Novel therapeutics against free living amoebae infections

Dr Jacob Lorenzo-Morales

University Institute of Tropical Diseases and Public Health of the Canary Islands

University of La Laguna

Free Living Amoebae

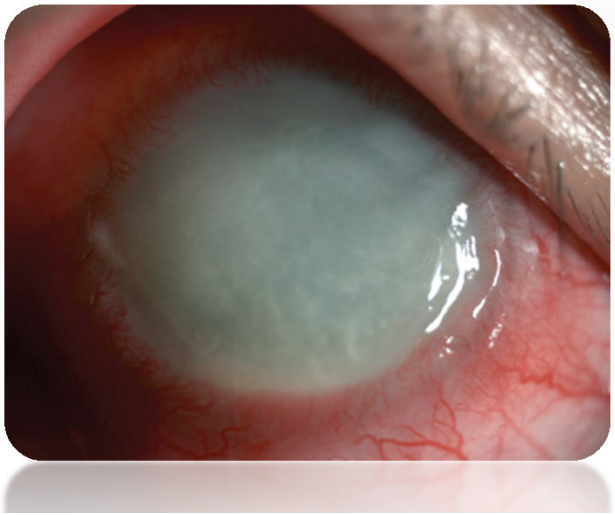
- Emerging Parasitic Protozoa.
- Life Cycle.
  - Trophozoite.
  - **Cyst**.
- Species/Genera:
  - *Naegleria fowleri*
  - *Balamuthia mandrillaris*
  - *Sappinia diploidea/pedata*
  - *Acanthamoeba* spp.



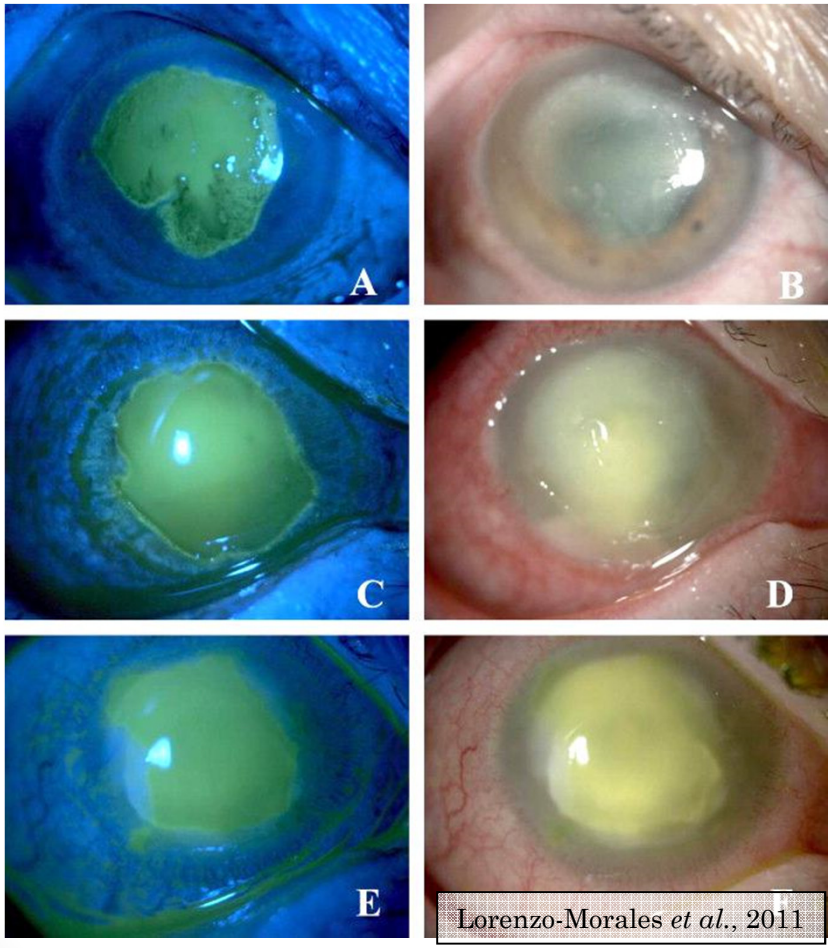
Pathology

- Encephalitis
  - Fatal in more than 90% of cases
- Keratitis
  - *Acanthamoeba* spp.
  - Issues:
    - Diagnosis
    - Therapy





Acanthamoeba spp.



Lorenzo-Morales *et al.*, 2011

- Invasive infection of the cornea.
- Non specific symptons.
- Incidence rising.

Main risk group:
Contact Lens Wearers

Acanthamoeba spp. Therapy

Current first line treatments:

- Topical application of **diamidines** (propamidine isotionate or hexamide)
- And cationic antiseptics: **biguanides** (polihexametilenbiguanide , (PHMB) or chlorhexidine digluconate)





WHAT WE DO IN OUR LAB

- Therapy
  - Chemotherapy→ novel active principles and products in the market
  - Molecular Therapy→ siRNA
  - Combination→ elucidation of drug targets
- Translational Medicine→ ULL-HRYC

ISSUES TO SOLVE

- FLA biology is still under study
- Axenic media are required for *B. mandrillaris* chemotherapy assays
- Pathogenecity is poorly understood.
- Cyst stage
- In vitro FLA chemotherapy assays → standardization
- *In vivo* models

TARGETS

Cytoskeleton (Actin, myosin, α & β tubulin)
Inhibitors: unknown (current known inhibitors have no effect)

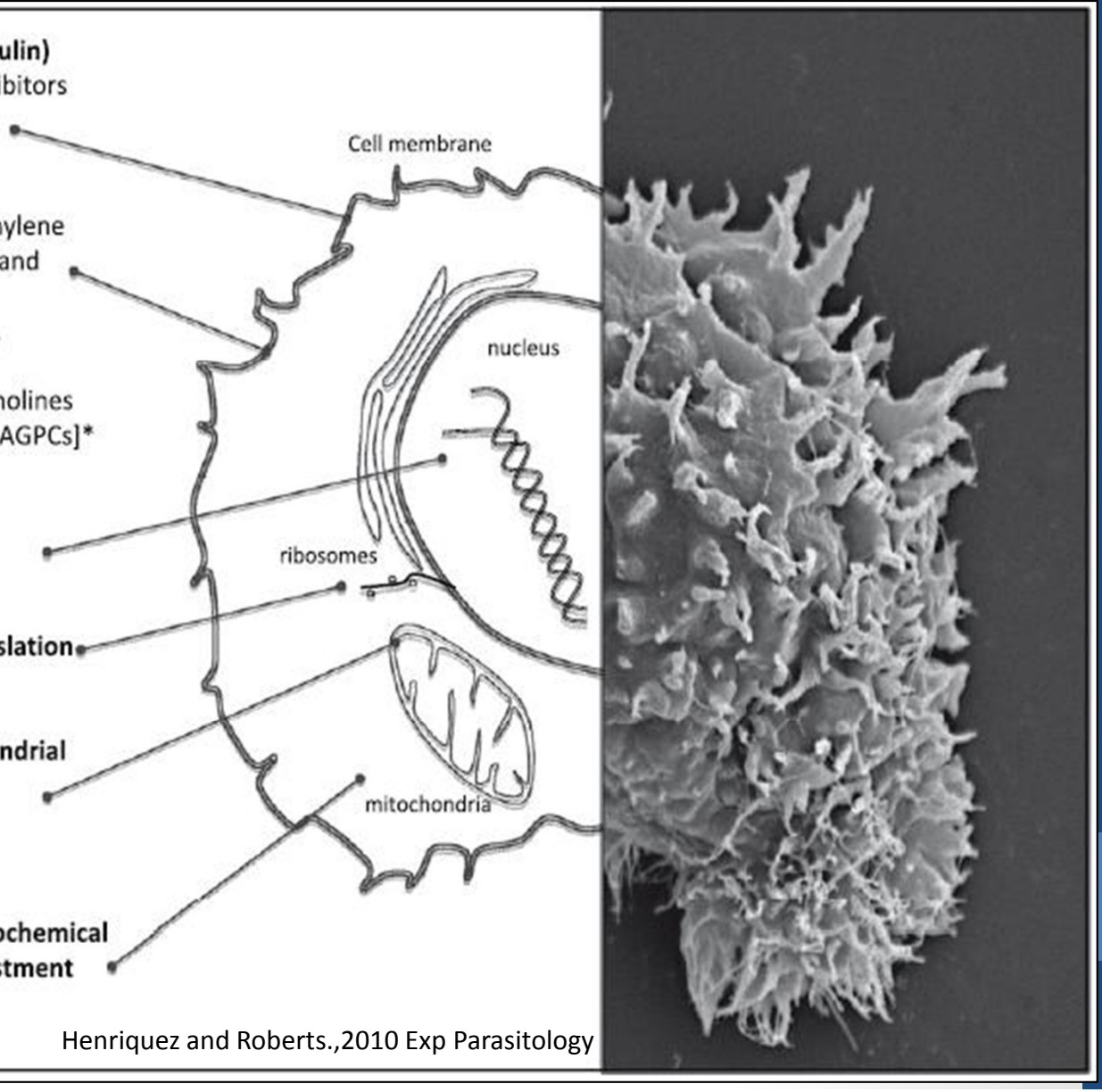
Membrane
Inhibitors: chlorhexidene, polyhexamethylene biguanide, acriflavine hydrodrochloride and proflavin, polymyxin B, amphotericin B, ketoconazole, miconazole, clotrimazole, fluconazole target sterols.
Phospholipid analogues (alkylphosphocholines [APCs] & alkylglycerolphosphocholines [AGPCs]*

Nucleic Acid
Inhibitors: 5-fluorocytosine (5-FC), RNAi

Polyamines biosynthesis (ornithine decarboxylase), effects on protein translation
Inhibitors: DFMO

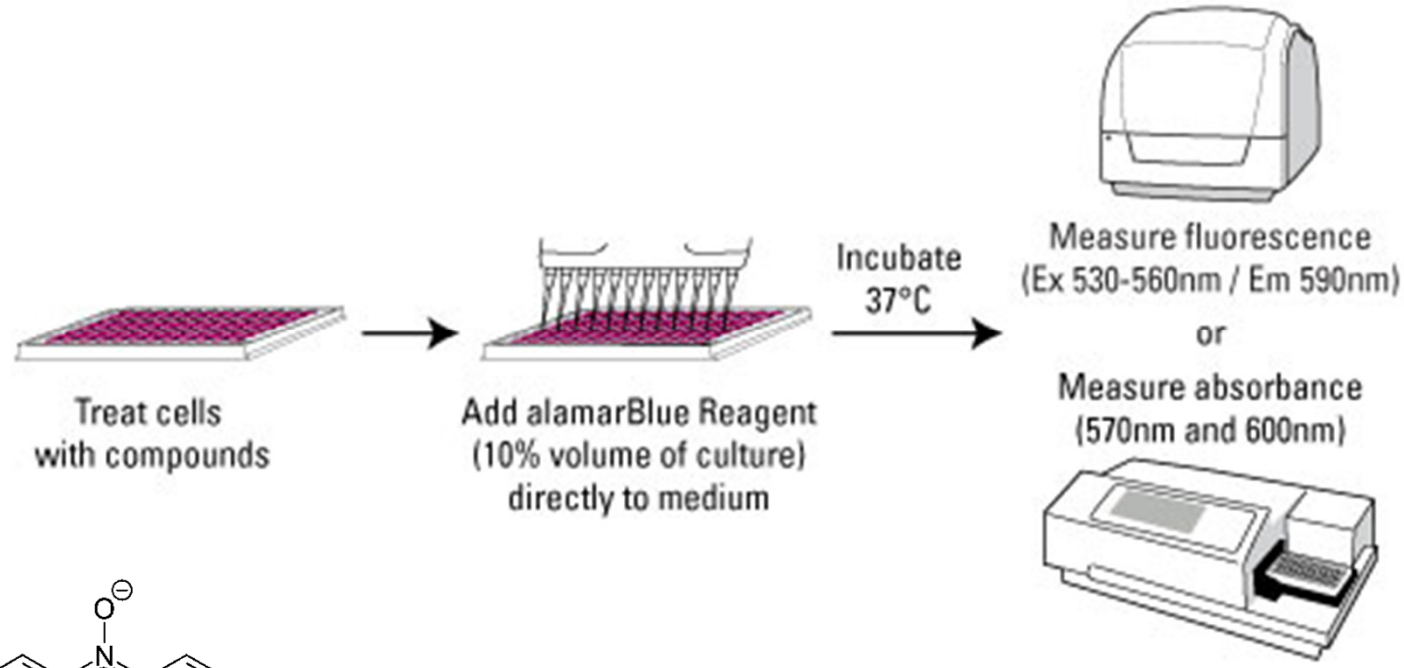
Alternative oxidase (AOX) and mitochondrial respiration
Inhibitors: SHAM, Ascofuronone,

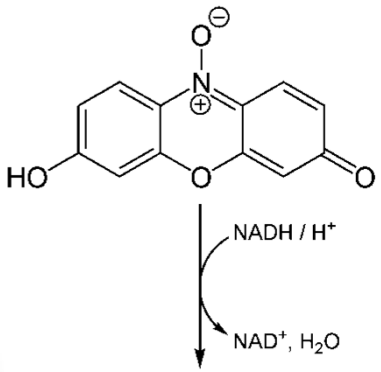
Differentiation to cysts
Cellulose Biosynthesis and Proximal Biochemical (e.g. glycogen metabolism) affect encystment
Inhibitors: 2,6-bichlorobenzonitrile



