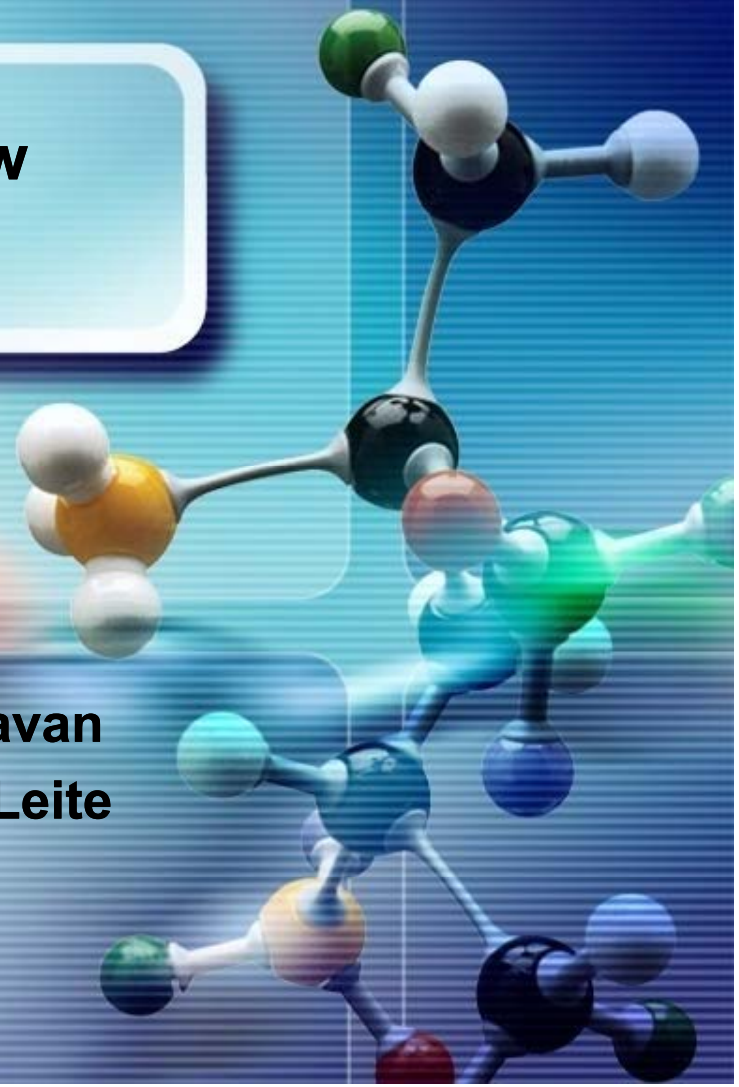
São Paulo State University "Julio de Mesquita Filho"
School of Phamaceutical Science

An approach to the search for new drugs against Tuberculosis



Researchers:

M.Sc. Fernando Rogério Pavan

Profª Drª Clarice Queico F. Leite

Remembering...

Who is the problem?
What we need to do?

- Tuberculosis (TB) is a preventable and curable infectious disease, transmitted through the air, the main etiologic agent is *Mycobacterium tuberculosis* (MTB)[1].
- In 2007 the mortality rate decreased to 1.3 million patients with TB and HIV-negative and 456,000 deaths among co-infected individuals[2].
- MDR-TB, XDR-TB and TB / HIV make it impossible to attempt to control TB[3].
- 1/3 of the population is infected with MTB in latency state.[4].
- Rifampicin – Discovery more than 40 years ago[2].

Enphasize

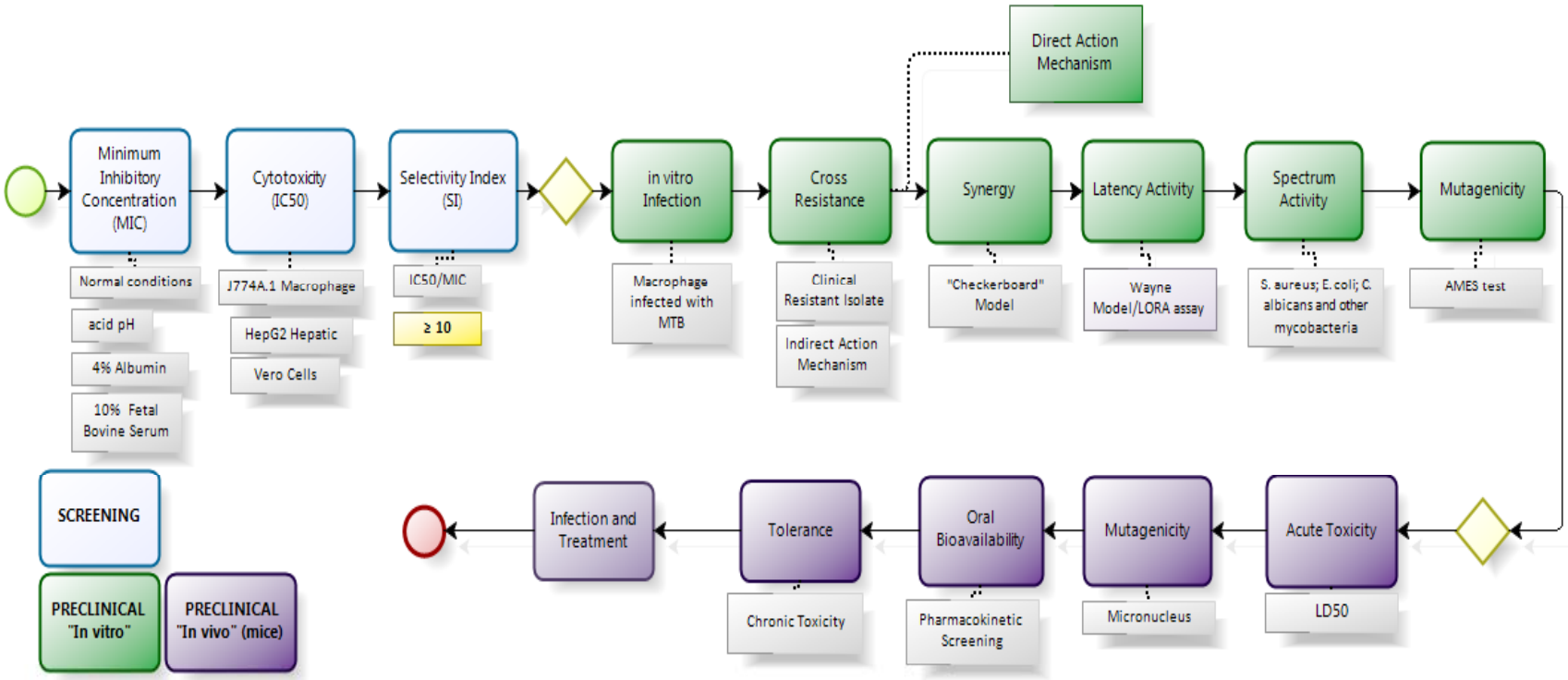
Need for a new agent[4]

- To Reduce the duration of treatment.
- To be active against resistant strains.
- Do not interfere with the retro-viral drugs.
- To be active against bacilli in latency state.

1Hunter, Pang et al., Kinetics and Ligand-Binding Preferences of MTB Thymidylate Synthases, ThyA and ThyX. **Plos One,** v. 3, n. 5, 2008.
2 Gandhi, Nunn et al., MDR and XDR tuberculosis: a threat to global control of TB. **Lancet,** v. 375, n. 9728, p. 1830-1843, May 2010.
3 Lalloo e Ambaram, New Antituberculous Drugs in Development. **Current HIV/AIDS Reports,** v. 7, n. 3, p. 143-151, 2010.
4 Ma, Lienhardt et al., Global tuberculosis drug development pipeline: the need and the reality. **Lancet,** v. 375, n. 9731, p. 2100-2109, 2010

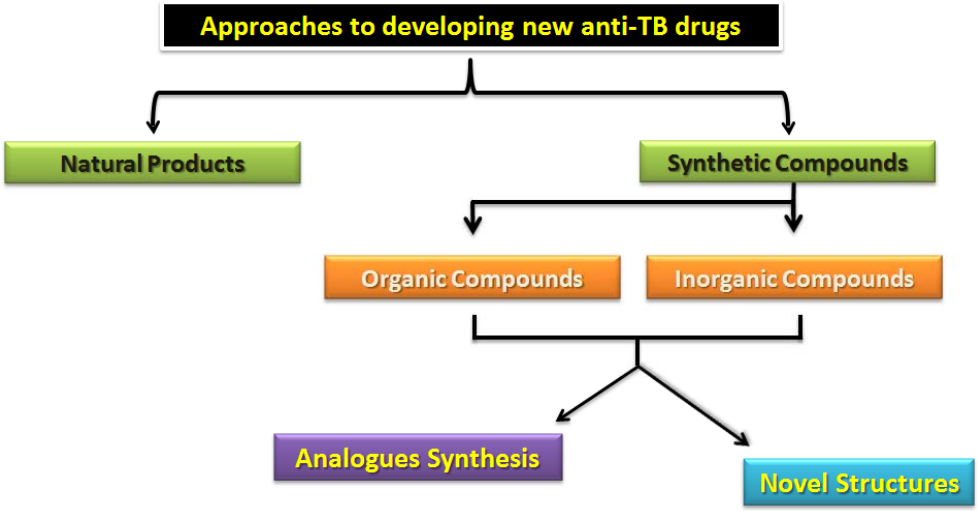
Anti-TB Drugs Pipeline

Pipeline to select new drugs against TB created at the "Hugo David" Laboratory, FCFAR/UNESP, Araraquara, Brazil.



Pavan et al., An Approach to research for new drugs against TB, **TUBERCULOSIS**, accept, 2011.

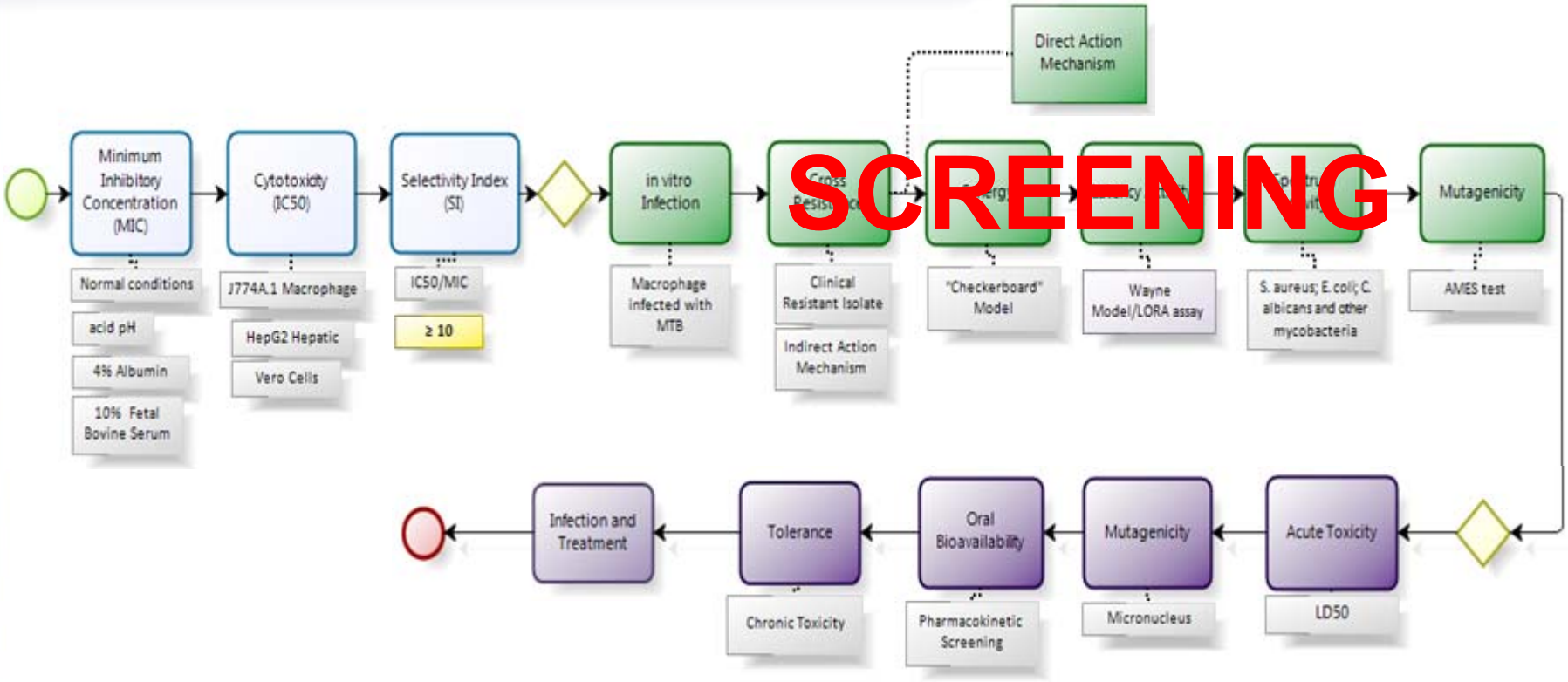
Anti-TB Drugs Pipeline



To date more than 2,000 compounds were analyzed in the stage of screening.

TARALLO, M. et al. Design of novel iron compounds as potential therapeutic agents against tuberculosis. **Journal of Inorganic Biochemistry,** v. 104, n. 11, p. 1164-1170, 2010.

Starting the Pipeline

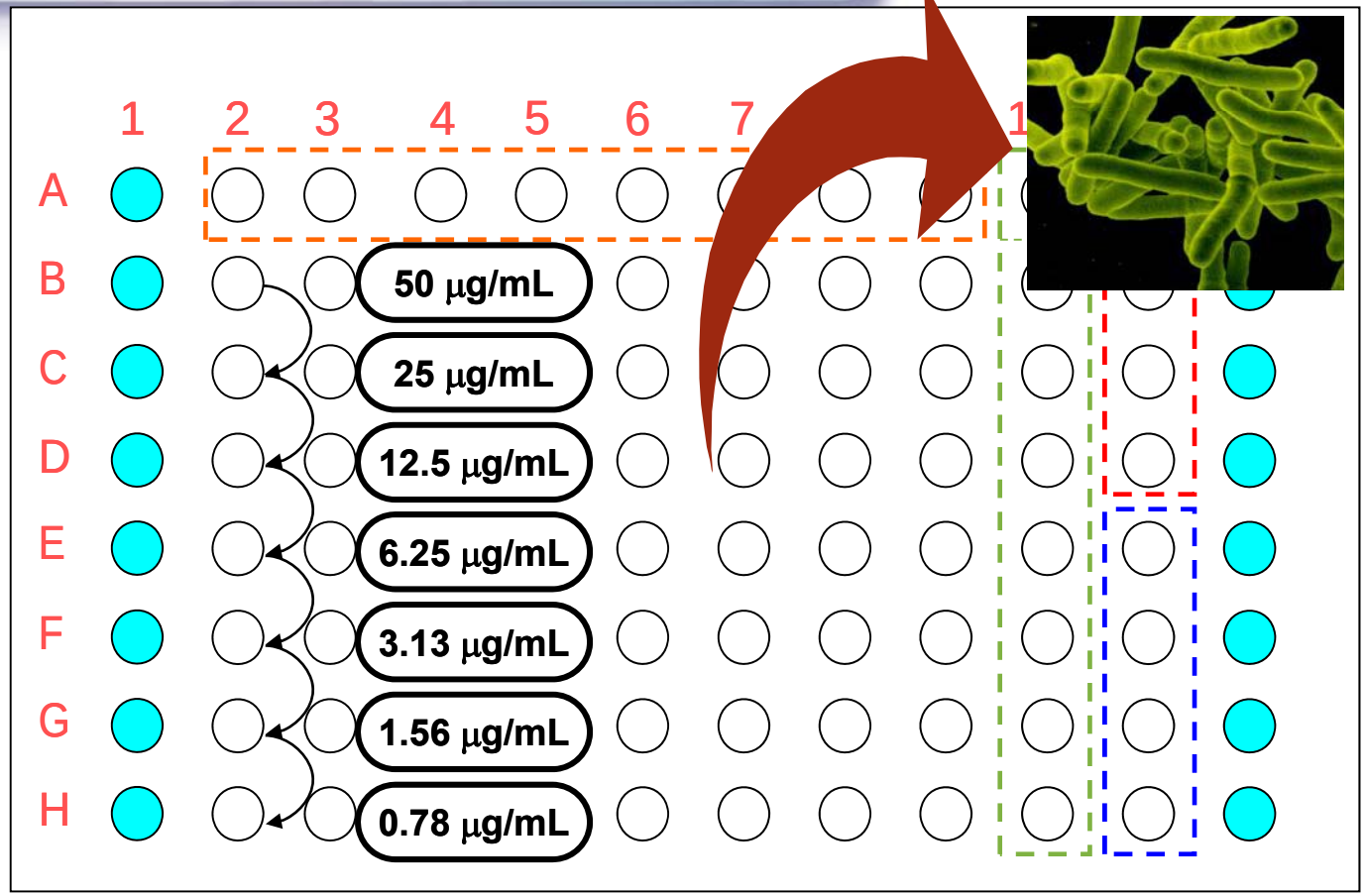


Screening

Determination of MIC against the MTB H37Rv in different environmental conditions (normal, pH 6.0, 4% ASB and 10% FBS)

