Universidade Estadual Paulista "Julio de Mesquita Filho"
Faculdade de Ciências Farmacêuticas
Pós-Graduação em Biociências e Biotecnologia aplicadas à Farmácia

Pre-Clinical Research

"New Drugs Against TB"

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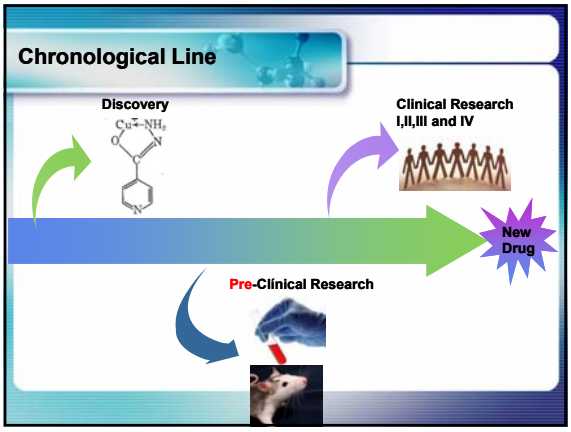
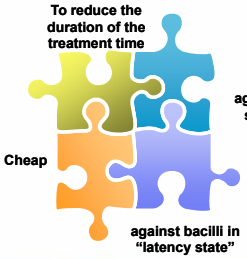
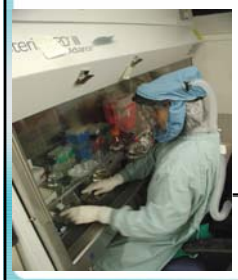
Chronological Line



What expect from a new drug?

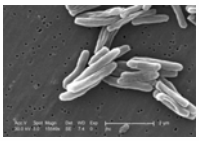




Biological Assays

In vitro

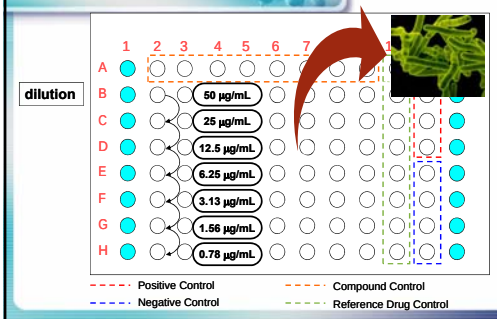
Screening - First Step



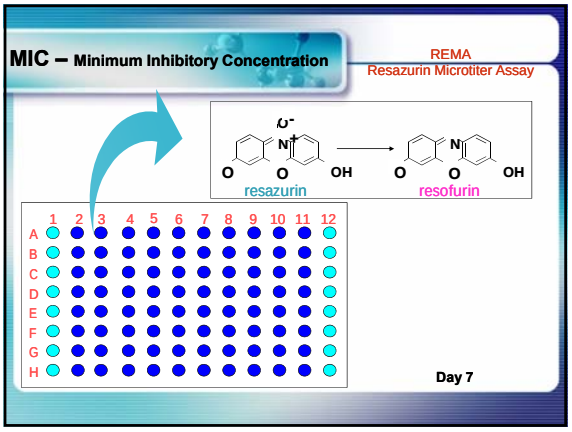
"The new compound is active against *Mycobacterium tuberculosis*?"