Henriquez and Roberts.,2010 Exp Parasitology

Alamar Blue Assay





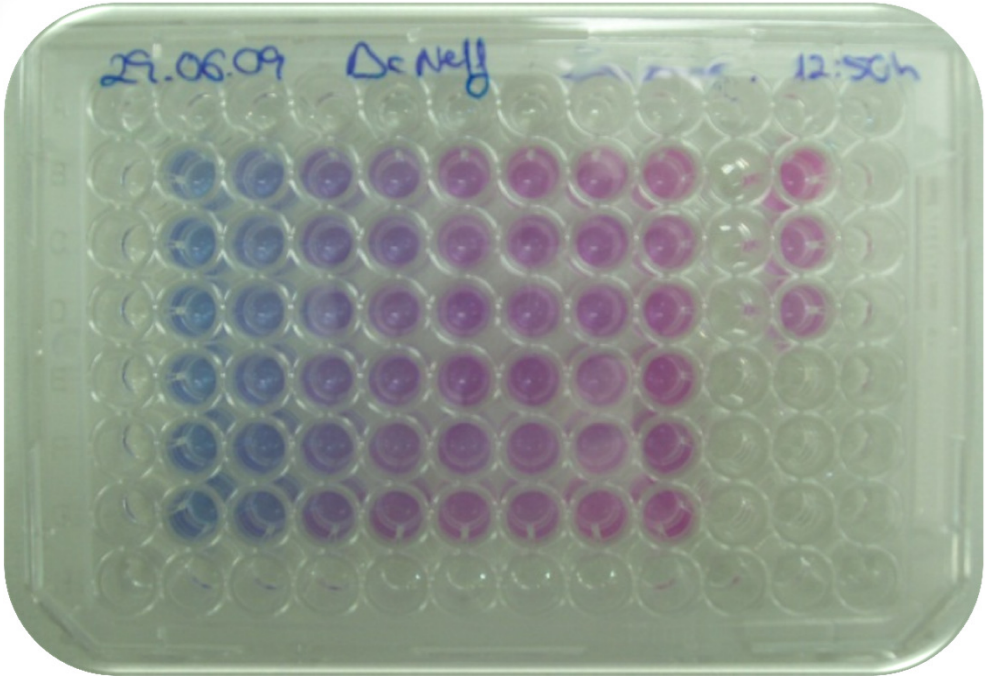
Resazurin blue

Resorufin pink

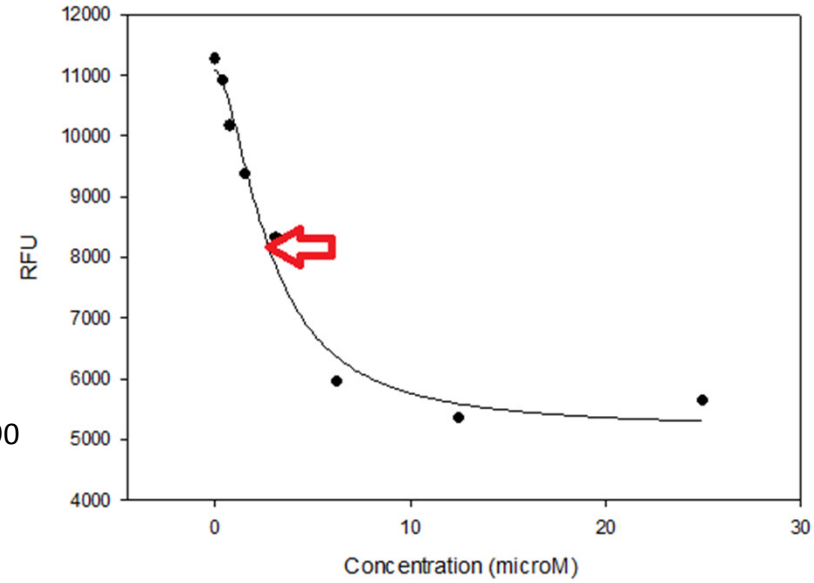


Universidad de La Laguna

Chemotherapy assays

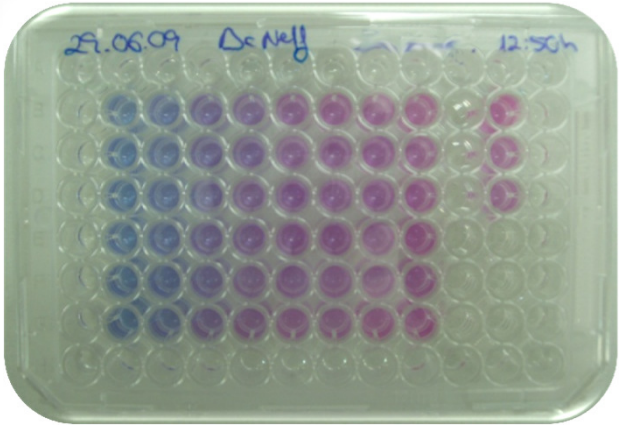


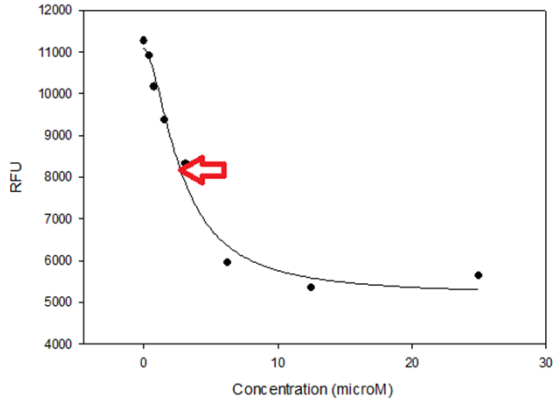
IC_{50} and IC_{90}

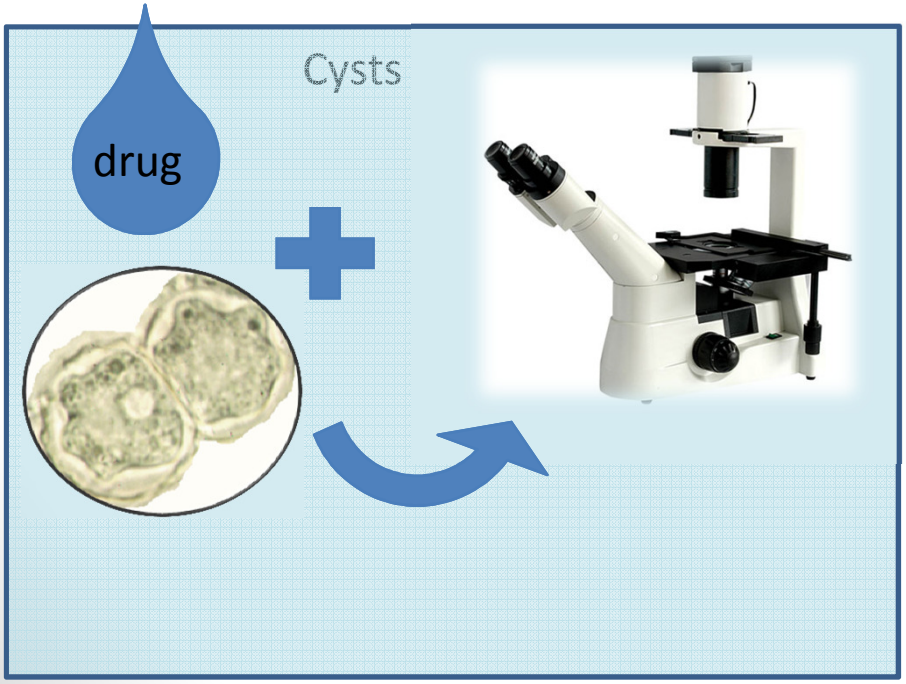


Universidad de La Laguna

Chemotherapy assays



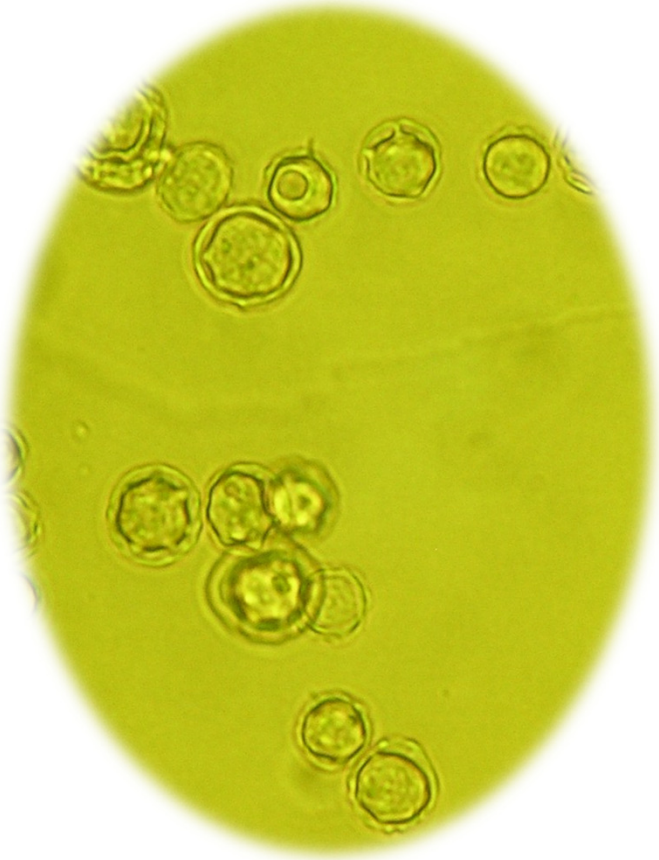


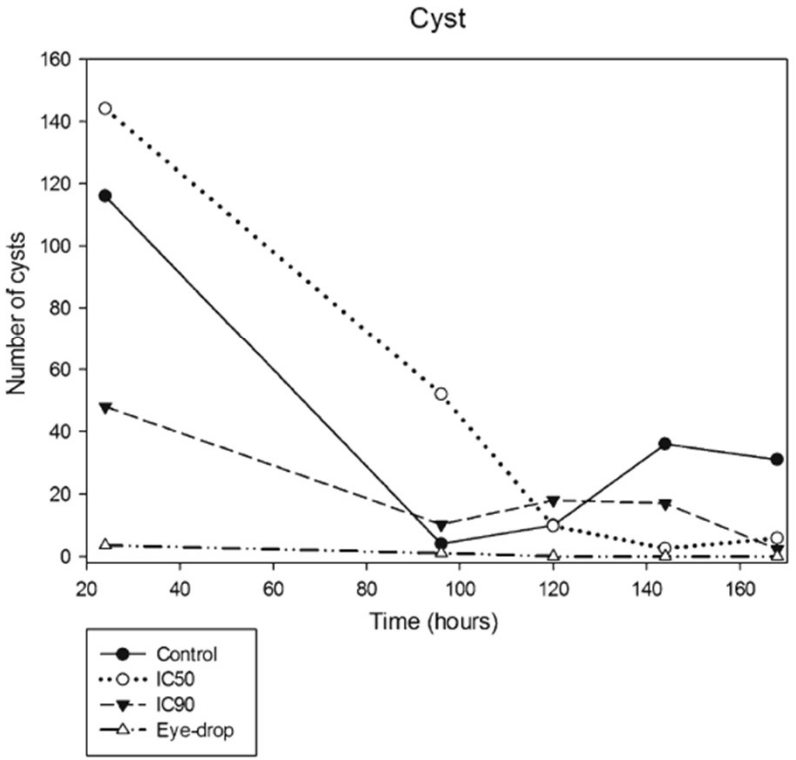




Universidad de La Laguna

Chemotherapy assays





Cysticidal activity

Universidad de La Laguna

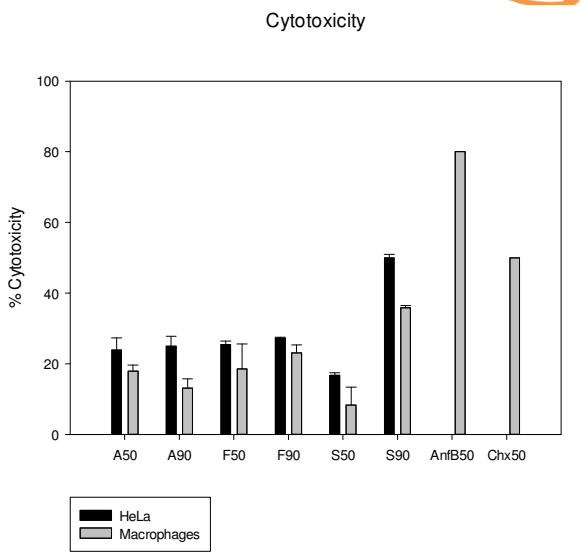
Cytotoxicity Evaluation

Cytotoxicity Detection Kit (LDH)®
Roche → LDH activity

IC_{50} and IC_{90}
Tested compounds

Macrophages J774.1A
HeLa





0-10 %: Non toxic
10-25 %: Low
25- 40 %: Moderated
>40 %: High

(Lorenzo-Morales *et al.*, 2010)

Our sources

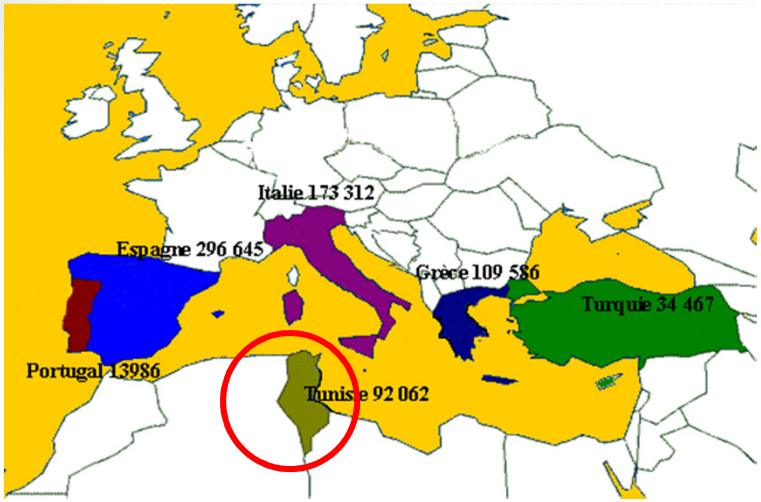
- Natural origin→ Plants (medicinal, folk/ traditional…)
- Synthetic
- Comercialized products→ eye drops, …