Screening

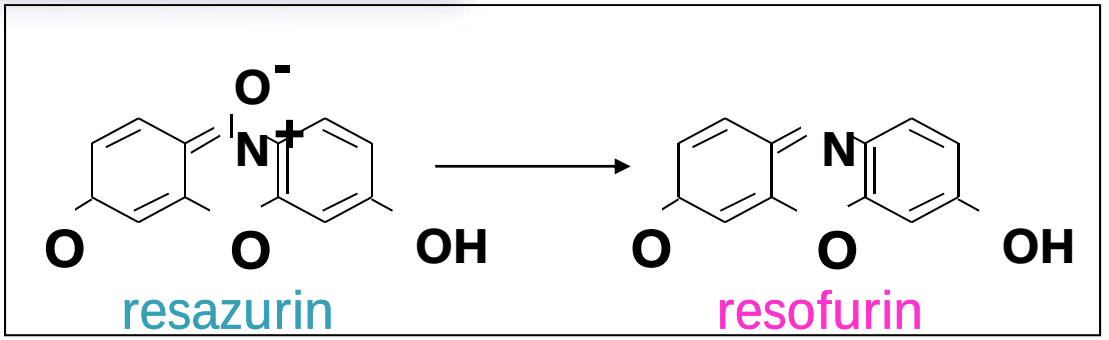
REMA
Resazurin Microtiter Assay

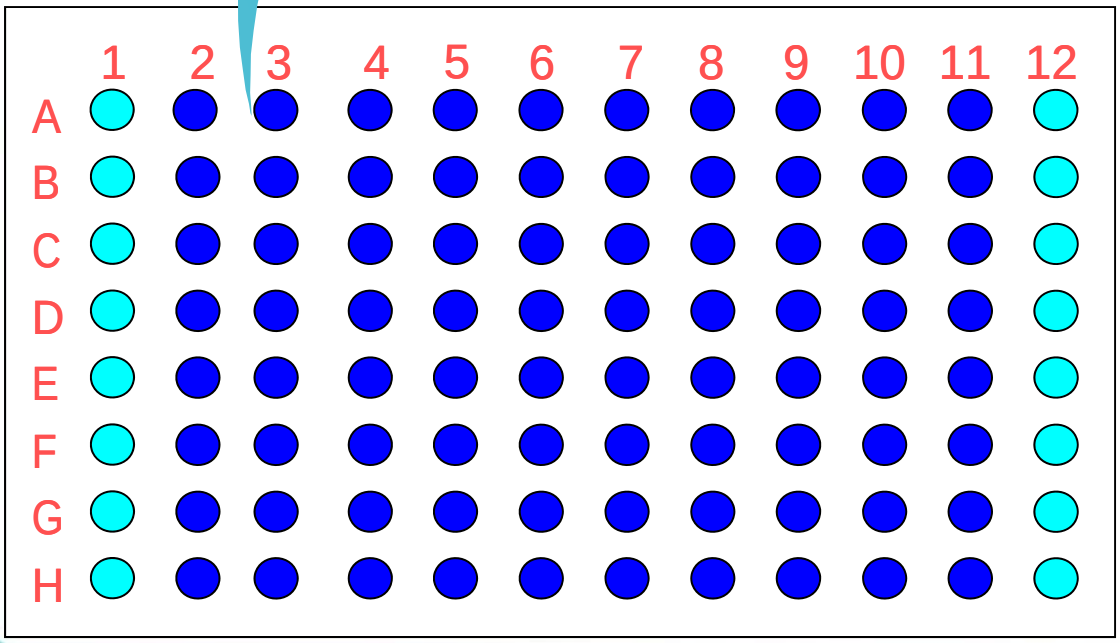


PALOMINO, J. C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 46, n. 8, p. 2720-2722, 2002.

Screening

REMA
Resazurin Microtiter Assay





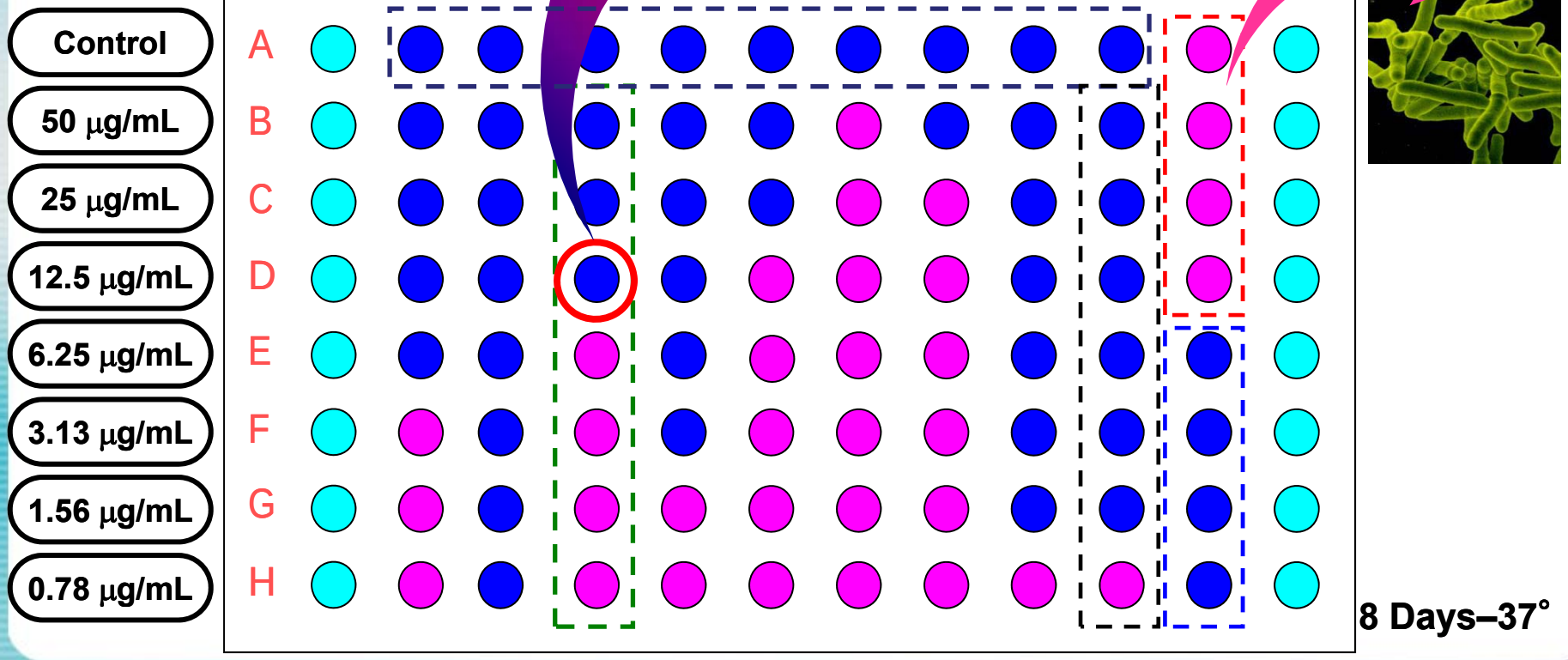
7 Days – 37° C

PALOMINO, J. C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 46, n. 8, p. 2720-2722, 2002.

Screening

REMA
Resazurin Microtiter Assay

MIC – 90% Inhibition of Bacterial growth



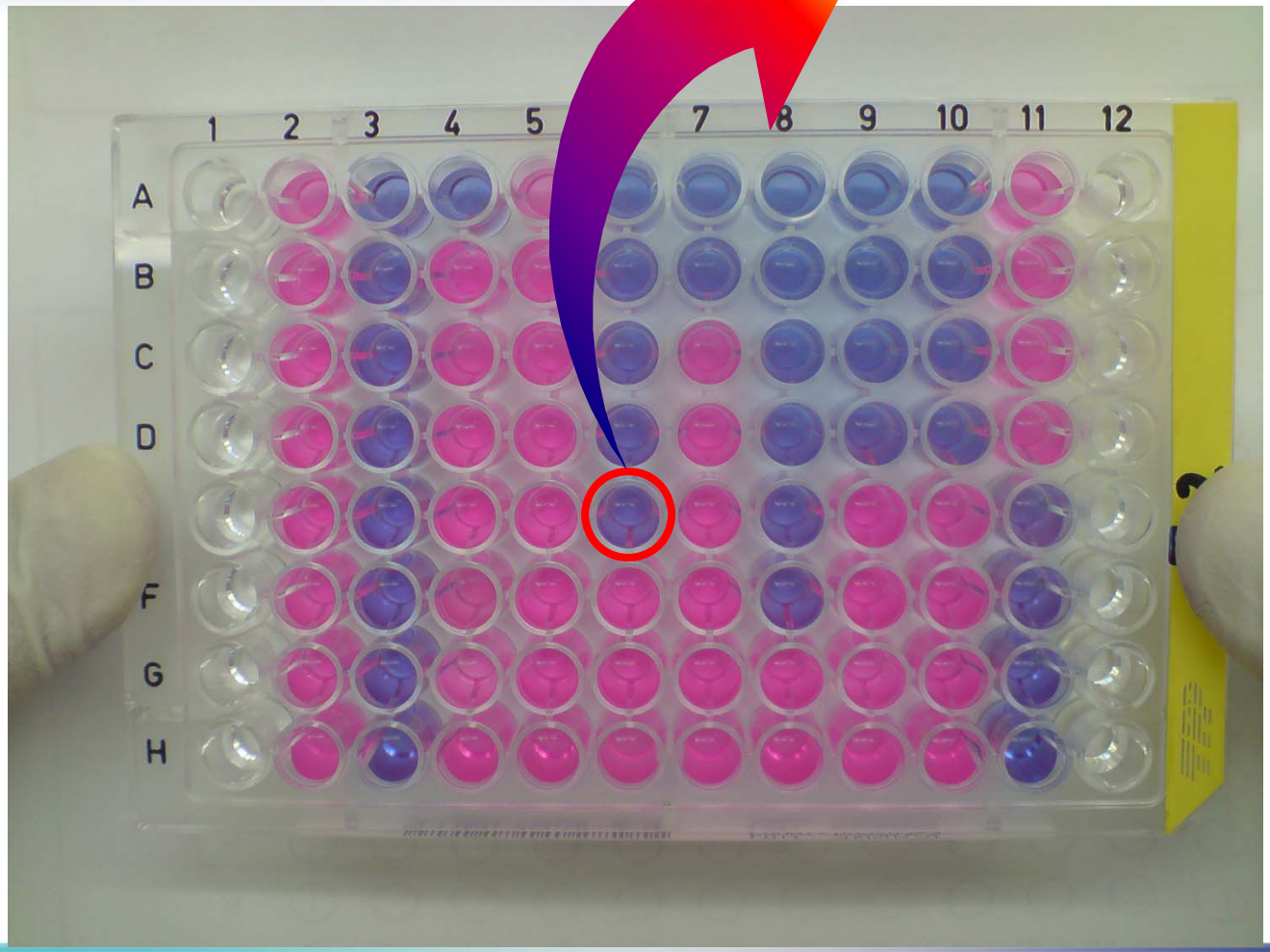
8 Days–37° C

PALOMINO, J. C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 46, n. 8, p. 2720-2722, 2002.

Screening

REMA
Resazurin Microtiter Assay

MIC – 90% Inhibition of Bacterial growth



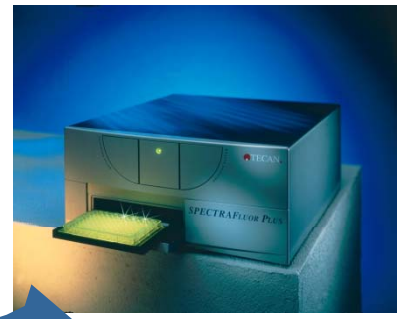
PALOMINO, J. C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 46, n. 8, p. 2720-2722, 2002.

Screening

REMA
Resazurin Microtiter Assay

INTERPRETATION

Fluorescence 530/590 nm



$$100 \times \left(1 - \frac{b}{a}\right)$$

MIC 90%

Visual

MIC 90%

PALOMINO, J. C. et al. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 46, n. 8, p. 2720-2722, 2002.

Screening

Determination of Cytotoxicity (IC50) front of VERO (normal cells);

HepG2 (hepatic cells) and J774A.1 (macrophage cells)

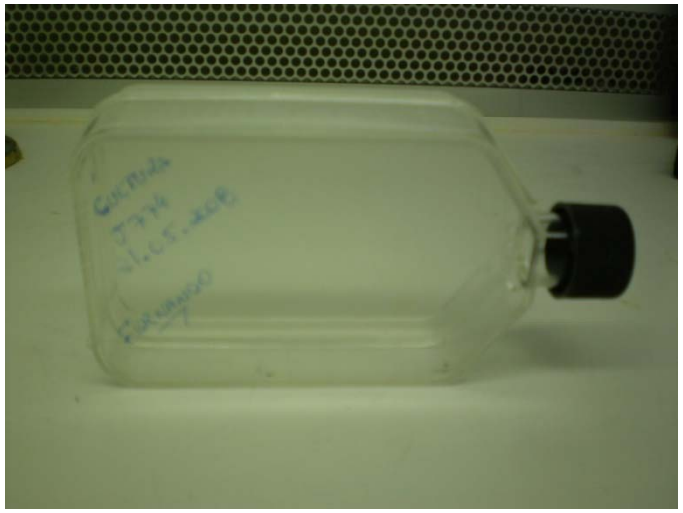
Screening

Cytotoxicity (IC50)

Cell Culture

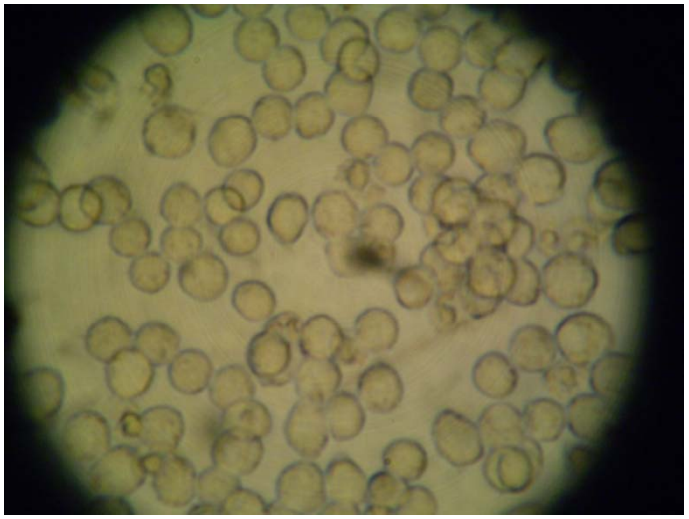
Cell Culture

Cells are incubated at 35° C with 5% CO2 in cell culture bottles in Eagle's medium supplemented with 10% fetal bovine serum and ATBs penicillin and amphotericin B.



