UNLV University Libraries
University of Nevada, Las Vegas

UNLV Theses, Dissertations, Professional Papers, and Capstones

2009

Protection of macrophages J774A.1 by purine nucleoside analogues from Bacillus anthracis mediated necrosis

Zadkiel R. Alvarez
University of Nevada Las Vegas

Follow this and additional works at: https://digitalscholarship.unlv.edu/thesesdissertations

 Part of the Bacterial Infections and Mycoses Commons, Bacteriology Commons, Biochemistry Commons, and the Cell Biology Commons

Repository Citation

Alvarez, Zadkiel R., "Protection of macrophages J774A.1 by purine nucleoside analogues from Bacillus anthracis mediated necrosis" (2009). *UNLV Theses, Dissertations, Professional Papers, and Capstones*. 141.
https://digitalscholarship.unlv.edu/thesesdissertations/141

This Thesis is protected by copyright and/or related rights. It has been brought to you by Digital Scholarship@UNLV with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Thesis has been accepted for inclusion in UNLV Theses, Dissertations, Professional Papers, and Capstones by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.

PROTECTION OF MACROPHAGES J774A.1 BY PURINE NUCLEOSIDE ANALOGUES FROM *B. ANTHRACIS* MEDIATED NECROSIS

by

Zadkiel R. Álvarez

Bachelor of Science
University of Navarra, Pamplona
2005

Master in Biotechnology
Aliter Business School, Madrid
2007

A thesis submitted in partial fulfillment of
the requirements for the

Master of Science in Biochemistry
Department of Chemistry
College of Sciences

Graduate College
University of Nevada, Las Vegas
December 2009

Copyright by Zadkiel Álvarez (2010)
All Rights Reserved

UNIVERSITY OF NEVADA LAS VEGAS

THE GRADUATE COLLEGE

We recommend that the thesis prepared under our supervision by

Zadkiel R. Álvarez

entitled

Protection of Macrophages J774A.1 by Purine Nucleoside Analogues from *B. Anthracis* Mediated Necrosis

be accepted in partial fulfillment of the requirements for the degree of

Master of Science
Biochemistry

Ernesto-Abel-Santos, Committee Chair

Chulsung Bae, Committee Member

Ronald Gary, Committee Member

Helen Wing, Graduate Faculty Representative

Ronald Smith, Ph. D., Vice President for Research and Graduate Studies
and Dean of the Graduate College

December 2009

ABSTRACT

Protection of Macrophages J774a.1 by Purine Nucleoside Analogues From *B. anthracis* Mediated Necrosis

by

Zadkiel R. Álvarez

Dr. Ernesto Abel-Santos, Examination Committee Chair
Professor of Biochemistry
University of Nevada, Las Vegas

Eight years after the lamentable anthrax attacks, major scientific effort continues to be done, in order to stop imminent acts of bioterrorism. Innovative ways of therapy against the anthrax disease are being investigated. *B. anthracis*, the etiological agent of the infection, has a dormant stage in its life cycle known as the endospore. When conditions become favorable spores germinate, transforming into vegetative bacteria. In inhalational anthrax, the most fatal manifestation of the disease, spores enter the organism through the respiratory track, and are phagocytosized by alveolar macrophages of the lungs. Spores are able to sense nutrient availability, activating their germination inside the phagosomal compartment. Germination is a crucial step for the commencement of the pathogenesis inside the host organism.

B. anthracis germination is activated by a wide variety of amino acids and purine nucleosides. Inosine and alanine are the two most potent nutrient germinants. Recent studies have shown that germination can be hindered by isomers or structural analogues of germinants. 6-thioguanosine, a guanosine analogue, is able to inhibit germination, and prevent *B. anthracis* toxin-mediated necrosis in murine macrophages.

Our investigation was focused on the screening of 43 different purine nucleoside analogues, to determine their protective effects on the macrophage cell line J774a.1. We have selected a group of 19 compounds that impeded the spore germination *in vitro,* and tested their protective effect in cells. Seven of these analogues exerted protective effect against *B. anthracis* mediated killing. Structure activity relationships analyses on those compounds has clarified the mechanisms of the inhibition in cells, and illustrated

a plausible model for the germinant-receptor recognition. Continuous research on this area should develop a novel antigerminant agent as an immediate prophylaxis to stop *B. anthracis* pathogenesis.

ACKNOWLEDGEMENTS

I am truly thankful with opportunity that was given to me by Dr. Ernesto Abel-Santos to expand my mind into a higher scientific knowledge. I would have never been able to reach my goals, without the perseverance and confidence that he has invested in me. Furthermore, he has shown me the ways of scientific ethics and discipline, and let me learned from mistakes committed in the past; this major experience in my life may serve as a gateway to improve myself, becoming an outstanding professional and provide service to mankind, whichever the route I decide to take in the near future.

I am thankful to Dr. Monique Akoachere, for she has given me guidance and help during the first stages of my degree. She has taught me the correct manners in all aspects of research, introducing me into the real laboratory work, which I had no experience. She was the pillar upon I built my lab-studentship.

I am indebted to Dr. Jürgen Brojatsch and Stephan Muehlbauer at the Albert Einstein College, NY, for their teachings on cell culturing techniques; they were essential for the lab work I have been performing these two years.

I am appreciative of the great friendship from all my lab partners during these years: Dr. Norma Ramírez, Andy Phui, Helen Luu, and Amber Howerton. They have made possible an excellent coexistence and pleasant working environment.

I salute all the members of my thesis committee: Dr. Ronald Gary, Dr. Chulsung Bae, and Dr. Helen Wing, for supporting this thesis project and maintain their confidence in me.

I am thankful to my mother and my father, for they have given me all the love that I needed throughout my period in the United States. Without such love, I would have never reached the finish line of this career.

But for above all things, I am thankful to God, which is the essence of my life. He is the essence that sustains all matter in the Universe, and beyond matter: even scientific knowledge. Nothing in life has a meaning if we do not find the path to God.

TABLE OF CONTENTS

ABSTRACT iii

ACKNOWLEDGEMENTS v

LIST OF FIGURES viii

LIST OF TABLES x

ABBREVIATIONS xi

CHAPTER 1 INTRODUCTION 1
1.1 Background and Significance 1
1.2 The Anthrax Disease 5
1.3 Biology of *Bacillus anthracis* 8
1.4 Spore Germination 21
1.5 Germination Inhibition 31
1.6 Research Questions and Hypothesis 40
1.7 Specific Aims of the Study 41

CHAPTER 2 MATERIAL AND METHODOLOGY 43
2.1 Bacterial strain and growth 43
2.2 Spore preparation 44
2.3 Analysis of the spore germination *in vitro* 45
2.4 Cell Line 47
2.5 Medium and Growth Conditions for Mammalian Cells 48
2.6 Nucleoside Analogues 49
2.7 Spore killing assay 53
2.8 Direct Quantification of Cell Viability 54
2.9 Fluorescence Quantification of Cell Viability 56
2.10 Fluorescence Microscopy 57
2.11 Half-maximal Inhibitory Concentration of the Protective Effect in Cells 58
2.12 Half-maximal Inhibitory Concentration of the Germination Inhibition *in vitro* 58

CHAPTER 3 RESULTS 60
3.1 Protection of J774a.1 by 6-thioguanosine from *B. anthracis* Mediated Necrosis 60
3.2 Half-maximal Inhibitory Concentration of 6-thioguanosine 68
3.3 Effect of 6-thioguanine 70
3.4 Effect of 6-chloroguanosine 73
3.5 Effect of 6-chloroguanine 75
3.6 Effect of 6-thionosine 76
3.7 Effect of 6-mercaptopurine 77
3.8 Effect of Allopurinol Riboside 79
3.9 Effect of Allopurinol 81
3.10 Effect of 6-benzylthioinosine 82
3.11 Effect of 6-methylmercaptopurine riboside 83
3.12 Effect of 6-*O*-methylguanosine 84
3.13 Effect of 2-aminoadenosine 85
3.14 Effect of PPER 86
3.15 Effect of 6-methylaminopurine riboside 87
3.16 Effect of 2-aminopurine purine riboside 88
3.17 Effect of Xanthosine 89

3.18 Effect of 6-*N*-benzylmethyladenosine..... 90
3.19 Effect of 6-*N*-benzoyladenosine..... 91
3.20 Effect of 6-*N*-benzylaminopurine..... 92
3.21 Effect of 6-(γ,γ-dimethylallylamino)purine riboside 93
3.22 Effect of 6-*N*-cyclohexyladenosine 94
3.23 Effect of 6-DPA..... 95
3.24 Effect of 6-ECA 96
3.25 Cytotoxic Compounds..... 97
3.26 Other Compounds..... 98
3.27 Compounds that Enhanced Cell Killing 101
3.27 Cooperative Effect by *D*-alanine with Nucleoside Analogues..... 102
3.28 Summary of results..... 106

CHAPTER 4 DISCUSSION..... 109
4.1 J774a.1 killing by *Bacillus anthracis*..... 109
4.2 Structure Activity Relationships..... 110
4.3 Exceptional cases: 6-TI and APR 118
4.4 Inhibitors or Germinants 121
4.5 Inhibitory Contribution by *D*-alanine 122
4.6 General Conclusions and Further Work 123

BIBLIOGRAPHY 125

VITA 131

LIST OF FIGURES

Figure 1 Stages of the sporulation of *Bacillus* sp..... 11
Figure 2 Morphology of the Endospore..... 14
Figure 3 Cellular mechanism of the anthrax toxins..... 17
Figure 4 Evolutionary strategies developed by *B. anthracis* in the interaction with the host cell..... 21
Figure 5 Sequential steps involving the germination of the endospore..... 22
Figure 6 Topology and localization of the tripartite protein encoded by the gerA operon in *B. subtilis* 26
Figure 7 Germination pathways for *B. anthracis*..... 31
Figure 8 Representation of the two enantiomers of alanine..... 35
Figure 9 Studies of McKevitt et al. with D-Ala preventing spore germination *in vitro*..... 36
Figure 10 Effect on the *B. anthracis* germination inhibition in vitro by 6-TG and 6-MMPR..... 38
Figure 11 Prevention of cell killing mediated by B. anthracis spores by 6-TG, revealed by fluorescence microscopy..... 40
Figure 12 Fractions obtained after centrifugation with Histodenz..... 45
Figure 13 Decrease in optical density observed after adding inosine (Ino) and *L*-alanine (Ala) to the spore suspension..... 46
Figure 14 Photography at phase contrast microscopy of the cell line J774a.1..... 48
Figure 15 Range selected for counting at 40X field..... 55
Figure 16 Photography of the different cellular stages of macrophages after infection with spores..... 55
Figure 17 Molecular structure of propidium iodide..... 56
Figure 18 Percentage of necrotic cells after infecting with *B. anthracis*..... 61
Figure 19 Fluorescence quantification on J774a.1 cell killing with *B. anthracis* at 6 hours..... 61
Figure 20 Fluorescence quantification on J774a.1 cell killing with *B. anthracis*..... 62
Figure 21 Protection by 6-TG at 4 and 6 hours..... 63
Figure 22 Microscopy observation at 3 hours..... 64
Figure 23 Effect of 6-TG after 6 hours..... 65
Figure 24 Effect of 6-TG on endospore germination in vitro..... 66
Figure 25 *B. anthracis* endospore germination observed at light microscopy..... 67
Figure 26 6-TG protection IC_{50} assay..... 68
Figure 27 Dose-response curve for 6-thioguanosine protective effect on macrophages J774a.1..... 69
Figure 28 Effect of 6-Tg in macrophages and *in vitro*..... 70
Figure 29 6-Tg protection on J774a.1 at 6 hours..... 71
Figure 30 6-Tg protection IC_{50} assay..... 72
Figure 31 Dose-response curve for 6-thioguanine protective effect on macrophages J774a.1..... 72
Figure 32 Effect of 6-CG on macrophages J774a.1..... 73
Figure 33 Dose-response curve for 6-chloroguanosine protective effect on macrophages J774a.1..... 74
Figure 34 IC_{50} assay for 6-Cg on macrophages J774a.1..... 75
Figure 35 Dose-response curve for 6-Cg on macrophages J774a.1..... 75
Figure 36 6-Tg does not inhibit germination *in vitro*..... 76
Figure 37 6-TI IC_{50} assay on macrophages J774a.1..... 76
Figure 38 Dose-response curve for 6-TI on macrophages J774a.1..... 77
Figure 39 IC_{50} assay for 6-Mp..... 78
Figure 40 Dose-response curve for the protection of 6-mercaptopurine in J774a.1..... 78
Figure 41 6-Mp does not inhibit germination *in vitro*..... 79
Figure 42 Protection J774a.1 macrophages by APR..... 79

Figure 43 Dose-response curve for the protection of APR in J774a.1..... 80
Figure 44 APR does not inhibit germination *in vitro*..... 80
Figure 45 Effect of Ap on J774a.1 cells..... 81
Figure 46 Ap does not inhibit germination *in vitro*..... 81
Figure 47 BTI protects J774a.1 cells from necrosis after 5 hours..... 82
Figure 48 BTI IC_{50} assay 83
Figure 49 BTI dose-response curve 83
Figure 50 Effect of 6-MMPR on J774a.1 cells after 6 hours 84
Figure 51 Effect of 6-OMG on J774a.1 cells after 6 hours 85
Figure 52 Effect of 2-AA on J774a.1 cells after 6 hours 85
Figure 53 Effect of PPER on J774a.1 cells at 4 and 5 hours 86
Figure 54 *In vitro* inhibition of PPER at different concentrations 86
Figure 55 Dose-response curve for the inhibition of PPER *in vitro* 87
Figure 56 Effect of 6-MAPR on J774a.1 cells at 6 hours 87
Figure 57 Dose-response curve for the inhibition of 6-MAPR *in vitro*..... 88
Figure 58 Effect of 2-APR on J774a.1 cells after 6 hours..... 88
Figure 59 Dose-response curve for the inhibition of 2-APR *in vitro*..... 89
Figure 60 Effect of Xanthosine (X) on J774a. 1 89
Figure 61 Effect of BMA on J774a.1 cells at 4 and 5 hours 90
Figure 62 Dose-response curve for the inhibition of BMA *in vitro*..... 90
Figure 63 Effect of NBA on J774a.1 cells at 4 and 5 hours..... 91
Figure 64 Dose-response curve for the inhibition of NBA *in vitro*..... 91
Figure 65 Effect of BAP on J774a.1 cells at 4 and 5 hours..... 92
Figure 66 Dose-response curve for the inhibition of BAP *in vitro*..... 92
Figure 67 Effect of DMAA on J774a.1 cells at 5 hours..... 93
Figure 68 Dose-response curve for the inhibition of *DMAA in vitro* 93
Figure 69 Effect of CHA on J774a.1 cells at 5 hours 94
Figure 70 Dose-response curve for the inhibition of CHA *in vitro* 94
Figure 71 Effect of 6-DPA on J774a.1 cells at 4.5 hours 95
Figure 72 Dose-response curve for the inhibition of 6-DPA *in vitro*..... 95
Figure 73 Effect of 6-ECA on J774a.1 cells at 4 and 5 hours..... 96
Figure 74 Dose-response curve for the inhibition of 6-ECA in vitro 96
Figure 75 Analogues with cytopathic effect on J774a.1 cells 97
Figure 76 Killing enhancement of J774a.1 cells 101
Figure 77 Dose-response curve for necrosis protection of J774a..... 102
Figure 78 Dose-response curve for the inhibition of *D*-Ala *in vitro*..... 103
Figure 79 Effect of D-alanine observed at light microscopy 103
Figure 80 Cooperative effect of *D*-Ala and 6-TG..... 104
Figure 81 Cooperative effect of two nucleoside anti-germinants and *D*-Ala..... 105
Figure 82 Killing of J774a.1 within time..... 110
Figure 83 Purine base derivates inhibit spore germination inside the cell..... 116
Figure 84 Model for nucleoside recognition by the receptor GerH in *B. anthracis*..... 118
Figure 85 Hypothetic mechanism for the enzymatic transformation of APR in mammalian cells..... 121
Figure 86 Eventual loss of protection by the nucleoside anti-germinants..... 122

LIST OF TABLES

Table 1 Heat resistance and optical density changes during germination..... 23
Table 2 Identified *ger* operon genes in different species of *Bacillus* 28
Table 3 Potential targets and agents that block germination of endospores 36
Table 4 Nucleoside analogues that inhibited *B. anthracis* germination *in vitro* 39
Table 5 Differences between Sterne and the virulent Ames strain of *B. anthracis* 46
Table 6 Nucleoside analogues derived from inosine and guanosine 52
Table 7 Purine bases derived from nucleoside analogues 54
Table 8 Analogues containing the six-member ring from the purine and other analogues of non-specific category 54
Table 9 Analogues that affected neither germination nor spore-mediated killing by *B. anthracis* 98
Table 10 Compounds that protect J774a.1 cells from *B. anthracis*-mediated killing..... 106
Table 11 Compounds that inhibited germination *B. anthracis* endospores *in vitro*..... 108
Table 12 Electronegativity of some C6-groups compared to IC50s *in vitro* 112

ABBREVIATIONS

Abbreviation	Definition
Ala	Alanine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
atm	Atmospheres
ATP	Adenosine triphosphate
ATR	Anthrax toxin receptor
AVA	Anthrax Vaccine Adsorbed
B.	*Bacillus*
BMM	Bone marrow derived macrophages
C	Concentration
C.	*Clostridium*
Ca	Calcium
$Ca(NO_3)_2$	Calcium nitrate
CaM	Calmodulin
CDC	Center of Disease Control
Cl	Chlorine
cm	Centimeter
CO_2	Carbon dioxide
D	Dextro enantiomer
ddH_2O	Double-distilled water
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
Dr.	Doctor
EDTA	Ethylenediaminotetraacetic acid
EF	Edema factor
FBI	Federal Bureau of Investigation
FDA	Food and Drug Administration
$FeSO_4$	Ferric sulphate
FU	Fluorescence Units
Gln	Glutamine
Glu	Glutamate
GMP	Guanosine monophosphate
GMPS	Guanosine monophosphate synthase
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H	Hydrogen
HBSS	Hanks Balanced Saline Solution
$HgCl_2$	Mercury chloride
HGPRT	Hypoxanthine-Guanine Phosporibosyl Transferase
His	Histidine
IMP	Inosine monophosphate
IMP-DH	Inosine monophosphate dehydrogenase
Inc.	Incorporated
Ino	Inosine
IgG	Immunoglobulin G
IL-1β	Interleukin-1-beta
IU	Inhibitory units

Abbreviation	Definition
L	Levo enantiomer
LB	Luria Bertani
LPS	Lipopolysaccharide
LF	Lethal factor
Log	Decimal logarithm
KCl	Potassium chloride
Ki	Inhibition constant
Km	Michaelis constant
MAPKK	Mitogen activated protein kinase-kinase
Met	Methionine
$MgSO_4$	Magnesium sulphate
m.o.i.	Multiplicity of infection
$MnCl_2$	Manganese chloride
mM	Millimolar
mL	Milliliter
N	Nitrogen
N/A	Not applicable
N/I	No inhibition
NaCl	Sodium chloride
NSRB	National Screening Laboratory in Biodefense
nm	Nanometer
O	Oxygen
OD	Optical density
PA	Protective antigen
PBS	Phosphate Buffered Saline
P_i	Inorganic phosphate
PI	Propidium iodide
pH	Co-logarithm of proton concentration
pp.	Pages
PP_i	Inorganic pyrophosphate
Pro	Proline
PRPP	Phophoribosyl pyrophosphate
R	Regression coefficient
®	Registered trademark
RI	Relative intensity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAPS	Small acid soluble proteins
™	Trademark
TNF-α	Tumoral Necrosis Factor-Alpha
USAMRIID	US Army Medical Research Institute for Infectious Diseases
UV	Ultra-violet
S	Sulphur
Ser	Serine
sp.	Species
v.	Version
Val	Valine
Zn	Zinc
°C	Celsius degrees
µg	Micrograms
µM	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Background and Significance

Bacillus anthracis is a gram-positive bacterium that has the ability of developing resistant forms in their life cycle called endospores. Such particular property is present in a diverse group of prokaryotes, included in the phylum *Firmicutes* [(1)]. Belonging to this group there are two relevant genera –*Clostridium* and *Bacillus*– that are important pathogens to humans. Endospore forming bacteria have developed several evolutionary strategies that make them prevail in a wide variety of niches, and infecting multiple hosts [(2,3)].

Many species of endospore-forming bacteria are causative agents of important diseases in humans and also in animals. The possibility of being infected by these bacteria is increased due to the fact that their spores may survive in almost any possible environment in our planet [(4)], and may easily disseminate in the environment through air and water. This added to the fact that many endospore-forming species produce severe toxins brings up the existence of major diseases: gas gangrene (*C. perfringens*), tetanus (*C. tetani*), pseudomembranous colitis and chronic diarrhea[1] (*C. difficile)*, food-borne and wound botulism (*C. botulinum*), edematous wound infestation and enterotoxemia (*C. sordelli*), food poisoning (*B. cereus*), and anthrax (*B. anthracis*) [(3)]. Many of these diseases can progress onto severe septicemias when infections are important, the patient does not receive the proper treatment, or if patient is immunocompromised. Anthrax is one of the most dangerous infectious diseases [(5)]. Only acute hemorrhagic fevers caused by the families of virus *Filoviridae* (Marburg,

[1] *Clostridium difficile* associated disease (CDAD) is a hospital-borne infection that takes place in patients that are depleted of the normal micro-flora of the colon due to antibiotic prophylaxis. It causes pseudomembranous colitis and necrotizing enterocolitis in humans.

Ebola) *Flaviviridae* (Yellow Fiver), or *Bunyaviridae* (Crimean-Congo fever) are considered more hazardous, since no specific treatment is available[2] [6].

Anthrax has existed in the World for centuries. The disease in cattle is described in Egyptian and Mesopotamian writings from 5000 B.C.[8] It appears in the Bible in the book of Exodus (chapter 9) as the fifth (death of livestock) and sixth (boils) plagues sent by God to Egypt [9]. Formerly, the disease was associated to a pest that affected primarily cattle. The appearance of a 'black eschar' was characteristic of cutaneous anthrax. Europe witnessed sporadic outbreaks of anthrax, with the most acute outbreaks occurring in 14th century in Germany and 17th century Russia and central Europe [10]. The people with higher risk of being infected were the farmers that work with animal hair and wool. In the 18th century inhalational anthrax was known in England as the 'woolsorters disease' [9]. On 1881, Louis Pasteur demonstrated on the 'Pouilly-Le-Fort trial' that cows infected with a attenuated strain survived after infecting with a virulent strain, leading to the first vaccination ever made[3,8-10]. In the early 1900s, human cases of inhalational anthrax appeared in United States among workers in textile industries processing wool. The incidence of the disease decreased considerably during the 20th [9].

The alarm for anthrax was activated in 1979 at the city of Sverdlovsk, Russia. During the 'Cold War' there were concerns on the investigation of anthrax spores as a possible biological weapons by both United States and the Soviet Union. The Army Medical Corps developed in the 1950s the vaccine of anthrax that is licensed at the present time, the AVA vaccine [9]. Meanwhile, the Soviet Union was developing highly virulent strains to use as weapons, until an accident occurred. One worker of the microbiology facility at Sverdlovsk removed an air filter from the spore drying facility, and forgot to notify it in the logbook [11]. Another researcher did not see anything

[2] Many RNA-viruses belong to the Biosafety Level 4 (BSL-4), which includes aerosol-transmitted agents causing severe to fatal disease in humans for which vaccines or other treatments are not available. Anthrax belongs to BSL-3, for there is a vaccine and antibiotic treatment available.

unusual and turned on the machine. After some days a terrible outbreak occurred, affecting people nearby the facility. 96 individuals developed anthrax by ingesting contaminated meat or touching infected animals, of which 64 resulted fatal [12]. This spore leak was one of the most important accidents related to bio-safety negligence in history. It has been considered as the 'Biological Chernobyl' [11].

The idea of using anthrax as a biological weapon is a potential threat to any country, specially the United States, victim of several terrorist attacks. Anthrax spores are very easy to handle and disseminate through the air. The Center of Disease Control and Prevention (CDC) has defined the different bioterrorism agents that are currently available [13]. *Bacillus anthracis* is included among the highest priority agents in Category A. This category refers to agents that can be easily disseminated and transmitted, may result in high mortality rate, and cause panic and awareness.

Just one week after 9-11 attacks, a new alarm broke out the panic in the US citizens. Letters containing anthrax spores were mailed to several news media offices and two democratic U.S Senators. The attacks came in two separate waves; the first five letters were dated on September 18th and were sent to several media offices in New York (ABC, NBC, New York Post), and Boca Raton, Florida (American Media Inc.). A second wave of to letters was sent on October 9th to the offices of two democratic senators. The event resulted in five fatalities and other seventeen people infected [14]. The case, also known as *Amerithrax*[3], was considered by the Federal Bureau of Investigation (FBI) the worst bioterrorist attack ever made inside the United States [15].

The terrorists were using the Ames strain A2012. This is one of the most virulent strains of *Bacillus anthracis*, and it is also vaccine resistant [16]. It is used in biodefense laboratories in the United States, in research to "challenge" vaccines[4] [16, 17]. After a long period of investigations, in 2008 the FBI found in that Bruce E. Ivins, a biodefense

[3] '*Amerithrax'* was the name that the FBI designated to the 2001 Anthrax attacks

[4] Ames strain was isolated in Texas (1981), as an AVA vaccine resistant strain. Its protective antigen was used in research to develop more efficient vaccines.

researcher at USAMRIID, was likely to be responsible for these attacks, and was charged with the death of five persons and injury of several others. After learning that federal prosecutors were preparing to indict him, Irvin committed suicide with an overdose of acetaminophen [18].

Since the anthrax event occurred in 2001, concerns for biodefense research have increased worldwide. A considerable number of research groups from public and private institutions are studying *Bacillus anthracis* for the good –to find more efficient vaccines and potential treatments and for the bad –weaponizing the bacteria to make more resistant and infective spores–[19]. The total budget spent by the U.S. Government in biodefense funding in 2001 was a total of US $576 millions. For 2008, this funding increased up to US $5,421 millions, almost five billion dollars more than 2001[20].

Many strains of *B. anthracis* has already been genetically engineered to escape immunization by vaccines (Ames strain) [16], and to generate antibiotic resistance [21, 22]. Nowadays, anthrax disease can only be treated with the different antibiotics approved by the Food and Drug Administration (FDA): ciprofloxacin, doxycycline, and penicillin. Nonetheless, when the infection progresses to the late stage, antibiotics do not seem to respond. Recent investigations are focused in finding novel ways of therapies different than antibiotics and vaccines. In the immediacy of bioterrorism, the development of new efficient ways of for prophylaxis against *B. anthracis* is essential, as a way to beat upcoming sinister attacks, and maintain welfare in World nations.

1.2 The Anthrax Disease

Bacillus anthracis is the etiological agent of anthrax. The disease is epizootic, occurring exclusively in mammals. Grazing herbivores are the most susceptible to the infection: they are more prone to ingest the spores from the soil, which is the main reservoir [3,5]. The infection is initiated by the entry of the spores into the host body. This takes place by four major ways: 1) entry into the tissues through skin abrasions, 2) via insect bite, 3) by direct ingestion of the spores in the soil or contaminated food sources, and 4) by inhalation airborne spores. Anthrax can be transmitted from animal to humans in rare occasions (zoonosis) by direct contact with infected animals or their products, or by ingestion of contaminated animal food sources. Human to human transmissions are rare [5,7].

Depending on the spores' portal of entry, the disease will progress in different ways leading to three types of infection: cutaneous, gastrointestinal and inhalational anthrax. If the infection is localized in one region or tissue, symptoms are minor and the disease can be controlled. The potential danger of this disease comes when the bacilli invade the bloodstream. Once the bacteria reach the blood, the infection becomes systemic, leading to a fatal bacteremia and toxemia that will jeopardize the life of the host organism.

1.2.1 Cutaneous Anthrax

In cutaneous manifestation of anthrax, *B. anthracis* endospores penetrate the tissues of the host organism through skin abrasions or cuts. In few occasions it has been transmitted through insect bites [3, 5, 7]. Spore germination will take place at the primary site, leading to local edema and necrosis. It is not clear, however, if the germination occurs inside or outside the cells, in the interstitial fluids. In any case, the spores will germinate, and the vegetative cells will produce the respective toxins.

The primary skin lesion is usually a painless, but pruritic papule that appears 3 to 5 days after the introduction of the spores. Within 24 to 36 hours, the lesion forms a vesicle that undergoes central necrosis. After drying it leaves a 'black eschar'

surrounded by edema, which characteristic and exclusive of cutaneous anthrax. This eschar must not be confused with a pustule, because is painless and non purulent. These lesions normally resolve without complications in near 90% of the cases. Nonetheless, antibiotic treatment is recommended. There is a risk of more severe complications, such as malignant edema, that is accompanied of shock symptoms and breathing difficulties when is located in neck or thoracic regions [5].

Untreated cases have a mortality rate from 10 to 20% in humans [23]. Cutaneous anthrax mortality is due to airway obstruction from malignant edemas or by septicemia. In rare occasions, the spores are phagocytosized by macrophages, and germinate inside them. The macrophages detach and migrate to regional lymph nodes carrying the bacilli. These will grow in the lymph node producing hemorrhagic lymphadenitis. Bacteria will spread through the lymph and blood, increasing rapidly to high numbers and causing a fatal septicemia and shock [5].

1.2.2 Gastrointestinal and Oropharyngeal Anthrax

The pathophysiology of gastrointestinal anthrax is very similar to that of cutaneous anthrax. Following ingestion of spores, the primary site of infection occurs on the epithelium of the gastrointestinal tract. It is likely that the bacterial inoculation takes place at a breach in the mucosal lining [7]. The exact site of germination of the spores remains unknown. A recent study suggests that *B. anthracis* endospores can be internalized by from epithelial cell line Caco-2 isolated from the intestine [24].

The most common symptom of gastrointestinal anthrax is ulceration accompanied with enteritis. Associated symptoms include fever, abdominal pain, diarrhea, and blood-tinged vomiting and stools. In some cases mediastinal widening has been observed, although this symptom is more proper of inhalational anthrax [3]. Anthrax death results from intestinal perforation and anthrax toxemia. Mortality rates are considerably higher than cutaneous anthrax: 25 to 60 percent of the cases that are non-treated. If the patient survives, the ulceration symptoms start to reside after 10 days [3, 23].

A less common for associated with ingestion of contaminated food is oropharyngeal anthrax is less common. It is normally milder than the intestinal form. Its symptoms include cervical edema and local lymphoadenopathy, causing respiratory difficulties. The lesions in the oropharynx have the appearance of pseudomembranous ulcerations[5].

1.2.3 Inhalational Anthrax

Inhalational anthrax is the most fatal form of infection. Even with aggressive antimicrobial therapy its mortality rates are high. If the infection becomes systemic, its mortality is near 100%. The only positive aspect about this manifestation of anthrax is that does not occur often. It only accounts for less than 5% of the cases usually, since the infective dose in human is high. In an estimate, a human needs to inhale least 50,000 spores for the infection, although this amount may vary among different individuals [3, 25].

After the spores are inhaled, they may reach the bronchioles and alveoli of the lungs. Alveolar macrophages can efficiently phagocytosize the *B. anthracis* spores, and they germinate inside the phagolysosome [26]. This is the one and only way that the bacteria will proliferate when spores are inhaled. Hence, germination inside the macrophages is the key step to initiate the pathogenesis inhalational anthrax.

Although the initial site of contact is the lungs, inhalational anthrax cannot be considered a true pneumonia. The macrophages will transfer the bacteria to the mediastinal and peribronchial lymph nodes, where the infection per se begins. When the macrophages are inside the lymph nodes, the germinated bacilli escape from the cells by necrosis via toxins or by other mechanisms not yet elucidated [27]. As it was mentioned with the other types of anthrax, the bacilli multiply inside the lymph nodes.

The incubation time for inhalational anthrax is from 10 days, but the onset symptoms do not begin until six weeks. However, the bigger amount of spores in the inocula, the less the time it will take for symptoms to occcur. After the incubation period, the bacteria proliferate and secrete their toxins causing a hemorrhagic

mediastinitis [5]. Subsequently the bacteria can disseminate through the bloodstream. In the earlier course of disease the initial symptoms are cough, myalgia, and fever, resembling those of a viral respiratory disease. Thoracic radiographs show a mediastinal widening, that is an evidence of hemorrhagic mediastinitis. Mediastinal widening is indicative of inhalational anthrax and can be used as differential diagnosis, although the infection its late stage by then. Inhalational and gastrointestinal anthrax are difficult to diagnose in the early stages of disease due to the nonspecific symptoms. One to three days after mediastinitis, the latter symptoms appear: acute dyspnea, cyanosis, diaphoresis, stridor, and coma followed by death [3, 7, 25].

1.3 Biology of *Bacillus anthracis*

1.3.1 Traits of the Species

The genus *Bacillus* is ubiquitous in nature. Most of the *Bacillus* species are facultative anaerobes and saprophytes. However, some are opportunistic or obligate pathogens of animals, including humans, other mammals, and insects [2, 3, 7, 28]. *Bacillus anthracis* was thought to be an obligate pathogen, but recent findings suggest that its vegetative stage can be present in soil and does not necessarily require an animal host to survive. The bacteria can be found as vegetative cell in soils of pH~6.0 and temperatures above 15.5°C (such as limestone) in a cell-spore cycle that may be maintained indefinitely [3, 29].

B. anthracis belongs to a highly homogeneous subdivision called the *Bacillus cereus* group of organisms that also includes *B. cereus* and *B. thuringiensis*. These three species probably belong to the same ancestor [1, 30]. *B. anthracis* and *B. cereus* are opportunistic pathogens in mammals, whereas *B. thuringiensis* is an insect pathogen. Even though their respective genomes are almost identical, they have notable differences in the phenotypes.

The most important difference between *B. cereus* and *B. anthracis* in their phenotype is the expression of the capsule of poly-γ-*D*-glutamic acid, and the exotoxin

complex. *B. anthracis* has two plasmids of virulence: pXO1 and pXO2. The ability to cause the anthrax disease is attributed to such plasmids. Both plasmids together are required for the pathogenesis. Plasmid pXO1 encodes the genes for the anthrax toxins (*cya*, *lef*, and *pagA*), whereas pXO2 contains the operon *capBCAD* that expresses enzymes for the biosynthesis of the capsule [31].

1.3.2 The Endospore

All members from the family Bacillaceae have an endosporal phase during their life cycle [1-3, 28]. The endospore is a resistant stage of bacteria, in which their cellular functioning and metabolism are deactivated, entering in a period of rest called 'dormancy'. Many living organisms enter in dormancy when conditions are unfavorable for their normal functioning and outgrowth. The normal mechanism of producing a spore is by a normal cellular division, or 'mitosis', in which the pre-sporal cell, or forespore, will undergo complex genetic changes to develop resistance and enter in dormancy. The organisms that produce the spore will eventually die. In spore-forming bateria, the sporal cell develops inside the mother cell, also known as the 'sporangial cell'. For that reason bacterial spores are named 'endo-spores' [32]. Endospores are formed in many bacteria in response to starvation. However, some *Clostridium* species, such as *C. acetobutylicum*, are sensitive to other stimulus; the agent that activates the spore formation in these species remains unknown [33].

The biological process of spore formation is known as 'sporulation' or 'sporogenesis'. The bacterial sporulation consist of two major stages: first, there is a process of cellular division, by which a protoplast is formed within the cytoplasm of the cell. Secondly, it is an ordered developmental process in which to main layers, cortex and coats are laid down on the spore protoplast [32].

Nutrient limitation seems to be the most significant stress that triggers spore formation in *Bacillus* [32, 34]. Different kinases can recognize nutrient limitation. In *Clostridium* sp., signals that trigger germination are diverse and are not necessarily caused by of nutrient limitation. Starvation by carbon, nitrogen, or phosphorus induces

the activation of a master transcription regulator called 'Spo0A'. Five different histidine kinases (KinA-E) are sensitive to oxygen levels and redox potential. The activation of these kinases starts up a phosphorylation cascade phophorylating the protein Spo0F, which subsequently phophorylates and activates Spo0A [34].

Spo0A activates multiple loci, required for the next step of sporulation: the asymmetrical division. First the bacterial chromosome replicates, forming two nucleoids disposed in an axial filament across the cell (Stage I). Afterwards, the cell membrane undergoes an invagination in and forms the spore septum that separates the two nucleoids (Stage II). Soon after the division, two distinct programs of gene expression are initiated: one for the pre-spore and another for the mother cell. These programs are directed by sporulation-specific RNA polymerase σ-factors; σ^E in the forespore (pre-spore), and σ^F in mother cell. After the division, subsequent proliferation of the cell membrane of the mother cell, will lead to the engulfment of the pre-spore. The immature spore is now completely surrounded by a double membrane, and is released as a protoplast into the mother cell (Stage III).

After the engulfment there is another change in the transcription, and the transcription factors σ^G and σ^K become active in the pre-spore and mother cell respectively. The pre-spore will eventually become the core of the spore and the mother cell is responsible of synthesizing the outer protective layers of the spore. Cortex material begins to be laid down between the double membranes of the protoplast. At the same time, 2,6-pyridinedicarboxilic acid (dipicolinic acid, DPA) is synthesized and accumulates inside the core associated calcium cations (DPA-Ca^{2+}). Stage IV is completed after the formation of the cortex [28, 32, 34].

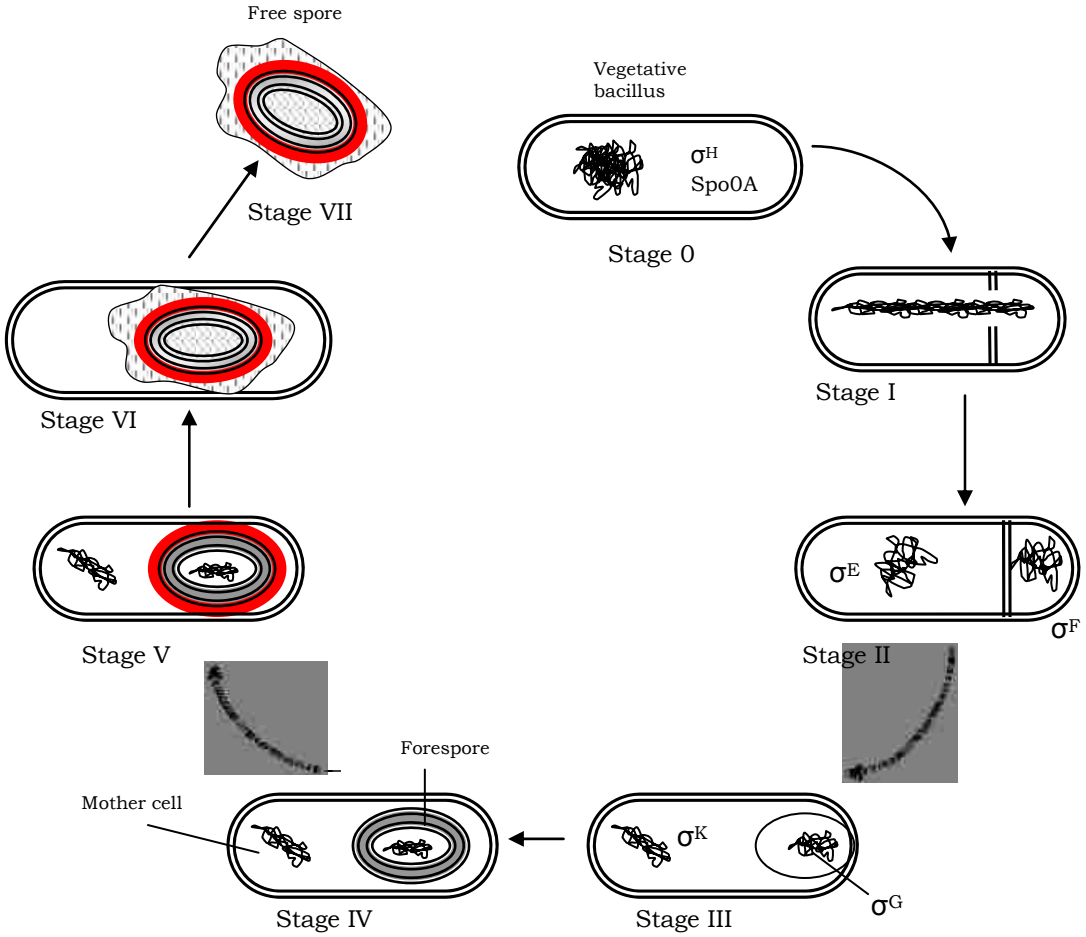


Figure 1. Stages of the sporulation of *Bacillus* sp. In the initial stage the vegetative cell will activate the factor Spo0A as a result of initiation signals (Stage 0). Spo0A combined with another factor (σ^H) will trigger the asymmetric division of the cell (Stages I-II). Subsequently two different factors (σ^E and σ^F) will lead a compartmentalized gene expression in the forespore and the mother cell. A series of proteins that will be encoded in the mother cell will trigger the migration of the membrane around the forespore has been engulfed (Stage III). A second set of transcription factors (σ^K and σ^G) will activate expression of new genes in each cell, leading the synthesis of the spore cover. First the cortex is synthesized (Stage IV), followed by the assembly of the coat proteins (Stage V). Finally the spore completes is maturation that is observed by the increase in refractivity (Stage VI) and the sporangial cell is lysed, releasing the mature spore (Stage VII). Adapted from Prescott, L., Harley, J. et Al (2000) "Microbiología". McGraw Hill Press. Spain.