Tunisia

Olive tree leaves,
Used in traditional medicine

Zarrazi, Limouni, Chemlali Tataouine, Dhokkar, and Toffehi



Dhokkar



Limouni

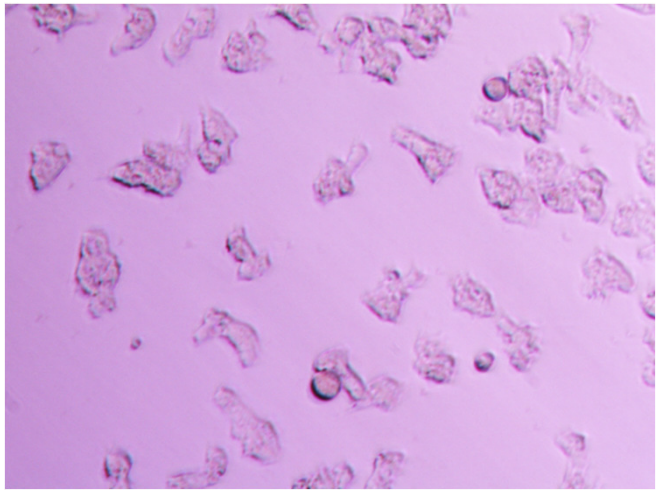


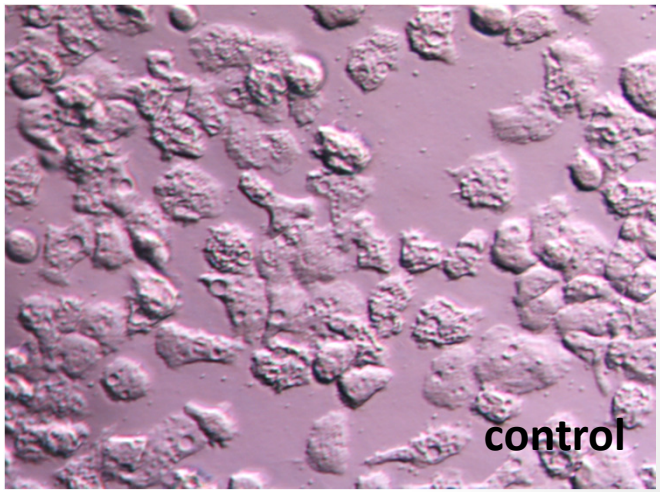
Chemlali Tataouine

Universidad de La Laguna

Total extracts of OLE against *A. castellanii* Neff

Cultivar		Limouni	Zarrazi	Dhokkar	Toffehi	Chemlali Tataouine
IC_{50} (µg/ml)	Methanol	12.585 ± 1.514	23.828 ± 0.732	10.108 ± 2.953	33.661 ± 1.398	22.083 ± 1.379
	Ethanol	17.505 ± 3.198 bcB	26.172 ± 1.367	10.126 ± 0.189	22.992 ± 1.703	19.495 ± 0.378
	Mixture	9.369 ± 1.325	19.397 ± 2.271	8.234 ± 1.703	24.402 ± 2.356 cB	19.617 ± 0.183





Parasitology Research
August 2013, Volume 112, Issue 8, pp 2825-2829
Activity assessment of Tunisian olive leaf extracts against the trophozoite stage of *Acanthamoeba*

Universidad de La Laguna

Dhokkar crude extract

Methanol fraction

Ethyl-acetate fraction

Chromatography

ARTICLE IN PRESS

Experimental Parasitology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr



L

Bioassay guided isolation and identification of anti-*Acanthamoeba* compounds from Tunisian olive leaf extracts

Ines Sifaoui [a,*], Atteneri López-Arencibia [b,2], Juan Carlos Ticona [c], Carmen Mª Martín-Navarro [b,d,2], María Reyes-Batlle [b,2], Mondher Mejri [a,1], Jacob Lorenzo-Morales [b,2], Antonio Ignacio Jiménez [c], Basilio Valladares [b,2], Isabel Lopez-Bazzocchi [c], Manef Abderabba [a,1], José E. Piñero [b,2]

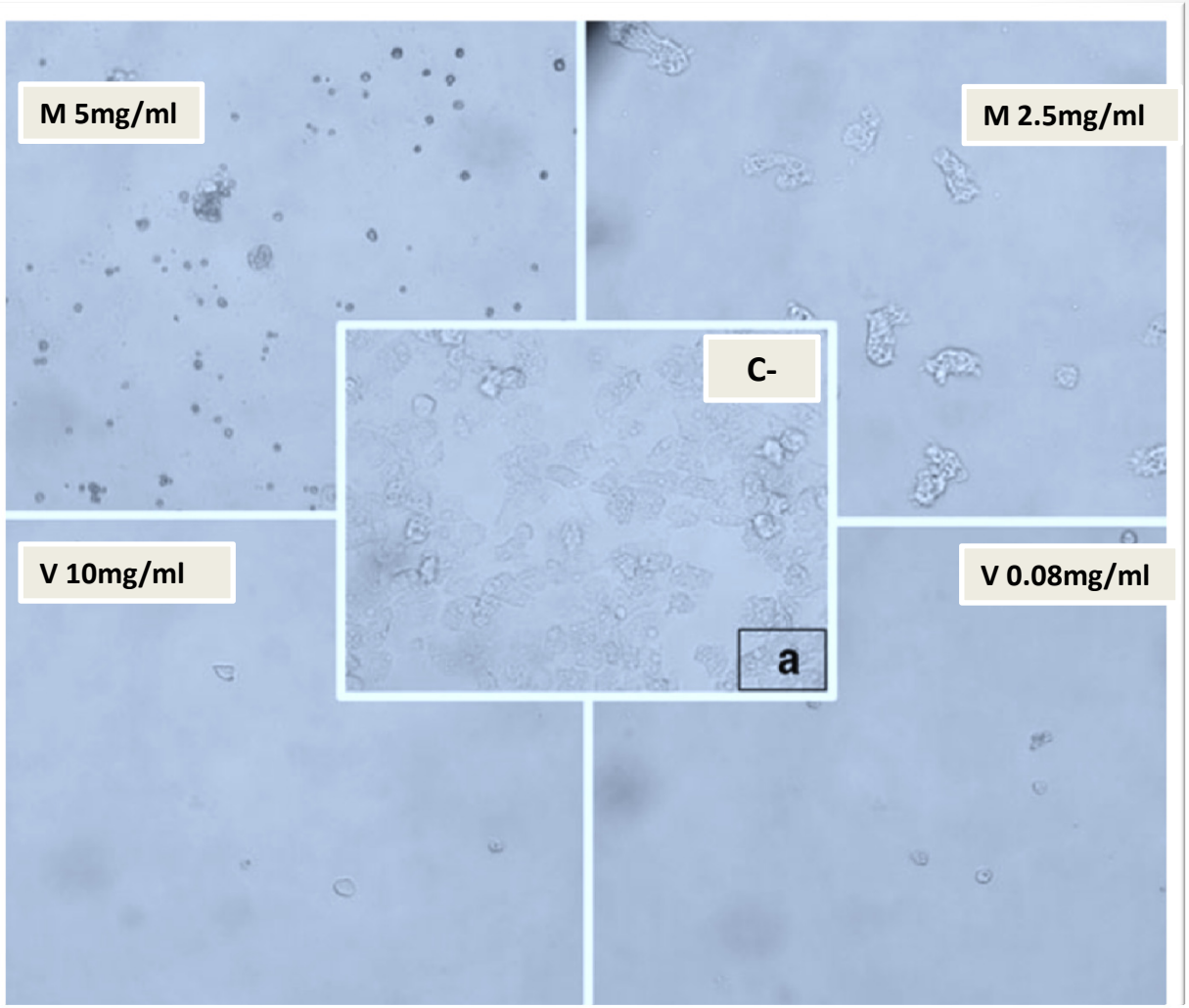
[a] *Laboratoire Matériaux-Molécules et Applications, IPEST, B.P. 51, 2070 La Marsa, University of Carthage, Tunisia*
[b] *University Institute of Tropical Diseases and Public Health, University of La Laguna, Avda Francisco Sanchez s/n, Campus de Anchieta, 38271 la Laguna, Tenerife, Canary Islands, Spain*
[c] *Instituto Universitario de Bio-Orgánica Antonio González, Universidad de La Laguna, Tenerife, Canary Islands, Spain*
[d] *Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh, Edinburgh, Scotland, United Kingdom*





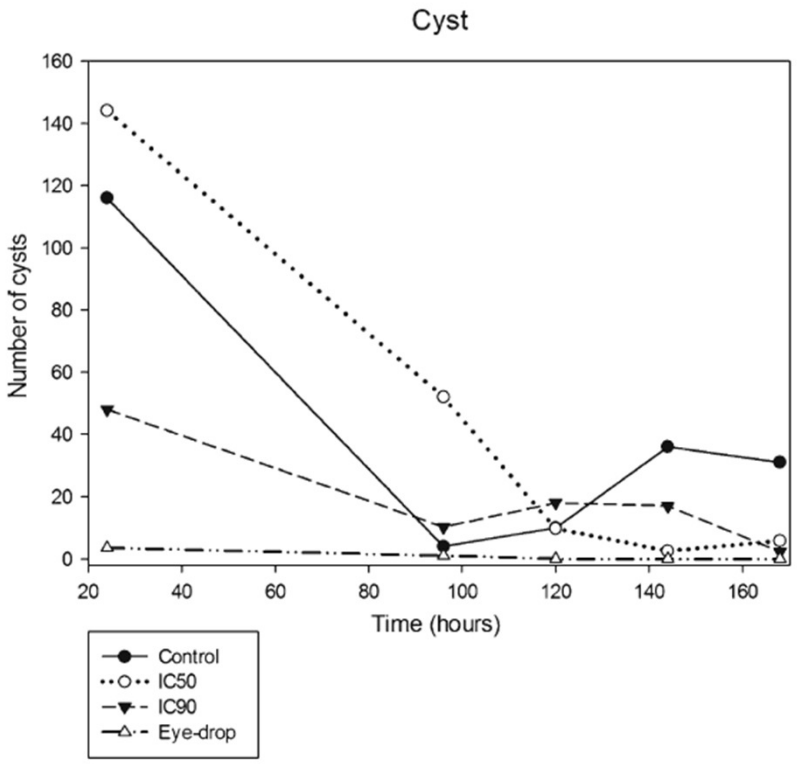
- **Moxifloxacin** eye drops solution (VIGAMOX®) Alcon (0.5 %, 5 mg/ml)
- **Voriconazole** (VFEND®) by Roerig/Pfizer Inc. (1%, 10 mg/ml)
- Clorhexidine digluconate , Alfa Aesar (0.1 %, 1 mg/ml)
- Anfotericine B**,** Sigma-Aldrich Chemistry Ltd (0.1 %, 1 mg/ml)

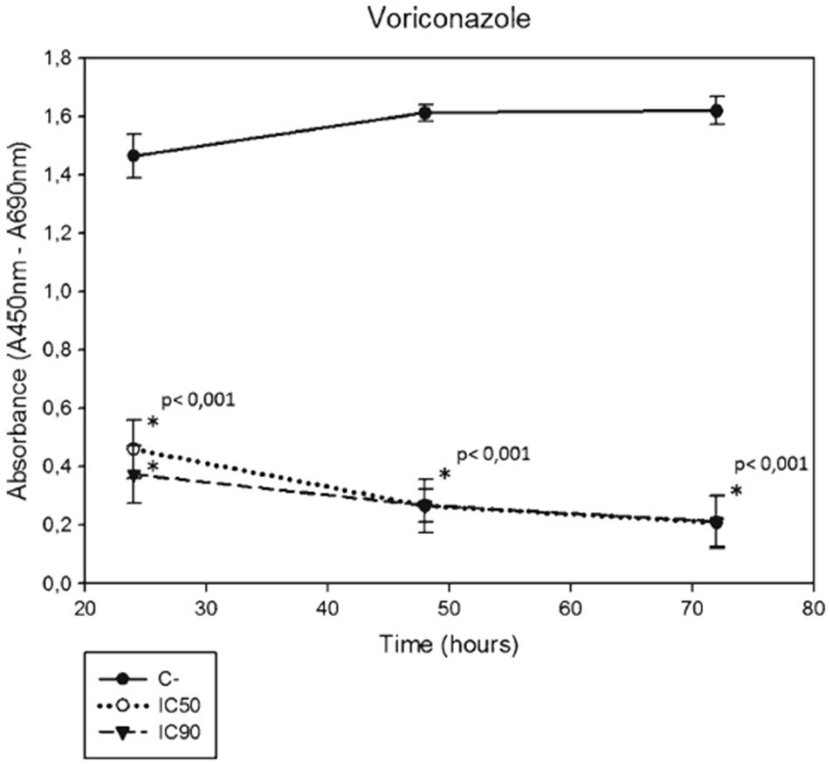
- Type strain: *Acanthamoeba castellanii* Neff (ATCC 30010, genotype T4)
- Clinical Strains (CLC-16, genotype T3 and CLC-51, genotype T1)



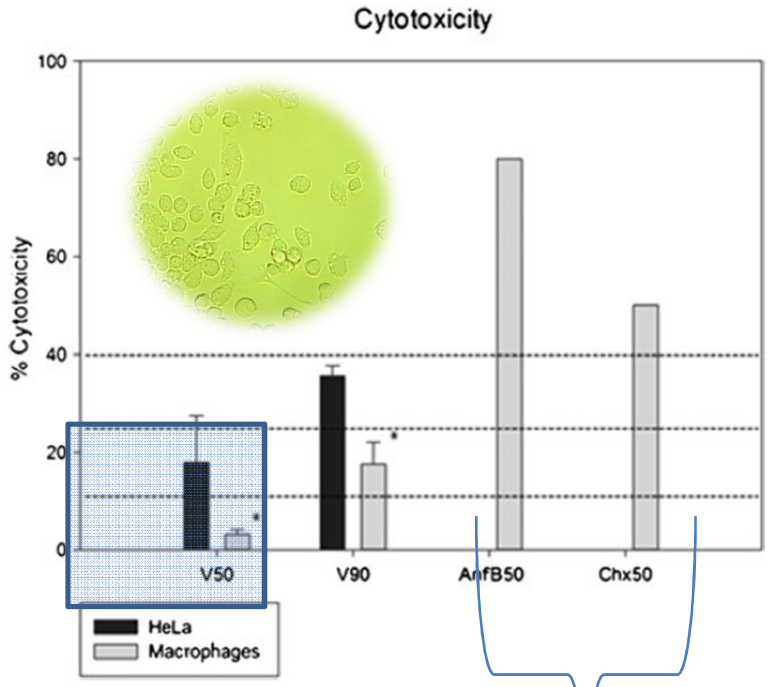
(µg/ml) 96h	AcNeff		CLC-16		CLC-51.1	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Voriconazole	4.59±0.24	10.63±0.46	7.66±2.05	15.43±4.00	4.59±0.59	10.48±1.37
Moxifloxacin	2,630.00±400.00	3,920.00±10.00	180.00±120.00	680.00±50.00	879.93±110.00	150.00±130.52
Chlorhexidine	0.92±0.10	8.16±0.45	6.01±1.92	17.42±1.23	5.29±0.75	14.26±0.91
Amphotericin B	36.61±0.52	92.36±8.79	22.82±1.18	66.39±3.14	62.15±5.24	157.09±12.67