A. Bottle used to cell culture

15 days

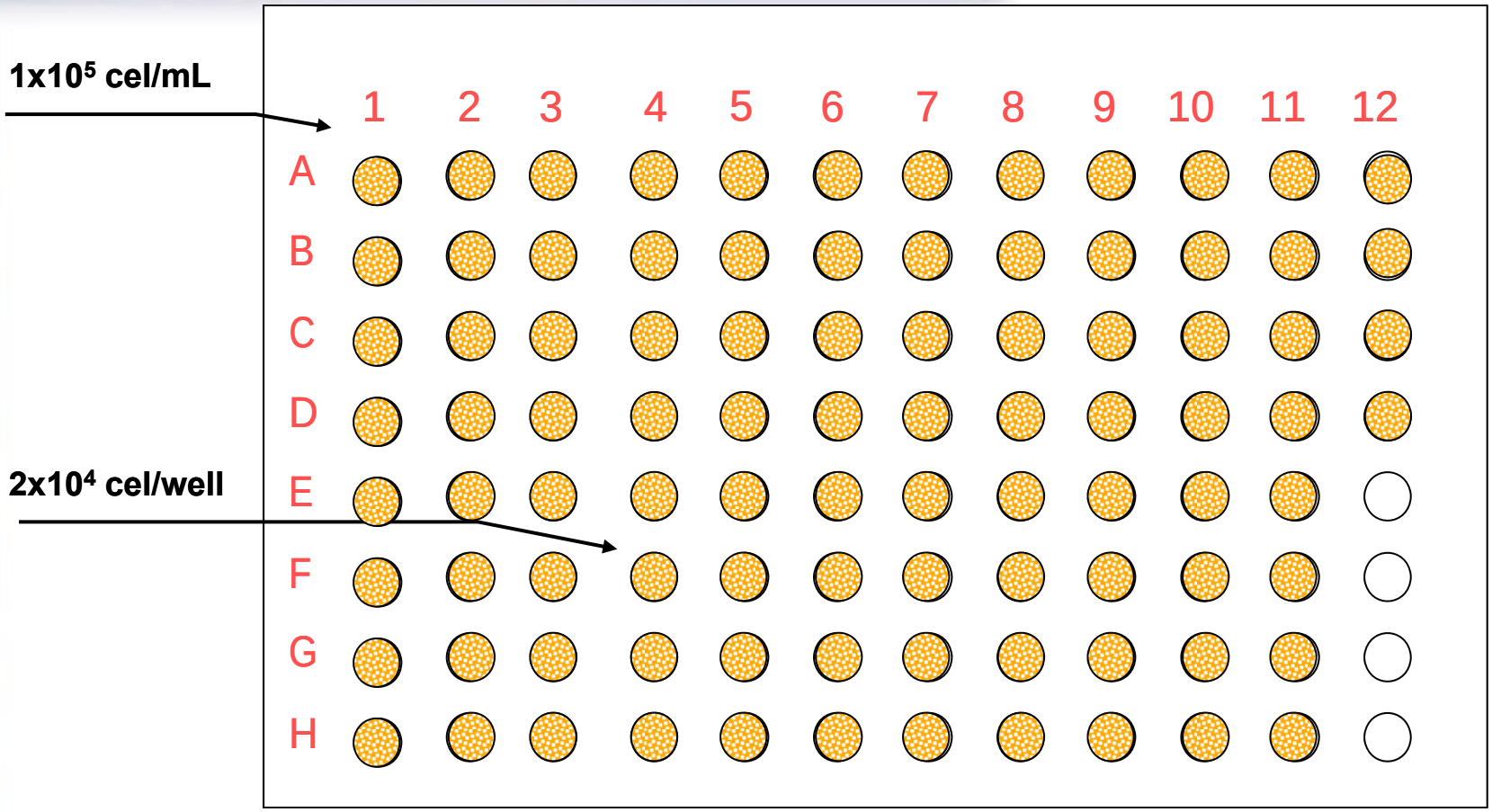


B. J774A.1 macrophage cells observed under an inverted microscope.

Screening

Cytotoxicity (IC50)

Methodology



24-48 hs

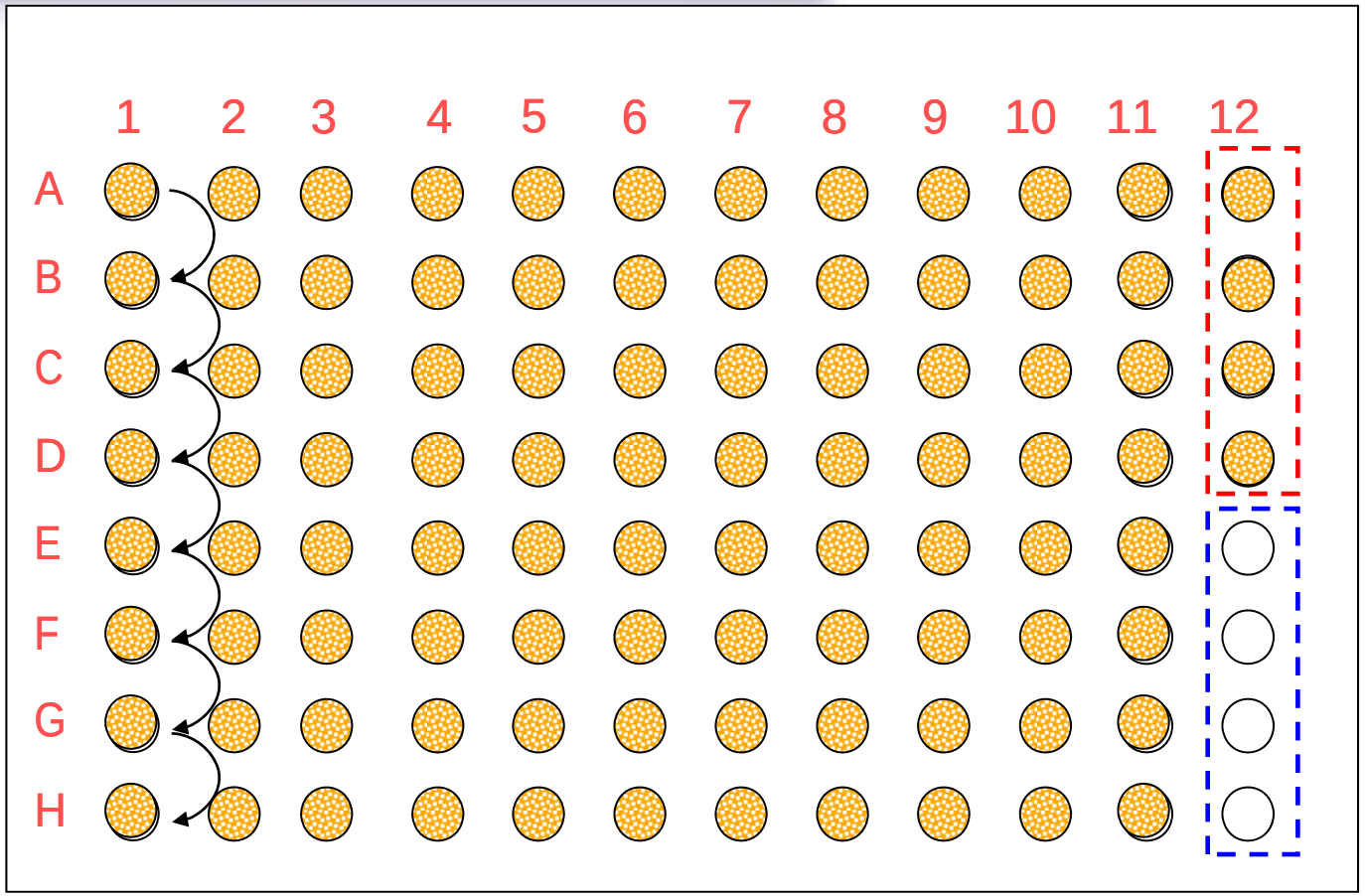
PAVAN, F.R. et al. Thiosemicarbazones, semicarbazones, dithio-carbazates and hydrazide/hydrazones: anti-Mycobacterium tuberculosis activity and cytotoxicity. **European Journal of Medicinal Chemistry,** v. 45, n. 11, p. 1898-1905, 2010.

Screening

Cytotoxicity (IC50)

Methodology

dilution

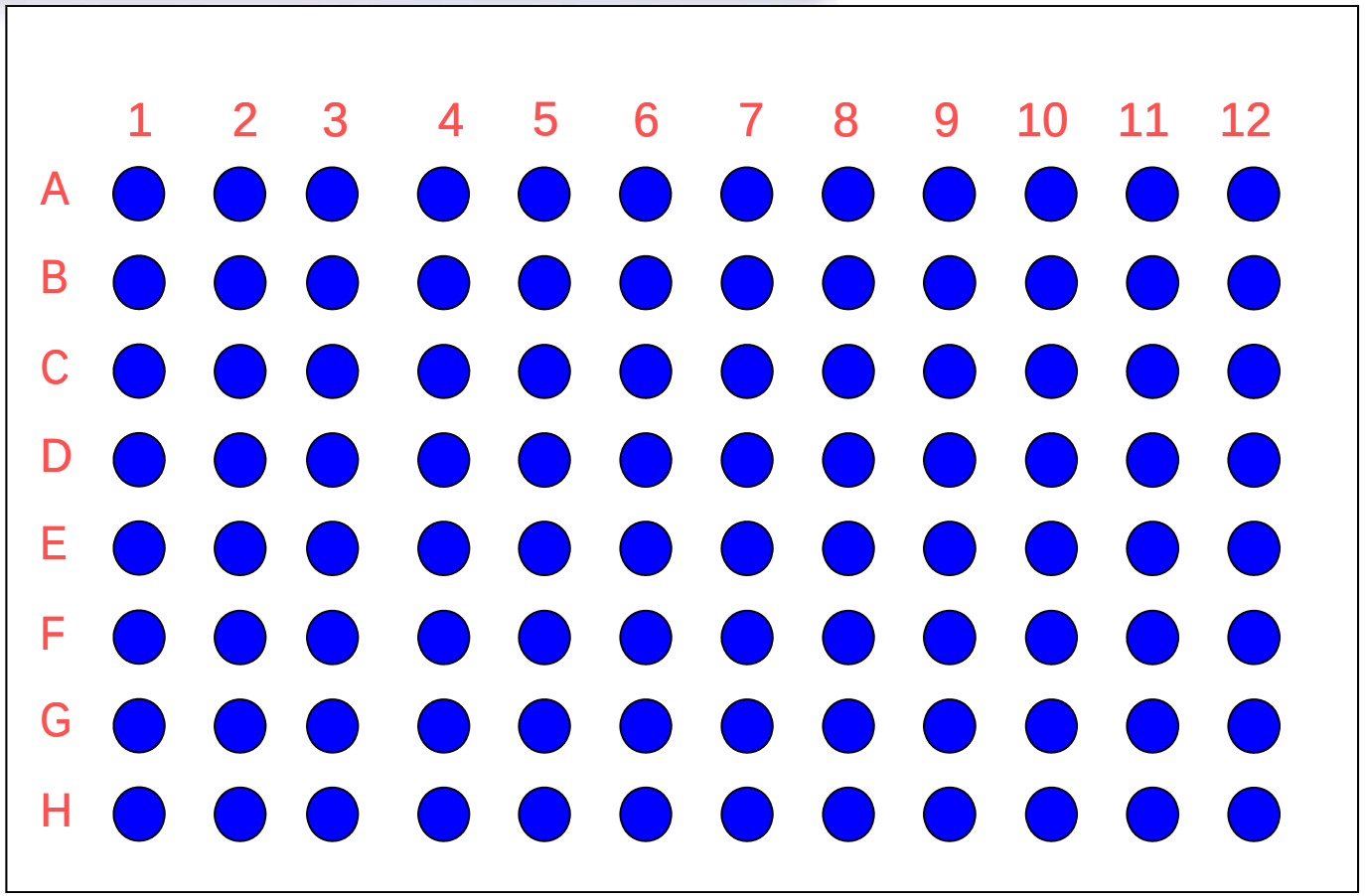


PAVAN, F.R. et al. Thiosemicarbazones, semicarbazones, dithio-carbazates and hydrazide/hydrazones: anti-Mycobacterium tuberculosis activity and cytotoxicity. **European Journal of Medicinal Chemistry,** v. 45, n. 11, p. 1898-1905, 2010.

Screening

Cytotoxicity (IC50)

Methodology



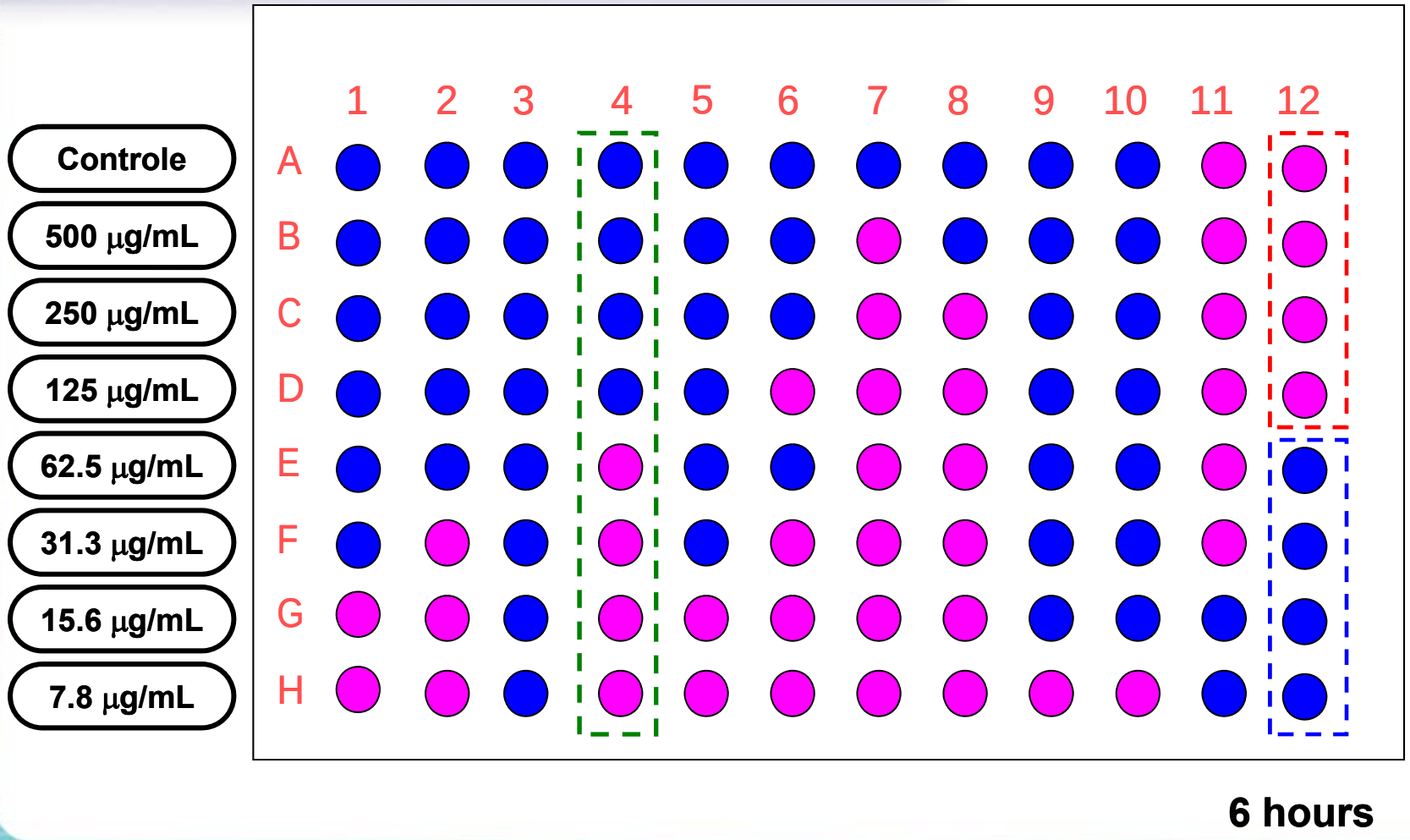
24 hours

PAVAN, F.R. et al. Thiosemicarbazones, semicarbazones, dithio-carbazates and hydrazide/hydrazones: anti-Mycobacterium tuberculosis activity and cytotoxicity. **European Journal of Medicinal Chemistry,** v. 45, n. 11, p. 1898-1905, 2010.