During stage V, coat proteins encoded by the *cot* genes of *B. subtilis* are synthesized. The coat proteins are deposited around the pre-spore. Coat assembly is an ordered complex process that involves several genes [35]. The proteins SpoIVA and CotE from *B. subtillis* have been well analyzed; SpoIVA function is related to attachment of the coat to the cortex, and CotE forms a shell around the coat to cover and harbor all

the coat proteins and enzymes that will be inactive during the spore dormancy. Some species, such as *B. anthracis,* form an additional covering for the coat, known as the exosporium; its function is not well known yet. Finally, the spore matures becoming resistant to heat, and organic solvents, and lytic enzymes lyse the sporangium or mother cell, liberating the spore [3].

The bacterial endospore is a structure of significant complexity, divided into several layers (see figure 2). A full mature spore is composed of parts that were originated in the forespore and in the mother cell. The main structural parts are detailed as follow:

Core.- It is the innermost part of the spore. It corresponds to the developing forespore, inside the sporangial cell. It contains the cytoplasm, DNA, ribosomes, enzymes and everything that is needed to function once returned to the vegetative state. It has a very low water content (25-50%), and large amounts of calcium cations and 2,6-pyridinedicarboxilic acid (DPA), that provides resistance to heat, desiccation, and reactive oxygen species (ROS) . One unique feature is the presence of SAPS proteins binding the DNA, crucial for the resistance to UV radiation [32, 36].

Inner membrane.- Is the former plasma membrane of the forespore. It contains several membrane proteins synthesized during the sporulation that contribute to the process of germination (ion channels, germination receptors).

Germ cell wall.- The cell wall corresponding to the forespore, and composed mostly by peptidoglycan, similar to the one of Gram-positive vegetative cells.

Cortex.- The cortex is a very thick low density layer that surrounds the core. It is mainly composed of modified peptidoglycan. This layer is synthesized during the sporulation, between the outer membrane from the mother cell, and the inner membrane of the forespore. It is important for the maintenance of the spore's

dehydrated state [37]. The cortex is also associated with the property of refractivity[5] of the spore [32].

Coat.- It is a dense proteinaceous multilayered-cover. It is synthesized in the late stages of sportulation on the outer membrane from the mother cell that engulfs the forespore. Al least 30 different protein species compose the coat [35]. It provides mechanical integrity and defense against toxic molecules, by excluding large-sized molecules and allowing small nutrients that are needed for activating the germination. It also carries several enzymes involved in the detoxification and a cortex-specific lytic enzyme (CwlJ) [36, 37]. It is divided into two layers of proteins (outer and inner coat) separated by a shell made of the protein CotE [35].

Exosporium.- In some species of *Bacillus,* including *B. anthracis*, the coat is supplemented with a glycoprotein cover. It serves as a semi-permeable barrier that excludes large, potentially harmful molecules, such as antibodies and hydrolytic enzymes. It has an external hair-like filament layer, composed mostly of the collagen-type glycoprotein BclA [38]. It has been found recently that the exosporium contains the enzyme alanine racemase (Alr), for the auto-regulation of the spore germination [39]. The exosporium is likely to be an important recognition site in the spore. BclA has ben shown to stimulate phagocytosis by macrophages [40].

[5] The refractivity or refractility of the spore is also known as the whitening of the spore. It is correlated in part to the calcium DPA intake but also with the synthesis of coat proteins. When a spore appears particularly bright when is viewed by phase-contrast or light microscopy it has become 'refractile'. This property is lost with cortex-less mutants. [3,32]

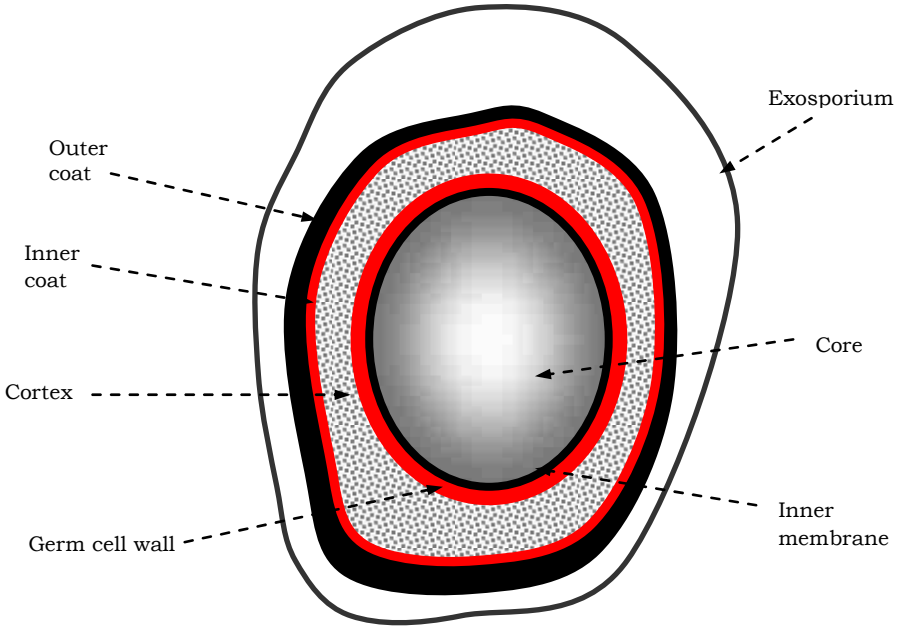


Figure 2. Morphology of the Endospore

Endospores are resistant to extreme conditions. They can survive to almost any environment present in our home planet, and it has been shown that they may resist environments outside the Earth [4]. They exhibit resistance to many agents physical agents, such as extreme heat of over 100°C, solar UV radiation (both UV-A and UV-B), γ-radiation, extreme desiccation, high hydrostatic pressure (near 200 atm), and even cosmic ionizing radiation. Even more, it can survive action of many toxic chemicals, including organic solvents, alkylating and oxidizing agents.

The spore coat is relatively impermeable, preventing the entry of damaging molecular agents. However, this impermeability is limited; some chemicals involved in the germination of the spore are able to penetrate the coat and the cortex binding receptor on inner membrane.

Besides the resistance to any hostile environment, endospores show an incredible longevity. They may remain dormant for hundreds of years. A few cases have obtained environmental samples of spores from up to 10^5 years, and they were able germinate. A

sample of viable *B. sphaericus* spores was obtained from fossilized Dominican amber dated of about 25 million years [41].

Several proteins, located in the core and coat, contribute to their resistance and longevity of the spores. Small acid soluble proteins play an essential role for the maintenance of DNA inside the core and the protection of dormant enzymes that need to be reactivated upon the germination [42]. Coat proteins are also crucial for the protection of the lytic enzymes associated with the coat and cortex degradation in the germination [35]. Other mechanisms of protection include DNA repairing enzymes, high cation concentration, low water content, and the presence of DPA. Mutant *B. subtillis* strains lacking the genes for the DPA sythetase (*dpaA*, *dpaB*) showed increased water content and less resistance to heat [43].

1.3.3 Pathogenesis and Host Cell Interactions

The pathogenesis of *B. anthracis* relies in the capsule and the tripartite toxin combination. The toxins belongs to the family of bacterial binary A-B toxins, characterized by a B moiety that binds the target cell, and an A moiety that translocates into the cytosol. The 'B' unit is called protective antigen or PA. The 'A' has two alternative subunits: the lethal factor (LF), and the edema factor (EF). The molecular mechanisms by which these toxins interact to produce the virulence in the host cells have been elucidated in the past two decades [44].

a) Protective antigen (PA):

The protective antigen binds the anthrax toxin receptor (ATR). ATR proteins are ubiquitous in different mammalian tissues. Two different forms of ATR have been found in humans: ATR1 and ATR2 [45]. Upon the binding, PA is activated proteolytically by a furin-like cell-surface membrane protease. The N-terminal fragment of 20 residues (PA_{20}) is cleaved. The C-terminal domains (PA_{63}) remain bound to the ATR and self-associate in the membrane forming symmetric, ring-shaped heptamers. The PA_{63} heptamer shows specific protein-protein

interaction with EF and LF, binding a maximum of three EF/LF molecules. The next step is the internalization of the toxins into the cells by ATR-mediated endocytosis. The endosomal compartment will be acidified by the lysosomal action inside the cell. This decrease in pH will trigger a conformational change in PA_{63} heptamer, allowing the insertion in the membrane and the formation of cation-selective channels. The channels will now lead the translocation of the EF/LF units into the cytosol [44].

b) The lethal factor or lethal toxin (LF):

LF is a four-domain Zn^{2+}-metalloprotease. The mature protein contains 776 residues. It has been found recently that LF cleaves the N-terminus of mitogen activated protein kinases (MAPKK) Mek1 and Mek2 [46]. This cleavage will result in the loss of function by the MAPKK and the alteration of several transduction pathways in the cell. This is correlated to the inhibition of the expression of tumoral necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), and release of pro-inflammatory mediators and nitric oxide. This will result in the loss of functioning and regulation inside the cell, triggering a rapid and selective apoptosis [44].

c) Edema factor or Edema Toxin (EF):

EF is a calcium-calmodulin dependent adenylate cyclase. The mature protein contains 776 residiues. Upon the translocation from the endosome, EF remains associated to the endosomal membrane. Eukaryotic adenylate cyclases are also membrane-associated. EF converts intracellular ATP into cAMP. This will lead to a substantial increase in the intracellular cAMP, upsetting water homeostasis and disrupting the signaling pathways, followed by membrane damage

and cell necrosis. EF is also the responsible for the edema symptom in cutaneous anthrax, therefore its name [7, 44].

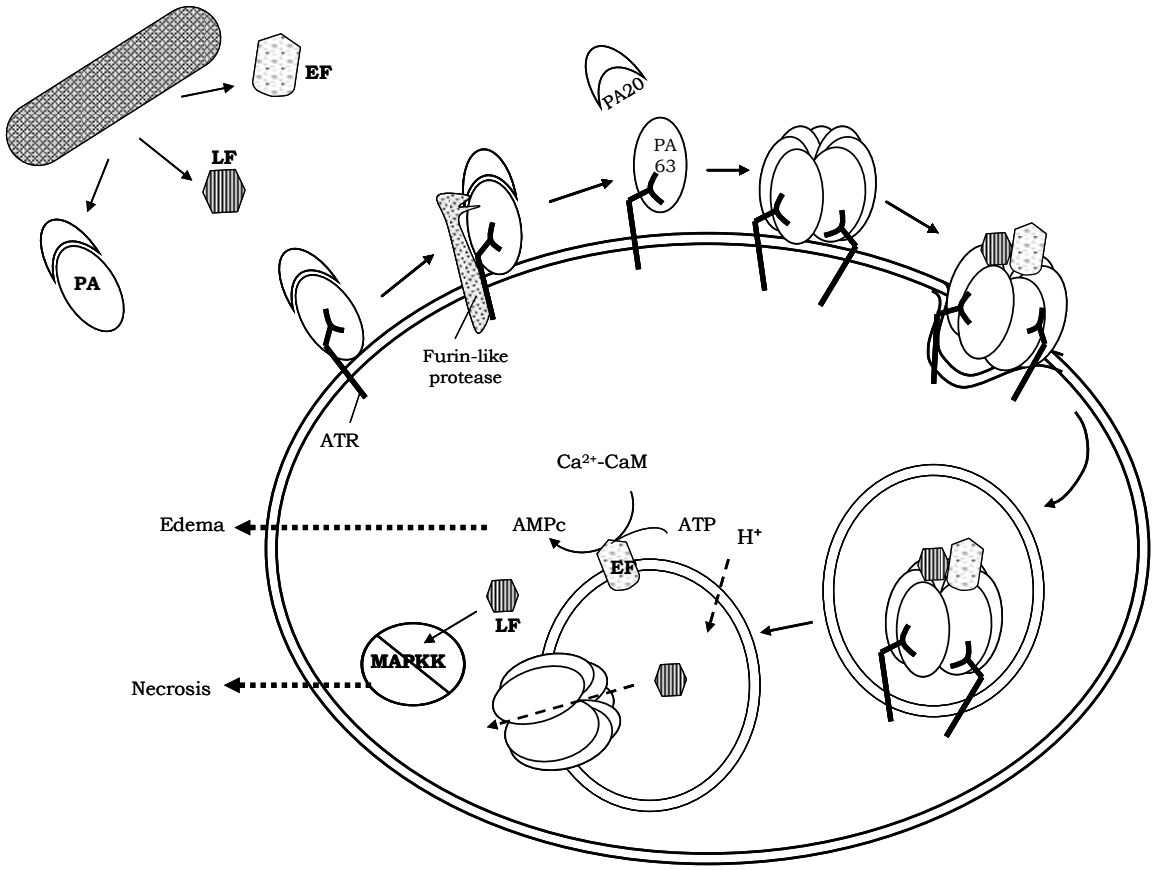


Figure 3. Cellular mechanism of the anthrax toxins. The toxins are released by the vegetative bacteria. The ATR receptors (ubiquitous in mammalian cells) will bind the protective antigen. A furin-like endoprotease in the membrane cleaves the PA, and the remaining fragment (PA_{63}) is oligomerized forming a heptamer. Toxins LF and EF bind the heptamer, and this complex enters the cell by endocytosis. The decrease in pH inside the lysosome will trigger a conformational change in the PA subunits, forming a pore in the membrane, and allowing the binary toxin to enter into the cytosol. EF will act as a membrane calmodulin-dependent adenylate cyclase, increasing dramatically the concentrations of AMPc, and upsetting cell signaling and homeostasis. LF cleaves the MAPKK resulting in the loss of functioning and subsequent apoptosis or necrosis. Adapted from Mock, M. et *al* (2001) *Annual Rev Microbiol* 55: 647-671

Bacillus anthracis is an extracellular pathogen. However, it appears to require an intracellular step to initiate infection, at least for inhalational anthrax. Macrophages serve the bacteria in two ways: as a medium for the spore to germinate, and as vehicle to penetrate the mucosal barrier and migrate to the lymph nodes. *In vivo* studies by

Guidi-Rontani and colleagues have shown that the germination of the endospore takes place inside the phagolysosome of alveolar macrophages. These cells play an important role in the cell-mediated immune response preventing the invasion of bacteria entering through the respiratory tract. Nonetheless, macrophages are 'deceived' by *B. anthracis* spores, becoming their allies. Guidi-Rontani refers to them as the 'Trojan horse' for the anthrax pathogenesis [47].

Anthrax spores that reach the alveoli can be readily phagocyted by the macrophages. Recent research has shown that macrophages show a tropism for the spores mediated by the integrin Mac-1. This protein is express by different macrophage cell lines and binds with a high affinity the exosporium protein BclA [40]. The spore protein can be considered as a virulence factor. Moreover, endospores can also be opsonized by C3 proteins, activating the classical pathway of the complement, upon the binding of IgG [48].The combination of the action of the integrin and the opsonization with C3 enhances phagocytosis of by macrophages.

The toxins that are produced by *B. anthracis* upon the germination may also enhance the leukocyte extravasation and further macrophage and neutrophil infiltration into the alveolus lumen. EF appears to stimulate the production of the pro-inflammatory IL-6 [49].LF is likely to stimulate the expression of IL-1β and TNF-α by macrophages.

The mechanism and germinants by which the endospore germinates inside the phagosomal compartment are not clear. Guidi-Rontani found that the *gerX* operon is not crucial but contributes considerably to germination in macrophages [26]. J. D. Ballard and colleagues, from the University of Oklahoma, have identified several genes in *B. anthracis* that are required for the germination *in vivo* [50]. Mutants in the *gerH* operon as well as in the *pga* gene are unable to germinate the cells. This shows that the protective antigen is also involved in the germination within the cell. Moreover they have identified five genes that are differentially expressed upon the phagocytosis. Many these

genes remain putative, and its function needs to be elucidated [50]. Thus, the germination mechanism in cells appears to be complex, involving several proteins.

Once the spores germinate, the vegetative bacilli can survive inside the phagolysosome [26, 51]. The bacteria need to escape from the action of digestive enzymes (lysozyme), oxidative stress (ROS), and extremely low pH; they must express genes to evade the lytic activity of the lysosomes. Other Gram-positive bacteria, such as *Streptococcus* sp. can be killed and digested inside the phagolysosome. Dixon and colleagues demonstrated that *B. anthracis* can germinate survive in the phagosome without pXO1 and pXO2, showing that neither the toxins nor the capsule are required for evading lytic action of the lysosomes [51]. The identity of the phagosomal protective genes remains to be elucidated.

Recent studies by Shatalin et *al.* propose a mechanism by which the bacteria escape the action of the lysosomal action. There is a latent oxidative stress system present in the spore and does not require *de novo* protein synthesis. The bacterial spores have their own bNOS enzyme that is activated upon the germination producing nitric oxide (NO). The spores have also the catalase enzyme present (Kat). Both Kat and bNOS will cooperate against antimicrobial ROS secreted by the macrophage. The NO produced by bNOS activates Kat and also prevents DNA damage from ROS by suppressing the Fenton reaction. Kat will catalyze the conversion of the damaging superoxide anions into water molecules. By studying defective mutants of bNOS, this NO-mediated defense system has been shown to be essential for the survival inside the phagosome [52].

B. anthracis pXO1/pXO2 defective mutant spores are capable of being phagocytosized and germinate inside the phagolysosome, but the vegetative cells cannot escape from this compartment. It has been shown that the phagolysosomal escape requires a gene from pXO1, which is the positive regulator *atxA* [51]. This gene regulates the expression of many other genes essential for the virulence: it activates the expression of the operon *capACB* for the capsule, and the gene *pga* for the protective

antigen; indirectly it also activates the expression of the remaining toxin genes, *let* and *cya* [53].

Dixon and colleagues have observed through microscopy the behavior of four different strains of *B. anthracis* spores. Dixon and colleagues have observed through microscopy the behavior of four different strains of *B. anthracis* spores. The first two strains were ΔSterne 7702 (plasmidless), and UT60 (pXO1$^+$, *atxA*-null). These strain are not able to escape efficiently from the phagolysosome and their vegetative cells proliferate and accumulate inside the macrophages until the overabundance of cells destroy the macrophage membranes by mechanical action. The third strain, RP-31, is pOX1$^+$/atxA$^+$, but does not carry the *let* genes for the LF and produces no detectable amounts of PA. In the observations with RP-31, the strain was released from the macrophages efficiently; due to the lack of virulence toxins the integrity of the macrophages was maintained. The last strain was a regular Sterne TCC-013 strain carrying pOX1$^+$/atxA$^+$ without alteration in any of the genes expressing the toxins. In this strain the vegetative bacilli escaped the phagolysosome via an *atxA*-regulated factor. When they were inside the cytosol the macrophage membrane integrity was disrupted, likely because of the action of the toxins. The studies show that *atxA* is required for the phagosomal escape, and the gene *let* of the letal factor is not involved directly in this process [51].

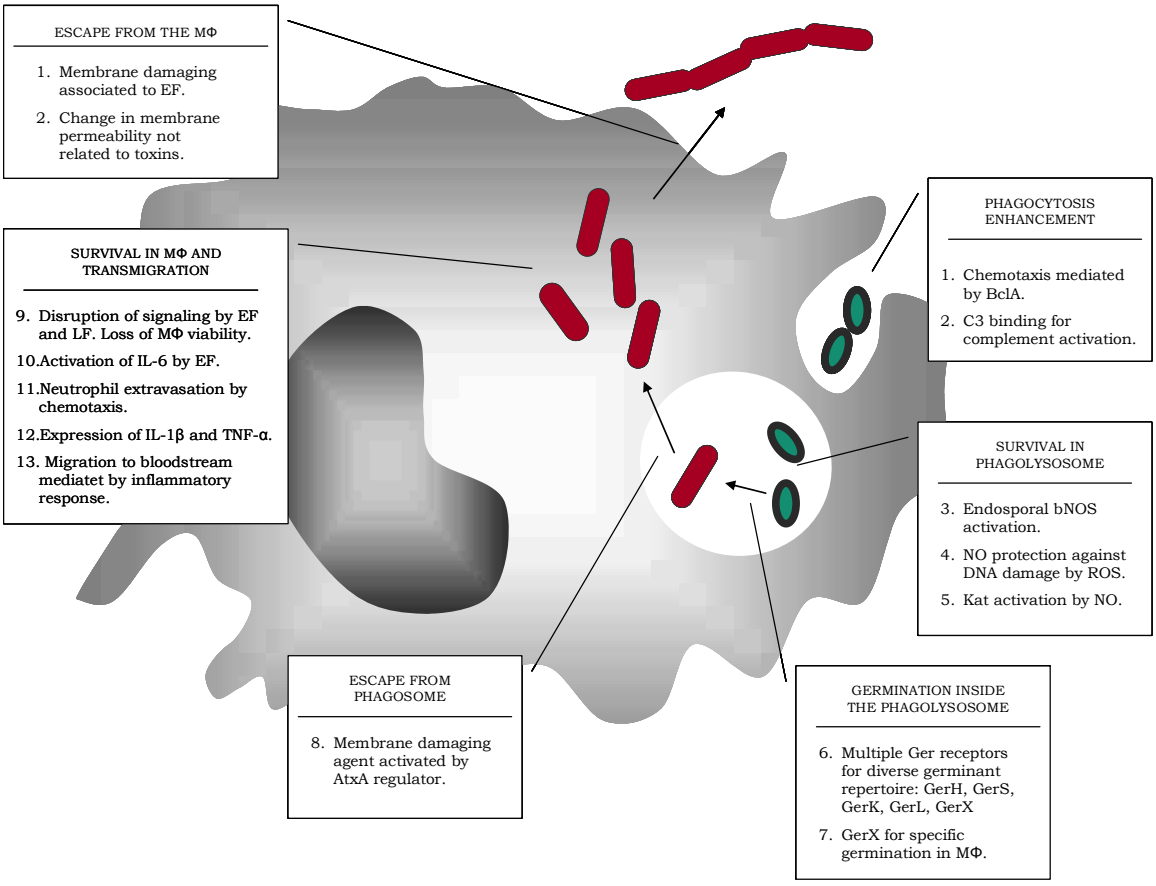


Figure 4. Evolutionary strategies developed by *B. anthracis* in the interaction with the host cell.

1.4 Spore Germination

1.4.1 Biochemical Stages of *Bacillus* Germination

Endospores are transformed into vegetative cells by a biochemical and physiological process called germination. *Bacillus* sp. endospores germinate when environmental conditions are favorable. These conditions are mainly nutrient availability, temperature, and pH [32]. Even though the spores are dormant and metabolically inactive, they must be sensitive to the stimulus of germination. This is stimulus is given by certain nutrients in the environment. These nutrients will act as signaling molecules for the germination pathway; therefore they are known as 'germinants'.

Germination is initiated by a signal transduction process that occurs in the endospore. Germinants will bind transmembrane proteins located around the core, and

beneath the coat and exosporium of the spore. This family of proteins is also known as Ger receptor proteins [54, 55].

The germination process can be summarized in five main events (figure 5):

1) Binding and recognition of signaling molecules to activate the germinaton receptors.
2) Signal transduction across the cell to produce the germination response.
3) Release of dipicolinic acid (DPA) and rehydration of the spore core.
4) Degradation of the spore cortex and core expansion.
5) Outgrowth and maturation of the core into vegetative cell.

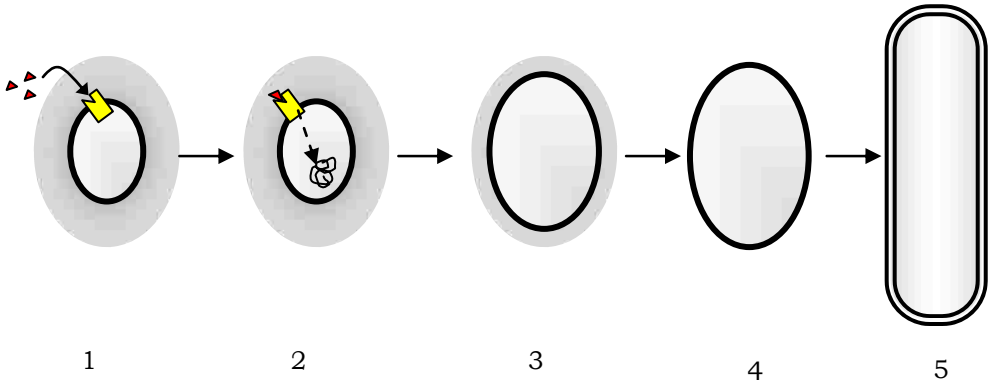


Figure 5. Sequential steps involving the germination of the endospore. When signaling molecules (amino acids and nucleosides) they will bind and activate the specific Ger receptor protein (1). The receptor triggers a signaling pathway (2) that leads to DPA release and rehydration of the core (3). Proteases and hydrolases are expressed for the degradation of the cortex (4). Finally cells recover their functionality, and acquire a morphology proper of the vegetative state.

Each of these events occurs in a sequential order. The first event is the recognition of the germinants (amino acids, monosaccharides, and/or nucleosides) that will bind and activate heterotrimeric receptor proteins. The mechanism for the activation and the functionality of each of the subunits of the *Ger* proteins are not yet well understood. It has been hypothesized that receptors may have more than one site of recognition, and may serve not only in the signal transduction, but also with an immediate response acting as a membrane transporters [55]. In wild type strains in normal physiological

conditions, once the receptors are activated, spores are committed to germination and all of the subsequent events will continue until the full maturation of the cell. Studies show that over 80% of *B. anthracis* Sterne strain endospores germinate within five minutes in nutrient broth at 37°C (see table 1) [56].

Table 1. Heat resistance and optical density changes during germination

Time germination (min)	Heat resistant CFU[6] (%)	OD^{7}_{550}
0	106.0	0.232
5	12.5	0.203
10	7.0	0.137
15	3.5	0.158
20	2.3	0.173
30	1.0	0.148
45	0.3	0.184

Source: E. Abel-Santos et Al (2006)

Ger proteins are located in the inner membrane. In order to initiate germination, germinant has to permeate the exosporium, coat and cortex layers of the spore and bind to a germination receptor located in the inner membrane. This requires the existence of special system. Recently, the role of the locus *gerP* of *B. subtilis* and *B. cereus* has been elucidated. The GerP protein plays an important role for the accessibility of nutrients into the spore core. Mutant strains lacking *gerP* show a poor rate of germination. When these strains are decoated, the germination is similar as the one of wild type strains, suggesting that GerP contributes to the access of germinants to the receptors [57].

Little is know on the mechanism of signal transduction that triggers expression of the cortex degradative enzymes and spore permeability. Signaling for germination must

[6] Heat-resistant CFU is a parameter that correlates directly with the concentration of endospore present in a suspension. After heat-treating a sample only endospores may survive, whereas vegetative cells are killed.

[7] Optical Density (OD) in the range of 520 to 600 nm is a parameter used to determine the spore concentration in one sample. OD

lead to two final responses in the spore: 1) rehydration of the core, after the dormancy accompanied with the release of DPA, and 2) expression and/or activation of enzymes for the degradation of the cortex, for the subsequent growth of the vegetative cell. These enzymes are mainly proteases and hydrolases [58, 59].

It is clear that water and cation transport is an early and likely essential event in the germination. Studies with specific drugs that block K^+, Na^+, and Ca^{2+} channels, inhibited the germination in *B. megaterium* [60]. It is hypotesized that the ion channel or at least one of them may be the Ger protein itself. *GerA* β-subunits have a sequence homology with integral membrane proteins that function as transporters.

Major effort to improve our understanding of the biochemical responses in the immediate events in the germination have been done in the recent years by Peter Setlow and colleagues. They developed mutants of *B. subtilis* that are unable to complete germination and are blocked at an intermediate stage. One of the very first responses is the release of protons (H^+), monovalent cations, and Zn^{2+}. This will lead to a change of pH in the core from ~6.5 to 7.7, essential for reactivating the spore metabolism [61]. Subsequently the large depot of DPA inside the spore is released associated with Ca^{2+} cations; this suggests the existence of DPA channels. Recently, it has been elucidated the identity of the gene *spoVA*, that is involved in the DPA uptake for the sporulation [59, 62]. It is unclear if these proteins are also involved in the DPA efflux outside the core. DPA release and water influx occur simultaneously through ion water exchange. GerN is a protein found in *B. megaterium*; it is a Na^+/H^+-K^+ antiporter. Mutants defective in *gerN* are able to grow and sporulate efficiently, but they are unable to germinate. It is believe that this antiporter plays a crucial role in the cation transport for the germination [63].

The second major response that follows ion transport and DPA release is the cortex degradation. The degradative enzymes are likely to be dormant state inside the core. It is not clear whether they are activated through rehydration, changes in pH or salinity, or by direct signal transduction led by Ger receptor. The possibility of expression of lytic

enzymes *de novo* is remote, since mRNA synthesis does not take place until the latter events of outgrowth and maturation. It has been that demonstrated that two cortex hydrolases are synthesized during sporulation in the forespore [55], confirming the idea that the enzymes are already present in the endospore.

There are two major group of lytic enzymes: spore cortex lytic enzymes (SCLEs) and cortical fragment lytic enzymes (CFLEs). SCLEs are hydrolases that target specific components of the peptidoglycan of the cortex. CFLEs are hydrolases of the polysaccharide moiety. Some of the enzymes have already been elucidated. The peptodoglycan layer is degraded by different hydrolases. In *B. subtilis* two enzymes CwlJ and SleB have been studied. Both required one component of the peptidoglycan for their action –muramic-δ-lactam–. SleB shows a transglycolase lytic activity, whereas CwlJ activity is unknown [59]. Once the cortex is fully degraded the core will complete its expansion; when this happens the spore starts to lose its dormancy and metabolic activity is reactivated.

In the outgrowth stage the cell begins to recover the rod shape characteristic of the vegetative bacteria. A large depot of small acid soluble proteins (SASP) that make up 10-20% of the content in the core, needs to be digested for full maturation. SASPs maintain the integrity of molecules in the core, such as DNA, and other proteins. SAPS degradation does not begin until the cortex is destroyed and the core is fully expanded [58, 64].

Macromolecular synthesis is reactivated also during the outgrowth. SAPSs are cleaved into oligopeptides by the germination protease (GPR). Other spore peptidases degrade the peptides into amino acids that will be used for the upcoming protein synthesis. The DNA shielding by the SAPSs is now removed. Transcription of mRNA begins within the first minutes of the germination. Protein synthesis immediately follows transcription taking place almost at the same time. However, DNA replication does start until 30-60 minutes later [58, 65].

One phenotypical trait of maturation, observable with optical microscopy, is the loss of refractivity, as well as the decrease in optical density in suspension. This is due to the degradation of the cortex. Loss of refractivity is correlated with a decrease in optical density; this is particularly useful to monitor spore germination *in vitro* [3, 32].

1.4.2 Germination Receptors

Ger receptors have been well studied in the present decade [66-71]. Ger receptors are encoded in tricistronic operons. It has been assumed that the three proteins encoded form a complex in the membrane for recognition of the ligand and initiation of the signaling cascade for the germination. All three receptor subunits are essential for the germination [55]. Mutational analysis of different *Bacillus* species has been done to determine relevance of each of the three subunits [67,68, 70].

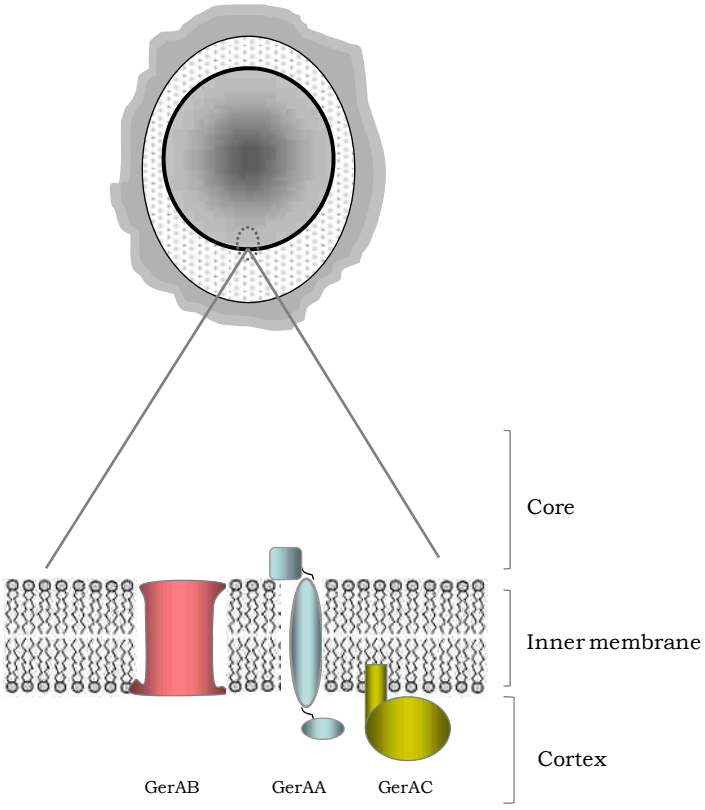


Figure 6. Topology and localization of the tripartite protein encoded by the *gerA* operon in *B. subtilis*. Adapted from Moir, A., Corfe, B. M., and Behravan, J. (2002) *Cell Mol Life Sci* 59, 403.

The *ger* operon family is ubiquitous among a wide range of species of endospore forming gram positives. Genes *ger* have been identified in *B. subtilis* [66, 70, 71], *B. cereus* [72], *B. anthracis* [68, 69] (table 2) *B. halodurans* [73]. With the local alignment search tool (BLAST) searches have revealed analogs of GerA in *Clostridum,* the other major genus of endospore forming bacterial: *C. difficile*, *C. acetobutylicum*, and *C. pasteurianum* [59].

Bacillus subtilis operon *gerA* (figure 6) serves as the quintessential model for being the first *ger* operon functioning to be elucidated [70, 71]. So far, five different putative operons for receptor proteins have been identified. Three of them (*gerA*, *gerB*, and *gerK*), have been characterized [66]. All three components encoded by this operon are membrane associated.

The protein GerAA has a domain of five or six membrane segments, plus a large N-terminal and C-terminal hydrophilic domains. The N-terminal domain is believed to be located inside the spore core, and it likely serves in signaling germination across membrane. The GerAB protein is predicted to have a motif with 10 transmembrane helixes. It is likely to be an integral membrane protein due to its high hydrophobic nature. Mutations within the membrane spanning elements affect the amount of concentration of germinant (*L*-alanine) required for germination. Therefore, this protein appears to affect the receptor-ligand binding. The GerAB and its homologues belong to a subfamily of the APC (amino acid/polyamine/organocation) superfamily of integral membrane transporters. The GerAC is a hydrophilic protein. However it is predicted to have a lipoprotein signal sequence. The protein will be anchored to the inner membrane due to an N-terminal lipid moiety attachment [58, 59, 66].

Table 2. Identified genes for the *ger* operon in different species of *Bacillus*

Organism	Chromosomal	Plasmidic
B. subtilis	*gerA, gerB, gerK, gerC*[†] *gerD*[†]*,gerF*[†]*, gerP*[‡]	
B. anthracis	*gerA*[††]*, gerH, gerK, gerL, gerS, gerY*[††]	*gerX*
B. cereus	*gerB*[*]*, gerH, gerI, gerK*[*]*, gerL, gerQ, gerR, gerN*[‡]*, gerS*[*]*, gerT*[‡]	

[†] Former putative genes first identified as involved in germination, but with a different function.
[††]Functionality remains putative.
[‡] Gene involved in the germination, but not as germination receptor.
[*] No direct phenotypic evidence on the function.

1.4.3 Germinants and Germination Pathways

Germinants are known as specific chemical compounds that trigger the germination of bacterial endospores. In 1949 Joan F. Powell performs the first published studies of the germination of *B. subtilis in vitro*, in a phosphate buffer containing *L*-alanine [(75)]. In the same year, Hills showed that the germination of *B. anthracis* was influenced by *L*-alanine, tyrosine, and adenosine [(76)].

Germinants are extremely diverse among the *Bacillus* and *Clostridium* species. This is due to the variety of receptors that perform the same function. Although amino acids are the most common among *Bacillus*, sugars and nucleosides may also trigger the germination. Receptors are triggered by one sole germinant, or by two acting together: co-germinants. Some receptors just require one of the co-germinant is required to trigger the signaling cascade, whereas some other need the presence of both co-germinants together [(58, 59)].

In each species of bacteria multiple Ger receptors recognize a diverse array of germinants. This diverse set of nutrients allows bacteria to germinate in different micro-environments and to distinguish between soil and host, to break the dormancy. There is a cooperative response when two or more receptors of different kinds are triggered at the same time [(59, 68)]. This cooperation may ensure that dormancy is broken, only when environmental conditions are favorable for growth. In *B. anthracis* efficient

germination is achieved only with two or more receptors acting together. Multiple germinants indicate the presence of multiple nutrients which will be needed for the vegetative cell.

P. Hanna and colleagues have done comprehensive studies on the germination receptors of *Bacillus anthracis* and their germinants during the present decade. The operon *gerH* was the first one to be studied, due to its significant homology with *gerI* of *B. cereus*. Even though the two receptors are quite similar and respond to the same germinant – inosine –, they do have some differences. *GerH* is unable to trigger germination with inosine only, but needs also an amino acid co-germinant, whereas *GerI* only requires the nucleoside. *GerH* is also required for the germination within macrophages. Other six *ger* operons have been indentified in *B. anthracis* (table 2): *gerA*, *gerK*, *gerL*, *gerS*, *gerX*, and *gerY*. Frame shift mutations of *gerA* and *gerY* have no detectable effect in the germination *in vitro*. The rest of the mutants recognize a different set of amino acid and nucleosides leading to different pathways for germination [68, 74].

P. Hanna has proposed a model of the different germination pathways *in vitro* by analyzing the kinetics of different mutants in each of the Ger receptors [68]. These pathways are the following:

a) L-Alanine mediated pathway.

This pathway is triggered by the presence of *L*-Ala alone in high concentration (1-10 mM), and requires both GerK and GerL receptors. This very same pathway will also take place with the prescence of *L*-Pro and *L*-Ala in lower concentrations (AP pathway).

b) Aromatic amino acid-enhanced alanine pathway (AEA).

The AEA response requires GerL, GerS, and GerH. GerS is a recognition site for aromatic amino acids, but it cannot be triggered alone. It requires the presence of GerH. This pathway also requires the activation of GerL with *L*-Ala to start up the signaling for germination.

c) Amino acid and inosine dependent pathway (AAID).