Voriconazole





Cytotoxicity Evaluation



Graefes Arch Clin Exp Ophthalmol
DOI 10.1007/s00417-013-2371-y

BASIC SCIENCE

Evaluation of the *in vitro* activity of commercially available moxifloxacin and voriconazole eye-drops against clinical strains of *Acanthamoeba*

C. M. Martín-Navarro · A. López-Arencibia · F. Arnalich-Montiel · B. Valladares · J. E. Piñero · J. Lorenzo-Morales

Received: 10 January 2013 /Revised: 8 April 2013 /Accepted: 25 April 2013
© Springer-Verlag Berlin Heidelberg 2013

Universidad de La Laguna

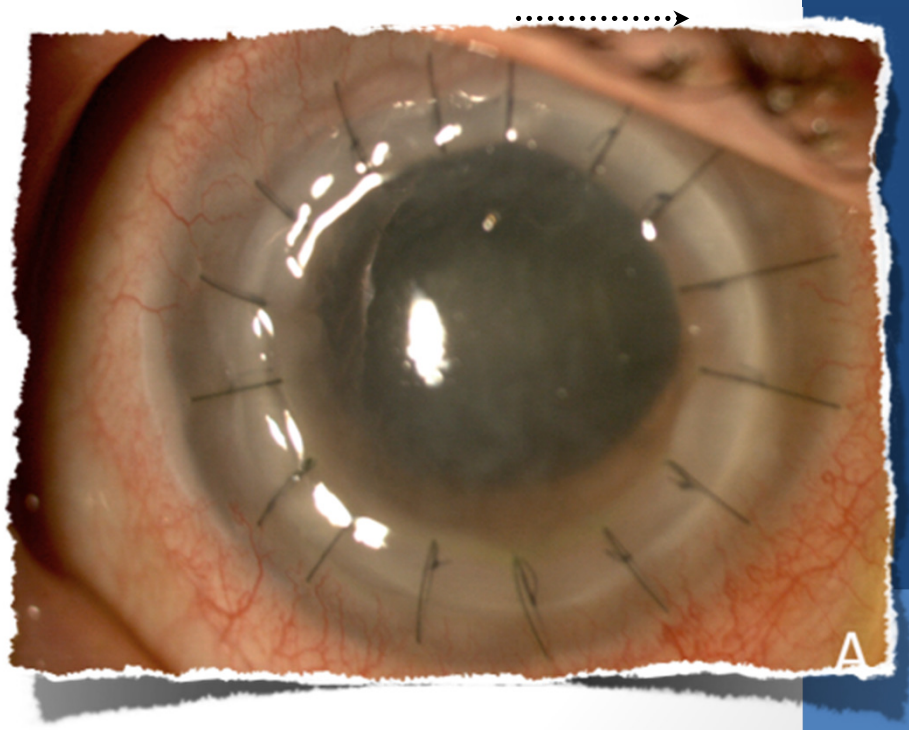
RESEARCH LETTERS

Successful Monitoring and Treatment of Intraocular Dissemination of *Acanthamoeba*

Francisco Arnalich-Montiel, MD, PhD
Carmen M. Martín-Navarro, PhD
Jorge L. Alió II, MD
Rogelio López-Vélez, MD, PhD
Enrique Martínez-Carretero, PhD
Basilio Valladares, PhD
José E. Piñero, PhD
Jacob Lorenzo-Morales, PhD

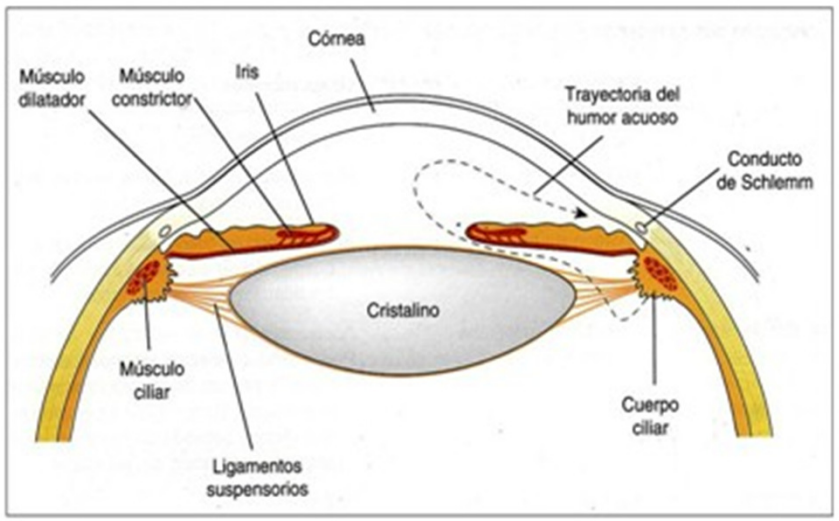
Successful customized voriconazole therapy for intraocular dissemination of *Acanthamoeba*.

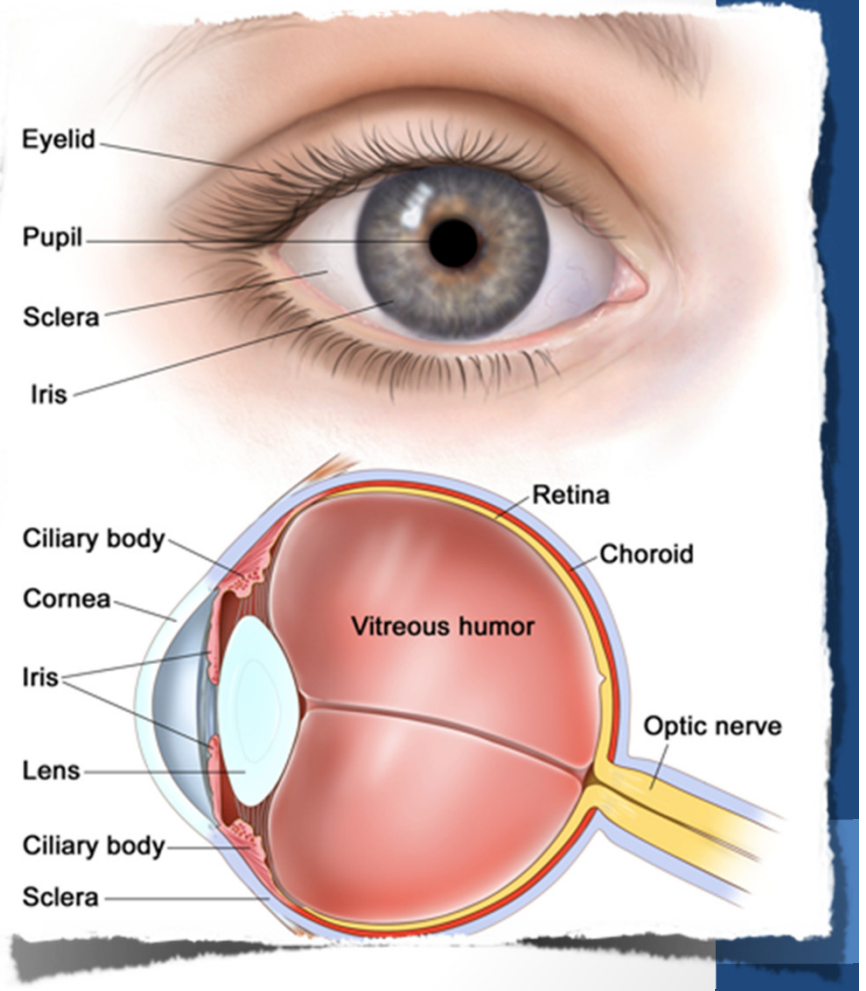
- AK → extracorneal, ocular perforation
- Keratoplastia
- Inflammation after 5 days



Successful customized voriconazole therapy for intraocular dissemination of *Acanthamoeba*. Case report and review of literature.

- Presence of *Acanthamoeba* Aqueous humor
- The clear fluid filling the space in the front of the eyeball between the lens and the cornea

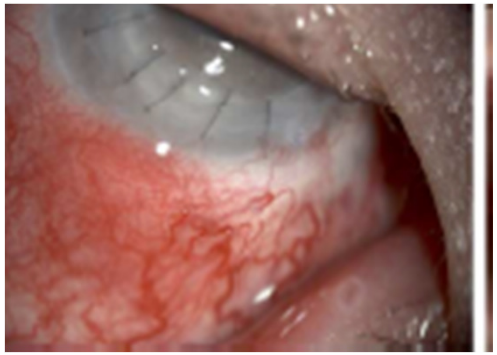


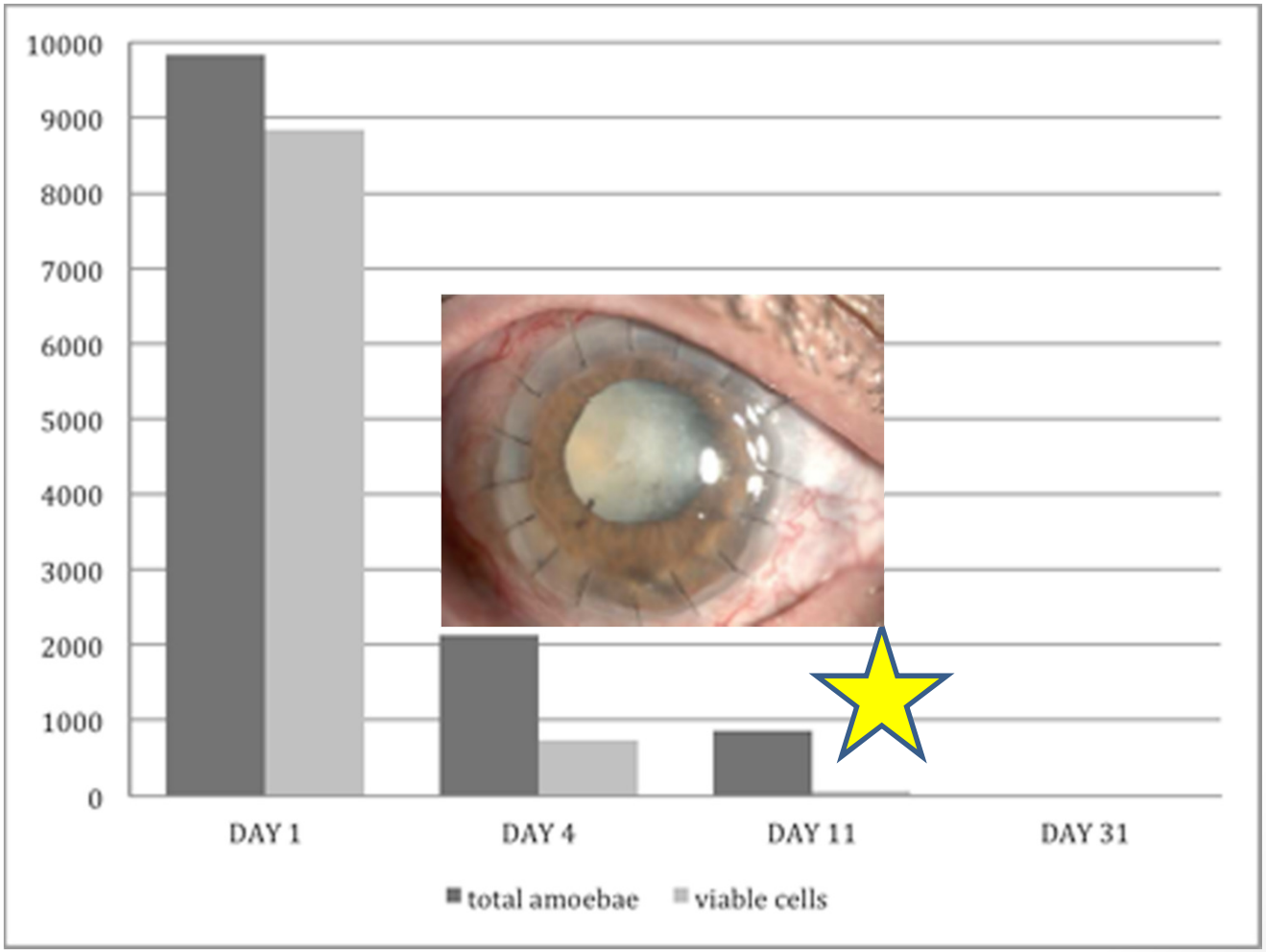


Successful customized voriconazole therapy for intraocular dissemination of *Acanthamoeba*. Case report and review of literature.