Screening

Cytotoxicity (IC50)

Methodology



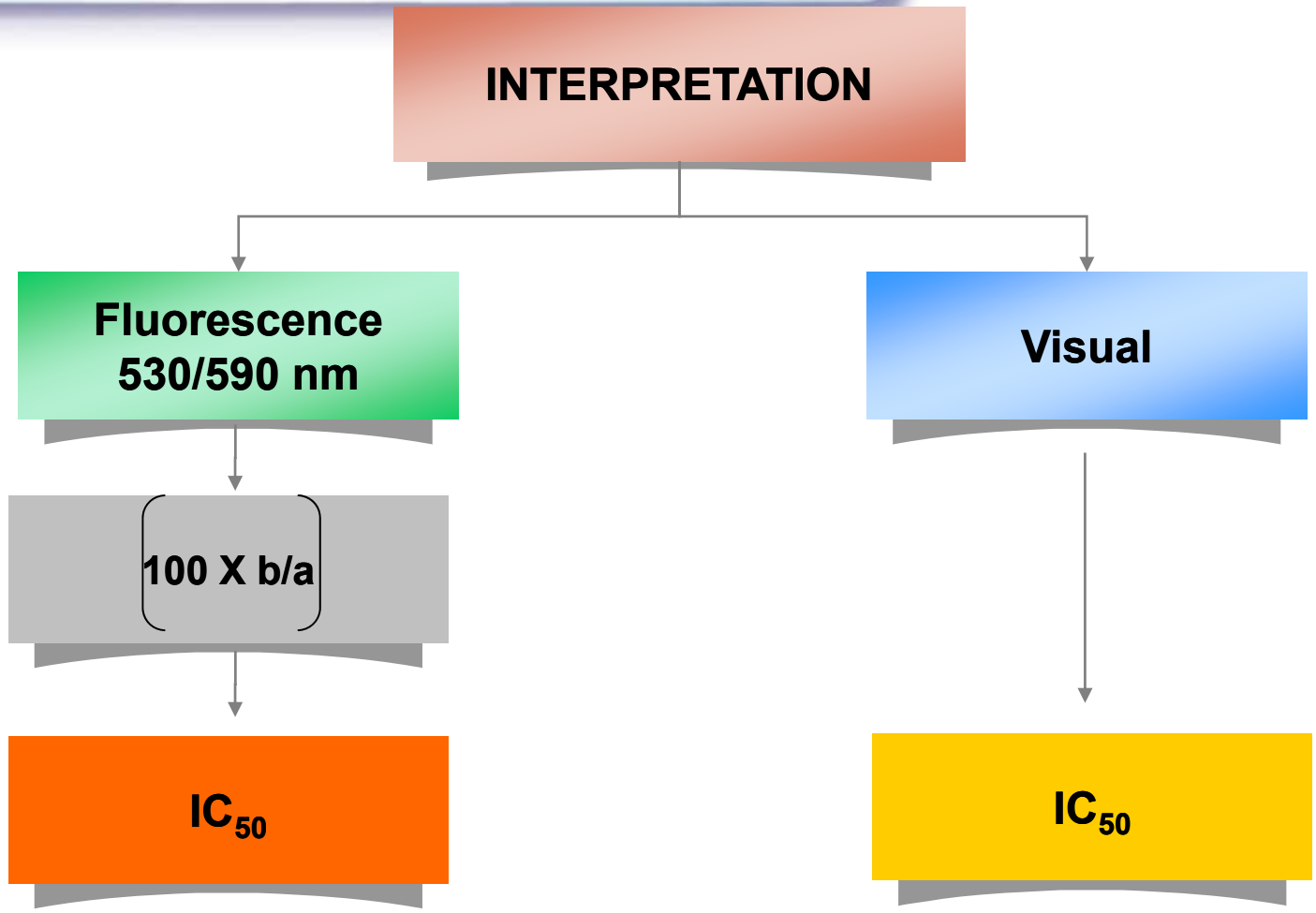
6 hours

PAVAN, F.R. et al. Thiosemicarbazones, semicarbazones, dithio-carbazates and hydrazide/hydrazones: anti-Mycobacterium tuberculosis activity and cytotoxicity. **European Journal of Medicinal Chemistry,** v. 45, n. 11, p. 1898-1905, 2010.

Screening

Cytotoxicity (IC50)

Methodology



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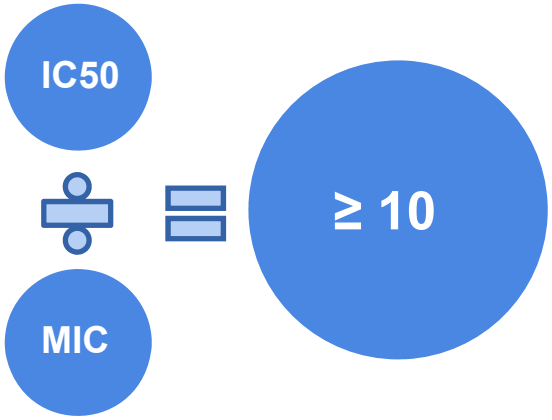
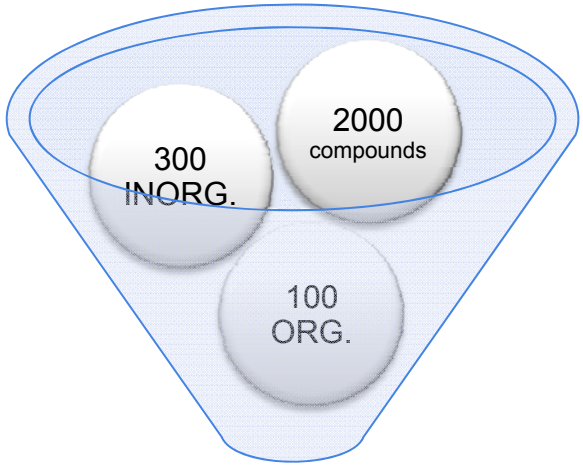
Screening

SELECTIVITY INDEX (SI)



OUR EXPERIENCE



90 SI ≥ 10

Ruthenium Compounds

Ruthenium (II) phosphine/diimines/picolinic acid

Screening Results

Table

Anti-MTB activity (MIC), cytotoxicity (IC50), and selectivity index (SI) of the ruthenium complexes and their free ligands

Identification	Compounds	MIC (μM)	IC50 (μM)	SI
SCAR01	$[Ru(pic)(dppb)(bipy)]PF_6$	1,2	23.6	34.20
SCAR02	$[Ru(pic)(dppb)(Me\text{-}bipy)]PF_6$	1,2	11.9	23.90
SCAR04	$[Ru(pic)(dppb)(phen)]PF_6$	1,4	30.4	40.10
SCAR05	$cis\text{-}[Ru(pic)(dppe)_2]PF_6$	0,8	23.0	31.20
SCAR06	$cis\text{-}[RuCl_2(dppb)(bipy)]$	1,6	3.35	15.20
SCAR07	Ru(pic)(dppe)(phen)	2,1	104	20.10

PAVAN, F.R. et al. Ruthenium (II) phosphin e/diimine/picolinate complexes: Inorganic compound s as agents against tuberculosis. **European Journal of Medicinal Chemistry,** IN PRESS, 2011.

Ruthenium (II) phosphine/diimines/picolinic acid

Screening Results

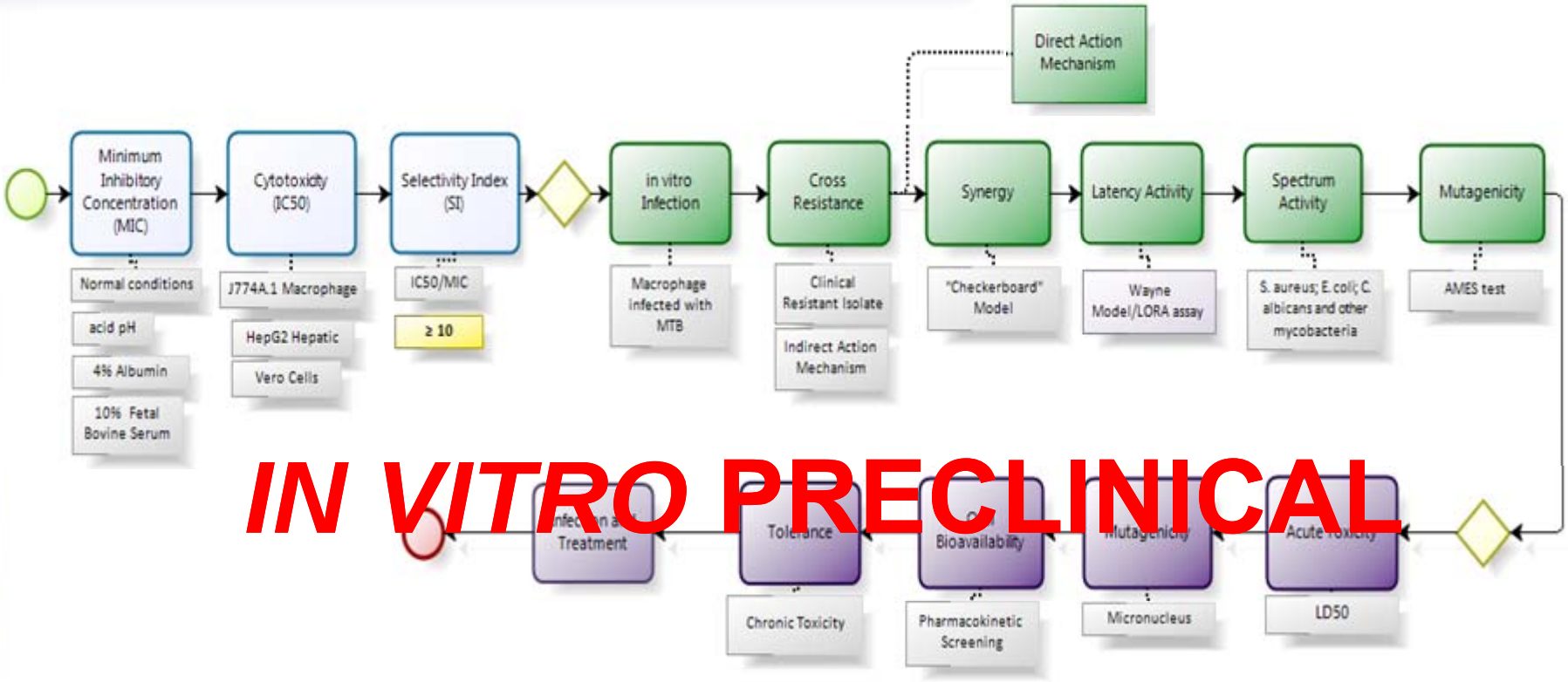
Table. Determination of MIC against the MTB H37Rv in different environmental conditions (normal, pH 6.0, 4% ASB and 10% FBS)

Identification	Compounds	REMA (µM)						
		Normal	pH 6,0		Protein Binding			
					4% ASB		10% SFB	
		MIC	MIC	Ratio	MIC	Ratio	MIC	Ratio
SCAR01	[Ru(pic)(dppb)(bipy)]PF_6	**1,2**	**2,4**	2,0	**2,5**	2,1	**1,3**	1,1
SCAR02	[Ru(pic)(dppb)(Me-bipy)]PF_6	**1,2**	**2,2**	1,8	**2,0**	1,7	**1,3**	1,1
SCAR04	[Ru(pic)(dppb)(phen)]PF_6	**1,4**	**2,7**	1,9	**3,5**	2,5	**1,9**	1,4
SCAR05	*cis*-[Ru(pic)$(dppe)_2$]PF_6	**0,8**	**1,7**	2,1	**1,7**	2,1	**0,8**	1,0
SCAR06	*cis*-[$RuCl_2$(dppb)(bipy)]	**1,6**	**4,9**	3,0	**3,2**	2,0	**3,1**	1,9
SCAR07	Ru(pic)(dppe)(phen)	**2,1**	**2,7**	1,3	**2,8**	1,3	**2,5**	1,2
Standard Drugs								
RMP	Rifampicin	**0,1**	**0,2**	2,0	**0,2**	2,0	**0,2**	2,0
INH	Isoniazid	**0,1**	**0,2**	2,0	**0,3**	3,0	**0,2**	2,0
STR	Streptomycin	**0,5**	**0,8**	1,6	**0,9**	1,8	**1,3**	2,6

"The complex did not show any loss in activity in the different conditions, indicating stability in acidic pH and no connection with serum proteins."

PAVAN, F.R. et al. Ruthenium (II) phosphin e/diimine/picolinate complexes: Inorganic compound s as agents against tuberculosis. **European Journal of Medicinal Chemistry,** IN PRESS, 2011.

Continuing the Pipeline



***IN VITRO* PRECLINICAL**

In vitro Preclinical

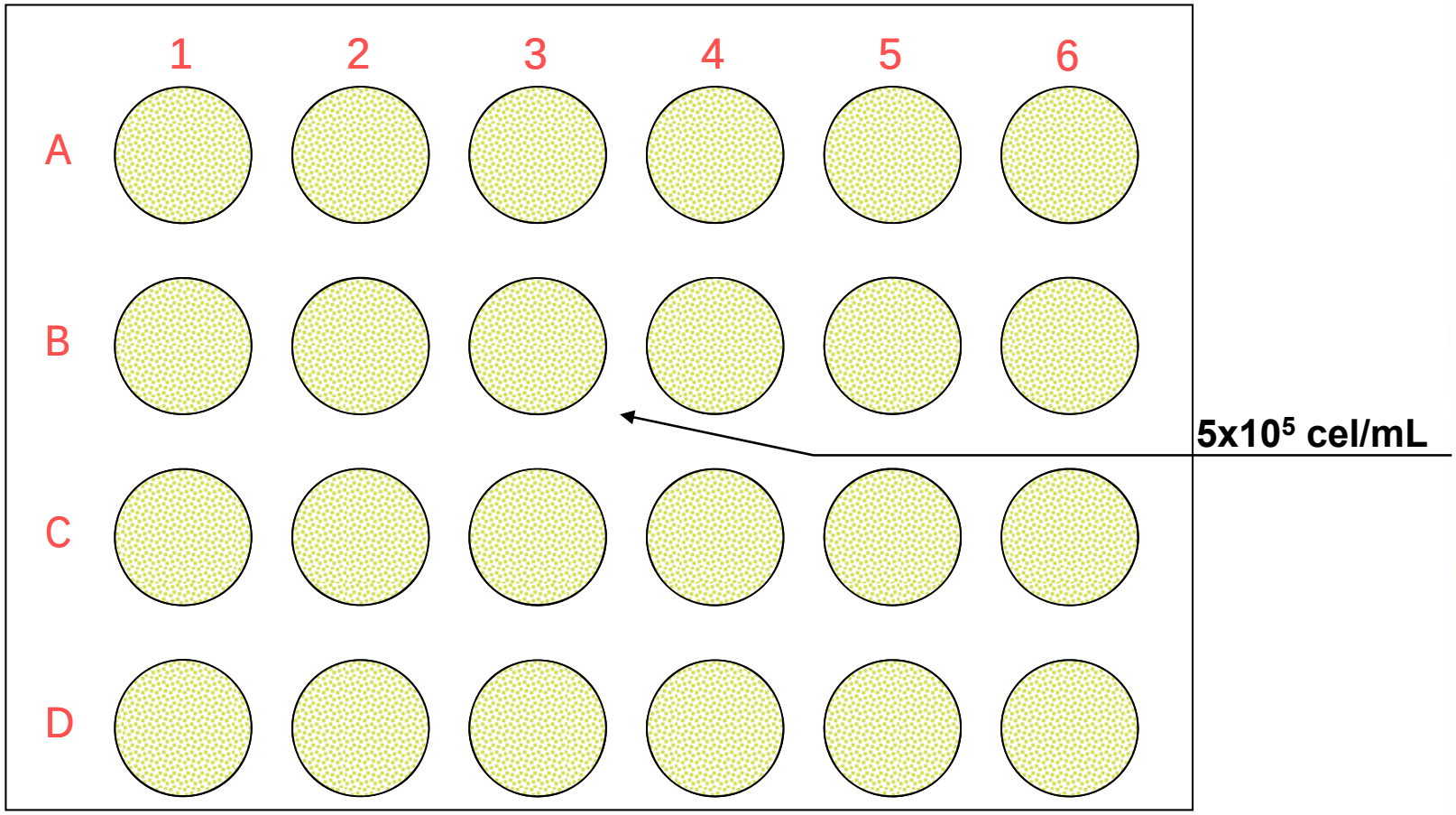
Intracellular activity against MTB Erdmann ATCC 35801

