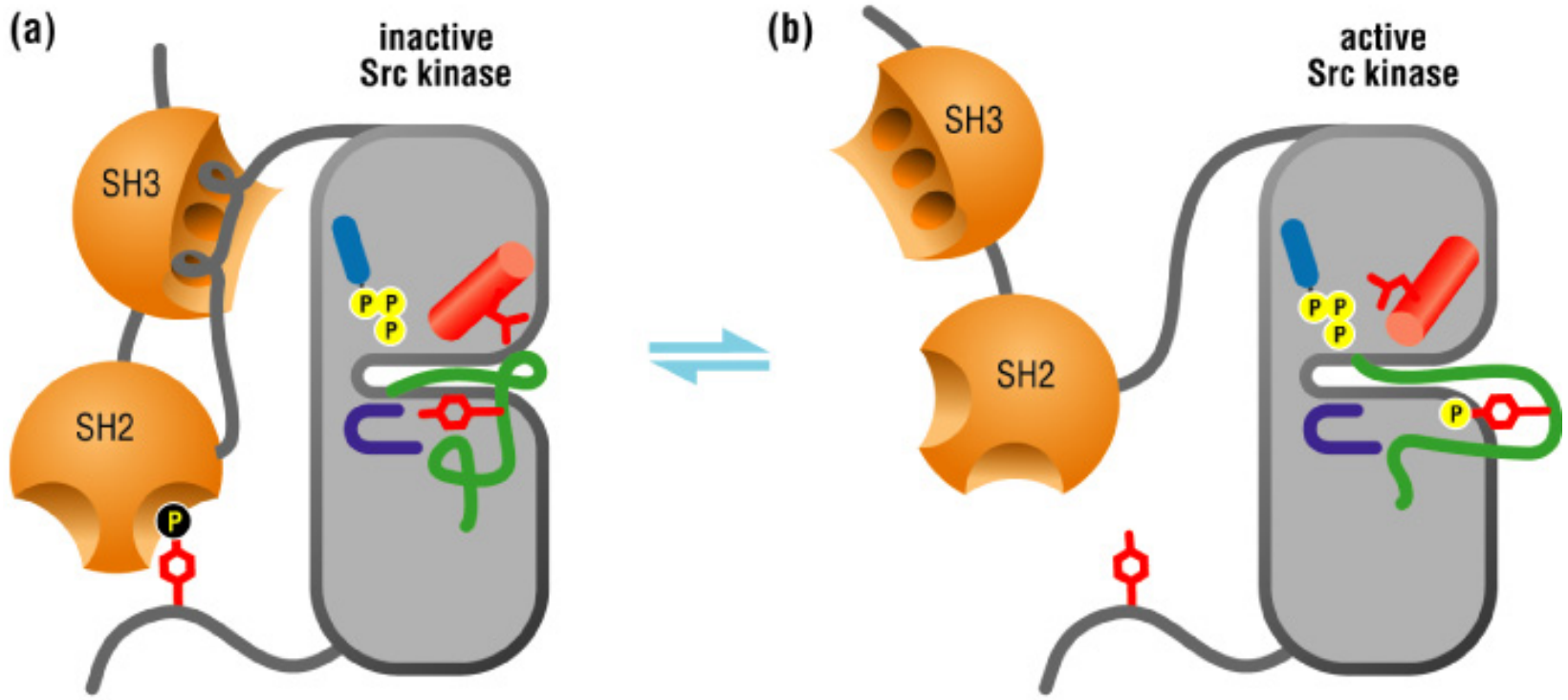
Closed vs open conformations

From **Protein Structure and Function** by Gregory A Petsko and Dagmar Ringe



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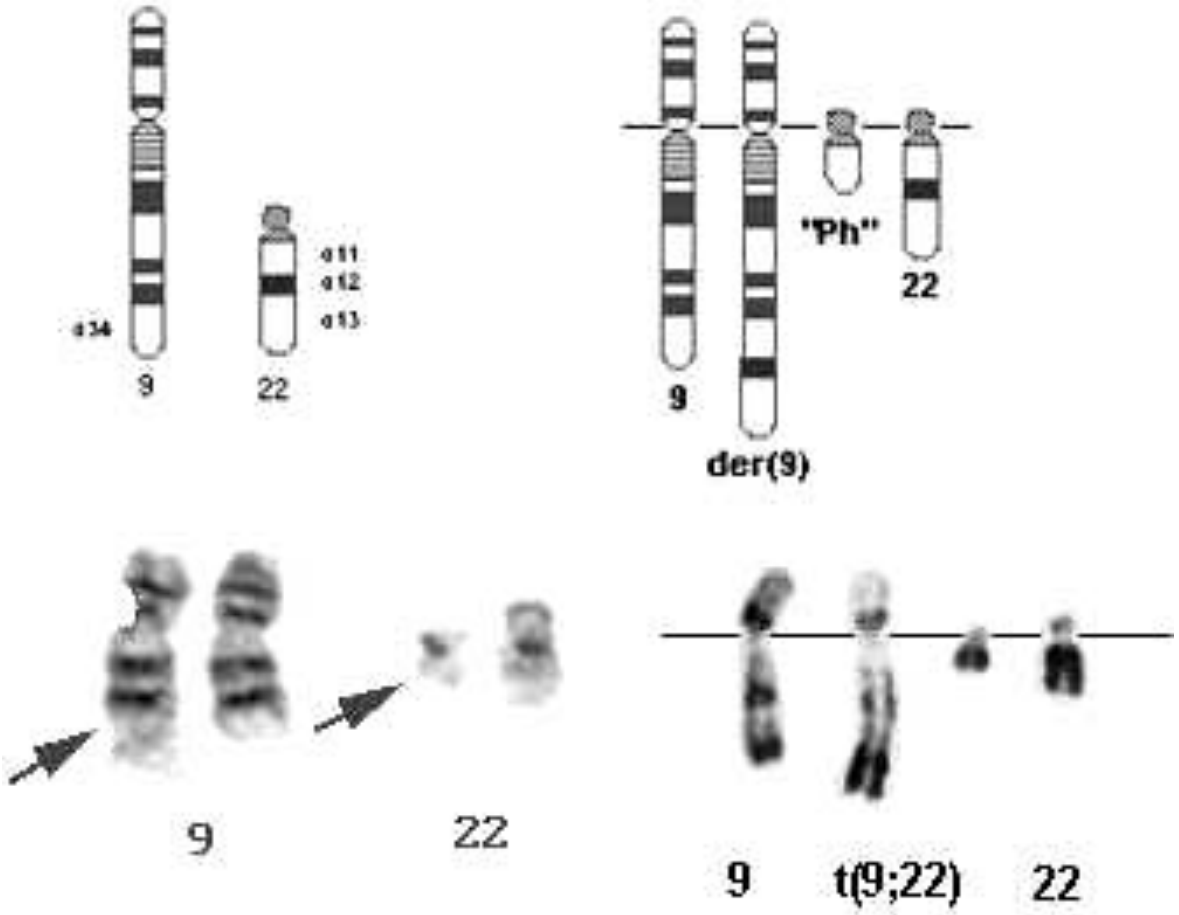
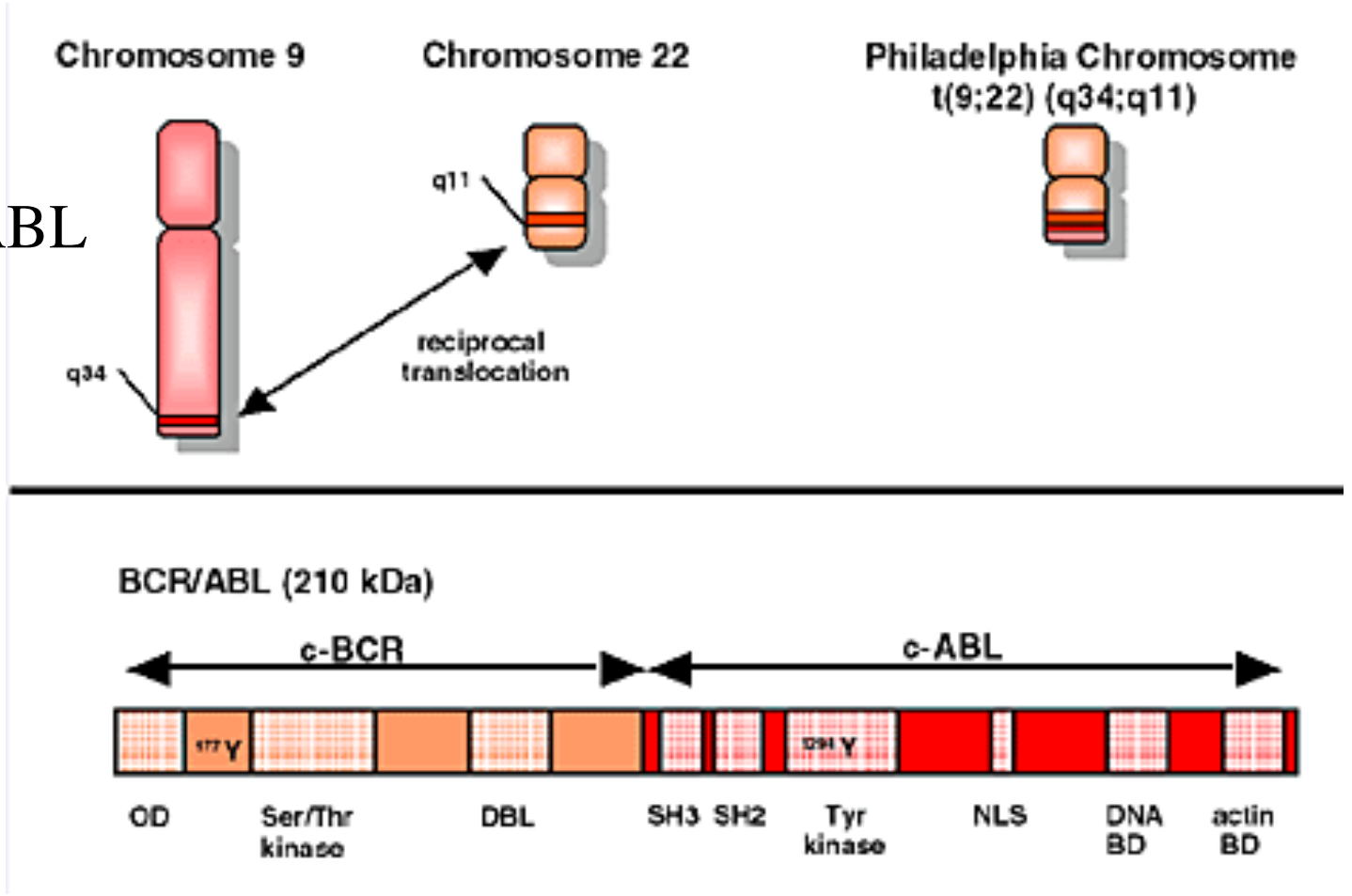
Closed vs open conformations