- T4 genotype Voriconazole sensitive
  - (IC90 10.63 µg/ml)
- Intraocular voriconazole 100 µg in serial 0.1 ml doses
- Oral voriconazol
- Chlorhexidine and topic voriconazol






10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
DAY 1
DAY 4
DAY 11
DAY 31
total amoebae
viable cells

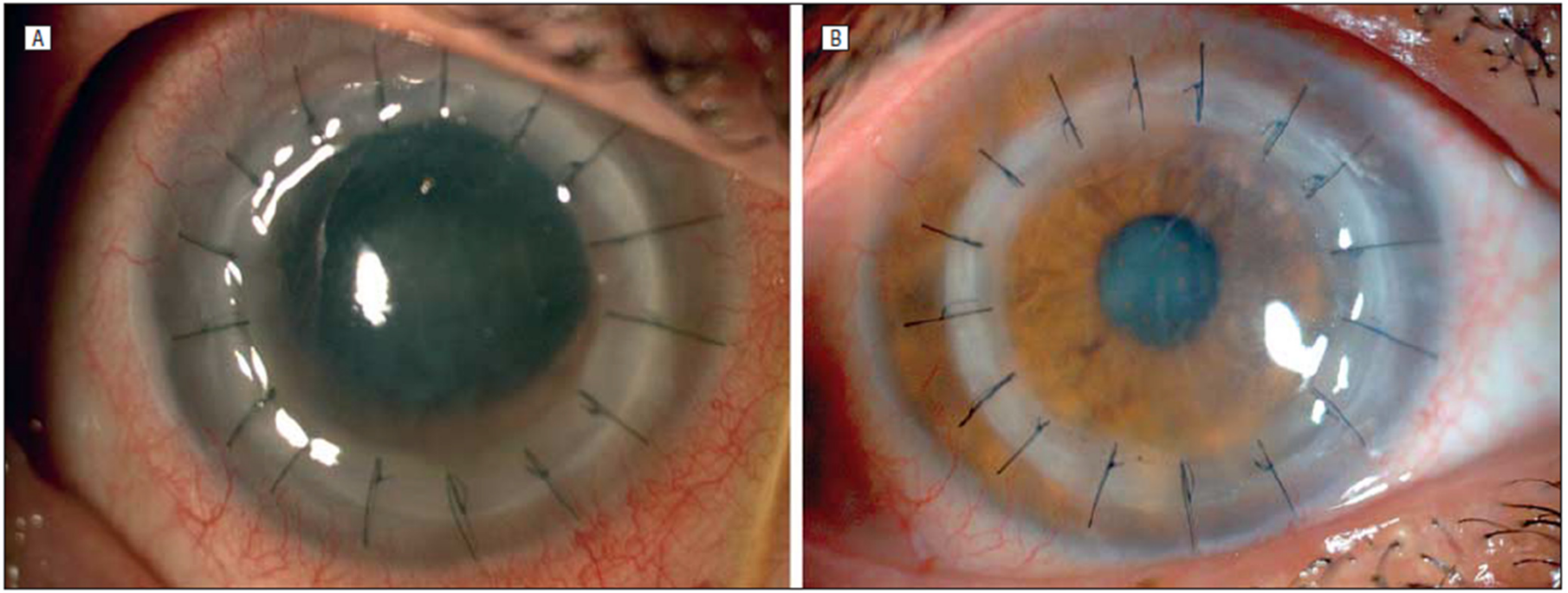
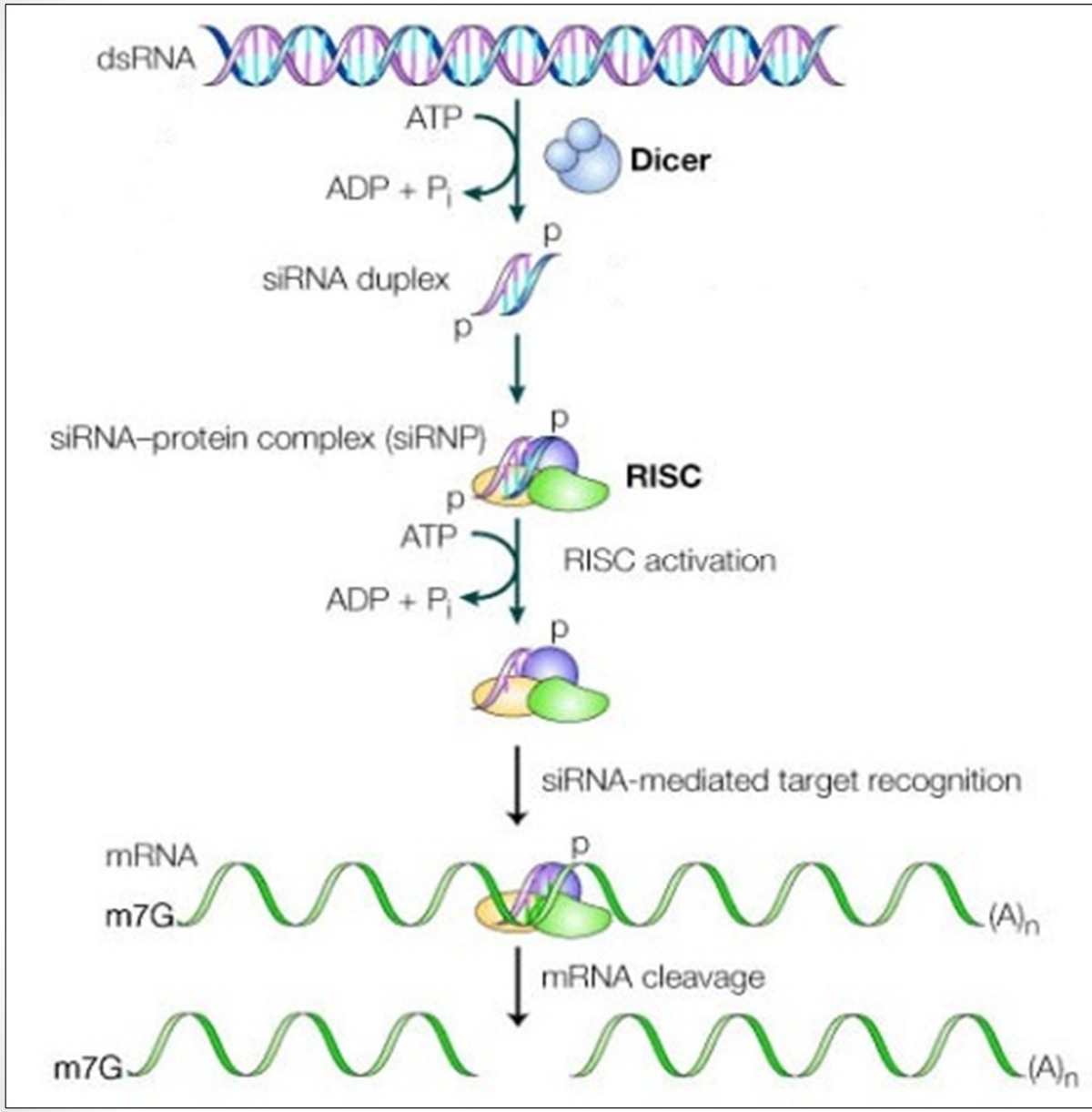
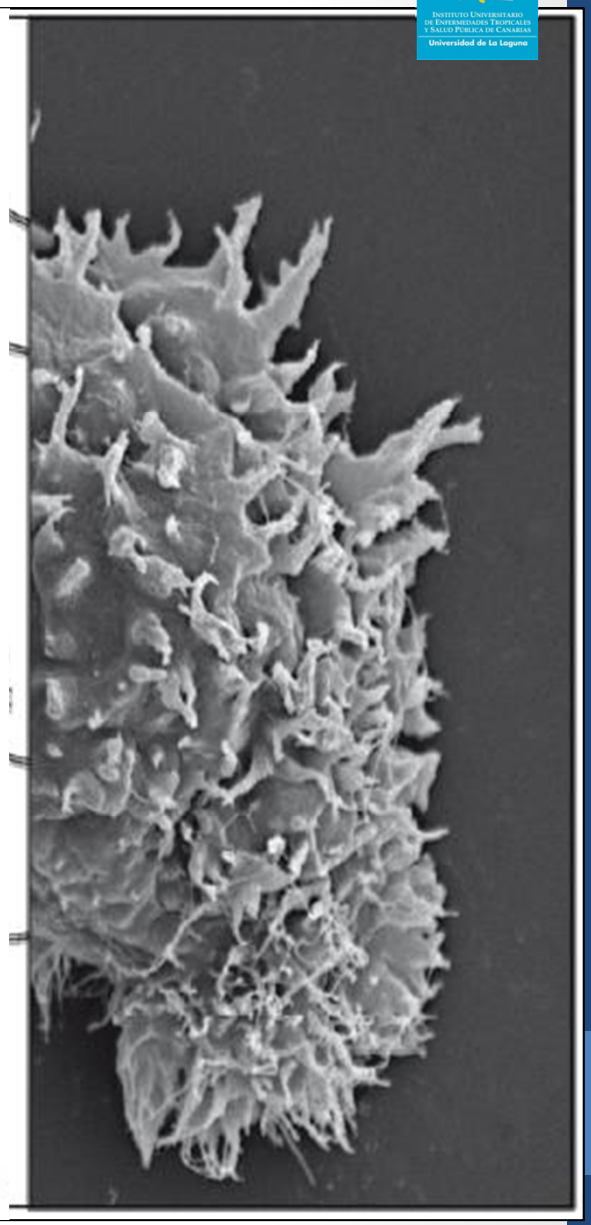


Figure 1. Anterior segment photograph of the patient's right eye 5 days after the therapeutic penetrating keratoplasty shows perikeratic hyperemia, corneal edema with Descemet folds, and mutton-fat keratic precipitates (A), and slitlamp examination after clinical resolution shows early signs of graft failure and pigmented keratic precipitates (B).

BEFORE

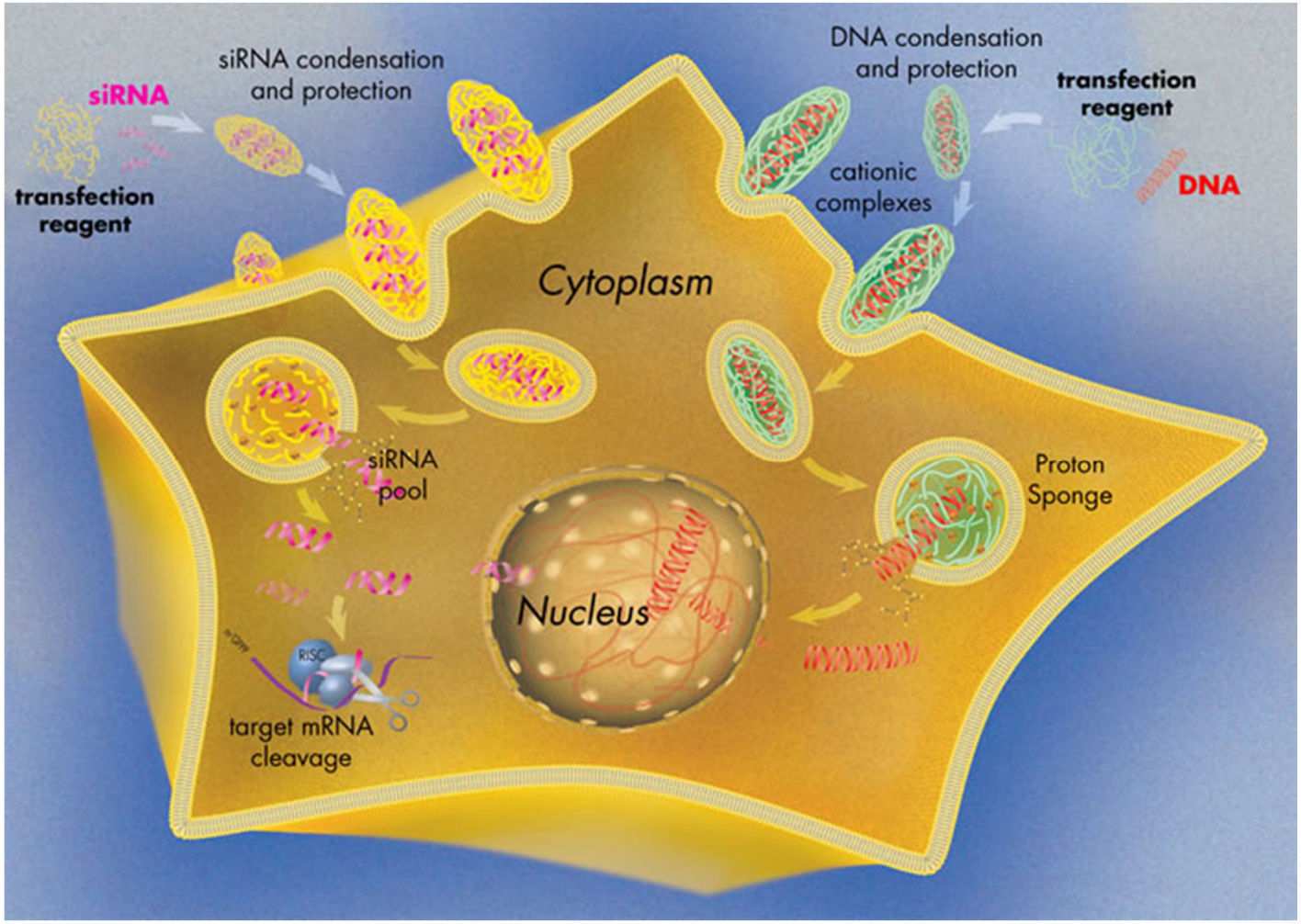
AFTER


dsRNA
ATP
ADP + Pi
Dicer
siRNA duplex
p
p
siRNA–protein complex (siRNP)
RISC
ATP
ADP + Pi
RISC activation
siRNA-mediated target recognition
mRNA
m7G
(A)n
mRNA cleavage
m7G
(A)n



Universidad de La Laguna

siRNAs



MOLECULAR THERAPY

- Extracellular serine proteases
  - Entry was blocked

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular & Biochemical Parasitology 144 (2005) 10–15

