

THE USE OF SYNTHETIC OLIGOSACCHARIDES AND GLYCOCONJUGATES TO
PROBE IMMUNOLOGICAL AND BIOLOGICAL PROCESSES

by

JINKENG ASONG

(Under the Direction of Geert-Jan Boons)

ABSTRACT

The importance of cellular oligosaccharides and glycoconjugates to critical biological processes such as bacterial and viral infection, leukocyte-endothelial cell adhesion, and immunological recognition of tumor cells, has made them attractive targets for synthetic medicinal chemists. Following microbial detection, over-activation of innate immune responses can lead to sepsis. The molecular basis for innate immune detection of bacterial cell wall peptidoglycan (PGN), a component of the cell wall of almost all bacteria, by the germline encoded innate immune cell expressed Toll-like receptor 2 (TLR2) is poorly understood. In this dissertation, it was demonstrated through surface plasmon resonance technology and cellular activation studies that TLR2 binds PGN fragments derived from both Gram- positive and -negative bacteria. Furthermore, the structural and functional significance of PGN partial structures were analyzed as potential indicator of bacterial endotoxicity. The established structure-activity relationships also demonstrated that host and pathogen can exploit structural differences between PGN of different bacterial strains to modulate or evade immune detection.

Next, a glycoconjugate construct of Mannostatin A was analyzed for selective inhibition of golgi α -mannosidase. Mannostatin A, originally isolated from the soil microorganism *Streptoverticillus*, is one of the most potent inhibitor of class II α -mannosidase known to interfere in the biosynthesis of oligosaccharides critical in tumor progression and metastasis. Here, a feedback pro-drug inhibitor of mannostatin A (aminocyclopentitetrol as control) was designed and screened for selective α -mannosidase inhibition. Through *in vitro* and cell based studies, it was determined that the enzyme dependent fragmentation of the pro-drug results to increase local concentration around the enzyme resulting in selective inhibition of mannosidases.

INDEX WORDS: Peptidoglycan, Toll-like receptor 2, Surface Plasmon Resonance Technology, Mannostatin A, Aminocyclopeptitetrol, Mannosidase, Pro-drug, Gold nanoparticles

THE USE OF SYNTHETIC OLIGOSACCHARIDES AND GLYCOCONJUGATES TO
PROBE IMMUNOLOGICAL AND BIOLOGICAL PROCESSES

by

JINKENG ASONG

B.S., University of Buea, Cameroon, 1999

M.S., University of Buea, Cameroon, 2003

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

© 2009

Jinkeng Asong

All Rights Reserved

THE USE OF SYNTHETIC OLIGOSACCHARIDES AND GLYCOCONJUGATES TO
PROBE IMMUNOLOGICAL AND BIOLOGICAL PROCESSES

by

JINKENG ASONG

Major Professor: Geert-Jan Boons

Committee: Jonathan Amster
Robert Phillips

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2009

DEDICATION

To my dear father, ASONGLEFAC LAWRENCE NJUZY and beloved mum, JULIE ACHA NWO, I am sincerely thankful to them for being such wonderful parents. Their unwavering belief in me has been a blessing throughout all these years.

ACKNOWLEDGEMENTS

This piece of work has been nurtured in its development by the contributions of many people. I remain forever grateful to Professor Geert-Jan Boons for proposing the research topics and allowing me to work in his group. Besides helping me overcome many difficulties, his useful criticisms and directives consistently challenged my thinking and expanded my understanding. To Dr. Margreet Wolfert, I am extremely thankful for having her as my Laboratory supervisor. Her contributions during challenging moments remain dear and invaluable.

I would also like to take this opportunity to express deep gratitude to my committee members, professors Jonathan Amster and Robert Phillips, for taking the time out of their busy schedules to attend my seminars and assist with my orals, dissertation and defense as well as other assistance they provide during my time at UGA.

I also want to thank many others who have helped my scientific career to this point. From the University of Buea, Cameroon, I am grateful to Prof. Vincent P.K. Titanji, Drs. Cho Ngwa Fidelis, Ngwa A. Alfred and Ngemenya Moses, together with Mr Micheal Yong who help in training me on basic scientific techniques. From the University of Georgia, Chemistry department, along with many friends, I would like to thank Monique Phillips, Dr. Austin yongye, Eric Mbua, Leopold Matamba for their friendship. I also profoundly thank Drs. Brian Ember and Sanjay Kumar who initially worked with me when I joined the Boons' group for their many words of advice. The immense

contributions of Drs Douglas Miller, Kaustabh Maiti and Jun Guo are greatly appreciated, as this work would not have been possible had they not synthesized the many compounds screened. I also extend a big thank you to Dr. Eric Roush from GE Healthcare for his willingness to answer many of my questions on SPR.

To my wife, the always calm and lovely Shirley Forchap, I say thank you for your warmth and unconditional love. I am blessed to have you in my life. To Fr. Keibi Jervis, Nfi Felix, Frida Takang, and Akompab Derick, thanks for your friendship throughout all these years. To my beloved aunts: Beatrice, Elizabeth, Judith, Vero and Stella Njuzy, and Florence Asonganyi, I appreciate the many instances you all stood by me. Finally, to pa and mami, I thank you both for your enormous and unwavering sacrifice on my person. As I grow older and wiser I continuously appreciate how lucky I am to have you as my parents. To my younger sisters: Atembeuh and Emeanyi Asong, brothers: Asongafac and Njukang Asong, I will forever treasure your prayers, care and sibling warmth, for it enriched my life with meaning.

This project received financial support in the form of grants made available to Professor Geert-Jan Boons by the institute of General Medicine of the National Institutes of Health (GM065248).

Finally, I thank God for allowing it all to happen.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
LIST OF TABLES	x
LIST OF FIGURES.....	xi
 CHAPTER	
1 Introduction: Literature Review.....	1
1.1 Innate Immunity and Pattern Recognition Receptors	1
1.2 Peptidoglycan/ Toll like receptor signaling cascade	4
1.3 Bacteria Cell wall Peptidoglycans	7
1.4 Peptidoglycan Heterogeneity	10
1.5 Peptidoglycan Turnover	12
1.6 Peptidoglycan and Innate Immune Response.....	12
1.7 Bacterial Pathogenesis and Control	14
1.8 Surface Plasmon Resonance (SPR) Technology.....	16
1.9 Glycosylation and Cancer	21
1.10 Inhibition of Mannosidases.....	24
1.11 Research Projects	26
2 Differential recognition of gram – positive and gram – negative bacterial peptidoglycan by Toll like Receptor 2.....	28
2.1 Abstract.....	28

2.2	Introduction	30
2.3	Goals.....	34
2.4	Experimental procedure	35
2.5	Results	50
2.6	Discussion.....	67
3	Selective inhibition of alpha-mannosidase by feedback pro-drug constructs of mannostatin A	71
3.1	Abstract.....	71
3.2	Introduction	72
3.3	Goals.....	74
3.4	Experimental procedure	75
3.5	Results	84
3.6	Discussion.....	91
4	Conclusions and Future Directions	93

APPENDICES	97
A Sensorgrams representing the concentration-dependent kinetic analysis of lysine- and DAP-containing PGN part structures with immobilized sTLR2 (5,000 RU).....	97
B Sensorgrams representing the concentration-dependent kinetic analysis of MTP-Lys (amide) 1 with immobilized biotinylated sTLR2	105
C Sensorgrams representing the concentration-dependent kinetic analysis of MTP-DAP (amide/acid) 5 with immobilized sTLR2 fitted with a Langmuir 1:1 binding model.....	106
D Response of HEK293T cells expressing human TLR4/MD2/CD14 to different PGNs	107
REFERENCES.....	108

LIST OF TABLES

	Page
Table 2.1: (A) An amino acid waveform with a Mode II current integration period used. (B) Gradient conditions for separations with AminoPac PA10	46
Table 2.2: Kinetic parameters for the interaction of TLR2 with synthetic compounds 1-7	56
Table 2.3: EC ₅₀ values of TNF- α production induced by DAP-containing PGNs	65
Table 3.1: pH-dependence of half-lives of self-fragmentation of 2	88

LIST OF FIGURES

	Page
Figure 1.1: The structure of Toll-like receptors.....	3
Figure 1.2: Interactions of pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs)	4
Figure 1.3: TLR signaling pathways results in the transcriptional regulation of immune responses.....	5
Figure 1.4: Toll-Like Receptor activation by Pathogen-Associated Molecular Patterns (PAMPs) and its role in response to infection.....	7
Figure 1.5: Cell wall structures of gram-positive and gram negative bacteria	8
Figure 1.6: Subunit structures of bacterial PGNs	9
Figure 1.7: Structures of Antibiotics.....	16
Figure 1.8: Schematic view of SPR Sensorgram (A) and detector (B)	17
Figure 1.9: Amine Coupling Chemistry.....	19
Figure 1.10: Synthesis of N-linked glycoproteins	23
Figure 2.1: Structure of PGN.....	33
Figure 2.2: Structures of synthetic mucopeptides	38
Figure 2.3: Electrophoretic analysis and Western blotting of sTLR2 protein	50

Figure 2.4: Sensorgrams representing the concentration-dependent kinetic analysis of lysine- and DAP-containing PGN part structures with immobilized sTLR2 (5,000 RU).....	54
Figure 2.5: A contact time experiment using TLR2 and compound 5 to confirm a two-state binding model	58
Figure 2.6: Thermodynamic Van't Hoff plots for MTrP-Dap-amide, 6a (A) and MTrP-Lys-amide, 2a (B).....	59
Figure 2.7: Sensorgrams for inhibition assay.	60
Figure 2.8: The structural identity of the PGNs	61
Figure 2.9: Induction of TNF- α production by MM6 cells treated with different PGNs. ...	63
Figure 2.10: TLR2 dependence of cellular activation by PGN.....	66
Figure 3.1: a) General strategy for the inhibition of enzymes by a feedback mechanism. b) A feedback inhibitor for mannosidases.....	74
Figure 3.2: Synthetic Scheme; Reagents and conditions	85
Figure 3.3: Release of pro-drugs 1 and 2 by human lysosomal mannosidase. HLM	87
Figure 3.4: Inhibition of polylactosamine formation by compounds 14 and 15.	90
Figure 3.5: Effect of Pro-drug on MDAY-D2 cell viability.....	91

LIST OF ABBREVIATIONS

ABTS	(2, 2'-azino-di (3- ethylbenthiazoline-6-sulfonate))
Ac	Acetyl
Ac ₂ O	Acetic anhydride
Ala	Alanine
BSA	Bovine serum albumin
CRs	C-type lectin receptors
CpG DNA	unmethylated CpG dinucleotides
Dap	<i>meso</i> -diamoinopimelic
dGMII	<i>Drosophila melanogaster</i> Golgi α-mannosidase II
EDC	ethyl-N-(3-diethylaminopropyl)carbodiimide
Fmoc	fluorenylmethoxycarbonyl
GlcNAc	<i>N</i> -Acetyl glucosamine
GlcNAc-TV	<i>N</i> -acetyl glucosaminyltransferase V
HEK297 cells	Human Embryonic Kidney 297 cells
HGMII	human Golgi α-mannosidase II
h-iFBS	heat-inactivated fetal bovine serum
HLM	Human lysosomal mannosidase
HPAEC	high pH anion exchange chromatography
HPLC	high performance liquid chromatography
iGlu	isoGlutamate

IL	interleukin
IRAK	IL-1 receptor– associated kinase
JBM	Jack Bean mannosidase
LED	Light-emitting diode
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
LSD	lysosomal storage disorder
Lys	Lysine
MI	α -mannosidase I
MII	α -mannosidase II
MALDI-TOF	matrix assisted laser desorption ionization time of flight
min	minute
mM	millimolar
MM6	human monocytic cell line MonoMac-6
4-MU-Man	4-Methylumbelliferyl- α -D-Mannopyranoside
MurNAc	N-acetylmuramic acid
MYD88	Myeloid differentiation primary response gene
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	N-hydroxysuccinimide
NOD	nucleotide-binding oligomerization domain
OT	Oligosaccharyl transferase
PAMPs	pathogen associated molecular patterns
PDEA	(2- (2-pyridinylthio)ethanamine hydrochloride)

PGN	peptidoglycan
PGRP	peptidoglycan recognition proteins
pI	isoelectric point
PMB	polymyxin B
Poly I:C	polyinosinic-polycytidylic acid sodium salt
PRRs	pattern- recognition receptors
RLRs	RIG-like helicases
RU	Resonance Unit
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
SPR	Surface Plasmon Resonance
TAK-1	transforming growth factor β -activated kinase -1
TFA	Trifluoroacetic acid
TICAM-2	TIR containing adaptor molecule-2
TIR	[Toll/IL (interleukin)-1 receptor] domain
TIRAP	TIR domain containing adaptor protein
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	Toll/IL-1R domain containing adaptor inducing IFN β
TLRs	Toll-like receptors
TNF- α	Tumor Necrosis Factor – alpha
χ^2	Chi square

CHAPTER 1

Introduction: Literature Review

1.1: Innate Immunity and Pattern Recognition Receptors

The innate and adaptive (acquired) immune systems are critical for the survival of multi-cellular organisms. In an infected state, eukaryotic host survival depends on its immune system to organize a battery of defense mechanisms to combat the invading microbe. The first line of defense is the phylogenetically older innate immune system, present in cartilaginous and bony fishes¹, amphibians, reptiles, birds and mammals^{1b}. However, if the pathogen evades the innate immune system, then the adaptive immune system is triggered, resulting to the generation of responses such as antibodies and cytokines, tailored to maximally eliminate the infecting pathogen². The innate immune system functions to control infection and eliminate pathogens, as well as direct T- and B-cell responses of the adaptive immune system³.

Macrophages and dendritic cells mediate the protective functions of the innate immune system^{1b}. These cells express germline encoded soluble cytoplasmic and surface proteins as their antigen recognition determinants also called pattern-recognition receptors (PRRs). Unlike the adaptive immune system whose antigen recognition determinants develop through clonal selection via gene rearrangement, thus endowing it with the capacity to recognize a vast number of antigens, PRRs only recognize conserved antigenic motifs⁴. A benefit of PRR recognition to conserved

antigenic motifs like lipopolysaccharides, peptidoglycans, double-stranded RNA, flagellin (Pathogen Associated Molecular Patterns, PAMPs), essential for the survival of the pathogen and hence less likely to change, allows detection by the host regardless of its stage in the life-cycle. In addition, the specificity and generation of immunological memory in the adaptive immune system augment the benefits of the innate immune response.

There are several classes of PRRs with distinct expression patterns. These include the Toll-like receptors (TLRs)⁵, the C-type lectin receptors (CLRs)⁶, mostly found on the cell surface. The RIG-like helicases (RLRs)⁷, DNA sensors⁸ and members of the NOD (Nucleotide Oligomerization Domain) -like receptor family are cytosolic PRRs^{4b}. Their interaction with a specific PAMP, results in the activation of specific signaling pathways which lead to distinct anti-pathogen responses like the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF- α) and interleukins^{4b, 5a}.

TLRs are type 1 transmembrane proteins (Fig.1.1) with an ectodomain containing a 19 to 25 leucine-rich repeat (LRR) motif and a TIR [Toll/IL (interleukin)-1 receptor] domain⁹. The LRR is mainly involved in ligand recognition and signal transduction. The intracellular TIR domain portion (Fig. 1.1), present in all TLRs and members of the IL-1 receptor family consists of approximately 200 amino acids with three highly conserved boxes. Box 1 is considered the signature sequence of the family, whereas boxes 2 and 3 contain amino acids critical for signaling^{9b}.

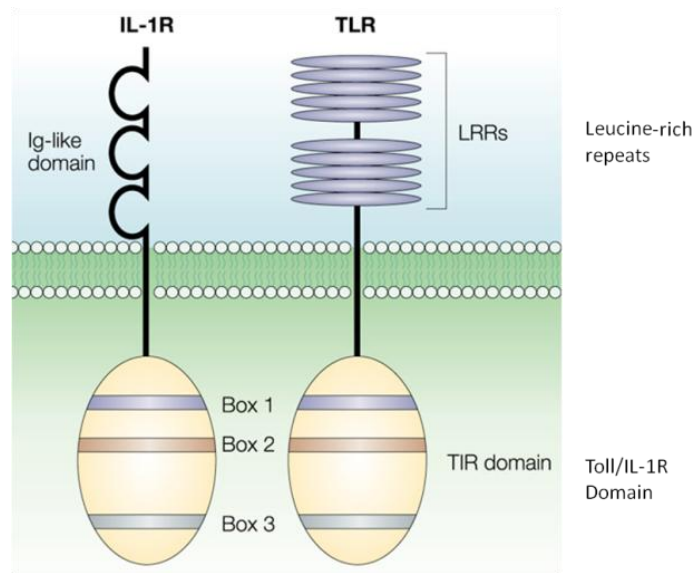


Fig.1.1: The structure of Toll-like receptors^{9b}

To date, about eleven TLRs have been identified in humans (Fig.1.2)^{9b}. An orthologue of *Drosophila* Toll, TLR4 was the first TLR to be identified and recognizes lipopolysaccharides¹⁰. TLR2 on the other hand, can form complexes with either TLR1 or TLR6 to bind to lipopeptides. The TLR1–TLR2 dimer binds to triacylated lipopeptides, whereas TLR2–TLR6 binds to diacylated lipopeptides¹¹. TLR3 recognizes double-stranded RNA¹² while TLR5 recognizes flagellin¹³. Single stranded RNA is recognized by both TLR7 and TLR8¹⁴ while TLR9 recognizes DNA¹⁵ originating from either the host or pathogen

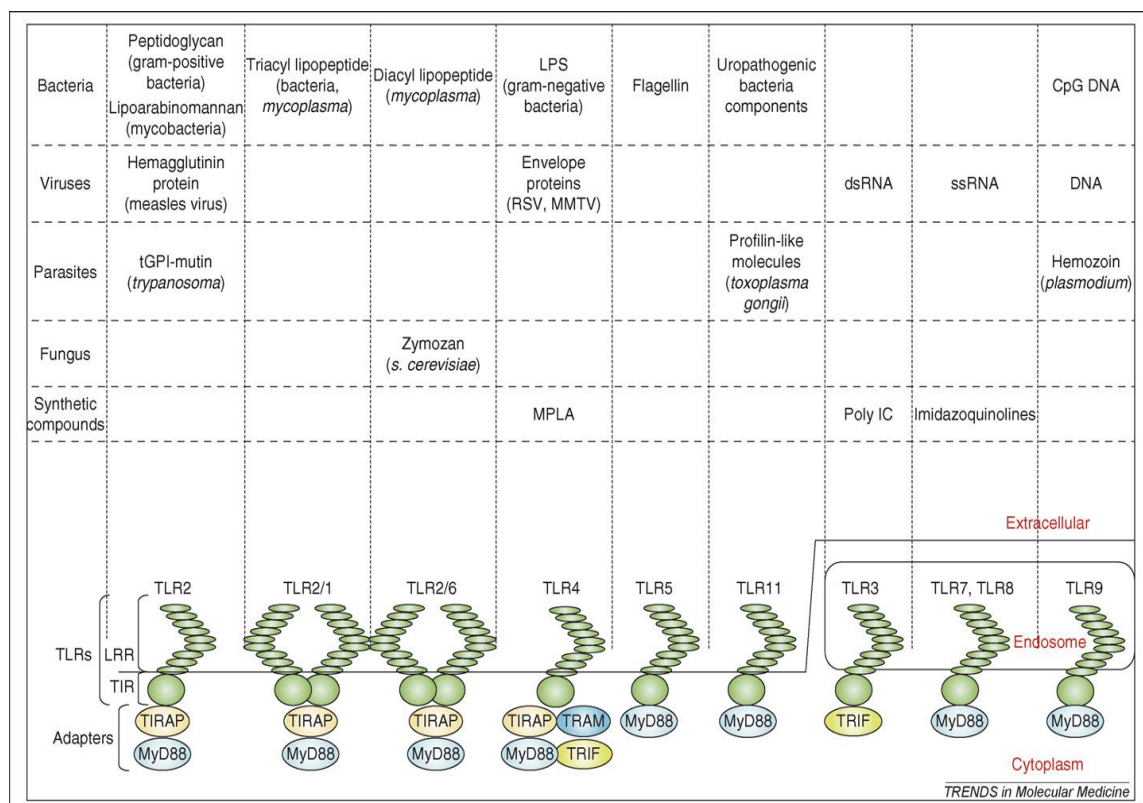


Fig. 1.2: Interactions of pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs)^{5a}.