with pSMT1 plasmid

In vitro Preclinical

Intracellular Activity

Methodology

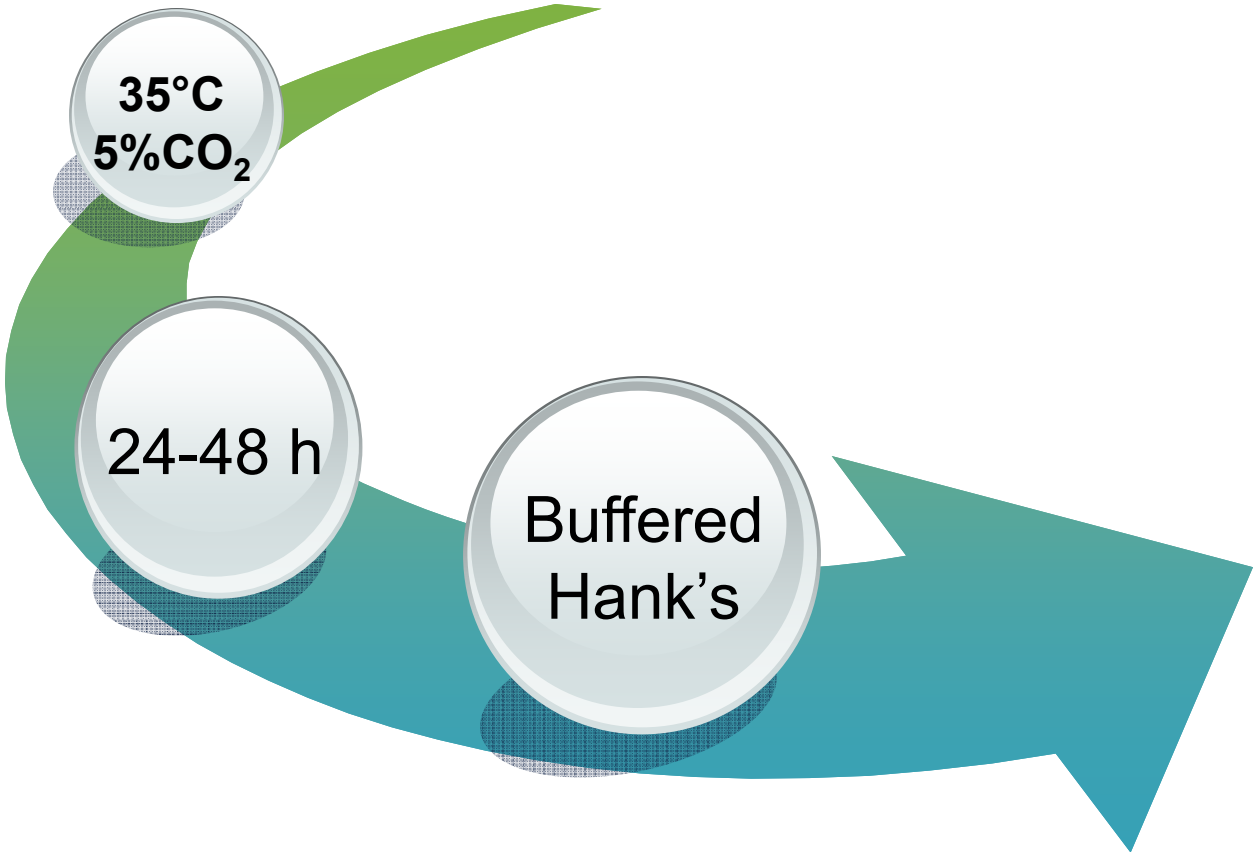


Snewin V.A. et al, Assessment of immunity to mycobacterial infection with luciferase reporter constructs. ***INFECTION AND IMMUNITY****, Sept. 1999, p. 4586–4593*

In vitro Preclinical

Intracellular Activity

Methodology



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In vitro Preclinical

Intracellular Activity

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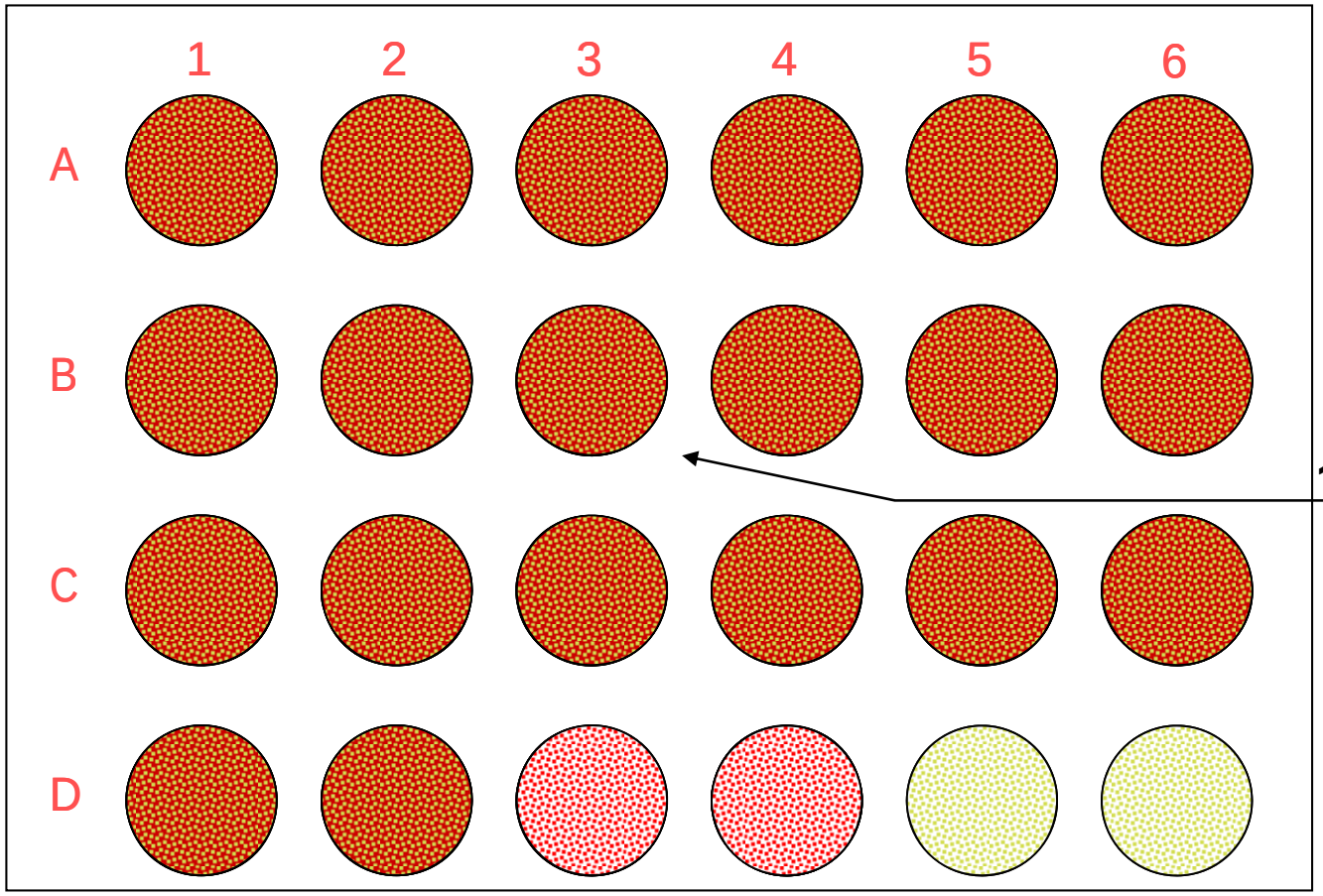

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In vitro Preclinical

Intracellular Activity

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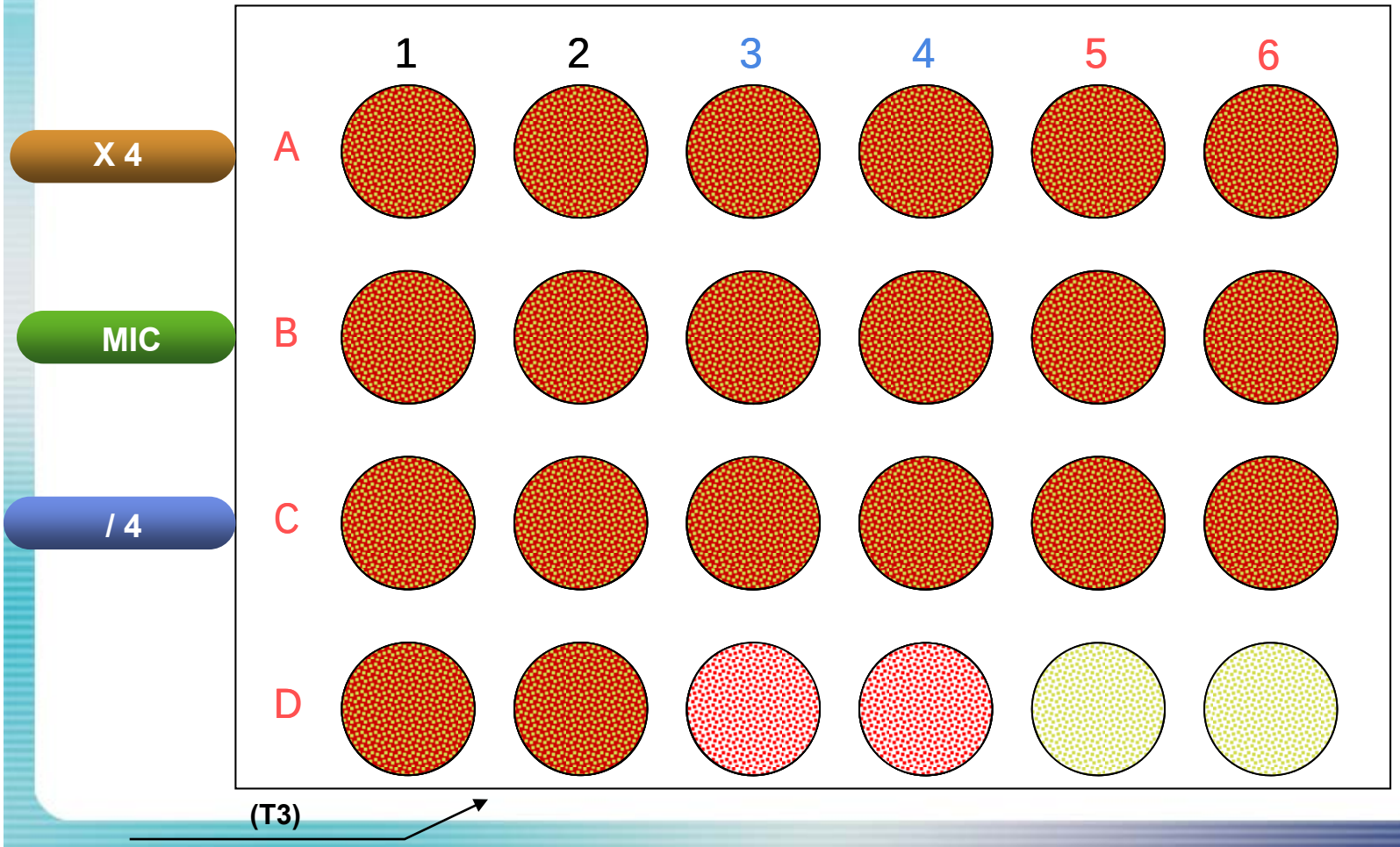

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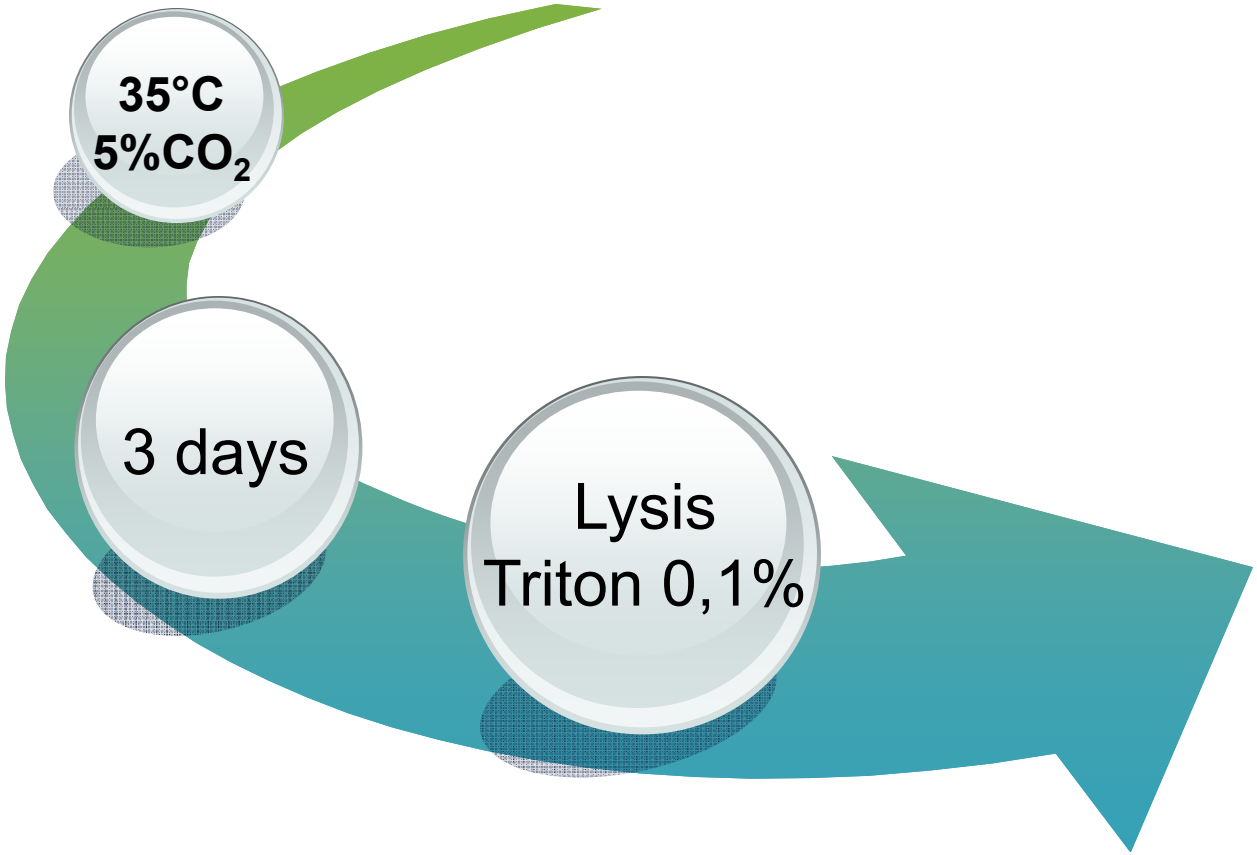
In vitro Preclinical