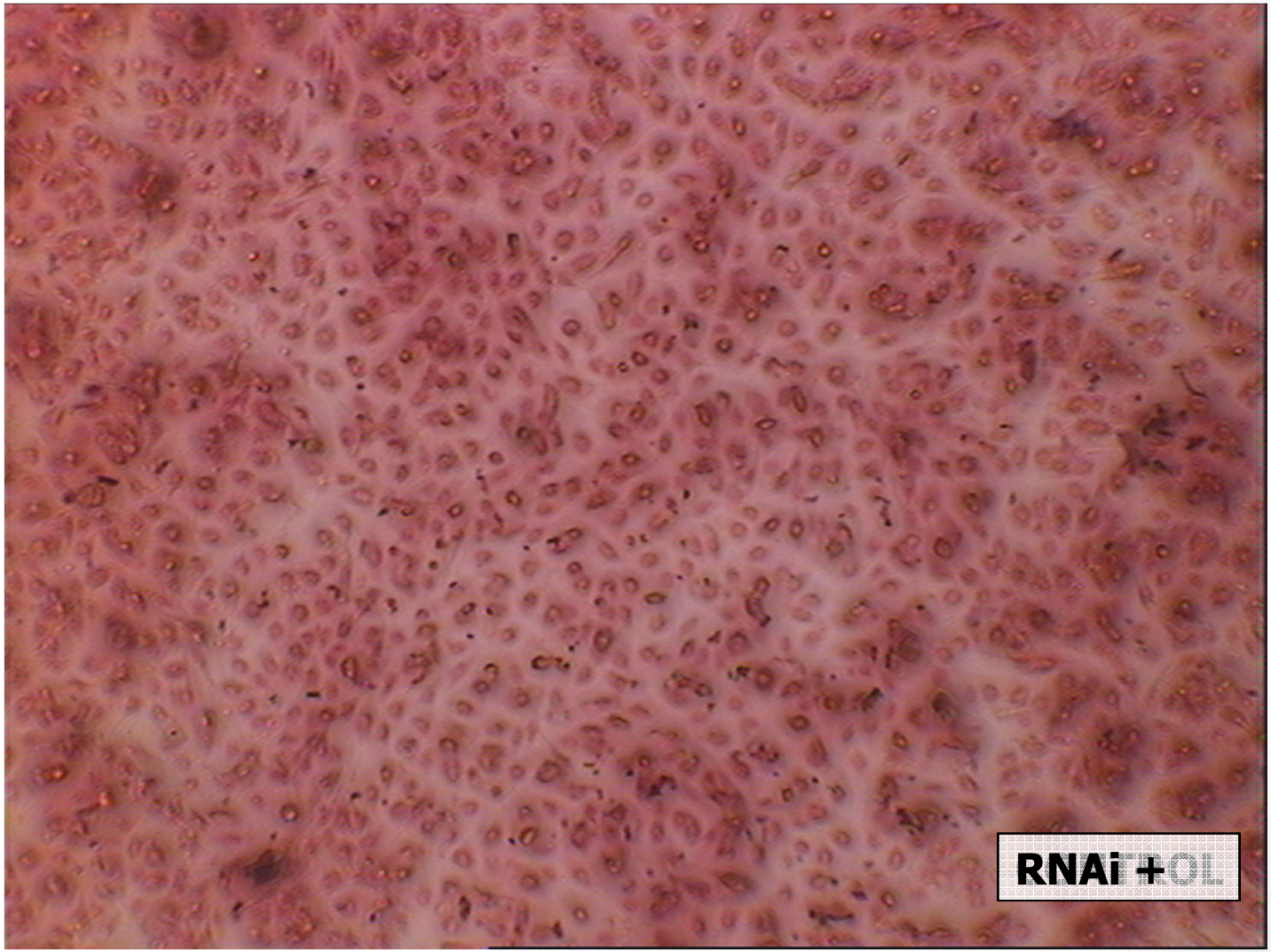
MOLECULAR & BIOCHEMICAL PARASITOLOGY

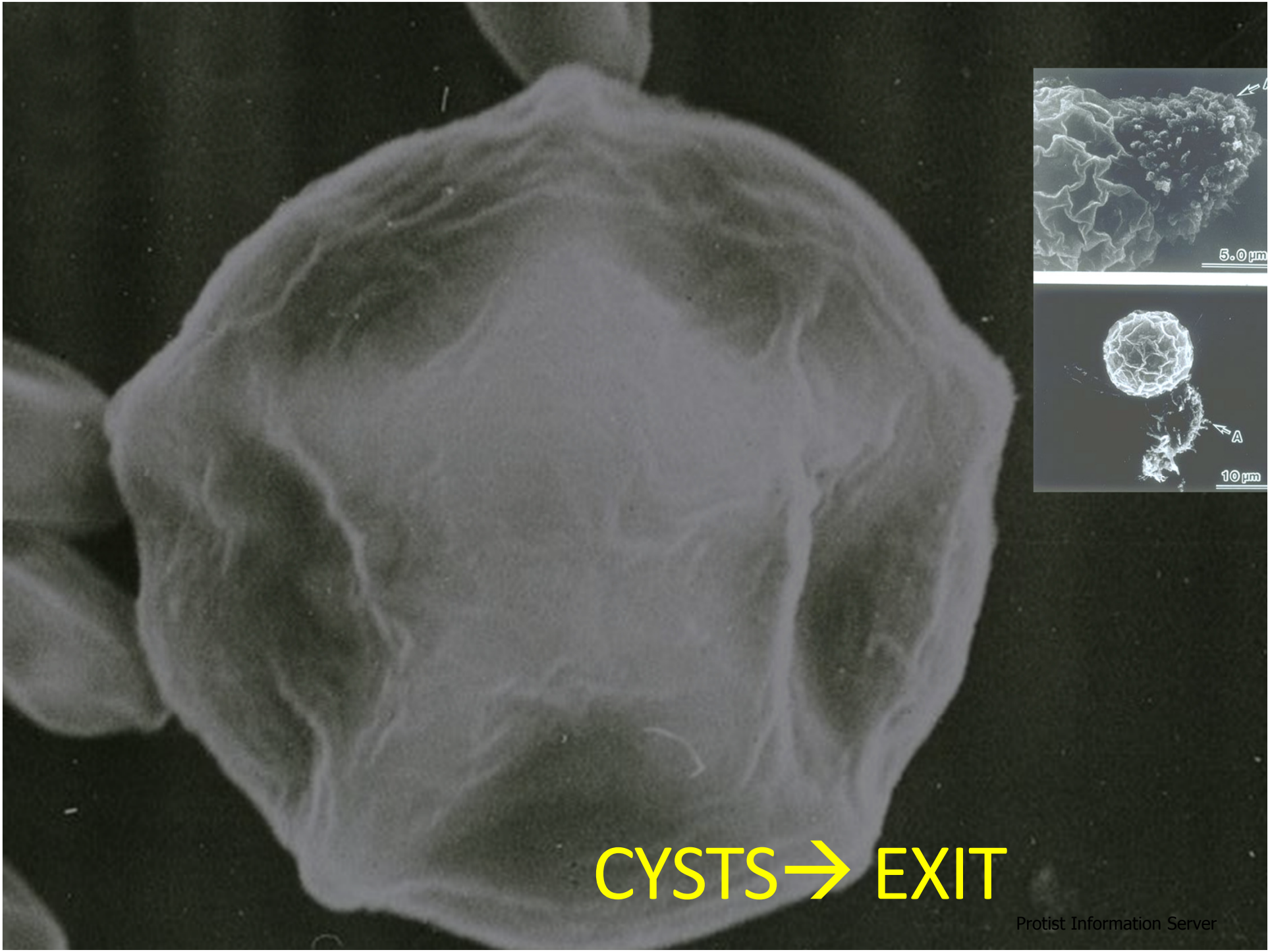
RNA interference (RNAi) for the silencing of extracellular serine proteases genes in *Acanthamoeba*: Molecular analysis and effect on pathogenecity

Jacob Lorenzo-Morales, Antonio Ortega-Rivas, Pilar Foronda, Néstor Abreu-Acosta, David Ballart, Enrique Martínez, Basilio Valladares *

Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, University of La Laguna, Avda. Astrofísico Fco. Sánchez, S/N 38203 La Laguna, Canary Islands, Spain

SERINE PROTEASES-siRNA



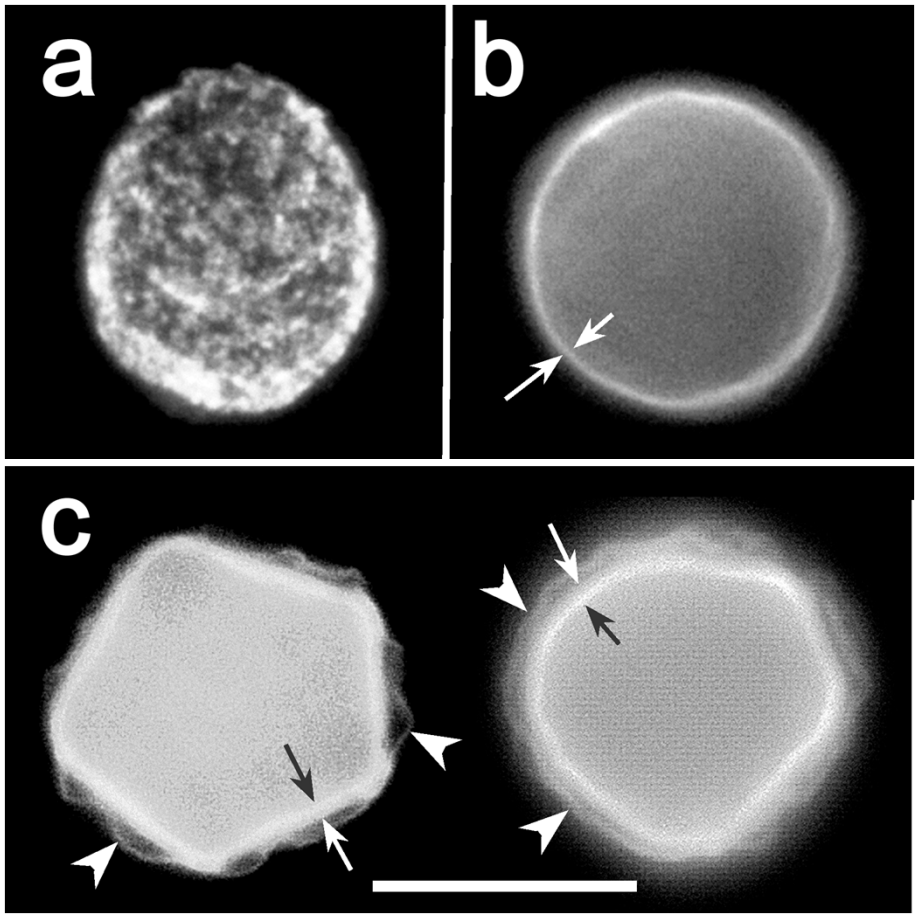


CYSTS→ EXIT

Protist Information Server

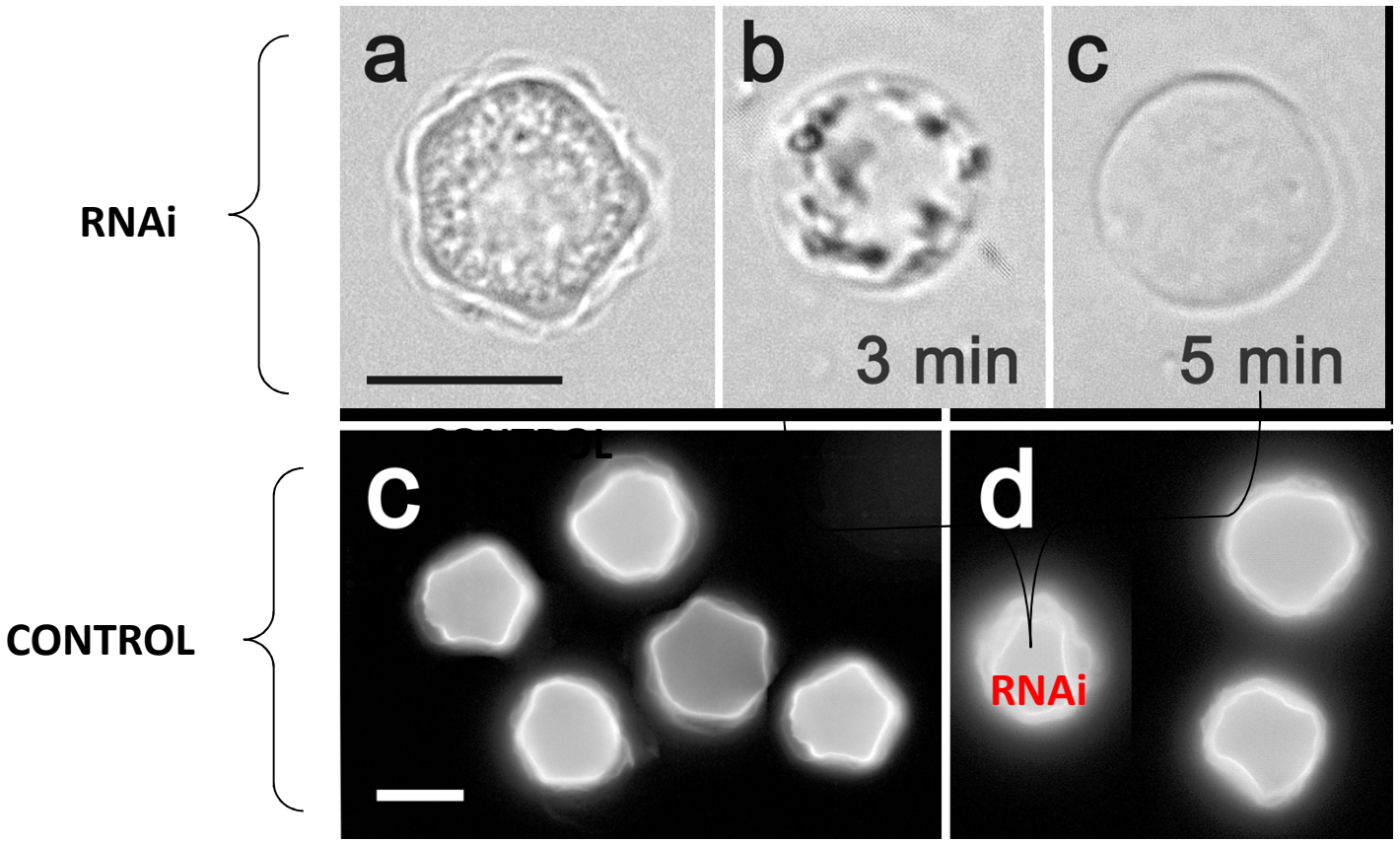
NORMAL ENCYSTMENT PROCESS

CELLULOSE→ GLYCOGEN PHOSPHORYLASE



Control
A. 12 h
B. 24 h
C. 48 & 72 h

CONTROL-RNAi GLYCOGEN PHOSPORYLASE



A. RNAi 48 hrs
B. RNAi 72 hrs
C. Control 48 hrs
D. Control 72 hrs

EUKARYOTIC CELL, Mar. 2008, p. 000 Vol. 7, No. 3
1535-9778/08/$08.00+0 doi:10.1128/EC.00316-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Glycogen Phosphorylase in *Acanthamoeba* spp.: Determining the Role of the Enzyme during the Encystment Process Using RNA Interference▽