1.2: Peptidoglycan/ Toll like receptor signaling cascade

Following ligand binding, it is proposed that TLR dimerizes^{9b} resulting in the activation of signaling pathways that culminate in transcriptional regulation of immune response genes (Fig. 1.3) of pro-inflammatory cytokines^{5a, 16}. Generally, the Toll/interleukin (IL)–1 receptor on TLRs promotes hetero- and homo-dimerization by interacting with adaptor proteins. These adaptor proteins include the Myeloid differentiation primary response gene (88) (MYD88), TIR domain containing adaptor protein (TIRAP or MAL), Toll/IL-1R domain containing adaptor inducing IFN β (TRIF or TICAM-1), TRIF-related adaptor molecule (TRAM or TIRP), TIR containing adaptor

molecule-2 (TICAM-2) and the SAM and ARM-containing protein (SARM)¹⁷. The differential recruitment of these adaptor proteins to the TLRs form the basis for specificity in the signaling processes activated by TLRs.

MyD88 is a universal adapter protein as it is used by all TLRs (except TLR3 which recruits only TRIF) to activate the transcription factor NF- κ B (nuclear factor κ B)¹⁶. However, TLR4 is unique among the TLRs as it recruits both MyD88/TIRAP and TRIF/TRAM adaptor proteins¹⁶. MyD88 contains both a TIR domain and a death domain. The association between TLR and MyD88 results in the recruitment of members of the IL-1 receptor– associated kinase (IRAK) family through death domain– death domain hemophilic interactions¹⁷. IRAK1 and IRAK4 (both serine/threonine kinases) phosphorylate and activate tumor necrosis factor receptor–associated factor 6 (TRAF6)^{9c}

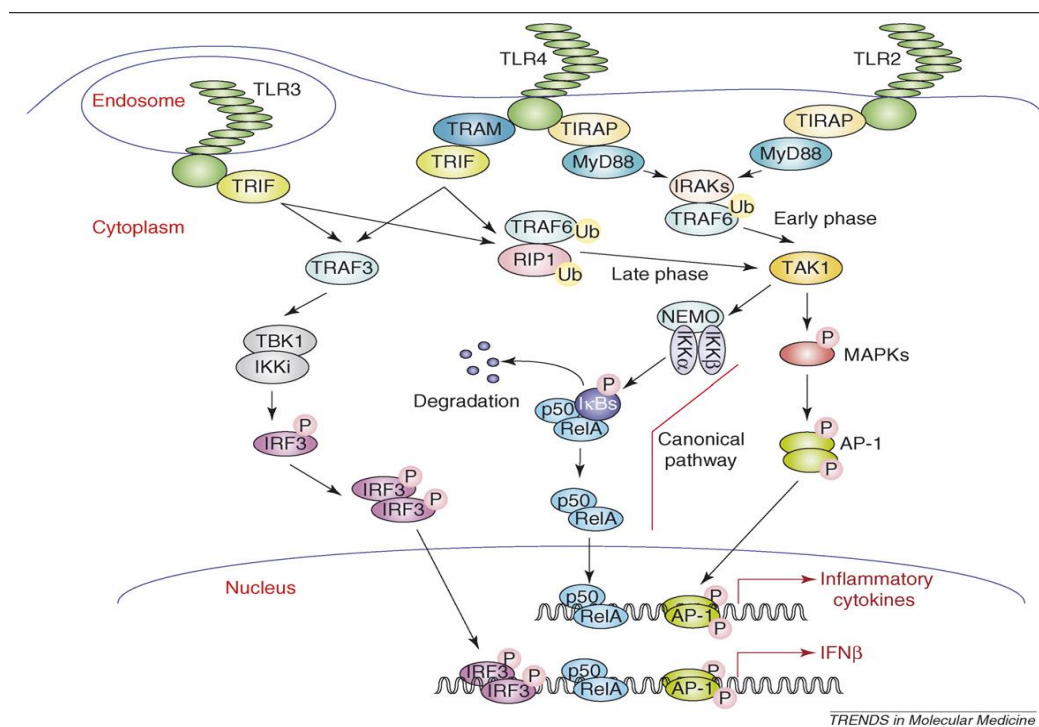


Fig. 1.3: TLR signaling pathways results in the transcriptional regulation of immune responses^{5a}

TRAF6, a RING-domain-containing E3 ligase forms a complex by polyubiquitination of itself through the E2 enzyme complex containing Ubc13 and Uev1A¹⁸. It then activates MAPK kinase kinase (MAPKKK) also called transforming growth factor β -activated kinase (TAK-1). The activated TAK-1, in turn, activates the I κ B α kinase complex (IKK), which consists of the kinases IKK α , IKK β and scaffolding protein IKK γ . The phosphorylation of I κ B leads to its degradation, the release of nuclear factor- κ B (NF- κ B) transcription factors, and the activation of NF- κ B- dependent genes, such as TNF- α , IL-1, and IL-6^{9c, 19}.

TRIF binds to TLR3 or TLR4 and recruits either TRAF3 or TRAF6 through binding to its N-terminal domain, leading to the activation of two distinct pathways. The binding to TRAF6 activates TAK1 in a manner similar to that in the MyD88-dependent pathway, leading to the NF- κ B stimulation²⁰. However, the binding to TRAF3 leads to the initiation of the TRIF-TRAF3-TBK1/IKKi-IRF3 axis to induce IFN- β . Briefly, the binding of ligand to TLR3 or TLR4 causes TBK1- and IKKi-mediated phosphorylation of the C-terminal regions of IRF3. This allows IRF3 to form a homodimer, translocate into the nucleus and bind its target sequences, such as IFN-stimulated response element (ISRE). TRAF3 serves to link these two molecules²¹ as it binds both TRIF and TBK1.

Tight control of the TLR activation pathway is essential to maintaining homeostasis as the over-activation of TLRs has been linked to inflammatory diseases such as sepsis and septicemia^{4a, 22}. The major hallmark of these diseases is the over-activation of NF- κ B, which regulates the induction of pro-inflammatory cytokines TNF- α ,

IL-1 and IL-6(Fig. 1.4), leading to cellular damage and multiple organ dysfunction syndrome.

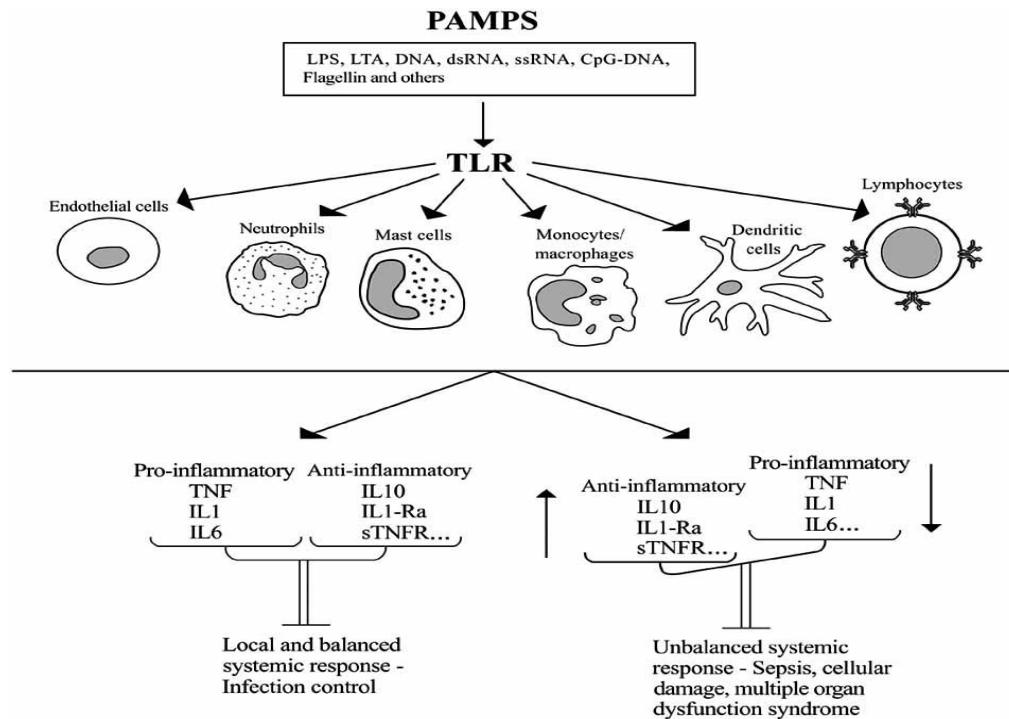


Fig.1.4: Toll-Like Receptor activation by Pathogen-Associated Molecular Patterns (PAMPs) and its role in response to infection²³

1.3: Bacteria Cell wall Peptidoglycans

In both gram-positive and gram negative bacterium, the cell wall (sacculi) is the main stress-bearing and shape-maintaining element that protects the underlying protoplast from lysing due to high internal osmotic pressure²⁴, and its integrity is of critical importance for cell viability. The cell wall consists of a cross-linked polymer called peptidoglycan (PGN) whose basic structure is very similar in both gram-positive and gram-negative bacteria (Fig. 1.5). The PGN in gram-positive bacterial (*Staphylococcus aureus*) cell wall is about 10 - 20 layers thick and only 1- 3 layers thick in gram-negative bacteria (*Escherichia coli*), making up 50% and 5% of their dry

weights, respectively. In *E. coli*, the PGN layer surrounding the cytoplasmic membrane is covalently attached to the outer membrane through lipoproteins whereas in *S. aureus*, it is covalently linked to charged polymers such as teichoic acid teichuronic acid, and proteins that are anchored to the cell wall^{24a, b, 25}.

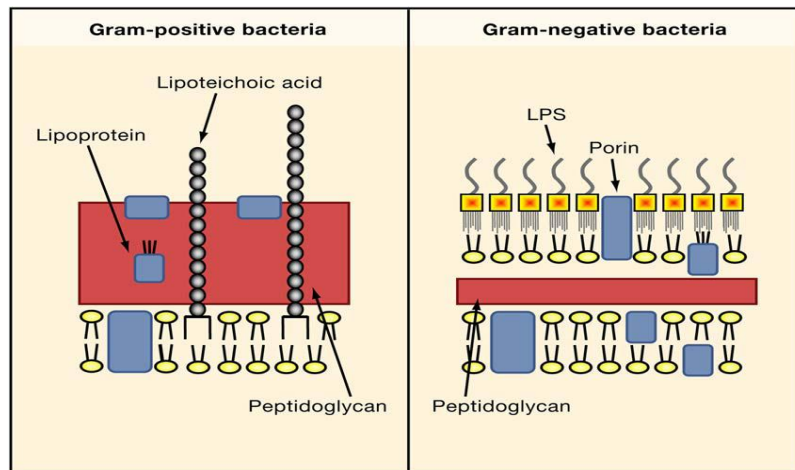


Fig. 1.5: Cell wall structures of gram-positive and gram negative bacteria^{4b}

Structurally, PGN consist of linear glycan strands cross-linked by short peptides (Fig. 1.6). These glycan strands are made up of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by β -(1- 4) glycosidic bonds^{24b}. The length distribution of these glycan chains varies for different bacterium^{24a, 26}. In *E. coli*, the glycan length is up to 80 disaccharide units, about 100 in *Bacillus subtilis* and up to 26 in *S. aureus*^{24c, 27}. These glycan strands form helical structures with the stem peptides protruding in all directions^{24a, 26, 28} and thus allowing cross bridge formation between PGN subunits (Fig. 1.6)^{24c}. In forming a PGN subunit, the D-lactoyl group of each MurNAc residue is attached to a peptide stem made of alternating L and D amino acids such as L-Alanine(Ala)-D-isoGlutamate (iGlu)- meso-diamoinopimelic

(Dap) (or L-Lysine (Lys))-D-Alanine-D-Alanine²⁴. In nascent PGN, the last D-Ala residue is lost in the transpeptidation process that links it to adjacent subunits^{24b, 29}. Cross-linking of PGN subunits generally occurs between the carboxyl group of D-Ala at position 4 and the amino group of the diamino acid or lysine at position 3, either directly or through a short peptide bridge (*S. aureus*, the cross bridge is a pentaglycine). Dap is often present in most gram-negative bacteria and some gram-positive *Bacillus* species. L-Lysine is present in most gram-positive bacteria^{24b}.

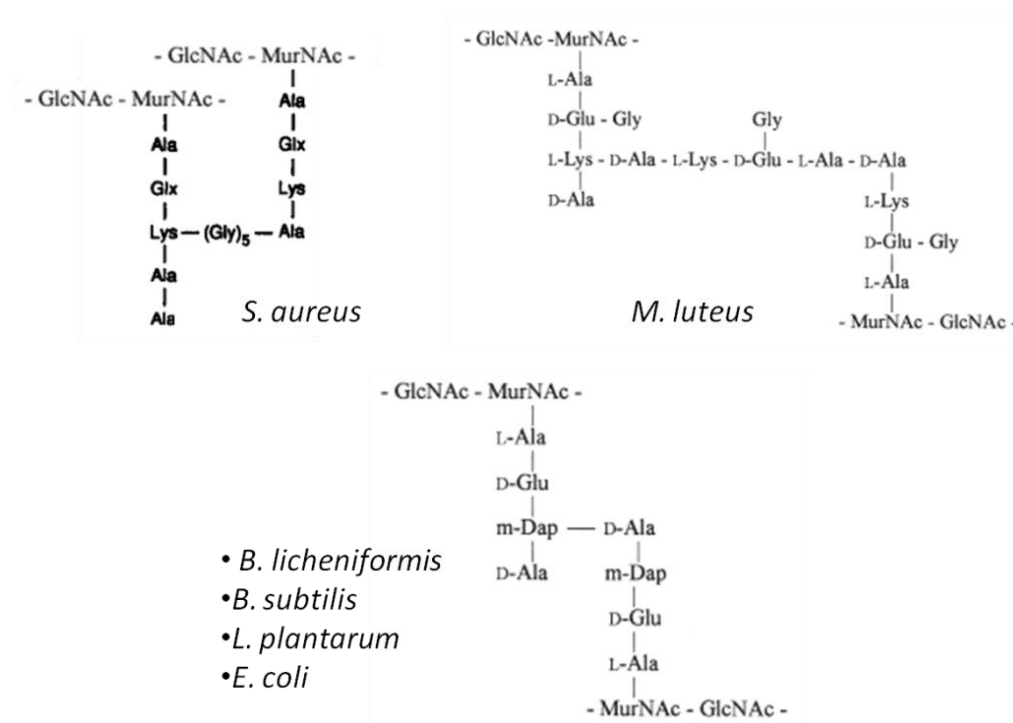


Fig. 1.6: Subunit structures of bacterial PGNs³⁰

However, cross-linking of PGN strands depends on the bacterial strain and growth conditions. In both *E. coli* and *B. subtilis*, up to 60% of their stem peptides are cross-linked, whereas it is about 90% in staphylococcal PGN³¹. The high degree of cross-linking found in *S. aureus* can be rationalized in a two-dimensional layer, as

resulting from its long and flexible pentaglycine cross bridge being able to span the distance between stem peptides from different PGN layers which would otherwise be too distant to be crosslinked^{24a, 26}. On the other hand, PGN cross linking in *E. coli* and *B. subtilis* relies on the fact that only every second peptide within its PGN strand is in the same plane and therefore sufficiently close to be directly cross-linked^{24a 26}. When a stem peptide is not cross-linked, both terminal D-Ala residues are usually cleaved by carboxypeptidases. However, in *S. aureus* these residues are not cleaved due to the lack of high activity, DD- and LD-carboxypeptidases^{24a}.

1.4: Peptidoglycan Heterogeneity

The main points of variation in bacterial peptidoglycan are found in the stem peptide, mode of cross-linking and the composition of the inter-peptide bridge^{24b, 31a}.

In the stem peptide, MurC ligase catalyses the addition of the first amino acid (L-Ala)³² in most bacterial species, but Gly (*Mycobacterium leprae*, *Brevibacterium imperial*) or L-Ser (*Butyribacterium rettgeri*) can be present in some bacteria spp.^{24b}. The amino acid at the second position is added by the MurD ligase³². In most gram-negative species this enzyme adds D-isoglutamate but in some gram-positive species, D-isoglutamine is the amino acid of choice. Catalyzed by the MurE ligase³², the most variation is found in the addition of amino acid at position 3 which is generally the diamino acid, *meso*-Dap (most gram-negative bacteria and gram-positive Bacilli) or L-Lys (most gram-positive bacteria). In a few bacteria species, the diamino acids (L-Ornithine, LL-Dap, *meso*-lanthionine, L-2,4-diaminobutyric acid, D-Lys) or monoamino acids (L-homoserine, L-Ala, L-Glu) are encountered^{24b}. Finally, in most cases, the

amino acids at positions 4 and 5 are added as a dipeptide (D-Ala-D-Ala). The integration of the dipeptide into the peptide stem is carried out by MurF ligase³². Thus the generated pentapeptide stem usually ends with D-Ala-D-Ala³³. D-Ala is predominantly found at position 4 in all species, however, D-Lactate (D-Lac) or D-Serine is found at position 5 in strains resistance to vancomycin³⁴. Uncommonly, Gly is found at position 4 or 5 in *E. coli* (1%) and *Caulobacter crescentus* (19%)^{35, 36}

Other variations on the stem peptide include amidation, hydroxylation, acetylation, and attachment of amino acids or protein at positions 2 and 3. Amidation of the α -carboxyl of glutamic acid yields D-isoglutamine found in *S. aureus* and *L. planterum*^{25c, 27c}. Also, the ϵ -carboxyl group of meso-DAP is amidated as found in *L. planterum* and *B. subtilis* bacteria. The hydrocarbon chain of D-Glu, meso-Dap or L-Lys is hydroxylated in some species like *Streptococcus pyogenes*³⁷. In *Corynebacterium insidiosum*, the γ -amino group of the diamino acid is acetylated³⁸. In other bacteria, an amino acid or amine-containing moiety, such as glycine (*Micrococcus luteus*, *Arthrobacter tumescens*), glycine amide (*Arthrobacter athrocyaneus*), D-alanine amide (*Arthrobacter sp.*), cadaverine (*Selenomonas ruminantium*) or N-acetylputrescine (*Cyanophora paradoxa*), is added to the α -carboxyl group at position 2^{24b, 39}.

Variations in the type of PGN cross-linkage and composition of the inter-peptide bridge (Fig. 1.6) add to the diversity of PGN heterogeneity. There are two main groups of cross-links^{24b}. In the first group which is the most common, the cross-linkage extends from the amino group of the side-chain of the residue at position 3 of one peptide subunit to the carboxyl group of D-Ala at position 4 referred to as the 3–4 cross-linkage. The 3–4 cross-linkage can be either direct as in most Gram-negative bacteria or

through an inter-peptide bridge as in most Gram positive bacteria^{24b}. In the second group, found mainly in the coryneform bacteria, the cross-linkage extends between the α -carboxyl group of D-Glu at position 2 of one peptide subunit and the carboxyl group of D-Ala at position 4, making the 2–4 cross-linkage. These cross-linking reactions are catalyzed by the transpeptidase domain of penicillin-binding proteins.