This pathway needs an amino acid and a purine nucleoside acting together as co-germinants. Inosine and adenosine are the most potent purine co-germinant. AAID pathway involves multiple receptors that are combined in pairs or triplicates for the response. There two AAID responses. AAID-1 includes the binary combination of inosine and either L-alanine, L-serine, L-valine, L-methionine, or L-proline. It requires the presence of GerH for nucleoside recognition in combination with GerK, or in combination with GerL, and another receptor that can be either GerS or GerX.

GerX is located in the plasmid of virulence pXO1 and plays an important role for the germination *in vivo*. It has been shown that *gerX* null mutants are less virulent. Their rate of germination in macrophages after 3 hours is 70% less than wild type Strene strains. GerX appears to be involved in the detection of specific germinants inside the macrophages [69].

AAID-2 needs the binary combination of *L*-histidine or aromatic amino acid and a purine. This route requires both GerH and GerS, with no detectable contribution from either GerK or GerL. AAID-1 and AAID-2 show different kinetics; the latter one also needs a higher concentration of the nucleoside to be effective.

Bacillus anthracis may germinate just in the presence of L-Ala, by triggering the alanine pathway. The rate of germination, however, is considerably slow. With 0.5 mM *L*-Ala the mean decrease in OD_{600} is only 2.5% after 30 minutes. When alanine is added in combination of inosine the decrease in OD600 is near 60%. In this case at least three more pathways are activated: AAID-1 activating the two different combinations of receptors (GerHSL and GerHSK), and AAID-2 (figure 7). The best rate of germination of B. anthracis is with the combination of a nucleoside such as inosine or adenosine, and alanine [74].

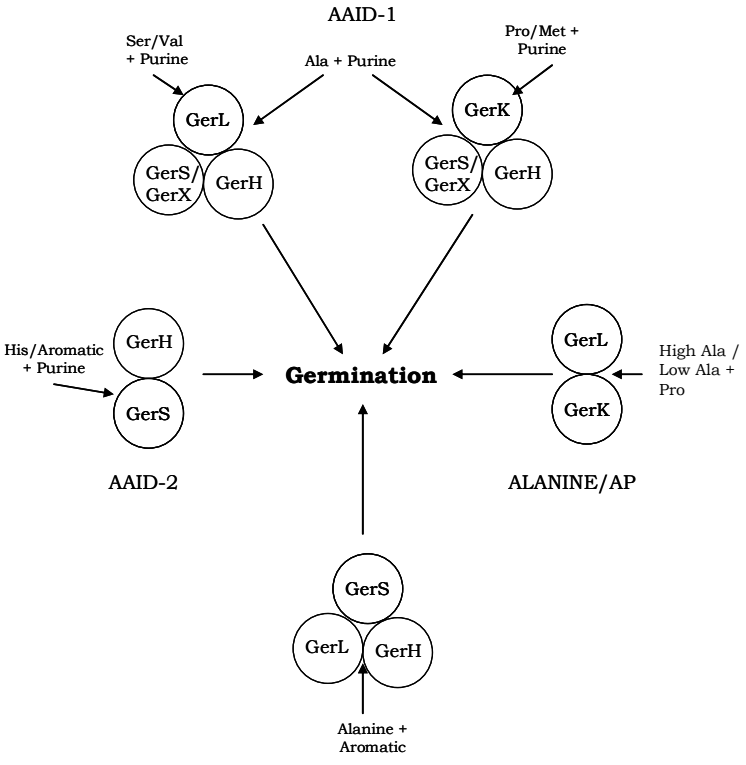


Figure 7. Germination pathways for *B. anthracis*. All the pathways need the combination of two or three Ger proteins to trigger the germination. Nucleoside dependent pathways are AAID-1/AAID-2; the remaining pathways require *L*-alanine alone or combined with other amino acids. Fisher, N., Hanna P. (2005) *J. Bacteriol.* 187 (23): 8055-8062.

1.5 Germination Inhibition

Germination is an essential step in *B. anthracis* for the development of the pathogenesis [27]. Inhibiting the germination is a potential target for anthrax prophylaxis. By understanding the mechanism and biochemistry of the germination, it is possible to alter the functioning of the proteins that are involved in the pathway. Blocking the germination is a way for preventing, not only anthrax, but any of the *Bacillus* and *Clostridium* associated diseases; germination inhibition in food-borne pathogens such as *B. cereus*[77], *B. megaterium* [78], *Clostridium botulinum* [79], *C. perfringens* [80], and *B. subtilis*[82] has been investigated previously.

Several ways for preventing germination have been studied. It has been shown that alkyl alcohols blocked the *in vitro* germination in *B. subtilis*, *B. cereus*, and *B.*

megaterium [82]. Many other ways of inhibition include protease inhibitors [83], ion-channel blockers [60], and sulphydryl reagents [84]. The most common way to block the germination is preventing the activation of the spore by nutrients or germinants [85].

D. Cortezzo and colleagues have studied the action several molecules that are able to inhibit the germination in *B. subtilis*. These compounds block the germination by nutrients before DPA release [85]. Such inhibitors can be grouped into two categories. The first group has compounds that inhibit *L*-alanine-triggered germination, but have no effect on *B. megaterium* or on germination with mixed nutrients; they respond to a specific nutrient receptor. In this division are many alkyl alcohols, N-ethylmaleimide, nifedipine, phenols, and potassium sorbate. The second group includes agents that prevent germination with multiple nutrients by either: 1) blocking multiple receptors, 2) Inhibiting an essential component in the signal transduction pathway between receptor activation to DPA release, or 3) obstructing the DPA release itself. Amiloride, $HgCl_2$, octanoic acid, octanol, phenylmethylsulphonylfluoride, quinine, tetracaine, tosyl-*L*-arginine methyl ester, and trifluoroperazine belong to this group. The other way of preventing germination by nutrients is by analogues of the nutrient themselves (analogues of germinants).

The current methods for preventing germination are grouped in the following brackets according to their specific action:

A) Agents that block activation of the receptor (analogues of germinants)

Detailed in section 1.5.1.

B) Enzyme inhibitors

Protease inhibitors and sulphydryl reagents are able to stop nutrient germination in several *Bacillus* species [83, 86-88]. Sulphydryl groups present in many enzymes involved in different steps of the spore germination. Most of these groups are located in membrane components. Bacteriostatic agents that can covalently modify sulphydryl can be considered germination inhibitors as well ($HgCl_2$, *N*-ethylmaleimide, cadmium acetate) [87]. Alkyl alcohols can

inactivate the lytic enzymes for the coat and cortex degradation [89]. Other enzyme inhibitors are calmodulin antagonists (trifluoroperazine). Inhibiting calmodulin can stop spore germination of *B. cereus* and *B. subtilis*, therefore this protein, triggered by Ca^{2+} as a second messenger, must be involved in the signal transduction in the spore [90].

C) Ion-channel blockers

Germination process requires water and cation transport as well as Ca^{2+}-DPA membrane translocators. Potential targets for inhibition are these membrane proteins. Ion channel blockers, such as amiloride, were shown to disable the germination in *B. subtilis* and *B. megaterium* [60, 85]. Unsaturated long-chain fatty acids have been tested with *Clostridium* sp., and may cause steric disorganization or inhibit substrate transport by altering the proton gradient and blocking the germination of the spores [91]. Nisin and subtilin are natural antibiotic peptides that also prevent germination in *C. perfring*ens and *Bacillus anthracis* [92, 93]. The mechanism on how nisin acts in spores is not clear, but it is correlated with a DPA release that can alter the dormancy of the spores. An analogue effect can be obtained in *C. difficile* with Ramoplanin, an antibiotic with nisin-like activity [94].

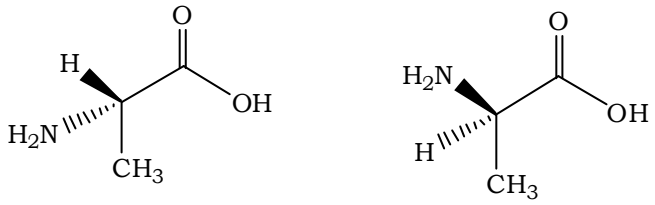
Table 3. Potential targets and agents that block germination of endospores

Ger receptor blockers	Enzyme inhibitors	Ion-channel blockers	Agents acting by non-specific mechanisms
6-thioguanosine	$HgCl_2$§	Nisin‡	Phenols
D-alanine	*N*-methylmaleimide§	Subtilin‡	Sodium hypophosphite
D-histidine	Cadmium acetate§	Ramoplanin‡	*N*-acetylmuramic acid
	Tosyl-*L*-arginine methyl ester†	Unsaturated long-chain fatty acids‡	Alkyl alcohols
	Leupeptine†	Amiloride**	
	Trifluoprezine*	Quinine**	
	Theophylline*	Tetracaine**	
	Potassium sorbate*	Procaine**	
	Sodium azide*		

* Calmodulin antagonists
** Na^+/K^+ and Ca^{2+} channels blockers
§ Sulphydryl reagents
† Protease inhibitors
‡ Agents disrupting membrane permeabilty

1.5.1 Inhibition by Analogues of Germinants

Investigations done by several research groups (P. Hanna , C. Guidi-Rontani, and L. Hornstra) have revealed the identity of different germination receptors in *Bacillus anthracis*, as well as their agonist (germinants). Ligand binding is the basis of the activation of the receptor and the initiation of the signaling pathways. Some molecules, however, may bind the receptors without triggering their activation. These molecules are known as 'antigerminants'. They have similar structure to the nutrients that trigger the germination but with modifications. Two types of germinants are found in *B. anthracis*: nucleosides and amino acid molecules. Thus, antigerminants will be derived either from a nucleoside, or from an amino acid. Antigerminants act as competitive inhibitors with the germinants: they will interact with the Ger receptors competing against the germinants for the binding site. The action of these molecules for preventing germination relies on the affinity to the receptor: the higher the affinity, the lower the concentration that is needed to bind the receptor and outweigh the germinant activation.



L-alanine *D*-alanine

Figure 8. Representation of the two enantiomers of alanine

The first anti-germinant that was found to prevent germination was the enantiomer of *L*-alanine: *D*-alanine (figure 8). *D*-alanine is not found in metazoans, but has been isolated from certain bacteria supernatant. In 1964, *D*-alanine was found to be an auto-inhibitor for the germination of *B. globigii* [95]. Previous works from Halvorson (1954) proposed a mechanism of germination auto-inhibition in bacteria by the enzyme alanine racemase[96]. H. Hu and colleagues demonstrated that *D*-alanine would hinder germination of *B. anthracis* spores. Addition of *D*-alanine to the culture medium resulted in a 75% inhibition of germination inside the macrophage cell line RAW 264.7. After adding *D*-histidine, the effect was improved, preventing germination in a 100%. They also showed that after adding L-alanine in higher concentrations than *D*-alanine, they were able to reverse this effect and resume the germination of the spores [97].

Subsequently, McKevitt and colleagues demonstrated that *D*-alanine also inhibits *B. anthracis* germination *in vitro*. *D*-alanine was effective even at concentrations 10-fold smaller than *L*-alanine (figure 8). M. McKevitt also found auto-inhibition by endogenous racemase activity in these bacteria [98].The mechanism of this racemase for the autoinhibition is not defined yet. The exosporium protein Alr has been associated with the racemase activity. A mutant strain *Δalr,* defective in this gene, germinates more efficiently in the presence of *L*-alanine than the regular Sterne strain [39].

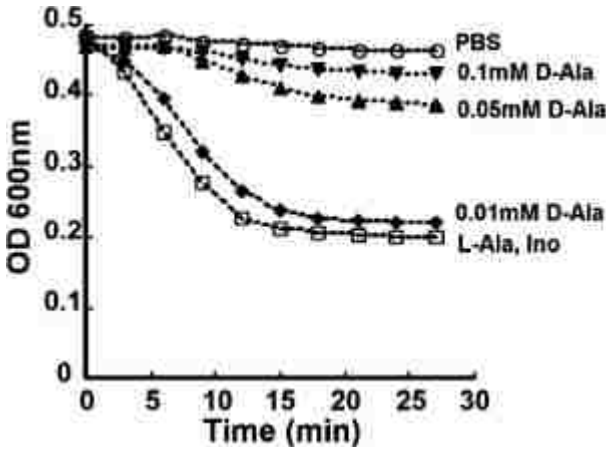


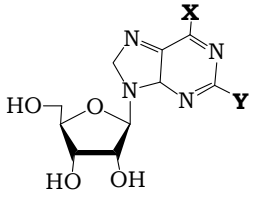
Figure 9. Studies of McKevitt et *al.* with *D*-Ala preventing spore germination *in vitro*. After 30 minuntes, there is no significant change in OD, after adding the highest concentration of *D*-Ala. McKevitt, M.T., et al. *Infect. Immun.* (2007) 75: 5726-5734.

The second group of anti-germinants corresponds to the nucleoside derivates. Based on the mutational analysis of Hanna there are four different pathways that cooperate synergistically for the germination of *B. anthracis* [68, 74]. The only germinant that is able to trigger germination by itself is *L*-alanine, but at un-physiologically high concentrations. After adding inosine, the most effective nucleoside germinant, the *in vitro* spore germination is optimal. It has been shown that germination pathways in both *B. cereus* and *B. anthracis* are synergic, so activating many pathways at the same time show an enhancement of the germination. *L*-alanine at high concentrations (10 mM) activates only one pathway via GerL/GerK. However, *L*-Ala and inosine together activate also the AAID-1 pathways, involving the two alternate set of receptors GerS/GerH/GerL and GerS/GerH/GerK. A nucleoside anti-germinant will block at least the three different pathways in which GerH is involved (see figure 7).

Abel-Santos and colleagues demonstrated the effect of different nucleoside analogues that inhibit the spore germination *in vitro* with *B. anthracis* Sterne strain. The analogues were screened from a large library of compounds derived from two germinants: inosine and guanosine. From this large number of analogues, only few compounds inhibited the germination *in vitro*. All the inhibitors have in common a

substitution in the position 6 of the purine base (derived from 6-thioinosine, 6-TI), or substitution at positions 2 and 6 (derived from 6-thioguanosine, 6-TG). The highest K_i corresponded to 6-methylmercaptopurine (6-MMPR) (table 4). Those that were able to inhibit the process of germination *in vitro* also were tested in macrophages RAW 264.7, but only 6-thioguanosine (6-TG) was able to protect the mammalian cells from *B. anthracis* killing [99].

Table 4. Nucleoside analogues that inhibited *B. anthracis* germination *in vitro*. Their corresponding inhibitions are also shown below. Akoachere, M. et *al* (2007) *J. Biol. Chem.* 282: 12112-12118



Substrate	X	Y	Ki (μM)
Inosine	OH	H	Germinant (Km = 270 μM)
6-thioinosine (6-TI)	SH	H	N/I
6-methylmercaptopurine riboside (6-MMPR)	SCH_3	H	31
6-thioguanosine (6-TG)	SH	NH_2	98
6-O-methylguanosine (6-OMG)	OCH_3	NH_2	34
6-chloroguanosine (6-CG)	Cl	NH_2	87
6-aminoguanosine (6-AG or 2-AA)	NH_2	NH_2	68
Xanthosine	OH	OH	777

The hypothesis made by Abel-Santos group is that the inhibitors are not actually anti-germinants, but very slow germinants, with a drastically lower efficiency than inosine. They have shown that 6-TG has a longer lasting effect than 6-MMPR in terms of OD decrease, even though that 6-MMPR has higher affinity than 6-TG (figure 10). However, even with 6-TG, there was a residual background of dead cells due to the ability of 6-TG to work as a very slow germinant.

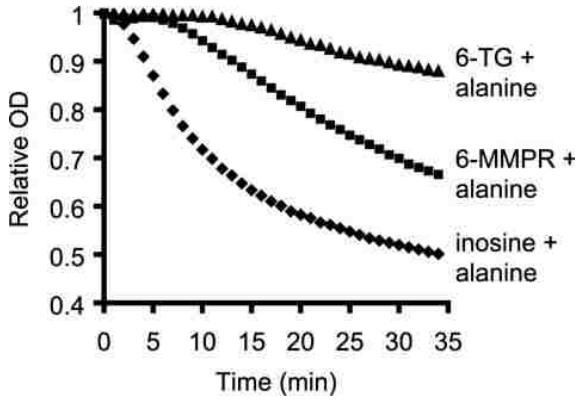


Figure 10. Effect on the *B. anthracis* germination inhibition *in vitro* by 6-TG and 6-MMPR (obtained by Abel-Santos et al). Both compounds acted as slow germinants in comparison with inosine, but 6-TG rate was considerably slower than 6-MMPR. Akoachere, M. et *al* (2007) *J. Biol. Chem.* 282: 12112-12118.

1.5.2 Protection of Macrophages by Antigerminants

Inhibiting the germination is a promising strategy for anthrax prophylaxis. Unfortunately the research done *in vivo* has not been as successful as expected. The low permeability of the spore membranes is one of the facts that may impede the use of anti-germinants as a potential target. Nevertheless, spores need to be sensitive to germination stimuli. It was mentioned on sub-chapter 1.4.1 that *B. subtilis* gene *gerP* was associated with a protein that is likely to enhance the diffusion of germinants through the outer layers of the spore. Besides the permeability of the spore there is another hindrance: changing from *in vitro* to *in vivo* conditions. What happens inside the cell is completely different to that in controlled conditions. The most notable aspect is the multiple combinations of nutrients present in the phagosome that may act as germinants. The different pH, ions, peptides and enzymes present in the cell can alter the kinetics of the process.

One of the promising aspects of the antigermination therapies is that they block a crucial step for the anthrax infection. It might be the best short-term prophylaxis, in combination with antibiotics to prevent the outbreak after inhaling or ingesting spores. Nowadays, there are only two efficient anti-germinants that protect murine

macrophages from *B. anthracis* mediated killing: *D*-Ala and 6-TG. Attempts for stopping the disease in mice have been unsuccessful so far.

At least one germination receptor is fully required for the germination of *B. anthracis* inside the macrophages: GerH [100]. Plasmid receptor GerX, located in virulence plasmid pOX1 can improve this germination significantly [69]. It has been demonstrated that spores that are not able to germinate efficiently (*ΔgerH*) lose their viability and are killed inside the phagolysosome of macrophages RAW 264.7 [101]. Spore that pre-germinate outside the phagolysosome are also readily killed [97]. These experiments illustrate the importance of an optimal germination, with the contribution of all germination receptors, for the spore survival inside the macrophages. The rapid maturation of the bacteria, the synthesis of toxins, and the expression of the atxA-activated membrane damaging agent are crucial to outdo the action of the phagolysosome.

H. Hu and colleagues have used a combination of two antigerminants to provide full protection to macrophages. The relevance of GerH for germination is a reason to consider this receptor as an important antigerminant target. This research group revealed that combining *D*-alanine and *D*-histidine blocks the main pathways for germination of *B. anthracis* (Alanine, AAID-1, AAID-2) resulting in 100% survival of the macrophages and the subsequent inactivation and killing of the spores by the phagosome [97, 101].

On the other hand, nucleoside anti-germinants are being studied by E. Abel-Santos and colleagues for the potential protection of macrophages. Previous work has shown that 6-thioguanosine (6-TG) gives almost 100% of protection after six hours of infection. Fluorescence microscopy revealed that cells infected with *B. anthracis* Sterne strain were killed starting at 3 hours, whereas cells treated with 6-TG were viable, even after 7 hours (figure 11). Interestingly, concentrations of 6-TG required for protection of macrophages were 100-fold smaller than those for inhibiting germination *in vitro* [99]. Further research is being conducted by this group with a broader spectrum of analogues for screening.

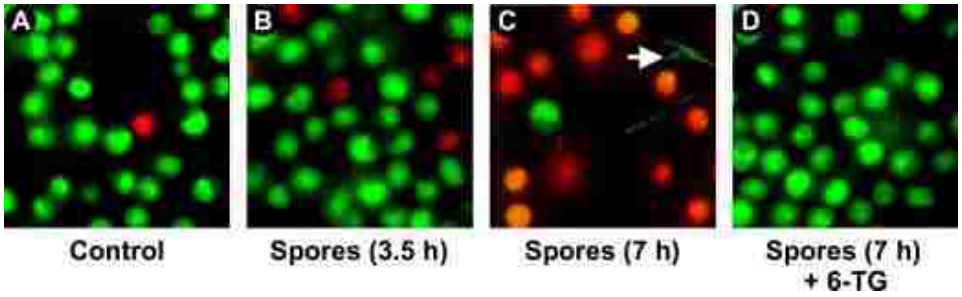


Figure 11. Prevention of cell killing mediated by *B. anthracis* spores by 6-TG, revealed by fluorescence microscopy. Cells were stain with Syto-13 (Green) and propidium iodide (Red). The last one is impermeable to membrane, and may only stain by binding to DNA from necrotic or apoptotic cells. These figures obtained by Abel-Santos et *al* illustrate that RAW 264.7 macrophages treated with remained viable after 7 hours, whereas non-treated macrophages were being killed within 3 and half hours. Akoachere, M. et *al* (2007) *J. Biol. Chem.* 282: 12112-12118.

1.6 Hypothesis and Research Questions

The recent findings suggest that nucleosides in combination with an amino acid are essential to trigger the germination of *B. anthracis* endospores [68, 74]. These compounds bind and activate protein receptors located in the inner membrane of the spore [54]. Among the nucleosides, inosine is the best germinant [74, 99]. 6-Thioguanosine (6-TG) is a nucleoside analogue that protects macrophages from killing by its anti-germinant effect on the spores [99]. Since the nucleoside receptor GerH is necessary for germination *in vivo* [97], we hypothesize that inosine analogues derived from 6-TG that interfere with the binding of nucleosides to the receptors will prevent germination of the spores and protect macrophages from an imminent necrosis mediated by the bacterial toxins.

The parameters for the efficacy of a good nucleoside anti-germinant were one of the matters of concern in the present study. The chemical and functional identity of the substituent that enhances the inhibitory properties of the compounds needed to be determined: certain chemical groups, substituting specific positions on the molecule, may interfere with the germination; some of them will not show any effect; while others could even act triggering the germination.

Another intriguing aspect of this research was answering why some anti-germinants are efficient *in vitro*, but they are not able to inhibit germination inside the macrophages. Binding affinity, defined by the inhibition constant (K_i), would not necessarily correlate with the efficacy in protecting cells. In addition, that binding affinity obtained *in vitro* can be different than the one of inside mammalian cells. Some molecules are not permeable to cell membranes, but even if they are permeable they must penetrate the thick outer layers of the spore and reach the inner membrane, where the receptors are. There are many more aspects that vary from a buffer to cells: acid pH, oxidative agents, enzymes, and many more nutrients that are competing with the anti-germinant.

Finally, it was necessary to clarify a possible interaction of two anti-germinants of different class, each one interacting with a different receptor. Previous work showed that there are four different germination pathways that can be activated in *B. anthracis*. The activation of two or more pathways together has a cooperative effect for the germination of the spore. Correspondingly, the addition of a combination of two anti-germinants targeting two different pathways must enhance the inhibitory effect.

1.7 Specific Aims of the Study

Based on the potential benefit that anti-germinants may represent as a novel target for anthrax prophylaxis, our study focused on the screening of an array of 43 different compounds derived from 6-thioguanosine (6-TG), one of the drugs that prevented cell killing by *B. anthracis.* The present work allowed the identification of different purine nucleoside derivatives that prevent the germination in macrophages. For each inhibitor, their respective half-maximal inhibitory concentration (IC_{50}) was obtained. These results were corroborated and compared by testing the rate of germination *in vitro*.

The effect on the germination by the different analogues was observed directly, through phase contrast and fluorescence microscopy, and measured by cell viability

assays. The data was obtained in intervals of thirty minutes to be able to see the process of inhibition through time. The results obtained contributed to the understanding on the kinetics of the spore mediated killing.

By analyzing structure activity relationships of these inhibitors it was possible to identify the parts of the molecule that are implicated with the receptor binding, and the nature of those chemical substitutes that interfere with the receptor activation.

CHAPTER 2

MATERIAL AND METHODOLOGY

2.1 Bacterial Strain and Growth

The bacterial strain used for the present studies was *Bacillus anthracis* ΔSterne $34F_2$. The strain was provided by Dr. Arturo Casadevall from the Albert Einstein College of Medicine, NY. These bacteria carry one of the plasmids of virulence pXO1, but lacks pXO2, for the synthesis of the poly-*D*-glutamic acid capsule. Plasmid pXO1 contains the genes for the expression of the three major toxins: PA (protective antigen), EF (calmodulin-dependent andenylate cyclase), and LF (Zn-metalloprotease). It also contains the gene for the germination operon *gerX*. Thus, the strain carries all the possible germination operons that have been identified (table 2).

The lack of capsule makes the vegetative bacteria vulnerable to the immune system response, with a subsequent killing led by macrophages, making this strain non-pathogenic to mammals. It is therefore considered a non-virulent, but toxigenic strain requiring a bio-safety level 2 for its shipping, manipulation, and use. Table 5 shows a comparison of the differences between the strain that is used and the pathogenic Ames Florida that was used for the 2001 attacks. For the latter one, its use is authorized only in certain institutions in the USA under BSL-3 [(6)].

The bacteria can be grown in aerobic conditions, in Luria-Bertani (LB) broth. The initial culture may be obtained by inoculating spores or from a frozen stock. The optimal growth is achieved at 37°C (mesophilic micro-organism). From this initial culture in LB broth, sub-cultures can be done in solid media, such as nutrient agar.

Table 5. Differences between Sterne and the virulent Ames strain of *Bacillus anthracis.*

Properties	Sterne $34F_2$	Ames Florida (A2012)
Virulence Plasmids	pOX1+/pOX2-	pOX1+/pOX2+
Capsule of poly-γ-*D*-glutamic acid	No	Yes
Expression of toxins (PA, EF, LF)	Yes	Yes
Toxin-mediated necrosis of macrophages	Yes	Yes
Pathogenesis and proliferation inside the host organism	No[†]	Yes
Penicillin resistant	Yes	Yes
AVA vaccine resistant	No	Yes

[†] There can be some exceptions of Sterne-related infections when patients are immuno-compromised or spores that make contact with a pre-existing wound. It is unlikely to acquire infection through oral or inhalational route. No human disease due to the Sterne strain has been reported [(6)].

2.2 Spore Preparation

For sporulation, the bacteria were incubated at 37°C for 5-7 days in a modified nutrient agar medium, containing the following minerals: KCl (0.1%), $MgSO_4$ (0.012%), $Ca(NO_3)_2$ (1 mM), $MnCl_2$ (0.1 μM), $FeSO_4$ (1 nM). The pH of the medium was adjusted to 7.6. Sporulation was confirmed through Schaeffer-Fulton[8] staining, before harvesting.

Plates were harvested by suspension of cells in double-distilled ice-cold water (ddH_2O). After three washing steps, endospores were separated through centrifugation by density using Histodenz™ (Sigma-Aldrich) at a 20 to 50% gradient (figure 12).

The spore pellet must be resuspended in ddH_20, and washed five more times, in order to remove cellular remnants and get a better degree of purity in the spores. After washing, spores were stored in aliquots of 0.5 mL, and kept at 4°C.

[8]Schaeffer-Fulton spore stain – A differential staining technique that uses malachite green (50 g/L) to stain spores, and a counter-stain of safranin or safarin (5 g/L) for vegetative cells

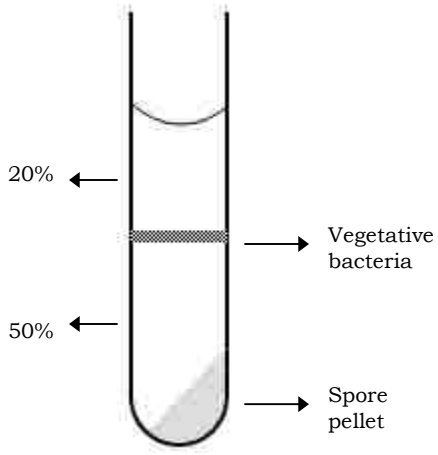


Figure 12. Fractions obtained after centrifugation with Histodenz. Vegetative bacteria remain at the inter-phase of the two densities, whereas spores precipitate, forming a thick pellet.

2.3 Analysis of the Spore Germination *In Vitro*.

The first step that must be done in any study of spore germination, before using the spores, is the heat activation at 70°C for 30 minutes. This heat activation has a dual purpose: 1) killing the non-sporulating organisms or vegetative cells that may be present in the sample and 2) heat-shocking the spores to enhance the predisposition of spores for germination. Sub-lethal heat seems to increase the ability of the spore to germinate [32]. The molecular basis of the heat-activation remains unknown.

Following the heat-activations, spores are washed, and resuspended in germination buffer (50 mM Tris-HCl; 10 mM NaCl; pH 7.5). This buffer provides the optimal pH and salt concentration for the germination of *B. anthracis* endospores. Spores are diluted in germination buffer in order to get an optical density of 1.00 (equivalent to a concentration of 10^8 spores/mL).

The germination of the spores is monitored by a decrease in optical density. The previous chapter addresses the property of 'whitening or refractivity' of the endospores given by the coat proteins. This property distinguishes spores from vegetative cells and

can be monitored with the absorption of light at 580 nm. The absorbance was registered by Biomate 5 spectrophotometer from ThermoSpectronics®.

In some cases minimal amounts of nutrients coming from cellular debris traces, may act as germinants, triggering the spore germination. A control must be run to see if there is no auto-germination: monitoring a decrease in OD_{580} for 15 minutes and variation should be less than ±0.05. If fluctuations exceed this interval, there is an auto-germination background that may affect the further studies.

After ensuring that no auto-germination is present, it must be confirmed that spores are able to germinate when germinants are added. For this purpose *B. anthracis* germination requires one nucleoside analogue and an amino acid acting as co-germinants. In the present studies we have used inosine at 0.25 mM and alanine at 0.04 mM to the spore suspension in germination buffer, in order to activate the spore germination *in vitro*. Healthy spores were able to germinate almost fully within the range of 15 minutes (figure 13). Germination is complete when it reaches the minimum possible optical density of the suspension, in the range of 0.45 to 0.48.

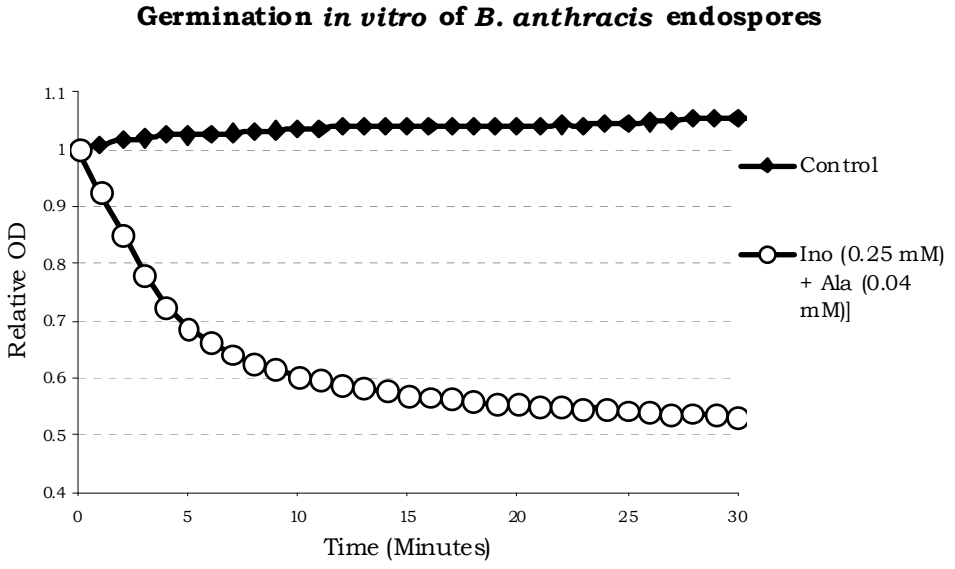


Figure 13. Decrease in optical density observed after adding inosine (Ino) and *L*-alanine (Ala) to the spore suspension.

The actual OD obtained is transformed to relative OD; a magnitude defined by the OD obtained at time 0 divided by the OD read at certain time. Relative OD is a measure that allows a better comparison between two different data sets, since the initial OD may vary considerably from one experiment to another.

For the analysis of nucleoside analogues on the germination *in vitro*, a kinetic assay was performed using Greiner Bio-one® flat transparent 96-well plates. Each well was filied up to a final volume of 0.2 mL of spore suspension. Nucleoside analogues were added from stock solutions to the desired concentration in volumes not higher than 10 μL. Analogues were incubated in the suspension at room temperature for 15 minutes. Subsequently, germinants (inosine and alanine) were added. Data was taken each minute for 45 to 60 minutes. To ensure accuracy in the results, samples were repeated in triplicates. Readings were done by the multi-well plate reader Tecan Infinite M200®. Germination was confirmed by light microscopy using the Schaeffer-Fulton endospore staining technique (figures 25 and 75).

2.4 Cell Line

The mammalian cell line used in the further studies is the J774a.1 murine monocyte/macrophages. The cell line was a gift from Dr. Jürgen Brojatsch, at the Albert Einstein College of Medicine, NY. The cell line is originated in the *Mus musculus* strain BALB/cN, from a reticulum cell sarcoma. The properties of this cell line (IL-1β production, complement receptor, and IgG receptor) enhance the antibody-dependent phagocytosis of the macrophages [102]. The endospore intake is therefore efficient in this cell line.

Initially we tested the cell line RAW 264.7, also murine macrophages. However the spore intake by RAW 264.7 was not as efficient as in J774a.1; thus, the germination process was slower and the amount of spores required in terms of multiplicity of infection (m.o.i.) was higher.

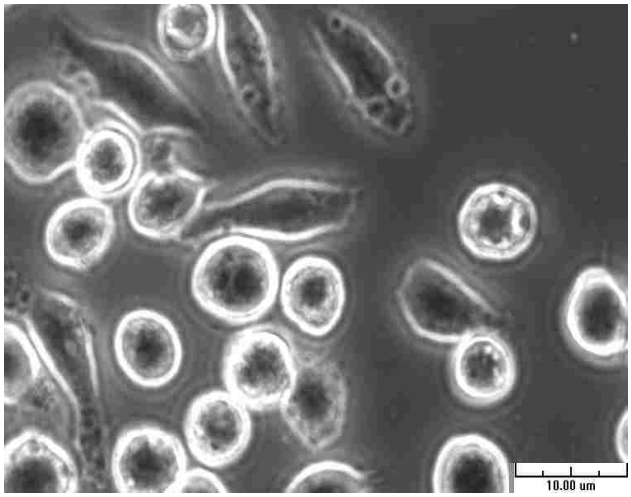


Figure 14. Photography at phase contrast microscopy of the cell line J774a.1

2.5 Medium and Growth Conditions for Mammalian Cells

For culturing the cell line J774a.1, the medium used was the Dulbecco Modified Eagle's medium (DMEM) without *L*-glutamine, obtained from Cellgro® Mediatech Inc. The medium was supplemented with 10% of dialyzed fetal bovine serum from HyClone®, GlutaMAX™ [9] from Invitrogen™, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The last two were obtained from Cellgro®.

Cells are incubated in tissue culture treated Petri dishes of 10 cm of diameter. The volume of media added to each plate was 10 mL. Plates are stored in an incubator, at 37°C, and a humidified atmosphere with 5% CO_2.

When cells reached a confluence[10] from 80-90%, sub-culturing is required for the continuity of the cell line from the primary culture. For sub-culturing, cells are detached from the plate using phosphate buffered saline solution (PBS) without calcium and magnesium (Cellgro®), supplemented with 5mM of EDTA. Cells are counted using a

[9] GlutaMAX is a substitute of *L*-glutamine that does not degrade in cell culture, and does not form ammonia as a byproduct. It improves cell viability and growth, and delays the phase of senescence in the cells (103).

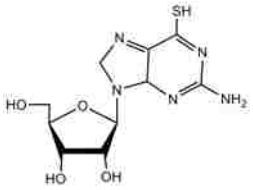
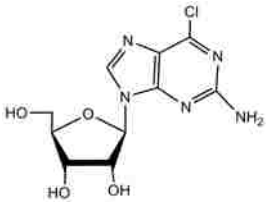
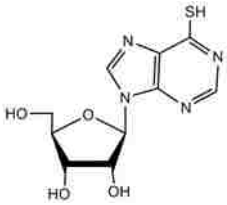
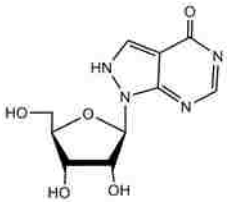
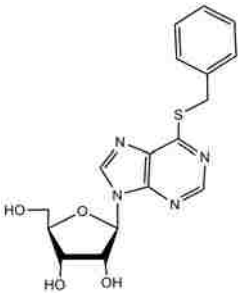
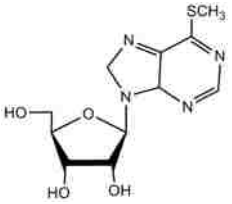
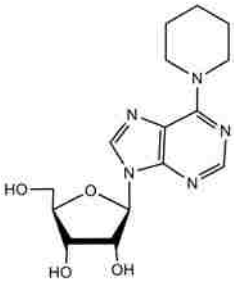
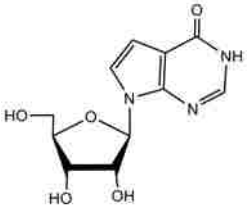
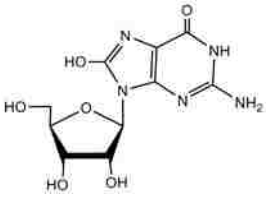
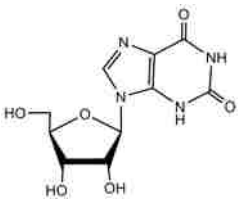
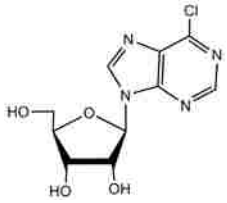
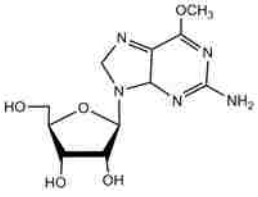
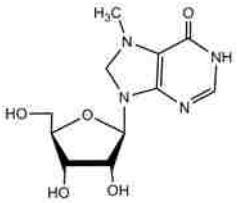
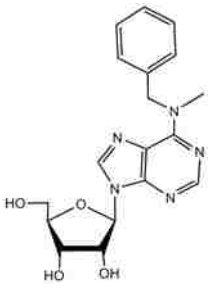
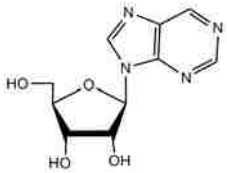
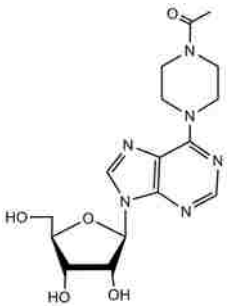
[10] Confluence refers to the extension of the cell monolayer on the plate. A confluence of 100% is reached when the monolayer covers the full area of the plate. After confluence reaches 90%, macrophages J774a.1 stop the exponential growth, entering the plateau phase.

hemocytometer. The cell suspension is centrifuged and resuspended in fresh medium. Sub-cultures are seeded to a final concentration between 50,000-100,000 cells/mL. The following sub-culture takes places within the next 4 to 5 days. After the 8th subculture, the health of the cell line decays, so a new primary culture is required directly from the frozen stock. Frozen stocks must be kept in liquid nitrogen at -180°C.