Philadelphia chromosome (translocation) chronic myeloid leukemia

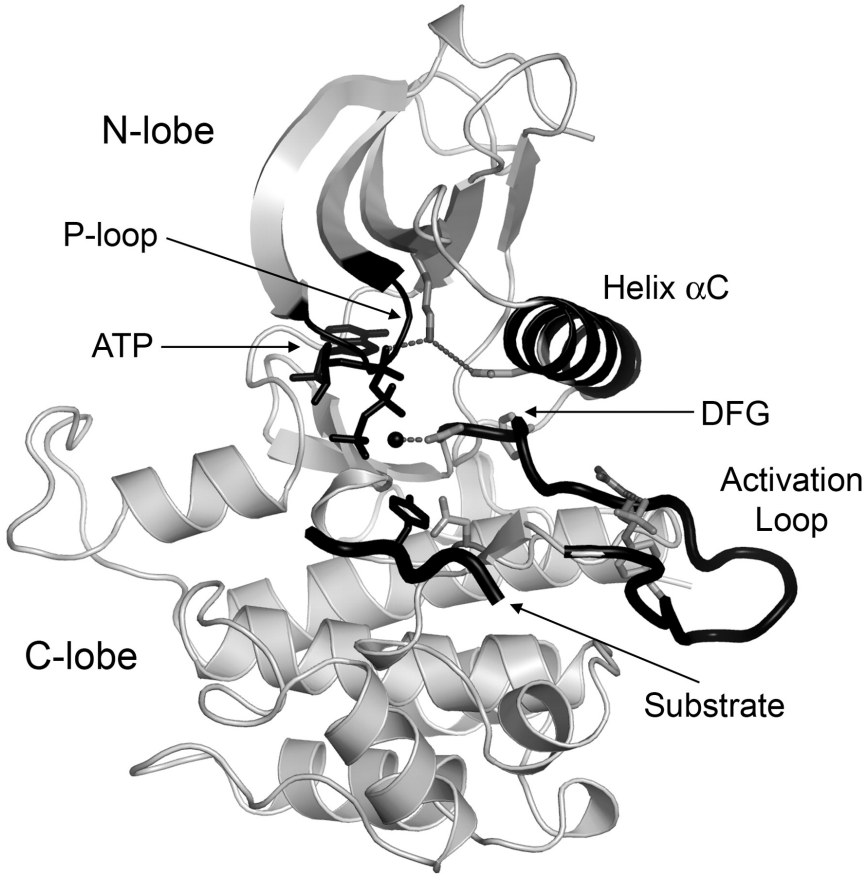


BCR/ABL chimeric protein kinase constitutively active

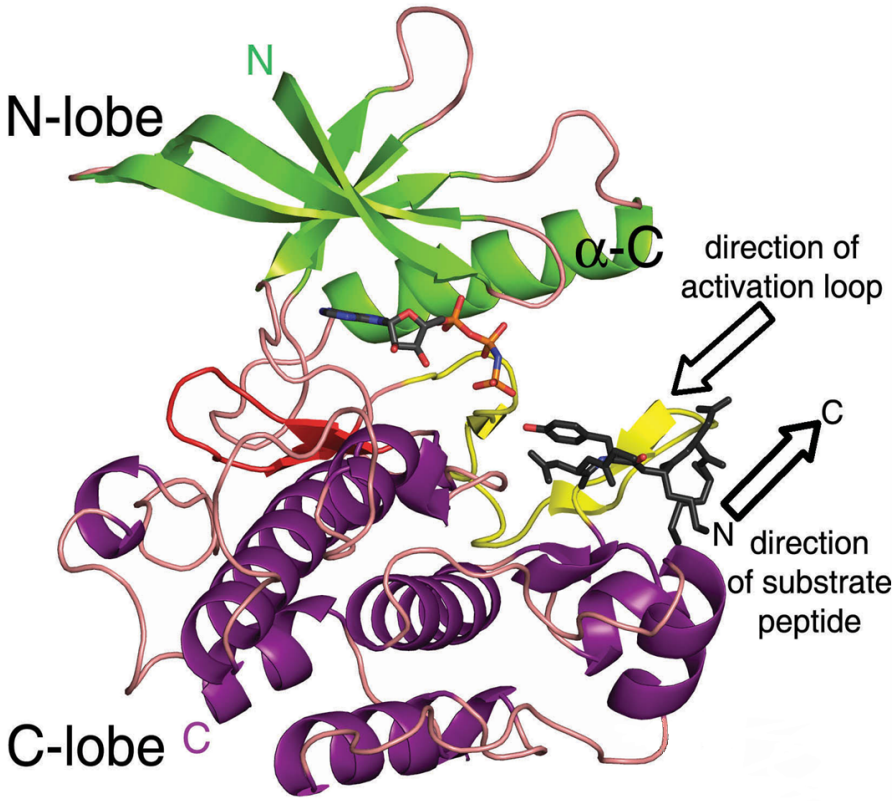
c-ABL



Crystal structure of ABL

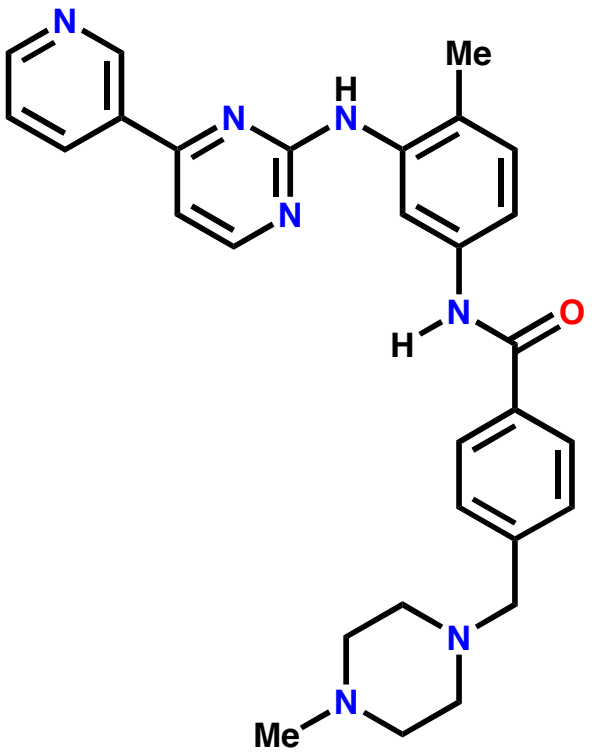


open active

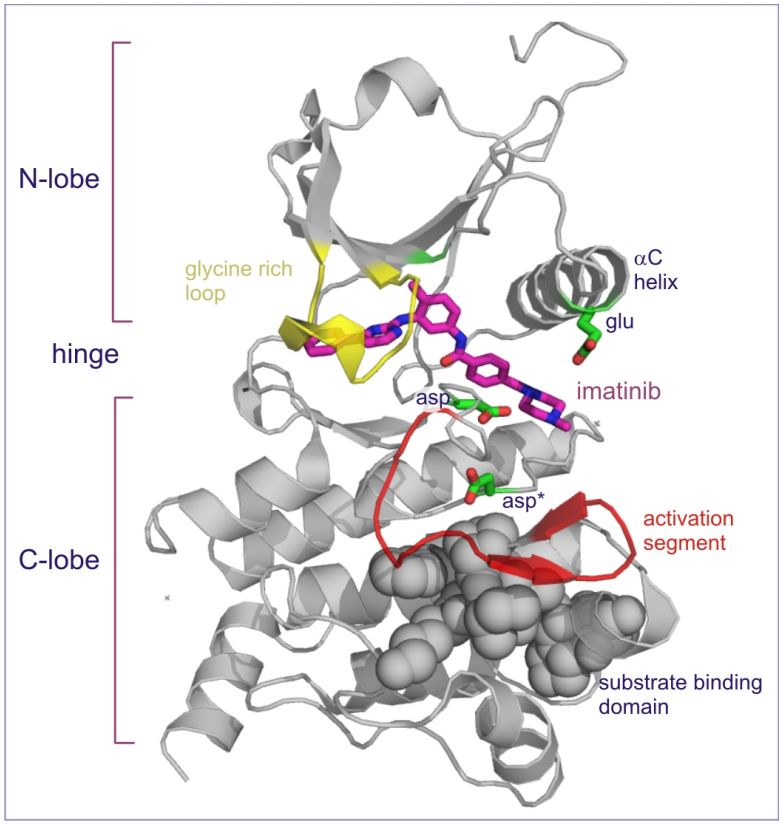


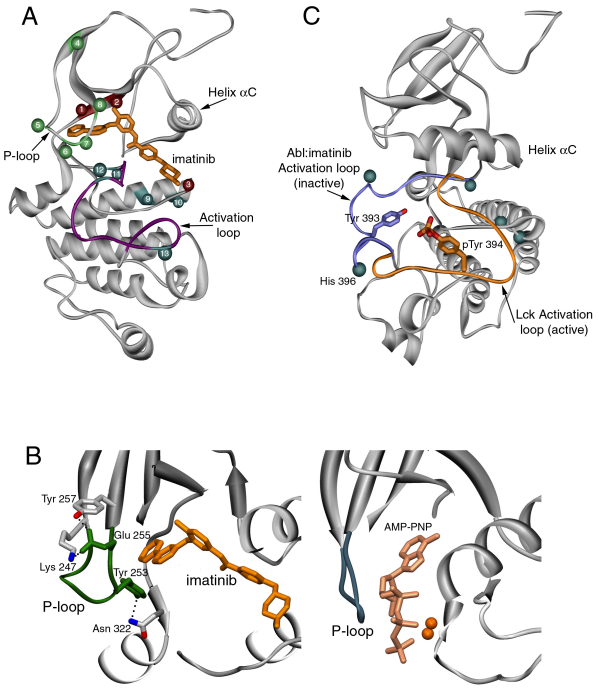
closed inactive

imatinib



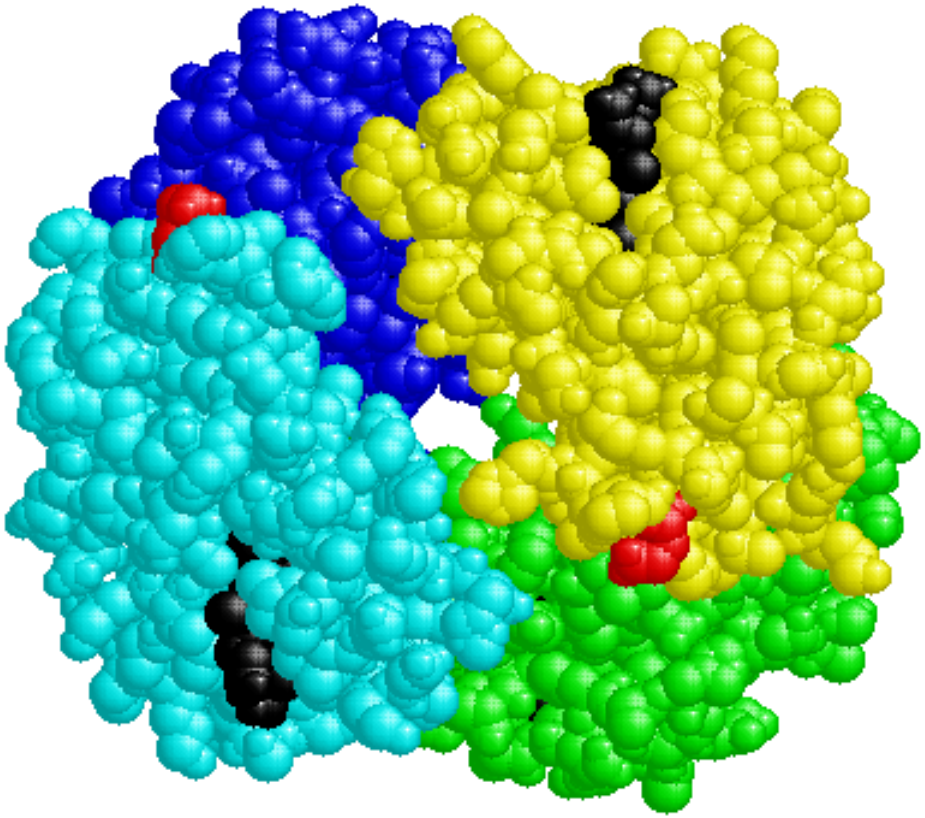
imatinib targets a closed conformation



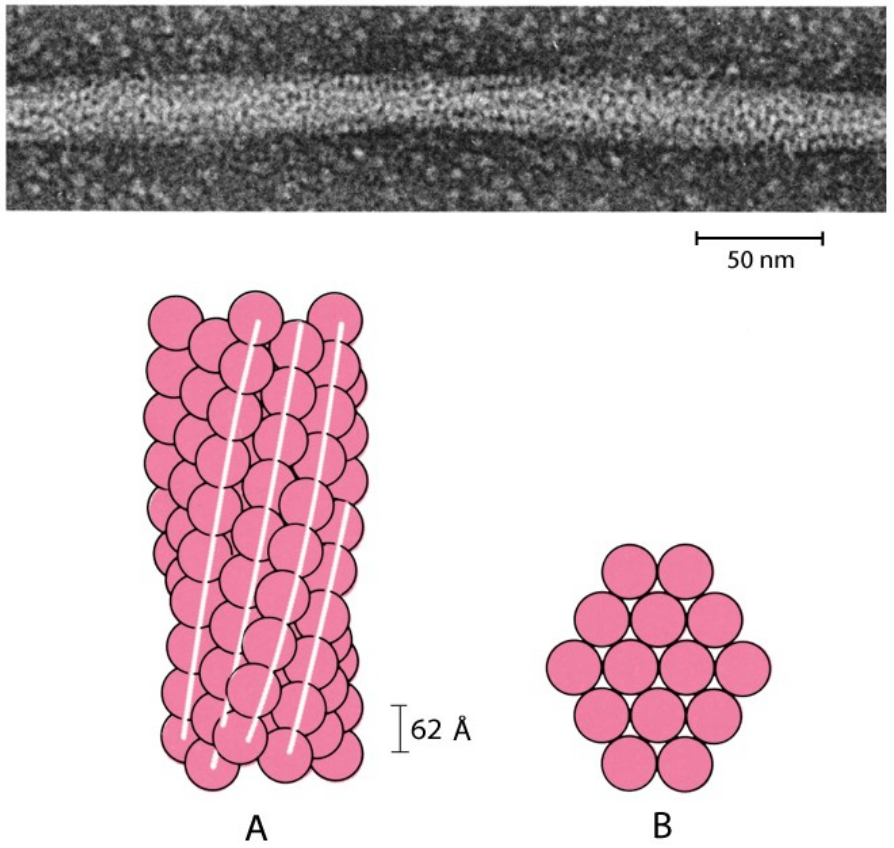


asciminib is an allosteric inhibitor

Sickle cell disease: Glu>Val^{6} variant alters the surface charge in hemoglobin S



Oxygen binding to Fe(II)



Biochemistry of neurodegenerative diseases and prions

Neurodegenerative disease	**Patologic protein (amyloid)**
Alzheimer‘s disease	Amyloid β
Parkinson disease	α-synuclein
Frontotemporal dementia	Tau
Hungtington disease	Huntingtin
Creutzfeldt-Jakob disease	Prion

Prions proteins: pathological conformation

PrP^C

The normal protein

is called PrP^C (for cellular)

has dominant secondary structure α-helix

is easily soluble

is monomeric and digested by proteases

is a transmembrane glycoprotein (neurons, lymphocytes); its function is unknown; it binds Cu^{2+} (regulation its homeostasis)

PrP^{Sc}

The abnormal, disease-producing protein

is called PrP^{Sc} (for scrapie)

has the same amino acid sequence

has dominant secundary structure β-sheets

is insoluble

is multimeric and resistant to proteases

When PrP^{Sc} comes in contact with PrP^C, it converts the PrP^C into more of itself These molecules bind to each other forming aggregates

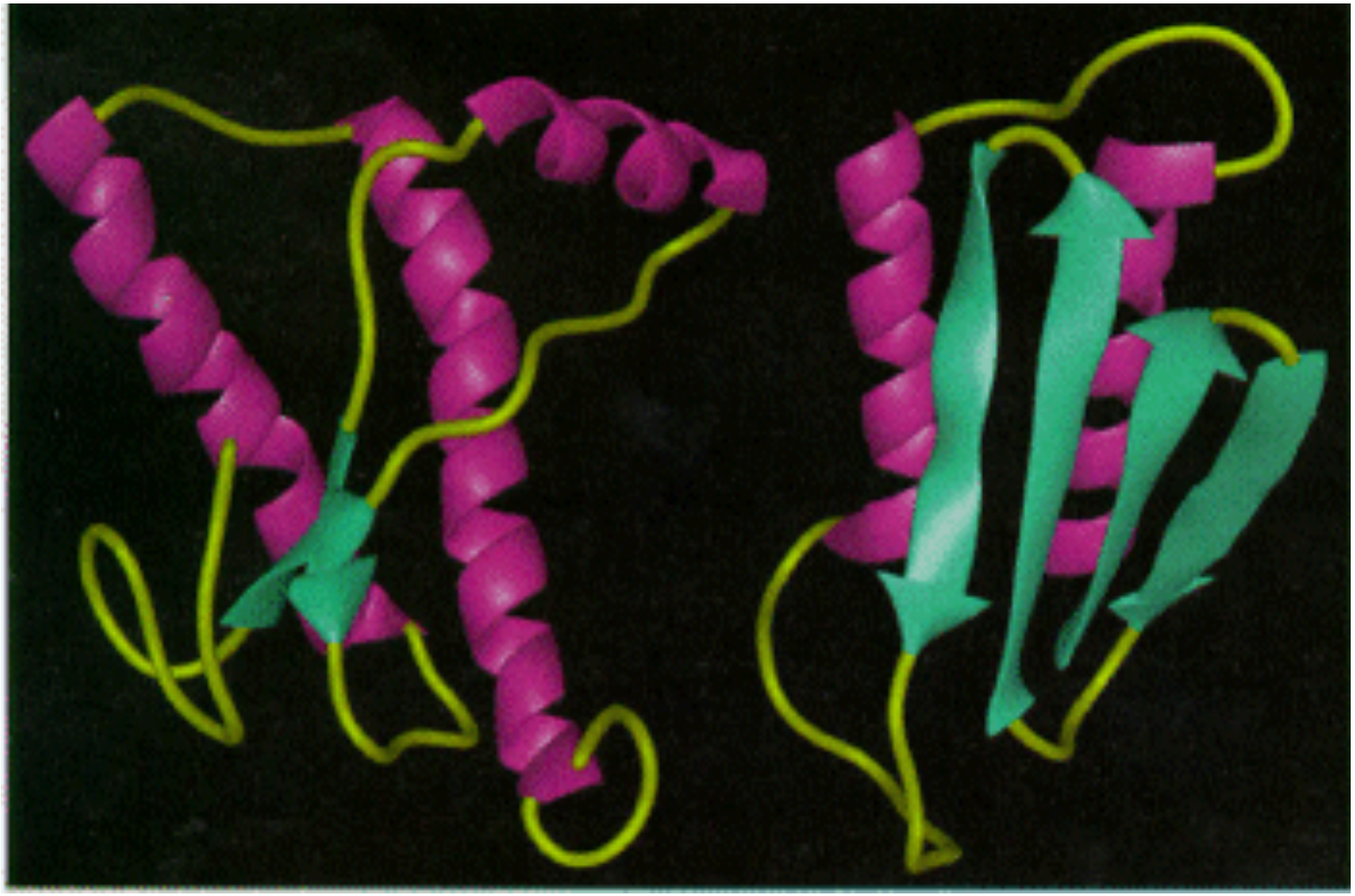
Prions proteins

PrP^{C}

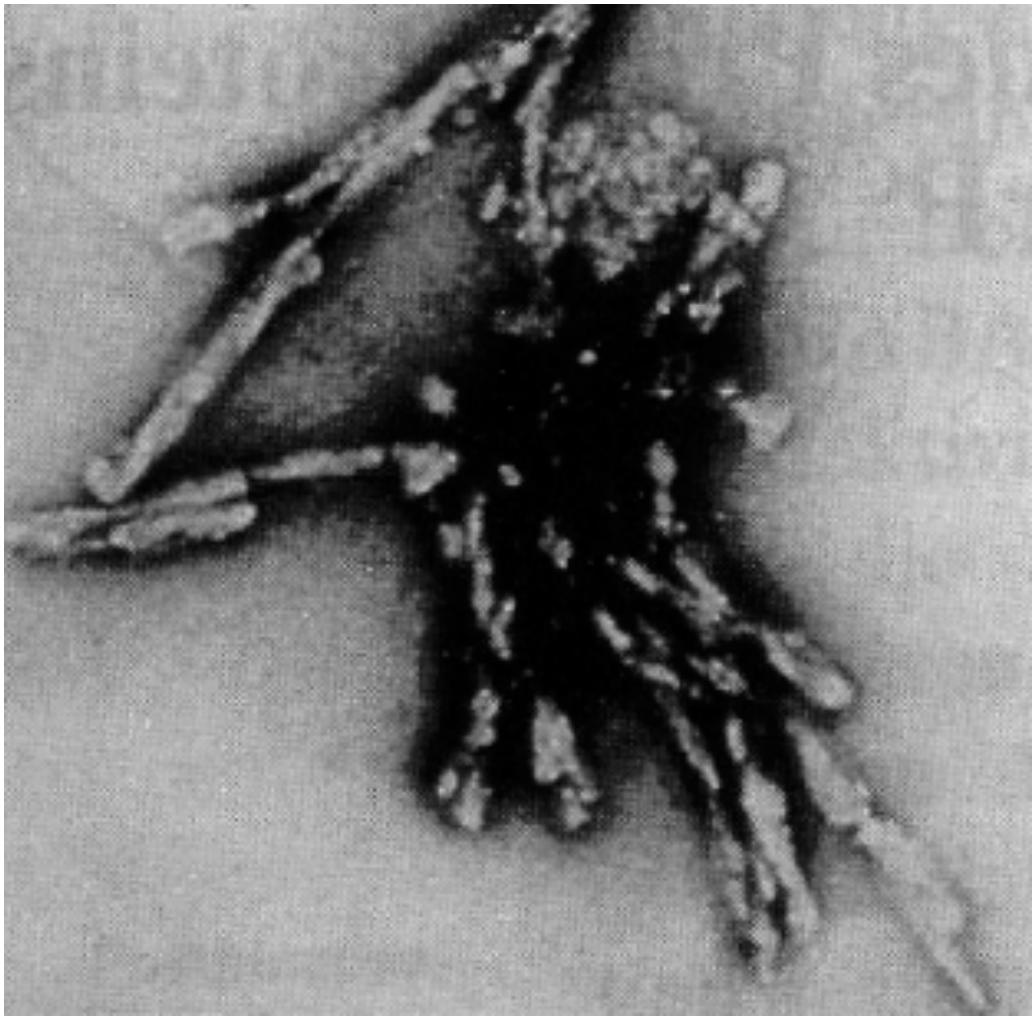
Predominantly α-helix

PrP^{Sc}

β-sheets (40%), α-helix (30%)



Prions aggregates



Originator vs Biosimilar

Sequence contains the information to guide folding into the native structure (conformation)

Altering the sequence can alter the structure (conformation)

Post-translation modifications can "alter" the structure (even only with local misfolding, e.g. in a loop)

Drug discovery & Structural Biology

- **medicine discovery (optimization)**
- **medicine safety (selectivity-stability)**
- **improve access to medicine**

UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Pharmaceutical Sciences
Biochemistry and Structural Biology Unit