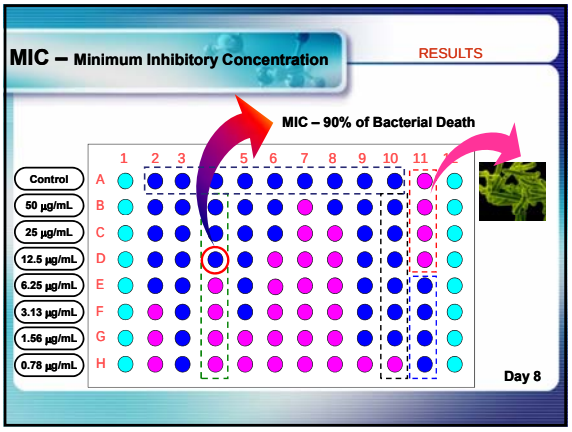
MIC – Minimum Inhibitory Concentration

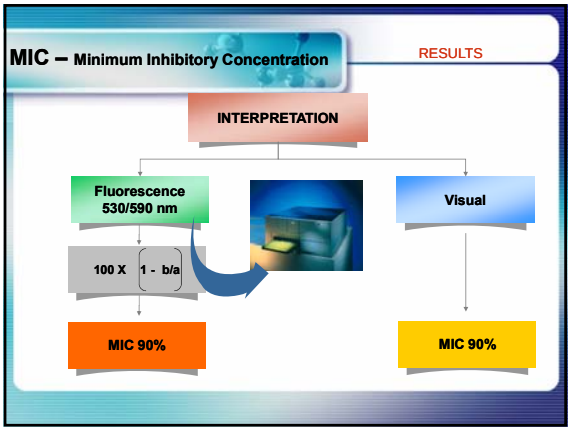
REMA
Resazurin Microtiter Assay



J.C. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, F. Portaels. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2002, p. 2720–2722


MIC – Minimum Inhibitory Concentration
REMA
Resazurin Microtiter Assay
resazurin
resofurin
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Day 7


MIC – Minimum Inhibitory Concentration
RESULTS
MIC – 90% of Bacterial Death
Control
50 µg/mL
25 µg/mL
12.5 µg/mL
6.25 µg/mL
3.13 µg/mL
1.56 µg/mL
0.78 µg/mL
Day 8


MIC – Minimum Inhibitory Concentration
RESULTS
INTERPRETATION
Fluorescence
530/590 nm
100 X 1 - b/a
MIC 90%
Visual
MIC 90%

Screening - Second Step

"The new compound is toxic against eukaryotic cells?"

IC_{50} – *In vitro* cytotoxicity

Murines tumor cell lines J774

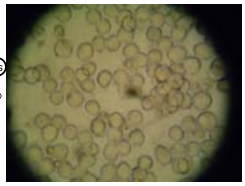
Cellular Culture

J774

Murines tumor cell lines J774

The cells were routinely maintained with RPMI medium supplemented with 10% fetal bovine serum (FBS), at 37°C in a humidified 5% CO2 atmosphere.