2.6 Nucleoside Analogues

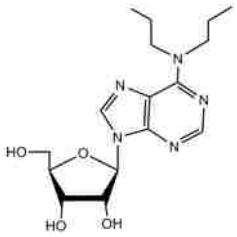
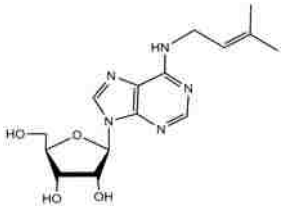
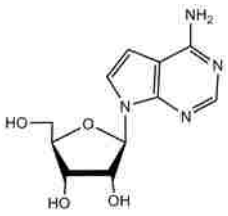
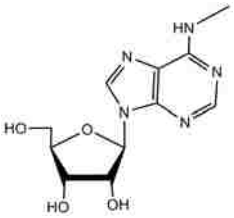
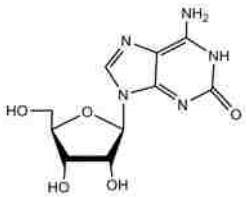
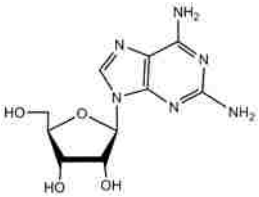
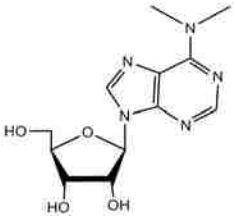
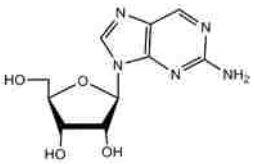
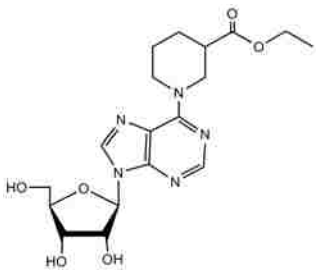
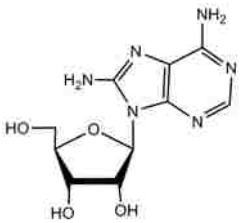
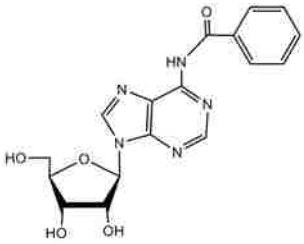
The different nucleoside analogues tested in the present study were purchased either from Sigma-Aldrich Corporation (Saint Louis, MO) or from Berry & Associates (Brea, CA); or synthesized by Dr.Kyungae Lee from the National Screening Laboratory in Biodefense (NSRB). The analogues used are grouped in three categories according to their structure: a) nucleoside analogues derived from inosine and guanosine, b) purine bases derived from the nucleoside analogues, and c) analogues containing only the six-member heterocyclic ring of the purine. The list of analogues is given in tables 6, 7, and 8.

Table 6. Nucleoside analogues derived from inosine and guanosine

 6-thioguanosine (6-TG)	 6-chloroguanosine (6-CG)	 6-thioinosine (6-TI)	 Allopurinol riboside (APR)
 6-benzylthioinosine † (6-BTI)	 6-methylmercaptopurine riboside (6-MMPR)	 N^6-cyclohexyladenosine (CHA)	 Immucillin H (IH)
 8-hydroxyguanosine (8-HG)	 Xanthosine (X)	 6-chloropurine riboside (6-CPR)	 6-*O*-methylguanosine (6-OMG)
 7-methylinosine (7-MI)	 6-*N*-benzylmethyladenosine † (BMA)	 Nebularine (Neb)	 1-(4-(9-(β)-ribofuranosyl)-9H-purin-6-yl)piperazin-1-yl)ethanone † (PPER)

† Compound synthesized by Dr. Kyungae Lee from the NSRB.

Table 6. (Coninued from page 50)

			
N^6,N^6-dipropyladenosine† (6-DPA)	6-(γ,γ-dimethylallyl amino)purine riboside (6-DMAA)	Tubercidin (Tu)	6-methylaminopurine riboside (6-MAPR)
			
Isoguanosine (IG)	2-aminoadenosine (2-AA)	6-*N*-dimethyladenosine † (6-DMA)	2-aminopurine riboside (2-APR)
			
6-*N*-((3-ethylacetyl)cyclohexyl) adenosine † (6-ECA)	8-aminoadenosine (8-AA)	6-*N*-benzoyladenosine (6-NBA)	

† Compound synthesized by Dr. Kyungae Lee from the NSRB.

Table 7. Purine bases derived from nucleoside analogues

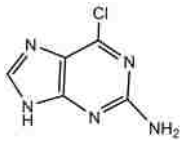
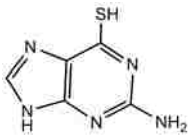
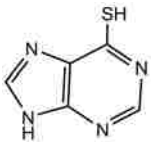
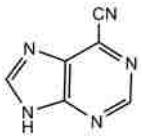
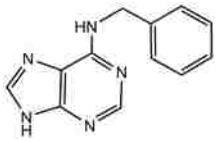
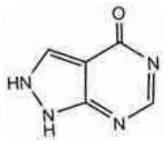
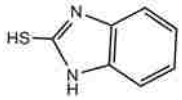
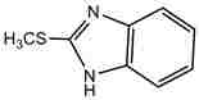
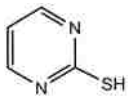
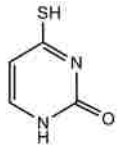
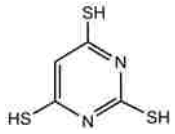
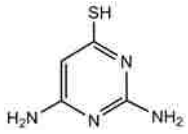
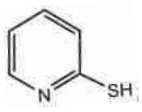
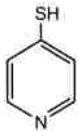
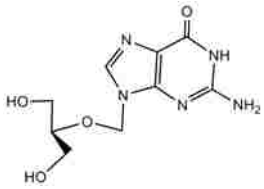
 6-chloroguanine (6-Cg)	 6-thioguanine (6-Tg)	 6-mercaptopurine (6-Mp)	 6-cyanopurine (6-CyP)
 6-N-benzylaminopurine (6-BAP)	 Allopurinol (Ap)	 2-mercaptobenzimidazole (2-MBI)	 2-methylmercapto-benzimidazole (2-MMBI)

Table 8. Analogues containing the six-member ring from the purine and other analogues of non-specific category.

 2-mercaptopyrimidine (2-MPm)	 2-thiouracil (2-TU)	 Trithiocyanuric acid (TTCA)	 2,4-diamino-6-mercaptopyrimidine (DAMPm)
 2-mercaptopyridine (2-MPy)	 4-mercaptopyridine (4-MPy)	 Gancyclovir (GCv)	 β-mercaptoethanol (BME)

2.7 Spore Killing Assay

The purpose of these assays is to assess the effect of the analogues in protecting macrophages from necrosis. The cell line J774a.1 will be treated with the respective compounds and then infected with endospores of *B. anthracis*. Cell necrosis is correlated with the germination of the spores inside the macrophages, and the production of the membrane-damaging toxins EF and LF. The more cell viability is observed, the better the protective effect of the anti-germinant has been. Cell viability will be monitored by direct observation through phase-contrast and fluorescent microscopies, and by quantification of fluorescence emission (sub-chapter 2.9).

Cells will be harvested after the culture reaches a confluence of 80-90% (beginning of the lag phase). For this assay is important to ensure that at least 95% of the cells are viable. Otherwise, the background of dead cells may affect the results. For this reason is important to count by direct observation through the hemocytometer, counting both viable and non-viable cells (Trypan-blue positive[11]). If the percentage of non-viable cells is above 5%, is recommendable not to perform the assay with these cells. In order to get a good percentage of cell viability, cells must be harvested just before reaching the maximum confluence (a considerable amount of cells enter senescence during lag phase), washed at least two times with PBS to remove floating dead cells; and detached with the least harsh methods (pipetting up and down the suspension instead of cell-scraping).

After counting, cells will be centrifuged and resuspended in new medium to a final concentration of 1 x 10^6 cells/mL. The medium used for this assay, must not contain antibiotics (streptomycin and penicillin), and must be free of any serum. Serum may induce germination outside the macrophages. The cell suspension is added in volumes of 80 μL to each well in a Greiner Bio-one® 96-well plate. Samples are repeated in triplicates. After incubating for 1 hour at 37°C and 5% CO_2, the corresponding

[11] Trypan blue is a stain used for animal cells, that selectively colours dead cells blue, while the living cells remain uncolored.

treatment is added to cells in volumes of 10 μL. Cells are now incubated for 30 minutes with the treatment. Subsequently spores are added in volumes of 10 μL, to get an m.o.i. of 5. The final volume of sample in each well should be 100 μL.

80 minutes after the inoculation of the spores, cells are washed two times with Hanks balanced saline solution (HBSS). After washing each sample is replaced with new medium containing the corresponding treatment. The purpose of this wash is to remove the majority of spores that were not taken by the macrophages. Such spores may germinate in time, giving a background of extracellular germination, and affecting the accuracy of the assay. After washing, the plate must be put inside the incubator again, until the time for measurements comes. Normally data acquisition starts at 3 hours after infection, which is the time when cell necrosis begins.

2.8 Direct Quantification of Cell Viability

Measuring the amount of living cells can be done by several techniques. Nowadays, the most efficient methods for quantification of cell viability are based on fluorescent or luminescent dyes. Our method of quantification is based on the fluorescence emission of propidium iodide (PI), which stains specifically necrotic cells. (See sub-chapter 2.9). In order to assess the accuracy of the fluorescence quantification, we have performed cell counts by direct observation at phase contrast microscopy.

The first step for counting is selecting a specific range of the field of vision. The field of view is based on observation at 40X lens. The area of field must have a number of 30 to 50 cells, to be representative statistically. In our case, we have selected to count 1/16 of field of view at 40X (figure 15). We count three random areas for each sample to get data in triplicates.

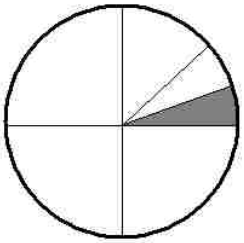
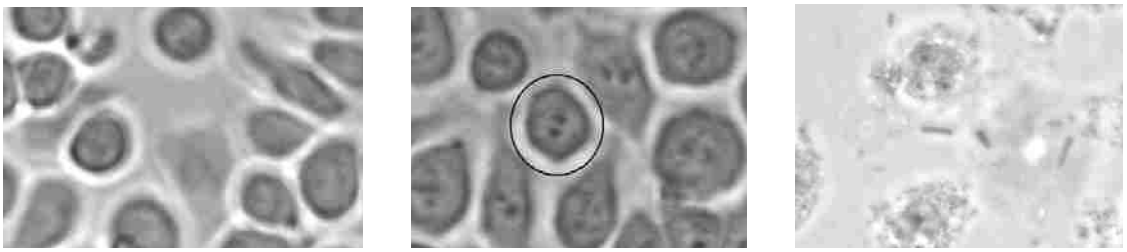


Figure 15. Range selected for counting at 40X field (highlighted in gray).

To distinguish necrotic from living cells the methodology performed is based on counting nuclei. Necrotic cells have a nucleus with a disrupted membrane. Membrane integrity can be observed through bi-refringence[12]. Cells that show bi-refringence are considered viable (figures 14 and 16).



Healthy Macrophages

Macrophages with phagosome containing endospores

Necrotic Macrophages

Figure 16. Photography of the different cellular stages of macrophages after infection with spores.

Counts have been done hourly after infecting with *B. anthracis* endospores. The data obtained after counting is transformed to percentages of dead cells vs. living cells. Results are shown in the next chapter.

[12] Bi-refringence or double-refraction is the property of deviate an incident ray of light into two polarized rays, perpendicular to each other. This can be observed in cell membranes at phase contrast microscopy; in an specific focus of the lens the membranes appear to be particularly bright.

2.9 Fluorescence Quantification of Cell Viability

For assessing the cytopathic effects after performing the spore killing assay the method that was chosen was by fluorescent staining with propidium iodide (PI) [104]. Propidium iodide is an intercalating agent and a fluorescent molecule. It binds DNA by intercalating between the bases. Once is bound to nucleic acid its fluorescence is enhanced by near 30-fold. PI is impermeable to cell membranes and may not reach the nuclear material in viable cells. Thus, the fluorescent stain is specific only for necrotic or apoptotic cells with damaged membranes. When bound to DNA, PI can be excited at a maximum of 535 nm wavelength, emitting at the peak wavelength of 617 nm.

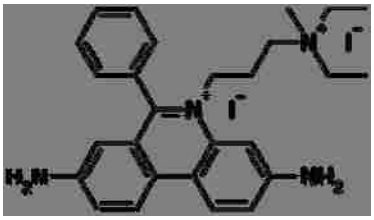


Figure 17. Molecular structure of propidium iodide.

A 10X solution of PI was prepared by dissolving the compound in phosphate-buffered saline solution. Just before taking the read-outs, PI was added to each well to a final concentration of 20 μM. The 96-well plate was shaken at dark for 10 minutes. Subsequently, readings were done in a TECAN™ Infinite® M200 cuvette/multi-well plate reader (with monochromator). After the respective data was acquired, the plate was put to the incubator again (37°C at 5% CO_2) until the next reading. Readings were normally taken every 30 minutes within 3 to 7 hours after infecting macrophages with the endospores. The obtained data in fluorescence units, was transformed into relative intensity. Relative intensity (RI) is magnitude used for standardizing all read-outs, since the background fluorescence may vary from one experiment to another. RI is defined as:

$$RI = \frac{FU_x - FU_c}{FU_c}$$

FU_x is the specific value of each well, measured in fluorescence units, and FU_c is the lowest possible value of the control (viable cells free of spores) or "zero value". By using RI it is possible to remove the residual fluorescence values and see the specific increase of dead cells in each well.

2.10 Fluorescence Microscopy

A direct observation on the cytopathic effect of was done by using the microscope Olympus BX51. J774a.1 cells were seeded to Lab-Tek® glass chambered cover slides. The cell killing assay was performed with the same procedure as in sub-chapter 2.7, but with a final volume of 500 μL of cell suspension in each chamber of the microscopy slide. Each slide carries four chambers. One chamber was the control of cells free of spores, the second had the infected cells without any treatment, and the remaining two were used to see the effect of two different treatments on cells: 6-thioguanosine and 6-thioguanine.

Two repeated slides were incubated, for two observations at 3 and 6 hours after infection. Before observing at the microscope, cell media was removed from each chamber, and cells were stained with PI at 20 μM concentration, and DAPI [13] (4',6-diamidino-2-phenylindole) at a 5 μM concentration. The latter one is used as a counter-stain for viable cells.

[13] DAPI is a commonly used dye in fluorescent microscopy. It binds DNA absorbing wavelength at a maximum 358 nm, and the maximum of emission is at 461 nm. It is permeable to cell membranes, staining either viable or non-viable cells.

2.11 Half-maximal Inhibitory Concentration of the Protective Effect in Cells

An IC_{50} was obtained for each of the nucleosides that showed a protective effect in the first screening. A spore killing assay was performed as is shown in sub-chapter 2.7, by adding the nucleoside analogues at varying concentrations (0.1, 0.5, 1, 5, 10, 50, 100, and 500 μM). Cell viability was measured by fluorescence readings after staining with PI (sub-chapter 2.9). The first reading was taken approximately 3.5 to 4 hours after infecting; time when necrotic cells start to appear. Successive readings were taken every 30 minutes until 6 to 6.5 hours. All of assays were carried out in triplicates.

An arbitrary time point was selected before reaching saturation[14]. Values given in RI were used for plotting against the decimal logarithm (log) of the concentrations. The data in semi-log plots was fitted into a non-linear regression of four-parameter logistic curve, using the software SigmaPlot v.11. The IC_{50} were automatically obtained by the regression analysis performed by the software. All the R squared (R^2)[15] values given for the different regressions were over 0.9.

2.12 Half-maximal Inhibitory Concentration of the Germination Inhibition *in vitro*

Assays of germination *in vitro* were performed in order to corroborate the results of the protective effect on cells: blocking pathogenesis on cells is directly related with inhibiting the germination of the spores *in vitro*. For obtaining IC_{50} of the inhibition *in vitro*, purified spores were diluted in germination buffer, and incubated with the nucleoside analogues for 15 minutes. The concentration selected for the analogues was

[14] At a given time, usually after 6 hours of infection, all cells will eventually become necrotic. The protective effect of the anti-germinant slows down the kinetic of the germination but does not stop it.

[15] R^2 is the square of the Pearson's correlation coefficient *r,* which is a statistical index that determines correlation between the data and the equation that fits the regression. "*r*" is defined by the quotient of the covariance of the two variables divided by the covariance of each dependent and independent variable for separate. R^2 has values between 0 and 1, being 1 the optimal fit for the regression.

in a range between 5 μM to 10 mM. All samples were repeated in triplicates. Germination was monitored using the TECAN™ Infinite® M200, reading absorbance at 580 nm, each minute for 45 minutes. An arbitrary time point was selected. Values of relative OD were plotted against the decimal logarithm of the respective concentrations. The data was fitted into a non-linear regression of four-parameter sigmoidal curve, using SigmaPlot v.11. The half-maximal inhibitory concentrations (IC_{50}) were given in the regression analysis. All R^2 values obtained were above 0.9.

CHAPTER 3

RESULTS

3.1 Protection of J774a.1 by 6-thioguanosine from *B. anthracis* Mediated Necrosis

To validate effectiveness of 6-TG on previous works done by Akoachere et *al* (2006) [99], studies on macrophages and *in vitro* were carried out. The first assessment was through direct observation of the necrosis by using phase contrast microscopy. Subsequently cell viability was measured by quantification of PI fluorescence emission, and observed through fluorescence microscopy. With these results we show that 6-TG efficiently protects the macrophage cell line J774a.1 from killing by *B. anthracis*.

3.1.1 Direct Counting

A cell killing assay was performed following the methods stated in sub-chapter 2.7, with the exception that there was no wash with HBSS at 80 minutes. For this assay, cells were treated with three different concentrations of 6-TG : 1, 10, and 100 μM. Cells were counted hourly 1-6 hours after infecting with the spores. Both viable and necrotic cells were counted in three different fields, and the average percentage of each was obtained. Only the highest concentration of 6-TG was showed a significant protection on the cells after six hours (figure 18).

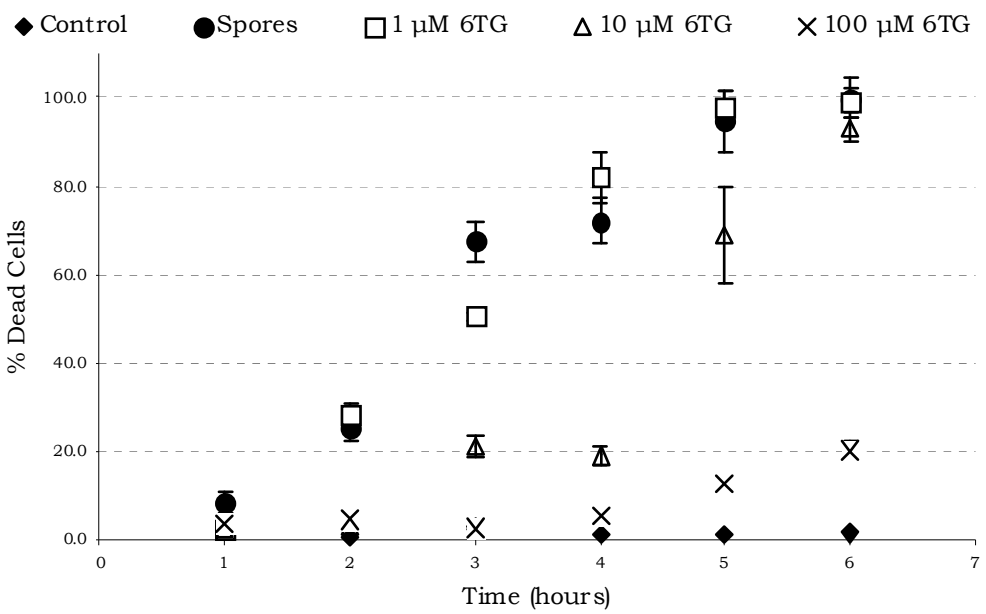


Figure 18. Percentage of necrotic cells after infecting with *B. anthracis*. J774a.1 cells were infected with *B. anthracis* endospores at an m.o.i. of 10. Necrosis started to be visible within the 1-2 hours after infecting cells. Cells that were uninfected (control) showed almost 100% viability throughout the whole experiment. 6-TG protected cells from necrosis at concentrations of 10 and 100 µM within the range of 2-4 hours. After 5 hours only the highest concentration showed full protection.

3.1.2 Quantification by Fluorescence

Another assay like in the previous sub-chapter was performed, but this time cell necrosis was monitored by the propidium iodide exclusion assay (sub-chapter 2.9). Results were similar to those obtained by direct cell counting (figure 19).

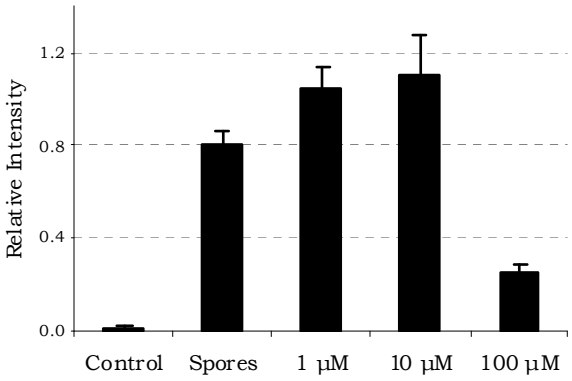


Figure 19. Fluorescent quantification on J774a.1 cell killing with *B. anthracis* at 6 hours. Necrosis was monitored by PI-DNA fluorescence. The highest concentration of 6-TG was the only to show protection after 6 hours.

In the previous figure we have shown that the PI-DNA fluorescence detection correlates with direct counting results; thus, it is an accurate procedure for measuring the amount of cell killing, and the method may be used in the further experiments.

After infecting our cell line, observations through phase contrast microscopy show considerable amount of spores germinating in the medium, before they undergo endocytosis by macrophages. After 75 minutes of infection, many spores have been already phagocyted by the macrophages. By performing a washing step at 80 minutes it was possible to remove many of the spores that are floating outside the cells. This washing step is crucial for getting a more specific response for the effect of the anti-germinant, since spore germination *in vivo* occurs within the macrophages. Figure 20 shows that protective effect by the anti-germinant was increased by performing a wash at 80 minutes.

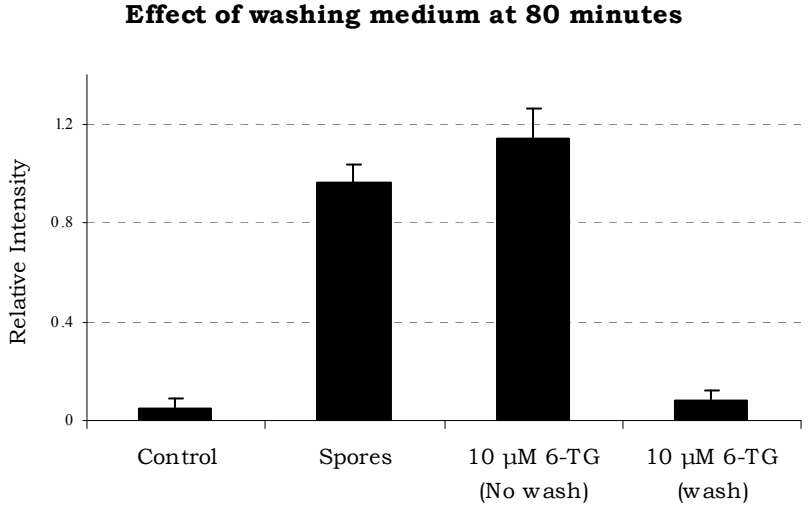


Figure 20. Fluorescent quantification on J774a.1 cell killing with *B. anthracis*. Protective effect of 6-thioguanosine is increased, by removing spores that germinate outside the cells. Readings were taken at 6 hours after infection. Samples that were not washed showed a necrosis rate near 1-fold higher.

These previous experiments were performed using TECAN GENios™ microplate reader. The machine does not have monochromator and detects fluorescence by irradiating and absorbing with filters for specific wavelengths. The filters available were 530 nm for excitation and 590 nm for absorbance. For propidium iodide the peak in emission is at 617 nm, so the signal registered from PI-DNA fluorescence is not the optimal. In the further experiments we have changed the reader for the one that is mention in chapter 2 (TECAN™ Infinite M200). By using this reader, that is equipped with monochromator, the emission was detected at is maximum wavelength (617 nm), and signal from PI-DNA fluorescence was enhanced. In figure 21, the signal coming from necrotic cells is increased to near a 3-fold of intensity, in comparison with previous graphs, where the increase is approximately 1-fold.

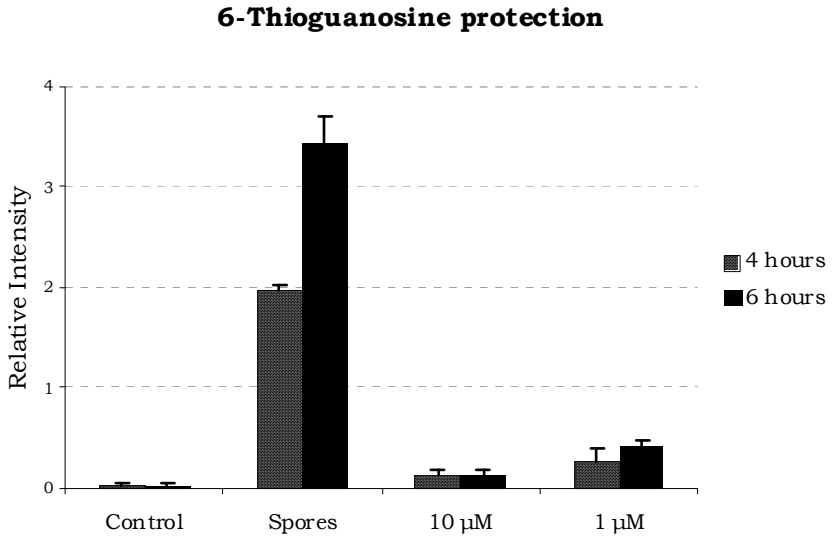
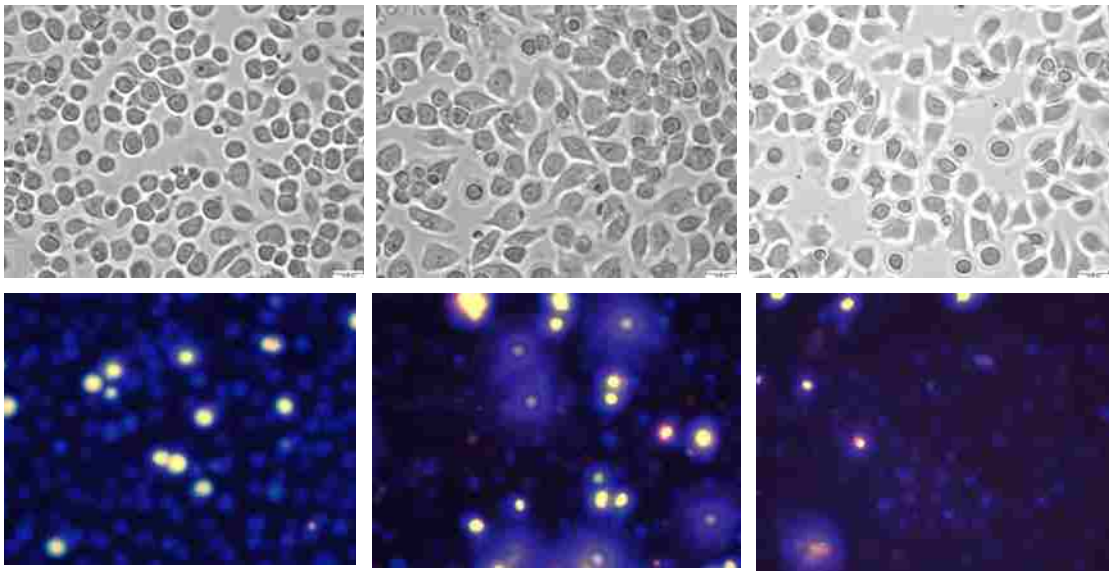


Figure 21. Protection by 6-TG at 4 and 6 hours. After removing spores at 80 minutes, protective effect by the nucleoside was enhanced, even at 1 µM. Signal was optimized by performing readings with the TECAN™ Infinite M200 with monochromator.

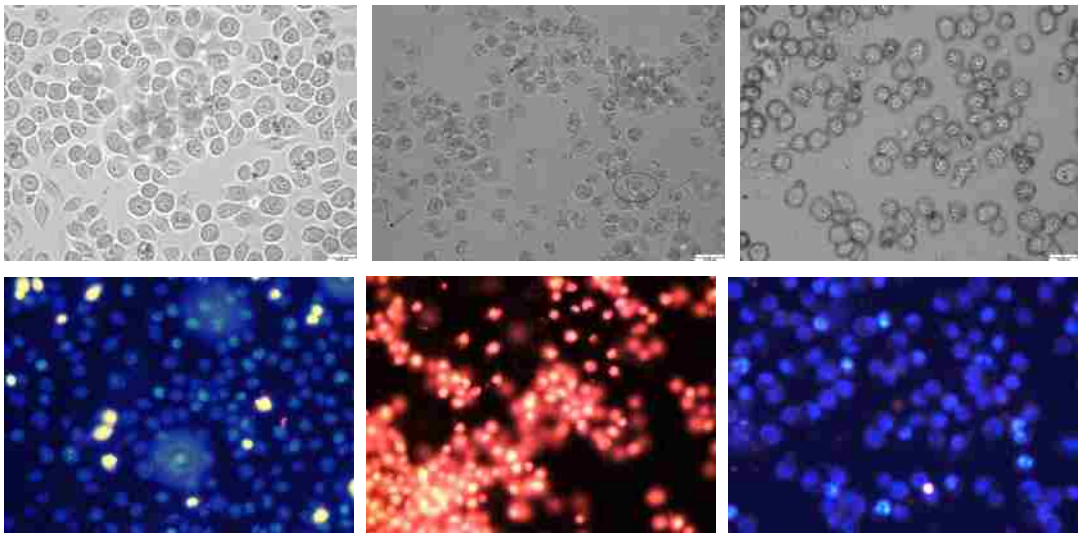
3.1.3 Fluorescence Microscopy

The protective effect by 6-TG was also observed by fluorescence microscopy. Observations at the microscope were used as qualitative assessment to corroborate the results obtained by the previous quantitative methodology. Propidium iodide was the exclusive stain for necrotic cells, whereas DAPI was used as counter-stain. The number of PI positive cells increased considerably in non-treated cells after 6 hours. (figures 22 and 23).



A B C

Figure 22. Microscopy observation at 3 hours. The three upper images correspond to phase contrast microscopy, and the bottom images to the respective fields with fluorescence microscopy. PI positive cells are bright-reddish, and the remaining cells stained with DAPI are blue. 'A' belongs to the control of non-infected macrophages; some residual background of non-viable cells is observed. 'B' shows the infected non-treated cells; the amount of necrotic cells is similar as in the control, since toxin-mediated killing has barely started. 'C' correspond to the cells treated with 10 μM 6-TG. The upper pictures B and C illustrate an efficient spore uptake by the J774a.1 cells.



A B C

Figure 23. Effect of 6-TG after 6 hours. The upper images correspond to phase contrast microscopy, and the bottom images to the respective fields with fluorescence microscopy. PI positive cells are reddish, and the remaining cells stained with DAPI are blue. 'A' belongs to the control of non-infected macrophages. 'B' shows the infected non-treated cells. A complete necrosis of these cells can be shown in both phase contrast and fluorescence microscopy (all cells are PI positive). Arrows indicate the outgrowing vegetative bacilli. 'C' belongs to the cells treated with μM 6-TG. The fluorescence microscopy picture shows a near complete protection of these cells from necrosis. Spores that were phagocytosized by J774a.1 did not germinate with the treatment at 6 hours (upper C picture).

3.1.4 Analysis of the Spore Germination *In Vitro*

An assessment for the effect of 6-TG in preventing spore germination was performed *in vitro* following the procedure in sub-chapter 2.3. 6-thioguanosine was added in combination with the germinants. Approximately a 25% of the spores treated with 6-TG germinated, in contrast with the spores with the germinants only. Figure 24 shows that 6-thioguanosine affected considerably the kinetics of *B. anthracis* germination, confirming the results obtained by M. Akoachere et al [99].

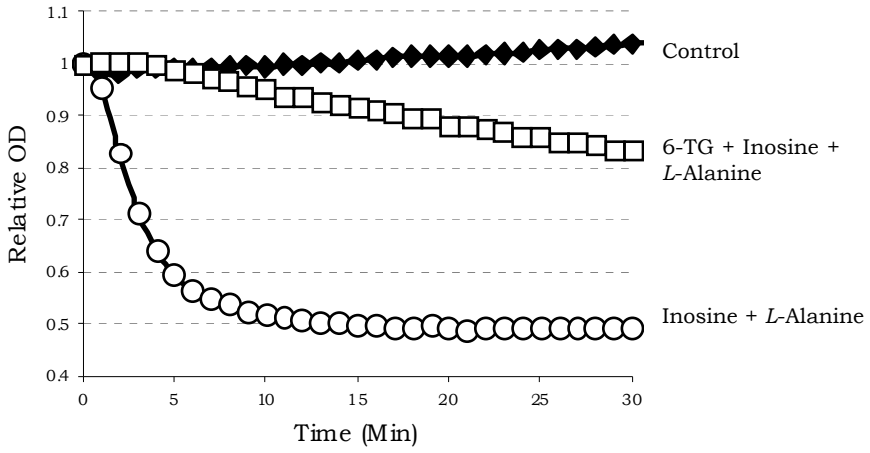
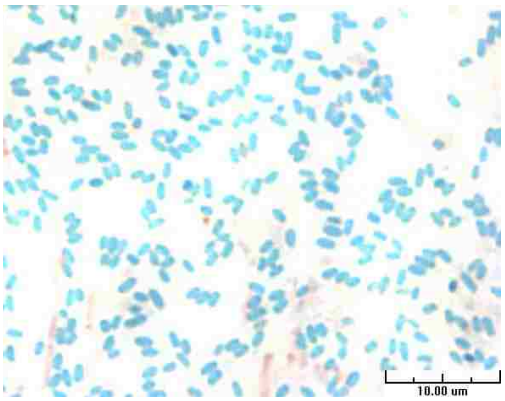
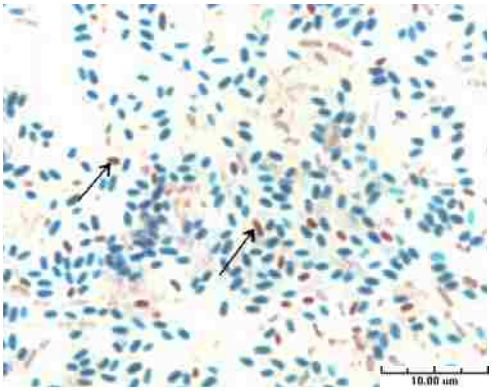


Figure 24. Effect of 6-TG on endospore germination *in vitro*. *L*-alanine (0.04 mM) and inosine (0.25 mM) triggered the germination of most of the spores within 5 minutes. When 6-TG (1 mM) was added the velocity of the germination decreased considerably, confirming that inhibits spore germination.

An observation through light microscopy was performed as a qualitative assessment. Spores were added to LB broth and incubated at 37°C in agitation. The Schaeffer-Fulton selective spore staining was applied to observe the germination at the microscope. Most the 6-TG-free spores germinated and matured after incubating 40 minutes. Treating spores with 6-TG did not stop germination *in vitro*, but slowed it down. At 40 minutes, the many treated spores showed an incomplete germination, and many did not even start germinating (figure 25).

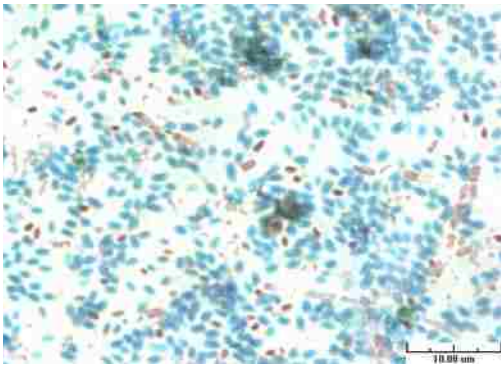


0 minutes

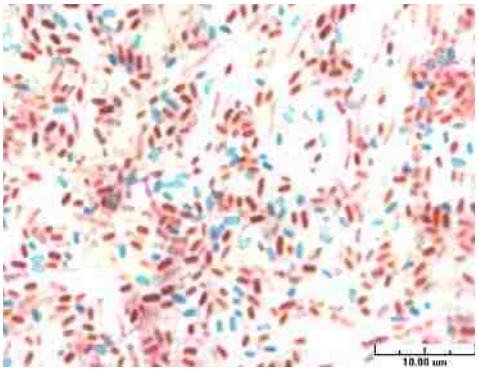


Broth – 5 minutes

Broth – 40 minutes



6-Thioguanosine – 15 minutes



6-Thioguanosine – 40 minutes

Figure 25. *B. anthracis* endospore germination observed at light microscopy (100X). Before incubating in broth most of the bacteria were in their endosporal state, and only few were dead vegetative bacteria. After five minutes, spores started to show signs of the first stages of germination, corresponding with the degradation of the cortex (safranine positive indicated with arrows). Within 40 minutes most of the cells were in their bacillar form. When 6-thioguanosine was added, several spores were still undergoing early stages of germination, and several did not start germinating yet (green malachite positive).

3.2 Half-maximal Inhibitory Concentration of 6-thioguanosine

Following the validation of the 6-thioguanosine protective effect on necrosis, the IC_{50} for this compound was obtained by the procedures mentioned in sub-chapter 2.11. Cell viability by PI-DNA fluorescence at four and six hours. The best separation time point was gotten at 6 hours (figure 26).

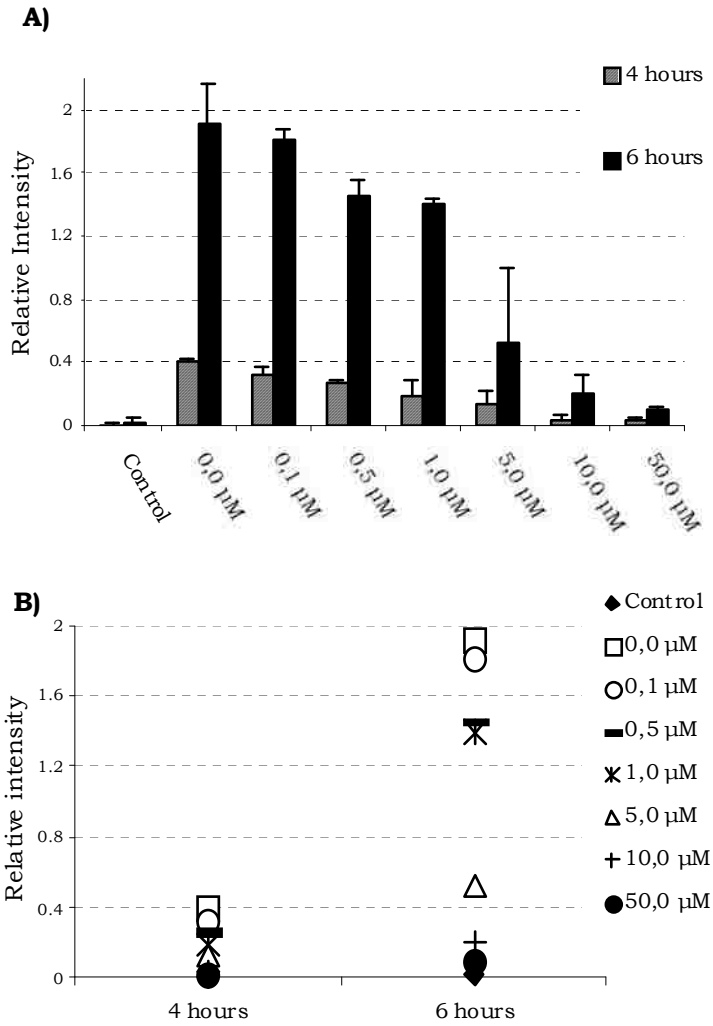


Figure 26. 6-TG protection IC_{50} assay. Six different concentrations of 6-thioguanosine were added to J774a.1 cells. Necrosis was monitored at 4 and 6 hours (A and B). The best separation response was obtained at 6 hours (B). Data corresponding to 6 hours was used for plotting IC_{50} regression graph.