Davide Ferraris

Silvia Garavaglia

Riccardo Miggiano

Menico Rizzi

Franca Rossi

Funding


EU


CSI-LTB


MINISTERO DELL'ISTRUZIONE,
DELL'UNIVERSITÀ E DELLA RICERCA


italian malaria network


SysteMTb


REGIONE
PIEMONTE

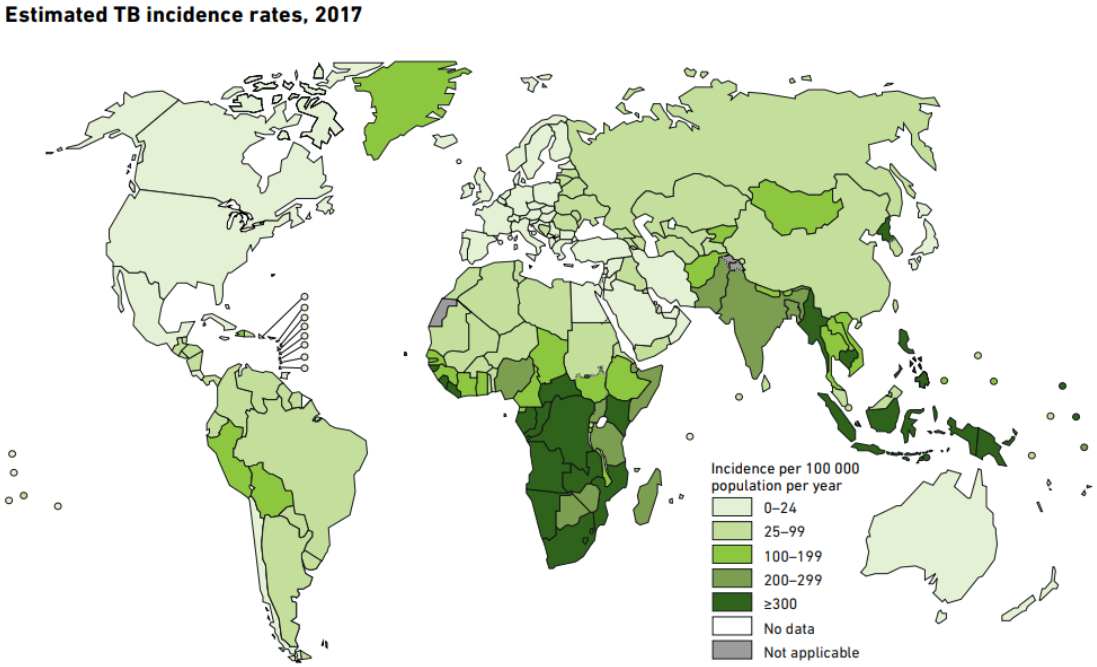

elettra


ESRF


MM4TB

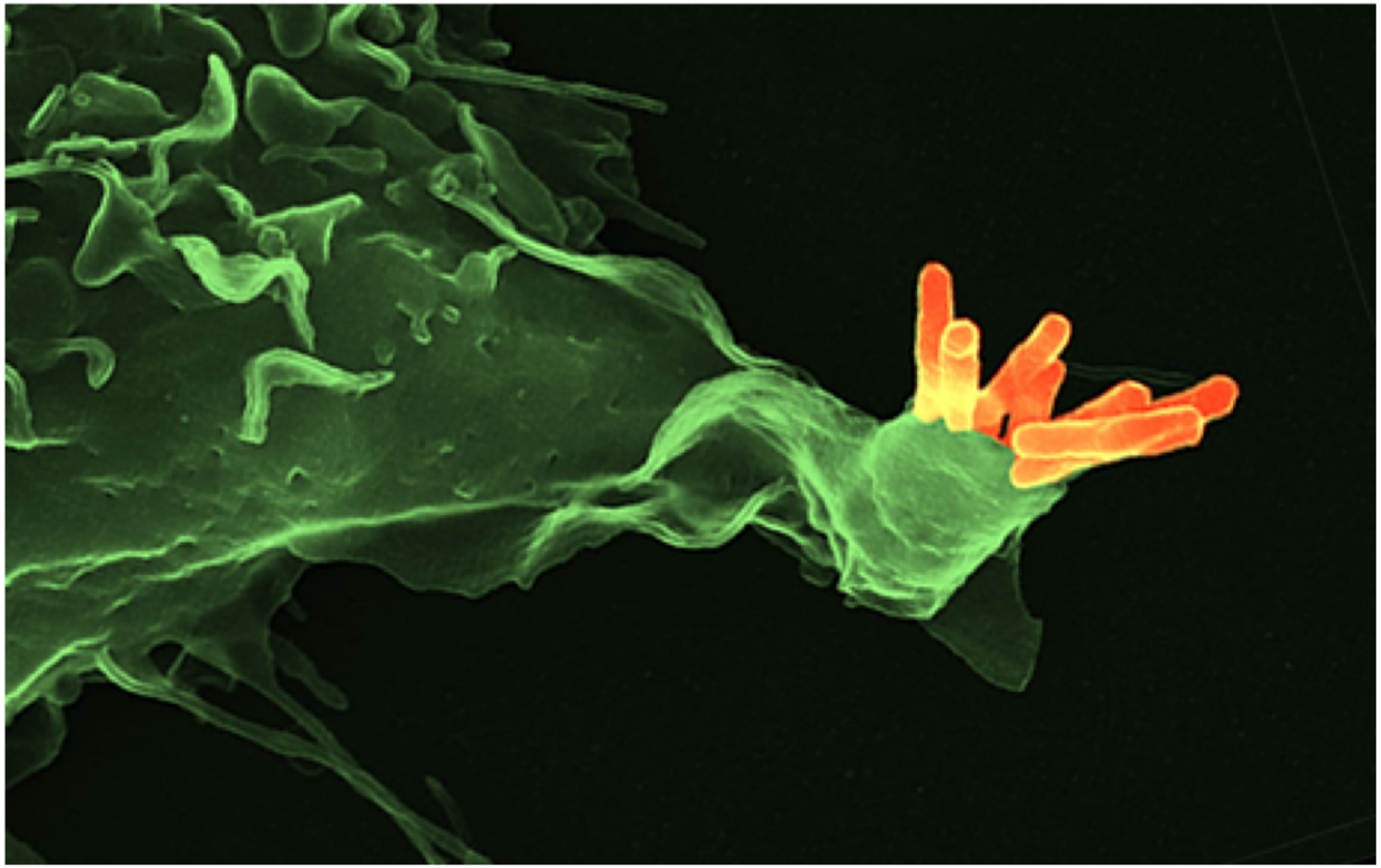

fondazione
cariplo

Tuberculosis: a global burden

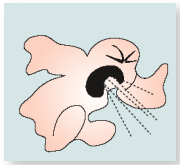


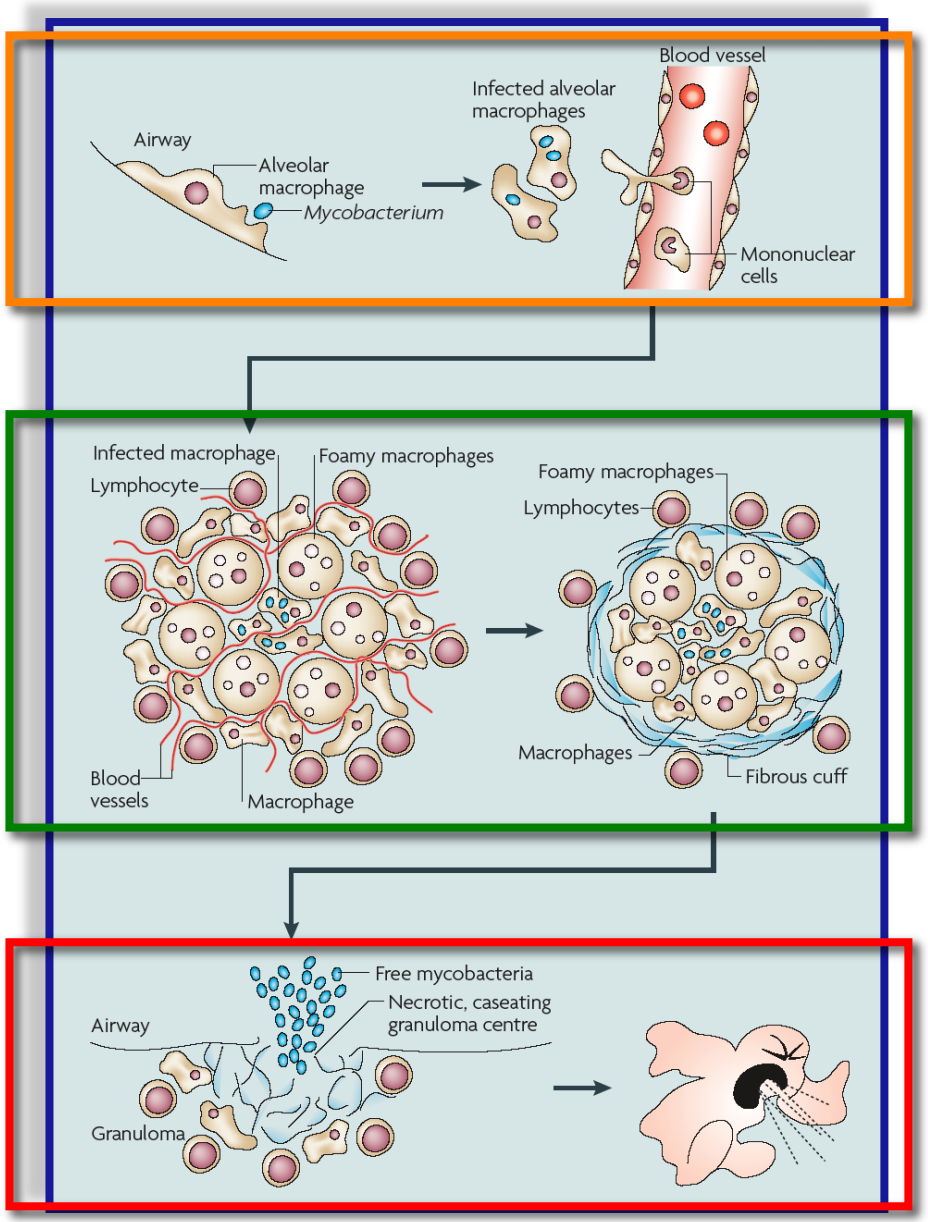
- The leading cause of death from a single infectious agent (above HIV/AIDS)
- Ninth cause of death worldwide
- 10.0 million people developed TB disease in 2017
- Estimated 1.3 million deaths in 2017 among HIV-negative people
- Additional 300 000 deaths from TB among HIV-positive people

Mycobacterium tuberculosis - Humankind: a long standing relationship



Infection by *Mycobacterium tuberculosis*



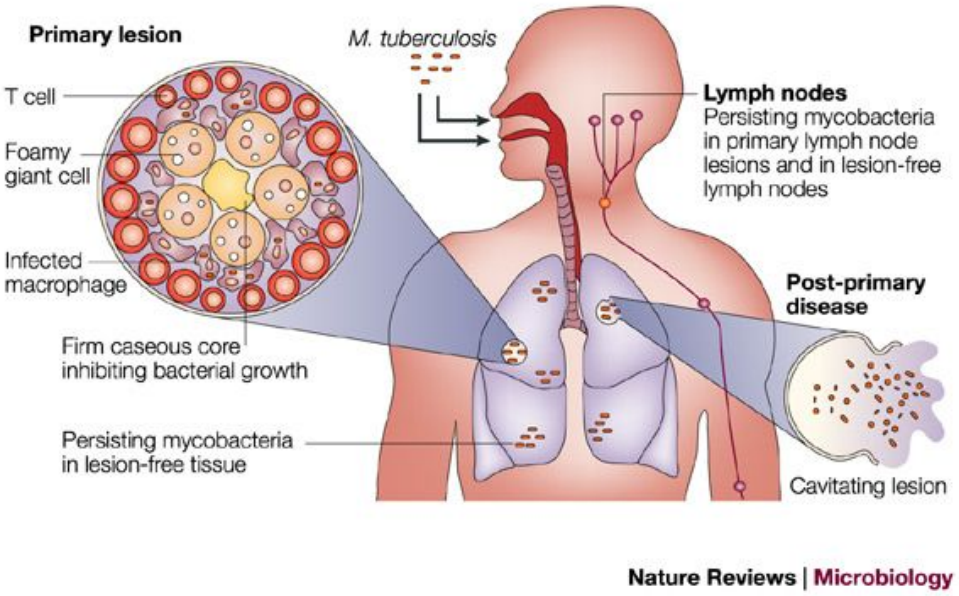


localized proinflammatory response

"containment" phase

Productive phase

Tuberculosis: a global burden



- **First line treatment (isoniazide and rifampicin)**
- **Second line treatments (Kan, fluoroquinolone)**

Major problems with fighting TB:

- **Multidrug resistant (MDR) strains**
- **Extensively drug resistant (XDR) strains**
- **Total drug resistant strains (TDR)**

➔ New antitubercular drugs are badly needed

Target-based vs. phenotypic screening

Target-based screening

Methods: HTS of large compound libraries for inhibition of a critical enzyme/protein

- **Uncertainty of molecule absorption and general biology**
- **Low success rate**
- **Multiple failures for such approach**

Whole-cell screening

Methods: HTS of molecules with bactericidal effects

- **Associated with whole genome sequencing**
- **Direct identification of the genes involved in pathogenisis**
- **Identification of the mutations that confer resistance**

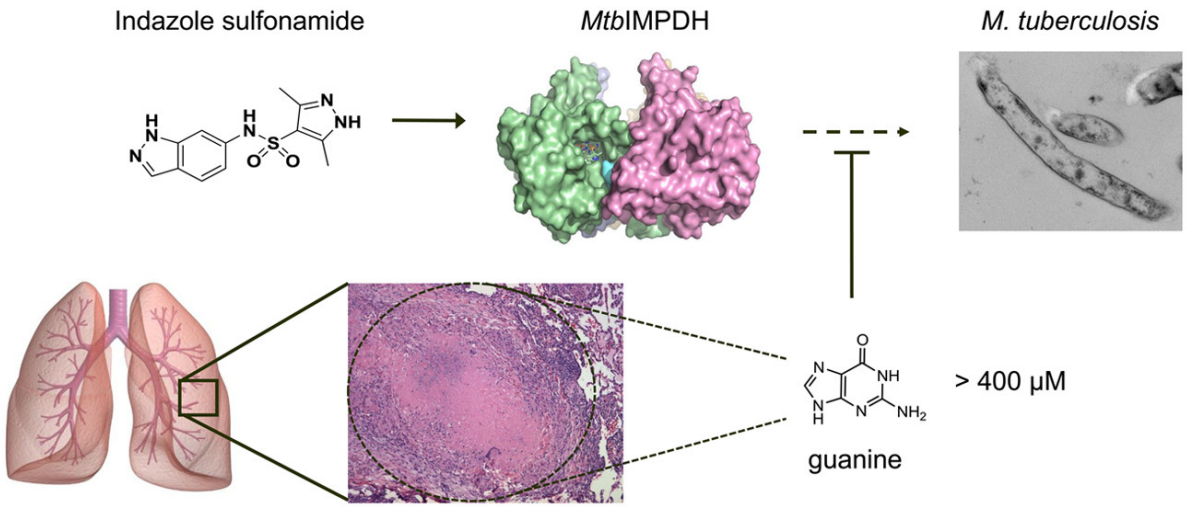
Valerie Mizhrai University of Cape Town (South Africa)

Tom Blundell, University of Cambridge, (UK)

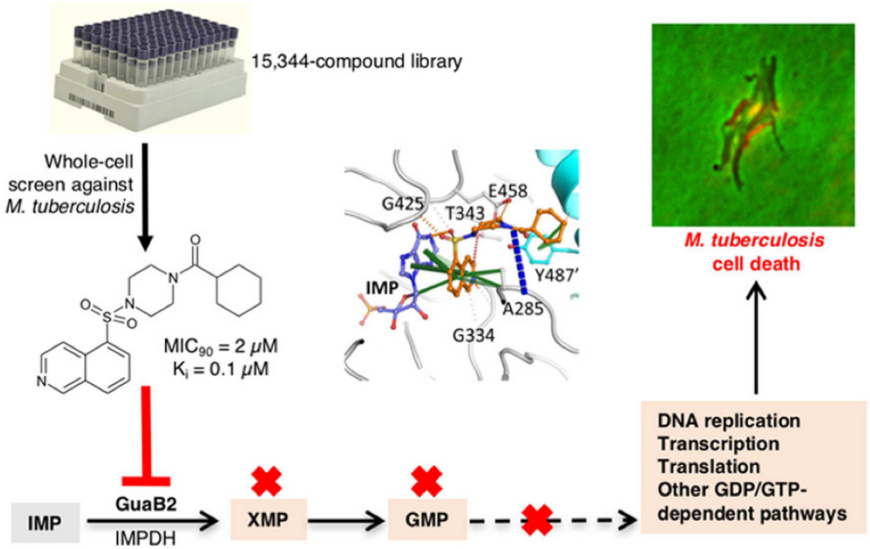
György Kéri, Vichem Ltd, Budapest (Hungary)

Barry Clifton III, NIAID, Bethesda (USA)

Menico Rizzi, UPO, Novara (Italy)

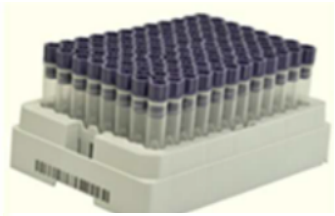


Park et al. Essential but not vulnerable: indazole sulfonamides targeting IMPDH as potential leads against *Mycobacterium tuberculosis*. *ACS Infect Dis,* 2017 Jan 13;3(1):18-33



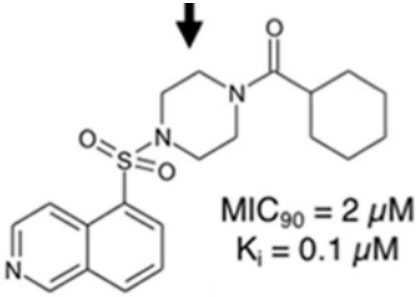
Singh et al. The inosine monophosphate dehydrogenase, GuaB2, is a vulnerable new bactericidal drug target for tuberculosis. *ACS Infect Dis*, 2017 Jan 13;3(1):5-17

Phenotypic screening – 2: 15344 compounds



15,344-compound library

Whole-cell screen against *M. tuberculosis*



VCC234718
(sulfonamide)

VCC234718-resistant *Mtb* mutant selection and characterisation

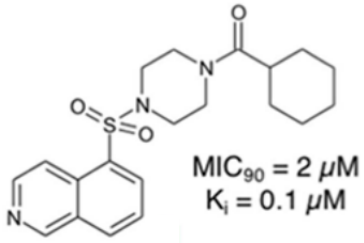


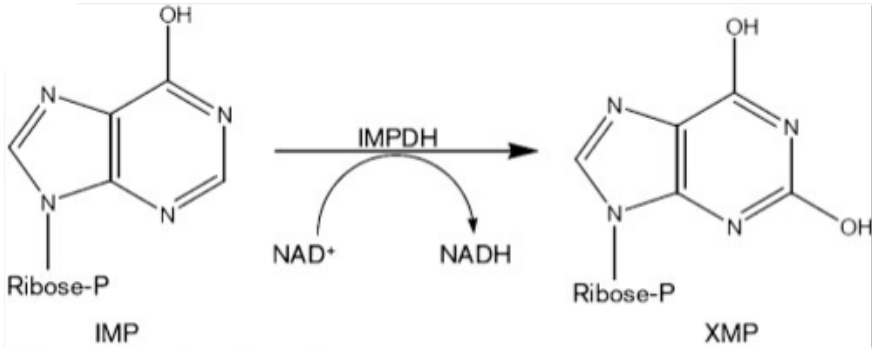
Table 1. Susceptibility of *Mtb* Strains to VCC234718

10xMIC$_{90}$

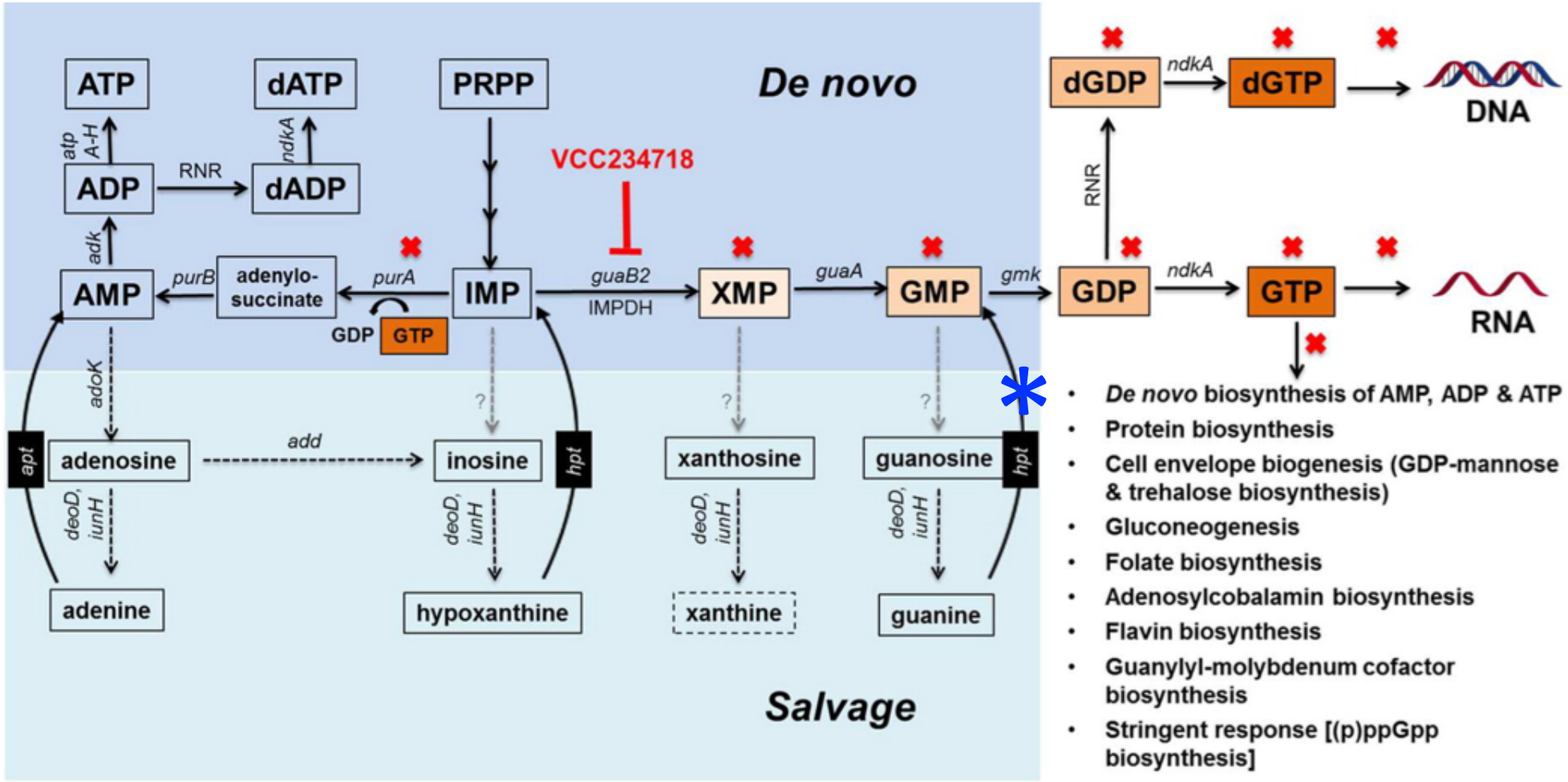
strain	MIC$_{90}$ (μM)
H37RvMA	2
SRMV2.6	>100