Intracellular Activity

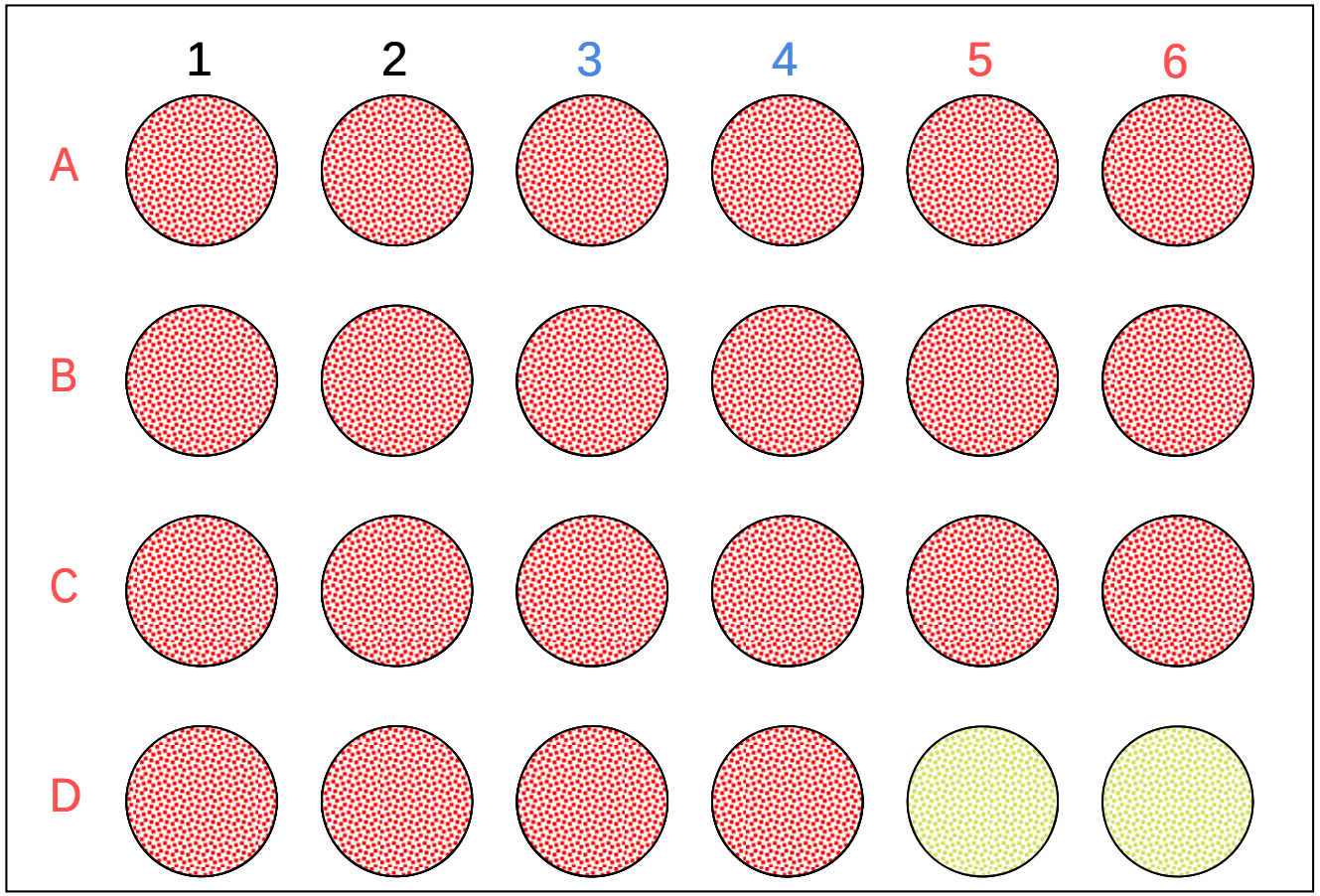
Methodology



In vitro Preclinical

Intracellular Activity

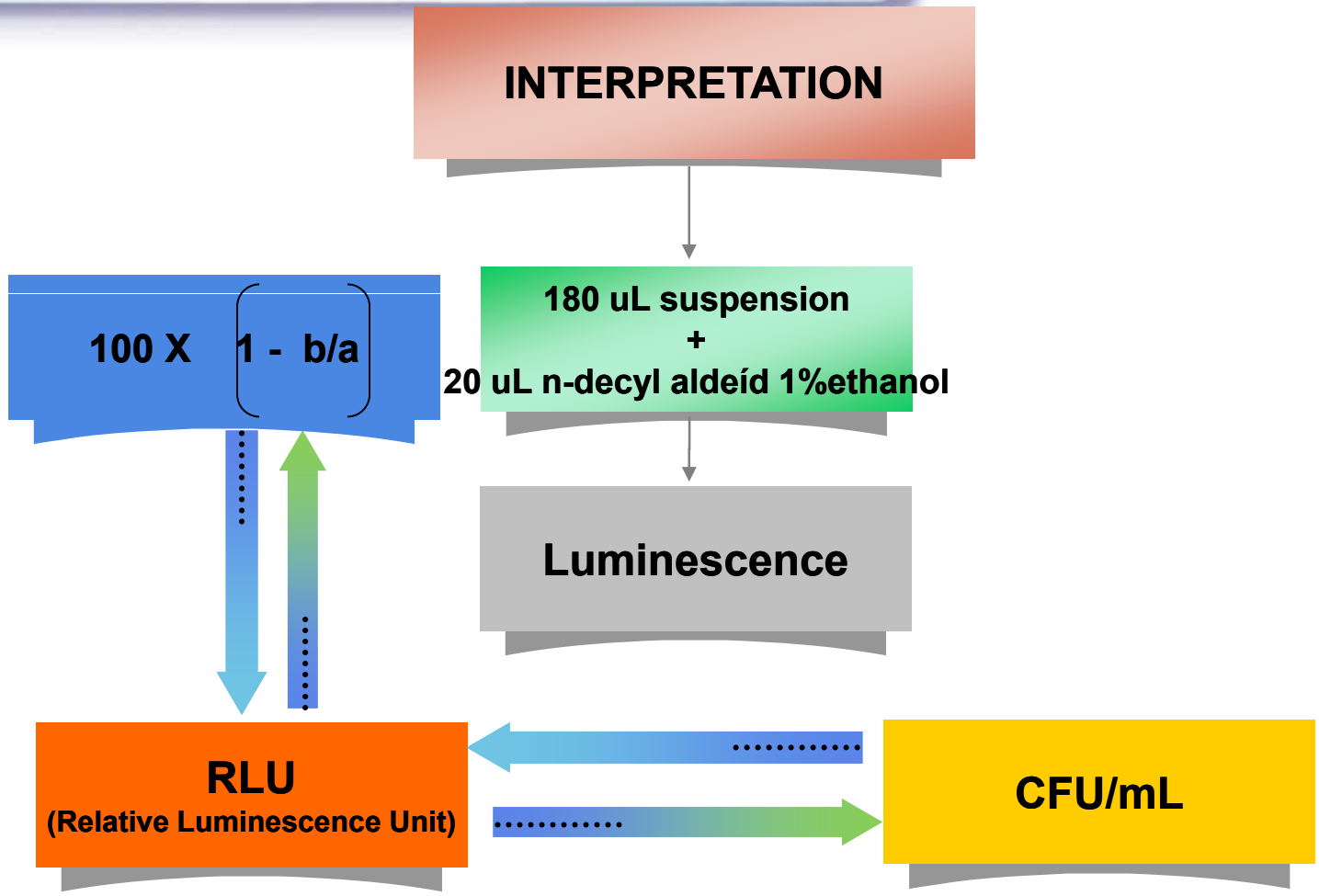
Methodology



In vitro Preclinical

Intracellular Activity

Methodology



Ruthenium (II) phosphine/diimines/picolinic acid

Intracellular Results

Table
Intracellular activity against MTB Erdmann ATCC 35801 with pSMT1 plasmid, of ruthenium compounds and rifampin at various concentrations

Identification	Compounds	Concentrations (μM)	Intracellular Inhibitory Activity (%)
SCAR1	cis-[Ru(pic)(dppe)$_2$]PF$_6$	3.80	77.5
		0.95	55.10
		0.24	48.30
SCAR2	cis-[RuCl$_2$(dppb)(bipy)]	2.00	82.30
		0.50	73.30
		0.13	70.50
SCAR3	[Ru(pic)(dppb)(Cl-bipy)]PF 6	3.04	62.30
		0.76	43.30
		0.19	31.90
SCAR4	[Ru(pic)(dppb)(phen)]PF$_6$	2.96	78.90
		0.74	75.30
		0.19	65.90
SCAR5	cis-[Ru(pic)(dppe)$_2$]PF$_6$	0.88	85.20
		0.22	80.50
		0.06	47.60
SCAR6	cis-[RuCl$_2$(dppb)(bipy)]	20.7	51.10
		5.17	33.40
		1.29	19.40
RMP	Rifampicin	0.49	80.40
		0.12	51.50
		0.04	24.00

PAVAN, F.R. et al. Ruthenium (II) phosphin e/diimine/picolinate complexes: Inorganic compound s as agents against tuberculosis. **European Journal of Medicinal Chemistry,** IN PRESS, 2011.

In vitro Preclinical

Cross Resistance

In vitro Preclinical

Cross Resistance

Strains

Available online at ScienceDirect www.sciencedirect.com
Elsevier Masson France EM|consulte www.em-consulte.com/en
ELSEVIER
BIOMEDICINE & PHARMACOTHERAPY

Original article

Drug resistance in *Mycobacterium tuberculosis* clinical isolates from Brazil: Phenotypic and genotypic methods

Marcelo Miyata [a,*], Fernando Rogério Pavan [a], Daisy Nakamura Sato [b], Leonardo Biancolino Marino [a], Mario Hiroyuki Hirata [c], Rosilene Fressati Cardoso [d], Fernando Augusto Fiúza de Melo [e], Cleslei Fernando Zanelli [a], Clarice Queico Fujimura Leite [a]

[a] School of Pharmaceutical Sciences, Biological Sciences Department, Paulista State University, Rodovia Araraquara-Jaú km 1, 14800-901 Araraquara, SP, Brazil
[b] Adolfo Lutz Institute, Ribeirão Preto Unit, Ribeirão Preto, SP, Brazil
[c] School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil
[d] Department of Clinical Analyses and Biomedicine, State University of Maringá, Maringá, PR, Brazil
[e] Clemente Ferreira Institute, São Paulo, SP, Brazil

58 clinical isolates
-Susceptible
- mono
- MDR-TB

OR

ATCC resistant Strains
www.atcc.org

In vitro Preclinical

Cross Resistance

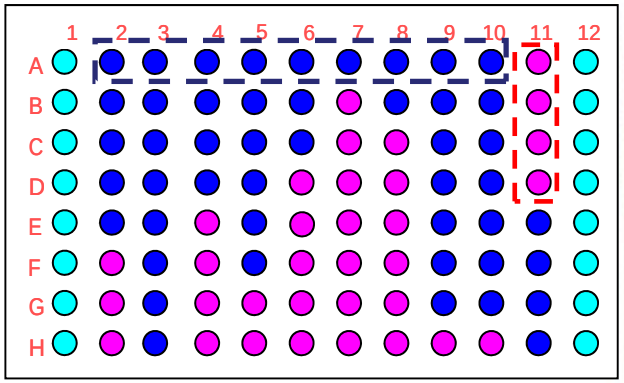
Methodology

58 Clinical Isolates

Fármaco	BACTEC™MGIT™960	
	Sensível	Resistente
INH	18	40
RMP	22	36
EST	32	26
EMB	39	19

Compounds Selected

Trying to understand possible diversity of resistance profiles.



MIC determination by REMA

MIYATA, M. PAVAN, F.R. et al. Drug resistance in *Mycobacterium tuberculosis* clinical isolates from Brazil: Phenotypic and genotypic methods. **Biomedicine & Pharmacotherapy**, DOI. 10.1016/j.biopha.2011.04.021, 2011.

Ruthenium (II) phosphine/diimines/picolinic acid

Cross Resistance

Table

Determination of anti-MTB activity of compounds against 25 clinical isolates selected panel built with profile of sensitivity/resistance.

Clinical Isolate nº	Standard Drugs BACTEC™ MGIT™ 960				SCAR compounds (REMA) µM					
	RMP	INH	STR	EMB	1	2	4	5	6	7
H37Rv	S	S	S	S	1,2	1,2	1,4	0,8	1,6	2,1
Susceptible isolates										
16	S	S	S	S	6,5	3,2	3,7	2,7	4,1	3,6
40	S	S	S	S	1,6	1,6	1,8	1,3	16,6	nd
48	S	S	S	S	13,1	3,2	3,7	1,3	>33,1	nd
66	S	S	S	S	>26,1	3,2	7,3	2,7	>33,1	nd
68	S	S	S	S	3,3	1,6	1,8	1,3	2,1	nd
71	S	S	S	S	3,3	1,6	1,8	1,3	>33,1	nd
72	S	S	S	S	3,3	3,2	3,7	1,3	>33,1	nd
75	S	S	S	S	26,1	1,6	1,8	1,3	>33,1	nd

nd – not determined

Ruthenium (II) phosphine/diimines/picolinic acid

Cross Resistance

Clinical Isolate nº	Standard Drugs BACTEC™ MGIT™ 960				SCAR compounds (REMA) µM					
	RMP	INH	STR	EMB	1	2	4	5	6	7
Mono-drugs-resistant TB										
15	S	**R**	S	S	6,5	1,6	3,7	1,3	4,1	3,6
77	S	**R**	S	S	6,5	6,4	7,3	5,3	nd	7,2
98	**R**	S	S	S	1,6	1,6	1,8	0,3	16,6	1,8
181	S	S	**R**	S	1,6	1,6	1,8	0,3	nd	1,8
Multi-drugs-resistant TB (MDR-TB)										
84	**R**	**R**	S	S	3,3	3,2	1,8	1,3	nd	1,8
145	**R**	**R**	S	S	1,6	1,6	1,8	0,7	nd	1,8
173	**R**	**R**	S	S	3,3	3,2	3,7	2,7	>33,1	3,6
176	**R**	**R**	S	S	26,1	1,6	3,7	2,7	>33,1	nd
46	**R**	**R**	**R**	S	6,5	1,6	3,7	2,7	16,6	7,15
142	**R**	**R**	**R**	S	26,1	1,6	1,8	2,7	4,1	nd
92	**R**	**R**	**R**	**S**	>26,1	6,4	7,3	5,3	>33,1	nd
93	**R**	**R**	**R**	**S**	26,1	3,2	3,7	2,7	>33,1	nd
59	**R**	**R**	**S**	**R**	13,1	1,6	1,8	0,3	>33,1	nd
61	**R**	**R**	**R**	**R**	6,5	1,6	3,7	1,3	>33,1	7,15
97	**R**	**R**	**R**	**R**	13,1	3,2	1,8	1,3	33,1	nd
104	**R**	**R**	**R**	**R**	3,3	0,8	1,8	0,3	8,3	0,9
185	**R**	**R**	**R**	**R**	25	0,39	0,39	0,39	>25	nd

nd – not determined

In vitro Preclinical

Drug Interaction

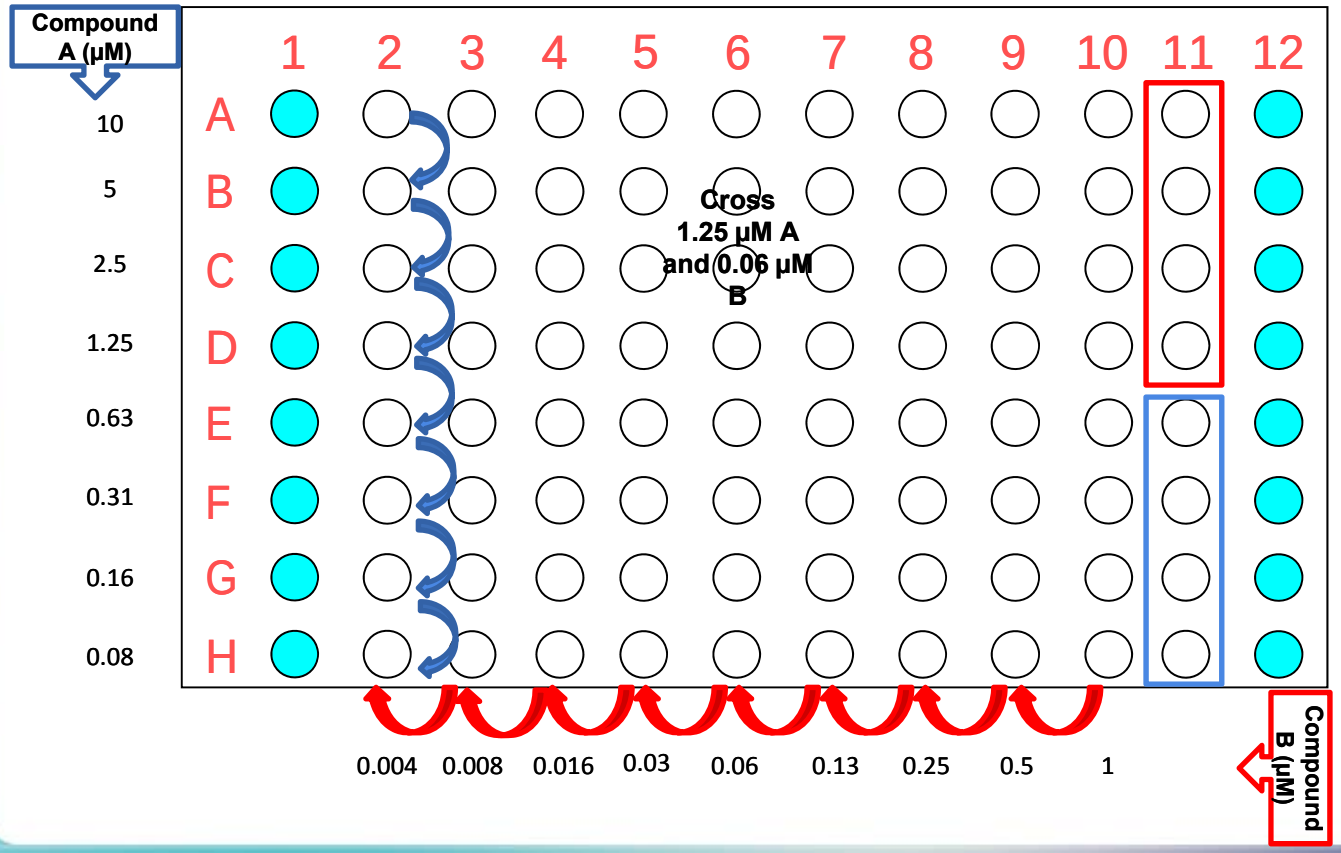
Evaluation of *in vitro* activity of combinations of candidate compounds using drugs between the 2D checkerboard methodology

In vitro Preclinical

Drug Interaction

Methodology

Checkerboarder 2D



LEGEND

H_2O

Compound A – Test??