Jacob Lorenzo-Morales,[1] Jarmila Kliescikova,[2] Enrique Martinez-Carretero,[1] Luis Miguel De Pablos,[3] Bronislava Profotova,[2] Eva Nohynkova,[2] Antonio Osuna,[3] and Basilio Valladares[1]*

University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Tenerife, Canary Islands, Spain[1]; Department of Tropical Medicine, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic[2]; and Institute of Biotechnology, Department of Parasitology, University of Granada, Campus de Fuentenueva 18071, Granada, Spain[3]

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2010, p. 5151–5155 Vol. 54, No. 12
0066-4804/10/$12.00 doi:10.1128/AAC.00329-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Therapeutic Potential of a Combination of Two Gene-Specific Small Interfering RNAs against Clinical Strains of *Acanthamoeba*▽

Jacob Lorenzo-Morales,[1,2]* Carmen M. Martín-Navarro,[1] Atteneri López-Arencibia,[1] María A. Santana-Morales,[1] Raquel N. Afonso-Lehmann,[1] Sutherland K. Maciver,[2] Basilio Valladares,[1] and Enrique Martínez-Carretero[1]

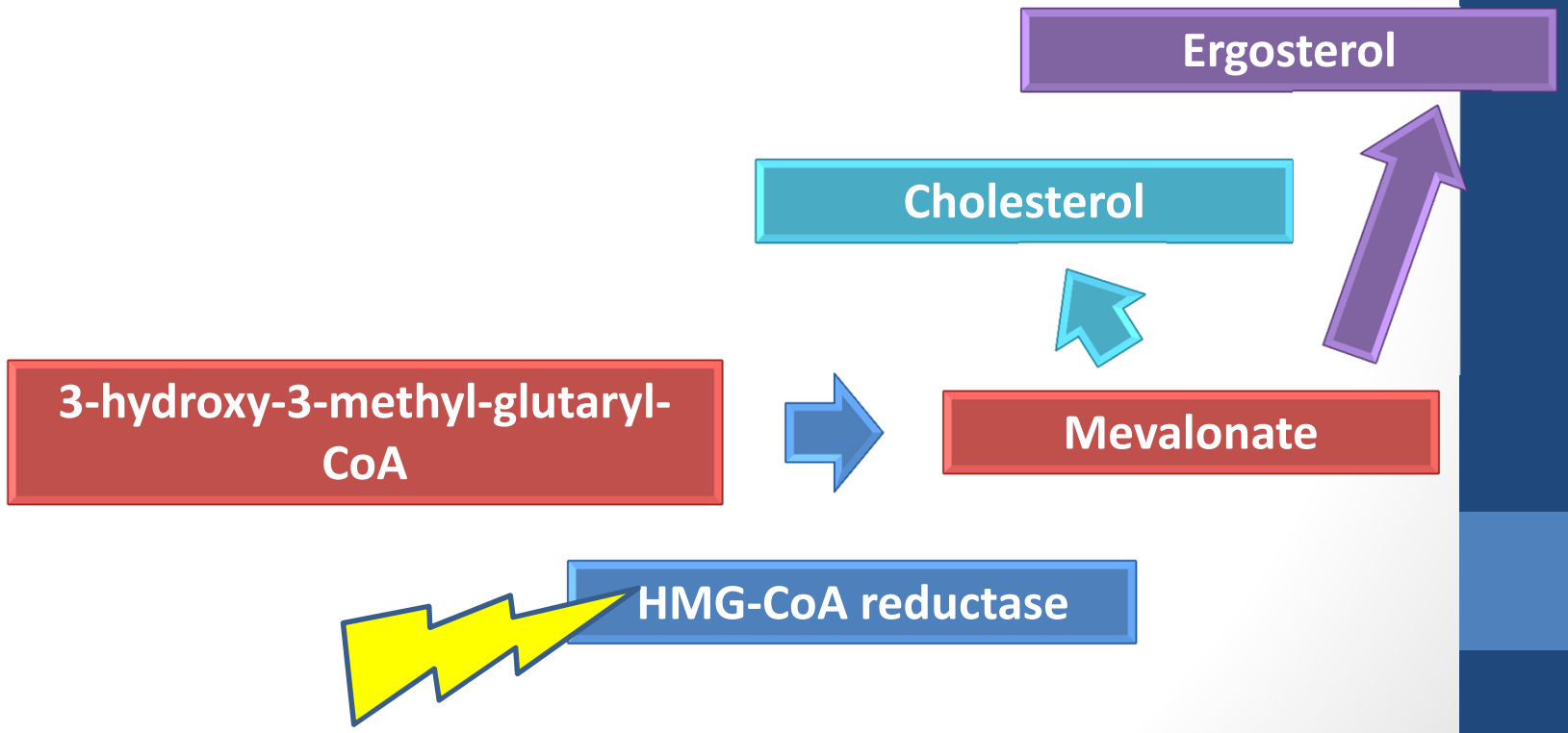
University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Avda. Astrofísico Fco. Sánchez, S/N 38203 La Laguna, Tenerife, Canary Islands, Spain,[1] and Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, United Kingdom[2]

Received 9 March 2010/Returned for modification 13 May 2010/Accepted 10 September 2010

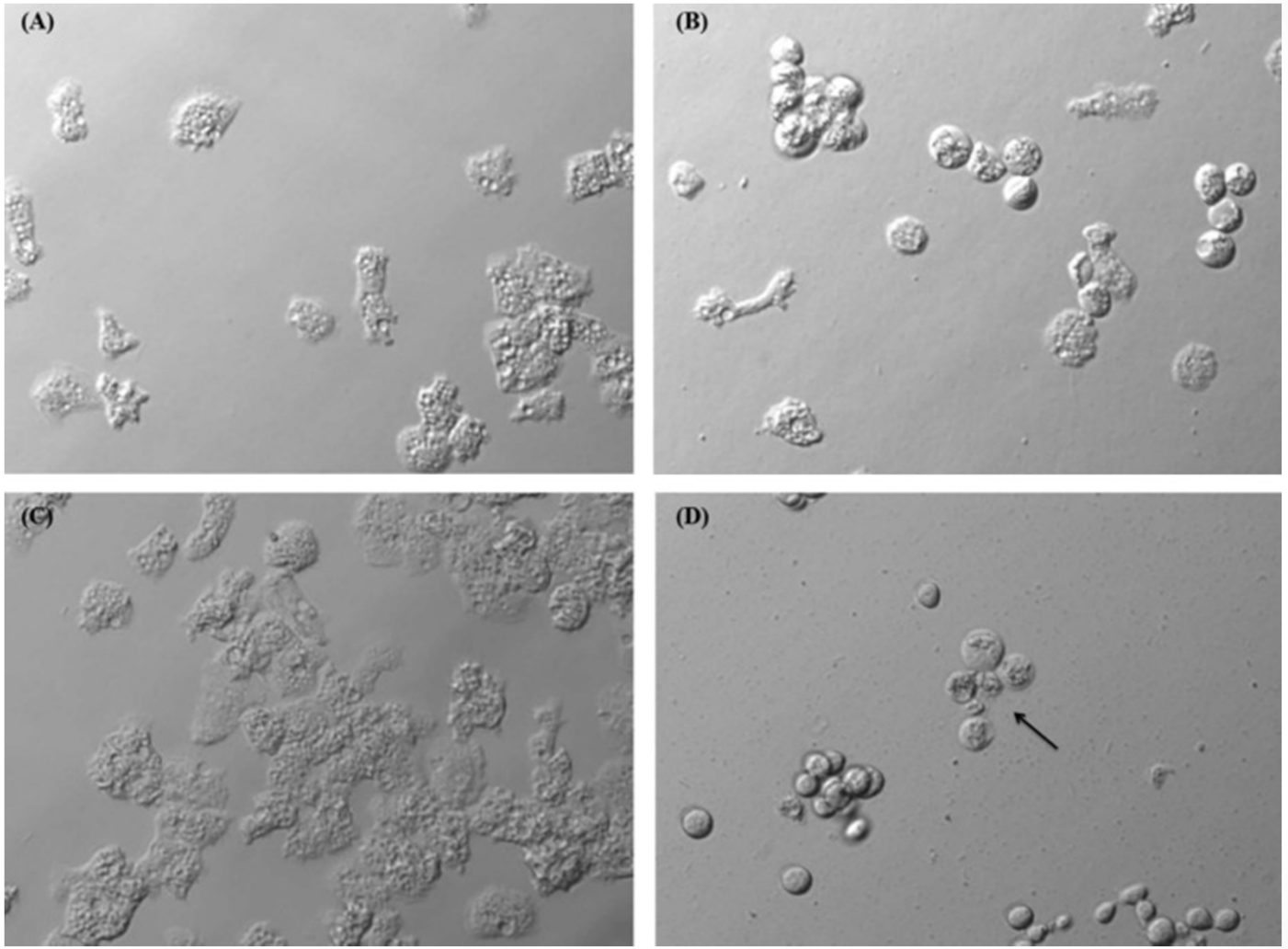
COMBINATION OF MOLECULAR THERAPY AND CHEMOTHERAPY

Sterol synthesis in amoeba

- Main sterol in the membranes→ ergosterol
- Same pathway as cholesterol synthesis in humans



siRNA HMCoA reductase



RNAi+→ non viable

Statins→ Inhibit HMCoA R

- Simvastatin
- Pravastatin
- Lovastatin
- Atorvastatin
- Fluvastatin

- Strains:
  - *Acanthamoeba castellanii* Neff (ATCC 30010)
  - Clinical strains:
    - CLC-16
    - CLC-41.r
    - CLC-51.l

Results

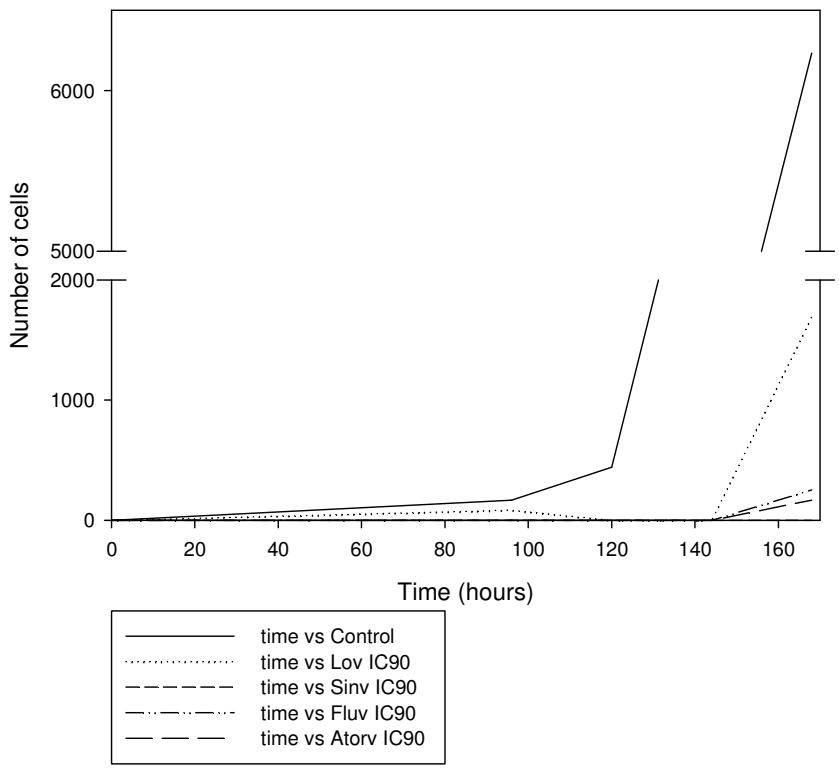
IC_{50} (mM) 96h	AcNeff	CLC-16	CLC-41.r	CLC-51.l
Lovastatin	17.14 ± 1.85	-	46.65 ± 4.50	-
Pravastatin	58.75 ± 11.01	-	-	-
Simvastatin	10.24 ± 1.09	31.44 ± 2.06	14.60 ± 0.59	39.73 ± 4.34
Fluvastatin	9.19 ± 0.98	54.64 ± 2.69	24.29 ± 0.97	16.50 ± 1.03
Atorvastatin	15.12 ± 2.19	33.34 ± 2.64	13.70 ± 0.81	26.63 ± 1.20

IC_{90} (mM) 96h	
Lovastatin	23.68 ± 0.32
Simvastatin	21.37 ± 1.51
Fluvastatin	20.70 ± 2.15
Atorvastatin	41.09 ± 0.00