Whole genome sequencing ➔ Resistance-conferring mutation: ***guaB2***^{Y487C}

SRMV2.6 *attB*::*guaB2*^{Y487C}	>200
H37Rv *attB*::*guaB2*^{Y487C}	>100

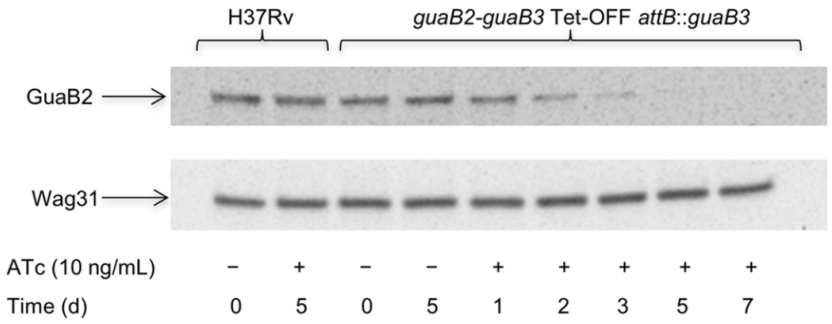


Purine de novo biosynthesis and salvage pathways in *Mtb* illustrating the impact of GuaB2 inhibition

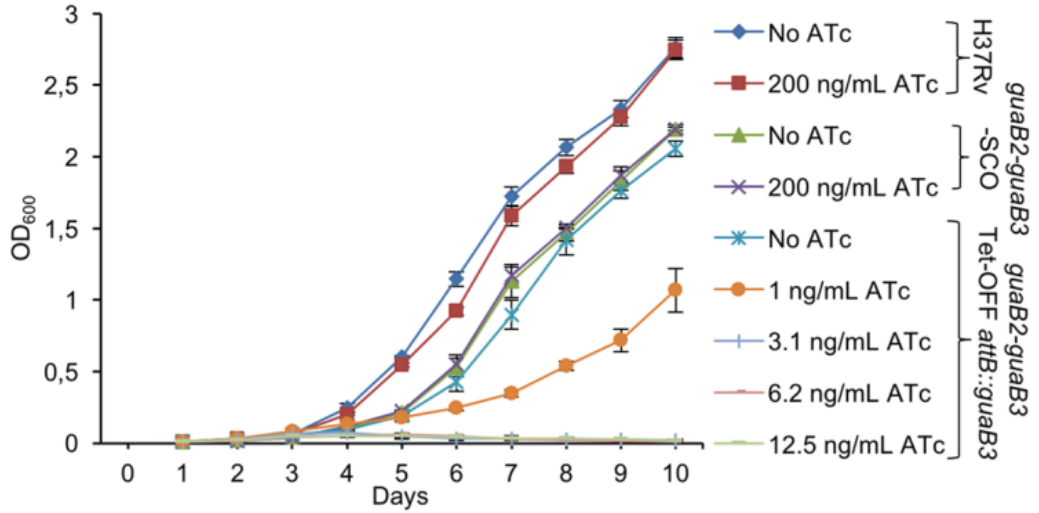


hypoxanthineguanine phosphoribosyl transferase *

Effect of *guab2* silencing: conditional *guaB2*-KO strain are growth impaired



Dose-dependent depletion of GuaB2 protein

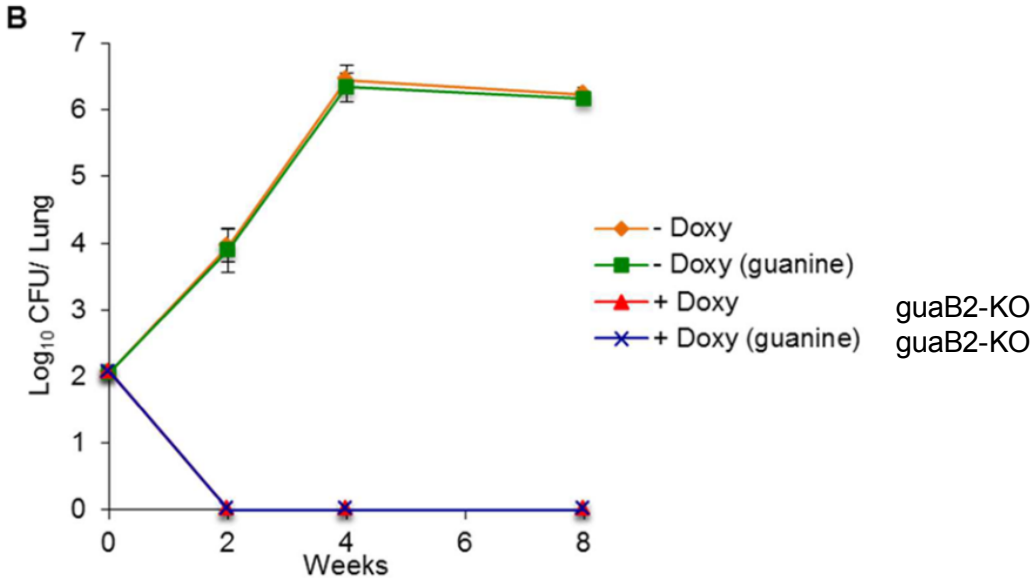


➔ **Dose-dependent impaired growth of conditional guaB2-KO strain**

➔ **GuaB2 is essential for growth**

Guanine does not rescue *Mtb* survival *guaB2*-silenced mice

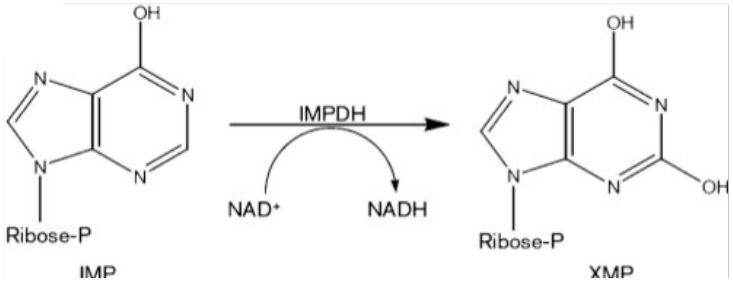
C57BL/6 mice were **infected by aerosol** with **conditional *guaB2*-KO mutant strain**.
Transcriptional silencing of *guaB2* was achieved by feeding the animals with **doxycycline-containing food**.
Lung homogenates were plated on agar plates **with or without guanine supplementation**.

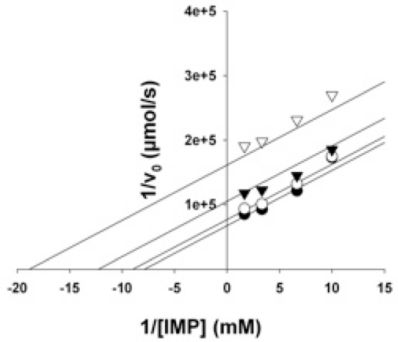


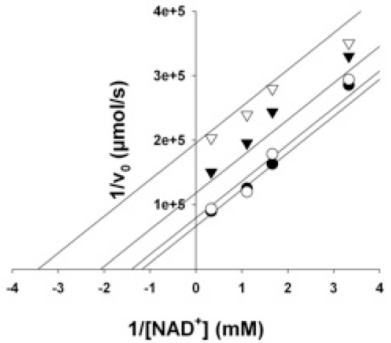
➔ **Guanine does not rescue *Mtb* survival in infected lung tissues**

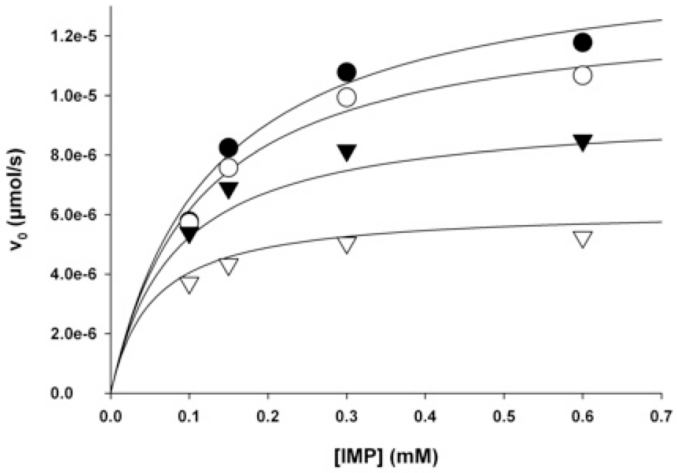
Depletion of GuaB2 is bactericidal in *Mtb* in vitro

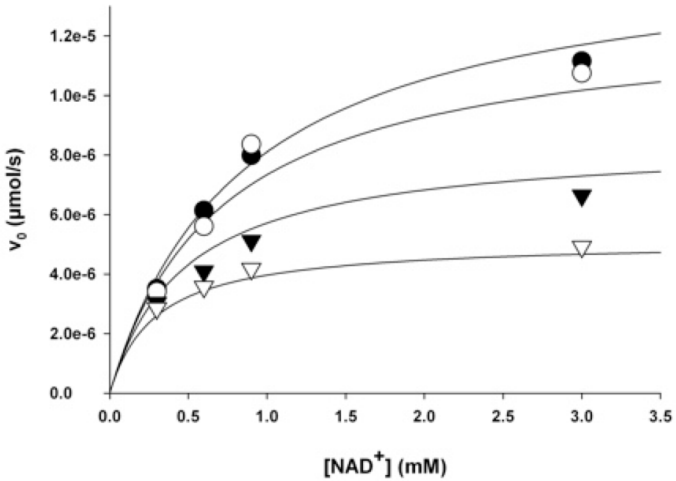
VCC718 is an uncompetitive Guab2 inhibitor





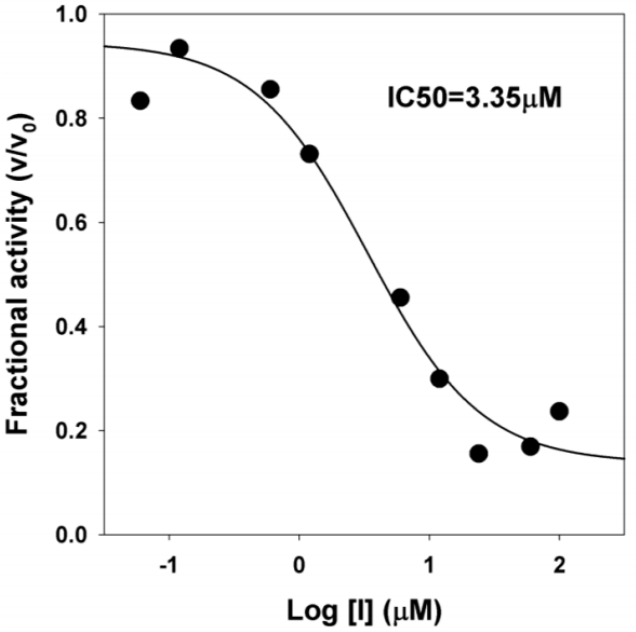


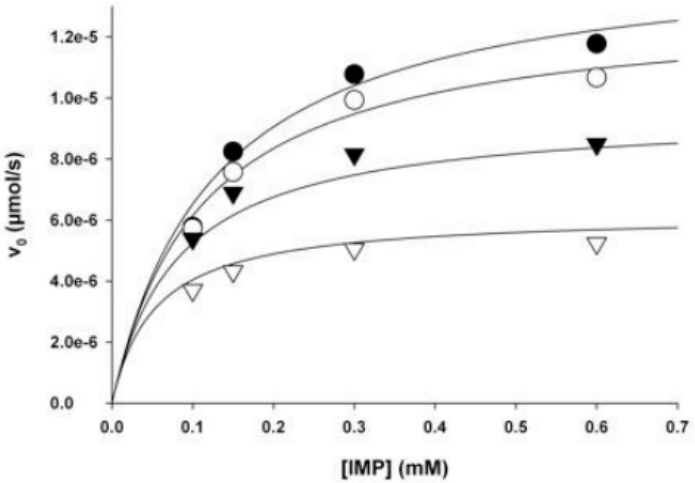




K*i* = 0.10 microM

VCC234718 is uncompetitive against *Mtb* IMPDH

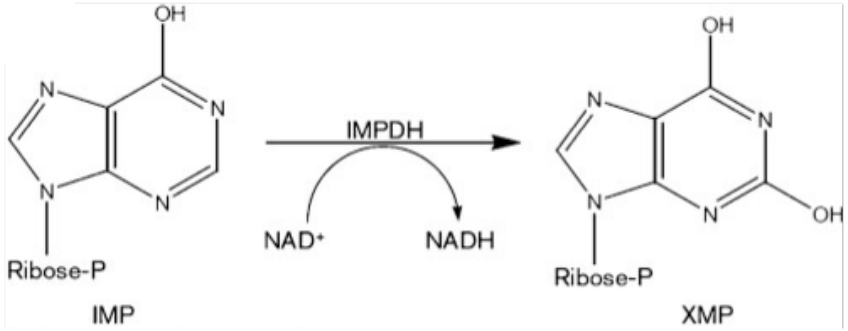


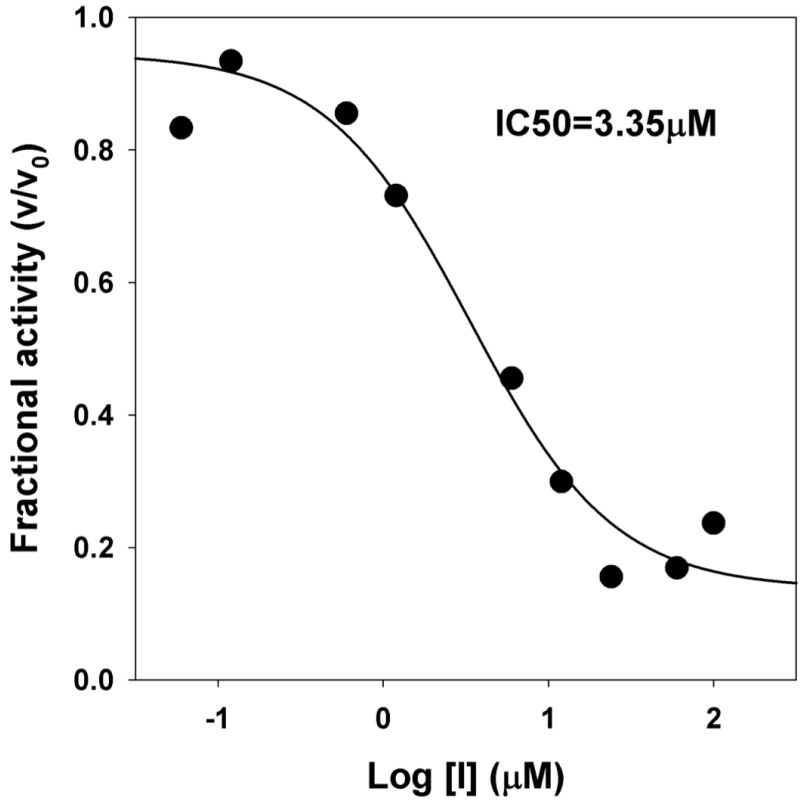


$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$

$$\mathrm{ES} + \mathrm{I} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{ESI}$$

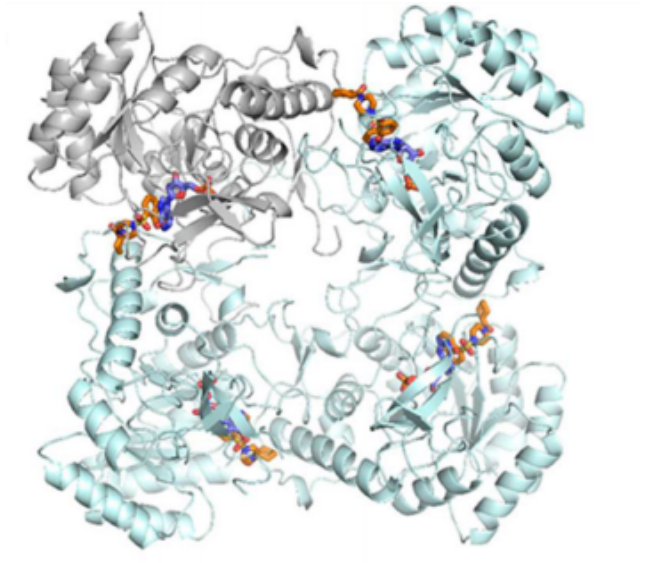
VCC718 is a selective uncompetitive Guab2 inhibitor