1.5: Peptidoglycan Turnover

Bacterial peptidoglycan is mechanically a very rigid structure and thus its remodeling is a prerequisite for bacterial replication as new PGN components are attached to existing cell walls⁴⁰. Recently, it was shown that cell separation may involve the removal of one or two layers of PGN between the two newly formed cell poles^{40b, 41}. The enzyme⁴² (amidases and peptidases) controlled hydrolysis of existing cell walls typically leads to loss of PGN. However, most of these products are captured and reutilized⁴³. This PGN recycling process is beneficial to the bacteria as an energy and carbon nutrient source, allowing it when faced with a sudden loss of nutrients to still have the necessary building blocks to complete a round of cell division and enter the stationary phase. This is particularly true for bacteria such as *E. coli* and *S. aureus* with an estimated PGN turnover per generation of about 5% and 50% respectively^{41, 44}.

1.6: Peptidoglycan and Innate Immune Response

Peptidoglycan has been shown to be a powerful biological effector^{22e}, thus it is critical to understand the biological role of PGN fragments resulting from turnover vis-à-vis pathogenicity and innate immunity^{22e, 44}. Its stimulatory activities include

adjuvanticity, activation of macrophages, and modulation of slow wave sleep⁴⁵. Until recently, TLR 2 was considered the mammalian receptor that binds to the active PGN fragments (Muropeptides) involved in pathogenicity⁴⁶. However, a report from 2004 show that TLR2 does not recognize PGN, but rather recognizes minor contaminants such as lipoproteins or lipoteichoic acids in PGN preparations⁴⁷.

However, other PGN receptors include cytosolic Nod 1 and Nod 2 proteins⁴⁸, known to recognize GlcNAc-MurNAc-L-alanine-γ-D-glutamate-meso-Dap (GMTriPDap) and GlcNAc- MurNAc-L alanine-D-glutamate (GMDiP) respectively. Though the involvement of TLR 2 as a PGN sensor is still unsettled, and Nod proteins detect the PGN agonists, the mechanistic implication for pathogenesis is that detection of PGN by the host must require processing of the PG layer. Thus bacterial endogenous hydrolases involved in turnover are crucial in the formation of the active small PGN fragments. Hence, mechanisms like PGN recycling that prevents release of PGN degradation products into the host constitute an escape mechanism, allowing the bacteria to evade detection from the host innate immune system.

Furthermore, another family of proteins shown to bind peptidoglycans is the Peptidoglycan recognition proteins (PGRPs)⁴⁹. Highly conserved from insects to mammals⁵⁰, members include PGRP-S, PGRP-L, PGRP-Iα, and PGRP-Iβ. The 19kDa PGRP-S binds to PGN and activates the prophenoloxidase cascade⁵¹ in insects, generating antimicrobial products that surrounds the pathogen with melanin and contains the infection⁵¹. On the other hand, PGRP-Ls are intracellular or membrane-spanning proteins which may function as a cell-surface receptor (or coreceptor) to activate the insect '*imd*' pathway which has similarities to the mammalian TNF-α

receptor-induced pathway⁵². In humans, PGRPs have highly differential expression in various organs and tissues. PGRP-S is highly expressed in the bone marrow (and to a lower extent in neutrophils and fetal liver); PGRP-L is highly expressed in the liver, and PGRP-I α and PGRP-I β in the esophagus, and to a lower extent in tonsils and thymus⁵⁰. This suggests that different mammalian PGRPs are likely to perform different functions unique to these organs, for example mammalian PGRP-S is bacteriostatic⁵³ and functions in intracellular killing of bacteria in neutrophils. While all PGRPs tested thus far (insect PGRP-S and mouse and human PGRP-S, -L and I α) bind PGN, the consequences of this binding and, thus, the exact functions of PGRP-L and PGRP-I α and-I β in innate immunity to bacteria in mammals are still being investigated⁴⁹.

1.7: Bacterial Pathogenesis and Control

The current control of bacterial infection involves the use of antibiotics like penicillins, cephalosporins, and glycopeptides which inhibit peptidoglycan synthesis (Fig. 1.7)⁵⁴. Once cell wall synthesis is inhibited, enzymatic autolysis of the bacteria occurs. Hence, the high osmotic pressure inside the cell bursts the inner and outer membranes of bacteria, eliminating it. These antibiotics exhibit different bactericidal mechanisms. The antibiotic cycloserine is an analog of D-alanine and interferes with enzymatic conversion of L-alanine to D-alanine by alanine racemase in the cytoplasm. It inhibits both the synthesis of the penultimate and terminal D- Alanines of the peptide stem in a PGN subunit⁵⁵.

During inter-peptide bridge formation, a nascent PGN subunit is passed across the cytoplasmic membrane attached to undecaprenol diphosphate carrier^{24a, b, 29}. It then

leaves the carrier on reaching the cell wall and the undecaprenol diphosphate is dephosphorylated to its monophosphate form. Bacitracin inhibits the dephosphorylation reaction thereby stopping PGN subunit synthesis⁵⁶.

The last step in peptidoglycan synthesis involves linking the sugar portion of the peptidoglycan subunit to the glycan backbone of the existing cell wall polymer^{24a, b, 29}. Cross-linking of the peptide portion of the subunit to a peptide in the cell wall then occurs. During this process D-alanine is enzymatically excised from the end of a pre-existing peptide side chain allowing it to be cross-linked to the recently synthesized peptidoglycan subunit. Vancomycin binds to D-alanine-D-alanine thus sterically inhibits transpeptidation⁵⁷.

The beta lactam antibiotics including penicillins like ampicillin, cephalosporins and monobactams also inhibit enzymes (penicillin binding proteins) involved in the transpeptidation of peptidoglycans. Common to all beta lactam antibiotic is the four member lactam ring. However, attached to the lactam ring, penicillins have an additional five membered ring, and cephalosporins a six member ring⁵⁵.

However, the emergence of antimicrobial-resistant microorganisms in both humans and food animals is a growing concern⁵⁸. Many bacteria display a range of mechanisms that protect them during antimicrobial exposure. These include production of β -lactamases and cephalosporinases, alterations in penicillin-binding proteins, multidrug efflux pumps, transferable resistance to vancomycin, and mutations in genes encoding DNA gyrase and DNA topoisomerase IV^{58b}. Furthermore, increase in both antibiotic usage, and frequency of immunosuppression from AIDS, transplant pharmacotherapy, and aging are some of the key factors driving this threat.

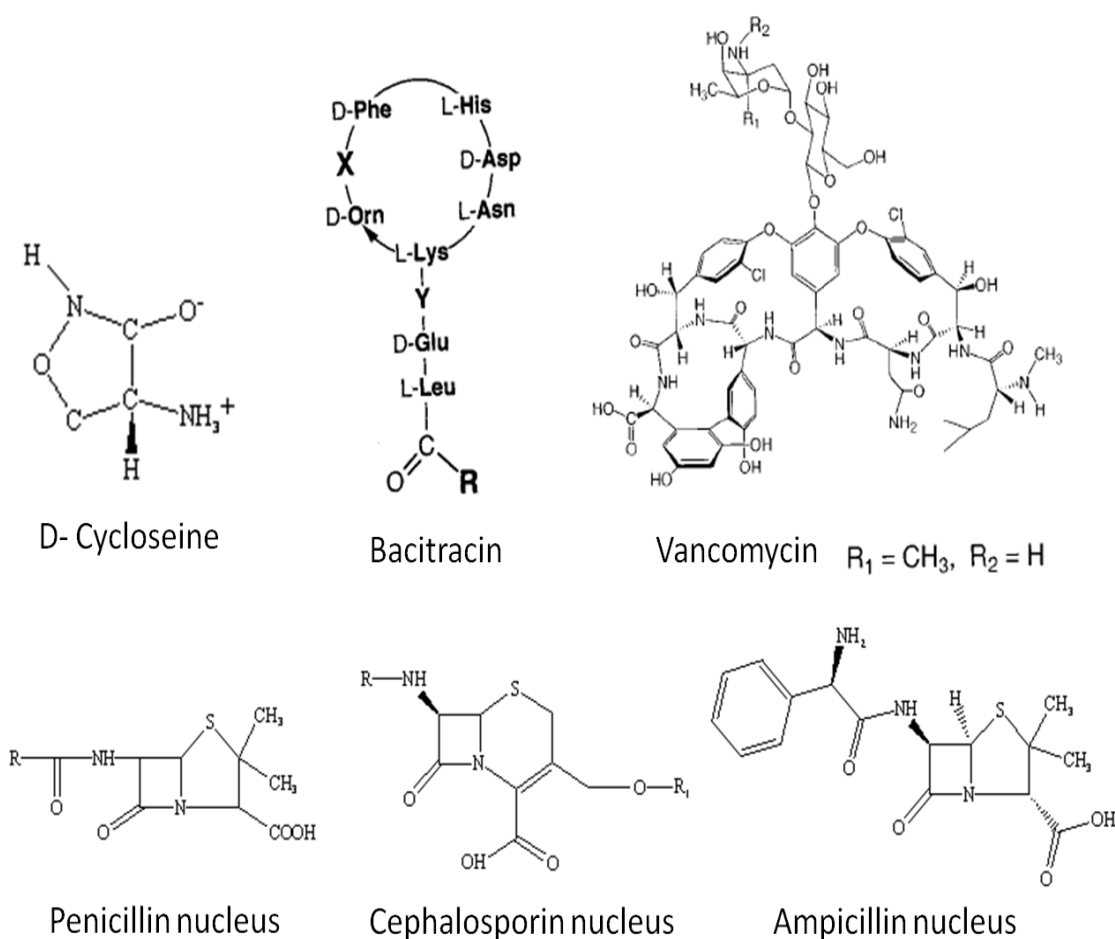


Fig. 1.7: Structures of Antibiotics^{56, 59}

1.8: Surface Plasmon Resonance (SPR) Technology

Surface Plasmon Resonance (SPR) is an optical biosensor technique that measures labeled free molecular binding events in real time⁶⁰. The fact is that SPR can provide affinity, kinetic and thermodynamic data; it is now widely used to screen drug candidates and monitor protein–protein/ peptide interactions, protein–DNA interactions, and DNA hybridization⁶¹.

The SPR biosensor consists of a light-emitting diode (LED, emitting near infrared light), a glass prism fixed to a sensor microchip, and a position-sensitive diode array detector^{60b}. Molecular binding events cause detectable changes in the local refractive index at the surface of the microchip, recorded as an SPR signal (Fig 1.8A). Usually, one reactant called the ligand is immobilization on a gold chip containing a thin dextran layer. The dextran layer extends approximately 100nm out from the sensor surface, making 0.2% of the height of the flow cell^{60a}. A second reactant called the analyte is injected to flow past the chip surface and interactions between both reactants are detected as association and dissociation events, resulting from mass changes at the sensor surface⁶².

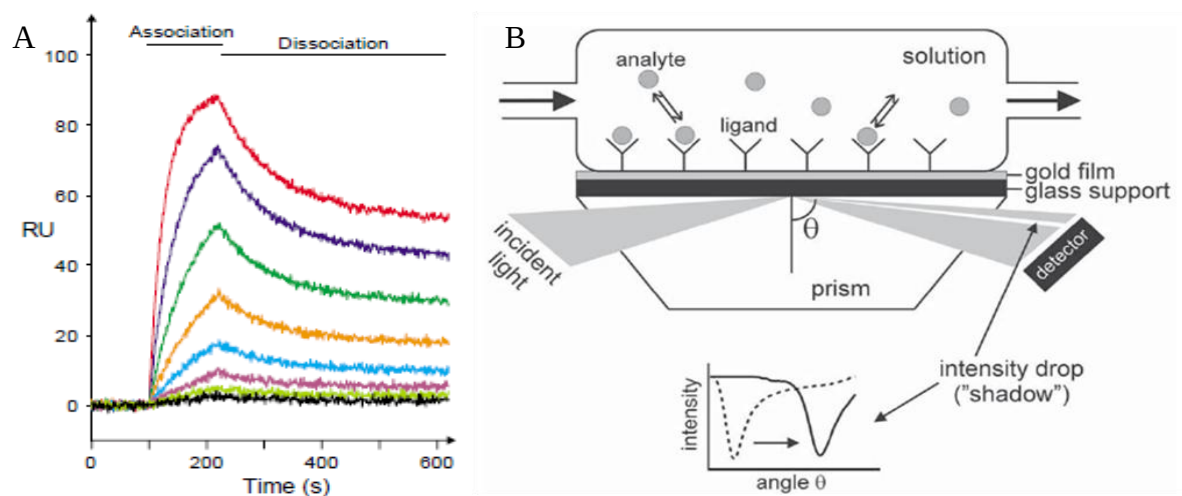


Fig 1.8: Schematic view of SPR sensorgram (A)⁶² and detector (B)⁶³

Theoretically, the SPR signal arises when incident light on a thin metal (aluminum, silver or gold) film generates a resonance event resulting from the interaction between electromagnetic vectors in the light and free electron clouds

(plasmons) in the metal conductor^{60b, 63}. By utilizing a prism, the resonant coupling at a specific angle θ of the incident light energy and the surface plasmon film causes the absorption of the light energy leading to a characteristic drop in the reflected light intensity at that specific angle (Fig 1.8B)⁶³. This resonance angle is sensitive to the wavelength of the incident light, the nature and thickness of the conducting metal film, and temperature⁶³. However if these factors are kept constant, the angle will depend mainly on the refractive index of the medium. Thus the resonance angle is a direct measure of the refractive index of the medium and is now mainly susceptible to changes from the interaction of the ligand and analyte^{60b}.

SPR is effective in studying interactions ranging from hundreds of Daltons⁶⁴ to whole-cell binding^{61c}. Its effective affinity range is from sub-picomolar to high millimolar binding affinity⁶⁵. However, it is difficult to measure very high affinity interactions because of their very slow dissociation rates ($k_{\text{off}} = 10^{-6} \text{s}^{-1}$, takes $\sim 10^4$ seconds for 1% of bound material to dissociate)⁶². Using long sample injection times and measuring equilibrium binding conditions over a range of concentrations greatly improves the accuracy of the measured high affinity⁶⁵. On the other hand, when measuring binding interactions with very low affinities, which are susceptible to non-specific binding of analyte to the sensor matrix, instrument noise and drift, proper controls are required for data-collection⁶⁶. The 'double referencing' technique, a method for subtracting reference data to remove small systematic signal deviations is particularly helpful^{64, 66}.

Binding affinities are also susceptible to the amount and method used to immobilize the ligand. There are many types of immobilization approaches. Amine coupling chemistry (Fig. 1.9) is commonly used as most proteins contain several

surface-exposed amine groups. The protein ligand is linked covalently via carboxyl groups on the dextran sensor chip surface⁶³. The immobilization is optimized with the protein prepared in a buffer having a pH below its isoelectric point (pI) but greater than 3.5⁶⁷. In cases where amine containing residues may be critical for activity of the protein binding site, an alternative coupling chemistry is used. One alternative is thiol-disulfide exchange⁶⁸. Here an active disulphide is first created on the sensor chip by derivatizing carboxyl groups with NHS/EDC followed by PDEA (2- (2-pyridinylthio)ethanamine hydrochloride) and exchange with intrinsic thiol groups on the ligand. Disulfides that remain active are then blocked with a cysteine solution. Ligand immobilization is also achieved with high-affinity capture methods involving streptavidin, antibodies, His tags, and specific fusion tag interactions⁶⁹. The desired level of ligand immobilized can be determined as a theoretical maximum response ($R_{\max}$), calculated using the formula⁷⁰.

$$R_{\max} = (\text{analyte MW})/(\text{ligand MW}) \times \text{ligand density} \times \text{stoichiometric ratio} \quad \text{Eq 1.1}$$

The theoretical $R_{\max}$ is often slightly higher than the experimentally obtained $R_{\max}$ due to steric interactions and partial inactivity of the ligand.

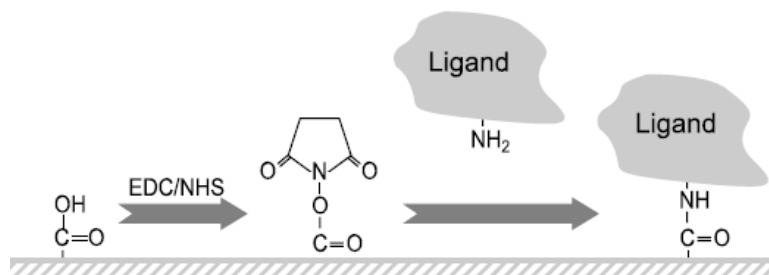
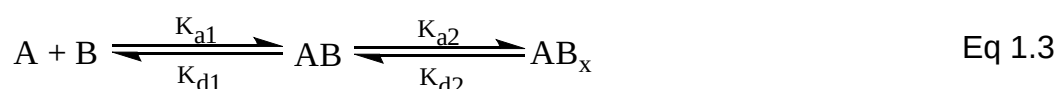


Fig. 1.9: Amine Coupling Chemistry

Once the ligand surface shows a stable baseline, the analyte is injected and both association and dissociation phases are determined and plotted in the form of a sensorgram (Fig 1.8A). The association rate ($k_a = \ln(dR/dt)$ vs. time) and dissociation rate ($k_d = \ln(dR_0/R_t)$ vs. time) constants are determined using mathematical operations that are incorporated into a BiaEvaluation software describing different types of Kinetics⁷¹. The function $\ln(dR/dt)$ and $\ln(R_0/R_t)$ are linear for Langmuir (1:1) interactions⁷². The 1:1 binding describes the simplest state of an interaction between analyte (A) and immobilized ligand (B).



However, the functions $\ln(dR/dt)$ and $\ln(R_0/R_t)$ are constant for mass-transfer-limited interactions, and curved for more complex systems involved with a conformation change⁷². The two-state (conformation) binding model can be described by the following equation^{70a, 73}.



The equilibrium constants of each binding step are $K_1 = k_{a1}/k_{d1}$ and $K_2 = k_{a2}/k_{d2}$, and the overall equilibrium binding constant is calculated as $K_A = K_1(1+K_2)$ and $K_d = 1/K_A$. In this model, the analyte (A) binds to the ligand (B) to form an initial complex (AB) which then undergoes subsequent binding or conformational change to form a more stable complex (AB_x)⁷⁰.

Also, equilibrium binding affinity in steady state kinetics can be determined using the binding responses in the steady-state region of the sensorgrams (R_{eq}) and plot

them against analyte concentration (C). The data is subjected to nonlinear regression fitting according to the following equation,

$$R_{eq} = CR_{max}/(C + K_d), \quad \text{Eq 1.4}$$

where R_{max} is the maximum binding response, and K_d is the dissociation constant^{70a}.

Thus, affinity values can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants in cases where the system does not reach steady state during the time frame of the experiment. However, it is recommended that the association times used be sufficient for at least one analyte concentration to reach steady state. Furthermore, to accurately determine dissociation rate constants, a measurable decrease in signal should occur during the dissociation period.