15 days

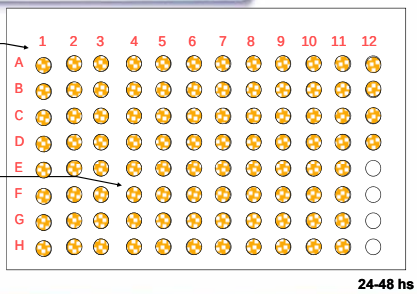


IC_{50} – *In vitro* cytotoxicity

Methodology

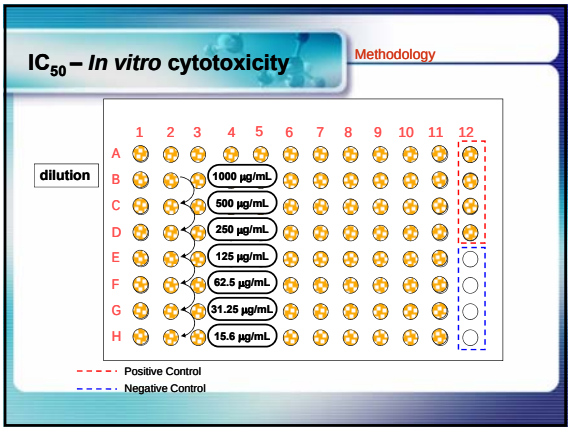
$1x10^5$ cell/mL

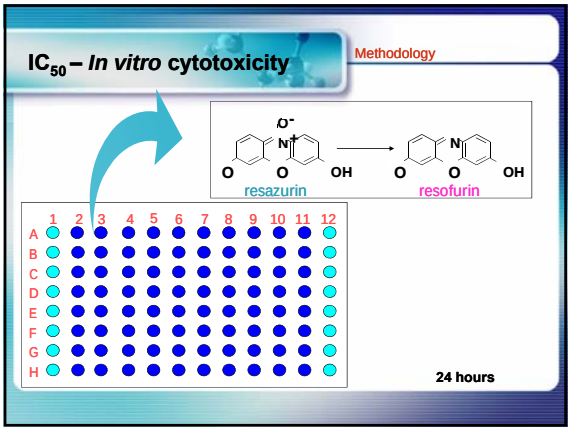
$2x10^4$ cell/well

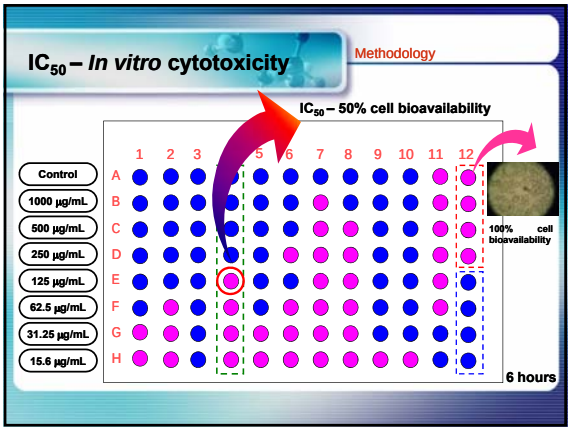


24-48 hs

S.A. Ahmed, R.M. Gogal, J. E. Walsh. J IMMUNOL METHODS, v. 170 (2): p. 211-224, 1994.


IC50 – In vitro cytotoxicity
Methodology
dilution
1000 µg/mL
500 µg/mL
250 µg/mL
125 µg/mL
62.5 µg/mL
31.25 µg/mL
15.6 µg/mL
Positive Control
Negative Control


IC50 – In vitro cytotoxicity
Methodology
resazurin
resofurin
24 hours


IC50 – In vitro cytotoxicity
Methodology
IC50 – 50% cell bioavailability
Control
1000 µg/mL
500 µg/mL
250 µg/mL
125 µg/mL
62.5 µg/mL
31.25 µg/mL
15.6 µg/mL
100% cell bioavailability
6 hours

IC_{50} – *In vitro* cytotoxicity

RESULTS

INTERPRETATION

Fluorescence 530/590 nm

100 X [1 - b/a]

IC_{50}

Screening - Third Step

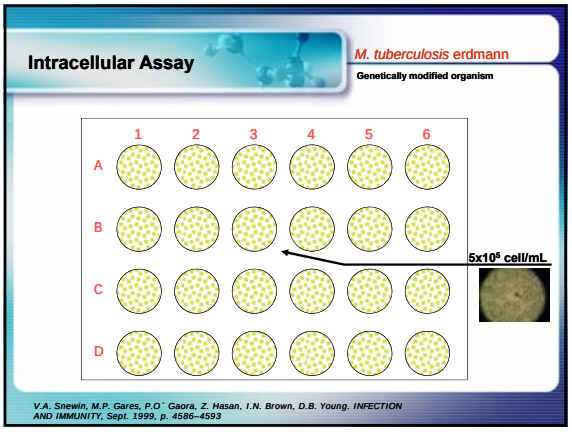
"The new compound is active against intracellular Bacteria?"