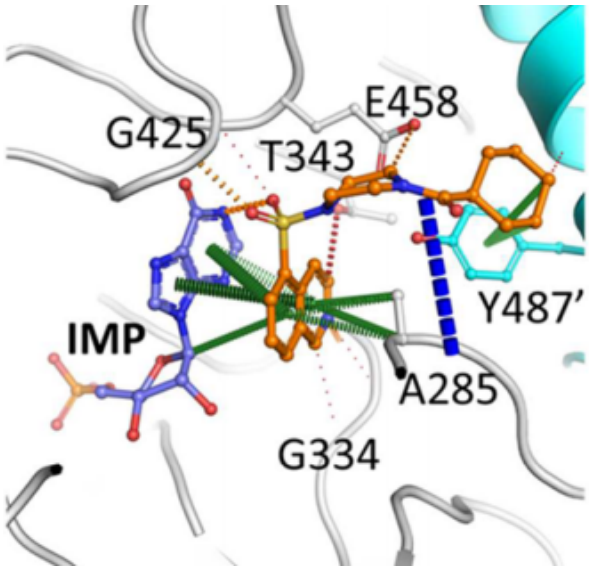




Binding of VC234718 to *M. thermoresistible* IMPDH

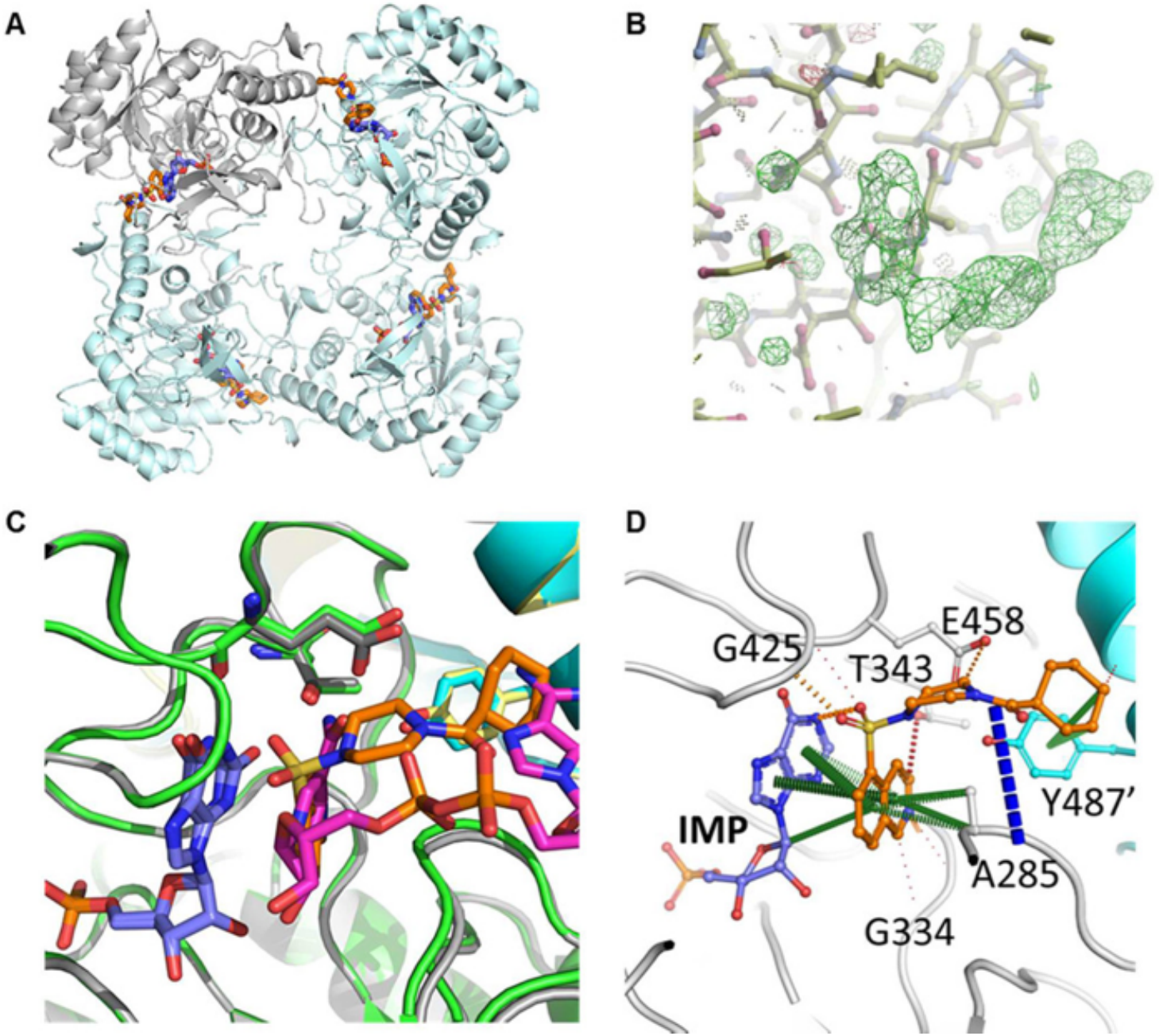
- 85% amino acid identity with the Mtb IMPDH
- 100% conservation of residues in the active site
- higher protein expression yields than the Mtb homologue. Mth
- ΔCBS domain (equally active as the wt)
- 1.6 Å resolution





Y487C
resistance-conferring mutation
➔ Disrupts the pi-stacking between Y and the cyclohexyl group of VCC234718

X-ray crystal structure of VCC234718 bound to *Mth* GuaB2



Conclusions

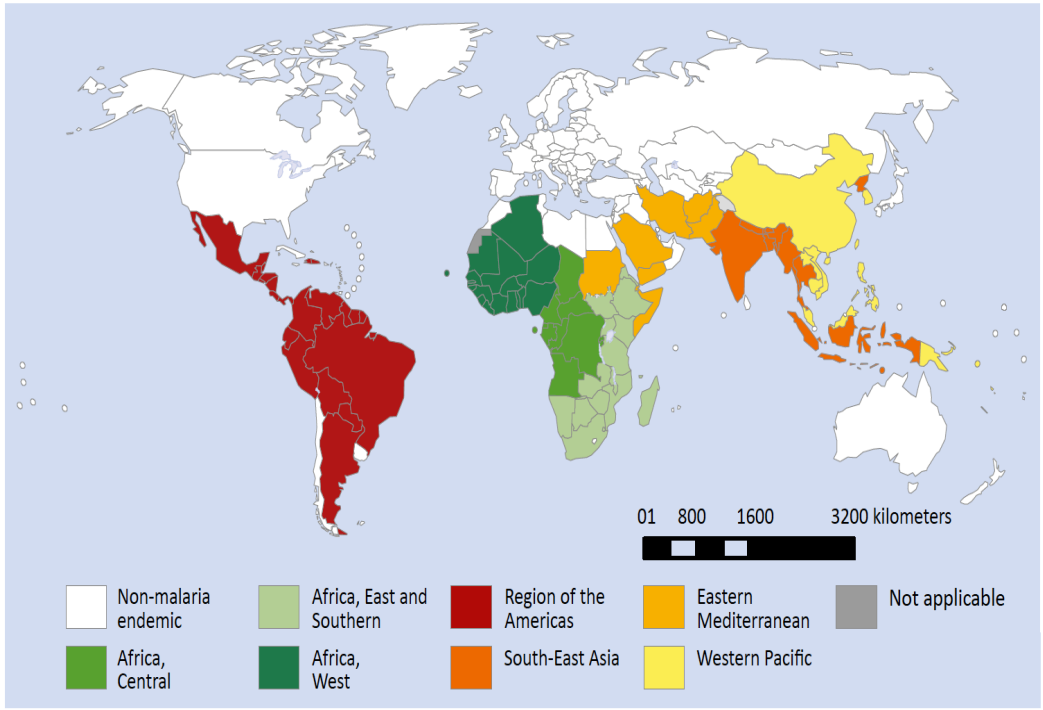
- Phenotypic screening has identifed **new sulfonamides Guab2 (IMPDH) inhibitors**
- Such inhibitors **target IMPDH activity**

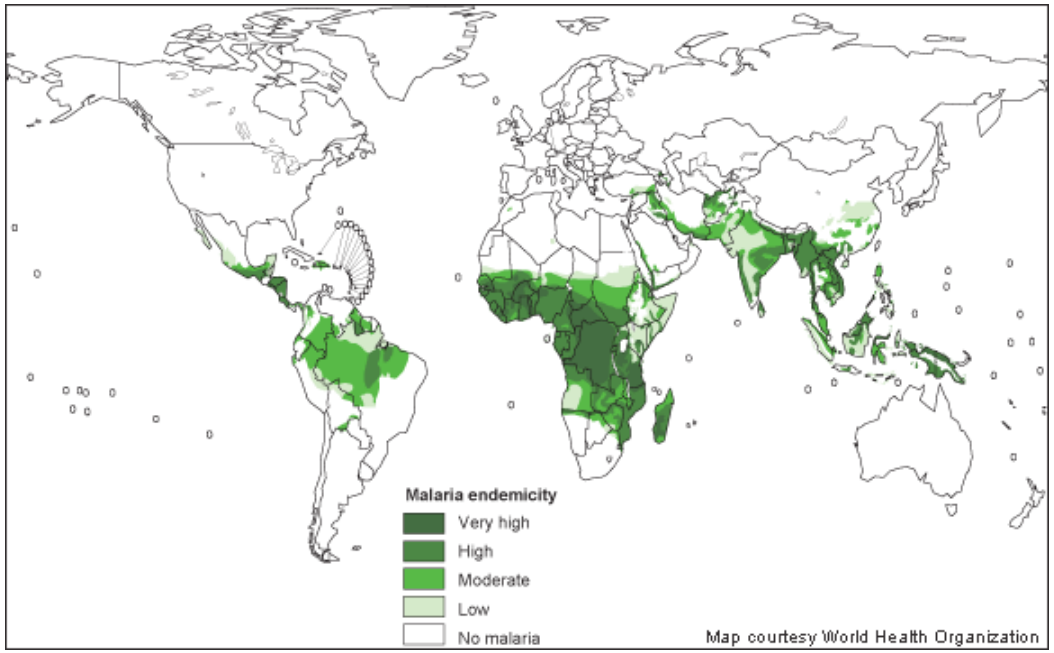
- IMPDH (Guab2) is **essential for *Mtb* infection**
- Guanine **could NOT** rescue *Mtb* in mice treated with *guaB2*-KO *Mtb* strains

➔ The vulnerability of GuaB2 still to be completely addressed

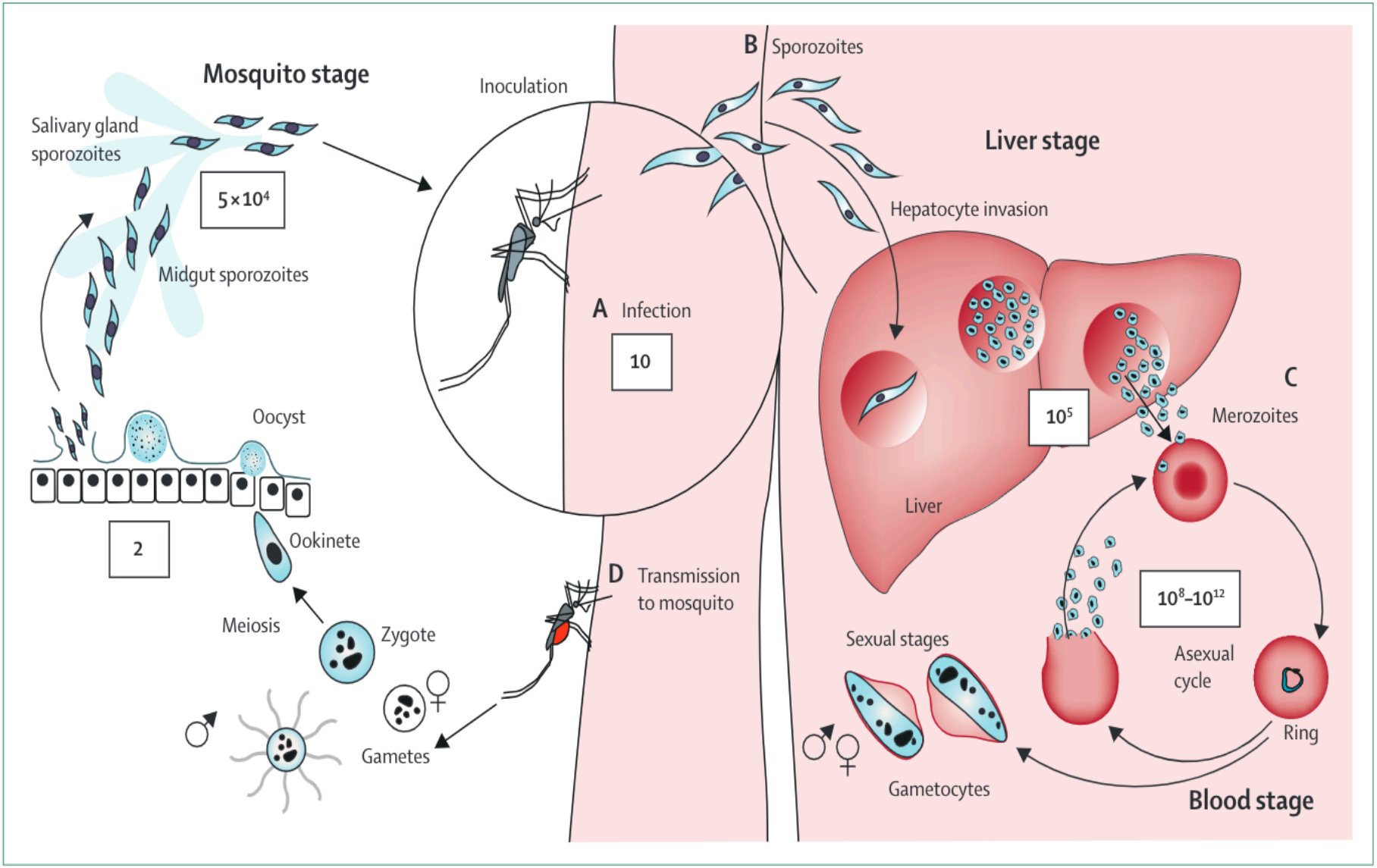
➔ Targeting of GuaB2 for the development of new anti-TB drugs can be considered.

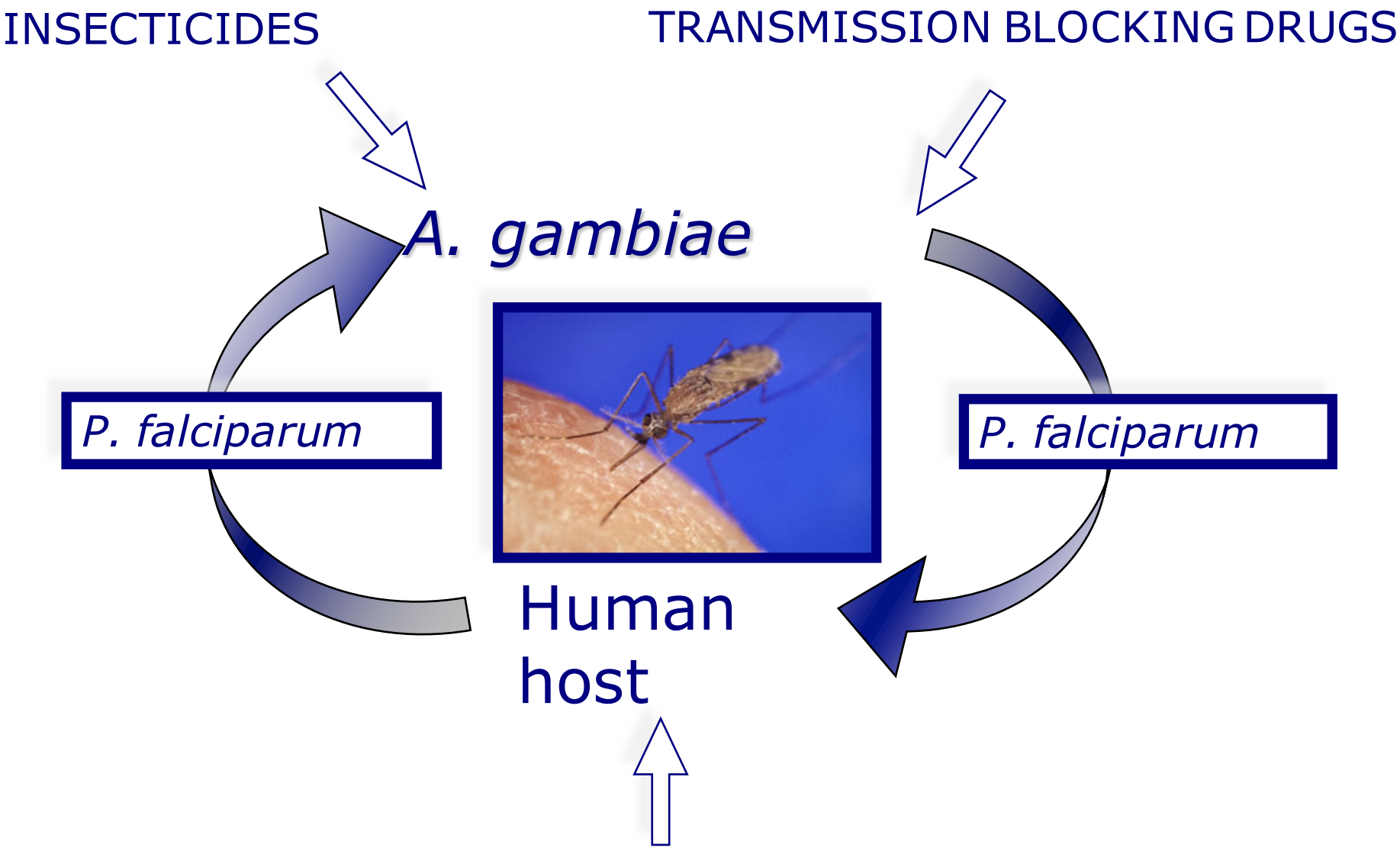
Malaria

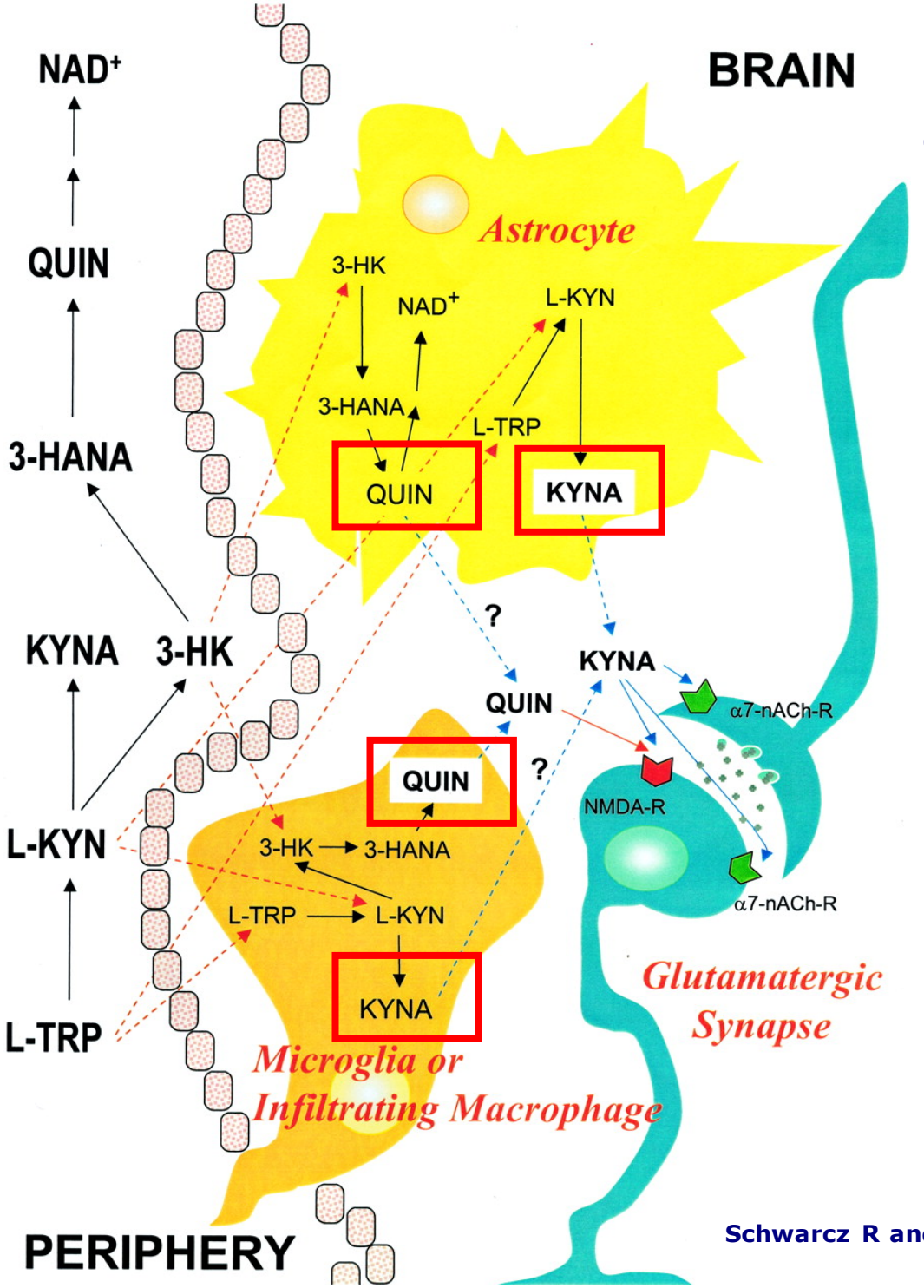

01 800 1600 3200 kilometers
Non-malaria endemic
Africa, East and Southern
Region of the Americas
Eastern Mediterranean
Not applicable
Africa, Central
Africa, West
South-East Asia
Western Pacific