1.9: Glycosylation and Cancer

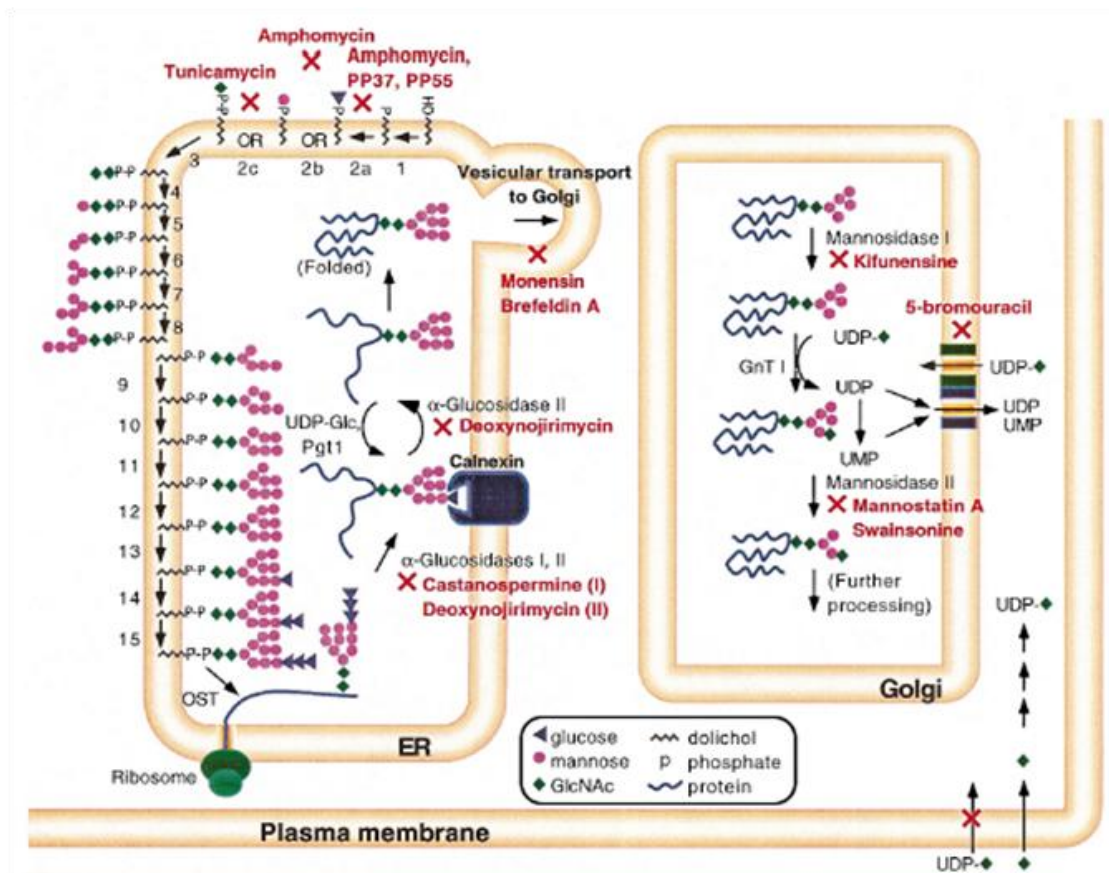
One out of every four deaths in the United States is due to cancer (breast, pancreatic, lung, and prostate), and globally, it is now the second leading cause of death each year⁷⁴. Beginning from the 1970s, the survival rate has only improved by about 10% which in part is due to the use of more powerful imaging tools for early detection. However, the major problem in cancer therapy is that most diagnosed cancers are typically in an advanced stage, in which the primary tumor has already metastasized, invading other organs and is beyond surgical intervention. This is

compounded by the fact that chemo- and radiation therapies have comparatively poor selectivity toward malignant tissue. Effective cancer control must address the development of new probes for early diagnosis, and the development of potent, targeted therapeutic regimes that would allow selective elimination of only diseased cells and tissue⁷⁴.

One of the major hallmarks of cancer is the phenotypic alteration in the (O- and N-) glycosylation pattern of cells⁷⁵. This understanding has led to the identification of glyco-biomarkers and potential targets for the development of therapeutic drugs. In the glycome of cancer patients, characterized N-glycosylation phenotypes include changes in the levels of tri- and tetra-antennary glycan structures, sialyl Lewis X epitopes and agalactosylated bi-antennary glycans⁷⁵⁻⁷⁶.

Briefly, in the eukaryotic N-glycosylation pathway, an oligosaccharyl transferase (OT) enzyme transfers an assembled donor oligosaccharide (Glucose₃Mannose₉ N-acetylGlucose₂, Glc₃Man₉GlcNAc₂) from dolichol to a specific asparagine within the Asn- Xaa-Ser/Thr sequon (where X can be any amino acid except proline) on a co-translated protein within the lumen of the endoplasmic reticulum (ER) (Fig.1.10)⁷⁷. A series of enzymes then trim the oligosaccharide from a high mannose structure to a hybrid and then to a complex chain glycan. The trimming process is initiated in the ER by glucosidases (I and II) which remove all three glucose residues to produce a glycoprotein with a Man₉GlcNAc₂ core structure⁷⁸. Mannosidase I (MI) then removes some of the mannose residues, allowing the conversion of high mannose by various GlcNAc-transferases to complex-type oligosaccharides in the medial golgi. GlcNAc-transferase I (GlcNAc-TI) adds a GlcNAc residue to form a hybrid-type N-glycan,

GlcNAc₁Man₅GlcNAc₂⁷⁹. Next, α -mannosidase II (MII) removes two mannosyl residues to form GlcNAc₁Man₃GlcNAc₂ (4, 5), which is further modified by GlcNAc-transferase II (GlcNAc-TII) to form GlcNAc₂Man₃GlcNAc₂, the precursor of complex-type *N*-glycans.^{77, 80} These branched *N*-glycans are further modified in the trans Golgi by β 1,4galactosyltransferases, elongated with poly *N*-acetylglucosamine (linear repeats of Gal β 1,4GlcNAc β 1,3) by β 1,3*N*-acetylglucosaminyltransferases (iGNTs), and capped with sialic acid and fucose⁸¹.



Enzymes: 1: dolichol kinase, 2a: mannosylphosphoryldolichol synthase, 2b: glucosylphosphoryldolichol synthase, 2c: dolichyl phosphate GlcNAc phosphotransferase, 3: GlcNAc-P-P-Dol GlcNAc transferase, 4–8: GDP-Man dependent mannosyltransferases; 6–12: Dol-P-Man-dependent mannosyltransferases; 13–15: Dol-P-Glc-dependent glucosyltransferases].

Fig.1.10: Synthesis of *N*-linked glycoproteins. The dolichol block is transferred to the protein and processing reactions begin. Enzyme inhibitors are shown in red⁷⁷.

Increased expression of *N*-acetyl glucosaminyltransferase V (GlcNAc-TV), leads to increased β 1, 6GlcNAc branching and subsequent polylactosamine extension⁸². This increased branching has been observed in human breast, colon and skin carcinomas and correlated with invasive and metastatic potential^{82a}. Due the lack of a crystal structure for GlcNAc-TV in drug development, the inhibition of the mannose trimming enzyme human Golgi α -mannosidase II (HGMII), provides a route to blocking additional branching of *N*-linked oligosaccharides⁸³. This prevents the formation of the biosynthetic precursor for GlcNAc-TV; hence the polylactosamine chain cannot be appended.

1.10: Inhibition of Mannosidases

Alpha-mannosidases (E.C. 3.2.1.24)⁸⁴ in mammalian cells belong to 2 main classes. The class 1 α -mannosidases are largely restricted to the early secretory pathway and are glycosylhydrolases of the family 47 class⁸⁵. They are involved in ER glycoprotein quality control where they regulate maturation and correct folding of nascent glycoproteins⁸⁶. These enzymes cleave four α 1,2-linked mannoside residues, converting the tri-mannosyl core of *N*-glycans $\text{Man}_9\text{GlcNAc}_2$ to $\text{Man}_5\text{GlcNAc}_2$ in the ER and Golgi complex⁸⁷.

The class 2 α –mannosidases are glycosylhydrolases of the family 38 class^{85b}. They are found in the Golgi complex, lysosomes, and cytosol, and are involved in both glycoprotein biosynthesis and catabolism^{85b}. Three sub-types have been identified, the acidic/ lysosomal (MAN2B1), intermediate/Golgi (GMII, MAN2A1), and neutral/cytosolic (MAN2C1/6A8) α –mannosidases⁸⁶. They have very similar sequences at their active sites and hydrolyze *N*-linked glycans from the non-reducing terminal α 1,3- and α 1,6-

linked mannose residues from GlcNAcMan₅GlcNAc₂ to produce GlcNAcMan₃GlcNAc₂, using a retention mechanism⁸⁷. The resulting sugar products have the same anomeric configuration as the original oligosaccharide. Human lysosomal α -mannosidase (HLM) has broad specificity and an increase in its expression has been previously demonstrated in extracts of glioma to correlate with malignancy⁸⁸. Human α -mannosidase II is inhibited by swainsonine with an IC₅₀ of 20–50 nM^{88a}. *Drosophila melanogaster* Golgi α -mannosidase II (dGMII) displays 61% sequence similarity with HGMII and has comparable substrate specificity, kinetic properties, and inhibitor sensitivity⁸⁹. Thus dGMII is a good model for structural and drug development studies of the mammalian enzyme.

Because most drug inhibitors of mannosidases are non-specific, targeting both the golgi and lysosomal mannosidases, this has led to the development of α -mannosidosis. Alpha-mannosidosis is a Lysosomal Storage Disorder (LSD) which results from the deficiency of lysosomal α -mannosidases to breakdown N-glycans in the excretory pathway⁹⁰. This results in the accumulation of glycan structures like high mannose N-glycans, and the tri-mannosyl core (Man α 1–3[Man α 1–6] Man β 1–4GlcNAc β 1–4GlcNAc) of the complex type structures⁸⁴. Alpha-Mannosidosis patients display hepatosplenomegaly and progressive neurological decline which may lead to mental retardation and ataxia⁸⁴. In human and animal models, α -mannosidosis has been treated with bone marrow transplantation⁹¹. However, the approach has shown only limited efficacy and there is a high risk of morbidity and mortality. Thus there is a need for the development of inhibitors that selectively target the Golgi GMII without targeting lysosomal mannosidase in order to prevent LSD development.

1.11: Research Projects

1.11.1: Characterization of PGN binding to TLR2

Contradictory results have been reported with regard to which molecular determinants are recognized by TLR2. In 2004, Travassos *et al*⁴⁷ published data that challenged the general view that TLR2 binds to PGN and a year later, the Dziarski group⁴⁶ provided further evidence showing that PGN is a TLR2-dependent cell activator. This contradiction in result is possible as the general approach to establish which microbial components are sensed by pattern recognition receptors is based on measuring cellular responses of macrophages from wild type or transgenic mice, or HEK297 cells transfected by genes of a PRR, after exposure of components isolated from microbes. The major limitation to these experimental approaches has been that they do not establish direct binding of PAMPs to relevant receptors, and the inherent structural heterogeneity of microbial products makes it impossible to identify specific structural features that are associated with biological responses. Also, misleading results have been obtained due to difficulties of removing contaminants that by themselves induce cellular responses.

To avoid these difficulties arising from the use of isolated bacterial compounds, we have synthesized a wide range of peptidoglycan derivatives and used SPR technology and cell based assays to determine intrinsic PGN binding specificities to human TLR2.

1.11.2: Inhibition of Glycosidases

Alterations in cell-surface carbohydrates are one of the hallmarks of tumor progression and metastasis. Compounds that inhibit the biosynthesis of these oligosaccharides are thus attractive leads for drug development. We report here a general approach for the design and synthesis of more selective glycosidase inhibitors based on feedback inhibition. This feedback inhibition mechanism allows a biosynthetic pathway to regulate itself through inhibition of the first committed step in the pathway by a down stream or final product.

The feedback inhibition pro-drugs described here are composed of an inhibitor that is modified by a linker that renders them inactive. The linker, in turn, is attached to a glycoside that can be hydrolyzed by a targeted glycosidase. The resulting intermediate is designed in such a way that it will undergo self-fragmentation to release the active inhibitor. Subsequently, the inhibitor will inhibit the enzyme that initiated its release. The attraction of this approach is that the inhibitor will only be released at the site of the enzyme. Selectivity is achieved due to the exquisite selectivity of glycosidases for their substrates. Furthermore, the release of the inhibitor is terminated when a sufficient quantity of the inhibitor is released. The remaining pool of pro-drug will be activated when active enzyme re-appears thereby approximating conditions of continuous infusion.

CHAPTER 2

Differential recognition of gram – positive and gram – negative bacterial peptidoglycan by Toll-like receptor 2

2.1 Abstract

Although much progress has been made towards the identification of innate immune receptors, far less is known about how these receptors recognize specific microbial products. Such studies have been hampered by the need to purify compounds from microbial sources and a reliance on biological assays rather than direct binding to monitor recognition. We have employed surface plasmon resonance (SPR) binding studies, using a wide range of well-defined synthetic muropeptides derived from Gram-positive (lysine-containing) and Gram-negative (diaminopimelic acid (DAP)-containing) bacteria, to demonstrate that Toll-like receptor 2 (TLR2) can recognize peptidoglycan (PGN). In the case of lysine-containing muropeptides, a limited number of compounds, which were derived from PGN remodeled by bacterial autolysins, were recognized. However, a wider range of DAP-containing muropeptides bound with high affinity and these compounds were derived from nascent and remodeled PGN. The difference in recognition of the two classes of muropeptides is proposed to be a strategy by the host to respond appropriately to Gram-negative and -positive bacteria, which produce vastly different quantities of PGN. It was also found that certain modifications of the carboxylic

acids of iso-glutamine and DAP can dramatically reduce binding, and thus, bacterial strains may employ such modifications to evade innate immune detection. Cellular activation studies employing highly purified PGN from *Bacillus licheniformis*, *B. subtilis*, *Escherichia coli*, *Lactobacillus plantarum*, *Micrococcus luteus*, and *Staphylococcus aureus* support the structure-binding relationship. The data firmly establishes TLR2 as an innate immune sensor for PGN and provides an understanding of host-pathogen interactions at the molecular level.

2.2 Introduction

The innate immune system is an ancient evolutionary system of defense against microbial infections that rapidly responds to highly conserved families of microbial components that are not produced by the host. These so called pathogen-associated molecular patterns (PAMPs)² include microbial structures such as lipopolysaccharide (LPS), lipoteichoic acid, mannans, flagellin, peptidoglycan (PGN) and DNA sequences containing unmethylated CpG dinucleotides (CpG DNA)⁹². The PAMPs are recognized by highly conserved pattern recognition receptors such as Toll-like receptors (TLRs), PGN recognition proteins (PGRPs), collectins, and nucleotide-binding oligomerization domain (NOD) proteins. Cellular activation by these receptors results in acute inflammatory responses such as the production of diverse sets of cytokines and chemokines, direct local attack against the invading pathogen, and initiation of responses that activate and regulate the adaptive component of the immune response. Although these responses are beneficial for host defense, the presence of a large amount of PAMPs can cause overproduction of the inflammatory mediators, which can lead to septic inflammatory response (SIR) syndrome and include life-threatening symptoms such as vascular fluid leakage, tissue damage, hypotension, shock, and organ failure⁹³.

Although much progress has been made towards the identification of key innate immune receptors, far less is known about how these receptors recognize and initiate responses to specific microbial products. In this respect, it is well known that structural variability is considerable among PAMPs of different microbial strains⁹⁴. Structural variability can be both advantageous and disadvantageous as it offers a microbe a

strategy to evade detection by the host innate immune system or presents the host an opportunity to mount a specific and appropriately measured immune response to particular microbial strains. Heterogeneity in the structure of PAMPs within a particular microbial strain and possible contaminations with other inflammatory mediators has made it difficult to identify biological responses initiated by specific PAMPs.

PGN is a highly complex structural component of the cell wall of almost all bacteria and has been implicated as a PAMP in innate immune responses. It is a large and complex polymer composed of alternating $\beta(1-4)$ -linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues cross-linked by peptide bridges (Fig. 2.1)^{94b, c, 95}. The carboxylic acid of *N*-acetylmuramic acid is linked to stem peptides consisting of four or five alternating L- and D-amino acids. Among different bacterial species, the structure of the sugar chain is rather conserved, while the composition of the stem peptide subunits varies considerably. For example, lysine is commonly the third amino acid of the peptide moieties of PGN of most Gram-positive bacteria, while Gram-negative and most rod-shaped Gram-positive bacteria have a diaminopimelic acid (DAP) residue at this position. Furthermore, the α -carboxylate of the iso-D-Glu moiety is often amidated or linked to additional amino acids such as Gly or D-Ser. Considerable heterogeneity has been found in the nature of the interpeptide bridge, which connects the ϵ -amino group of the lysine or DAP moiety to the penultimate D-Ala of another PGN chain, resulting in the loss of terminal D-Ala.

Additional structural heterogeneity is introduced during bacterial growth and division, which requires remodeling of cell wall PGN by bacterial autolysins such as D,D- and D,L-carboxypeptidases, endopeptidases, and *N*-acetylmuramidases. These

enzymes cleave peptide and glycosidic linkages to generate muramyl tri- and tetrapeptides, tetrapeptides extended by glycine moieties, and shorter polysaccharide chains (Fig. 2.1A)⁹⁶. The remodeling process leads to a loss of PGN fragments to the bloodstream that can be detected by innate immune receptors.

The *intracellular* NOD receptors (NOD1 and NOD2) can detect small monovalent PGN fragments resulting in the production of (pro) inflammatory mediators^{43b-d, 43f}. PGRPs are a relatively new class of pattern recognition receptors that are highly conserved from insects to mammals and can bind or enzymatically process PGN^{43e, 43g}. Mammalian PGRPs do not induce inflammatory responses but exert their roles by amidase or antibacterial activity. The involvement of TLR2 in the recognition of PGN for the initiation of inflammatory responses has been controversial⁹⁷. Furthermore, it is not known which structural elements of PGN can be recognized by TLR2. The involvement of TLR2 in PGN sensing is, however, critical for understanding host-pathogen interactions and may provide opportunities for the development of new therapeutic strategies for the treatment of SIR^{43j}.

We have employed a wide range of well-defined synthetic PGN derivatives combined with surface plasmon resonance (SPR) measurements to establish whether human TLR2 can detect PGN. The binding data has been validated by cellular activation studies using highly purified and chemically modified PGN. It has been found that PGN derived from Gram-positive and -negative bacteria can be detected by TLR2. In the case of Gram-positive bacteria, the initial linear biosynthetic product and cross-linked PGN are poorly recognized and active fragments are only formed after remodeling of PGN by bacterial autolysins. A wider range of PGN fragments derived

from Gram-negative bacteria could be complexed with high affinity, including the nascent muramyl pentapeptide. The structure-activity relationship also demonstrates that modifying the structure of PGN can be a strategy used by bacteria to evade immune detection by TLR2. Cellular activation studies employing highly purified and chemically modified PGN support the established structure-binding relationship.