Compound B – Standard Drugs

Positive Control

Negative Control

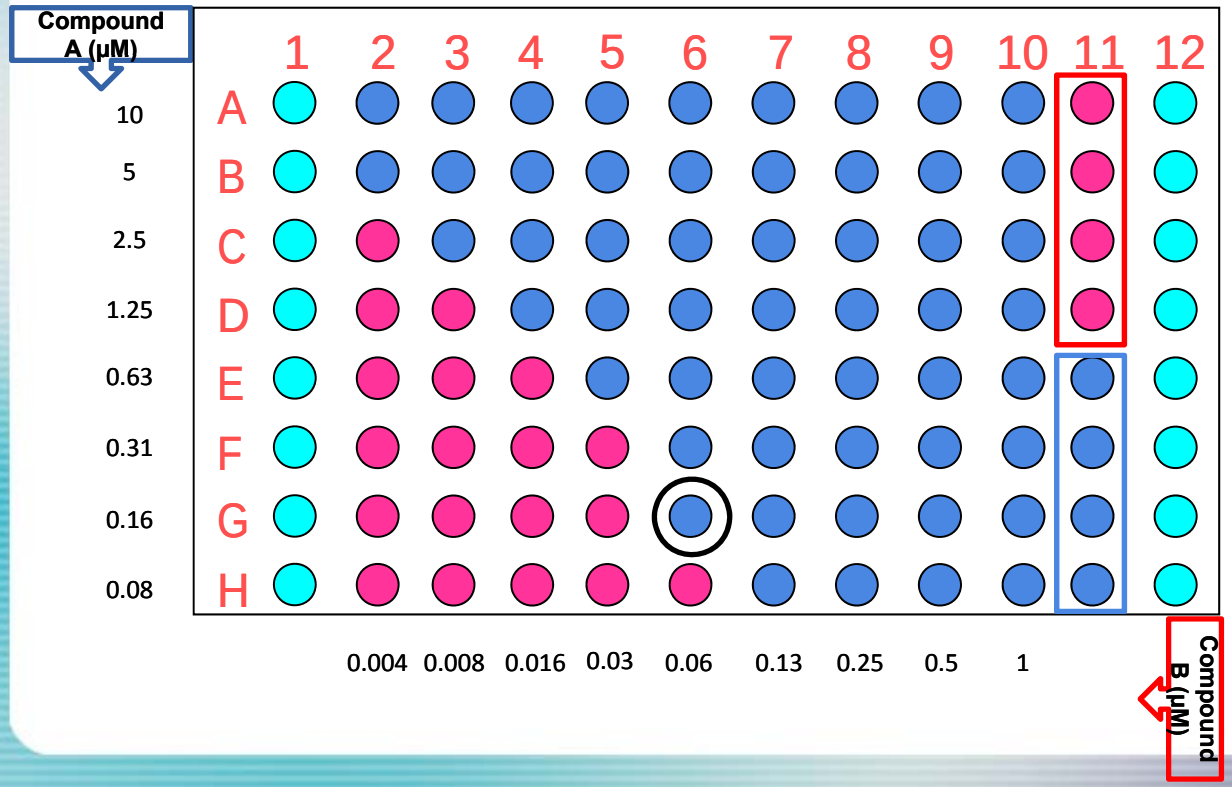
15 LUNA-HERRERA, J. et al. Synergistic antimycobacterial activities of sesquiterpene lactones from Laurus spp. **Journal of Antimicrobial Chemotherapy,** v. 59, n. 3, p. 548-552, 2007

In vitro Preclinical

Drug Interaction

Methodology

FIC = MIC **[A]** combination / MIC **[A]** alone + MIC **[B]** combination / MIC **[B]** alone = **FIC index**



Lower FIC

Parameters

≤ 0,75 Sinergismo

0,75-4 indiferente

> 4 antagonismo

LEGEND

H_2O

Compound A – test??

Composto B – Standard drugs

Positive Control

Negative Control

Ruthenium (II) phosphine/diimines/picolinic acid

Drug Interaction

Table

Evaluation of *in vitro* activity of combinations of compounds between the candidate drugs using checkerboard methodology.

Compounds/ Standard drugs	FIC	Interaction	Compounds/ Standard drugs	FIC	Interaction	Compounds/ Standard drugs	FIC	Interaction
Rifampicin			Ethambutol			Moxifloxacino		
SCAR1	1,0	Sinergismo	SCAR1	1,6	Indiferente	SCAR1	2,5	Indiferente
SCAR2	2,0	Indiferente	SCAR2	1,6	Indiferente	SCAR2	1,3	Indiferente
SCAR4	1,0	Sinergismo	SCAR4	3,3	Indiferente	SCAR4	1,3	Indiferente
SCAR5	1,1	Indiferente	SCAR5	1,7	Indiferente	SCAR5	2,6	Indiferente
SCAR6	1,0	Sinergismo	SCAR6	1,6	Indiferente	SCAR6	0,8	Sinergismo
SCAR7	1,0	Sinergismo	SCAR7	2,4	Indiferente	SCAR7	2,5	Indiferente
Isoniazid			Streptomycin					
SCAR1	1,4	Indiferente	SCAR1	1,3	Indiferente			
SCAR2	1,0	Sinergismo	SCAR2	1,3	Indiferente			
SCAR4	1,0	Sinergismo	SCAR4	1,3	Indiferente			
SCAR5	1,0	Sinergismo	SCAR5	1,4	Indiferente			
SCAR6	0,5	Sinergismo	SCAR6	0,5	Sinergismo			
SCAR7	1,0	Sinergismo	SCAR7	1,3	Indiferente			

In vitro Preclinical

Latent Assay

Evaluation of activity of new compounds against the MTB persistent without replication, using recombinant MTB H37Rv (pFCA-luxAB) and recovery test with low oxygen

(LORA-*Low Oxygen Recovery Assay*)

In vitro Preclinical

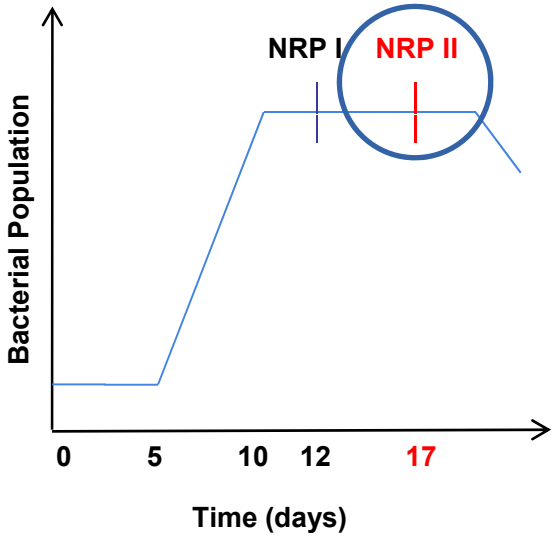
Activity on the latency model (NRP II)

Wayne Model (Obtain of NRP II)

MTB $H_{37}Rv$ ATCC 27294 (pFCA-luxAB)

37ºC

Stirring constantly/without disturbing the environment.



1. • Harvest
2. • Washing
3. • Freezing – 80ºC

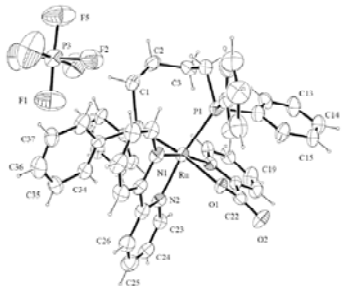
16 WAYNE, L. G.; HAYES, L. G. An in vitro model for sequential study of shiftdown of Mycobacterium tuberculosis through two stages of nonreplicating persistence. **Infection and Immunity,** v. 64, n. 6, p. 2062-2069, 1996.

In vitro Preclinical

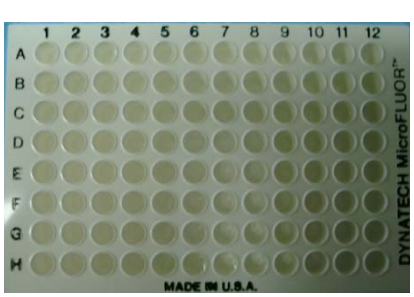
Activity on the latency model (NRP II)

Methodology

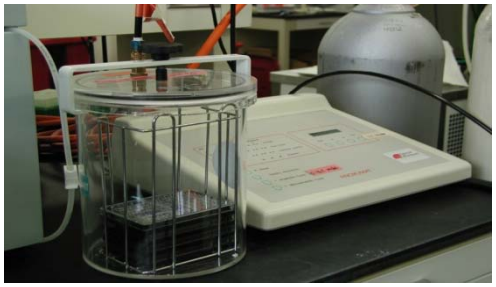
LORA – Low Oxygen Recovery Assay



2×10^5 **(Wayne)**



Anoxomat WS-8080

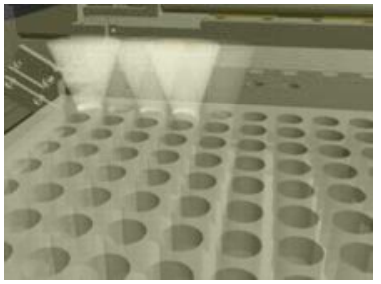


$10\%H_2$, $5\%CO_2$ e N_2 balanced

10 days of incubation at 37ºC under aerobic conditions.

28h "recovery" aerobic

Calculate MIC in NRP II



CHO, S. H. et al. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 51, n. 4, p. 1380-1385, 2007.

Ruthenium (II) phosphine/diimines/picolinic acid

Activity in the latency stage

Table. Evaluation of activity of new compounds against the MTB persistent without replication, using recombinant MTB H37Rv (pFCA-luxAB) and recovery test with low oxygen **(LORA-*Low Oxygen Recovery Assay*)**

Identification	Compounds	LORA MIC (µM)
SCAR01	$[Ru(pic)(dppb)(bipy)]PF_6$	0,55
SCAR02	$[Ru(pic)(dppb)(Me\text{-}bipy)]PF_6$	0,46
SCAR04	$[Ru(pic)(dppb)(phen)]PF_6$	0,53
SCAR05	$cis\text{-}[Ru(pic)(dppe)_2]PF_6$	0,31
SCAR06	$cis\text{-}[RuCl_2(dppb)(bipy)]$	0,42
SCAR07	Ru(pic)(dppe)(phen)	1,21
Standard Drugs		
RMP	Rifampicin	0,84
INH	Isoniazid	> 507
EST	Streptomycin	3,52
EMB	Ethambutol	> 24,2
MOX	Moxifloxacin	6
PA-824	PA-824	0,65

In vitro Preclinical

Spectrum Activity

In vitro Preclinical

Spectrum Activity

Methodology

Mycobacterium smegmatis → *REMA*

S. Aureus 5x10^5 CFU/mL

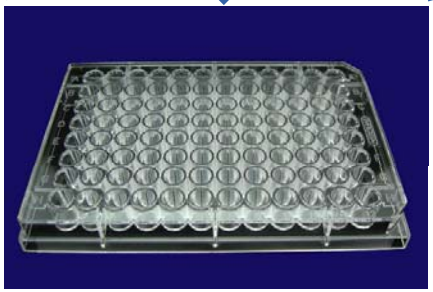
E. coli 5x10^5 CFU/mL

C. albicans 1,5x10^6 CFU/mL

Staphylococcus aureus

Escherichia coli

Candida albicans



Microdilution in browth

Incubation at 37 ºC
24 h – *S. aureus e E. coli*
48 h – *C. albicans*

D.O. 492 nm → **MIC calculated**

18 MOODY, J. A. Synergism testing: broth microdilution checkerboard and broth macrodilution methods. In: HD, I. (Ed.). **Clinical microbiology procedures handbook**. Washington, DC: American Society for Microbiology, 1992. p.1-28.

Ruthenium (II) phosphine/diimines/picolinic acid

Spectrum Activity

Table. Spectrum activity determination

Identification	Compounds	Spectrum Activity (μM)			
		E. coli	*S. aureus*	*C. albicans*	*M. smegmatis*
SCAR01	$[Ru(pic)(dppb)(bipy)]PF_6$	> 10,4	5,1	> 10,4	5,2
SCAR02	$[Ru(pic)(dppb)(Me\text{-}bipy)]PF_6$	> 10,2	2,4	> 10,2	5,0
SCAR04	$[Ru(pic)(dppb)(phen)]PF_6$	> 11,8	4,6	> 11,8	5,7
SCAR05	$cis\text{-}[Ru(pic)(dppe)_2]PF_6$	> 8,5	0,3	1,9	5,3
SCAR06	$cis\text{-}[RuCl_2(dppb)(bipy)]$	> 13,3	5,9	> 13,3	> 13,3
SCAR07	Ru(pic)(dppe)(phen)	> 11,4	5,5	> 11,4	5,6
Standard Drugs					
GTM	Gentamicin	1,0	0,6		>100
RMP	Rifampicin				>100
INH	Isoniazid				0,9
SM	Streptomycin				0,2
MOX	Moxifloxacin				>512
MET	Metronidazol				0,06
TMC	TMC-207				>100

In vitro Preclinical

unesp

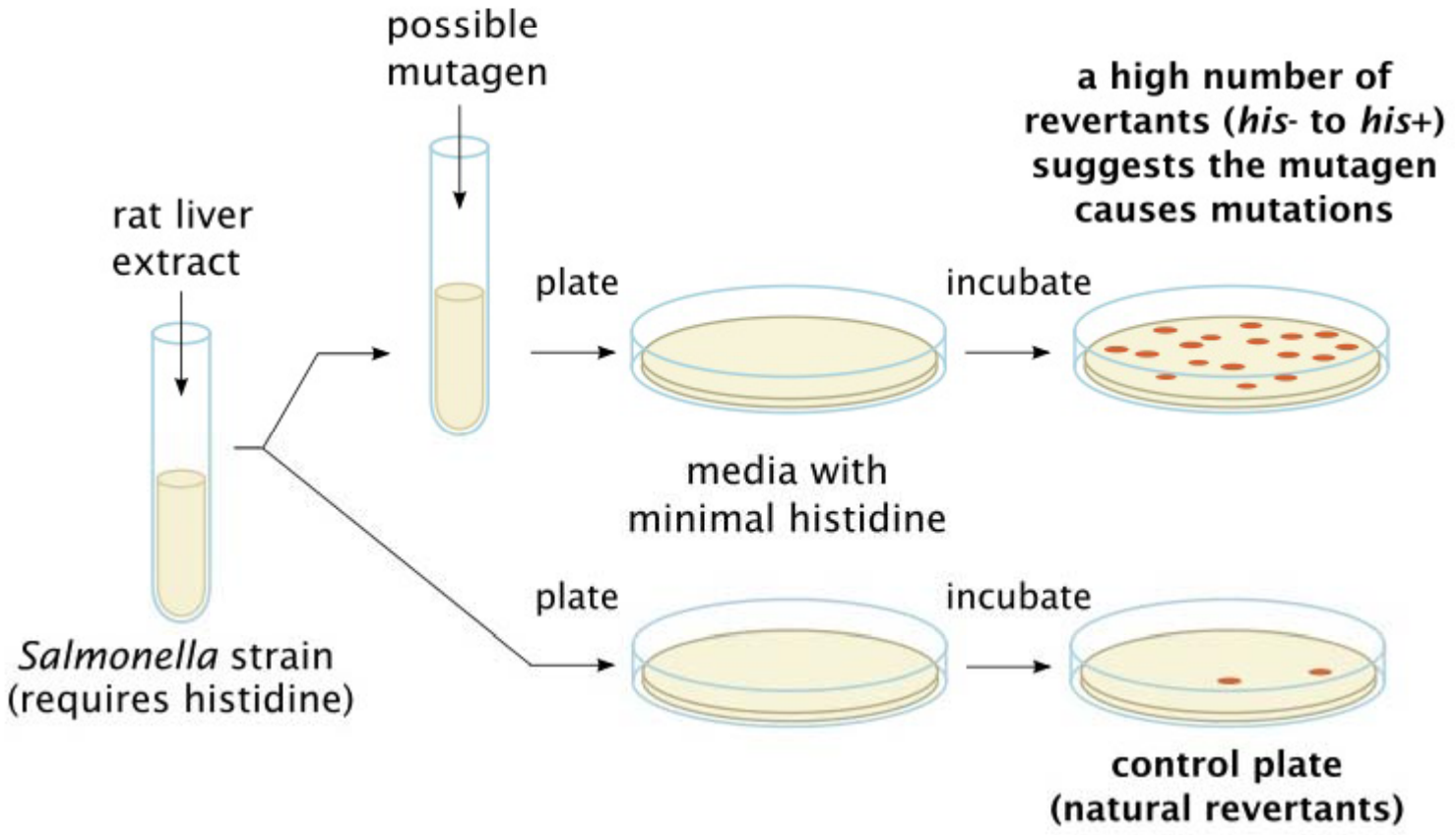
Profª Drª Eliana Aparecida Varanda

Mutagenicity

In vitro Preclinical

Mutagenicity

Methodology



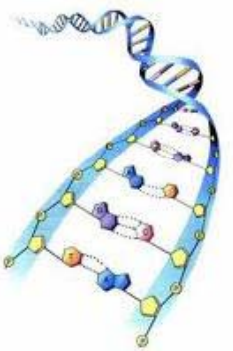
In vitro Preclinical

Profª Drª Rosilene Fressati Cardoso

Action Mechanism

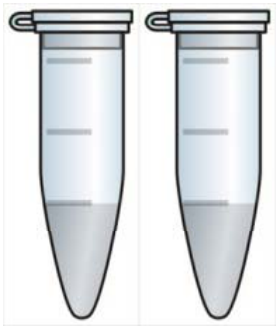
In vitro Preclinical

Action Mechanism



RDA - Representational difference analysis

RDA 1



Tester 1 **Driver 1**

✓**RNA EXTRACTION**

✓**cDNA Synthesis**

***Tester* 1**: *M. tuberculosis* H37Rv ATCC 27294 + test compound.
***Driver* 1**: *M. tuberculosis* H37Rv ATCC 27294

In vitro Preclinical

Action Mechanism

✓CLONING

Fragments of interest

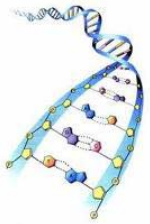
\+ tampão ligase
DNA ligase
ATP

vector pGEM-T-Easy (Promega)

Transformation into *Escherichia coli* cells by electroporation.