Malaria endemicity
Very high
High
Moderate
Low
No malaria
Map courtesy World Health Organization

Malaria life cycle




INSECTICIDES
TRANSMISSION BLOCKING DRUGS
A. gambiae
P. falciparum
P. falciparum
Human host
ANTIMALARIAN DRUGS



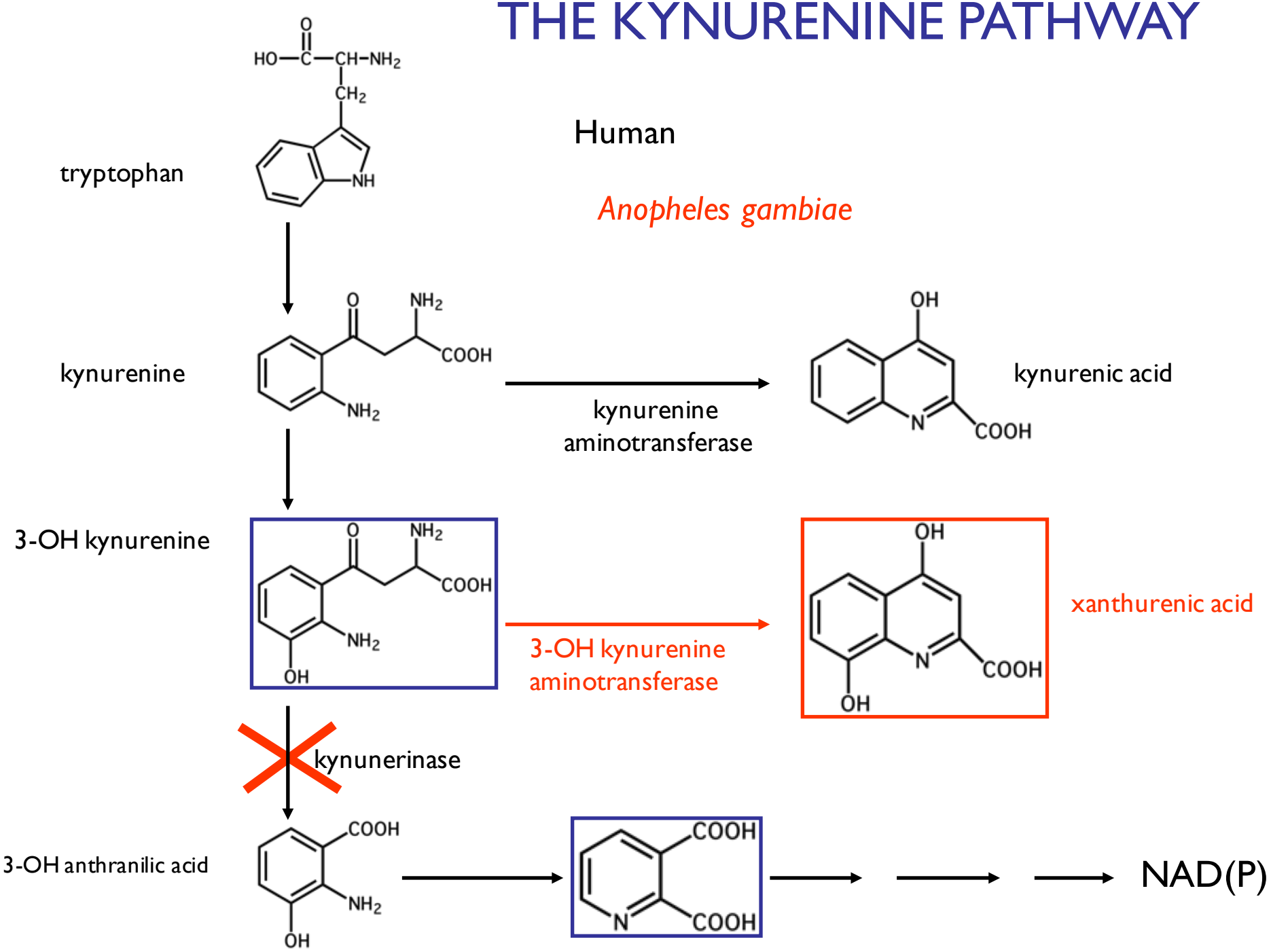
CEREBRAL AND EXTRACEREBRAL KYNURENINE PATHWAY

TRYPTOPHAN METABOLITES ARE NEUROACTIVE

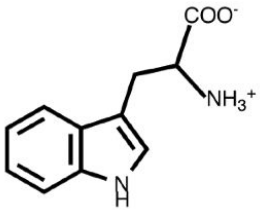
KYNURENINE PATHWAY: ATTRACTIVE TARGET FOR SEVERAL NEUROLOGICAL DISORDERS

Schwarcz R and Pelliciari R. J. Pharmacol Exp Ther. 2002, 303, 1-10

THE KYNURENINE PATHWAY



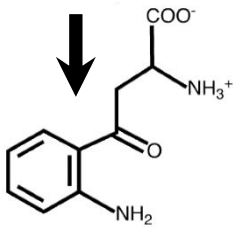
The kynurenine pathway in *A. gambiae*



Tryptophan

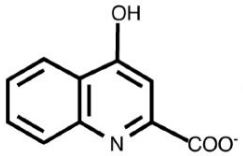
- PROTEIN SYNTHESIS
- NEUROACTIVE COMPOUND (*per se*)
- PRECURSOR OF SEROTONIN AND MELATONIN
- *NEUROTOXIN (in excess)*

TDO



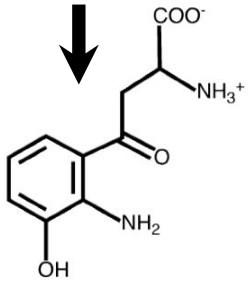
L-kynurenine

KAT



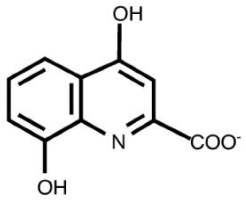
Kynurenic acid

- NEUROPROTECTION
- MODULATOR OF LEARNING



3-OH-kynurenine

3-HKT



Xanthurenic acid

DETOXIFICATION PRODUCT

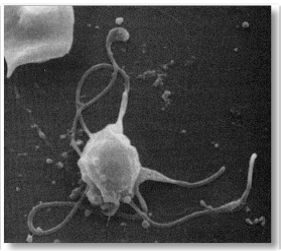
During pre-adult stages

- PRECURSOR OF EYE PIGMENTS
- PROMOTES CELL DEATH DURING METAMORPHOSIS

In adult insects

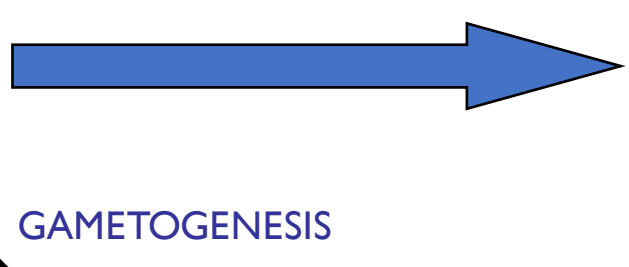
- *NEUROTOXIN (in excess)*

In infected mosquitoes: trigger of parasite male gametogenesis



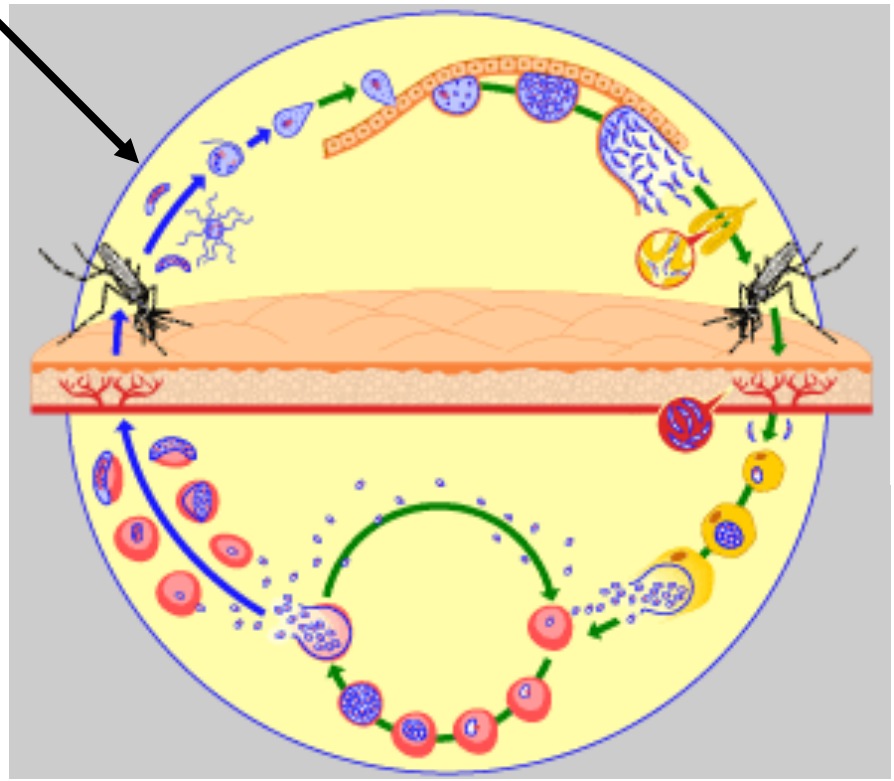
THE MALARIA CYCLE

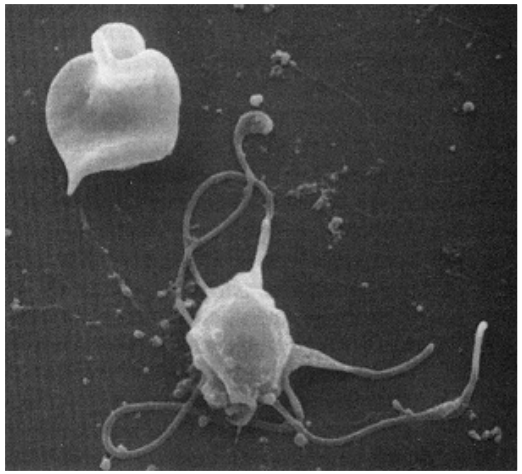
GAF



xanthurenic acid

GAMETOGENESIS





Garcia GR, et al J. Biol. Chem., 1996

Billker O et al. Nature, 1998,

XA concentration required to sustain half-maximal gametogenesis: *P. berghei* 9.0 μM; *P. falciparum* 2.0 μM

XA concentration in a typical human blood meal: 0.6 – 0.8 μM

The *Anopheles gambiae* 3HKT

3-hydroxykynurenine
(3-HK)

Xanthurenic Acid
(XA)

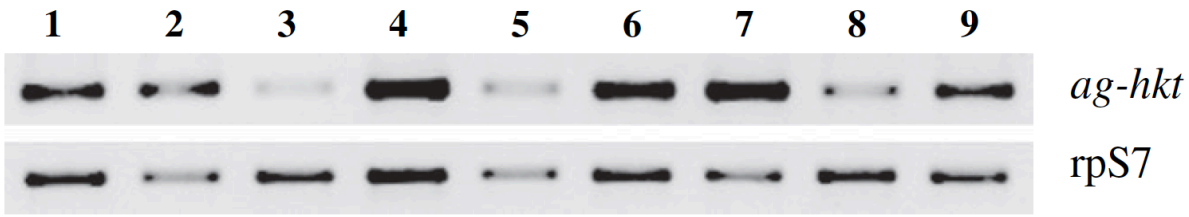
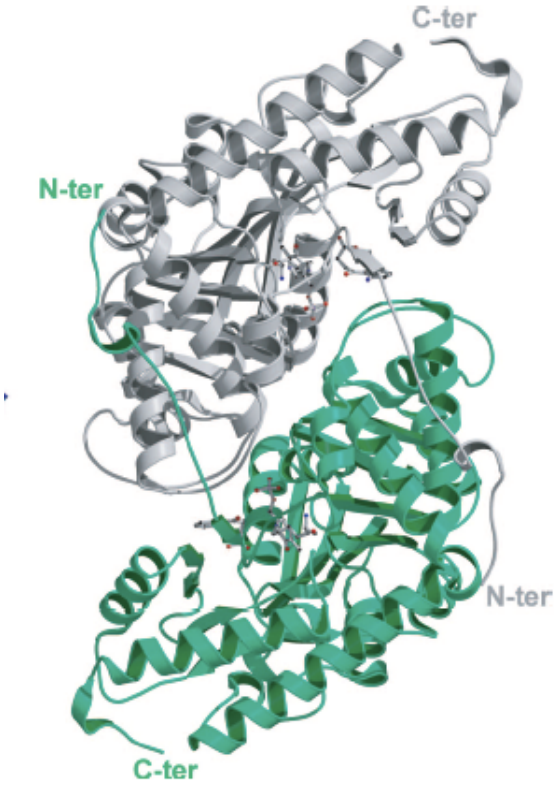
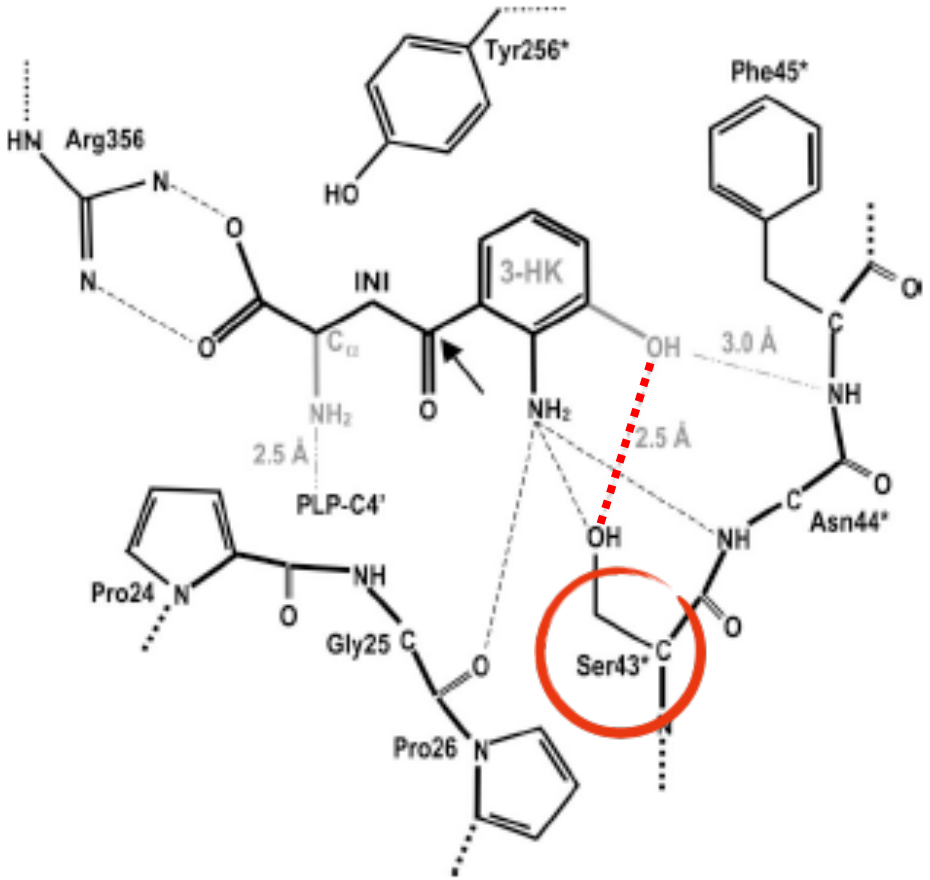


Fig. 2. Developmental- and tissue-specific *Ag-hkt* expression analysis. RT-PCR amplification of total RNA from different tissues and developmental stages with *Ag-hkt*- and *rpS7*-specific primers. Lanes: 1, embryos; 2, larvae; 3, pupae; 4, adult females; 5, salivary glands; 6, ovaries; 7, midgut; 8, carcasses (adult females with salivary gland, ovaries and gut removed); 9, adult males.

Rossi F., *et al.* (2005) *FEBS Journal* **272**: 5653–5662
Identification and biochemical characterization of the *Anopheles gambiae* 3-hydroxykynurenine transaminase.