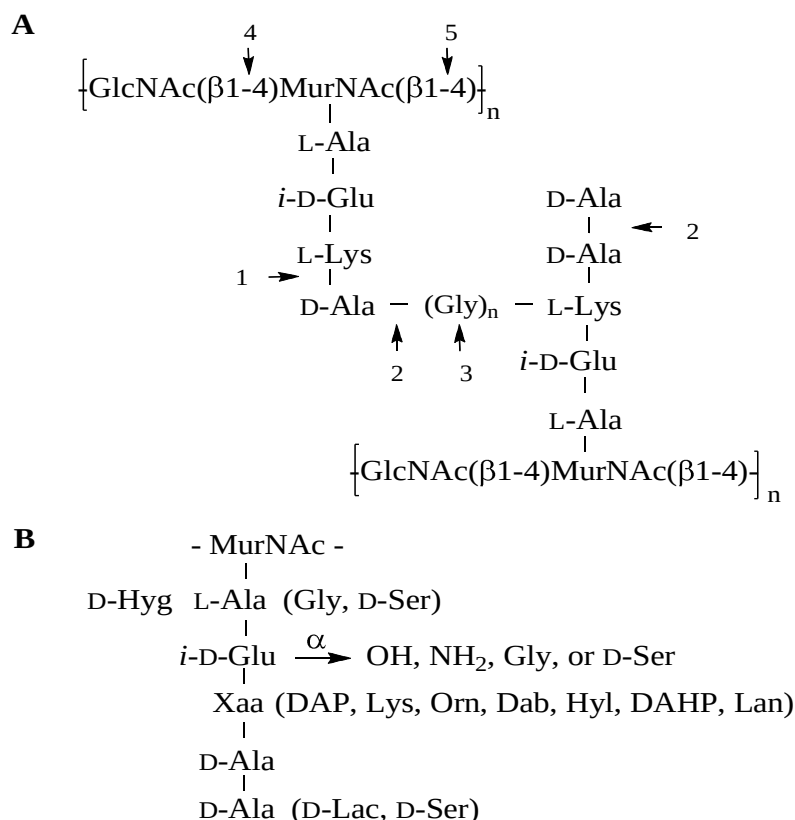


Fig. 2.1: Structure of PGN. Common primary structure of Gram-positive PGN (A). Sites where autolysins may act are indicated: 1. D,L-carboxypeptidase, 2. D,D-carboxypeptidase, 3. endopeptidase, 4. N-acetylglucosaminidase, and 5. N-acetylmuramidase. Variations in the peptide chain of PGN (B). Residues in parentheses may replace corresponding amino acids. The α -carboxylic acid of iso-D-Glu may be modified by an amide, Gly, or D-Ser. (Dab: 2,4-diaminobutyric acid, DAHP: 2,6-diamino-3-hydroxypimelic acid, Hyg: threo-3-hydroxyglutamic acid, Hyl: hydroxylysine, Lan: lanthionine, Orn: ornithine).

2.3 Goals

Amidst the current controversy involving TLR2/PGN interactions, this study seeks to address the following concerns:

- 1) Investigate the immunogenic properties of bacterial peptidoglycans.
 - a) By studying the binding affinities between synthetic PGN partial structures and TLR2.
 - b) By studying the interaction between soluble polymeric PGN and TLR2.
 - c) By studying the type of binding kinetics between PGN and TLR2. This could either involve a more complex binding or a simple 1:1 binding.
- 2) Investigate the functional group significance of PGN part structures and relate them to their capacity to elicit the production of cytokines that are important in innate immunity. This will exploit functional group differences within PGN monomeric unit of different bacteria strains. The PGNs will be selected, extracted, characterized, and then used for cell activation studies. The levels of TNF- α cytokine produced will be measured and used to determine the capacity of these polymeric PGNs to activate the innate immune system.

2.4 Experimental procedure

Reagents: *E. coli* 055:B5 LPS from List Biological Laboratories, polymyxin B from Bedford Laboratories, Pam₃CysSK₄ from Calbiochem, poly(I)·poly(C) from Amersham Biosciences, recombinant human tumor necrosis factor alpha (TNF-α) from Endogen, rat polyclonal antibody to human TLR2 (pAb hTLR2) from InvivoGen, rat polyclonal IgG from Sigma-Aldrich, and mouse monoclonal anti-poly-histidine antibody from Qiagen. The sTLR2 plasmid⁹⁸ was a kind gift from Y. Kuroki (Sapporo Medical University School of Medicine, Japan). Soluble polymeric Peptidoglycans (sPGN) obtained from *S. aureus* were provided as a gift from the Dziarski group⁴⁶.

2.4.1 Chemical Synthesis of PGN Partial Structures

Compounds (Fig 2.2) were synthesized by the Boons group using classical fluorenylmethoxycarbonyl (Fmoc) chemistry and standard manual solid-phase peptide synthesis techniques. Compounds **1**, **2a,b**, **3a**, **4**, **5**, **6a,d**, and **7** were prepared as described previously⁹⁹. However, in the preparation of compound **2c**, Sieber Amide resin (100 mg, 42 μmol) was swelled in dimethylformamide (5 ml) for 45 min and then treated with piperidine in DMF (20%, 3x 3 ml). After a reaction time of 30 min, the solvents were removed by filtration and the resin washed with DMF (3x 3 ml), and then treated with Fmoc-D-alanine (26.1 mg, 84 μmol) in DMF in presence of HATU (31.9 mg, 84 μmol) and DIPEA (29 μl). The progress of the reaction was monitored by Kaiser test. After completion of the coupling, the resin was washed with DMF (3 x 3 ml), and the Fmoc protecting group removed by treatment with piperidine in DMF (20%, 3x 2 ml, 3 x 10 min). The reaction cycle was repeated using Fmoc-L-Lys(Boc)-OH (39.3 mg, 84

μmol), Fmoc-D-isoglu(Gly-OBn) (43.4 mg, 84 μmol), Fmoc-L-alanine (26.1 mg, 84 μmol), and 2-*N*-acetyl-1- β -O-allyl-4,6-benzylidene-3-muramic acid (38.1 mg, 84 μmol). The resulting resin bound glycopeptide was washed with DMF (3x 5 ml), dichloromethane (7x 3 ml), and methanol (3x 3 ml), dried *in vacuo* (10 h), rewelled in DCM (5 ml) and filtered. The glycopeptide was released from the resin by treatment with trifluoroacetic acid in DCM (2%, 10x 2 ml). The resin washing were combined, concentrated under reduced pressure, and the residue coevaporated with toluene. The crude product was subjected to TFA in DCM (20%, 3 ml) to ensure complete removal of the benzylidene and Boc protecting groups. Pd/C (10%, 35 mg) was added to a solution of the crude compound in a mixture EtOH/H₂O/1N HCl (4:2:0.02, v/v/v, 1.5 ml) and the reaction mixture was stirred for 40 h, after which it was passed through an acrodisc syringe filter (0.2 μm supor membrane, PALL Life Sciences) and the resulting filtrate concentrated under reduced pressure. The benzyl ester of the resulting compound was removed by stirring under an atmosphere of H₂ (1 atm) in presence of Pd/C (20 mg) in mixture of EtOH/H₂O/HOAc (4:2:0.2, v/v/v, 1.6 ml) for 20 h. After completion of the reaction, Pd/C was filtered off through acrodisc syringe filter (0.2 μm supor membrane), and the solvents were evaporated *in vacuo*. After purification by LH-20 size exclusion column chromatography using MeOH/dichloromethane as the eluent (1/1, v/v), the glycopeptide was further purified by semi-preparative HPLC (Eclipse XDB-C18 column, 5 μm , 9.4 x 250 mm, eluent: water/acetonitrile/0.1% TFA) to afford, after lyophilization of the appropriate fraction, the target compound **2c** as a mixture of α/β anomers (8.1 mg, 64%). $[\alpha]^{22}_{\text{D}} = +52.23$. ¹H NMR (500 MHz, D₂O): δ 5.11 (d, *J* = 3.0 Hz, 1H; H-1), 4.21-4.44 (m, 5H; α -CH, Lys, α -CH, Glu, α -CH, Ala, α -CH, Lac), 3.33-3.91 (m, 6H; H-2, H-

3,H-4, H-5, H-6ab), 3.00 (t, $J = 8.0$ Hz, 1H), 2.86 (t, $J = 7.8$ Hz, 2H; ϵCH_2 , Lys), 2.20-2.41 (m, 2H, γCH_2 , Glu), 1.98-2.18 m, 2H, βCH_2 , Glu), 1.78 -1.88 (m, 4H, NHCH_3 , CH, Lys), 1.58-1.72 (m, 3H, 3 x CH, Lys), 1.22 - 1.42 ppm (m, 9H, CH_3 , Lac, 2 x CH_3 , 2 Ala); ^{13}C (HSQC): δ 89.23 ($\alpha\text{C-1}$), 87.65 ($\beta\text{C-1}$), 84.28, 82.67, 79.67, 78.96, 74.43 (αC , Ala, Glu, Lys, Lac), 80.23, 76.65, 69.78, 69.31, 63.52, 62.32, 60.54, 55.76, 54.38, 53.62, 50.83, 46.87, 40.37 (ϵC , Lys), 32.65 ($\gamma\text{C-Glu}$), 31.20, 28.21, 27.42 ($\beta\text{C-Glu}$), 26.31, 24.82, 23.76, 22.37, 22.22, 18.79, 17.65, 16.75 ppm; HRMS-MALDI-TOF calcd for $\text{C}_{30}\text{H}_{52}\text{N}_8\text{O}_{14}$ [$\text{M}+\text{Na}$]: 771.36; found 771.35.

Compound **3b** was prepared by a similar approach, however, instead of Fmoc-D-isoglu(Gly-OBn), Fmoc-D-isoglutamine, was employed as the third amino acid and the first coupling amino acid was Fmoc-Glycine. Each amino acid component was used in two-fold excess with respect to loading of the resin. The target compound was obtained as a mixture of α/β anomers (10.6 mg, 71%). $[\alpha]^{22}_{\text{D}} = +23.12$. ^1H NMR (500 MHz, D_2O): δ 5.02 (d, $J = 3.4$ Hz, 0.70H; H-1- α anomer), 4.56 (d, $J = 8.4$ Hz, 0.30H; H-1- β anomer), 4.20-4.34 (m, 4H; $\alpha\text{-CH}$, Lys, $\alpha\text{-CH}$, Glu, $\alpha\text{-CH}$, Ala, $\alpha\text{-CH}$, Lac), 3.31-4.00 (m, 8H; H-2, H-3, H-4, H-5, H-6ab, 2H, Gly), 2.88 (t, $J = 9.0$ Hz, 2H, ϵCH_2 , Lys), 2.21-2.34 (m, 2H, γCH_2 , Glu), 1.78-1.97 (m, 4H, NHCH_3 , CH, Lys), 1.51-1.76 (m, 8H,), 1.23-1.42 ppm (m, 6H, CH_3 , Lac, CH_3 , Ala); HRMS-MALDI-TOF calcd for $\text{C}_{30}\text{H}_{53}\text{N}_9\text{O}_{13}$ [$\text{M}+\text{Na}$]: 770.37; found 770.35.

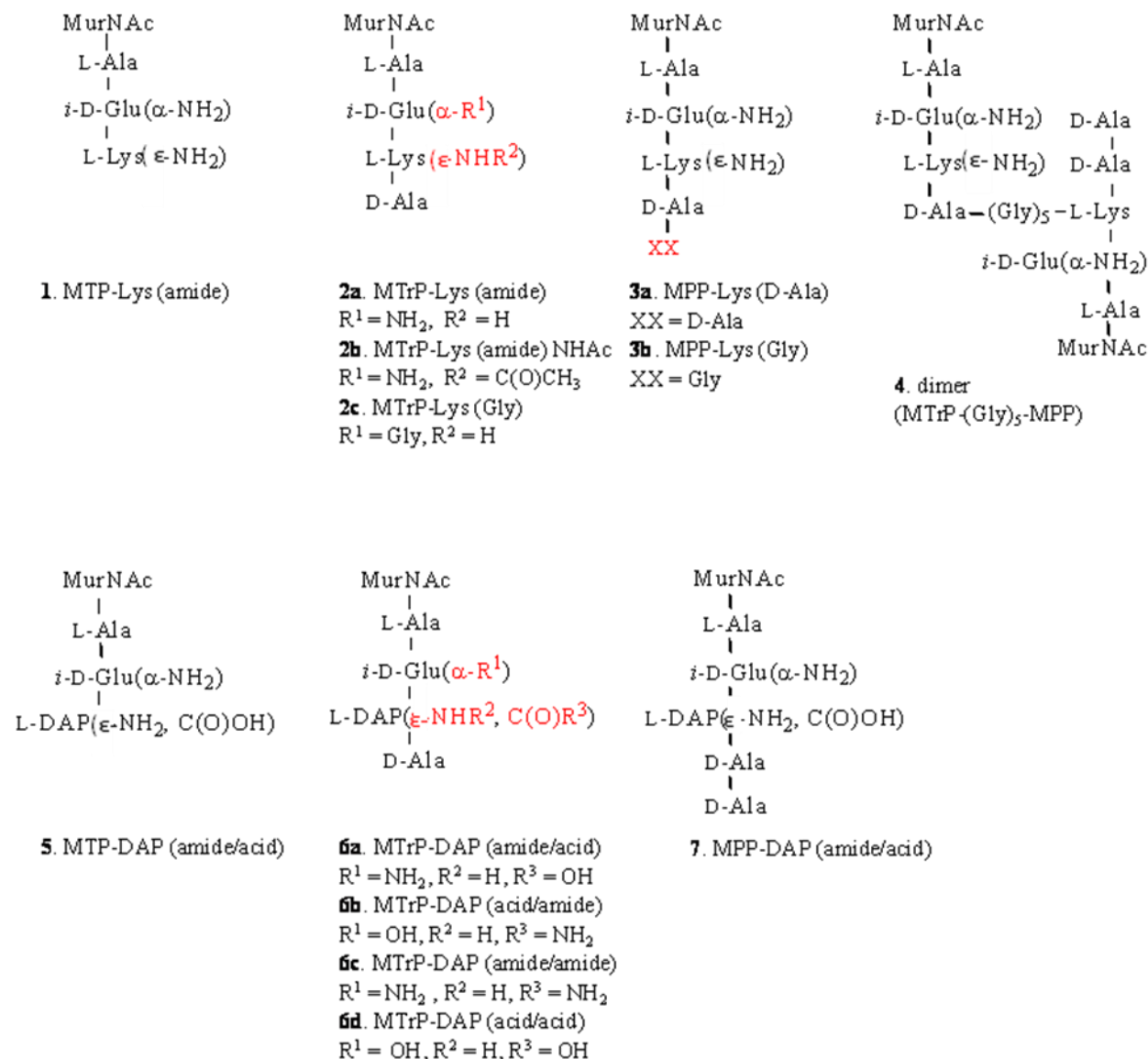


Fig 2.2: **Structures of synthetic muopeptides.** Lysine-containing compounds **1-4** derived from PGN of Gram-positive bacteria and DAP-containing compounds **5-7** derived from Gram-negative and rod-shaped Gram-positive PGN.

Compound **6b** was prepared by a similar approach, however, instead of Fmoc-L-Lys-OH, Fmoc-DAP(δ -CONH₂)-OH was used as third amino acid and Fmoc-D-Glu-OBn as the second amino acid. Each amino acid component was used in two-fold excess with respect to the loading resin. The target compound was obtained as a mixture of α/β

anomers (2.1 mg, 42%). ^1H NMR (500 MHz, D_2O): δ 5.22 (d, J = 2.8 Hz, 0.64H; H-1- α anomer), 4.71 (d, J = 8.2 Hz, 0.36H; H-1- β anomer), 4.20-4.48 (m, 6H, α,ϵ -CH, DAP, α -CH, Glu, α -CH, Ala, α -CH, Lac), 3.70-4.00 (m, 4H; H-2, H-3, H-6a,b), 3.50-3.66 (m, 2H, H-4, H-5), 3.20 (t, J = 8.0 Hz, 1H), 2.88-3.10 (m, 2H, γ -CH₂, Glu), 2.44 (t, J = 8.2 Hz, 1H), 2.09-2.40 (m, 5H, β -CH₂, Glu, NHCOCH₃), 1.38-1.61 (m, 9H, CH₃, Lac, 2 x CH₃, 2 Ala); HRMS-MALDI-TOF calcd for C₂₉H₅₀N₈O₁₄Na [M+Na]: 757.34; found 757.31.

Compound **6c** was prepared by a similar approach, however, instead of Fmoc-L-Lys-OH, Fmoc-DAP(δ -CONH₂)-OH was used as third amino acid. Fmoc-DAP(δ -CONH₂)-OH was prepared by a cross metathesis reaction of suitably protected vinyl glycine and allyl glycine followed by reduction of the double bond of the resulting compound. Each amino acid component was used in two-fold excess with respect to loading of the resin. The target compound was obtained as a mixture of α/β anomers (6.2 mg, 62%). ^1H NMR (500 MHz, D_2O): δ 5.01 (d, J = 3.2 Hz, 0.60H; H-1- α anomer), 4.55 (d, J = 8.2 Hz, 0.40H; H-1- β anomer), 4.02-4.46 (m, 6H; α,ϵ -CH, DAP, α -CH, Glu, α -CH, Ala, α -CH, Lac), 3.31-3.92 (m, 6H, H-2, H-3, H-4, H-5, H-6a,b), 2.75-2.81 (m, 2H, γ -CH₂, Glu), 2.22-2.36 (m, 5H, β -CH₂, Glu, NHCOCH₃), 1.97-2.09 (m, 2H, CH₂, DAP), 1.24-1.38 ppm (m, 9H, CH₃, Lac, 2 x CH₃, 2 Ala); HRMS-MALDI-TOF calcd for C₂₉H₅₁N₉O₁₃Na [M+Na]: 756.77; found 756.65.

2.4.2: Expression and Purification of a Soluble Form of Recombinant Extracellular Human TLR2 Domain (sTLR2)

The recombinant soluble form of human TLR2 consisting of the putative

extracellular domain (Met¹-Arg⁵⁸⁷) and a His₆ tag at the C-terminal end was expressed in a baculovirus-insect cell expression system. Monolayers of *Spodoptera frugiperda* (Sf9) cells (Invitrogen) were co-transfected with linearized baculovirus DNA (BaculoGold; BD Biosciences Pharmingen) and the pVL1393 plasmid vector (Invitrogen) containing cDNA for sTLR2⁹⁸. Recombinant plaques were isolated and viral titers were amplified to $\sim 1-10 \times 10^7$ plaque-forming units/ml. The recombinant viruses were used to infect monolayers of *Trichopulsia ni* (High Five) cells (Invitrogen) in serum-free medium at a multiplicity of 2-5. After 3-days of incubation, the medium was collected after low-speed centrifugation. Soluble TLR2 was purified by nickel-NTA (Qiagen) chromatography according to the manufacturer's instruction using 300 mM imidazole to elute the sTLR2 from the resin. The sTLR2-containing fractions were concentrated using Nanosep centrifugal devices (Pall Life Sciences). The purified protein was dialyzed against phosphate buffered saline (0.01 M; pH 7.4) and stored at – 80 °C. The sTLR2 protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce). The purity of the protein was analyzed by SDS-PAGE (10% gel) under reducing conditions with Coomassie G-250 (GelCode Blue Stain Reagent; Pierce) and silver (GelCode SilverSNAP Stain Kit II; Pierce) staining. The identity of the protein was confirmed by Western blotting using a rat polyclonal antibody to human TLR2 (pAb hTLR2) and an anti-histidine (penta-His HRP) antibody. Approximately 20 µg of sTLR2 was obtained from 1 ml culture medium.

2.4.3: Kinetic Analysis using SPR Technology

Binding interactions between sTLR2 and various analytes were examined using a Biacore T100 biosensor system (Biacore Inc. - GE Healthcare). Soluble TLR2 was immobilized by standard amine coupling using an amine coupling kit (Biacore Inc. - GE Healthcare). The surface was activated using freshly mixed N-hydroxysuccinimide (NHS; 100 mM) and 1-(3-dimethylaminopropyl)-ethylcarbodiimide (EDC; 391 mM) (1/1, v/v) in water. Next, sTLR2 (50 µg/ml) in aqueous NaOAc (10 mM, pH 4.5) was passed over the chip surface until a ligand density of approximately 5,000 RU was achieved. The remaining active esters were quenched by aqueous ethanolamine (1.0 M; pH 8.5). The control flow cell was activated with NHS and EDC followed by immediate quenching with ethanolamine. HBS-EP (0.01 M HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20; pH 7.4) was used as the running buffer for the immobilization and kinetic studies. Analytes were dissolved in running buffer and a flow rate of 20 µL/min was employed for association and dissociation at a constant temperature of 25 °C. A double sequential 60 s injection of aqueous NaOH (50 mM; pH 11.0) at a flow rate of 50 µL/min followed by 3 min stabilization with running buffer was used for regeneration and achieved prior baseline status. The same experimental surface was used for approximately 2 weeks and maintained under running buffer conditions. MTP-DAP (amide/acid) (**5**) was used as a positive control in each experiment to check the stability of the TLR2 surface activity during the course of the experiments.