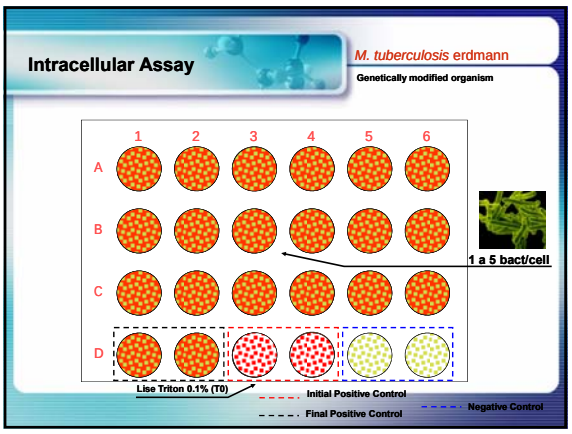
Intracellular Assay

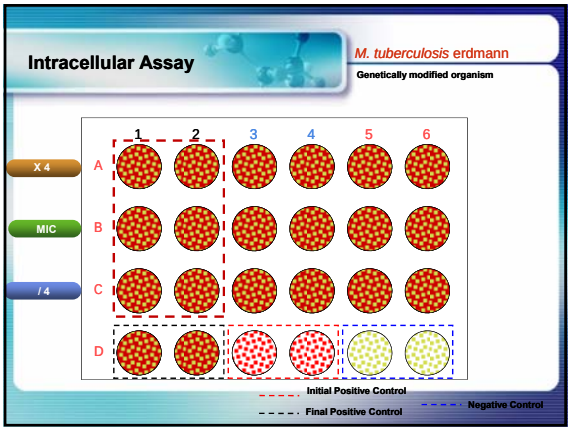
M. tuberculosis erdmann

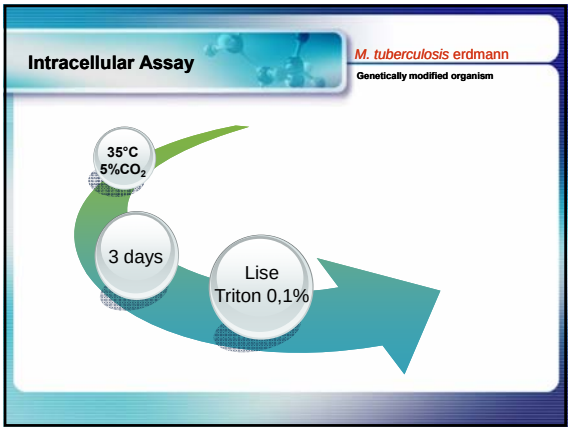
Genetically modified organism

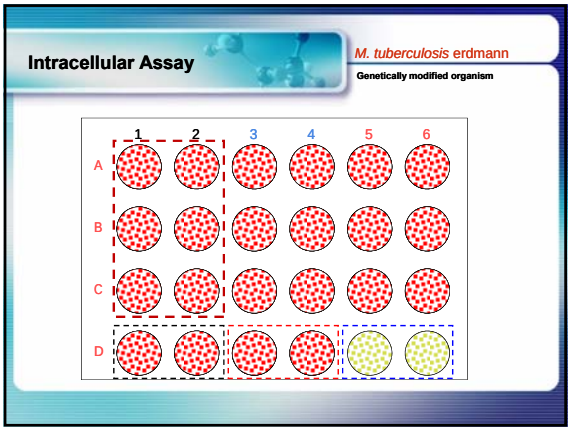
Living Cells + ATP or FMNH2 = "Light"

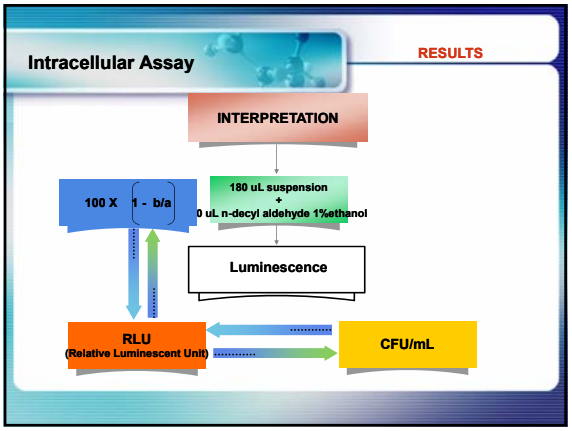

Intracellular Assay
M. tuberculosis erdmann
Genetically modified organism
1 2 3 4 5 6
A
B
C
D
5x10⁵ cell/mL
V.A. Snewin, M.P. Gares, P.O´ Gaora, Z. Hasan, I.N. Brown, D.B. Young. INFECTION AND IMMUNITY, Sept. 1999, p. 4586–4593


Intracellular Assay
M. tuberculosis erdmann
Genetically modified organism
1 2 3 4 5 6
A
B
C
D
1 a 5 bact/cell
Lise Triton 0.1% (T0)
Initial Positive Control
Final Positive Control
Negative Control


Intracellular Assay
M. tuberculosis erdmann
Genetically modified organism
1 2 3 4 5 6
X 4
MIC
/ 4
A
B
C
D
Initial Positive Control
Final Positive Control
Negative Control


Intracellular Assay
M. tuberculosis erdmann
Genetically modified organism
35°C
5%CO2
3 days
Lise
Triton 0,1%

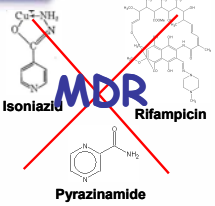

Intracellular Assay
M. tuberculosis erdmann
Genetically modified organism
1
2
3
4
5
6
A
B
C
D


Intracellular Assay
RESULTS
INTERPRETATION
100 X
1 - b/a
180 uL suspension
+
0 uL n-decyl aldehyde 1%ethanol
Luminescence
RLU
(Relative Luminescent Unit)
CFU/mL

Pre-Clinical Research

National Institutes of Health

- *In vivo*
  - LD $_{50}$
  - Pharmac okinetic
  - Infection
- *In vitro*
  - MIC
    IC$_{50}$
    Intr. At.
  - MDR
  - Sinergism
  - Latent

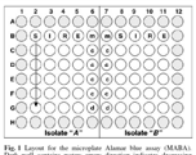


"The new compound is active against MDR TB?"