Data points obtained for 6 hours were used to plot four parameter regression curve using SigmaPlot v. 11. Relative intensity was plotted against the decimal logarithm of the concentration of 6-TG (figure 27). Results obtained by regression analysis indicated an IC_{50} of 3.5 μM, with an R^2 of 0.963, and a standard error of the estimate (*SE*) of 0.221.

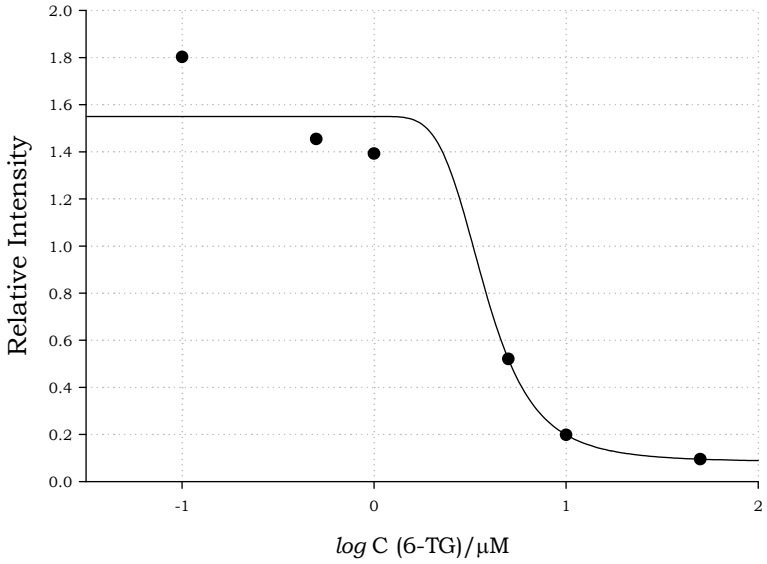


Figure 27. Dose-response curve for 6-thioguanosine protective effect on macrophages J774a.1. The half-maximal inhibitory concentration corresponded to 3.5 μM.

3.3 Effect of 6-thioguanine

6-thioguanine (6-Tg) is the corresponding base of the nucleoside 6-TG. Analysis of the effect of 6-Tg and other bases were performed to determine whether the rybosyl group was essential for Ger receptor binding or not. Cell viability assays were performed on J774a.1 cells, and by monitoring germination *in vitro*. 6-Tg protected germination after 6 hours (figure 28).

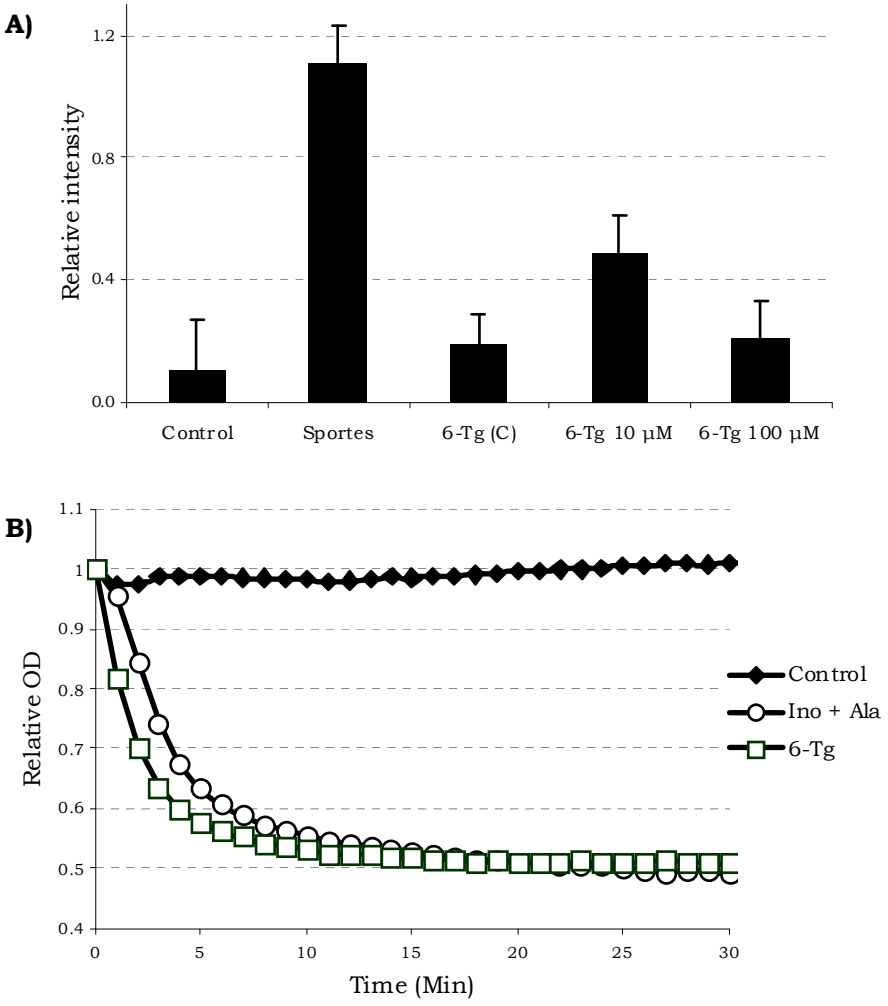


Figure 28. Effect of 6-Tg in macrophages and *in vitro*. 6-thioguanine protects macrophages from *B. anthracis* mediated killing (A). '6-Tg (C)' is the control for treatment without spores; it showed that the nucleoside analogue was not cytotoxic. Surprisingly, after the analysis *in vitro* 6-Tg did not inhibit germination, when Ino and *L*-Ala were added (B).

The results of figure 28 confirm that 6-Tg protected macrophages from necrosis, but did not prevent germination of the endospores *in vitro.* The effect of 6-Tg on J774a.1 was corroborated by a direct observation on phase contrast and fluorescence microscopy (shown in figure 29).

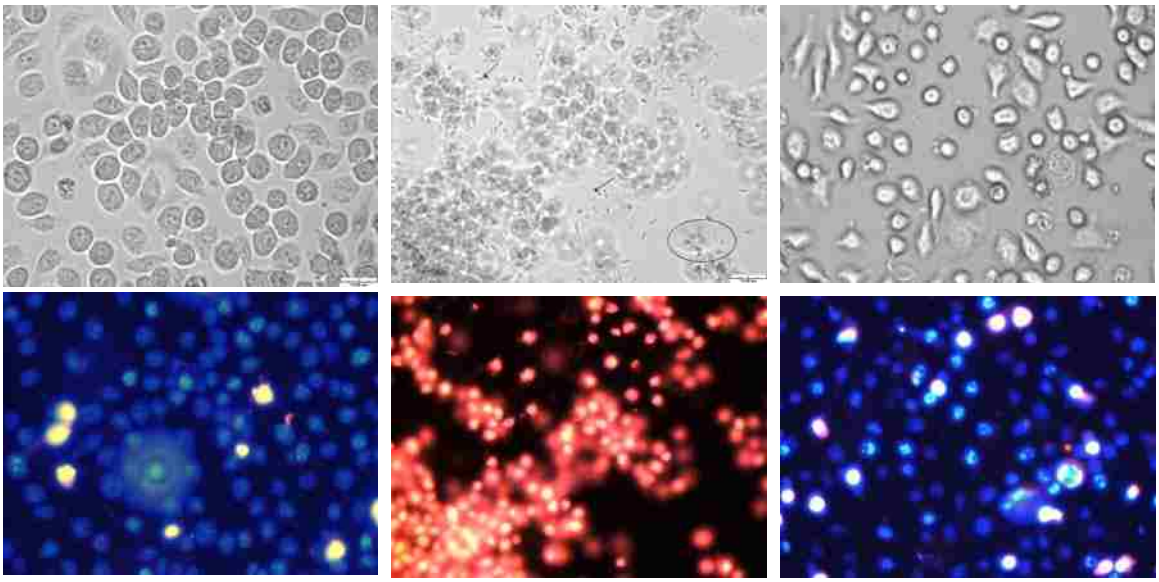


Figure 29. 6-Tg protection on J774a.1 at 6 hours. Upper figures correspond to phase-contrast microscopy, and the bottom ones to fluorescence microscopy of the corresponding field. Fluorescent dyes were PI (reddish), and DAPI (blue). 'A' is the control of non-infected cells. 'B' shows a general necrosis (PI- positive cells) in non-treated cells after 6 hours of infection with *B. anthracis*. 'C' images correspond to the protective effect of 6-Tg. The majority of cells in C was viable; unaltered membranes and no PI staining.

The half-maximal inhibitory concentration was obtained for 6-TG protective effect in cells. Data obtained at 6 hours was used for plotting regression curve. The value of this one was obtained by a regression analysis on SigmaPlot, and corresponded to 1,9 μM with an R^2 of the regression of 0.997 (figures 30 and 31).

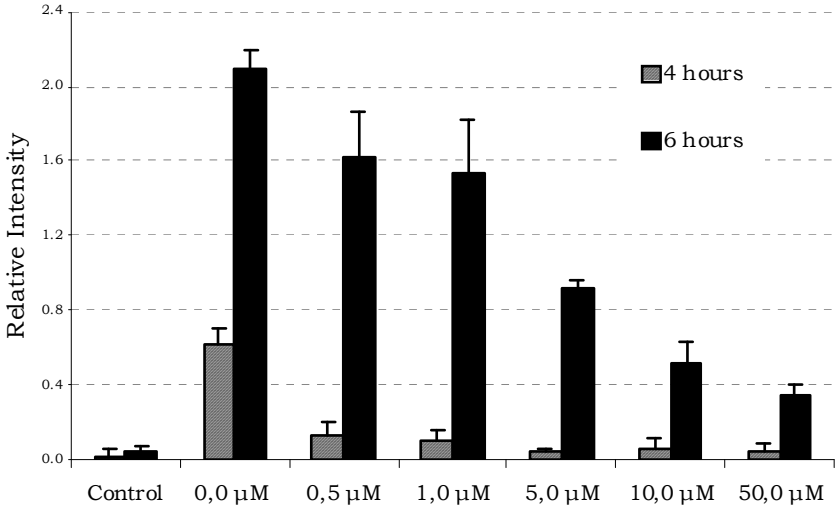


Figure 30. 6-Tg protection IC_{50} assay. Six different concentrations of 6-thioguanine were added to J774a.1 cells. Necrosis was monitored at 4 and 6 hours, by PI exclusion assay.

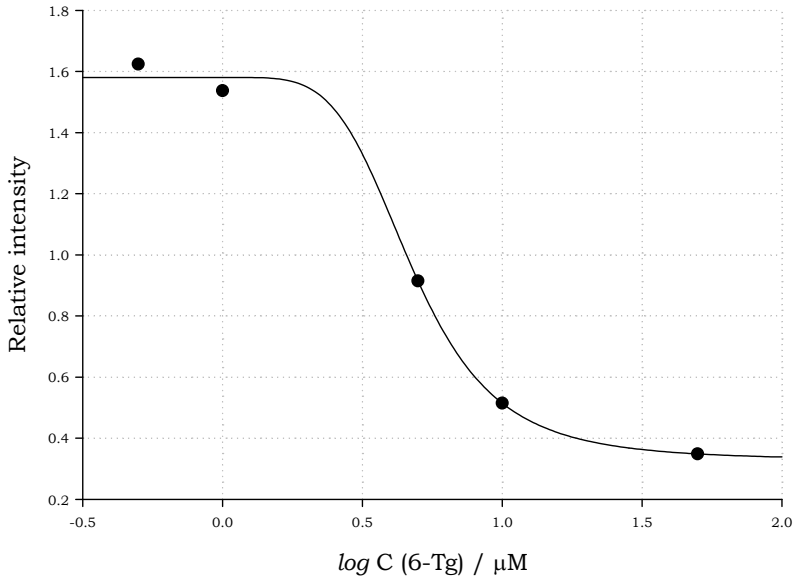


Figure 31. Dose-response curve for 6-thioguanine protective effect on macrophages J774a.1. The half-maximal inhibitory concentration corresponded to 1.9 µM.

3.4 Effect of 6-chloroguanosine

In the following results, it has been confirmed that 6-chloroguanosine (6-CG) has a protective effect in J774a.1. After performing a cell killing assay monitored by PI exclusion assay, there was protection by 6-CG at four hours (figure 32); after 5 hours treated cells became necrotic. 6-CG protective effect was weaker than the one of 6-TG and 6-Tg.

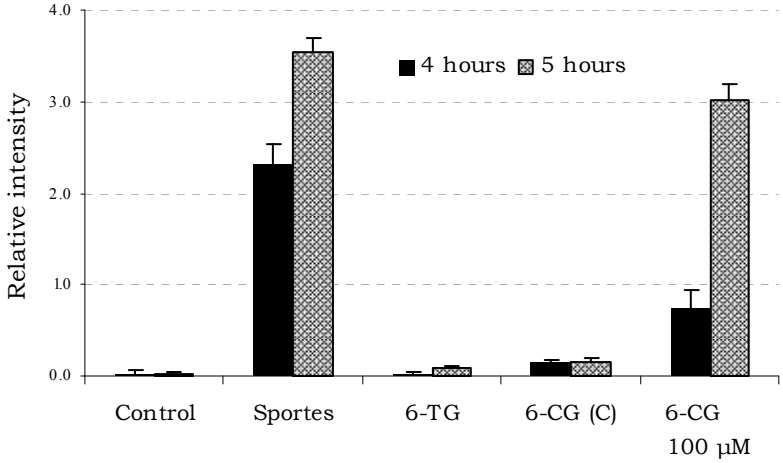


Figure 32. Effect of 6-CG on macrophages J774a.1. After 4 hours 6-CG protective effect of the analogue was lost and cells became necrotic, shown by the increase of fluorescence in PI. '6-TG (C)' shows that the analogue was not toxic to cells.

An IC_{50} for the effect of 6-CG was obtained as in previous experiments. The data was obtained after 4 hours with concentrations of 1, 5, 10, 50, 100, and 500 μM. The regression analysis indicates an IC50 of 0.19 mM, near 100-fold higher than the ones of 6-TG and 6-Tg. The R^2 for the regression was of 0.998, with an *SE* of 0.0612 (figure 33).

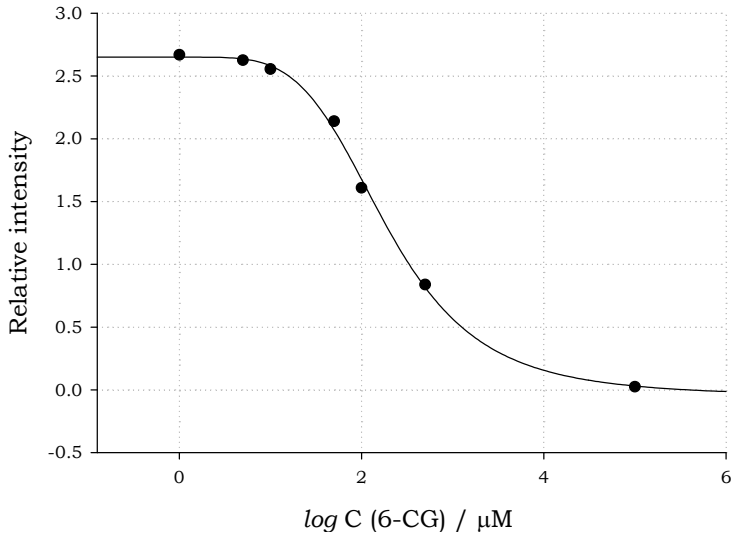


Figure 33. Dose-response curve for 6-chloroguanosine protective effect on macrophages J774a.1. Data for plotting was obtained from the 4 hour time point. The half-maximal inhibitory concentration corresponded to 190 μM.

The IC_{50} of 6-CG *in vitro* was obtained by Akoachere et *al* [99]. The value of K_i was used to obtain the IC_{50} through the modified Cheng-Prusoff equation [104]. The IC_{50} of the inhibition *in vitro* corresponded to 0.89 mM. K_i value is given in table 4.

3.5 Effect of 6-chloroguanine

6-chloroguanine (6-Cg) is the base of the nucleoside 6-CG. Cell killing assays and analysis of germination *in vitro* were performed with 6-Cg, in the same way as previous experiments. 6-Cg did not prevented the germination *in vitro*, but protected macrophages from necrosis with a similar IC_{50} as 6-CG. These results are given in (figures 34 to 36).

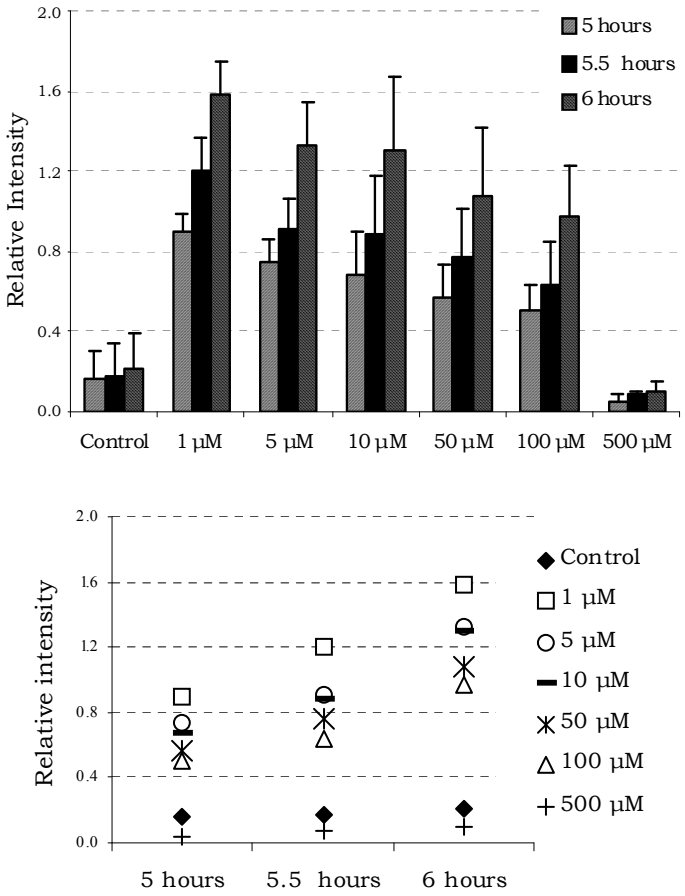


Figure 34. IC_{50} assay for 6-Cg on macrophages J774a.1. The best separation time point corresponded to 6 hours.

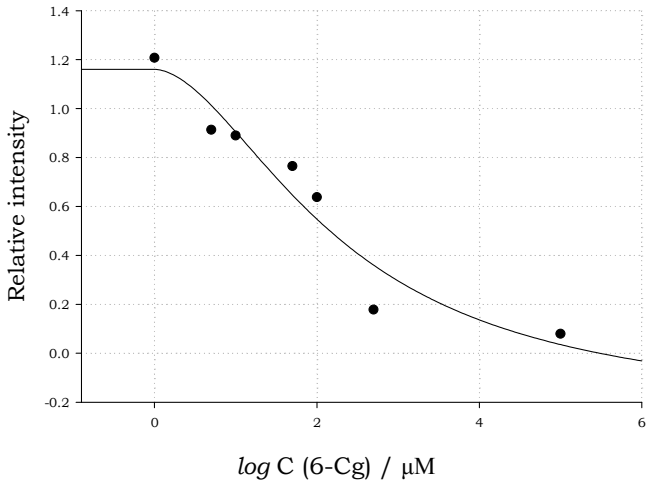


Figure 35. Dose-response curve for 6-Cg on macrophages J774a.1. The IC_{50} of the regression corresponded to 204 µM. Data points were taken at 6 hours after infection.

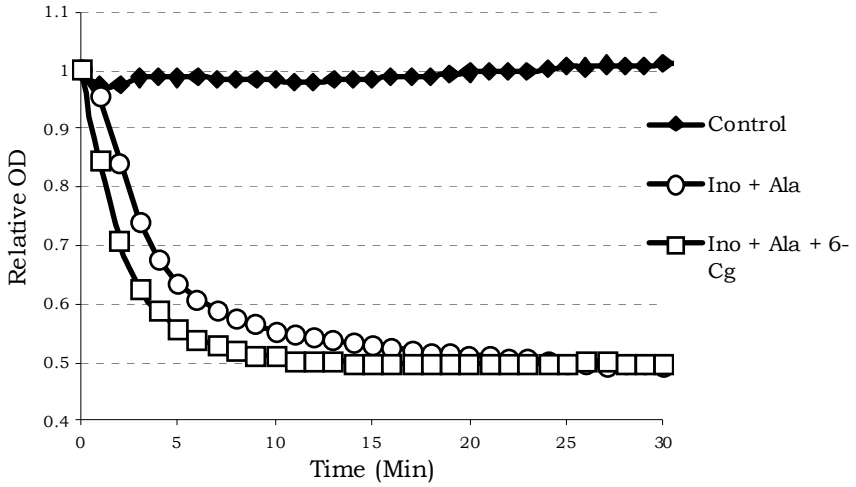


Figure 36. 6-Tg does not inhibit germination *in vitro*. The rate of germination after treating with 1 mM 6-Tg was the same as with Ino (0.25 mM) and *L*-Ala (0.04 mM) alone.

3.6 Effect of 6-thioinosine

6-thioinosine prevented cell killing and inhibited the germination in vitro. Both IC_{50}s were obtained in each case. Results are shown in figures 37 and 38.

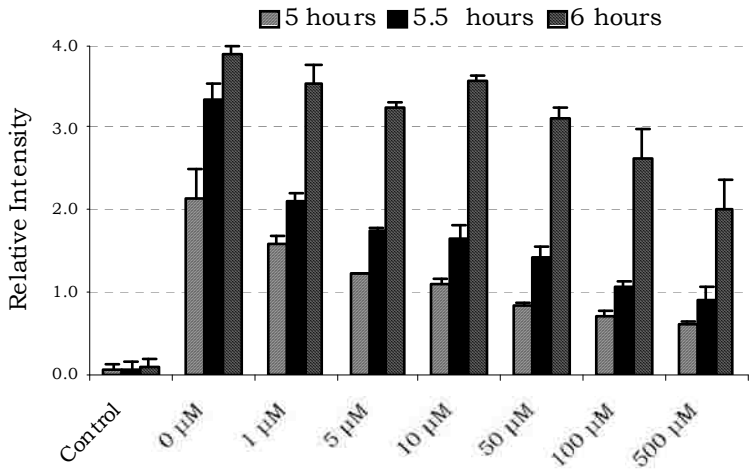


Figure 37. 6-TI IC_{50} assay on macrophages J774a.1. Data for the IC50 regression plot was taken at 5 hours.

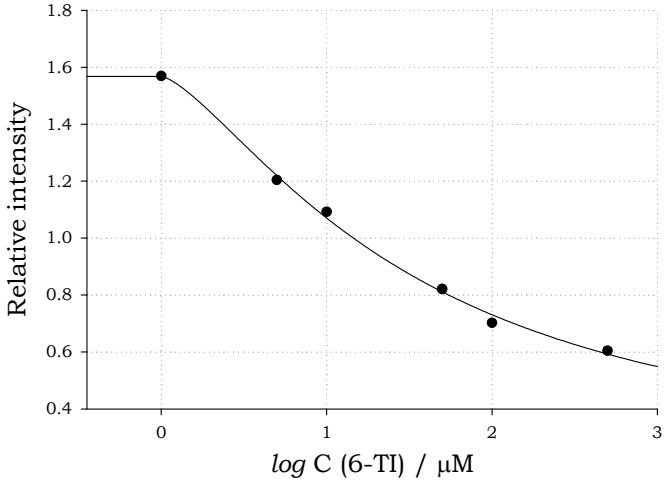


Figure 38. Dose-response curve for 6-TI on macrophages J774a.1. The IC_{50} of the regression corresponded to 35.2 μM. Data points were taken at 5 hours after infection. R^2 of regression was 0.997.

6-TI was ineffective in inhibiting the germination in vitro K_i for the inhibition *in vitro* [99]. Thus, no IC_{50} was obtained for 6-TI inhibitory effect on endospore germination.

3.7 Effect of 6-mercaptopurine

6-mercaptopurine or 6-Mp is the base that belongs to the nucleoside 6-thioinosine (6-TI). 6-mercaptopurine showed protection of J774a.1 from spore-mediated killing in the range of 3-5 hours (figure 39). The IC_{50} obtained was higher than the one of the corresponding nucleoside, approaching 0.4 mM. 6-Mp was tested *in vitro* and did not alter the germination of the spores with inosine and *L*-alanine.

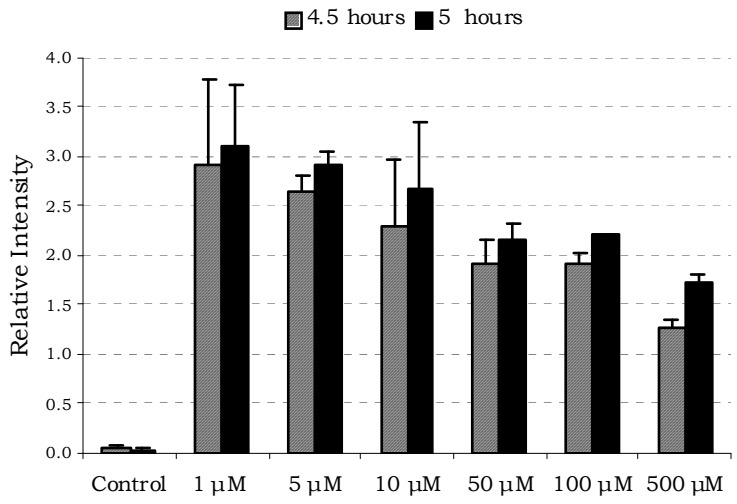


Figure 39. IC_{50} assay for 6-Mp. The cell killing was monitored between 4-6 hours. The time point that best fitted the IC_{50} plot was at 4.5 hours.

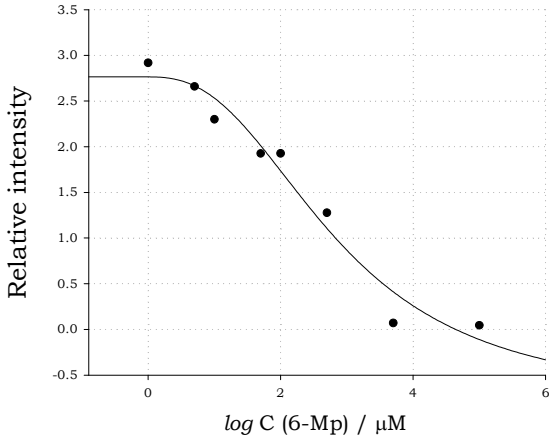


Figure 40. Dose-response curve for the protection of 6-mercaptopurine in J774a.1. The IC_{50} estimated by the regression analysis was 398 µM. The R^2 of the regression was 0.954 with an *SE* of 0.31.

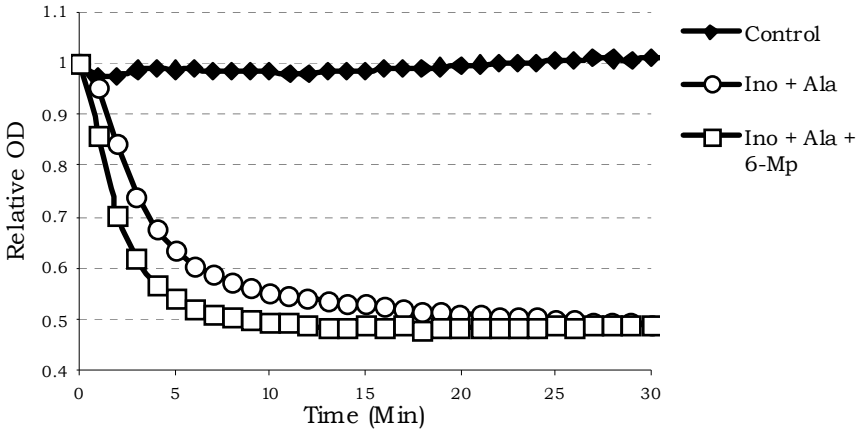


Figure 41. 6-Mp does not inhibit germination *in vitro*. The rate of germination after treating with 1 mM 6-Mp was the same as with Ino (0.25 mM) and *L*-Ala (0.04 mM) alone.

3.8 Effect of Allopurinol riboside

The inosine analogue allopurinol riboside (APR) showed a significant protection to macrophages J774a.1. Contrary to the expectations, it did not inhibit the germination of the endospores *in vitro*.

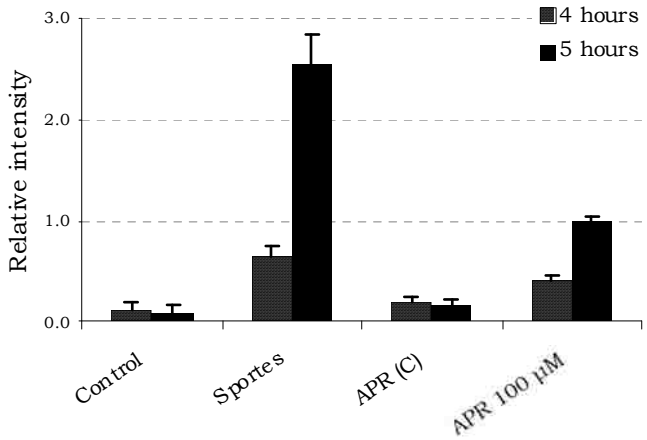


Figure 42. Protection J774a.1 macrophages by APR. After 5 hours APR showed a significant protection from necrosis, in contrast with the non-treated cells. APR was not cytotoxic by itself (APR (C)).

A dose-response assay for the protection was performed. 5 hours time point was selected to plot the relative intensities with their respective concentrations. The IC_{50} obtained by the regression analysis was 646 μM, with an R^2 of 0.978 and a *SE* of 0.159.

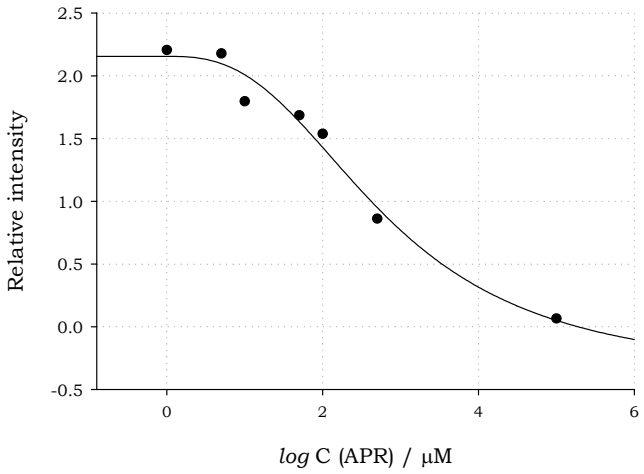


Figure 43. Dose-response curve for the protection of APR in J774a.1. The IC_{50} estimated by the regression analysis was 646 μM. The R^2 of the regression was 0.979, with an *SE* of 0.16.

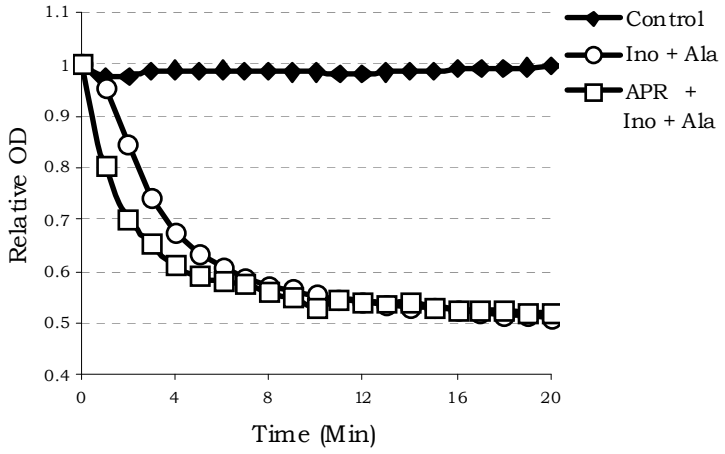


Figure 44. APR does not inhibit germination *in vitro*. Germinants (Ino and Ala) were added with 1 mM APR. No significant effect on the kinetics of the germination was observed.

3.9 Effect of Allopurinol

Allopurinol (Ap) is the base of the nucleoside APR. This compound did not either affect the germination of the spores *in vitro*, or protect the J774a.1 cells from spore-mediated killing.

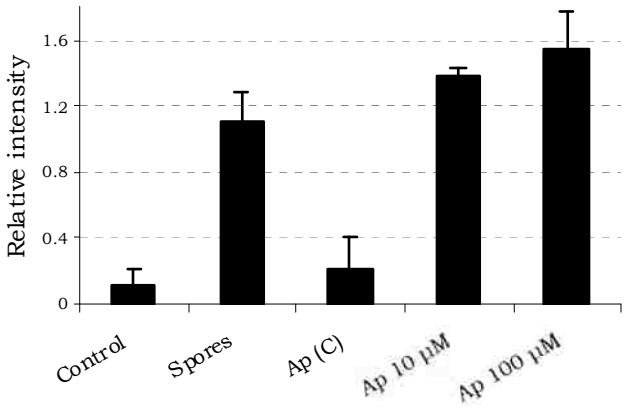


Figure 45. Effect of Ap on J774a.1 cells. After five hours of treatment Ap did not prevent macrophages from necrosis. PI-DNA fluorescence was higher for the two different concentrations of Ap than the non-treated control. Ap did not show cytotoxicity.

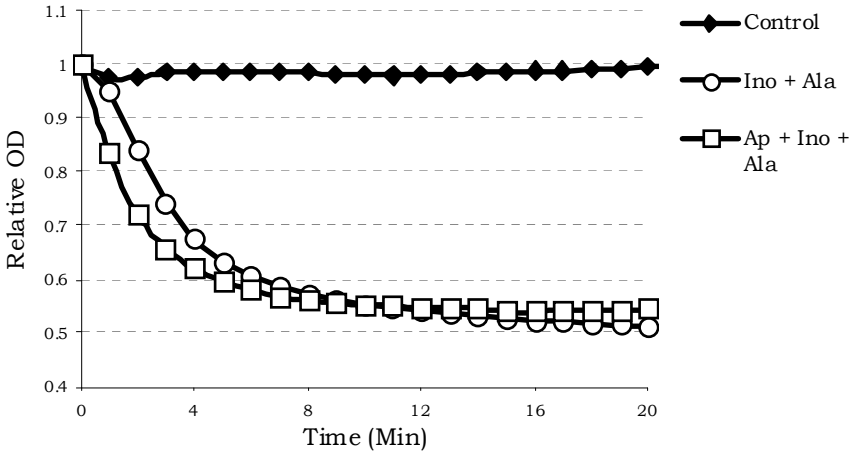


Figure 46. Ap does not inhibit germination *in vitro*. Germinants (Ino and Ala) were added with 1 mM Ap. No significant effect on the kinetics of the germination was observed.

3.10 Effect of 6-benzylthioinosine

The next inosine derivative, 6-benzylthioinosine (BTI), showed a considerable protection from cell killing after 5 hours. BTI is also an inhibitor of the germination in vitro. M. Akoachere (University of Nevada Las Vegas, 2006) has obtained the IC_{50} of the inhibitory effect *in vitro*, which is 0.0506 mM. This inhibitory effect *in vitro* can be correlated with the protection of the macrophages (figure 47).

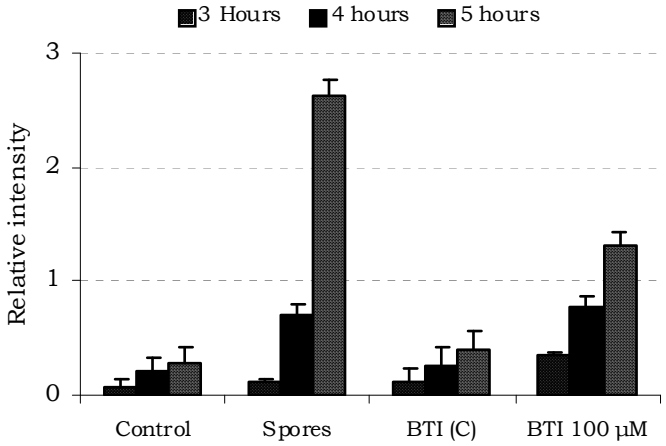


Figure 47. BTI protects J774a.1 cells from necrosis after 5 hours. PI fluorescence in non-treated cells was 1-fold higher than cells treated with 100 µM BTI. Cytotoxicity by the nucleoside is not present or minimal; 'BTI (C)' shows a similar response to the non-infected cells.

In addition, a dose-response assay was performed to assess the IC_{50} of the protective effect on cells. The selected concentrations were 0.5, 1, 5, 10, 50, and 100 µM. A regression curve was obtained with an R^2 of 0.996 and *SE* of 0.166 (figures 48 and 49).

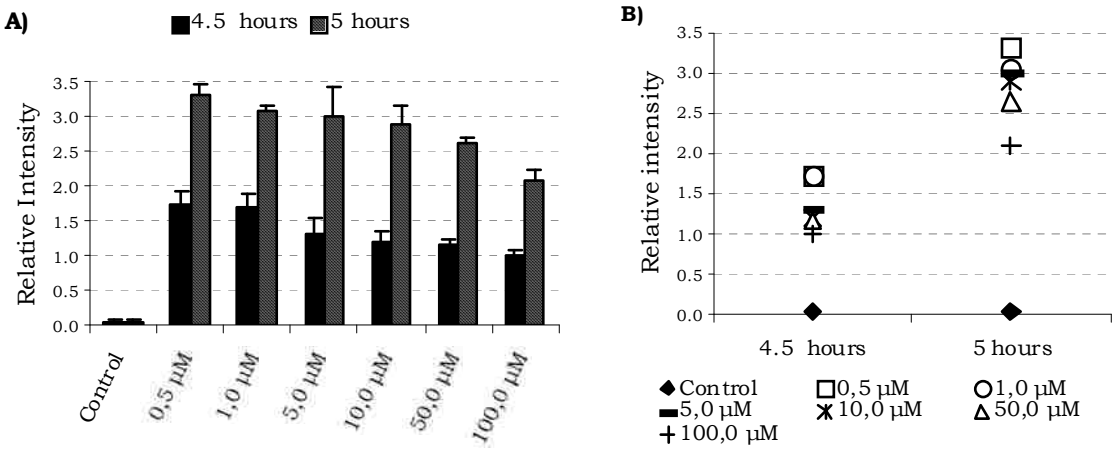


Figure 48. BTI IC_{50} assay. Data was obtained in 4.5 and 5 hours (Figure A). The best separation corresponded to 5 hours; valid for calculating the regression plot (Figure B).

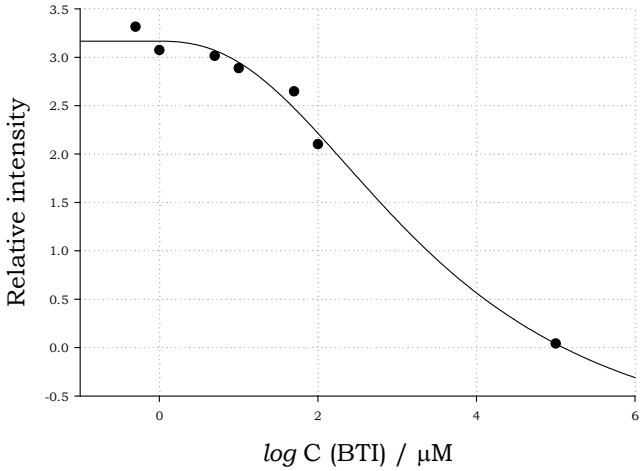


Figure 49. BTI dose-response curve. IC_{50} was obtained by regression analysis and corresponded to 1.04 mM.

3.11 Effect of 6-methylmercaptopurine riboside

The compounds analyzed from sub-chapter 3.11 to sub-chapter 3.25 did not protect macrophages from B. *anthracis* mediated killing, but did inhibit the spore germination *in vitro*. The first of these compounds is 6-methylmercaptopurine riboside (6-MMPR). Akoachere et *al* [99] showed that 6-MMPR acts as a competitive inhibitor of inosine for the spore germination, with a K_i of 31 µM (table 4). Its IC_{50} can be derived from Cheng-

Prusoff[16] equation, and has the value of 0.32 mM. 6-MMPR, however, showed little to no effect in protecting J774a.1 from necrosis by *B. anthracis* (figure 50).