To minimize bulk refractive index changes, nonspecific binding and instrument drift on the generated binding sensorgrams, a double referencing of the data was performed¹⁰⁰. First, bulk refractive index change effects were minimized by preparing all

analyte in the HBS-EP buffer. Then, the binding responses over the reference surface were subtracted from the active surface to correct for nonspecific binding, while a blank analyte run of only running buffer was also subtracted from the specific binding sensorgrams to minimize instrument noise¹⁰⁰. Using Biacore T100 evaluation software (Biacore Inc. - GE Healthcare), the response curves of various analyte concentrations were globally fitted to either the 1:1 Langmuir model or the two-state binding model¹⁰¹.

2.4.4: Biotinylation of sTLR2 and Immobilization of Biotinylated sTLR2

sTLR2 (40 µg/ml) was mixed with EZ-linkTM TFP-PEO-biotin (Pierce Biotechnology) in PBS (1:2 eq) and incubated at 4 °C overnight. Unreacted EZ-linkTM TFP-PEO-biotin was removed by filtration of the mixture over a Nanosep centrifugal device with cut-off of 3 KDa (Pall Life Sciences). The biotinylated sTLR2 was re-suspended in HBS-EP and immobilized by passing over a streptavidin-coated chip (Series S Sensor Chip SA (certified); Biacore) until a ligand density of approximately 2,000 RU was achieved. Next, a mixture of ethanolamine and biotin (10:1 eq) was passed over the ligand and control flow cells to block free streptavidin binding sites.

2.4.5: Conformational Change Analysis

A binding concentration at which a PGN subunit fragment approaches steady state was selected and injected over an immobilized TLR2 (5000RU) at 3 different contact times of 2, 5 and 10 min. All other conditions for analysis were the same as described for the kinetic measurements above. The binding responses were normalized such that for each curve, the maximum amount of sample bound, at the point where the

injection ended, was set to equal 100 units on the y-axis. The dissociation phases of the curves was compared for dissociation rate change as a function of time¹⁰⁰. In cases where no conformational change occurred, the curves were expected to overlap. Deviations are indicative of a conformational change.

2.4.6: Thermodynamic Analysis of PGN/TLR2 binding

A two-fold serial dilution of both Lys- and Dap-containing compounds was diluted and injected over immobilized sTLR2 (5000 RU). Using the Thermodynamic Wizard on the T100 BIAcore instrument, kinetic analyses were performed at temperatures of 13 °C, 19 °C, 25 °C, 31 °C, and 37 °C. All conditions for the association, dissociation and regeneration phases were the same as described for the kinetic measurements above. The thermodynamic parameters for the analysis including the binding affinity constants (K_D) for each compound determined at several temperatures was used to determine Van't Hoff free energy changes (ΔG) by plotting $\ln K_D$ as a function of the inverse absolute temperature ($1/T$). This relates the Van't Hoff equation ($\ln K_D = \Delta H^\circ/RT - \Delta S^\circ/R$) to standard free energy change ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_D$) of a system, and allows the standard enthalpy (ΔH°) and standard entropy (ΔS°) for each binding profile to be calculated^{64, 102}. The correlation coefficients (R^2) were used to determine the linearity of each Van't Hoff plot while R is the universal gas constant.

2.4.7: SPR Inhibition Assay

A two-fold serial dilution of Lys-containing PGN fragment at concentrations of 200 to 3.13 μ M were each incubated with a fixed concentration of TLR2 (0.125 μ M) at 25 °C for 30mins. The SPR binding response analysis was performed by injecting the mixture over immobilized biotinylated sPGN at a flow rate of 20 μ l/min. The association and dissociation times were 180 seconds each. The surface was regenerated with 10 mM Glycine-HCl, pH 2.5.

2.4.8: Isolation and Purification of Insoluble PGN

B. licheniformis, *E. coli*, and *L. plantarum* were cultivated in Nutrient Broth (Difco), LB Broth (Sigma), and Lactobacilli MRS Broth (Difco), respectively. Freshly prepared media were inoculated with the bacteria and incubated at 37 °C under aerobic conditions for 12-18 h. One liter of the grown bacterial cell culture was harvested by centrifugation for 30 min at 10000 rpm (4 °C). The pellets were transferred into 4% (final concentration) boiling sodium dodecyl sulfate (SDS)^{30a}. The cells were then boiled for 30 min. and the bacterial cell walls (sacculi) concentrated by centrifugation at RT for 30 min at 10000 rpm (4 °C). The sacculi was washed (8x) with water until no more SDS could be detected by the method of Hayashi¹⁰³. The cell walls were further broken with glass beads (0.1 mm) on a Vortex at maximal speed for 30 min. The suspensions were centrifuged at 2,000 rpm for 5 min to remove the glass beads.

The collected broken walls were treated overnight at 37 °C in 100 mM Tris-HCl (pH 7.5) with α -amylase (EC 3.2.1.1; Sigma; 100 μ g/ml). DNase (EC 3.1.21.1; 10 pg/ml; Sigma) and RNase (EC 3.1.27.5; 50 pg/ml; Sigma) and incubated overnight at 37 °C.

Finally, all the PGN suspensions from *B. subtilis*, *M. luteus*, *S. aureus*, *B. licheniformis*, and *L. plantarum* were treated with trypsin (EC 3.4.21.4; 100 µg/ml; Worthington) for 16h. The enzymes was inactivated by boiling for 15 min. in 1% (final concentration) SDS. The PGNs were then pelleted and washed sequentially with water (2x), 8 M LiCl (1x), 100 mM EDTA (1x), water (2x) and acetone (1x). The PGNs were re-suspended in water and lyophilized.

To remove the TA, the PGNs were stirred in 2 ml of hydrofluoric acid (48%) for 48 h at 4 °C, recovered by centrifugation at 10,000 rpm for 60 min. and re-suspended in water. The remaining acid was neutralized with ice-cold 30% ammonium hydroxide (NH₄OH) and then washed with water (8x), pelleting each time at 10,000 rpm for 30 min. The pellets were then lyophilized, weighed, re-suspended in sterile distilled water and stored at -80 °C until used.

A similar method was followed for the preparation of the peptidoglycan from *Escherichia coli*, a Gram-negative bacterium. The only alteration was due to the fact that the lack of TA in its cell walls made the hydrofluoric acid treatment step irrelevant.

2.4.9: N-Acetylation of PGN

The purified PGNs of *M. luteus* and *S. aureus* (150 µg) were suspended in a cooled (4 °C) mixture of aqueous saturated NaHCO₃ (100 µl) and Ac₂O (1 µl, 10 mmol). After agitation for 18 h at 4 °C, the reaction mixture was centrifuged (13,000 rpm, 30 min) and the solvents removed by decanting. The resulting pellet was washed five times by re-suspension in deionized water (100 µl) followed by centrifugation and decanting. A Kaiser test showed the absence of free amine groups.

2.4.10: Determining the Purity of polymeric insoluble Peptidoglycans (iPGN) from *S. aureus*, *B. subtilis*, *M. luteus*, *L. plantarum*, *B. licheniformis*, and *E. coli*, using high performance anion exchange chromatography (HPAEC)¹⁰⁴

An HPAEC IPAD system (Dionex ICS-3000) consisting of a SP-1 low-pressure gradient pump, an electrochemical detector and an AminoPac PA10 column set were used (Table 2.1)¹⁰⁴. The condition for analysis was set as shown in table 2.1A and 2.1B. The analysis time was 74 min and 4 degassed solvents *i.e.* distilled water, 250 mM sodium hydroxide, 1.0 M sodium acetate and 0.1M acetic acid were used. Briefly, each PGN sample was hydrolyzed by adding 6N HCL and then allowed the reaction mixture at 100 °C overnight. The mixture was lyophilized and then re-dissolved with degassed distilled water containing sodium azide (20 mg/L). Twenty- five microliter (25 µl) of each sample was injected into the Dionex machine for PGN composition analysis.

Table 2.1: (A) An amino acid waveform with a Mode II current integration period was used. The time is given in milliseconds and the potentials are in Volts¹⁰⁴. (B) Gradient conditions¹⁰⁴ for separations with AminoPac PA10: E1: water, E2: 250 mM sodium hydroxide, E3: 1.0 M sodium acetate, E4: 0.1M acetic acid. Flow rate: 250 µl/min

A) Waveform with a Mode II current integration period was used

Time (millisecond)	Potential	Mode II
0	-0.2	
40	-0.2	
50	-0.05	
110	-0.05	Start
210	-0.05	
220	0.28	
460	0.28	
470	-0.05	
560	-0.05	End
570	-2	
580	-2	
590	0.6	
600	-0.2	

B) Gradient conditions for separation with AminoPac PA10

Time (min)	E1 (%)	E2 (%)	E3 (%)	E4 (%)	Curve	Comments E4: 0.1M acetic acid
Init.	76	24	0	0		
0.0	76	24	0	0		
2.0	76	24	0	0		
8.0	64	36	0	0	8	
11.0	64	36	0	0		
18.0	40	20	40	0	8	
21.0	44	16	40	0	5	
23.0	14	16	70	0	8	
45.0	14	16	70	0		
45.1	0	0	0	100	8	This segment cleans the column
47.1	0	0	0	100		
47.2	20	80	0	0	8	Removal of acetate from the column
49.2	20	80	0	0		
49.3	76	24	0	0	5	Re-equilibration
74.0	76	24	0	0		

E1: water, E2: sodium hydroxide (250 mM), E3: sodium acetate (1.0 M), E4: acetic acid (0.1 M).

2.4.11: The evaluation of Cytokine production using Cellular activation studies with purified and chemically modified PGN

The HEK 293T and MM6 cells were maintained in a humid 5% CO₂ atmosphere at 37 °C and used for this study. MM6 cells were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with penicillin (100 u/mL) / streptomycin (100 µg/mL; Mediatech), OPI supplement (containing oxaloacetate (1 mM), pyruvate (0.45 mM) and bovine insulin (0.2 u/mL); Sigma) and fetal bovine serum (10%; HyClone). Before each experiment, MM6 cells were incubated with calcitriol (10 ng/mL; Sigma) for 2 days to differentiate into macrophage-like cells. The human embryonic kidney cell line (HEK 293T), obtained from ATCC, was grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g/L) and sodium bicarbonate (1.5 g/L) supplemented with penicillin (100 u/mL) / streptomycin (100 µg/mL; Mediatech),

Normocin (100 µg/mL; InvivoGen) and FBS (10%). HEK293T cells, stably transfected with human TLR2 and human TLR4/MD2/CD14, was obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with blasticidin (10 µg/mL) and for HEK cells w/ hTLR4/MD2/CD14 also with HygroGold (50 µg/mL).

The Measurement of cytokine from MM6 was done through a TNF-α ELISA. Differentiated MM6 cells were incubated with stimuli for 5.5 h in the presence of polymyxin B (PMB) to ensure that any TNF-α production was not caused by LPS contamination. Concentrations of TNF-α in culture supernatants was determined in duplicate by a solid phase sandwich ELISA. Briefly, 96-well plates (Nalge Nunc International) were coated with purified mouse anti-human TNF-α monoclonal antibody (mAb; Pharmingen). TNF-α in standards and samples were allowed to bind to the immobilized mAb for 2 h at room temperature, after which biotinylated mouse anti-human TNF-α mAb (Pharmingen) was added. After addition of avidin-horseradish peroxidase conjugate (Pharmingen) and ABTS (2, 2'-azino-di (3- ethylbenthiazoline-6-sulfonate)) peroxidase substrate (Kirkegaard & Perry Laboratories) the absorbance was measured at 405 nm using a microplate reader (BMG Labtech). PGN concentration-response data was analyzed using nonlinear least-squares curve fitting in GraphPad Prism (GraphPad Software, Inc.). These data was fitted with the following logistic equation: $Y = E_{\max} / (1 + (EC50/X)^{\text{Hill slope}})$, where Y is the TNF-α response, X is the PGN concentration, $E_{\max}$ is the maximum response and EC50 is the concentration of PGN producing 50% stimulation. The Hill slope was set at 1 to be able to compare the EC50 values of different inducers. TNF-α value was presented as the means + SD of duplicate cultures and the experiments were repeated three times.

2.4.12: Transfection and NF- κ B Activation Assay

The receptor specificity of highly purified PGN was also investigated by employing HEK 293T cells. Highly purified PGN was used to induce activation of NF- κ B in a TLR2- dependent manner. HEK 293T wild type cells and HEK 293T cells stably transfected with human TLR2 or human TLR4/MD2/CD14 was transiently transfected in 96-well tissue culture plates (16,000 cells/well) using PolyFect Transfection Reagent (Qiagen) with expression plasmids pELAM-Luc (NF- κ B-dependent firefly luciferase reporter plasmid, 50 ng/well) S1 and pRL-TK (*Renilla* luciferase control reporter vector, 1 ng/well; Promega) as an internal control to normalize experimental variations. Forty-four h post-transfection, cells were exposed to the stimuli at the indicated concentrations in the presence of FBS for 4 h. The luciferase activity was measured in cell extracts using the Dual-Luciferase Reporter Assay System (Promega) and a combination luminometer/fluorometer microplate reader (BMG Labtech). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data was reported as the means + SD of triplicate treatments, with each transfection experiment being repeated three times.

2.5 Results

2.5.1 Purification of soluble sTLR2 protein

The soluble sTLR2 protein (fig. 2.3) was modified by a His₆-tag, which facilitated purification by nickel-nitrilotriacetic acid (NTA) column chromatography. SDS-PAGE analysis and Western blotting confirmed the homogeneity of the protein

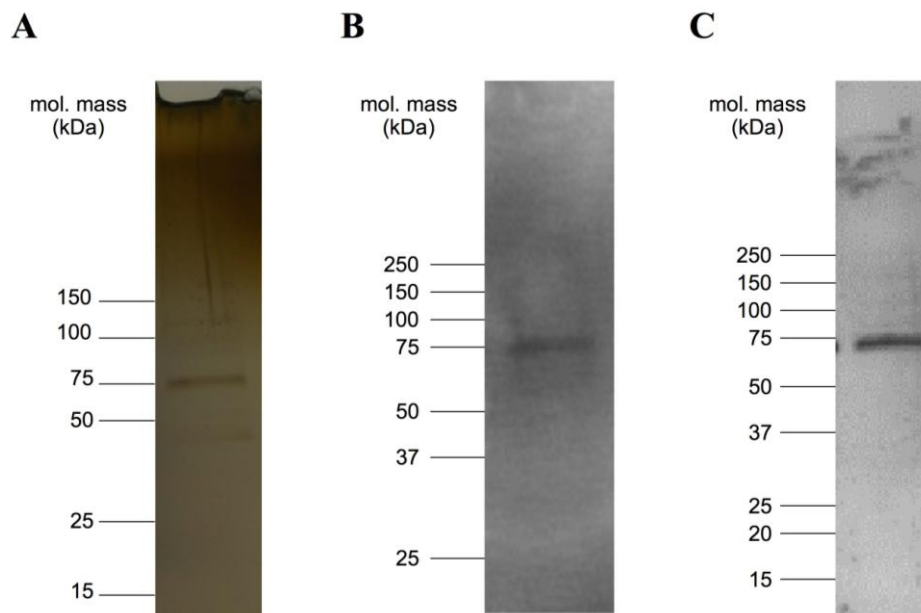


Fig. 2.3: **Electrophoretic analysis and Western blotting of sTLR2 protein.** Purified sTLR2, subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions, was visualized by silver staining (A) and identified by Western blotting using a rat anti-human TLR2 polyclonal antibody (B) and a mouse anti-poly-histidine monoclonal antibody (C).

2.5.2 Analysis of the Binding of Human Soluble TLR2 to Synthetic PGN Part Structures

SPR was employed to study the interactions of the soluble extracellular domain of human TLR2 with synthetic lysine- and DAP-containing PGN fragments **1-7** (Fig. 2.2). The lysine-containing muramyl tripeptide **1** (MTP-Lys), muramyl tetrapeptides **2a-c** (MTrP-Lys), muramyl pentapeptides **3a,b** (MPP-Lys), and dimer **4** are derived from PGN of Gram-positive bacteria. On the other hand, compounds **5-7** are part structures of Gram-negative and rod-shaped Gram-positive PGN and structurally similar to muropeptides **1-3**, with the exception that the lysine residue is replaced by a DAP moiety. SPR studies using these derivatives, established the importance of each amino acid of the stem peptide for binding and uncovered that TLR2 can recognize PGN derived from Gram-positive and -negative bacteria. Furthermore, muramyl pentapeptides **3a** and **7** are derived from nascent PGN whereas the muramyl tri- (**1**, **5**) and tetra-peptides (**2**, **6**) represent structures that can be formed during PGN remodeling by bacterial autolysins. The use of these compounds uncovered the importance of nascent and remodeled PGN for TLR2 recognition.

Cross-linking of Gram-positive PGN results in the loss of a terminal D-Ala moiety to generate a tetrapeptide, which is linked to an ϵ -amino group of the lysine of another PGN polymer through a glycine-containing interpeptide bridge (Fig. 2.1A). Compound **4**, which is composed of a muramyl tetrapeptide linked through a pentaglycine bridge peptide to a muramyl pentapeptide, was employed to study the influence of PGN cross-linking on TLR2 recognition. Bacterial endopeptidases can cleave the glycine interpeptide bridge to generate a stem tetrapeptide extended by glycine moieties (Fig.

2.1A). Muramyl pentapeptide **3b** represents such a structure and contains a tetrapeptide extended by one Gly moiety. Derivative **2b** was used to establish the influence of acylation of the lysine side chain amino group (R^2) for binding. This modification was deemed important because it mimics the attachment of this functionality to the penultimate D-Ala of another PGN chain as in *S. aureus* PGN.

Many bacterial strains modify the α -carboxylic acid of iso-glutamic acid of PGN by amidation or by addition of Gly^{94b, c}. The importance of the chemical nature of the ϵ -carboxylic acid of iso-D-Glu (R^1) of lysine containing PGN was investigated by employing compound **2c**, which has a glycine linked to iso-D-Glu as in *M. luteus* PGN. Bacteria that biosynthesize DAP-containing PGN can have the α -carboxylic acid of iso-D-Glu (R^1) and the γ -carboxylic acid of DAP (R^3) as an amide or acid. The importance of these modifications was investigated using muramyl tetrapeptides **6a-d**, which are derived from *B. licheniformis* (**6a**), *B. subtilis* (**6b**), *L. plantarum* (**6c**), and *E. coli* (**6d**) and have the carboxylic acids in the amide or acid form.

SPR is a rapid and sensitive method for the evaluation of affinities of biomolecular interactions¹⁰⁵ that has as a benefit that it relies exclusively on mass changes, thus allowing the study of interactions in real time without the need for external labels such as fluorophores, which in some cases can alter the nature of the interaction. Collecting SPR data for low molecular weight analytes such as compounds **1-7** is challenging because the refractive index monitored during a binding event is relatively small, and thus results in responses with much lower magnitudes than those observed in typical protein-protein interactions. In spite of these challenges, the high sensitivity and reproducibility of modern instruments combined with proper experimental

design permits the direct monitoring of the binding of low molecular weight analytes to immobilized proteins¹⁰⁶.