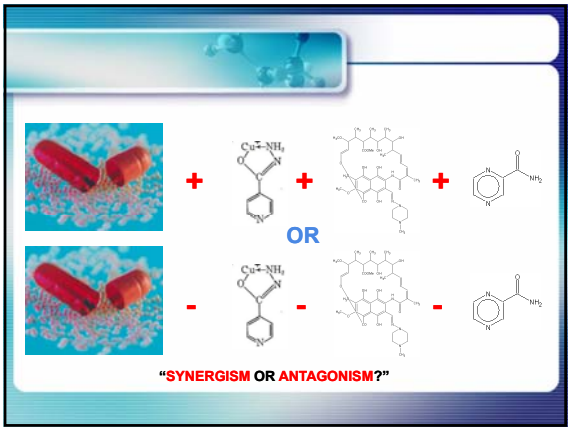
MDR Assays

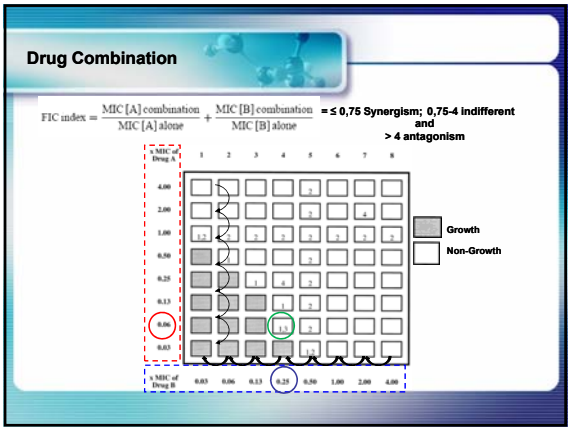
REMA – Resazurin Microtiter Assay

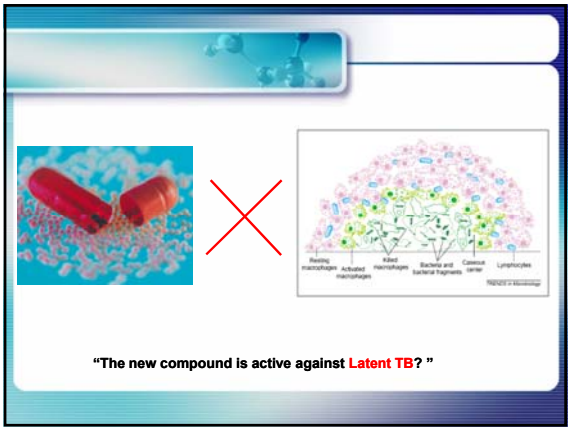
MDR Clinical Isolates
Isoniazid
Rifampicin
Pyrazinamide
Streptomicyn



HERRERA, J.L. et al. Use of Receiver Operating Characteristic Curves to Assess the Performance of a Microdilution Assay for Determination of Drug Susceptibility of Clinical Isolates of Mycobacterium tuberculosis **Eur J Clin Microbiol Infect Dis**, *22, p. 21-27, 2003.*

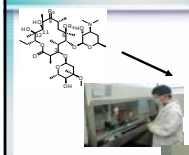

+
+
+
OR
-
-
-
"SYNERGISM OR ANTAGONISM?"