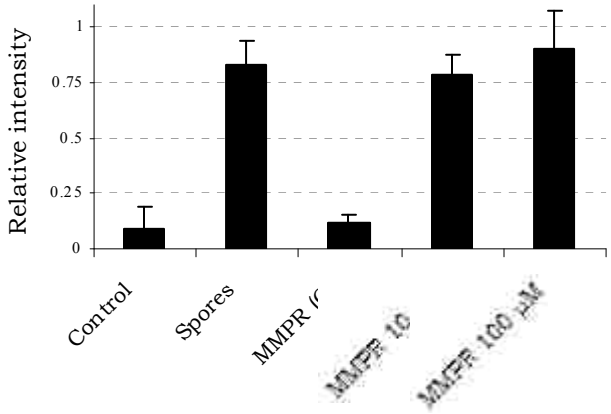


Figure 50. Effect of 6-MMPR on J774a.1 cells after 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in the treated cells was similar to the non-treated ones.

3.12 Effect of 6-*O*-methylguanosine

6-*O*-methylguanosine (6-OMG) inhibited the germination of the spores in vitro. The K_i obtained by Akoachere et *al*, was useful to determine the IC_{50}, which belongs to 0.35 mM. 6-OMG does not have a notorious effect on protecting macrophages from necrosis (figure 51).

[16] The Cheng-Prusoff equation correlates the kinetic inhibition constant (K_i) with the half-maximal inhibitory concentration by expressing that $K_i = IC_{50} / (1 + S/K_m)$, where S is the substrate concentration, and K_m the Michaelis constant of such substrate [(104)].

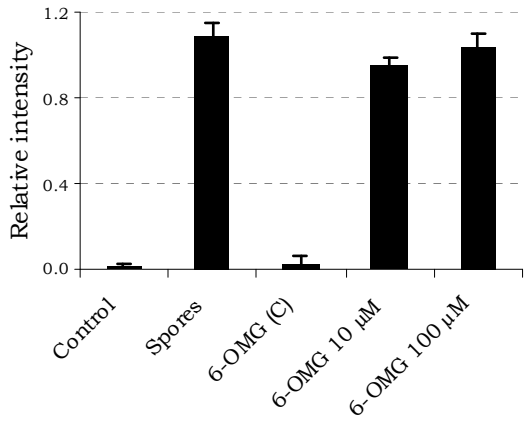


Figure 51. Effect of 6-OMG on J774a.1 cells after 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in the treated cells was similar to the non-treated ones.

3.13 Effect of 2-aminoadenosine

2-aminoadenosine (2-AA) or 6-aminoguanosine inhibited the spore germination *in vitro* with a K_i of 68 µM, corresponding to an IC_{50} of 0.69 mM [99]. 2-AA did not prevent the anthrax necrosis within 6 hours (figure 52).

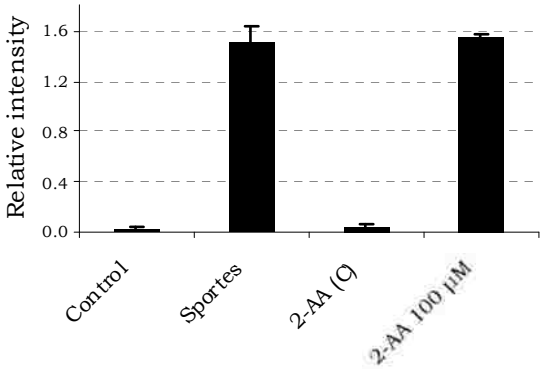


Figure 52. Effect of 2-AA on J774a.1 cells after 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in the treated cells was similar to the non-treated ones.

3.14 Effect of PPER

1-(4-(9-(β)-ribofuranosyl)-9H-purin-6-yl)piperazin-1-yl)ethanone (PPER) did not show protection for J774a.1, but inhibited the germination *in vitro* (figures 53 and 54). Its IC_{50} was obtained following the procedures on sub-chapter 2.12 (figure 55).

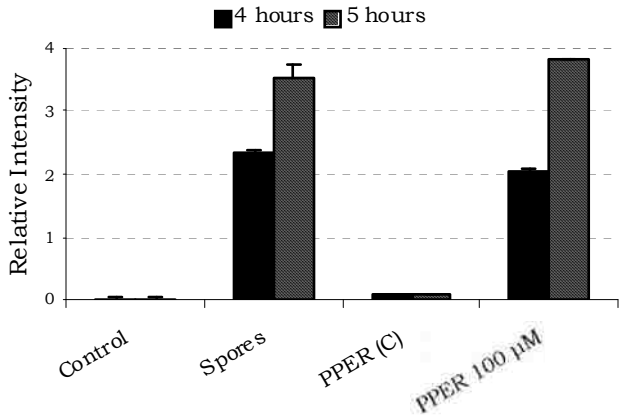


Figure 53. Effect of PPER on J774a.1 cells at 4 and 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

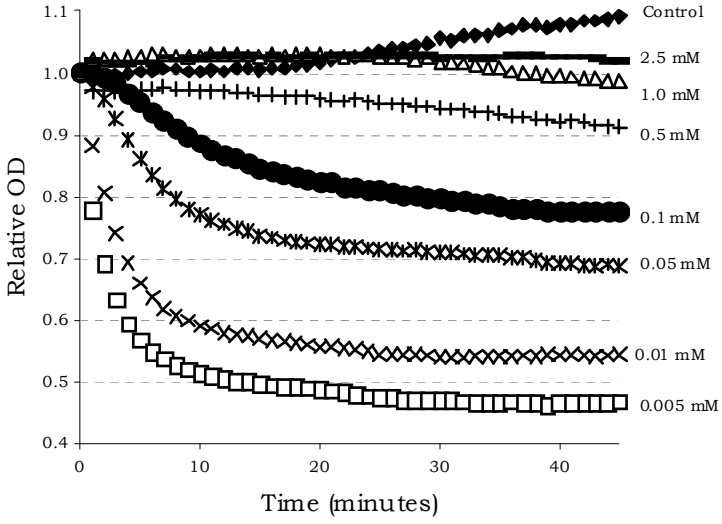


Figure 54. *In vitro* inhibition of PPER at different concentrations. Germinants added were inosine (0.25 mM) and *L*-alanine (0.04 mM). A control with no germinant was run to test for possible auto-germination. The maximum concentration of PPER prevented the germination process almost completely within 45 minutes.

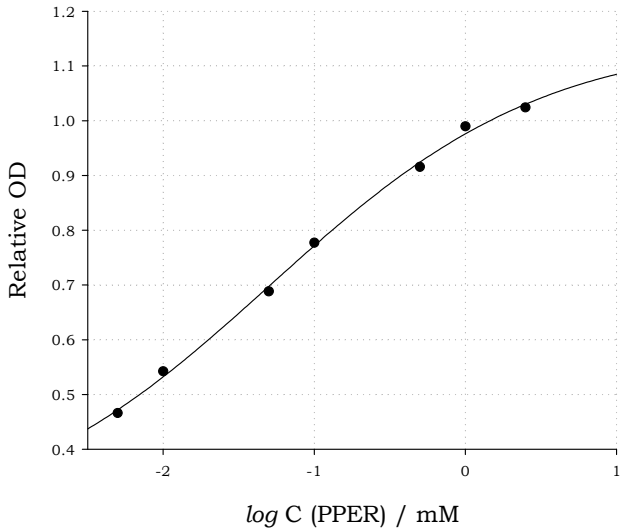


Figure 55. Dose-response curve for the inhibition of PPER *in vitro*. Data was selected for an arbitrary time point. An IC_{50} of 0.016 mM was obtained by the regression (R^2=0.998 and *SE* = 0.014).

3.15 Effect of 6-methylaminopurine riboside

6-methylaminopurine riboside (6-MAPR) did not prevent spore-mediated killing on J774a.1. Germination *in vitro* was inhibited by 6-MAPR. The following figures show the IC_{50} plot as well as the treatment in cells.

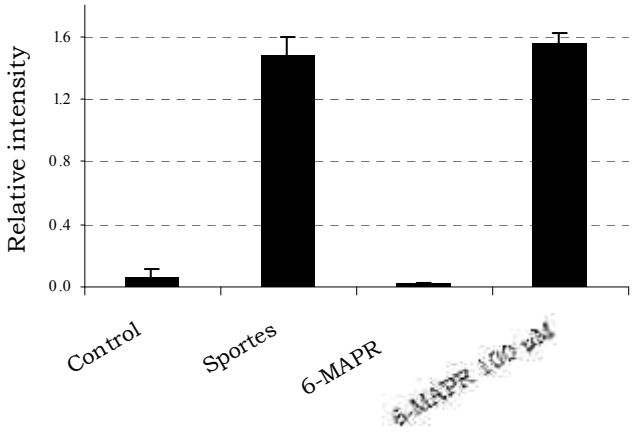


Figure 56. Effect of 6-MAPR on J774a.1 cells at 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

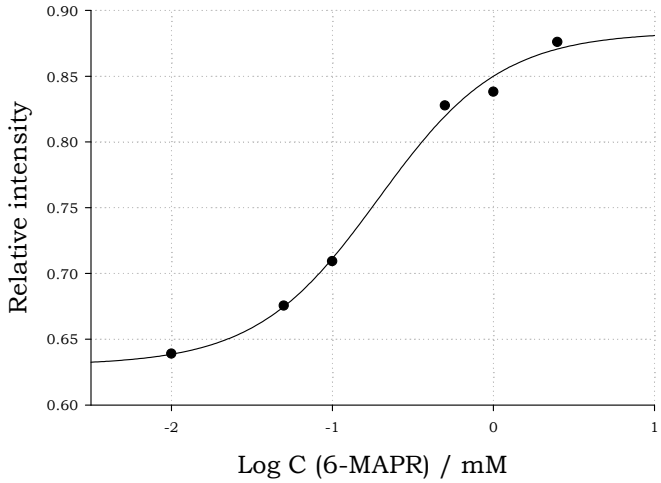


Figure 57. Dose-response curve for the inhibition of 6-MAPR *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.193 mM was obtained by the regression (R^2=0.995 and *SE* = 0.011).

3.16 Effect of 2-aminopurine riboside

The present analogue (2-APR) inhibited the spore germination *in vitro* but did not prevent cell necrosis of J774a.1 cells. Results including the IC_{50} *in vitro* are shown in the following figures.

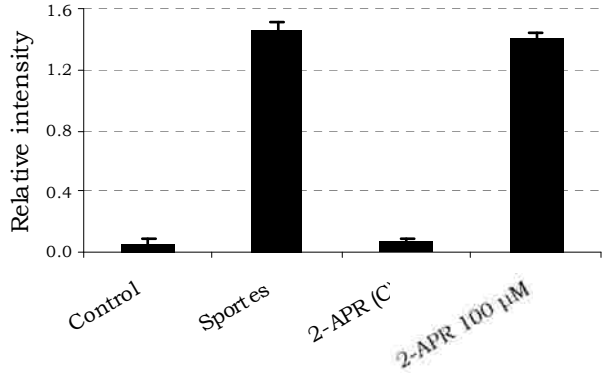


Figure 58. Effect of 2-APR on J774a.1 cells after 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

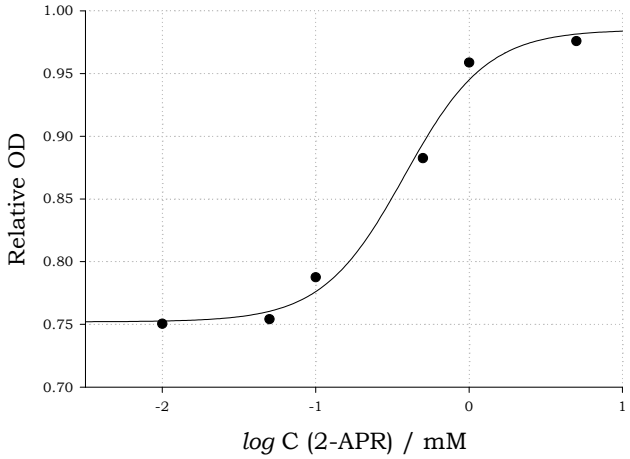


Figure 59. Dose-response curve for the inhibition of 2-APR *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.379 mM was obtained by the regression (R^2=0.990 and *SE* = 0.016).

3.17 Effect of Xanthosine

Xanthosine (X) did not protect J774a.1 cells from necrosis within 6 hours. Previous work by Akoachere et *al*, showed that X is a weak inhibitor in the presence of inosine and *L*-alanine; the K_i (777 μM) was significantly higher than other inhibitors (table 4). The IC_{50} that correspond to this constant is 7.2 mM. Inhibitory effect from X is only noticeable at extremely high, non-physiological concentrations.

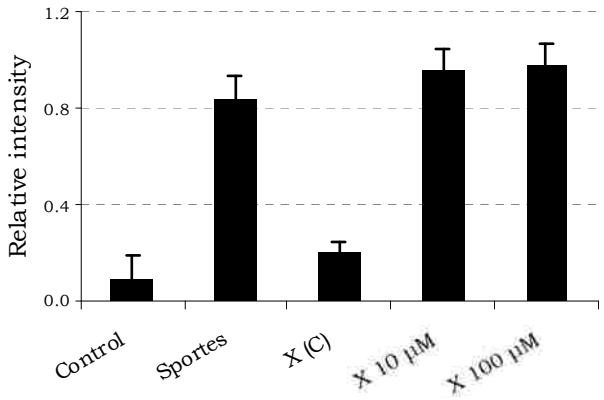


Figure 60. Effect of Xanthosine (X) on J774a.1 cells after 6 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

3.18 Effect of 6-*N*-benzylmethyladenosine

6-*N*-benzylmethyladenosine (BMA) did not protect macrophages from necrosis, but acted inhibiting the germination of the endospores *in vitro*. An IC_{50} of the inhibition was obtained for this compound.

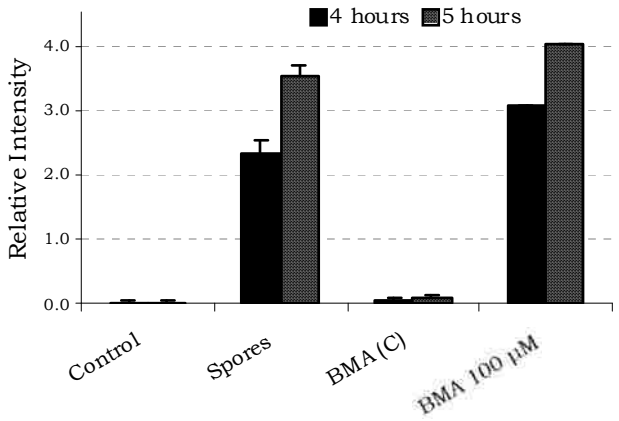


Figure 61. Effect of BMA on J774a.1 cells at 4 and 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

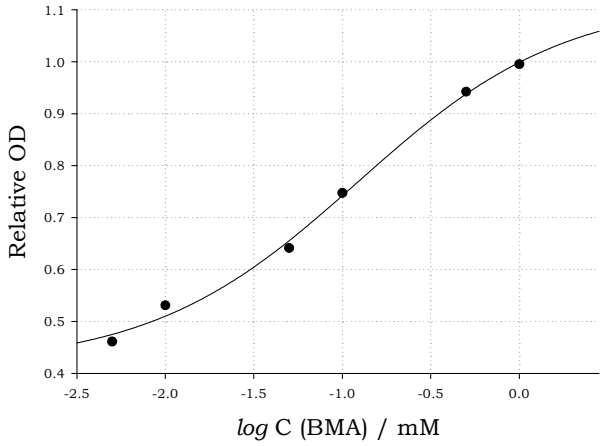


Figure 62. Dose-response curve for the inhibition of BMA *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.122 mM was obtained by the regression (R^2=0.996 and *SE* = 0.021).

3.19 Effect of 6-*N*-benzoyladenosine

6-*N*-benzoyladenosine (NBA) inhibited the germination of *B. anthracis* spores *in vitro*. The corresponding IC_{50} for the inhibition was obtained. No effect regarding J774a.1 necrosis protection was observed.

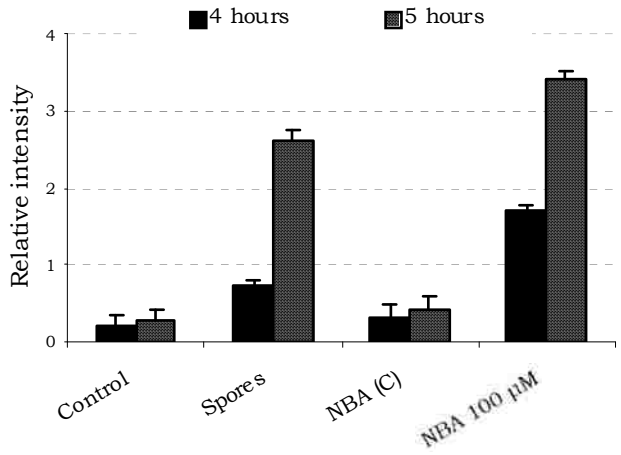


Figure 63. Effect of NBA on J774a.1 cells at 4 and 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar or slightly higher than the non-treated ones.

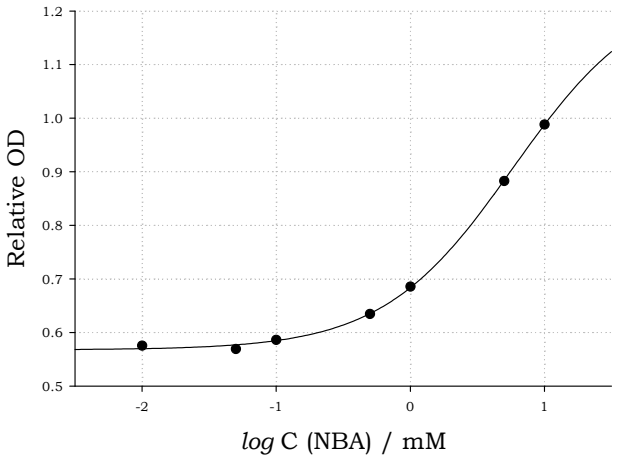


Figure 64. Dose-response curve for the inhibition of NBA *in vitro*. Data was selected for an arbitrary time point. An IC_{50} of 1.142 mM was obtained by the regression (R^2=0.997 and *SE* = 0.008).

3.20 Effect of 6-*N*-benzylaminopurine

This compound (BAP) was able to inhibit the germination of the spores in vitro, even though it was lacking the ribosyl group. This is the only 'base-derivative' from the set of compounds that affected the germination rate *in vitro*. Unfortunately, there was no correlation with the protective effect on macrophages. No protection was observed within 4 to 6 hours of infection.

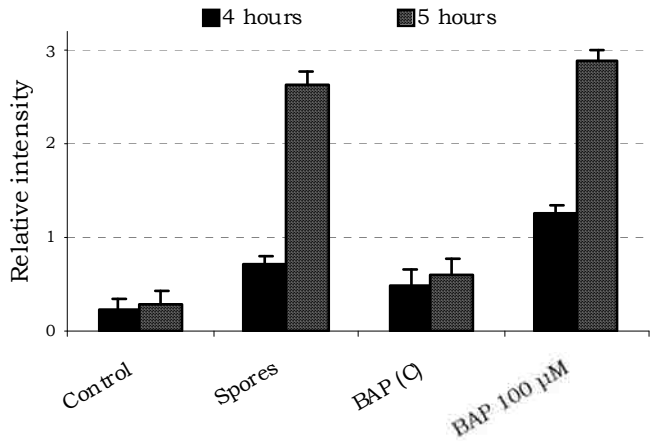


Figure 65. Effect of BAP on J774a.1 cells at 4 and 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar or slightly higher than the non-treated ones.

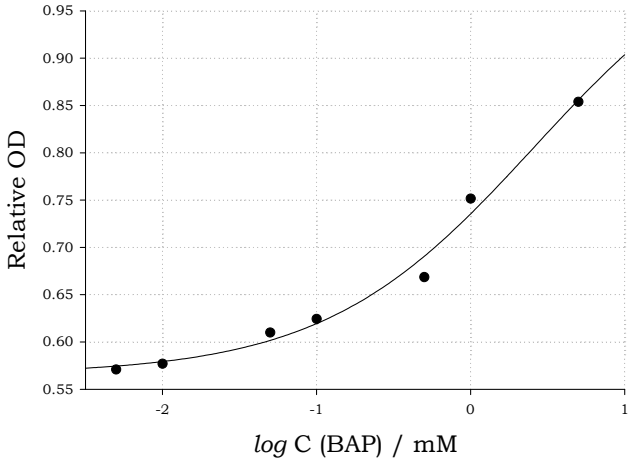


Figure 66. Dose-response curve for the inhibition of BAP *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 1.552 mM was obtained by the regression (R^2=0.985 and *SE* = 0.020).

3.21 Effect of 6-(γ,γ-dimethylallylamino)purine riboside

The present compound (DMAA) also inhibited the germination of *B. anthracis* endospores in vitro. No effect was shown in protecting J774a.1 cells (figures 67 and 68).

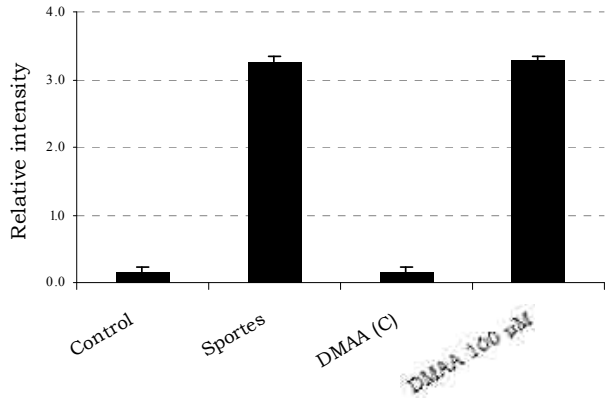


Figure 67. Effect of DMAA on J774a.1 cells at 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar to the non-treated ones.

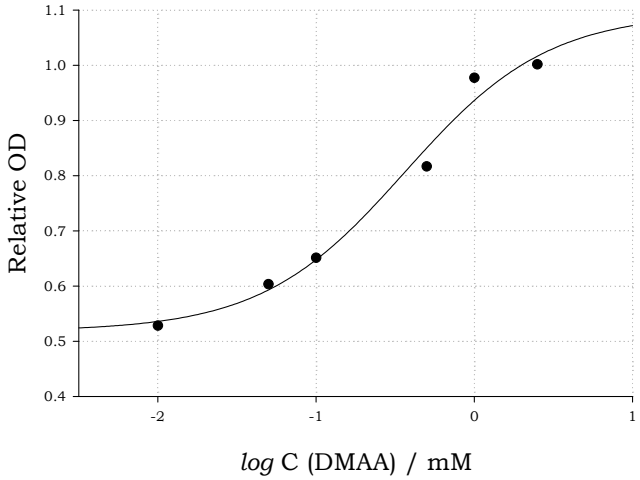


Figure 68. Dose-response curve for the inhibition of DMAA *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.364 mM was obtained by the regression (R^2=0.984 and *SE* = 0.040).

3.22 Effect of 6-*N*-cyclohexyladenosine

6-N-cyclohexyladenosine (CHA) showed an inhibitory effect on spore germination *in vitro*. An IC_{50} for the present analogue was obtained. Treatment with this compound did not affect the necrotic response on J774a.1 cells (figures 69 and 70).

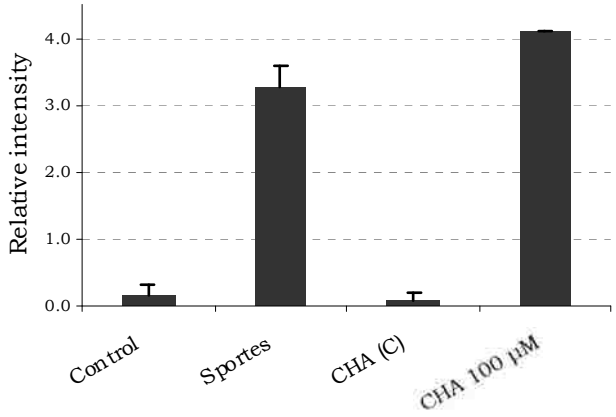


Figure 69. Effect of CHA on J774a.1 cells at 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar or slightly higher than the non-treated ones.

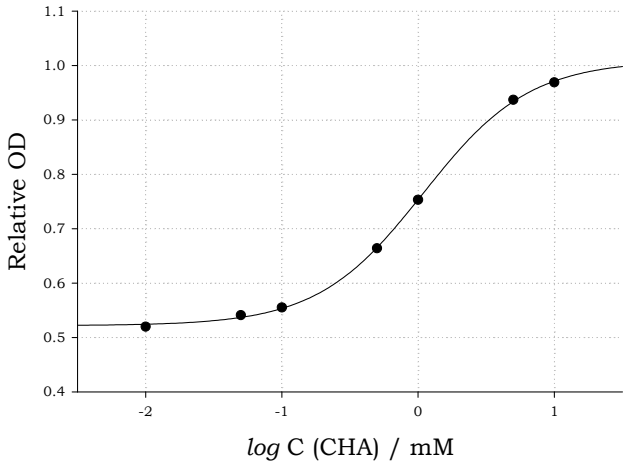


Figure 70. Dose-response curve for the inhibition of CHA *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 1.089 mM was obtained by the regression (R^2=0.999 and *SE* = 0.004).

3.23 Effect of 6-DPA

6-DPA (6-N-dipropyladenosine) inhibited the germinations of the endospores in vitro, but was unable to protect macrophages from necrosis. The half-maximal inhibitory concentration was obtained with a dose-response assay (figures 71 and 72).

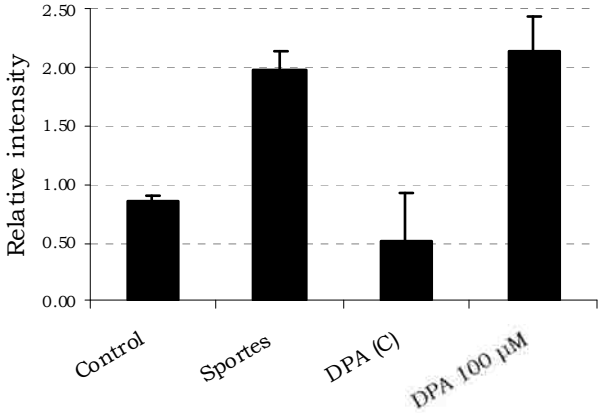


Figure 71. Effect of 6-DPA on J774a.1 cells at 4.5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar or slightly higher than the non-treated ones.

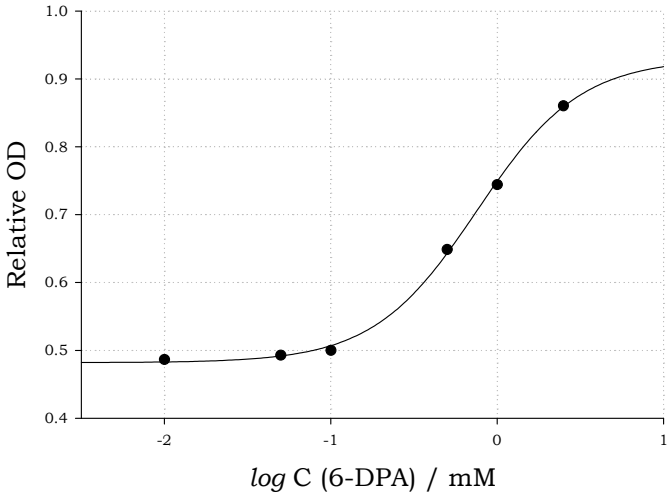


Figure 72. Dose-response curve for the inhibition of 6-DPA *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.756 mM was obtained by the regression (R^2=0.999 and *SE* = 0.008).

3.24 Effect of 6-ECA

6-ECA (6-*N*-((3-ethylacetyl)cyclohexyl)adenosine) inhibited the germination of *B. anthracis* endospores *in vitro* and its corresponding IC_{50} was calculated. No noticeable protection on J774a.1 necrosis was brought out by this analogue (figures 73 and 74).

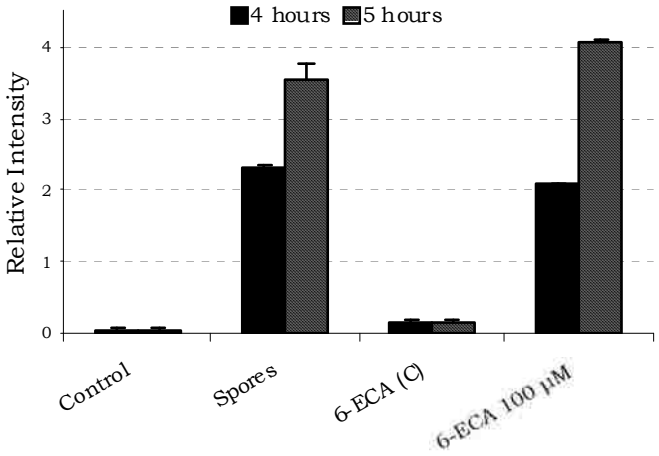


Figure 73. Effect of 6-ECA on J774a.1 cells at 4 and 5 hours. Cell necrosis was monitored by PI-DNA fluorescence. The rate of killing in treated cells was similar or slightly higher than the non-treated ones.

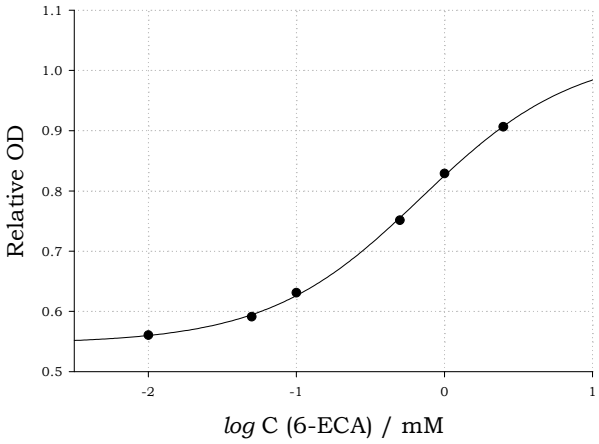


Figure 74. Dose-response curve for the inhibition of 6-ECA *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.697 mM was obtained by the regression (R^2=0.999 and *SE* = 0.006).

3.25 Cytotoxic Compounds

The following compounds selected from the screening have exerted a moderate to notorious cytopathic effect on J774a.1 macrophages. Cytotoxicity was assessed through PI-DNA fluorescence emission, in samples containing non-infected viable cells, with 100 μM of the respective treatment.

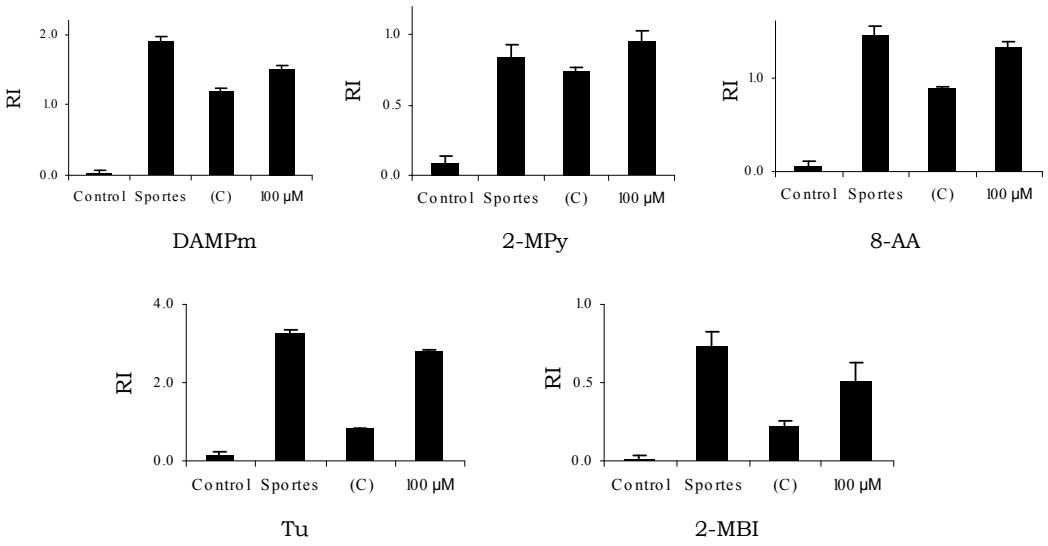


Figure 75. Analogues with cytopathic effect on J774a.1 cells. Fluorescence emission was taken at 6 hours after infecting with spores. An increase in relative intensity is observed in treated non-infected cells '(C)', in comparison with the control non-treated control.

3.26 Other Compounds

The remaining analogues used for the screening did not alter either the germination of the spores *in vitro*, or prevented necrosis in the macrophage cell line. The following table shows the results obtain for each of the compounds in the cell killing assay (monitored by fluorescence intensity), and in the analysis of germination *in vitro*. Concentrations of treatment were 100 μM and 1 mM respectively. Fluorescence readings were all taken at 6 hours after infecting cells.

Table 9. Analogues that affected neither germination nor spore-mediated killing by *B. anthracis*

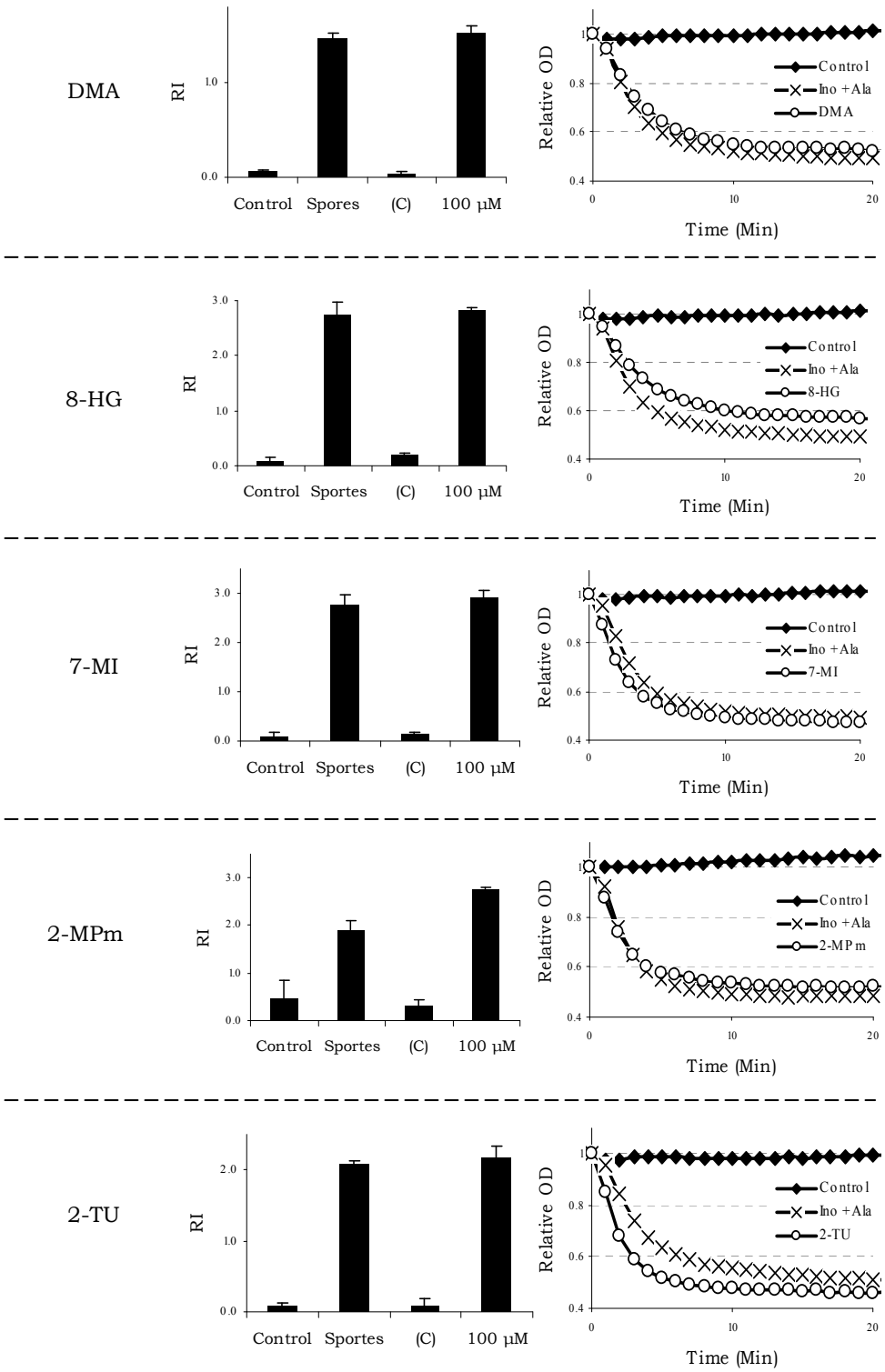
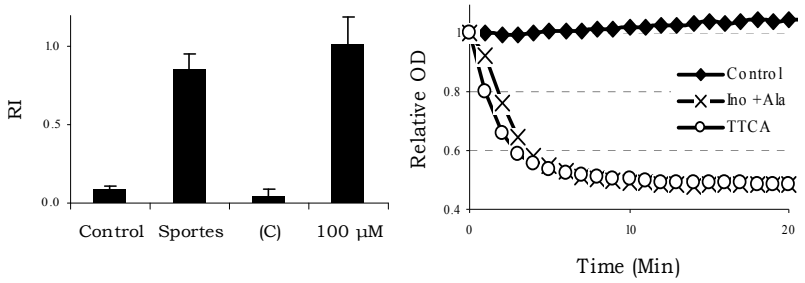
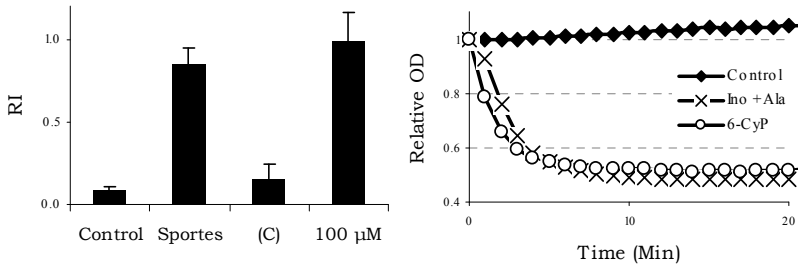


Table 9. (Continued from last page)

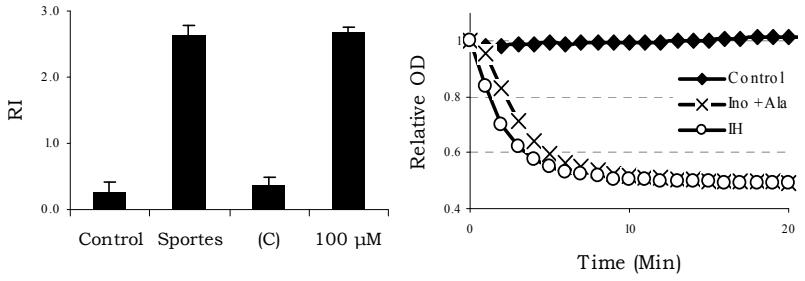
TTCA



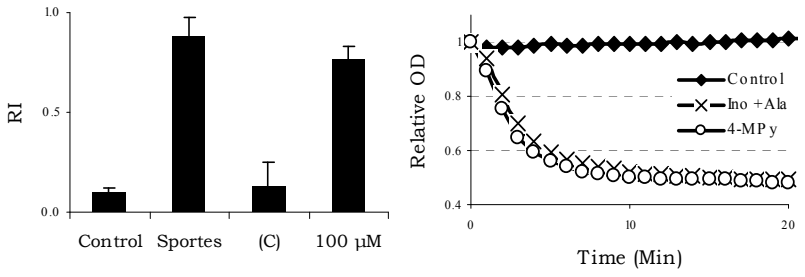
6-CyP



IH



4-MPy



6-CPR

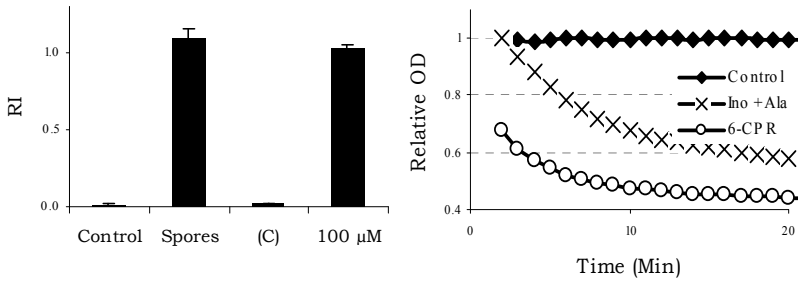
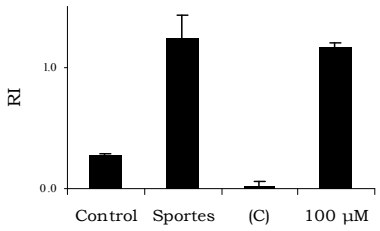
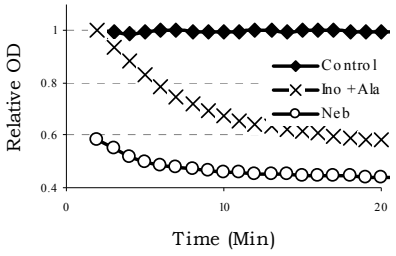


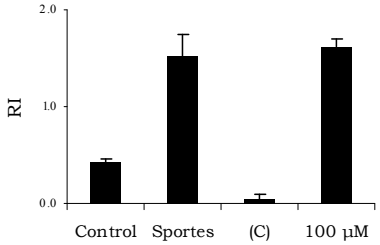
Table 9. (Continued from last page)

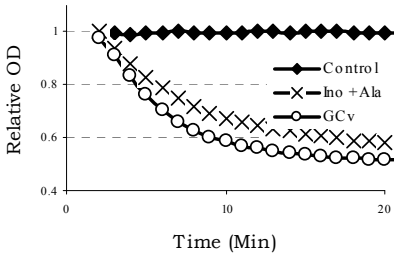
Neb



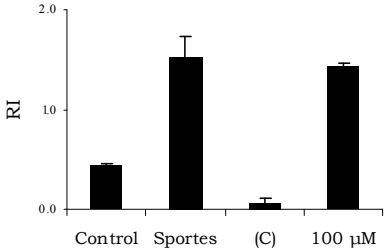


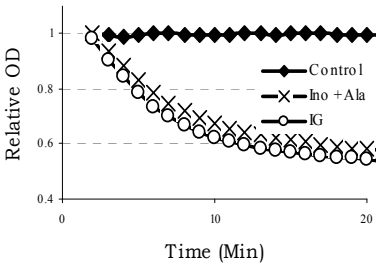
GCv



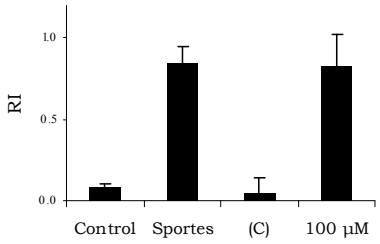


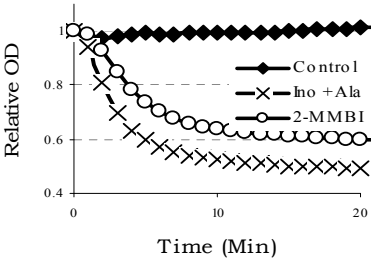
IG



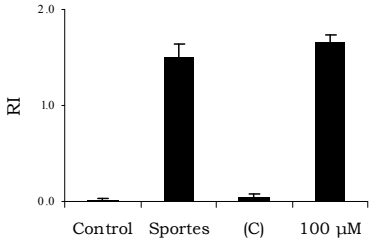


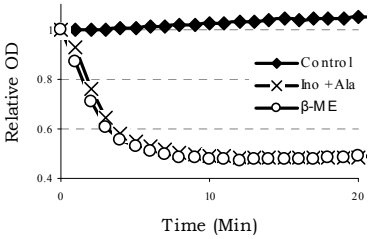
2-MMBI





BME





3.27 Analogues that enhanced cell killing

We have identified compounds that upon their addition the macrophages cell killing rate was moderately enhanced. These molecules did not act as either as 'germinants' or 'inhibitors' for the germination *in vitro,* and were not toxic to the cell line. The most notable compounds in this group were 6-NBA, BMA, IH, and 8-HG (figure 76). The enhancement is noticeable before they reached the signal saturation time (5 -6 hours).