Approximately 5,000 RU of sTLR2 were immobilized on NHS-activated groups of a CM-5 research grade sensor chip surface and titration experiments were performed with the synthetic compounds. Good agreements between the theoretically predicted and experimentally observed maximal specific binding ($R_{\max}$) parameters indicated that amine immobilization of the protein yielded fully active surfaces. The high mass transport coefficients ($> 10^{10}$) obtained from the kinetic model fit with mass transport limitation (MTL) experiments using the MTL wizard were indicative of insignificant mass transport effects and negligible rebinding of the analyte during the post injection phase (data not shown). For all SPR experiments, bulk refraction caused by the difference in the refractive index of the running buffer and sample injection was negated by using a control cell functionalized by ethanolamine.

Representative sensorgram for the interaction of lysine-containing muramyl tripeptide **1** with immobilized sTLR2 are shown in Fig. 2.4A. A 1:1 Langmuir binding model gave an excellent fit of the sensorgrams ($\chi^2 = 0.26$) and provided a K_D of 78 mM (Table 2.2). Furthermore, a slow association and dissociation characterized the binding. Some heterogeneity was observed at the highest concentration of analyte tested, and therefore TLR2 was immobilized by an alternative method at a lower loading (2,000 RU) by biotinylation of TLR2 using EZ-linkTM TFP-PEO-biotin and a streptavidin-coated chip. Binding experiments gave very similar sensorgrams (Appendix B) and kinetic analysis provided a dissociation constant of 97 μ M, underscoring that heterogeneity at the highest concentrations of analyte does not significantly affect the binding affinity.

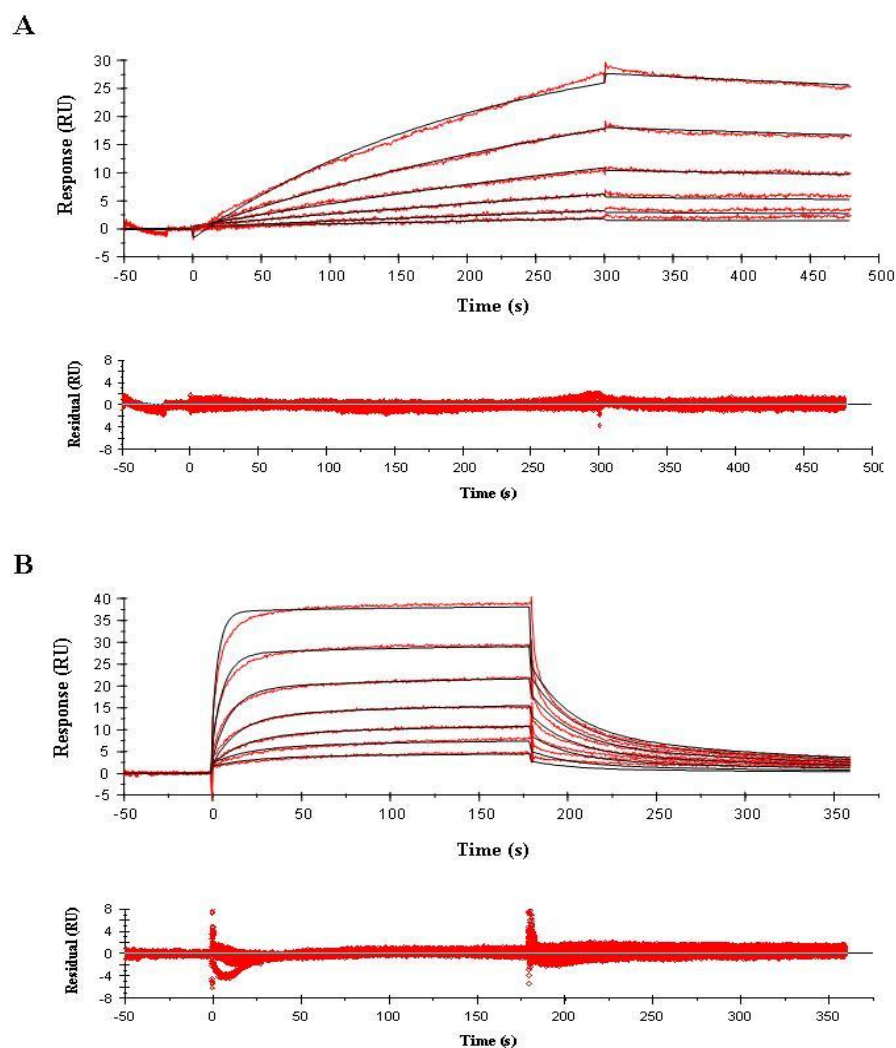


Fig. 2.4: **Sensorgrams representing the concentration-dependent kinetic analysis of lysine- and DAP-containing PGN part structures with immobilized sTLR2 (5,000 RU).** Shown are simultaneous kinetic analysis of two-fold serial dilutions of MTP-Lys (amide) **1** at concentrations of 500 to 15.6 μM , fitted with a Langmuir 1:1 binding model (black lines) (A) and MTP-DAP (amide/acid) **5** at concentrations of 100 to 1.6 μM , fitted with a two-state binding model (black lines) (B). The corresponding residual values are plotted below the individual sensorgrams. RU indicates resonance units. Triplicate measurements provided K_D values of $78 \pm 6 \mu\text{M}$ and $11 \pm 2 \mu\text{M}$ for **1** and **5**, respectively. As negative controls, *E. coli* LPS and Poly (I).Poly (C), which are agonists of TLR4 and TLR3, respectively, were used and gave no binding (Appendix A). The TLR2 agonist Pam₃CysSK₄ was employed as a positive control, which showed binding in a dose-dependent manner but an affinity constant could not be determined due to a poor fit of the sensorgrams, probably as a result of aggregation of the analyte.

Muramyl tetrapeptide **2a** exhibited a similar kinetic profile as **1** (Table 2.2, Appendix A), however, in this case a slightly smaller K_D was determined; indicating that the D-Ala moiety attached to iso-glutamate contributes somewhat to binding. Muramyl pentapeptide **3a** showed binding only at very high concentrations (> 500 mM), which made it difficult to accurately determine a binding constant, and established that TLR2 cannot accommodate the second D-Ala moiety of **3a**. Dimer **4** was also poorly recognized by TLR2; however, muramyl pentapeptide **3b**, which contains one glycine moiety linked to the stem peptide, was recognized with modest affinity. These results indicate that nascent PGN (muramyl pentapeptide) and the initial cross-linked product are poorly recognized by TLR2 (Table 2.2, Appendix A). However, PGN remodeling products such as compounds **1**, **2a**, and **3b** are recognized by the receptor.

Muramyl tetrapeptide **2b**, which has a lysine with an acetylated side chain, was found to bind with a 10-fold reduced affinity, compared to amine-containing mucopeptide **2a** further demonstrating that crosslink leads to loss of activity. Compound **2c**, which contains an isoglutamic acid moiety extended by glycine, was poorly recognized by TLR2, indicating that modification of the α -carboxylic acid of iso-glutamine is a strategy by microbes to avoid innate immune detection by TLR2.

TABLE 2.2: Kinetic parameters for the interaction of TLR2 with synthetic compounds **1-7**

Analyte	k_{a1} (10^{-3} s^{-1})	k_{d1} (10^{-3} s^{-1})	k_{a2} (10^{-3} s^{-1})	k_{d2} (10^{-3} s^{-1})	K_D μM	χ^2 (RU^2)
MTP-Lys (amide) 1	10 ± 4	0.80 ± 0.31	-	-	78 ± 6	0.26
MTrP-Lys (amide) 2a	24 ± 1	0.43 ± 0.01	-	-	18 ± 1	0.35
MTrP-Lys (amide) NHAc 2b	2.1 ± 0.7	0.52 ± 0.04	-	-	241 ± 64	0.32
MTrP-Lys (Gly) 2c	-	-	-	-	> 1,000	-
MPP-Lys (D-Ala) 3a	-	-	-	-	> 1,000	-
MPP-Lys (Gly) 3b	9.7 ± 2.4	2.4 ± 0.3	-	-	258 ± 96	0.21
MTrP-(Gly) ₅ -MPP 4	-	-	-	-	> 1,000	-
MTP-DAP (amide/acid) 5	2269 ± 7 ---	48 ± 18	3.5 ± 1.7	4.1 ± 1.5	11 ± 2	0.57
MTrP-DAP (amide/acid) 6a	3796 ± 580	89 ± 42	3.9 ± 1.2	5.0 ± 2.1	12 ± 1	0.88
MTrP-DAP (acid/amide) 6b	66 ± 15	0.22 ± 0.10	-	-	3.6 ± 2.0	0.25
MTrP-DAP (amide/amide) 6c	696 ± 205	95 ± 18	5.0 ± 1.0	3.7 ± 1.5	56 ± 12	0.40
MTrP-DAP (acid/acid) 6d	-	-	-	-	NB	-
MPP-DAP (amide/acid) 7	3599 ± 182	69 ± 3	3.8 ± 0.5	4.4 ± 0.2	10 ± 1	1.0

The recombinant extracellular domain of human sTLR2 was immobilized on NHS-activated groups of a CM-5 sensor chip surface (5,000 RU) and titration experiments were performed with the synthetic compounds **1-7**. The binding constants of compounds **1**, **2a,b**, **3b**, and **6b** were determined by fitting the data using a 1:1 binding model. For compounds **5**, **6a**, **6c**, and **7**, a two-state binding model was employed to determine binding constants. NB indicates no binding at 100 mM. Sensorgrams representing the kinetic analysis are presented in Fig. 3 for compounds **1** and **5** and in Fig. S2 for compounds **2a-c**, **3a,b**, **4**, **6a-d**, and **7**. RU indicates resonance units. Data represent mean values $\pm$ s.d ($n = 3$).

Surprisingly, the titration sensorgram of the DAP-containing compound **5** showed a very distinct binding profile in which an initial fast association was followed by slow increase in binding (Fig. 2.4B). A 1:1 binding model gave an unsatisfactory fit due to nonrandom distribution of residuals during the dissociation phase (Appendix C). A two-state binding model provided, however, a good fit ($\chi^2 = 0.57$), and a K_D of 11 mM was established. A contact time experiment, in which the dissociation phase of binding sensorgrams is analyzed as a function of time, was performed to confirm the two-state binding model (Fig. 2.5A)¹⁰¹. As expected, the dissociation rate became progressively slower after a longer injection (contact) time (120, 300, and 600 sec. gave 6.2×10^{-3} , 4.5

$\times 10^{-3}$, and $3.1 \times 10^{-3} \text{ s}^{-1}$, respectively), which is in agreement with a two state-binding model. Furthermore, simulation of the rate constants of the experimentally determined response curve for a 600 s contact time experiment, using the two-state binding model equation, showed that fast complexation between TLR2 and compound **5** (**5**-TLR2) is followed by a slow conformational change to give **5**-TLR2* (Fig. 2.5B). The initial complex (**5**-TLR2) dissociates fast whereas the complex that has undergone a conformational change (**5**-TLR2*) has a delayed release of bound compound and is more stable. A two-state binding model can also be rationalized by a sequential two-step binding process; however, the small size of the ligand makes such a process unlikely. Finally, the association constant established by kinetic analysis was validated by an experiment that employed an extended contact time of 15 min to achieve a state close to equilibrium. The latter feature made it possible to analyze the data using a steady state equilibrium model, which provided a K_D of 21 mM; a value that is in close agreement with that obtained by kinetic analysis.

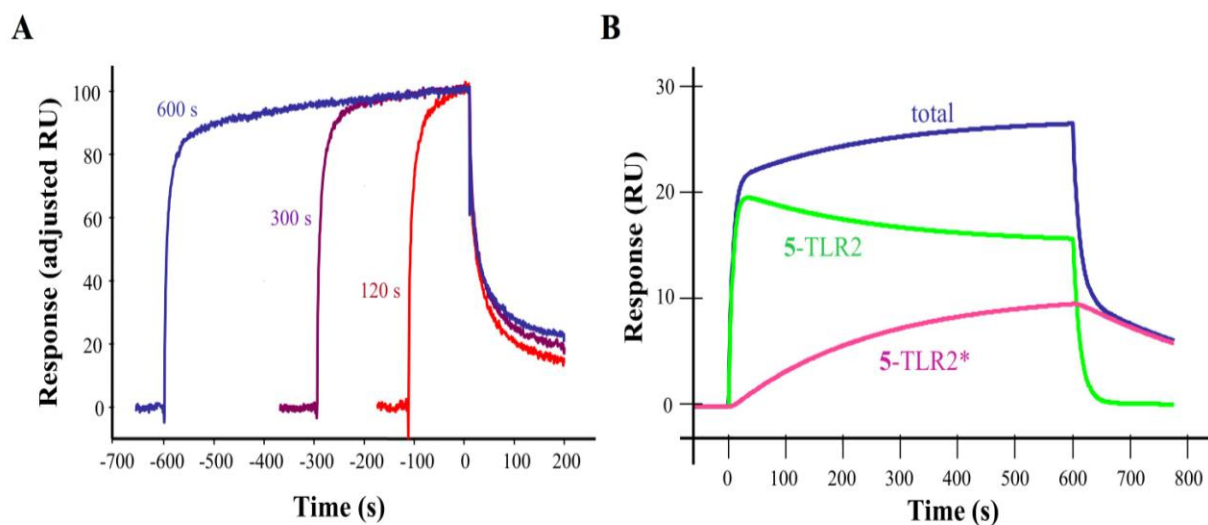


Fig. 2.5: **A contact time experiment using TLR2 and compound 5 to confirm a two-state binding model.** Adjusted binding curves for the MTP-DAP (amide/acid) (**5**; 50 μM) for exposure times of 120 s (red), 300 s (purple), and 600 s (blue) are shown (A). The analyte injection endpoint of each time point was set to the same value on the y-axis (100) and x-axis (0) to indicate the point at which the comparison of the various dissociation phases began. The dissociation rates of triplicate measurements were $6.2 \pm 0.6 \times 10^{-3}$, $4.5 \pm 1.1 \times 10^{-3}$, and $3.1 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ at analyte exposure times of 120, 300, and 600 s, respectively. A statistically significant difference ($P < 0.05$) was observed between 120 versus 600 s. The total binding (blue) represents the observed data for a 600 s exposure using **5** at 50 μM (B). The formation of the initial complex **5**-TLR2 (green) and the complex, which has undergone a conformational change (**5**-TLR2*; pink), are obtained by simulating the equation for two-state binding (see Experimental Procedures section). RU indicates resonance units.

Muramyl tetrapeptide **6a** and muramyl pentapeptide **7** bound with similar affinities as **5** indicating that in the case of DAP-containing Muropeptides (Table 2.2), the TLR2 protein can accommodate the D-Ala-D-Ala moiety but does not contribute to binding. In these cases, the sensorgrams could only be properly fitted using a two-state binding model. Finally, it was observed that the chemical nature of the α -carboxylic acid of iso-Glu(n) and the γ -carboxylic acid of DAP can have a dramatic impact on binding. Thus,

compounds **6b** and **6c** bound in the low- and mid-micromolar range, respectively whereas binding was abolished when compound **6d** was employed.

2.5.3 Thermodynamic Analysis of PGN/TLR2 binding

As noted, the Dap and Lys containing compounds had very distinct binding profiles as shown in figure 2.4; thermodynamic analysis was performed to determine the various forces driving the binding interactions. The free energy changes (ΔG) for the binding of MTrP-Lys-amide (**2a**) and MTrP-Dap-amide (**6a**) to sTLR2 were -26.9 kJ/mol and -29.51 kJ/mol respectively. This showed that sTLR2 binding of PGN part structures was favorable. However, the SPR analysis revealed that binding of MTrP-Lys-amide was driven by enthalpy (ΔH^0) whereas that for MTrP-Dap-amide was driven by entropy (ΔS^0) (Fig. 2.6). Enthalpies of -34.18 kJ/mol and 64.4 kJ/mol were obtained, whereas entropies of -24.42 J/K*mol and 315 J/K*mol were obtained for MTrP-Lys-am and MTrP-Dap-am respectively.

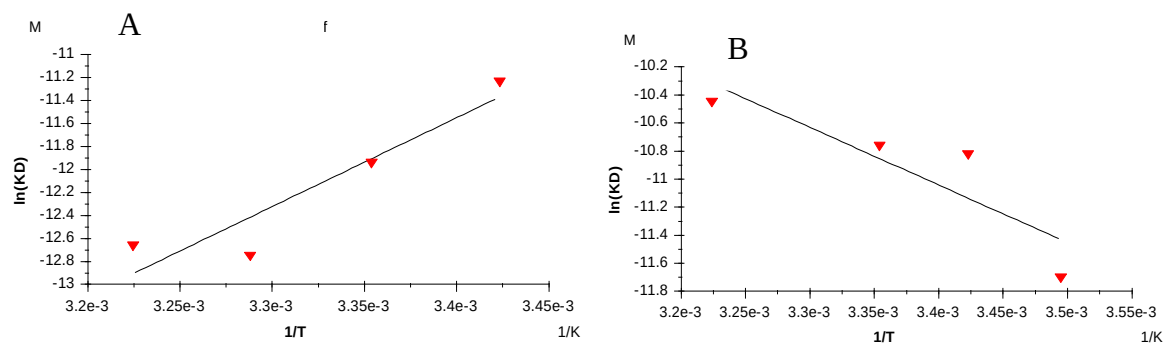


Figure 2.6: Thermodynamic Van't Hoff plots for MTrP-Dap-amide, **6a** (A) and MTrP-Lys-amide, **2a** (B). The analysis yielded $\Delta G = -29.51$ kJ/mol, $\Delta H^0 = 64.4$ kJ/mol, $\Delta S^0 = 315$ J/K*mol and a correlation coefficient (R^2) of 0.87 for MTrP-Dap-am. With a correlation coefficient (R^2) of 0.77 for MTrP-Lys-am plot, the thermodynamic parameters where $\Delta G = -26.9$ kJ/mol, $\Delta H^0 = -34.18$ kJ/mol, $\Delta S^0 = -24.42$ J/K*mol.

2.5.4 SPR Inhibition Assay was done to determine if small synthetic PGN fragments and polymeric PGN bind to the same site on TLR2

The data showed that incubating 200 μ M MTrP-Lys-amide (**2a**) with 0.125 μ M sTLR2 prevented its binding to immobilized biotinylated sPGN by 38%. PGRP-I α C was used as a positive control and incubating it with 100 μ M MtrP-Lys-amide led to a 35% binding inhibition (Fig. 2.7). It was expected that incubating sTLR2 with a much higher concentration of MTrP-Lys-amide could completely inhibit its binding to immobilized sPGN otherwise any other observed binding could indicate a secondary binding site for the PGN to sTLR2.