Ag3HKT: structure-based site-directed mutagenesis





Crystal structure of the *Anopheles gambiae* 3-hydroxykynurenine transaminase

Franca Rossi[†], Silvia Garavaglia[†], Giovanni Battista Giovenzana[†], Bruno Arcà[‡], Jianyong Li[§], and Menico Rizzi[†¶]

PNAS | April 11, 2006 | vol. 103 | no. 15 | 5711–5716

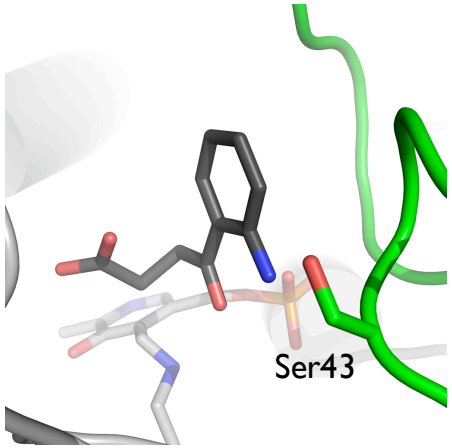
Ag3HKT: structure-based site-directed mutagenesis

Ag3HKT mutant S43A

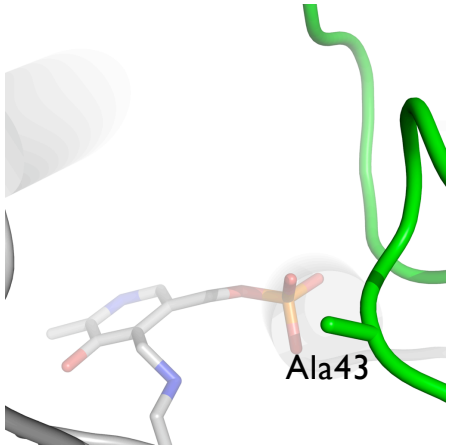
steady-state kinetics

		Wild-type	S43A
D,L 3OH kynurenine	*kcat* (sec^{-1})	5.8	**20.3**
	K_M (mM)	0.6	**6.7**
	kcat/K_M (sec^{-1}/mM)	9.6	**3.0**
D,L kynurenine	*kcat* (min^{-1})	5.8	**12.1**
	K_M (mM)	2.0	**6.3**
	kcat/K_M (sec^{-1}/mM)	2.9	**1.92**
L kynurenine	*kcat* (min^{-1})	6.0	**14.8**
	K_M (mM)	1.0	**4.9**
	kcat/K_M (sec^{-1}/mM)	6.0	**3.0**

Ag3HKT, wt

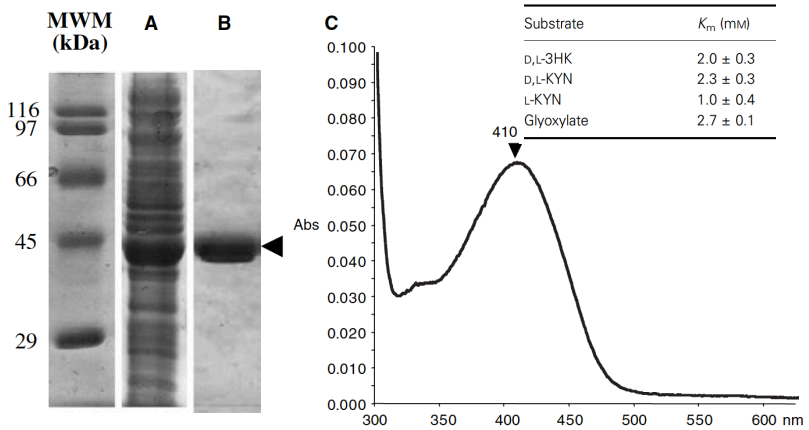


Ag3HKT-S43A



Biochemical and structural analysis of Anopheles gambiae 3-HKT (Ag-HKT)

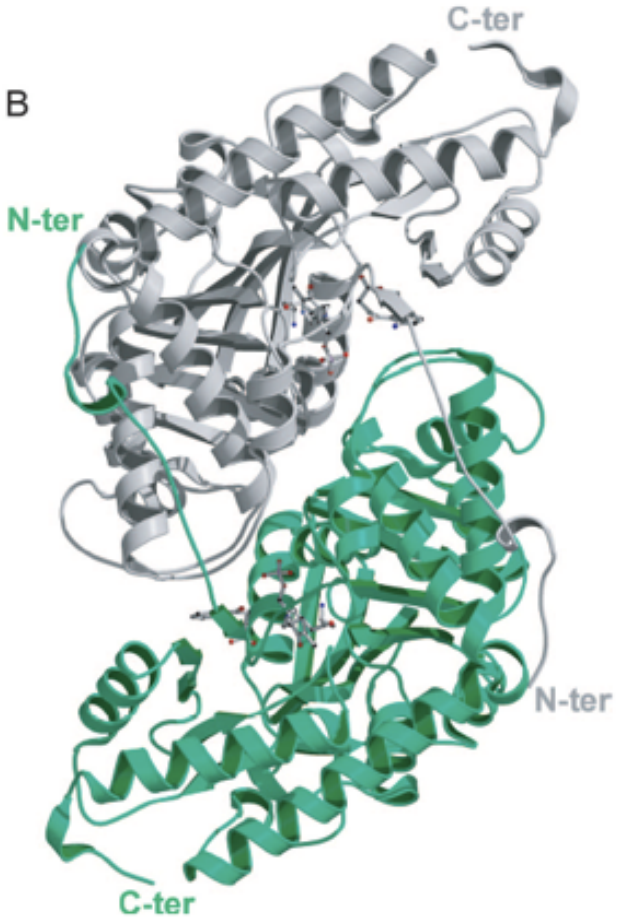
Recombinant Ag-HKT expression, purification and characterization



Substrate	K_m (mM)
D,L-3HK	2.0 ± 0.3
D,L-KYN	2.3 ± 0.3
L-KYN	1.0 ± 0.4
Glyoxylate	2.7 ± 0.1

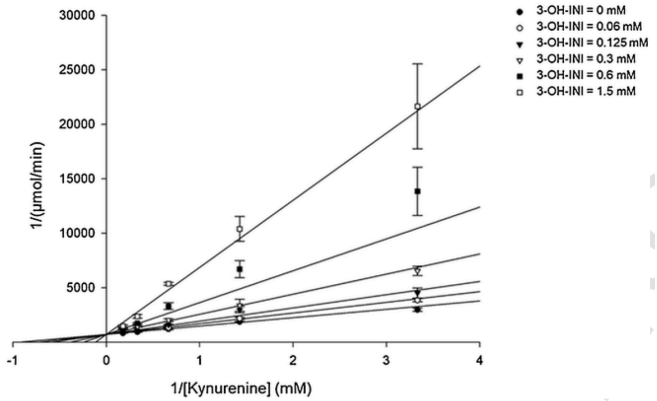
Rossi F., *et al.* (2005) *FEBS J* **272**: 5653–5662

X-ray crystallography-based study of Ag-HKT structure



Rossi F., *et al.* (2006) *Proc Natl Acad Sci U.S.A.* **103**: 5711–5716

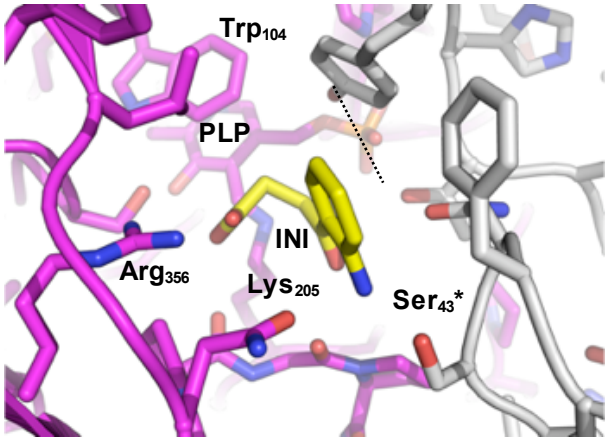
HPLC-MS/MS-based study of Ag-HKT inhibition



Canavesi R., et al. (2019) J Pharm Biomed Anal **173**:154-161

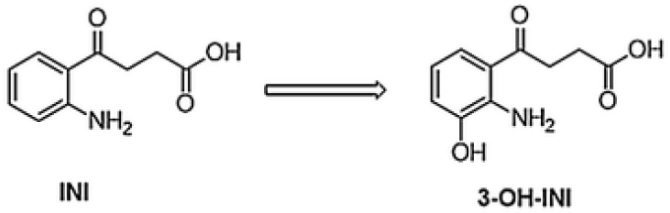
close-up of Ag-HKT ligand binding site

crystal structure of Ag-HKT::INI

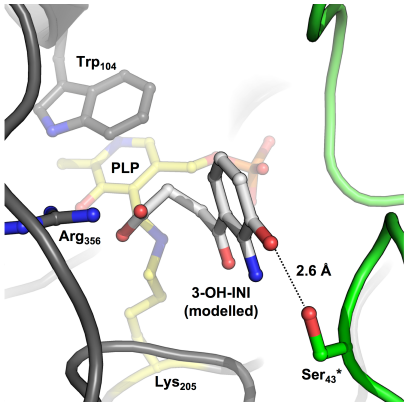


PDB code: 2ch2

structure-based
rational inhibitor modification



(model)

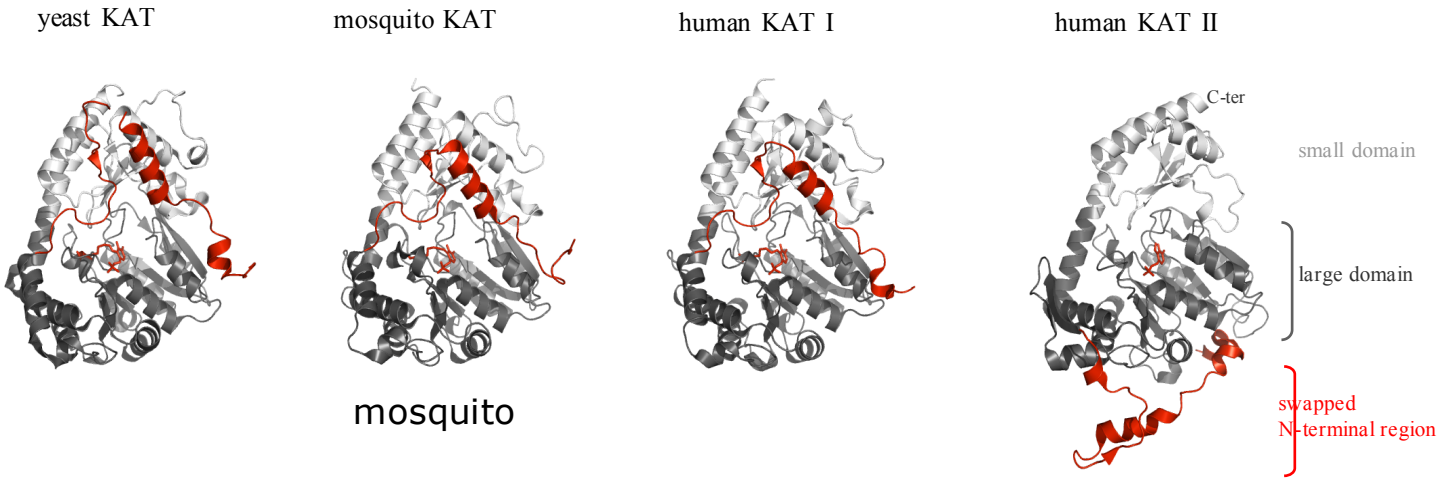


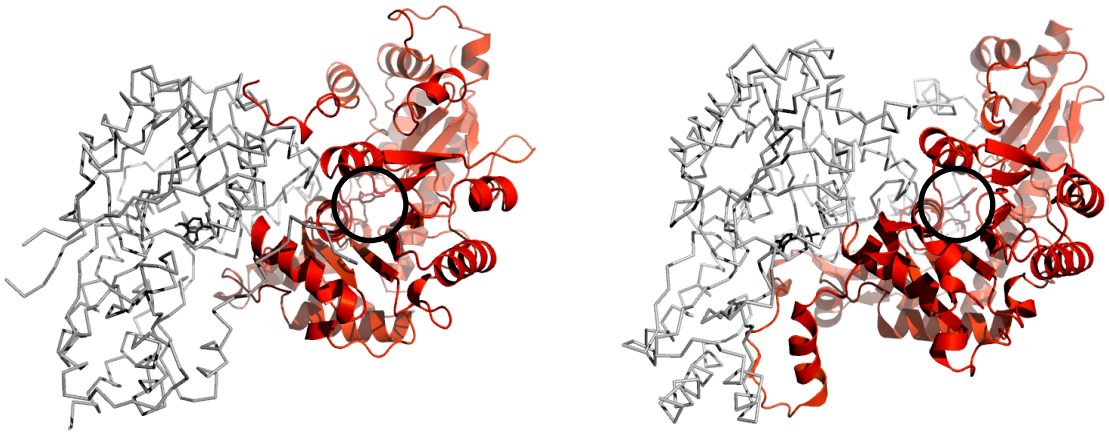
chemical synthesis

3-OH-INI displays "improved" Ki
compared to the INI molecule,
although still in the high micromolar range (125 μM)