Agar LB (Luria-Bertani)

selection of recombinant clones.



Extraction of plasmid DNA

✓SEQUENCING

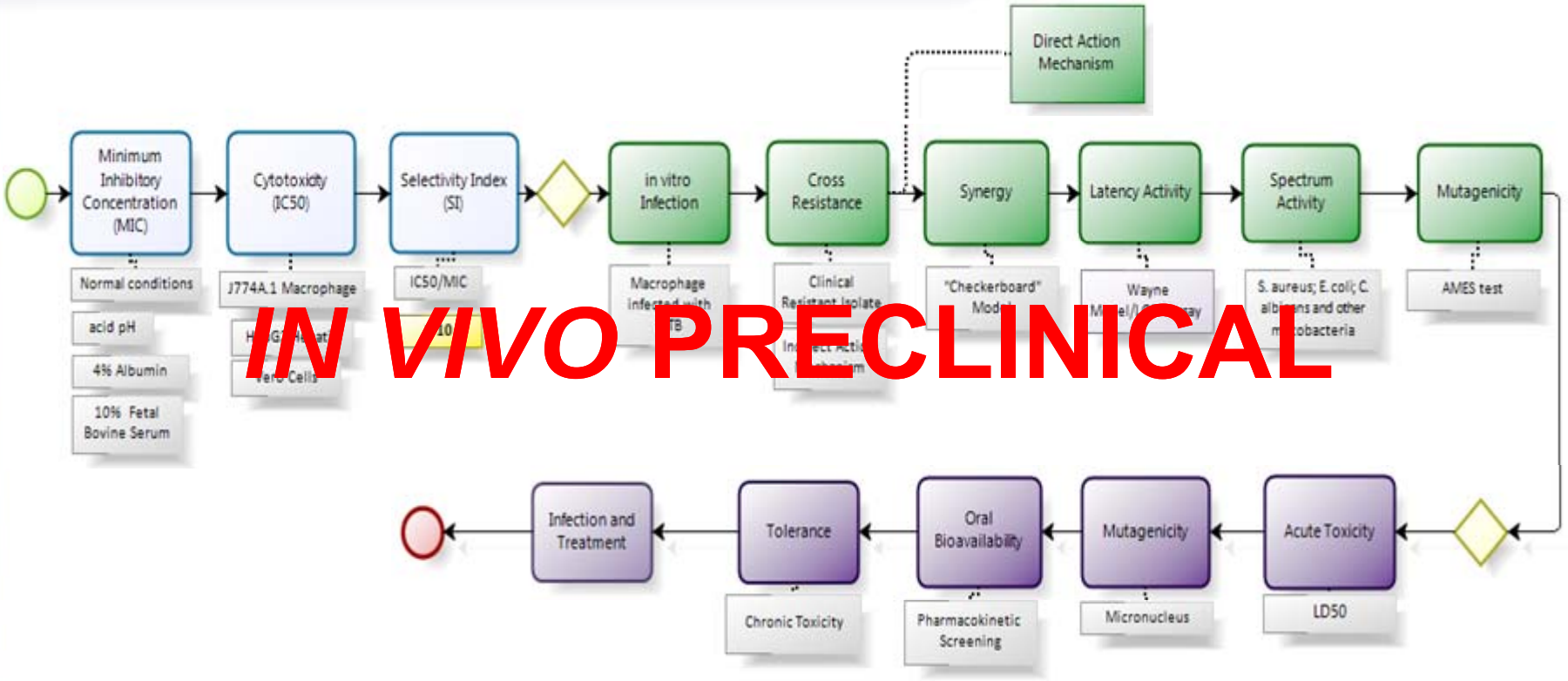
✓**Automatic Sequencer MegaBACE 1000** (Amersham Biosciences).

✓**kit *DYEnamic ET* e *Dye Terminator Cycle Sequencing*** (Amersham Biosciences)

✓The observed sequences are compared with the GenBank database (www.ncbi.nlm.nih.gov) using the Blastx program (Altschul et al., 1997).

✓Comparative analysis by subtraction between the driver and the tester.

Continuing the Pipeline



In vivo Preclinical

Acute Toxicity

In vivo Preclinical

Acute Toxicity

Animals to Experimentation

- C57BL/6 female, black, aged 8-10 weeks, with average weight of 15-20 grams..

- BALB/c mice, white, aged 8-10 weeks with an average weight of 15-20 grams.

In vivo Preclinical

Acute Toxicity

"Must be approved by the ethics committee on animal Research"

- 3 animals/group, 1 compound per group
- Single dose of 2000 mg/kg per body weight (gavage)
  - Doses of 100, 300, 1000 mg / kg per body weight (if necessary)
- Control Group - Feeding *ad libitum*
- Observations after administration of 4 to 6 hours during the first 24 hours – "Hippocratic Screening" (any behavioral change)
- Until 14 days.

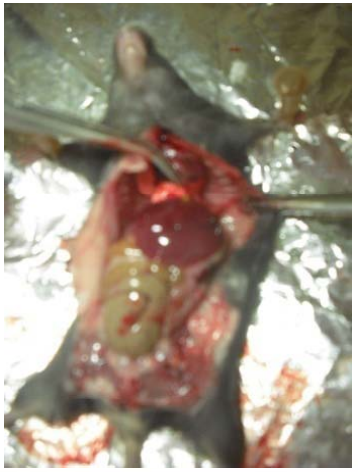
OECD 2001. Guideline 423: Acute Oral Toxicity - Acute Toxic Class Method. http://www.oecd.org/publications. Paris: Head of Publications Service.

In vivo Preclinical

Acute Toxicity

Methodology

- The surviving animals are sacrificed and the following organs examined macroscopically and weighed:
  - Heart, Liver, Pancreas, Kidneys and lungs.
  - Statistical analysis
- Acute Toxicity:
  - LD_{50} > 2000 per body weight = Low Toxicity
  - LD_{50} 500 - 2000 per body weight = Moderately Toxicity
  - LD_{50} 100 - 500 per body weight = Highly Toxicity
  - LD_{50} ≤ 25 per body weight = Extremely Toxicity



19 OECD 2001. Guideline 423: Acute Oral Toxicity - Acute Toxic Class Method. http://www.oecd.org/publications. Paris: Head of Publications Service.

Ruthenium (II) phosphine/diimines/picolinic acid

Acute Toxicity

Table. Evaluation of Acute Toxicity of the compounds in C57BL/6.

Identification	Compounds	Doses (mg/kg per body weight)	Losses(%)
SCAR01	$[Ru(pic)(dppb)(bipy)]PF_6$	2.000	0
		1.000	-----
SCAR02	$[Ru(pic)(dppb)(Me\text{-}bipy)]PF_6$	2.000	83,33
		1.000	16,6
SCAR04	$[Ru(pic)(dppb)(phen)]PF_6$	2.000	16,6
		1.000	-----
SCAR05	$cis\text{-}[Ru(pic)(dppe)_2]PF_6$	2.000	50
		1.000	-----
SCAR06	$cis\text{-}[RuCl_2(dppb)(bipy)]$	2.000	0
		1.000	-----
SCAR07	Ru(pic)(dppe)(phen)	2.000	nd
		1.000	nd
RMP	Rifampicina	2.000	0
		1.000	0

nd – not determined

In vivo Preclinical

Tolerance

In vivo Preclinical

Tolerance

Methodology

- BALB/c or C57BL/6 mice
  - 2 animals/group, 1 compound per group

Single dose (gavage) Starting of 50 mg/kg per body weight → **Cycle of 5 days** - Analisys of Hipocratic *Screening* → Final Cycle doses are increased. → **Ideal Dose determined**

Tolerance in BALB/c mice

Daily doses, oral way, 5 days cycles

SCAR4 - $[Ru(pic)(dppb)(phen)]PF_6$

Ideal concentration of 75 mg/kg per body weight

SCAR7 - $Ru(pic)(dppe)(phen)$

Ideal concentration of 50 mg/kg per body weight

RMP - Rifampicin

Ideal concentration of 15 mg/kg per body weight

In vivo Preclinical

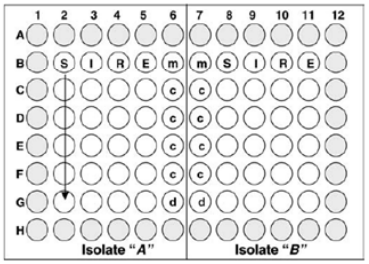
Pharmacokinetic Screening

In vivo Preclinical

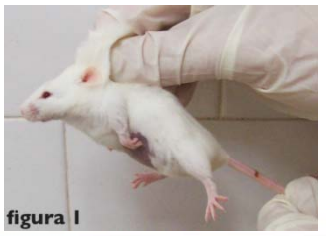
Pharmacokinetic Screening

Determination of active concentrations the blood.

REMA



Serum separated and keep at -70°C



3 mice have blood collected by the tail.

Lower Concentrations than LD_{50}

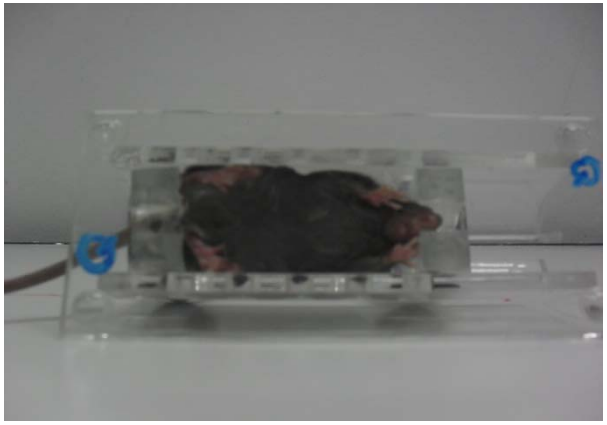
LD_{50} Detemined

Oral Bioavailability

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 Preclinical

Pharmacokinetic Screening



In vivo Preclinical

Infection Mice

In vivo Preclinical

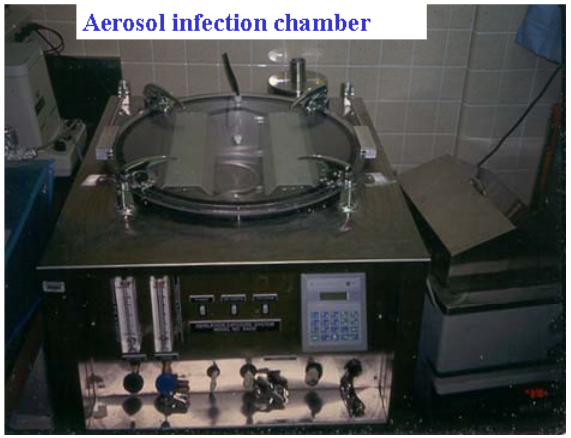
Infection Mice

Animal Infection

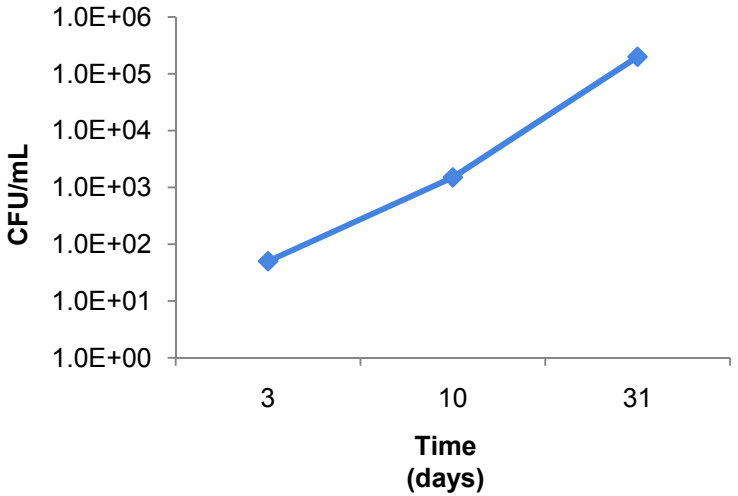
n=100
BALB/c

5x106
CFU/mL

30min



Multiplication of MTB Erdman in the lungs of BALB/c



In vivo Preclinical

Mice Treatment

Animal Treatment

BALB/c mice, female
6 animal/group, 1 compound per group

10º dia
Start the treatment

31º dia
End of the treatment
Single daily doses
(gavage)

Compounds	[ ] mg/kg per body weight
SCAR4	75
SCAR7	50
RMP	15

End of the treatment

CFU/mL of the lung
treatment animals

×

CFU/mL of the lung treatment
animals with RMP

×

CFU/mL of the lung
treatment animals
with the compounds

20 FALZARI, K. et al. In vitro and in vivo activities of macrolide derivatives against Mycobacterium tuberculosis. **Antimicrobial Agents and Chemotherapy,** v. 49, n. 4, p. 1447-1454, 2005.

Ruthenium (II) phosphine/diimines/picolinic acid

Animals treatment

Treatment of animals infected with MTB Erdman

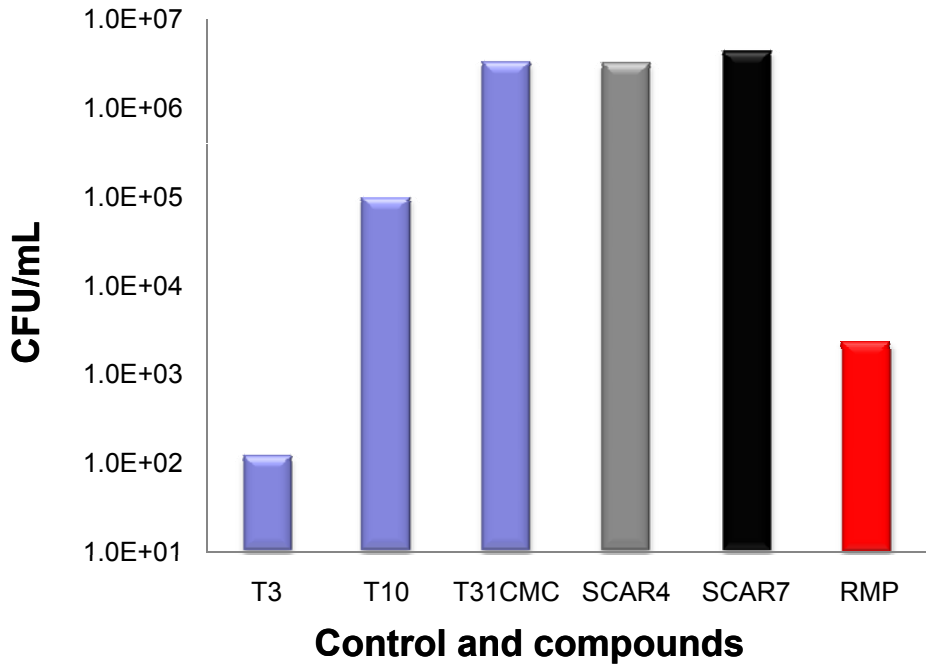


Figure. Analysis of the quantity of bacilli from the lungs of BALB/c controls and treated with compounds

Muito Obrigado!

pavanfer@yahoo.com.br