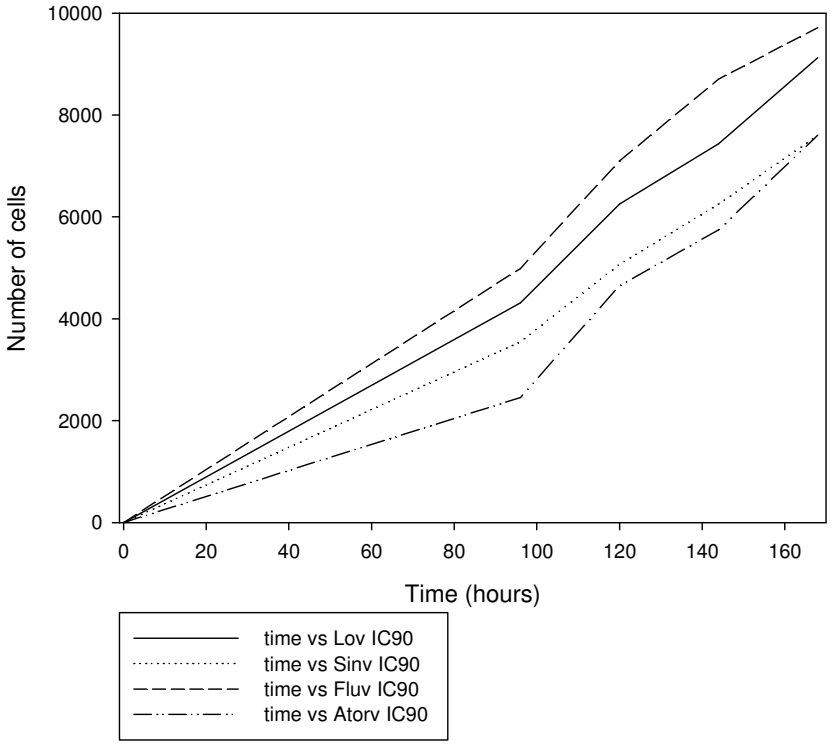
Results

→ Cysticidal activity /Toxicity

Trophozoites when IC_{90} is used



Unviable cyst when IC_{90} is used



Conclusions

- Statins are amoebicidal and cysticidal (10X lower humans)
- Future therapy?→ elucidated target using siRNA



Editor: Edlind | Section: Experimental Therapeutics | Designation: T

Inhibition of 3-Hydroxy-3-Methylglutaryl–Coenzyme A Reductase and Application of Statins as a Novel Effective Therapeutic Approach against *Acanthamoeba* Infections

Carmen María Martín-Navarro,[a] Jacob Lorenzo-Morales,[a] Rubén P. Machín,[b] Atteneri López-Arencibia,[a] José Manuel García-Castellano,[b] Isabel de Fuentes,[c] Brendan Loftus,[d] Sutherland K. Maciver,[e] Basilio Valladares,[a] José E. Piñero[a]

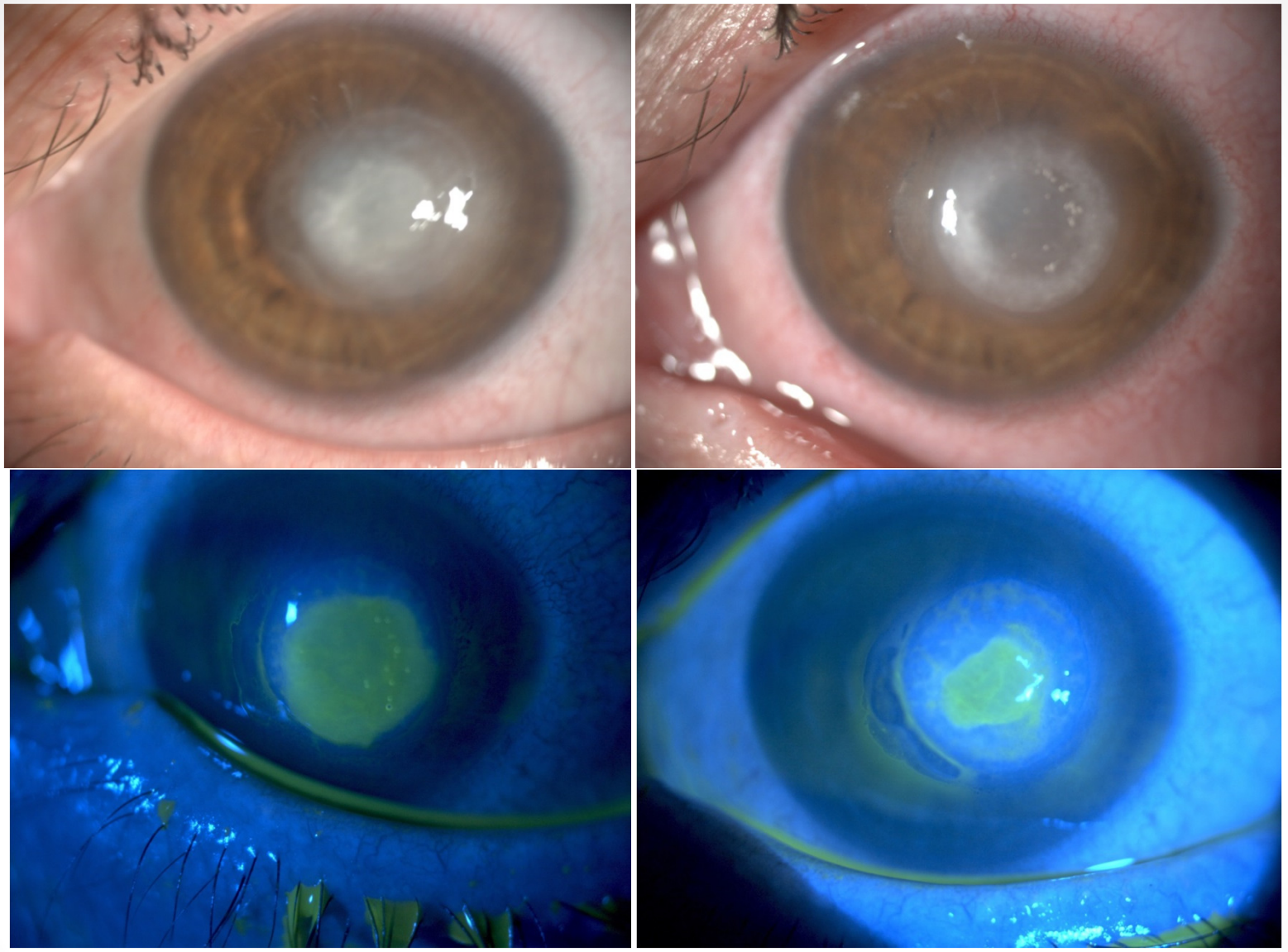
University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, La Laguna, Tenerife, Canary Islands, Spain[a]; Molecular Oncology Group (G-OncoMol), Research Unit, University Hospital of Gran Canaria Dr. Negrín, Barranco La Ballena, Las Palmas de Gran Canaria, Canary Islands, Spain[b]; Carlos III Health Institute, Microbiology National Center, Majadahonda, Madrid, Spain[c]; School of Medicine and Medical Science, Conway Institute, University College Dublin, Belfield, Dublin, Ireland[d]; Centre for Integrative Physiology, School of Biomedical Sciences, University of Edinburgh, Edinburgh, Scotland, United Kingdom[e]

Acanthamoeba **is an opportunistic pathogen in humans, whose infections most commonly manifest as *Acanthamoeba* keratitis or, more rarely, granulomatous amoebic encephalitis. Although there are many therapeutic options for the treatment of *Acanthamoeba*, they are generally lengthy and/or have limited efficacy. Therefore, there is a requirement for the identification, validation, and development of novel therapeutic targets against these pathogens. Recently, RNA interference (RNAi) has been widely used for these validation purposes and has proven to be a powerful tool for *Acanthamoeba* therapeutics. Ergosterol is one of the major sterols in the membrane of *Acanthamoeba*. 3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase is an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, one of the precursors for the production of cholesterol in humans and ergosterol in plants, fungi, and protozoa. Statins are compounds which inhibit this enzyme and so are promising as chemotherapeutics. In order to validate whether this enzyme could be an interesting therapeutic target in *Acanthamoeba*, small interfering RNAs (siRNAs) against HMG-CoA were developed and used to evaluate the effects induced by the inhibition of *Acanthamoeba* HMG-CoA. It was found that HMG-CoA is a potential drug target in these pathogenic free-living amoebae, and various statins were evaluated *in vitro* against three clinical strains of *Acanthamoeba* by using a colorimetric assay, showing important activities against the tested strains. We conclude that the targeting of HMG-CoA and *Acanthamoeba* treatment using statins**

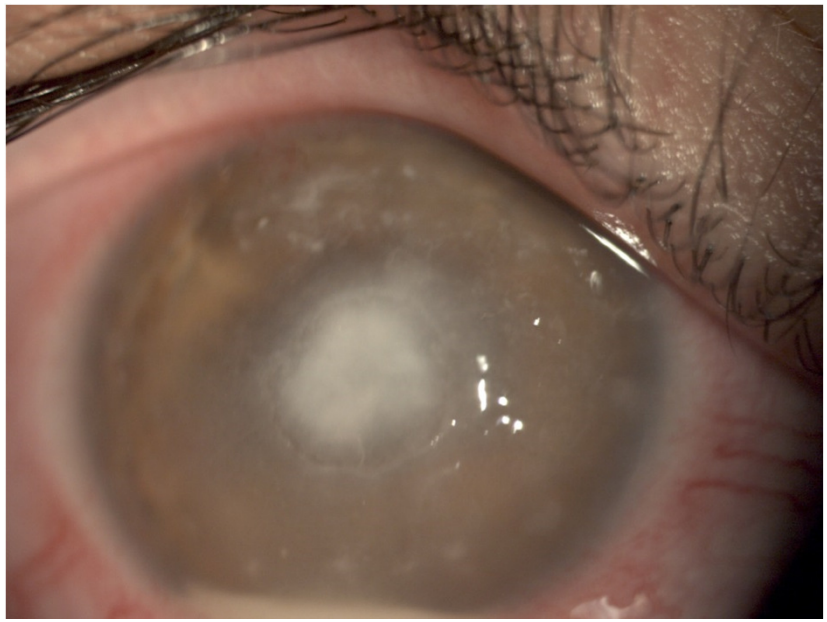
First trials in AK Patients

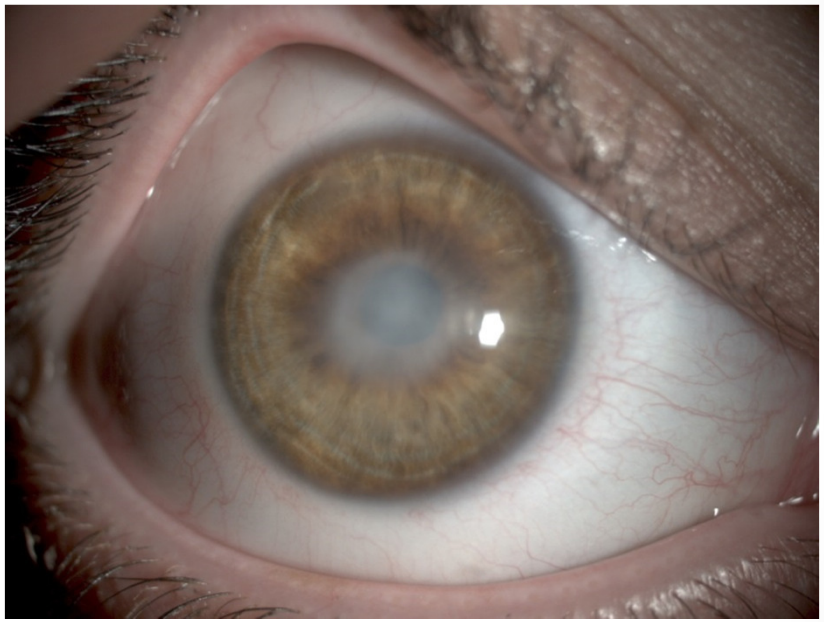


- March 2015-May 2015
- 4 patients not responding to AK therapy (chlorhexidine 0.02%)
- 2 Contact lens wearers and 2 non-contact lens wearers
- Once *Acanthamoeba* was diagnosed → atorvastatin 20mg



Patient 1





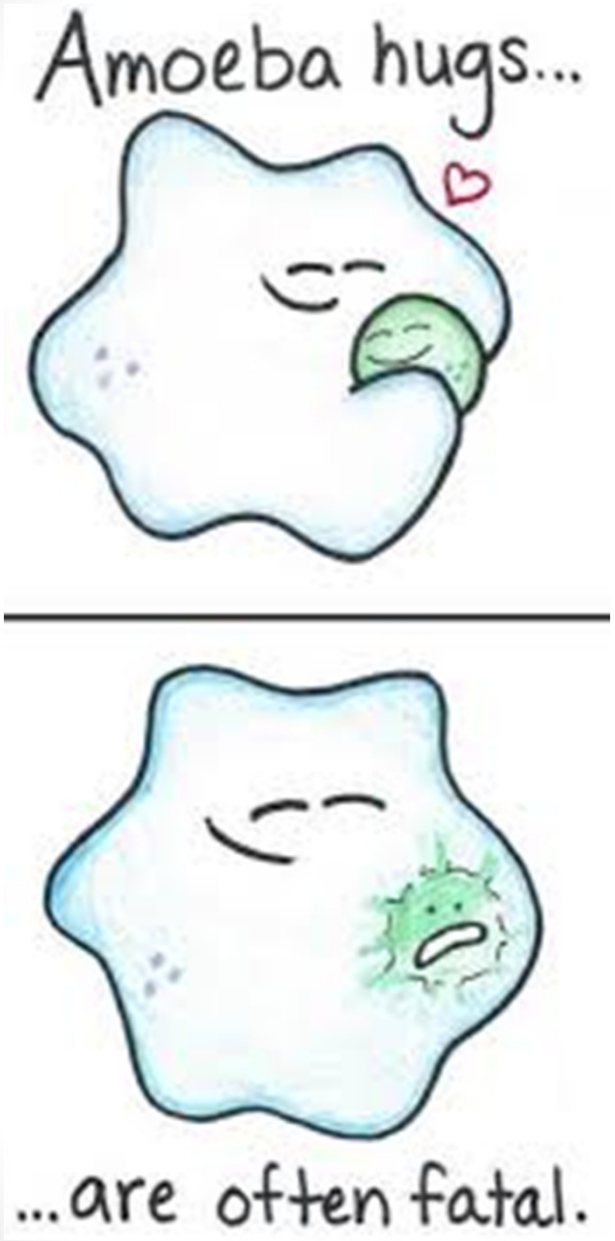
Patient 4

Acknowledgments

GOBIERNO DE ESPAÑA
MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD
Instituto de Salud Carlos III
ricet
Hospital Universitario Ramón y Cajal
SaludMadrid
Comunidad de Madrid
Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias
Universidad de La Laguna







THANK YOU!