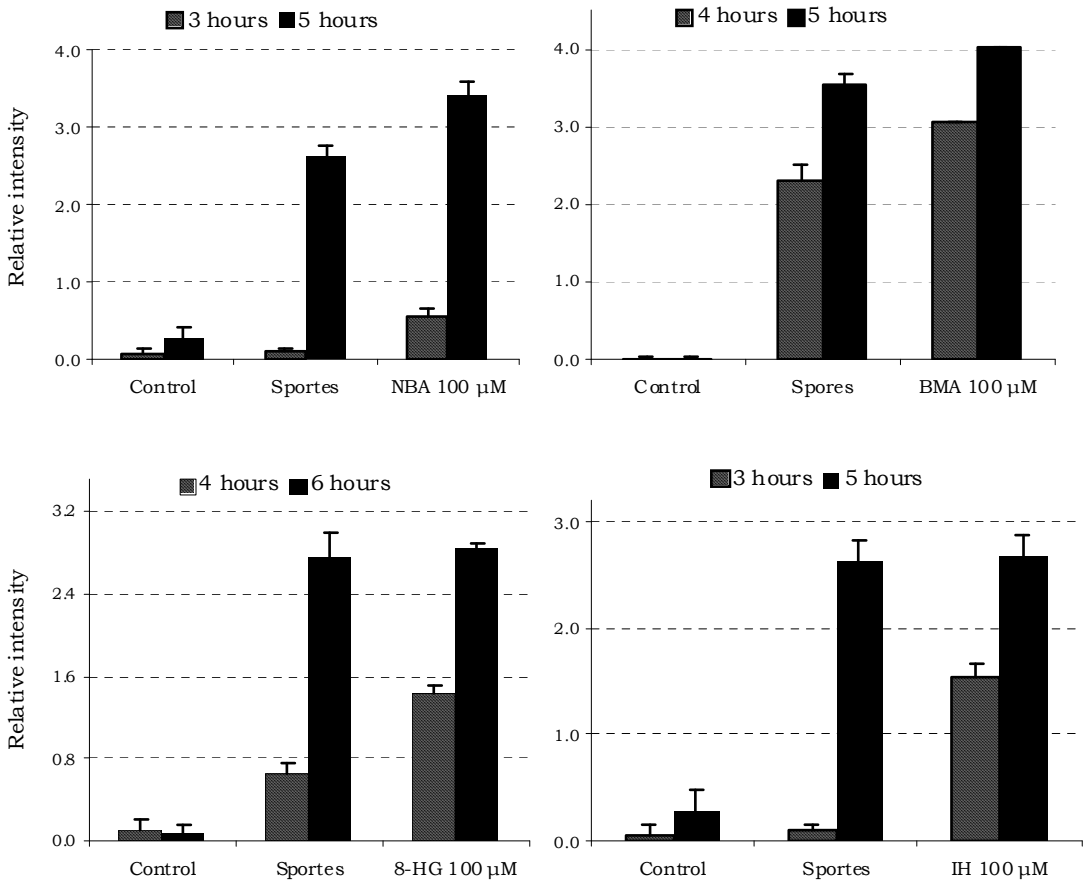


Figure 76. Killing enhancement of J774a.1 cells. Macrophages were treated with the respective nucleoside analogues at the dosis of 100 µM (6-NBA, BMA, 8-HG, and IH). The amount of non-viable cells was higher in the treated samples, in contrast with the non-treated control. The effect is observable before reaching the saturation time point (5-6 hours).

3.28 Cooperative Effect by *D*-alanine with Nucleoside Analogues

D-alanine is a natural anti-germinant for endospores of *B. anthracis*. This molecule can be synthesized by the alanine racemase present in the spore, and block germination pathways involving *L*-alanine as a germinant. Two research groups have already shown that *D*-alanine protects macrophages from *B. anthracis* toxin-mediated necrosis, by inhibiting the germination of the endospores inside the phagosome, and subsequent killing of the spores by the macrophages [97,98]. If *L*-alanine triggers the germination receptor GerL, then *D*-alanine will likely bind the same receptor. In the present investigations we corroborate the protecting results of *D*-Ala by obtaining half-maximal inhibitory concentrations for the germination *in vitro* and for the J774a.1 killing protection. IC_{50} assays were performed as in previous experiments (figures 77 and 78).

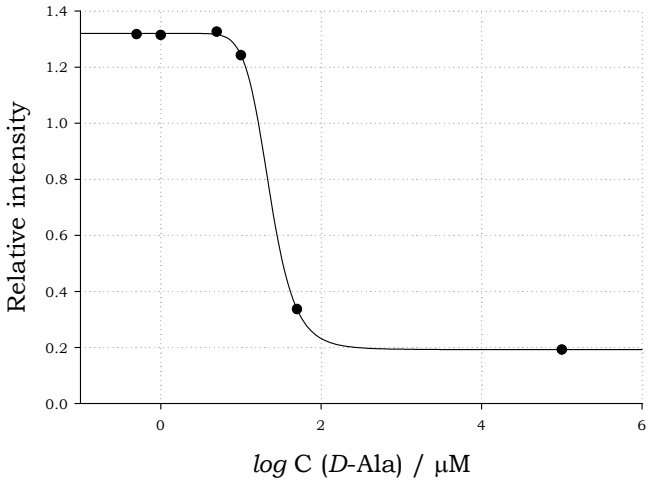


Figure 77. Dose-response curve for necrosis protection of J774a.1 by *D*-alanine. Data was taken at 4.5 hours of infection. An IC_{50} of 22.9 μM was obtained by regression analysis (R^2=0.999 and *SE* = 0.008).

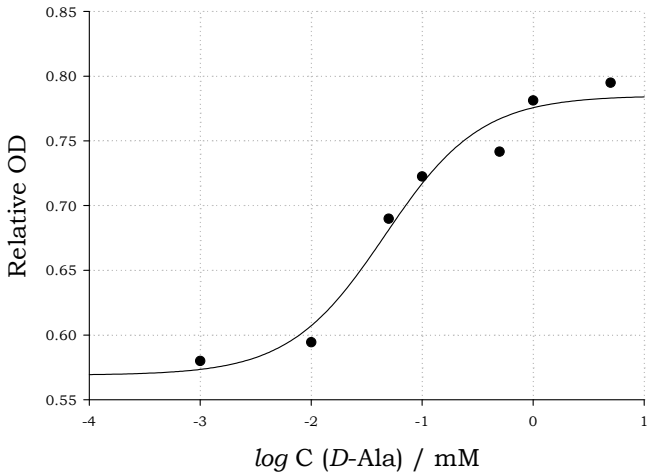


Figure 78. Dose-response curve for the inhibition of *D*-Ala *in vitro*. Data was selected from an arbitrary time point. An IC_{50} of 0.016 mM was obtained by regression analysis (R^2=0.974 and *SE* = 0.019).

The germination inhibition by *D*-Ala was also observed at light microscopy (figure 79). The following pictures illustrate the efficiency of the amino acid in preventing the germination after incubating the spores for 40 minutes in Luria-Bertani broth.

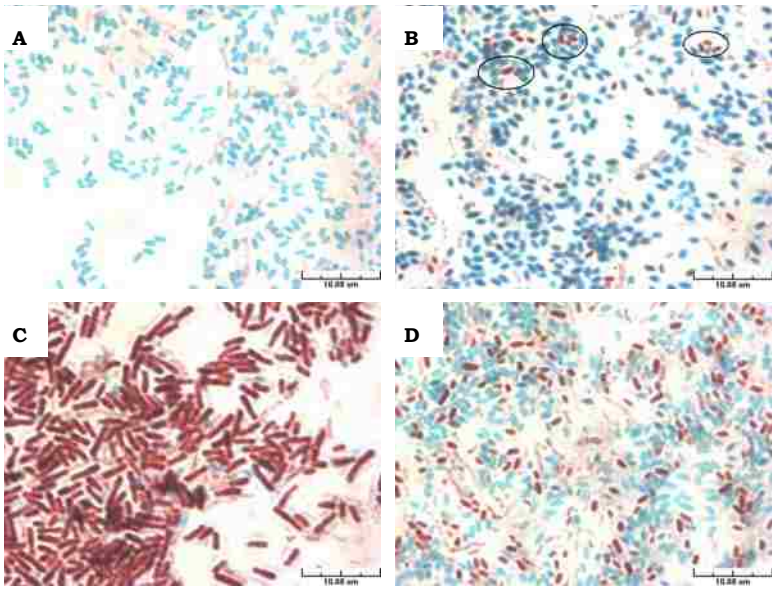


Figure 79. Effect of *D*-alanine observed at light microscopy. *B. anthracis* spores were incubated in LB broth, and samples were stained by Schaeffer-Fulton method. At time 0 (figure A) no germinating spores were observed. After five minutes in broth (B) some spores were already undergoing the first stages of germination. After 40 minutes in broth germination was completed for the majority of spores (C). Broth containing 10 mM *D*-alanine inhibited the germination considerable after 40 minutes (D).

The germination of *B. anthracis* takes place in the presence of extremely high concentrations of *L*-alanine. Nevertheless, only when *L*-Ala is combined with a nucleoside or another amino acid, germinants may act at physiological concentrations. A combination of two inhibitors, one nucleoside (6-TG) and one amino acid (*D*-Ala), could act in synergy inhibiting different pathways of germinations combined. A cell killing assay was performed with *D*-Ala and 6-TG at sub-optimal concentrations. Each of these in inhibitors for separate was not able to protect cells from necrosis at 6 hours. Combined together they showed a cooperative effect, protecting J774a.1 cells from toxin-mediated killing (figure 80).

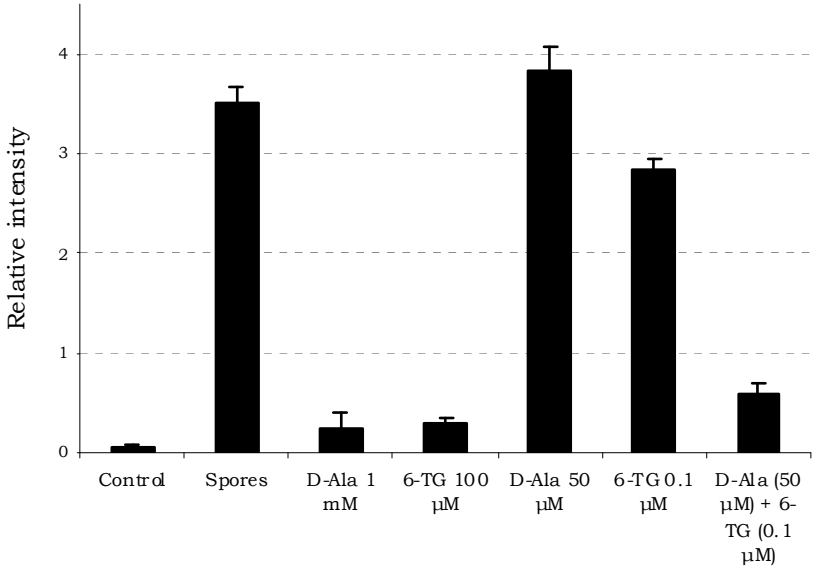


Figure 80. Cooperative effect of *D*-Ala and 6-TG. Six hours after infecting macrophages each of these inhibitors at sub-optimal concentrations failed to protect cells from necrosis (PI-DNA fluorescence increased near 4-fold from non-infected control). These compounds combined together at the same concentrations protected cell damage considerably.

Similar assays were performed with *D*-alanine in combination of two other nucleosides that prevented necrosis in cells: 6-TI and 6-CG (figure 81). A moderate cooperation with the amino acid was observed; the synergy shown was not as important as the one in previous experiment with 6-TG. These results confirm the hypothesis that

two anti-germinants of different nature (nucleoside and amino acid) enhance the protection of each of the anti-germinants alone.

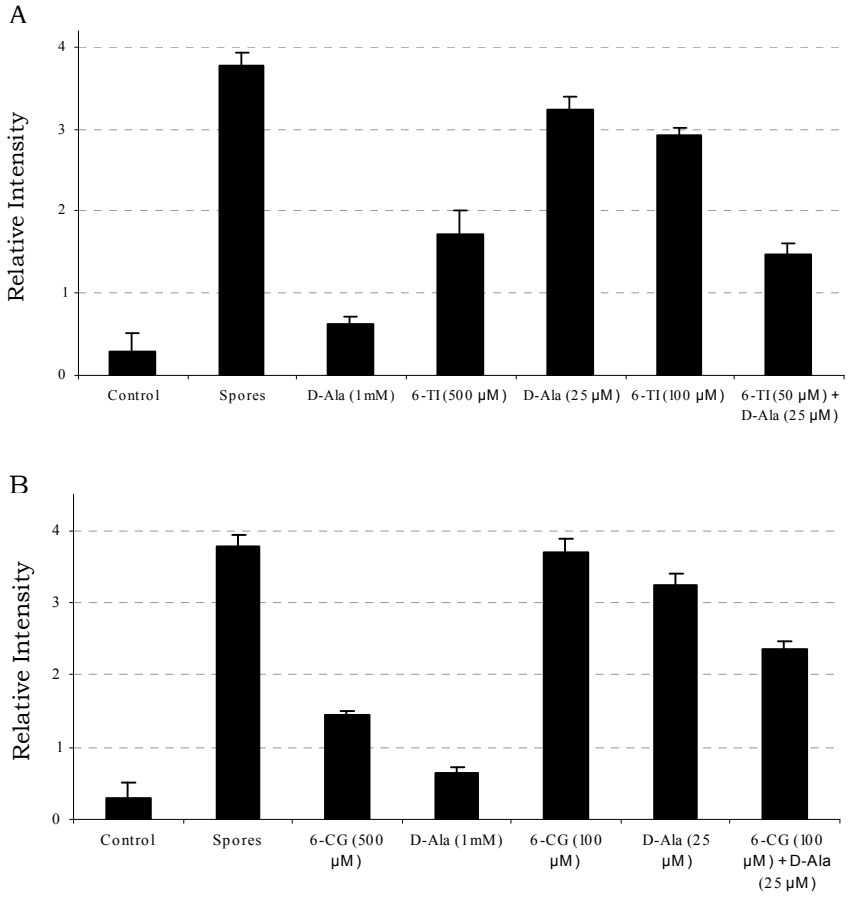
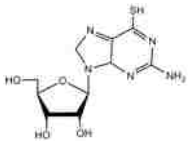
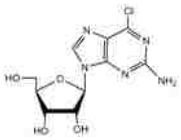
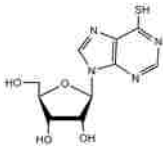
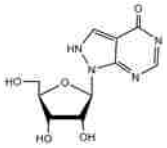
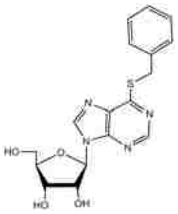
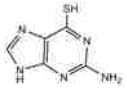
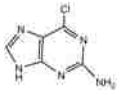
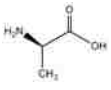


Figure 81. Cooperative effect of two nucleoside anti-germinants and *D*-Ala. Chart A shows effect of 6-TI, and chart B the effect of 6-CG. Five hours after infecting macrophages, each of these inhibitors at sub-optimal concentrations failed to protect cells from necrosis. When the nucleosides were treated in combination with *D*-Ala, necrosis response was diminished at that time.

3.29 Summary of Results

The following table represents the half-maximal inhibitory concentrations obtained for each of the analogues that protected J774a.1.

Table 10. Compounds that protect J774a.1 cells from *B. anthracis*-mediated killing.

Name	Acronym	Structure	IC_{50} (mM)
6-thioguanosine	6-TG		0.0035
6-chloroguanosine	6-CG		0.1894
6-thioinosine	6-TI		0.0351
Allopurinol riboside	APR		0.6461
6-benzylthioinosine	BTI		1.0449
6-thioguanine	6-Tg		0.0018
6-chloroguanine	6-Cg		0.2040
6-mercaptopurine	6-Mp		0.3981
D-alanine	*D*-Ala		0.0229

The following table represents the half-maximal inhibitory concentrations obtained for each of the analogues that inhibited the spore germination *in vitro*.

Table 11. Compounds that inhibited germination *B. anthracis* endospores *in vitro*.

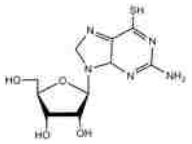
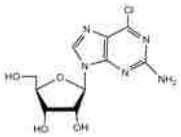
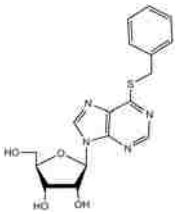
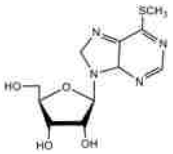
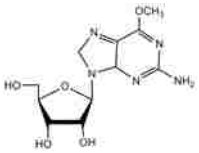
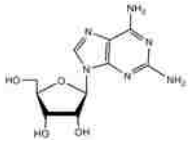
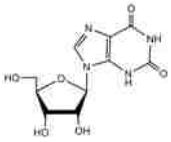
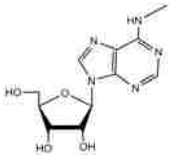
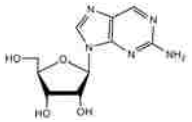
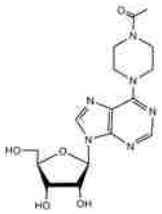
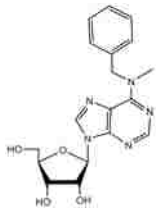
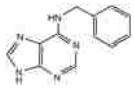
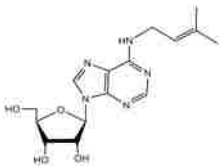
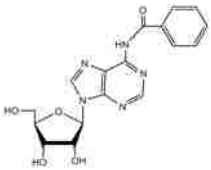
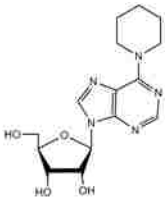
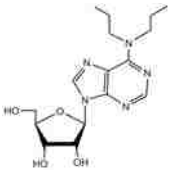
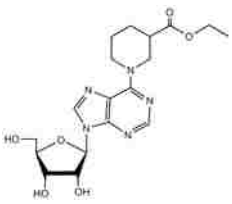
Name	Acronym	Structure	IC_{50} (mM)
6-thioguanosine	6-TG		1.0054
6-chloroguanosine	6-CG		0.8925
6-benzylthioinosine	BTI		0.0506
6-methylmercaptopurine riboside	6-MMPR		0.3180
6-*O*-methylguanosine	6-OMG		0.3488
2-aminoadenosine	2-AA		0.6977
Xanthosine	X		7.1914
6-methylaminopurine riboside	6-MAPR		0.1932
2-aminopurine riboside	2-APR		0.3793

Table 11. Compounds that inhibited germination *B. anthracis* endospores *in vitro* (continued from last page).

Name	Acronym	Structure	IC_{50} (mM)
1-(4-(9-(β)-ribofuranosyl)-9H-purin-6-yl)piperazin-1-yl)ethanone	PPER		0.0511
6-*N*-benzylmethyladenosine	6-BMA		0.1218
6-*N*-benzylaminopurine	BAP		1.5524
6-(γ,γ-dimethylallyl amino)purine riboside	DMAA		0.3642
6-*N*-benzoyladenosine	NBA		1.1426
6-*N*-cyclohexyladenosine	CHA		1.0890
6-*N*-dipropyladenosine	6-DPA		0.7556
6-*N*-((3-ethylacetyl)cyclohexyl) adenosine	6-ECA		0.6966

CHAPTER 4

DISCUSSION

4.1 J774a.1 killing by *Bacillus anthracis*

The present study confirms that mammalian macrophages J774a.1 can readily phagocyte endospores of *B. anthracis*. Direct observation through phase-contrast microscopy shows that after infecting with an m.o.i. of 5, phagocytosis takes place in a time of 60 to 90 minutes. Washing cells and replacing medium at 80 minutes removes most of the free spores in the medium that were not phagocytosized. Spore germination within the macrophages occurs within 2-3 hours after infecting. Germinated spores lose their characteristic birefringence, but their oval-shaped morphology remains the same. It is not until the spore maturation when necrotic macrophages started to appear, after 3-4 hours; protein expression starts at stage 5 of germination (subchapter 1.4.1). Toxin activity does not come until spores are germinated. Action by LF/EF is essential for the survival of the germinated spore inside the macrophage. [27]. Germinated bacilli were able to kill and escape from the cell. An ultimate cell-lysis led by the action of the anthrax toxins allows freedom of vegetative bacteria, and their rapid proliferation in DMEM medium.

It is not clear, however, if killing took place by necrosis, apoptosis, or a combination of both. Recent studies found that LF can induce either caspase-1 mediated necrosis or apoptosis, depending on the macrophage genotype. Bone marrow derived macrophages (BMMs) from mouse strain BALB/c undergo a rapid necrosis by LF [105]. J774a.1 comes from the same mouse strain. Killing response in these cells is fast: when necrosis starts there is a lag-phase from 3 to 4 hours. Within an hour the signal from PI-DNA reaches saturation at 5 hours (figure 82). Thus, most of the cells are killed within one hour approximately. This fast killing is more likely to be a necrosis instead of apoptosis. Rapid apoptosis led by LPS or disruption Mek3/6 MAP-kinases pathways occurs in certain macrophages or dendritic cells within 8 hours at least. On the other hand, EF is

not cytotoxic by itself, but alters cell homeostasis by increasing cAMP [46]. Both toxins together contribute synergistically to cytotoxicity inside the macrophages.

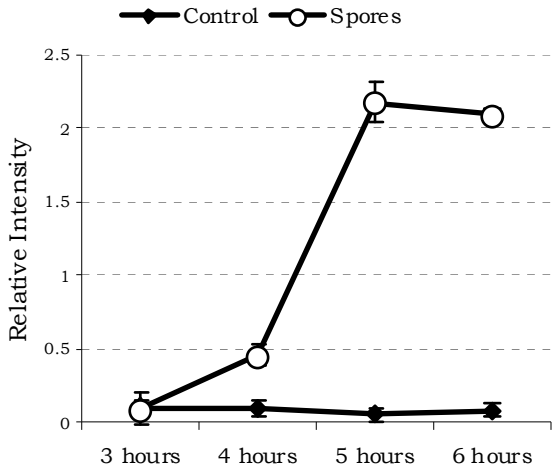


Figure 82. Killing of J774a.1 within time. After 3 hours of infection, there is no significant increase in fluorescence intensity by PI-DNA. Some killing started at 4 hours and within an hour a maximum fluorescence signal is reached, that correlates with a fast necrotic response led by the anthrax toxins.

4.2 Structure activity relationships

The data in chapter 3 confirms the protective action of the nucleoside 6-TG against the spore germination and subsequent toxin-mediated killing of macrophages. Few analogues derived from 6-TG have also exerted protection with higher IC_{50} than the first one. Among the nucleoside compounds, 6-CG, 6-TI, APR, and BTI were found to show a protective effect in cells. These compounds all have in common a *sp3* hybridized exocyclic atom in position 6 of the purine base, with the exception of APR. 6-TG and 6-CG are guanosine derivatives with an amino group as substituent in position 2 of the ring.

Analysis of half-maximal inhibitory concentrations of compounds that inhibit cell necrosis shows that those compounds having an exocyclic 2-amino are stronger inhibitors than those that do not have. The difference between 6-thioguanosine and 6-

thioinosine is the 2-amino of the ring; the first one has the amino group and an IC_{50} 100-fold smaller than the last one. An analogous example is the effect that exerts 6-CG and 6-CPR. Both have an exocyclic atom of chlorine in position 6, although the first has also the 2-amino. The first one protects cell killing at an IC_{50} near 0.9 mM, where as the other has no effect in cells or *in vitro*. We may conclude that 2-amino shows a significant interaction with the receptor that enhances inhibitory effect. Compounds with an un-substituted 2 position may act as inhibitors, depending on the effect exerted by the 6 position group. On the other hand mono-substituted purine with 2-amino inhibits germination *in vitro* but not in cells (2-APR), which means that 2-amino exerts inhibitory action by its own, without the contribution by the group at position 6.

An amino group in position 2 suggests the possibility of a dipole-dipole electrostatic interaction with residues in the binding pocket of the receptor. Other groups in this position, such as 2-oxo, (X, IG) had little to no effect in the germination inhibition *in vitro*. The type of interaction should be a bifurcated hydrogen bonding, being the two hydrogens of the amino groups the possible donors. We hypothesized the hydrogen bonding type because it is a strong intermolecular interaction; adding a 2-amino increases the inhibitory potency considerably. Other possible lower energy forces electrostatic can be ruled out in this case.

Results show that position 6 is even more relevant for the inhibition than 2-amino. All of the compounds that prevented spore germination in cells have an exocyclic group in position 6. This group contains an electronegative atom bonded to the C_6 of the purine with *sp3* hybridization. Chemical properties of this group will contribute or hinder the inhibitory effect. The facts that affect the inhibition potency conferred by 6-position of the ring are the following:

a) *Electronegativity*. – The atom linked to C_6 from the exocyclic group is usually an electronegative element (O, Cl, N, or S). E. Abel-Santos et *al* (in press) showed that 6-group electronegativity is correlated to the inhibition constant (K_i) of *B. cereus* spore germination; Log (K_i) decreased in a lineal relationship with electro-

negativity of the group. Results obtained in *B. anthracis* showed no correlation (table 12).

Table 12. Electronegativity of some C_6-groups compared to IC_{50}s *in vitro*

Chemical group	Electronegativity†	K_i /mM (*B. cereus*)§	IC_{50} / mM (*B. anthracis*)
$-OCH_3$	3.09	0.112	0.35
$-N(CH_3)_2$	2.78	0.025	N/A
$-N(H)CH_3$	2.74	0.017	0.19
$-SCH_3$	2.52	0.008	0.31

† Values given by 'super atom' approximation based on the Pauling scale.
§Data provided by Dr. E. Abel-Santos, University of Nevada Las Vegas (not published yet).

Although electronegativity is relevant, we conclude that there was no direct relationship between electronegativity and IC_{50} for inhibiting *B. anthracis* endospore germination. Furthermore, electronegativity-IC_{50} cannot be correlated for protective effects in cells either. The results showed that any *in vitro* inhibitor having nitrogen as the electronegative element (adenosine derivates) did not protect macrophages from necrosis. Compounds that protected cells from killing have S, Cl, or O. The most notable difference between these elements and nitrogen is that the latter one has a valence of 3. Compounds having protective effect contained electro-poor atoms with a valence of 1 or 2. Nitrogen may be linked to the C_6 and have other two side chains; the remaining electronegative elements will have one or no side chains. It would be possible to consider that there is steric hindrance for this fact; but even the 6-*N* derivatives that have one side chain solely (6-MAPR) did not show any effect on cells as they did *in vitro*. A hypothesis to explain this situation is that adenosine derivatives show affinity for another enzyme implicated in the nucleoside metabolism of the mammalian cell; these analogues will be either chemically transformed, or will bind irreversibly to a protein or enzyme in the cell, obstructing them from inhibiting Ger receptor activation.

These premises lead to hypothesize that 6-group inhibitory potency relies on the strength of the dipole moment created by the electronegative element. The best inhibitors have a relatively low electronegativity in 6-group (C^6-SR, C^6-SH, C^6-NR) leading to dipole-dipole interactions. On the other hand, germinants have a strong polarity in the 6-substituent conferred by the presence of highly electronegative atoms (C^6=O, C^6-NH_2) leading to stronger electrostatic interactions of the hydrogen bonding type. 6-group is likely to be a hydrogen bond acceptor of a donor belonging to side chains of residues from the binding pocket. This hydrogen bonding will induce a conformational change in the receptor, triggering its activation and germination signaling.

b) *Sp3 hybridization*. – The electro-poor atom that is bonded to C_6 of the ring must have a *sp3* hybridization to inhibit receptor activation. When the orbital hybridization of this atom is of *sp2*, such as in the 6-oxo group (guanosine, inosine, xanthosine, immucillin H), the analogue will trigger, more or less, spore germination *in vitro* (APR is the only exception to this rule). Even though 6-oxo shows keto-enol tautomerism in purine bases, 3*H*-enol forms are less abundant in physiological conditions [107]. Enol forms (-OH) have a *sp3* orbital hybridization but concentrations are minimal, showing no significant inhibitory effect *in vitro* or in cells. In the case of 6-OMG, there is an oxygen atom bonded to a methyl group (-OCH_3), and having a *sp3* orbital: this compound decreases the germination rate *in vitro*. We conclude that *sp3* hybridization of the atom directly connected to C_6 of the purine is required for 6-groups to hinder the activation of the Ger receptor.

c) *Length and morphology of the side chain*. – The 6-exocyclic side chain extension does not necessarily cause an steric hindrance for ligand-receptor binding. Most of the compounds having side chains at 6-position, bound to an electronegative atom (C_6-S-R, C_6-N-R, C_6-O-R) inhibited the *in vitro* endospore germination efficiently, no matter the length of these chains. Bulky side chains from

adenosine derivatives hindered germination *in vitro* but not in cells, whereas long side chains of 6-thioinosine derivatives (6-BTI) also prevented germination within the macrophages.

Strikingly, analogues with small side chains (6-MAPR, 6-OMG, 6-MMPR) showed no protection on cells, even though they hampered *in vitro* germination. 6-MMPR has a methyl side chain, and 6-BTI has benzyl group; the first one does not prevent germination within macrophages, in contrast with the second one. *In vitro,* their IC_{50} are approximately 50 µM for 6-BTI, and 320 µM for 6-MMPR; this suggests the existence of interactions of either London dispersion forces, or π-π stacking with the benzyl group, that increase affinity of the analogue with the receptor.

Analogues having tertiary amine at C_6, inhibited germination with the presence of bulky side-chains (6-DPA, BMA, ECA, CHA, PPER), but showed little to no effect with small side groups, like methyl (6-DMA). Tertiary amines have lower polarity. The fact of having bulky groups as substituent is possibly adding more interactions (London dispersion forces, π-π stacking), that increase ligand-receptor affinity. 6-DMA (C^6-N-$(CH_3)_2$) does not hinder germination, contrary to 6-MAPR (C^6-NH-(CH_3)): the latter one has a higher polarity, allowing a stronger dipole-dipole interaction. 6-DPA has similar polarity to 6-DMA, although the first inhibits germination (IC_{50}=0.75 mM). 6-DPA propyls have a stronger Van Der Waals interactions than metyhls from 6-DMA. To summarize these ideas, we conclude that the size of the 6-exocyclic group does not add steric hindrance for the binding with the germination receptor, and interactions from additional forces by bulky groups may increase the affinity of the analogue.

d) *Hydrophobicity*. – Solubility and size of the molecule seems to be relevant for the diffusion of the compound in cells. The best example is given by the protective action of 6-TG and 6-BTI, both having an exocyclic sulfur group. 6-TG, with a non-substituted thiol at position 6, has an IC_{50} of 3.5 x 10^{-3} mM for the

inhibition of cell necrosis. 6-BTI has a benzyl side chain, and its IC_{50} is near 1 mM; a thousand times higher than 6-TG. Partition coefficients (*Log*P)[17] are -0.43 and 1.18 for 6-TG and 6-BTI respectively. Strikingly, *in vitro*, this inhibitory potency is inverted, with 6-BTI having and IC_{50} 1000-fold smaller than 6-TG (50 to 1000 μM). Hence, 6-BTI has a higher affinity for the receptor, since more intermolecular forces are implicated, albeit it has a limited diffusion or solubility in cell medium due to its high hydrophobicity and size. 6-MMPR has IC_{50} of 0.32 mM *in vitro*, but a *Log*P of 0.02; this implies that its solubility in cell medium limits the concentration available for cell diffusion. Even though 6-BTI has less solubility, its IC_{50} is 100-fold smaller than the one of 6-MMPR.

In addition to the 2 and 6 substitutions in the purine, other structural facts from the nucleosides are to be considered. The importance of the ribosyl sugar that binds the N^9 of the purine was assessed by using ribosyl-free compounds and a modified 9-*N* non-sugar substituent (GCv). It was found that the 9-*N*-ribosyl is required for the inhibition of the germination *in vitro*. None of the purine base derivates studied (6-Tg, 6-Cg, 6-Mp, Ap, 6-CyP, 2-MBI, 2-MMBI) were able to affect germination with only one exception: benzylaminopurine (BAP). BAP was the only base screened having a side chain attached to N^6. Compared to the other bases, BAP is likely to have additional interactions of the type of π-π stacking by the benzyl group, or induced dipoles (London dispersion forces). Surprisingly, results *in vitro* did not correlate with the screening in J774a.1 macrophages. 6-Tg, 6-Cg, and 6-Mp are the bases that correspond to the nucleosides 6-TG, 6-CG, and 6-TI respectively. These bases exerted a moderate to high protection of cells from necrosis. Some studies have found that 6-Tg and 6-Mp are transformed into 6-TG-monophosphate (6-TGMP), and 6-TI-monophosphate (6-TIMP) in lymphoma cell lines, by the enzyme hypoxanthine-guanine phosphoribosyl transferase

[17] *Log*P is defined as the partition coefficient of an un-ionized compound, given by the ratio of the concentration of solute in octanol divide by the concentration of the solute in water. (*Log*P = *Log* $([solute]_{octanol}/[solute]_{water})$. It is used in organic chemistry and pharmaceutical sciences to measure the hydrophobicity of a substance.

(HGPRT) [108, 109]. We suggest that also 6-Cg may be transformed to 6-CGMP. The presence of the 5'-nucleotidase-I in the cytososol [110], promotes the degradation of monophosphate nucleotides to nucleosides. Thus 6-TGMP, 6-CGMP, and 6-TIMP are converted to the nucleoside 6-TG, 6-CG, and 6-TI respectively, which inhibit spore germination inside the phagolysosome (figure 83).

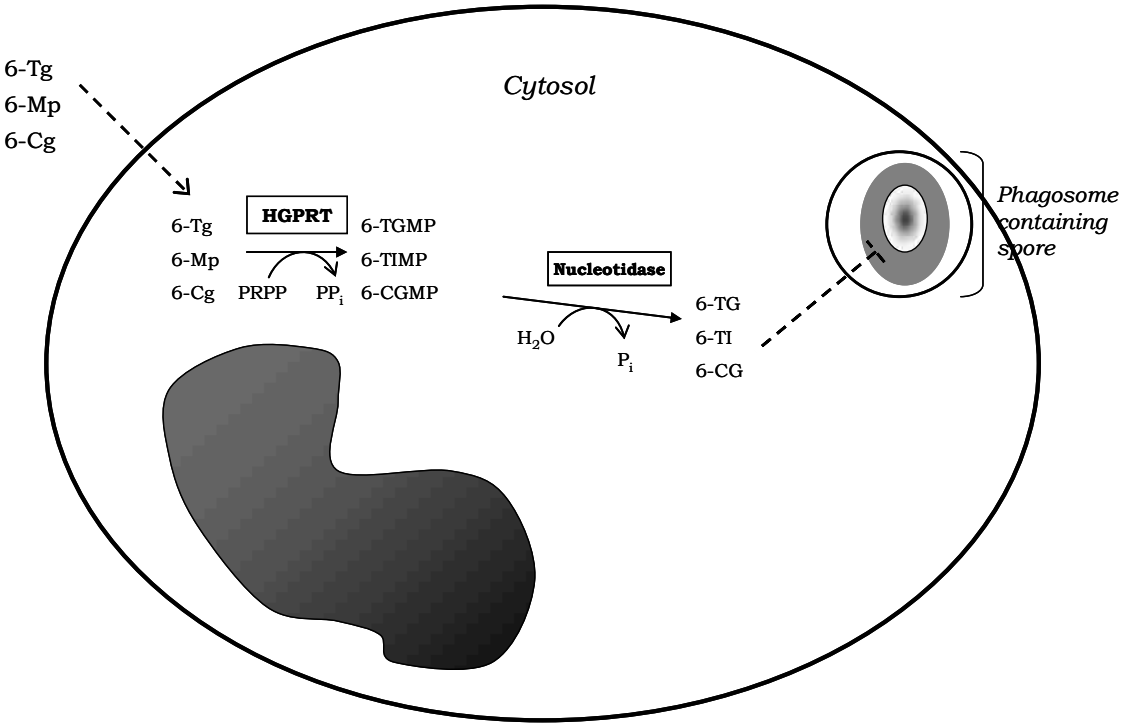


Figure 83. Purine base derivates inhibit spore germination inside the cell. Bases are converted into their respective nucleosides, by a two-step enzymatic catalysis.