Drug Combination
FIC index = MIC [A] combination / MIC [A] alone + MIC [B] combination / MIC [B] alone
= ≤ 0,75 Synergism; 0,75-4 indifferent and > 4 antagonism
Growth
Non-Growth


"The new compound is active against Latent TB? "

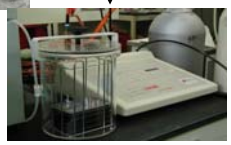
Low Oxygen Recovery Assay (LORA)

UIC Institute for Tuberculosis Research



Low oxygen-adapted *M. tuberculosis* carrying luciferase gene

2×10^4



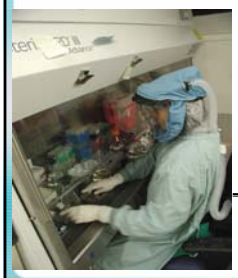
28h "recovery" in air

Cfu in 24-well plates

luciferase

10 days under <0.16% oxygen

*CHO, S.H.; et al. Low-Oxygen-Recovery Assay for High-Throughput Screening of Compounds against Nonreplicating Mycobacterium tuberculosis. **Antimicrob Agents Chemother**, 51 (4), p. 1380-1385, 2007.*



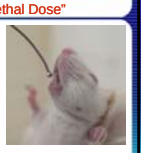
Biological Assays

In vivo

"The new compound is TOXIC? "

Lethal Dose - LD_{50}

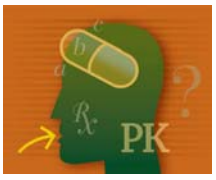
"Lethal Dose"



"Need ethic committee approval"
CEP/FCF/CAr. n° 41/2008

- Female Swiss mice
  - 6 animals/group, 1 compound per group
- Only dose of 2.000 mg/Kg of corporeal weight
  - Doses of 100, 300, 1000 mg/Kg of corporeal weight (if necessary)
- Control Group – Feeding of *ad libitum*
- Observation after administration of 4 to 6 hours and 2 times for day

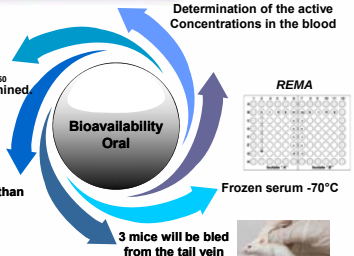
Gruppo et al.; Rapid Microbiologic and Pharmacologic Evaluation of Experimental Compounds against Mycobacterium tuberculosis. ***Antimicrobial Agents and Chemotherapy****, 50(4), 1245-1250, 2006.*



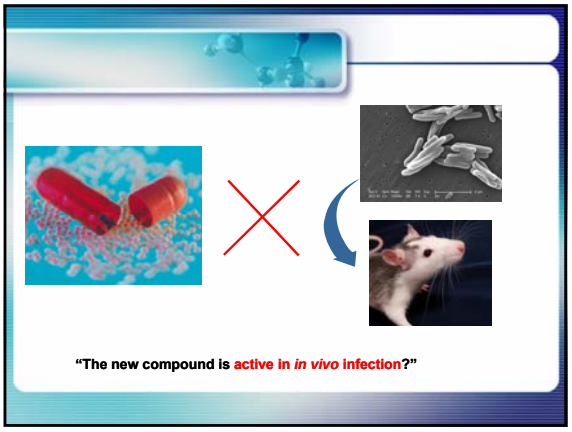
"PHARMACOKINETIC"

PHARMACOKINETIC

Bioavailability Oral



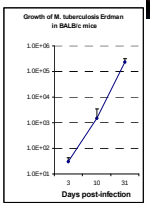
Gruppo et al.; Rapid Microbiologic and Pharmacologic Evaluation of Experimental Compounds against Mycobacterium tuberculosis. ***Antimicrobial Agents and Chemotherapy****, 50(4), 1245-1250, 2006.*



In vivo Infeccion

UIC Institute for Tuberculosis Research

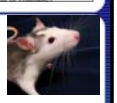
Gamma Interferon Gene-Disrupted Mice



LENAERTS, A.J.M. et al. Rapid in vivo screening of experimental drugs for Tuberculosis using Gamma Interferon gene-disrupted mice. ***Antimicrob Agents Chemother****, 47(2), p. 783-785, 2003.*

In vivo Infeccion

UIC Institute for Tuberculosis Research



- 18 days after infection, will start the treatment and continuous at 9 days with daily doses.
- Negative Group Control – Without drugs administration
- Positive Group Control – Daily doses at 25 mg/Kg/day with Isoniazid
- 1 group per compound, 5 animals per group,
- After treatment, the animals will be sacrificed, the lungs and spleen removed aseptically and CFU realized from homogenized tissue


Muchas gracias!!!!!!!!!!!!!!!!!
Phytomedicine
METAL IONS
pavanfer@yahoo.com.br
AQA
Inorganic Biochemistry
POLYHEDRON