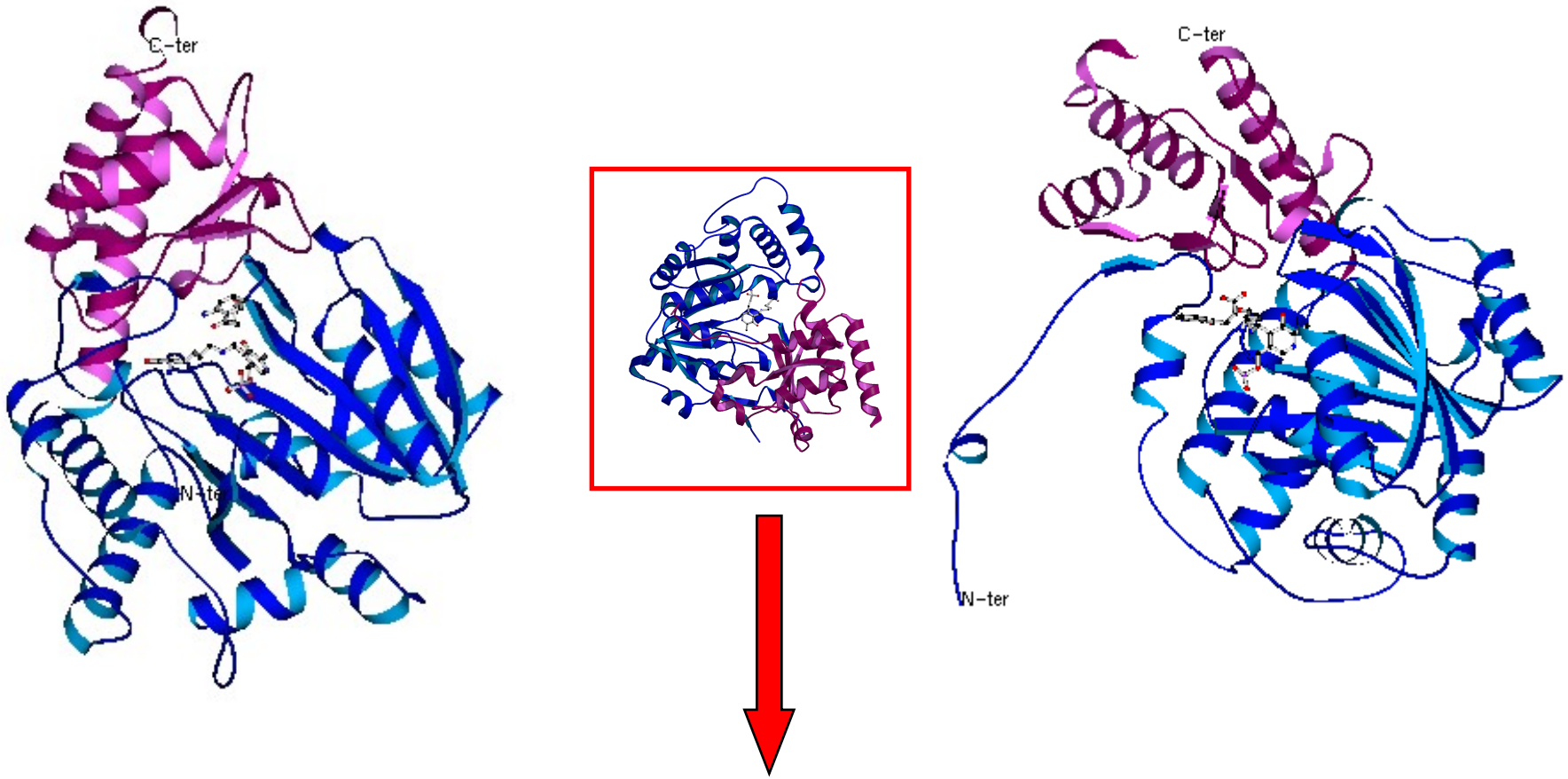
KAT I vs KAT II

kynurenine aminotransaminase



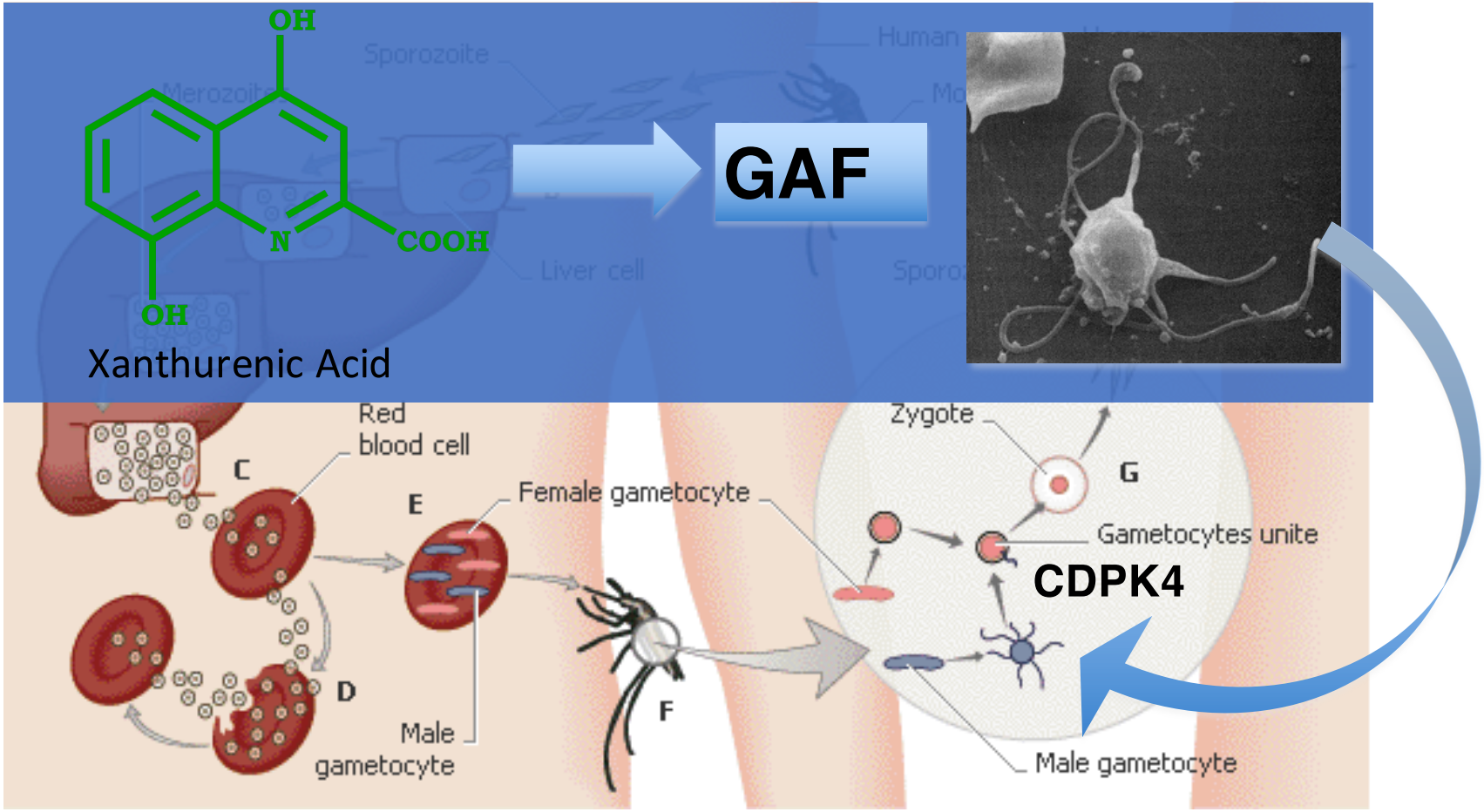


Rossi et al. Curr. Opin. Struct. Biol., 2008, 6, 748-55

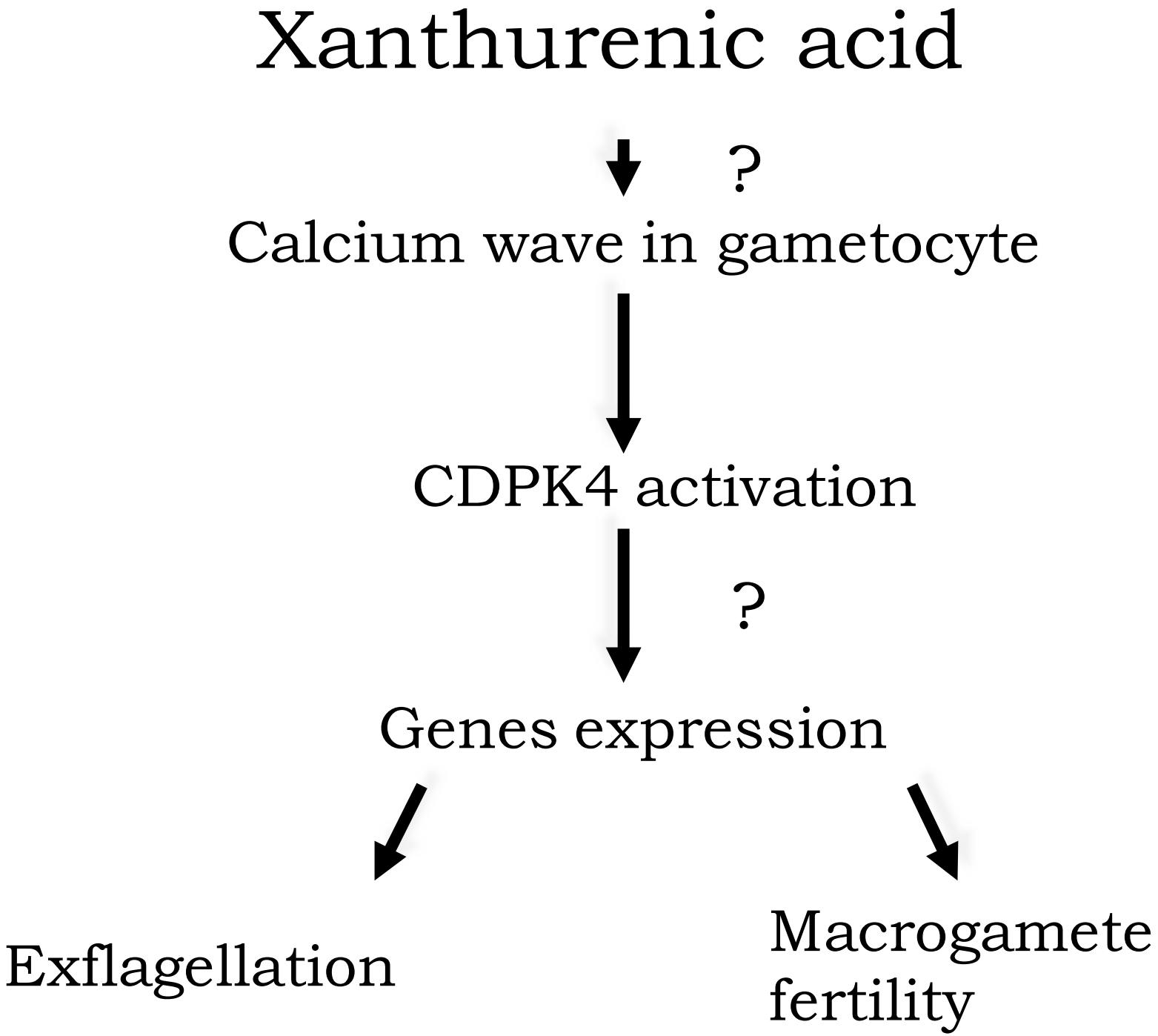


Human KatI *vs A. gambiae* 3-HKT 5.1 Å rmsd over 320 aa

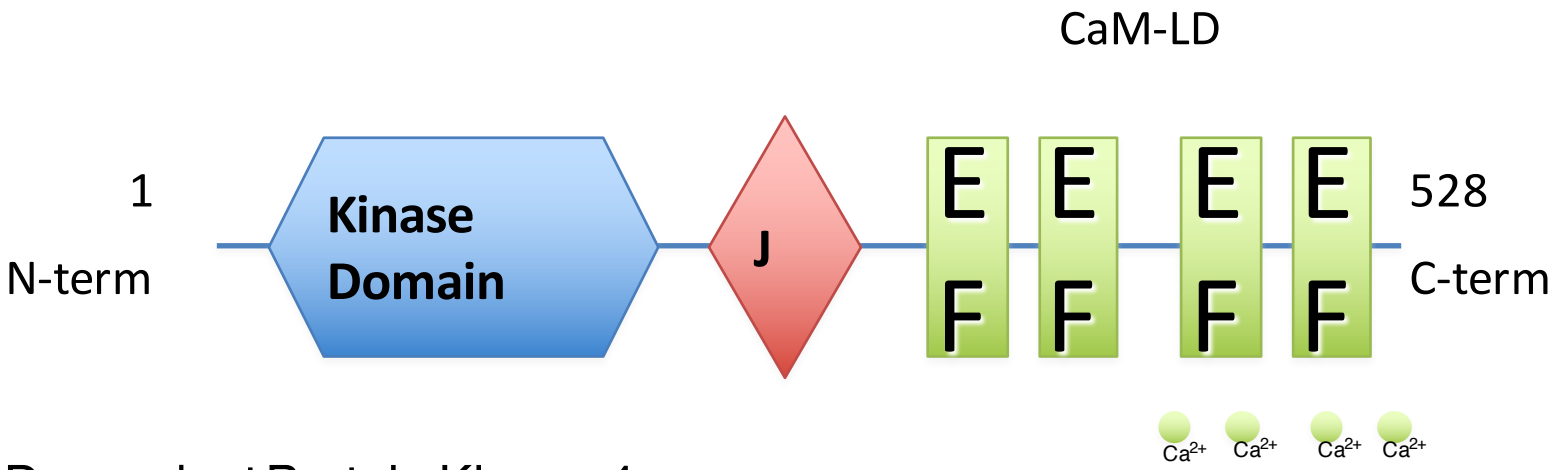
The Xanthurenic Trigger



Calcium and a Calcium-Dependent Protein Kinase Regulate Gamete Formation and Mosquito Transmission in a Malaria Parasite
O. Billker et al.; **Cell**, Vol. 117, 503–514, May 14, 2004


Xanthurenic acid
?
Calcium wave in gametocyte
CDPK4 activation
?
Genes expression
Exflagellation
Macrogamete
fertility

Pf-CDPK4



Calcium-Dependent Protein Kinase 4

CDPKs family (plants, Apicomplexa)

Absence in human kinome

CDPK4 K.O. → **NO EXFLAGELLATION**

INHIBITOR → **TRASMISSION BLOCKER**

Looking for CDPK4 inhibitors

Biochemistry **1994,** *33,* 15259−15265

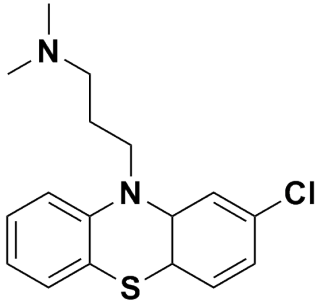
Drug Binding by Calmodulin: Crystal Structure of a Calmodulin−Trifluoperazine Complex[†,‡]

William J. Cook,[*,§,||] Leigh J. Walter,[||] and Mark R. Walter[||,⊥]

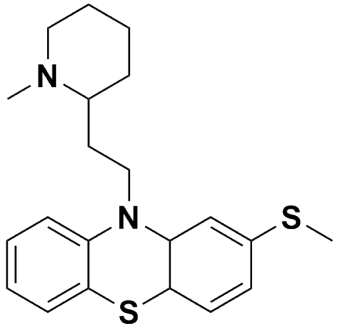
Departments of Pathology and Pharmacology and Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received July 19, 1994; Revised Manuscript Received October 18, 1994[⊗]

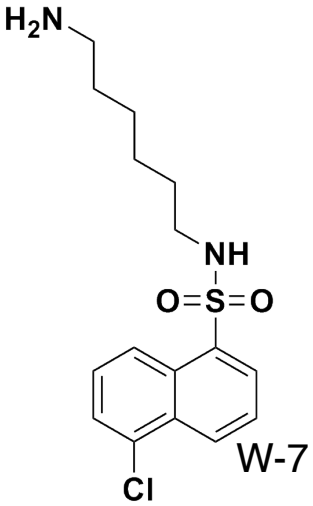
Looking for CDPK4 inhibitors

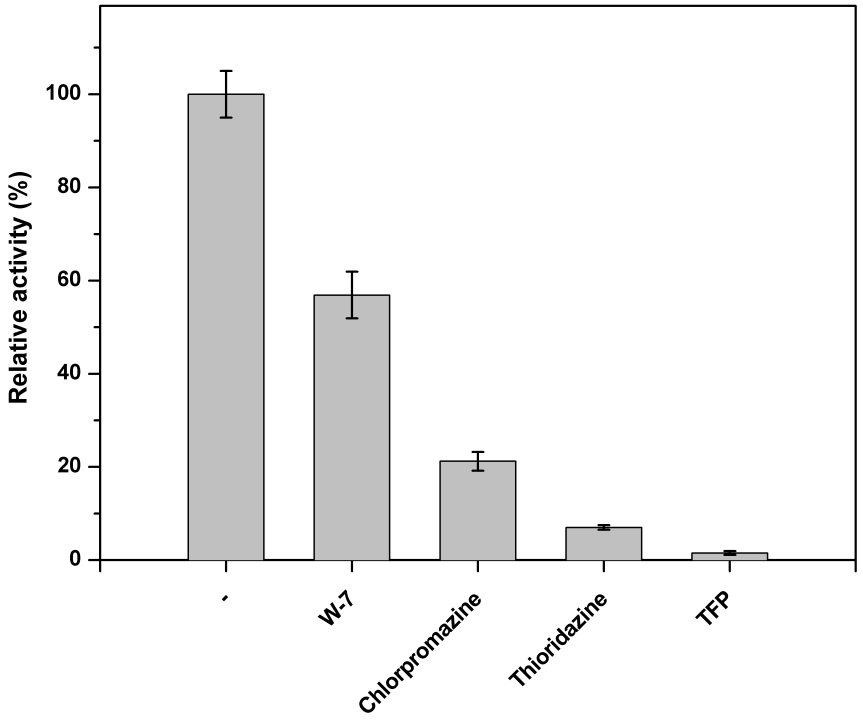


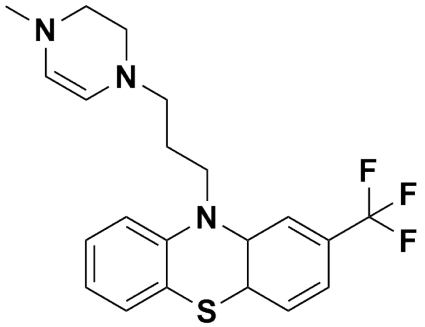
Chlorpromazine



Thioridazine







Trifluoperazine (TFP)

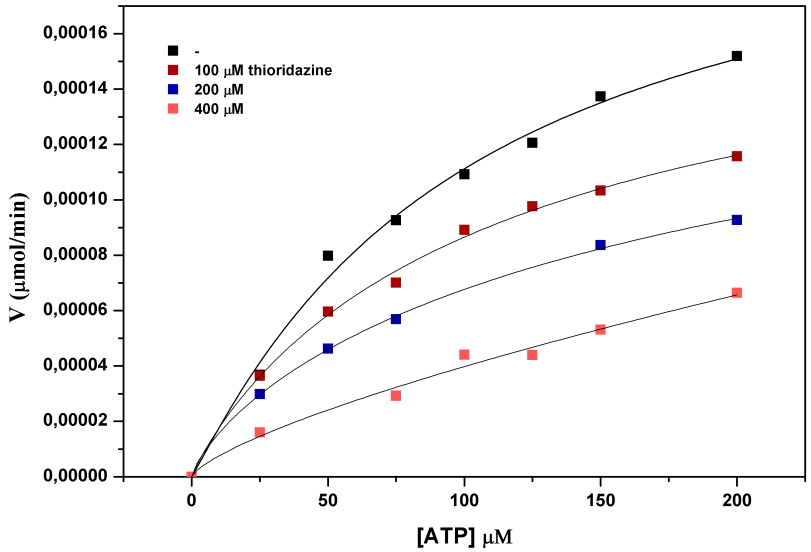
Inhibition mechanism characterisation

Kinetic parameters: V_{max}= 0,155 nmol/min; K_M= 106 µM; k_{cat}= 48,9 min^{-1}

Assay conditions: 50 mM Tris-HCl pH 7.5; 4 mM $MgCl_2$; 2 mM $CaCl_2$; 150 µM substrate; Δ [ATP]; 0.5 µg CDPK4

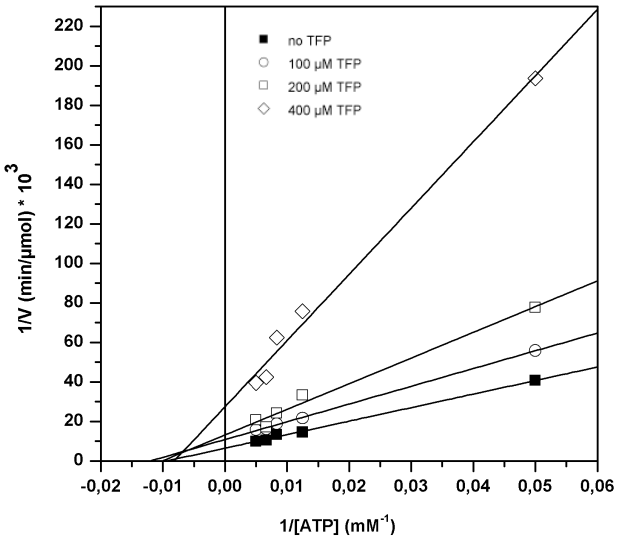
Time: 10’ Temperature: 30°C

Thioridazine



K_i > 200 µM

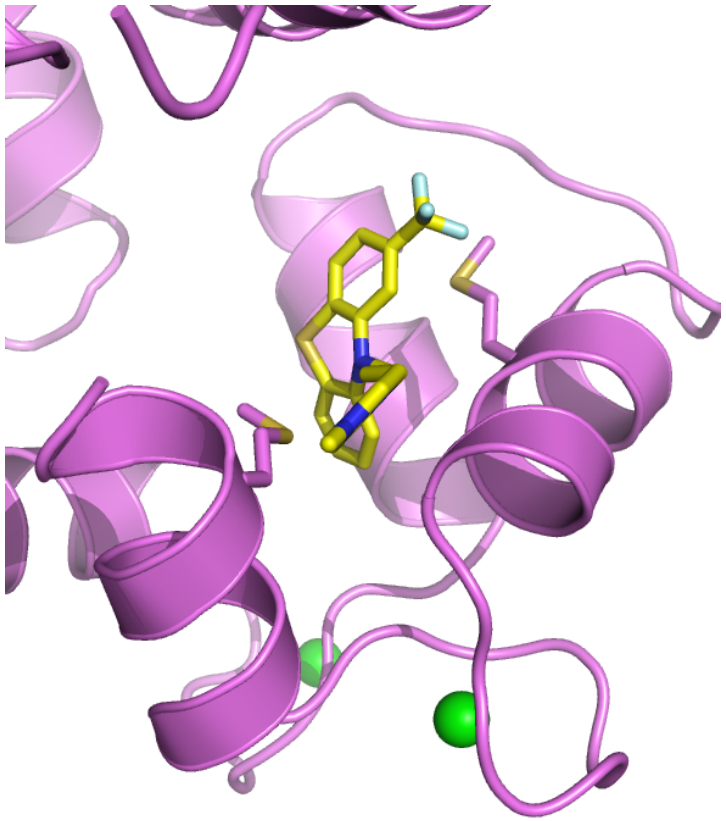
Trifluoperazine (TFP)



K_i = 150 µM

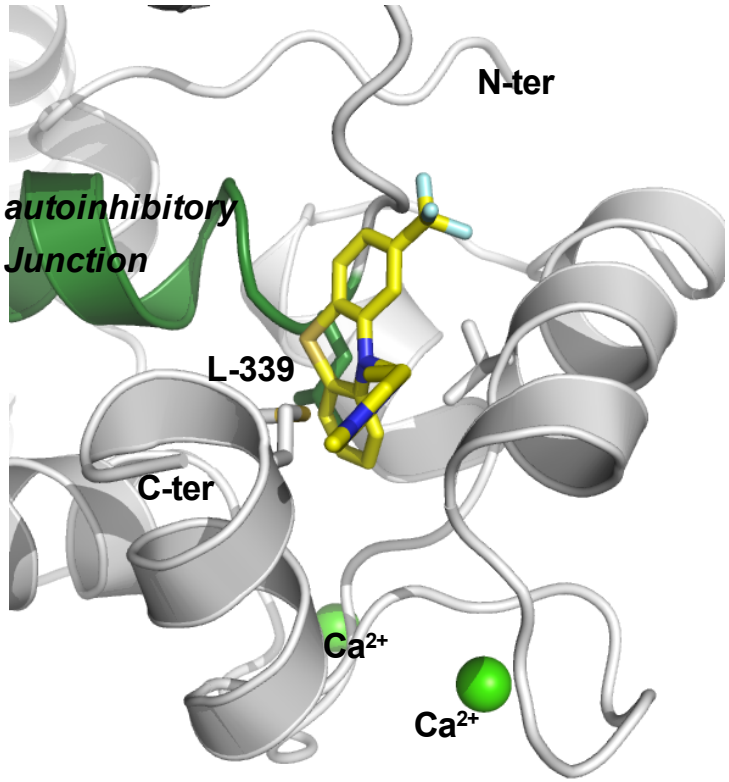
Inhibitory theory

CaM – TFP complex



Identity > 70 %

*Tg*CDPK1



Identity 74.4 %

Acknowledgments

- **ORGANIZERS !!!**
- **ALL OF YOU FOR YOUR ATTENTION !!**