The ribosyl groups are necessary for the optimal ligand-receptor binding. Hydrogen bonding between the hydroxyl groups and amino acid residues is possible. Gancyclovir (GCv) contains a 9-*N* substituent different than a ribose sugar. It has 2-hydroxy groups that may interact electrostatically, in a similar way to hydroxyl groups of the ribose. Analysis *in vitro* did not show a significant contribution by GCv; it did not trigger or inhibit germination. The plane formed ribose is perpendicular to the base, and distance

between hydroxyl groups is different than in GCv. Thus, conformational disposition of hydroxyls is relevant for the hydrogen bonding interactions.

Substitutions in 7 or 8 position of the purine ring (7-MI, 8-HG) had little to no effect on germination. Groups in those positions could present steric hindrance for the binding with the catalytic pocket or the receptor, impeding the binding to the receptor. Disposition of nitrogen hetero-atoms among the ring does not necessarily hinder binding, but decreases affinity considerably. APR and IH had no significant effect on spore germination *in vitro*; both have a 6-oxo (similar to inosine) but lacking N hetero-atom at position 7. Then, N^7 of the purine should contribute to interactions with the binding site.

Finally, the possibility that the six-member pyridine type ring has inhibitory effect by its own can be ruled out. Pyridine derivatives (2-MPm, 2-TU, TTCA, DAMPm, 2-MPy, 4-MPy) did not affect spore germination or protected cells from killing. The sulfur group does not inhibit germination by its own either (β-mercaptoethanol). Therefore, the integrity of the purine ring is essential for triggering or blocking the germination receptor activation.

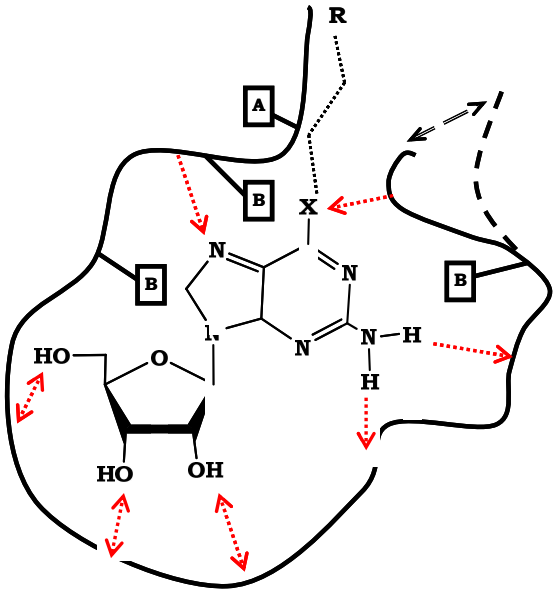


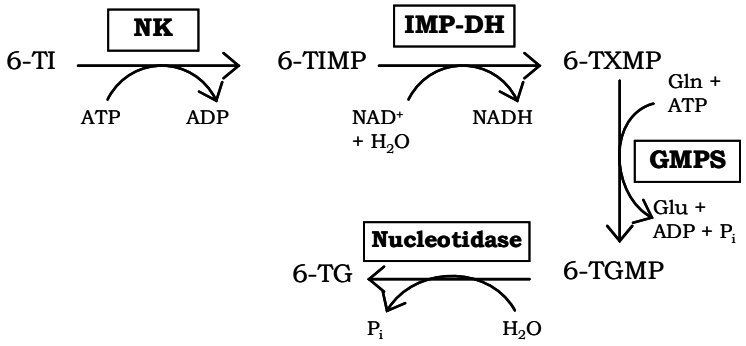
Figure 84. Model for nucleoside recognition by the receptor GerH in *B. anthracis*. Hydrogen bonding interactions are represented by the red arrows. Direction of these arrows indicates the hydrogen acceptor. Double-sense arrows show a hypothetical bonding in which acceptor-donor is not defined. 'B' refers to bulky residues that sterically restrict nucleoside binding. 'A' represents an aromatic residue for a potential π-π stacking with the C^6 side chain. 'X' is an electronegative element and 'R' is a bulky side chain. The presence of a H-bond with X induces a conformational change in the catalytic pocket, triggering Ger receptor activation.

4.3 Exceptional Cases: 6-TI and APR

The obtained data reveals that 6-thioinosine (6-TI) hindered cell necrosis, with an IC_{50} of 35 µM approximately. *In vitro*, 6-TI does not have a significant interaction with the spore germination of *B. anthracis* [99]. These results suggests that 6-TI itself have little to no affinity to the receptor. Electronegativity of the 'mercapto' group and thio-derivates is moderately smaller than alcohols or amines. A decreased dipole moment of 'mercapto' groups diminishes the dipole-dipole interactions with the receptor. In addition, the lack of amino group substituting the C^2 of the ring reduces hydrogen bonding interactions with the binding site. 6-TI and 6-MMPR are very close structures. Although electronegativity of their 6-substituent is similar, 6-TI have a higher solubility in medium (LogP = -0.81) than 6-MMPR (LogP = 0.02). 6-TI can be diffused easier than 6-MMPR due to a lower molecular weight and higher solubility in in water; however 6-TI

does not inhibit spore germination. Since there is no possible steric or solubility hindrance, we suggest that 6-TI will actually bind the Ger receptor with a lower affinity than germinants and slightly trigger spore germination, due to a weak dipole interaction by the 'thio' group.

6-TI exerts protection in cells in contrast with the results obtained *in vitro*. 6-TI is likely to undergo enzymatic transformation. 6-TI is very similar to inosine. In the metabolism of nucleotides, inosine mono-phosphate (IMP) is oxidized to xanthosine monophosphate (XMP) by IMP-Dehydrogenase. XMP is subsequently transformed to guanosinie monophosphate (GMP) by GMP-synthase [111]. Nucleosides entering to the cytosol are converted to mono-phosphate nucleotides by the nucleotide kinases: 6-TI is converted to 6-TIMP. 6-TIMP may undergo the same reactions as IMP, due to a minimal structural difference. 6-TIMP is oxidized to 6-thioxanthosine mono-phosphate (6-TXP), and finally converted to 6-TGMP. 6-TGMP is degraded to 6-TG by cytosolic or lysosomal nucleotidases. 6-TG will then inhibit spore germination. The mechanism is shown below:



Allopurinol riboside (APR) is a peculiar case. This purine nucleoside analogue shows a moderate protective effect in the macrophage cell line, having an IC_{50} close to 0.65 mM. APR shows no effect on the germination *in vitro*, and its base (Ap) does not hamper spore germination *in vitro* or inside the cells. APR is likely to be modified ezymatically *in vivo* into another compound that binds and inhibit spore germination.

APR has been used a drug to inhibit the growth of the protozoans *Leishmania* sp. and *Trypanosoma* sp. in humans [112, 113]. This compound is converted to 4-aminopyrazolo(3,4-*d*)pyrimidine riboside or aminopurinol riboside (AmPR), which is incorporated to the RNA, being toxic for the promastigotes. APR is selectively toxic because it is not catalyzed by the same enzymes in humans [112], or in mouse [114]. In mammalian cells, APR is metabolized to IMP analogues, whereas in the protozoan APR is converted either to ATP or GTP analogues. We suggest that APR, which has a strong homology with inosine, will be catalyzed by the same enzymes that convert IMP into GMP (IMP-deydrogenase and GTP-synthase), leading to the analogue 2-aminoallopurinol riboside mono-phosphate (2A-APRMP). Alternatively, APRMP could be catalyzed into aminopurinol mono-phosphate (AmPRMP) by adenylsuccinate synthetase and adenylsuccinate lyase. Either 2A-APR or AmPR may be acting as inhibitors for *B. anthracis* germination. Further investigations with these two analogues *in vitro* will be required.

Allopurinol (Ap), the corresponding base for APR, is a drug used for the treatment of hyperuricemia [115]. The base has no effect regarding the germination triggering or inhibition. It is a structural isomer of hypoxanthine, and efficiently inhibits the enzyme 'Xanthine oxidase', obstructing the pathway for the synthesis of uric acid. Ap may also undergo the savage pathway catalyzed HGPRT, and be converted into APRMP [116]. Ap is mostly converted into oxypurinol by xanthine oxidase, rather than APR. Only 10% of the Ap is converted to APR [117]. These findings demonstrate that only extremely high concentrations of Ap will lead to significant concentrations of APR to prevent spore germination inside the macrophages.

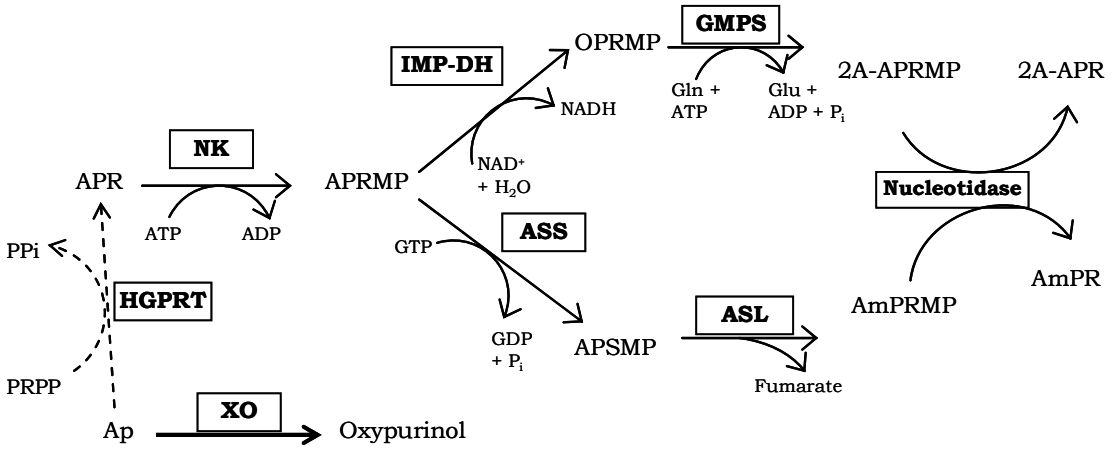


Figure 85. Hypothetic mechanism for the enzymatic transformation of APR in mammalian cells. The corresponding abbreviations are Ap (allopurinol), APR (allopurinol riboside), PRPP (5-phospho-α-*D*-ribosyl-1-pyrophpate), NK (Nucleotide kinase), XO (xanthine oxydase), HGPRT (hypoxanthine-guanine phophoribosyl transferase), APRMP (allopurinol mono-phosphate), ORPMP (oxypurinol mono-phosphate), IMP-DH (inosine mono-phosphate dehydrogenase), GMPS (guanosine mono-phosphate synthetase), ASS (adenylo-succinate synthetase), APSMP (allopurinoyl succinate mono-phosphate), ASL (adenylosuccinate lyase), 2A-APR (2-aminoallopurinol riboside), AmPR (Aminopurinol riboside).

4.4 Inhibitors or Weak Germinants

It was previously mentioned that nucleoside derivatives are competitive inhibitors of germinants (sub-chapter 1.5.1). In our studies, anti-germinant protection in J774a.1 only lasted for a period comprehended in 6 hours approximately. After certain amount of time, endospores in treated cells started to germinate, eventually leading to a rapid necrosis of the macrophages. Nucleoside anti-germinants are not fully inhibiting but slowing the kinetics of the germination. Each compound shows a varying pattern of protection in time. 6-TG and 6-Tg were the stronger inhibitors. Event though they impeded cellular necrosis within 6 hours of infection, after that time was passed, there was an increasing number of necrotic cells. Weaker inhibitors, such as 6-TI or 6-CG, protected more or less within 5 hours, and at 6 hours most of the treated cells lost their viability (figure 86).

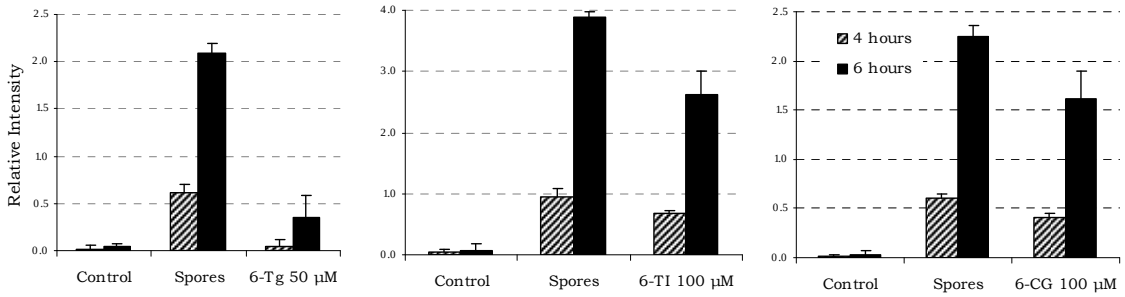


Figure 86. Eventual loss of protection by the nucleoside anti-germinants. Protective effect may last up to 6 hours in strong inhibitors (6-Tg). In weaker inhibitors (6-CG, 6-TI), the kinetics of cell killing slowed down in contrast with the non-treated cells; after 6 hours, most of the cells were killed.

From these observations, we propose that nucleoside inhibitors can be considered weak germinants. Since the hydrogen bonding interaction with the 6-exocyclic group is the key to trigger receptor activation, it can be inferred that anti-germinants have a group with less electronegativity and lower dipole moment that diminishes the strength of the hydrogen bonding interaction. Nevertheless, dipole-dipole electrostatic interactions are possible; this will trigger certain conformational change in the pocket to activate the receptor. This activation is, however, considerably less than the one by natural nucleoside germinants (inosine, guanosine, and adenosine), and other nucleoside analogues having a higher polarity in the 6-group (IG, X, APR, 6-CPR).

4.5 Inhibitory Contribution by *D*-alanine

Results included in sub-chapter 3.27 demonstrate a cooperative effect existing with the interaction of *D*-alanine and nucleoside inhibitors: 6-TG protected macrophages at sub-optimal concentrations in combination with *D*-alanine, in contrast with the nucleoside alone. This finding fits into the multiple-pathway model for *B. anthracis* germination activation proposed by P. Hanna and colleagues [68], addressed in sub-chapter 1.4. *D*-alanine inhibits the GerL receptors, which contribute to at least three different pathways: Alanine pathway, AAID-1 (amino acid and inosine dependent pathway), and AEA (aromatic amino acid enhanced alanine pathway). Nucleoside

inhibitors bind the receptor GerH, hindering AAID-1 and AAID-2 pathways. Then, we have an additional pathway (AAID-2) that is blocked by the nucleoside analogues. Furthermore, the mixture of nucleoside anti-germinants and *D*-alanine will block the AAID pathway via GerX activation, considering that contributions by this receptor are relevant for spore germination within the macrophages [26].

In addition, Hanna's multiple-pathway model states that two or more pathways activated by germinants contribute synergistically to the rate of germination of the endospore. The present work corroborates such principle with the finding that two inhibitors affecting more than one pathway will show a cooperative effect in preventing germination and cell killing of macrophages.

4.6 General Conclusions and Further Work

The present study has conducted an extensive screening in a wide variety of nucleoside analogues, to identify their effect on the germination of *B. anthracis* endospores. Besides clarifying previous work on the germination inhibition *in vitro*, new insights have been gained on the mechanism of germination inhibition inside mammalian cells. An exhaustive analysis of the data provided sufficient information to determine relevant molecular interactions involved in the binding of the ligand with the germination receptor. Our findings elucidate a plausible model for receptor activation, in which the conformational change in the receptor is induced by a crucial hydrogen bonding interaction with the 6-exocyclic group of the purine ring.

This model corresponds with the idea that nucleoside inhibitors do not impede the germination *per se*, but affect the velocity of the receptor activation, behaving as weak germinants. The establishment of a hydrogen bonding between C^6-electronegative element of the nucleoside and the residue of the active site is given by the dipole moment of such element. When such polarity is not enough to create an electrostatic interaction of hydrogen bond type, the molecule will bind the active site without

triggering the conformational change in the receptor. Thus, we consider inhibitors as weak germinants with light dipole interactions may lead to sporadic activation

Further work on this area should be done for validating these hypotheses. The characterization of GerHA protein is recommended, in order to elucidate the three-dimensional conformation and motifs that shape the active site. Characterization should be achieved by the performance of studies of x-ray crystallography and mass spectrometry. Such information will reveal the identity of the residues that are involved in the binding with the purine nucleosides.

Investigation on antigerminants for *B. anthracis* should continue. This work must be aimed in the finding of a stronger and non-toxic inhibitor as an immediate prophylaxis to stop anthrax pathogenesis. It is recommended a chemical synthesis for a nucleoside derivate having a strong affinity for the GerH receptor, but without polarity in the 6-exocyclic chain; lacking the electro-poor element. Such molecule should be able to block the binding site without interacting with the catalytic residues that trigger the receptor activation.

Perfecting anti-germination drugs will contribute significantly with biodefense concerns. These drugs could represent the must immediate antidote against the commencement of anthrax pathogenesis upon the inhalation of the spores. The development of novel antigerminant agents may even serve as new approach for prophylaxes against diseases caused by other sporulating bacteria. Such treatment could be efficient for preventing severe systemic infections by *Bacillus* and *Clostridium* genera, including tetanus and CDAD.

BIBLIOGRAPHY

1. Berkeley, R., M. Heyndrickx, et al. (2002). "Application and systematics of *Bacillus* and Relatives." (C. 1 and 5) pp. 8-22; 47-63.
2. Doi, R. H. and M. McGloughin (1992). "Biology of Bacilli: Applications to Industry." (C. 1): 1-18.
3. Balows, A., H. G. Truper, et al. (1981). "The Prokaryotes." (C. 76): 1662 - 1768.
4. Nicholson, W. L., N. Munakata, et al. (2000). "Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments." *Microbiol. Mol. Biol. Rev.* 64(3): 548-572.
5. Dixon, T. C., M. Meselson, et al. (1999). "Anthrax." N Engl *J Med* 341(11): 815-826.
6. "Biosafety in Microbiological and Biomedical Laboratories" (1999) Center of Disease, Control and Prevention.
7. Mock, M. l. and A. s. Fouet (2001). "Anthrax." *Ann Rev Microbiol* 55(1): 647-671.
8. Dürst, U., Spiegel M., et *al* (1968). "Anthraxmeningitis" *Scheiz Med Woch* 116: 1222-122
9. Sternbach, G. (2003). "The History of Anthrax." *J Emerg Med* 24(4): 463-467.8.
10. Alexandra, M. (2001) "A Brief History of Anthrax." http://lhncbc.nlm.nih.gov/apdb/phsHistory/resources/pdf/anthrax.pdf
11. Alibek, K., Handelman, S. (1999) "Biohazard: The Chilling True Story of the Largest Covert Biological Weapons Program in the World". Delta Press.
12. Meselson, M., Guillemin, J. et al. (1994). "The Sverdlovsk anthrax outbreak of 1979." *Science* 266(5188): 1202-8.
13. Center of Disease Control and Prevention (2007) "Bioterrorism" http://emergency.cdc.gov/bioterrorism/
14. Atlas, R. M. (2002). "Bioterrorism: From Threat to Reality." *Ann Rev Microbiol* 56(1): 167-185.
15. Federal Bureau of Investigation. (2006) "Amerithrax Fact Sheet". http://www.fbi.gov/anthrax/amerithrax_factsheet.htm
16. Welkos, S. L., N. J. Vietri, et al. (1993). "Non-toxigenic derivatives of the Ames strain of Bacillus anthracis are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence." *Microb Pathog* 14(5): 381-388.
17. Little, S. F. and G. B. Knudson (1986). "Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in the guinea pig." *Infect Immun* 52(2): 509-512.
18. Willman, David (2008). "Apparent suicide in anthrax case". *Los Angeles Times*
19. Knight, J. (2001). "Bioweapons: Delivering death in the mail." *Nature* 414(6866): 837-838.
20. Franco, C., Deitch, S. (2007) "Billions For Biodefense: Federal Agency Biodefense Funding, Fy2007–Fy2008" *Biosec Bioterr* 5 (2): 117-133.
21. Metcalfe, N. (2002). "A short history of biological warfare." *Med Confl Surviv* 18(3): 271-82.
22. Athamna, A., M. Athamna, et al. (2004). "Selection of *Bacillus anthracis* isolates resistant to antibiotics." *J Antimicrob Chemother* 54(2): 424-428.
23. Davies, J. (1982) "A major epidemic of anthrax in Zimbabwe" *Cent Afr J Med* 28: 291-298.
24. Russell, B. H., et al. (2007). "*Bacillus anthracis* internalization by human fibroblasts and epithelial cells." *Cell Microb* 9(5): 1262-1274.
25. Brachman, P. S. (1980) "Inhalational anthrax" *Ann NY Acad Sci* 353: 89-93.
26. Guidi-Rontani, C., et al. (1999). Germination of *Bacillus anthracis* spores within alveolar macrophages. *Mol Microbiol* 31(1): 9-17.
27. Guidi-Rontani, C., et al. (2001). Fate of germinated Bacillus anthracis spores in primary murine macrophages. *Mol Microbiol* 42(4): 931-8.

28. Prescott, L., Harley, J et Al (2000) “Microbiología” McGraw Hill, Spain. Capítulo 23: 507-524.
29. Saile, E. and Koehler, T. M. (2006). “*Bacillus anthracis* Multiplication, Persistence, and Genetic Exchange in the Rhizosphere of Grass Plants” *Appl Environ Microbiol* 72(5): 3168-3174
30. Helgason, E., et al. (2000). “Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis: one species on the basis of genetic evidence”. *Appl Environ Microbiol* 66(6): 2627-30.
31. Read, T. D., et al. (2003). The genome sequence of Bacillus anthracis Ames and comparison to closely related bacteria. *Nature* 423(6935): 81-86.
32. Gould, G. W., Hurst, A. (1969) “The Bacterial Spore” Academic Press, NY.
33. Zickner B, Dürre P. (2002) “Changes in protein synthesis and identification of proteins specifically induced during solventogenesis in Clostridium acetobutylicum”. *Electrophoresis* 23 (1):110-21.
34. Piggot, P. J. and Hilbert, D. W. (2004). “Sporulation of Bacillus subtilis”. *Curr Op Microbiol* 7(6): 579-586.
35. Driks, A. (2002). "Maximum shields: the assembly and function of the bacterial spore coat." *Trends Microbiol* 10(6): 251-254.
36. Setlow, P. (2006). "Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals." *J Appl Microbiol* 101(3): 514-525.
37. Moir, A., B. M. Corfe, et al. (2002). "Spore germination." *Cell Mol Life Sci* 59(3): 403-9.
38. Sylvestre, P., Couture-Tosi, E., and Mock, M. (2005) “Contribution of ExsFA and ExsFB Proteins to the Localization of BclA on the Spore Surface and to the Stability of the Bacillus anthracis Exosporium”. *J. Bacteriol.* 187: 5122-5128.
39. Chesnokova, O. N., S. A. McPherson, et al. (2009). "The Spore-Specific Alanine Racemase of Bacillus anthracis and Its Role in Suppressing Germination during Spore Development." *J. Bacteriol.* 191(4): 1303-1310.
40. Oliva, C. R., M. K. Swiecki, et al. (2008). "The integrin Mac-1 (CR3) mediates internalization and directs Bacillus anthracis spores into professional phagocytes." *Proc Natl Acad Sci* 105(4): 1261-1267.
41. Greenblatt, C. L., A. Davis, et al. (1999). "Diversity of Microorganisms Isolated from Amber." *Microbial Ecology* 38(1): 58-68.
42. Setlow, P. (2007). "I will survive: DNA protection in bacterial spores." *Trends in Microbiology* 15(4): 172-180.
43. Paidhungat, M., B. Setlow, et al. (2000). "Characterization of Spores of Bacillus subtilis Which Lack Dipicolinic Acid." *J. Bacteriol.* 182(19): 5505-5512.
44. Ascenzi, P., P. Visca, et al. (2002). "Anthrax toxin: a tripartite lethal combination." *FEBS Letters* 531(3): 384-388.
45. Bradley, K. A., J. Mogridge, et al. (2001). "Identification of the cellular receptor for anthrax toxin." *Nature* 414(6860): 225-229.
46. Moayeri, M. and S. H. Leppla (2009) "Cellular and systemic effects of anthrax lethal toxin and edema toxin." *Molecular Aspects of Medicine* In Press, Corrected Proof.
47. Guidi-Rontani, C. (2002). "The alveolar macrophage: the Trojan horse of Bacillus anthracis." *Trends in Microbiology* 10(9): 405-409.
48. Premanandan, C., C. A. Storozuk, et al. (2009). "Complement protein C3 binding to Bacillus anthracis spores enhances phagocytosis by human macrophages." *Microbial Pathogenesis* In Press, Corrected Proof.
49. Hoover, D. L., A. M. Friedlander, et al. (1994). "Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP." *Infect. Immun.* 62(10): 4432-4439.

50. McKevitt, M., S. Barua, et al. (2009). "The Mechanism of Bacillus anthracis Intracellular Germination Requires Multiple and Highly Diverse Genetic Loci." *Infect. Immun.* 77(1): 23-31.
51. Dixon, T. C., A. A. Fadl, et al. (2000). "Early Bacillus anthracis and macrophage interactions: intracellular survival and escape." *Cell Microbiol* 2(6): 453-463.
52. Shatalin, K., I. Gusarov, et al. (2008). "Bacillus anthracis-derived nitric oxide is essential for pathogen virulence and survival in macrophages." *Proc Natl Acad Sci* 105(3): 1009-1013.
53. Michèle Mock, T. M. (2003). "Anthrax toxins and the host: a story of intimacy." *Cell Microbiol* 5(1): 15-23.
54. Hanna, P. C. and J. A. W. Ireland (1999). "Understanding Bacillus anthracis pathogenesis." *Trends Microbiol* 7(5): 180-182.
55. Moir, A., B. M. Corfe, et al. (2002). "Spore germination." *Cell Mol Life Sci* 59(3): 403-409.
56. Ireland, J. A. and P. C. Hanna (2002). "Amino acid and purine ribonucleoside induced germination of Bacillus anthracis DeltaSterne endospores: gerS mediates responses to aromatic ring structures." *J Bacteriol* 184(5): 1296-1303.
57. Behravan, J., H. Chirakkal, et al. (2000). "Mutations in the gerP Locus of Bacillus subtilis and Bacillus cereus Affect Access of Germinants to Their Targets in Spores." *J. Bacteriol.* 182(7): 1987-1994.
58. Alvarez, Z., Abel-Santos, E. (2007). "Potential use of inhibitors of bacteria spore germination in the prophylactic treatment of anthrax and Clostridium difficile-associated disease." *Exp Rev Anti Infect. Ther.* 5(5): 783-792.
59. Setlow, P. (2003). "Spore germination." *Curr Opin Microb* 6(6): 550-556.
60. Mitchell, C., J. F. Skomurski, et al. (1986). "Effect of ion channel blockers on germination of Bacillus megaterium spores." *FEMS Microbiol Letters* 34(2): 211-214.
61. Setlow, B., E. Melly, et al. (2001). "Properties of Spores of Bacillus subtilis Blocked at an Intermediate Stage in Spore Germination." *J. Bacteriol.* 183(16): 4894-4899.
62. Tovar-Rojo, F., M. Chander, et al. (2002). "The Products of the spoVA Operon Are Involved in Dipicolinic Acid Uptake into Developing Spores of Bacillus subtilis." *J. Bacteriol.* 184(2): 584-587.
63. Southworth, T. W., A. A. Guffanti, et al. (2001). "GerN, an Endospore Germination Protein of Bacillus cereus is a Na+/H+-K+ Antiporter." *J. Bacteriol.* 183(20): 5896-5903.
64. Setlow, P. (2007). "I will survive: DNA protection in bacterial spores." *Trends Microbiol* 15(4): 172-180.
65. Harry, E. J. (2001). "Coordinating DNA replication with cell division: lessons from outgrowing spores." *Biochimie* 83(1): 75-81.
66. Moir, A., E. H. Kemp, et al. (1994). "The genetic analysis of bacterial spore germination." *Soc Appl Bacteriol Symp Ser* 23: 9S-16S.
67. Hornstra, L. M., Y. P. de Vries, et al. (2006). "Characterization of germination receptors of Bacillus cereus ATCC 14579." *Appl Environ Microbiol* 72(1): 44-53.
68. Fisher, N. and P. Hanna (2005). "Characterization of Bacillus anthracis Germinant Receptors In Vitro." *J. Bacteriol.* 187(23): 8055-8062.
69. Guidi-Rontani, C., Y. Pereira, et al. (1999). "Identification and characterization of a germination operon on the virulence plasmid pXO1 of Bacillus anthracis." *Mol Microbiol* 33(2): 407-14.
70. Corfe, B. M., A. Moir, et al. (1994). "Analysis of the expression and regulation of the gerB spore germination operon of Bacillus subtilis 168." *Microbiology* 140 (11): 3079-83.
71. Zuberi, A. R., I. M. Feavers, et al. (1985). "Identification of three complementation units in the gerA spore germination locus of Bacillus subtilis." *J. Bacteriol.* 162(2): 756-762.

72. Clements, M. O. and A. Moir (1998). "Role of the gerI Operon of Bacillus cereus 569 in the Response of Spores to Germinants." *J. Bacteriol.* 180(24): 6729-6735.
73. Takami, H., K. Nakasone, et al. (2000). "Complete genome sequence of the alkaliphilic bacterium Bacillus halodurans and genomic sequence comparison with Bacillus subtilis." *Nucl. Acids Res.* 28(21): 4317-4331
74. Ireland, J. A. W. and P. C. Hanna (2002). "Amino Acid- and Purine Ribonucleoside-Induced Germination of Bacillus anthracis Delta-Sterne Endospores: gerS Mediates Responses to Aromatic Ring Structures." *J. Bacteriol.* 184(5): 1296-1303.
75. Powell, J. F. (1950). "Factors Affecting the Germination of Thick Suspensions of Bacillus subtilis Spores in L-Alanine Solution." *J Gen Microbiol* 4(3): 330-338.
76. Hills, G. M. (1949). "Chemical Factors in the germination of spore bearing aerobes. The effects of amino acids on the germination of Bacillus anthracis, with some observations on the relation of optimal form to biological activity. ." *Biochem J* (45): 8.
77. Abriouel, H., M. Maqueda, et al. (2002). "Inhibition of bacterial growth, enterotoxin production, and spore outgrowth in strains of Bacillus cereus by Bacteriocin AS-48." *Appl. Environ. Microbiol.* 68(3): 1473-1477.
78. Foster, S. J. and K. Johnstone (1986). "The use of inhibitors to identify early events during Bacillus megaterium KM spore germination." *Biochem J* 237(3): 865-70.
79. Rowley, D. B., and Feeherry, F. (1970) "Conditions affecting germination of Clostridium botulinum 62A spores in a chemically defined medium" *J. Bacteriol.* 104, 1151-1157.
80. Sanchez-Plata, M. X., et al. (2005). "Predictive model for Clostridium perfringens growth in roast beef during cooling and inhibition of spore germination and outgrowth by organic acid salts". *J Food Prot* 68(12): 2594-605.
81. Yasuda-Yasaki, Y., Namiki-Kanie, S. and Hachisuka, Y. (1978). "Inhibition of Bacillus subtilis spore germination by various hydrophobic compounds: demonstration of hydrophobic character of the L-alanine receptor site". *J. Bacteriol.* 136(2): 484-490
82. Trujillo, R. and Laible, N. (1970). "Reversible inhibition of spore germination by alcohols". *Appl Microbiol* 20(4): 620-624.
83. Boschwitz, H., et al. (1983). "Effect of inhibitors of trypsin-like proteolytic enzymes Bacillus cereus T spore germination". *J. Bacteriol.* 153(2): 700-708.
84. Foster, S. J. and Johnstone, K. (1986). "The use of inhibitors to identify early events during Bacillus megaterium KM spore germination". *Biochem J* 237(3): 865-70.
85. Cortezzo, D. E., Setlow, B. and Setlow, P. (2004). "Analysis of the action of compounds that inhibit the germination of spores of Bacillus species". *J Appl Microbiol* 96(4): 725-741.
86. Brown, W. C. M., D. Sanford, W. Granada, S. (1990). "N-Acetylmuramic acid inhibits spore germination and germination enzymes". *J. Basic Microbiol* 30(2): 67-72
87. Morris, S., Walsh, R. and Hansen, J. (1984). "Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action". *J. Biol. Chem.* 259(21): 13590-13594.
88. Yasuda, Y., et al. (1982). "Quantitative structure-inhibitory activity relationships of phenols and fatty acids for Bacillus subtilis spore germination". *J. Med. Chem.* 25(3): 315-320.
89. Craven, S. E. and Blankenship, L. C. (1985). "Activation and injury of Clostridium perfringens spores by alcohols". *Appl. Environ. Microbiol.* 50(2): 249-256.
90. Sacks, L. E. (1990). "Chemical germination of native and cation-exchanged bacterial spores with trifluoperazine". *Appl. Environ. Microbiol.* 56(4): 1185-1187.

91. Seward, R. A., Deibel, R. H. and Lindsay, R. C. (1982). “Effects of potassium sorbate and other antibotulinal agents on germination and outgrowth of Clostridium botulinum type E spores in microcultures”. *Appl. Environ. Microbiol.* 44(5): 1212-1221.
92. Mazzotta, A. S. and Montville, T. J. (1999). “Characterization of fatty acid composition, spore germination, and thermal resistance in a nisin-resistant mutant of Clostridium botulinum 169B and in the wild-type strain”. *Appl. Environ. Microbiol.* 65(2): 659-664.
93. Gut, I. M., et al. (2008). “Inhibition of Bacillus anthracis Spore Outgrowth by Nisin. Antimicrob”. *Agents Chemother.* 52(12): 4281-4288.
94. Mantipragada, S. B., et al. (2003). “Lipid-protein interactions and effect of local anesthetics in acetylcholine receptor-rich Membranes from Torpedo marmorata electric organ”. *Biochemistry* 42(30): 9167-9175.
95. Fey, G., Gould, G. W. and Hitchins, A. D. (1964). “Identification of d-Alanine as the Auto-Inhibitor of Germination of Bacillus globigii Spores”. *J Gen Microbiol* 35(2): 229-236.
96. Church, B. D., Halvorson, H. and Halvorson, H. O. (1954). “Studies On Spore Germination: Its Independence From Alanine Racemase Activity”. *J. Bacteriol.* 68(4): 393-399.
97. Hu, H., Emerson, J. and Aronson, A. I. (2007). “Factors involved in the germination and inactivation of Bacillus anthracis spores in murine primary macrophages”. *FEMS Microbiology Letters* 272(2): 245-250.
98. McKevitt, M. T., et al. (2007). “Effects of Endogenous D-Alanine Synthesis and Autoinhibition of Bacillus anthracis Germination on In Vitro and In Vivo Infections”. *Infect. Immun.* 75(12): 5726-5734.
99. Akoachere, M., et al. (2007). “Identification of an in Vivo Inhibitor of Bacillus anthracis Spore Germination”. *J. Biol. Chem.* 282(16): 12112-12118.
100. Hanna, P. C. and Weiner, M. A. (2003). “Macrophage-Mediated Germination of Bacillus anthracis Endospores Requires the gerH Operon”. *Infect. Immun.* 71(7): 3954-3959.
101. Hu, H., et al. (2006). “Inactivation of Bacillus anthracis spores in murine primary macrophages”. *Cell Microbiol* 8(10): 1634-1642.
102. Ralph, P. and Nakoinz, I. (1975). “Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line”. *Nature* 257(5525): 393-394.
103. Christie, A. and Butler, M. (1994) “Growth and Metabolism of a Murine Hybridoma in Cultures Containing Glutamine-based Dipeptides”. *FOCUS*® 16, 1, 9.
104. Brojatsch, J., et al. (2006). “Mitochondrial Impairment Mediates Cytolysis in Anthrax Lethal Toxin-Treated Murine Macrophages”. *Cell Cycle* 6(1): 6-12.
105. Cheng, H. C. (2001). “The power issue: determination of KB or Ki from IC50: A closer look at the Cheng-Prusoff equation, the Schild plot and related power equations”. *J Pharm Toxicol Met* 46(2): 61-71.
106. Alileche, A., et al. (2005). “Anthrax lethal toxin-mediated killing of human and murine dendritic cells impairs the adaptive immune response”. *PLoS Pathog* 1(2): e19.
107. Chung, G., Oh, H. and Lee, D. (2005). “Tautomerism and isomerism of guanine-cytosine DNA base pair: Ab initio and density functional theory approaches. Journal of Molecular Structure” *The O Chem* 730(1-3): 241-249.
108. Brockman, R. W. (1963). “Biochemical Aspects of Mercaptopurine Inhibition and Resistance”. *Cancer Res* 23: 1191-1201.
109. Elgemeie, G. (2003). “Thioguanine, mercaptopurine: their analogs and nucleosides as antimetabolites”. *Curr Pharm Des* 9(31): 2627-42.
110. Hunsucker, S. A., Spychala, J. and Mitchell, B. S. (2001). “Human Cytosolic 5'-Nucleotidase I”. *J Biol Chem* 276(13): 10498-10504.
111. Elion, G. (1989). The purine path to chemotherapy. *Science* 244(4900): 41-47.

112. Nelson DJ, L. S., Elion GB, Marr JJ, Berens RL (1979). Comparative metabolism of a new antileishmanial agent, allopurinol riboside, in the parasite and the host cell. . *Adv Exp Med Biol.* 1979(122B): 7-12.
113. Were, J. and Saphiro, T. (1993). "Effects of Probenecid on the Pharmacokinetics of Allopurinol Riboside. *Antimicrob. Agents Chemother*". 37(5): 1193-1196.
114. LaFon, S. W., et al. (1985). "Inosine analogs. Their metabolism in mouse L cells and in Leishmania donovani". *J Biol Chem* 260(17): 9660-9665.
115. Pacher, P., Nivorozhkin, A. and Szabo, C. (2006). "Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol". *Pharmacol Rev* 58(1): 87-114.
116. Reiter, S., et al. (1983). "On the metabolism of allopurinol: Formation of allopurinol-1-riboside in purine nucleoside phosphorylase deficiency". *Biochem Pharmacol* 32(14): 2167-2174.
117. Nelson, D. J., et al. (1979). "Metabolism of pyrazolo(3,4-d)pyrimidines in Leishmania braziliensis and Leishmania donovani. Allopurinol, oxipurinol, and 4-aminopyrazolo(3,4-d)pyrimidine". *J Biol Chem* 254(10): 3959-3964.

VITA

Graduate College
University of Nevada, Las Vegas

Zadkiel R. Álvarez

Degrees:

Bachelor of Science in Biochemistry, 2005
University of Navarra. Pamplona, Spain

Master of Biotechnology, 2007
Aliter Business School. Madrid, Spain

Publications:

Alvarez, Z. and Abel-Santos, E. (2007). Potential use of inhibitors of bacteria spore germination in the prophylactic treatment of anthrax and *Clostridium difficile*-associated disease. Expert Review of Anti-infective Therapy 5(5): 783-792.

Thesis Title:

Protection of macrophages J774a.1 by purine nucleoside analogues from *B. anthracis* mediated necrosis.

Thesis Examination Committee:

Chairperson, Ernesto Abel-Santos, Ph.D.
Committee Member, Chulsung Bae, Ph. D.
Committee Member, Ronald Gary, Ph. D.
Graduate Faculty Representative, Helen Wing, Ph. D.