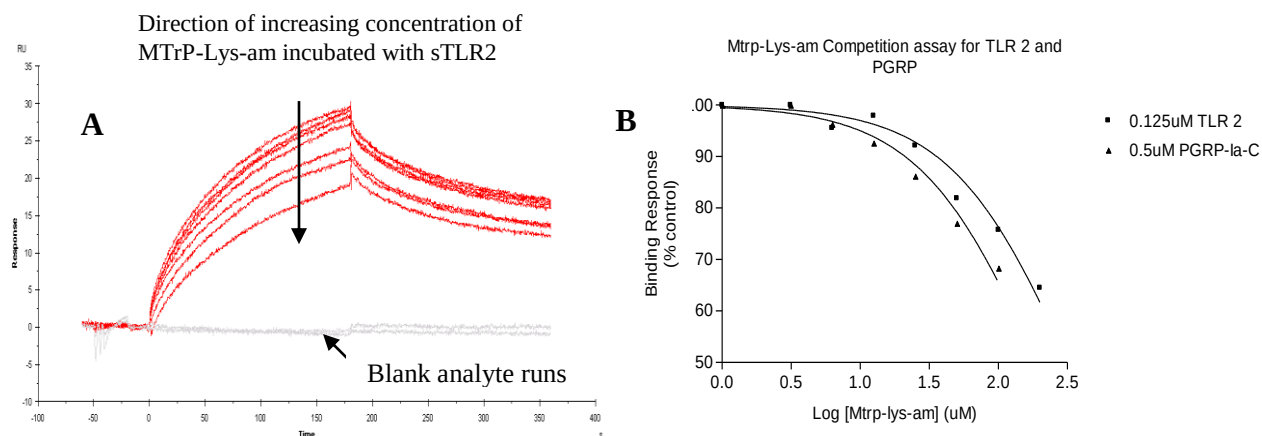


Fig. 2.7: Sensorgrams for inhibition assay. Concentrations from a two fold serial dilution of 200 μ M MTrP-Lys-am solution were each incubated at 25 $^{\circ}$ C for 30mins with a fixed concentration of 0.125 μ M TLR2. Graph A, shows the SPR binding responses obtained after injections of MTrP-Lys-am/TLR2 mixtures over immobilized biotinylated sPGN. The highest concentration of 200 μ M MTrP-Lys-am incubated with sTLR2 produced the least binding. Graph B shows the percent inhibition of sTLR2 binding to immobilized sPGN relative to the control (0.125 μ M TLR2). PGRP-I α C (0.5 μ M) was used as the positive control.

2.5.5 Determining the Purity of polymeric insoluble Peptidoglycans (iPGN) from *S. aureus*, *B. subtilis*, *M. luteus*, *L. plantarum*, *B. licheniformis*, and *E. coli*¹⁰⁴

The structural identity of the PGNs was determined by acid-catalyzed hydrolysis of the amide and glycosidic linkages followed by amino acid and monosaccharide analysis using high performance anion exchange chromatography and integrated pulsed amperometric detection (HPAEC IPAD). A high level of purity was obtained for the various bacterial PGN screened. After resolution of components, very few extra peaks were detected (Fig. 2.8)

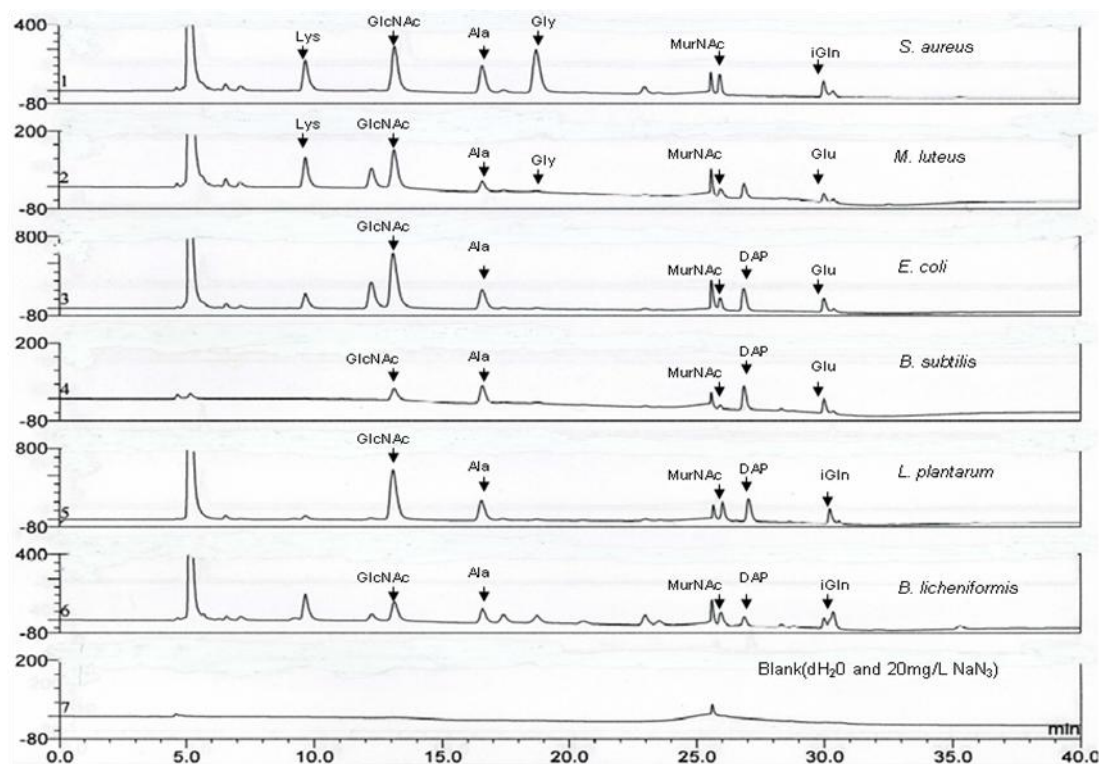


Fig. 2.8: The structural identity of the PGNs. Determined by acid-catalyzed hydrolysis of the amide and glycosidic linkages followed by amino acid and monosaccharide analysis using HPAEC IPAD. Shown are the HPAEC IPAD profiles of the bacterial PGN. The HPAEC IPAD

2.5.6: Cellular Activation Studies with Purified and Chemically Modified PGN

A number of cellular activation studies were performed to validate the biological significance of the *in vitro* binding studies. First, cellular responses induced by highly purified *S. aureus* PGN were compared with those of a PGN preparation, in which free amino functionalities were acetylated with acetic anhydride. It was expected that acetylation of the free amines would lead to a reduction of biological activity because the SPR studies had shown that this functionality of lysine contributes significantly to binding (compound **2a** vs. **2b**). Furthermore, the activity of *M. luteus* PGN was investigated because this Gram-positive bacterium modifies the α -carboxylic acid of iso-glutamine with glycine and our binding studies have shown that such a modification dramatically reduces the affinity for TLR2 (compound **2a** vs. **2c**). Thus, it was expected that PGN from *M. luteus* would display low biological activity. In addition, PGN from *B. licheniformis*, *B. subtilis*, *L. plantarum*, and *E. coli* were studied because these bacterial strains express DAP-containing PGN that differ in chemical nature of the α - and γ -carboxylic acid of iso-Glu(n) and DAP. The SPR studies had established that these modifications can have a dramatic impact on binding to TLR2 (compounds **6a-d**).

The various PGNs were purified by treatment with pronase or trypsin to remove proteins and lipoproteins, followed by washing with SDS to remove noncovalently bound lipophilic residues, and finally exposure to HF (48% v/v) to cleave phosphodiester of teichoic acids^{97b}. The free amino groups of lysine-containing PGNs from *S. aureus* and *M. luteus* PGN were acetylated by reaction with acetic anhydride in aqueous saturated sodium bicarbonate. The absence of any free amino groups was confirmed by Kaiser test.

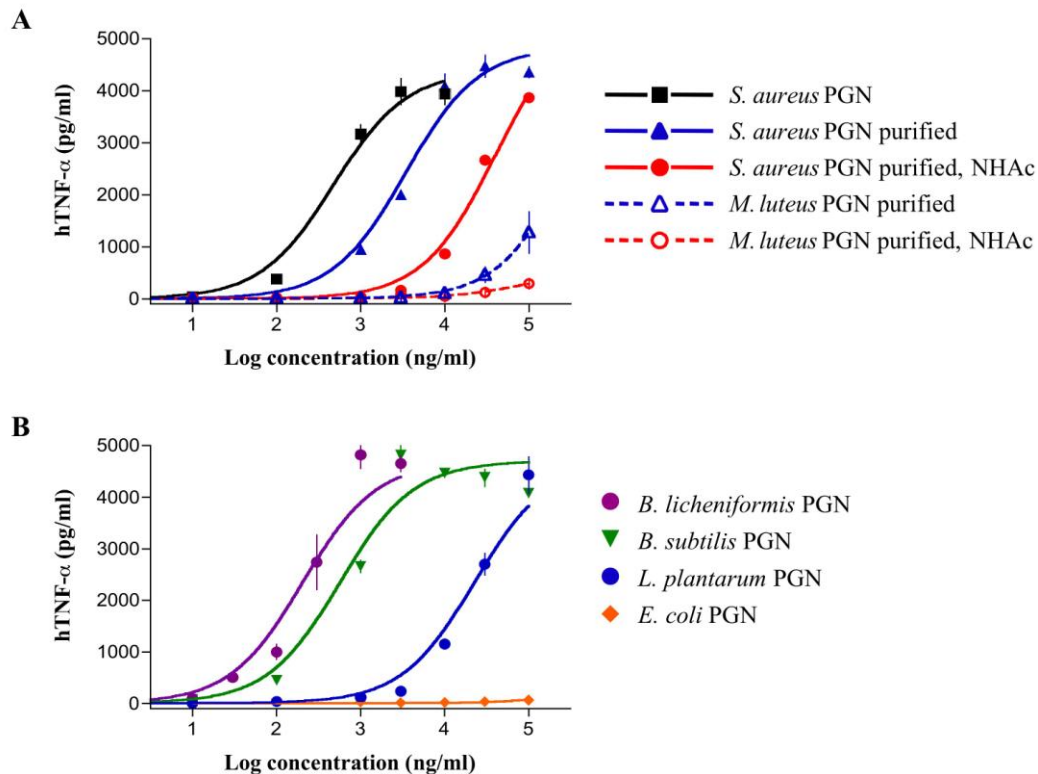


Fig. 2.9: Induction of TNF- α production by MM6 cells treated with different PGNs. TNF- α production after treatment with lysine-containing PGNs (A). MM6 cells were incubated with increasing concentrations of unpurified and purified *S. aureus* PGN before and after *N*-acetylation and purified *M. luteus* PGN before and after *N*-acetylation for 5.5 h. TNF- α protein in cell supernatants was determined using ELISA. The best-fit EC₅₀ values are 0.5, 3.4, and 40.2 μ g/ml for *S. aureus* PGN, purified *S. aureus* PGN, and purified / *N*-acetylated *S. aureus* PGN, respectively. An estimated EC₅₀ value of 184 μ g/ml was obtained for purified *M. luteus* PGN. All combinations of EC₅₀ values gave statistical significant differences ($P < 0.05$). TNF- α production after treatment with DAP-containing PGNs (B). MM6 cells were incubated with increasing concentrations of purified *B. licheniformis*, *B. subtilis*, *L. plantarum*, and *E. coli* PGN for 5.5 h. The best-fit EC₅₀ values and statistical analysis are reported in Table 2. Pronase-treated PGNs gave similar dose-response curves. Data represent mean values $\pm$ s.d. ($n = 3$).

Next, a human monocytic cell line (Mono Mac 6; MM6) was exposed over a wide range of concentrations to the various PGN preparations. After 5.5 hours, the supernatants were harvested and examined for TNF- α using capture ELISA. Potencies

(EC₅₀, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose-response curves to a logistic equation using PRISM software. Interestingly, each *S. aureus* PGN preparation yielded clear dose response curves (Fig. 2.9A). The purification protocol led, however, to a significantly lower potency demonstrating that PGN contains contaminants that induce TNF- α production. *S. aureus* PGN in which the free amines were acetylated gave an EC₅₀ that was ~10-times larger compared to unmodified PGN, which is in agreement with the finding that acetylation of ϵ -amine of the lysine moiety of (**2a** vs. **2b**) resulted in a significant reduction in binding affinity (Table 2.2). Importantly, purified PGN from *M. luteus* induced TNF- α only at very high concentrations and the estimated EC₅₀ value is at least 2-orders of magnitude larger than that of *S. aureus*. This observation is in agreement with the results of the binding studies, which showed that glycine modification of iso-glutamine of PGN, as observed in *M. luteus*, leads to a significant reduction in binding (Table 2.2). As expected, acetylation of PGN of *M. luteus* abolished activity.

The biological activities of the DAP-containing PGNs modeled the findings of the binding studies. Thus, PGN obtained from *B. licheniformis*, *B. subtilis*, and *L. plantarum*, which contain iso-Glu and DAP moieties similar to compounds **6a**, **6b**, and **6c**, respectively induced the secretion of TNF- α (Fig 2.9B and Table 2.3). *L. plantarum* PGN was less active than the two other preparations and contains a mucopeptide (**6c**) that bound with lower affinity with TLR2. Importantly, *E. coli* PGN did not induce the secretion of TNF- α , which is in agreement with the finding that mucopeptide **6d**, which has iso-Glu and DAP moieties similar to *E. coli* PGN, does not bind with TLR2.

TABLE 2.3: EC₅₀ values of TNF- α production induced by DAP-containing PGNs

PGN	EC ₅₀ (μ g/ml) ^a	
<i>B. licheniformis</i>	0.21	0.12 to 0.36
<i>B. subtilis</i>	0.57	0.28 to 1.18
<i>L. plantarum</i>	23	16 to 33
<i>E. coli</i>	NA ^b	NA

MM6 cells were incubated with increasing concentrations of purified *B. licheniformis*, *B. subtilis*, *L. plantarum*, and *E. coli* PGN for 5.5 h. TNF- α protein in cell supernatants was determined using ELISA. The dose-response curves are presented in Fig. 5B.

^a Values of EC₅₀ are reported as best-fit values and as 95% confidence intervals of the EC₅₀. A statistical significant difference ($P < 0.05$) was observed between *B. licheniformis* versus *L. plantarum* PGN and *B. subtilis* versus *L. plantarum* PGN.

^b NA indicates no activity at 100 μ g/ml.

Next, the receptor specificity of the responses was investigated by pretreatment of MM6 cells with an anti-human TLR2 antibody followed by exposure to purified PGNs (Fig. 2.10A). Rat pAb hTLR2 was able to abolish TNF- α secretion demonstrating that the PGN preparations mediate biological responses through TLR2. This conclusion was further validated by employing HEK 293T cells stably transfected with various immune receptors and transiently transfected with a plasmid containing the reporter gene pELAM-Luc (NF-kB-dependent firefly luciferase reporter vector) and a plasmid containing the control gene pRL-TK (*Renilla* luciferase control reporter vector). As can be seen in Fig. 2.10B, highly purified PGN could induce activation of NF-kB in a TLR2-dependent manner. No activation was observed in wild-type HEK293T cells and cells transfected with human TLR4/MD2/CD14 (Appendix D). Previously, it was observed that treatment of PGNs with trypsin resulted in a loss of NF-kB activation when exposed

to HEK 293T cells expressing TLR2^{97b}. The discrepancy with our results is most likely due to the fact that the previous study examined cellular activation only at a relatively low concentration of 1 µg/ml, at which PGN does not induce cellular activation.

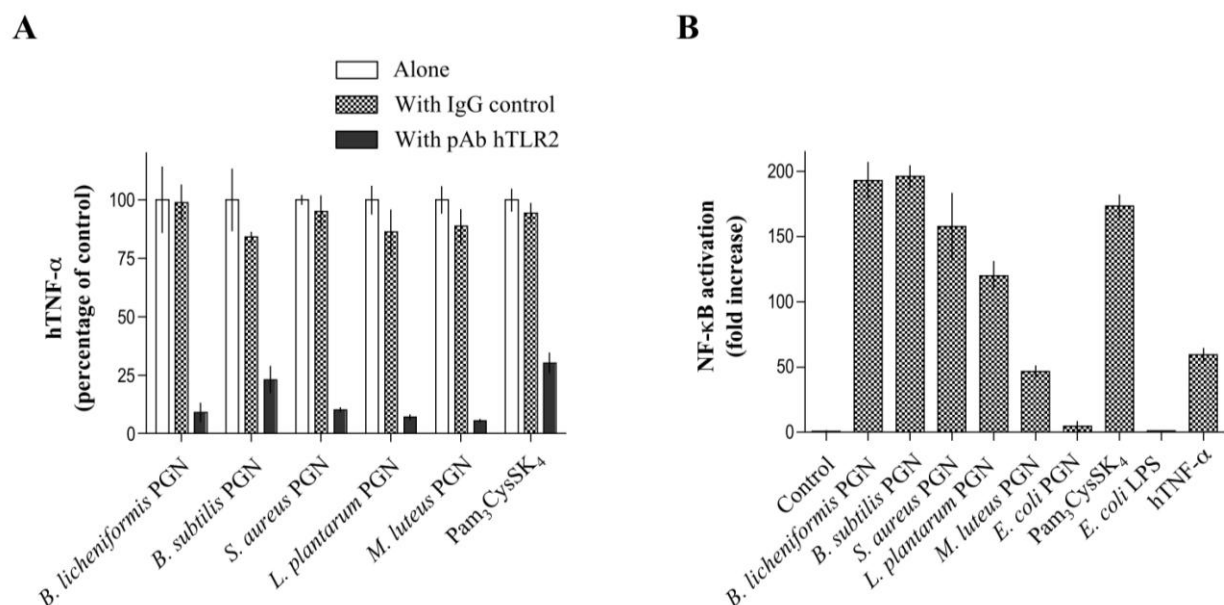


Fig. 2.10: TLR2 dependence of cellular activation by PGN. Effect of anti-TLR2 pAb on activity of PGNs in MM6 cells (A). MM6 cells were preincubated with a rat anti-human TLR2 pAb (5 µg/ml), a non-specific rat IgG control (5 µg/ml), or medium for 30 min at 4 °C. TNF-α was measured after 5.5 h of stimulation with different PGNs or Pam₃CysSK₄ at their IC₅₀ concentration (Fig. 9 and Table 2.3). Results are expressed as percentage of TNF-α concentration of control cells, which are incubated only with the various stimuli. In each case, no significant difference was observed between exposure to PGN or Pam₃CysSK₄ alone and in the presence of rat IgG control. Response of HEK293T cells expressing human TLR2 to different PGNs (B). Induction of NF-κB activation was determined in cultures of HEK293T cells stably transfected with human TLR2 and transiently transfected with pELAM-Luc and pRL-TK plasmids. Forty-four h post-transfection, cells were treated with purified PGNs (100 µg/ml each) or were left untreated (control). Forty-eight h post-transfection, NF-κB activation, as firefly luciferase activity relative to *Renilla* luciferase activity, was determined. As positive controls Pam₃CysSK₄ (1 µg/ml) and human TNF-α (10 ng/ml) and as negative control *E. coli* LPS (1 ng/ml) were used. Forty-eight h post-transfection, NF-κB activation was determined. Fold change is fold increase over control (cells exposed to medium only is arbitrarily set at 1). Data represent mean values ± s.d. (n = 3).

2.6 Discussion

Bacterial growth and division involves the remodeling of the very strong PGN layer by bacterial autolysins such as *N*-acetyl muramidases, *N*-acetyl muramoyl-L-alanine amidases, and carboxypeptidases⁹⁶. During this process, soluble PGN fragments are lost to the bloodstream and a growing body of literature indicates that these turnover products can initiate innate immune responses⁴³. The structural complexity of PGN and covalent modification with inflammatory mediators such as lipoproteins and teichoic acids has made it difficult to firmly establish TLR2 as an innate immune receptor of PGN^{97b, c}.

Previously, it was observed that purification of PGN by pronase or trypsin, followed by treatment with SDS and exposure to HF led to loss of TLR2-mediated activity. This study did, however, not consider that cross-linked PGN isolated from bacterial cell walls may be of low activity. Furthermore, it also did not account for the importance of modification of carboxylic acids of iso-Glu and DAP for biological activity.

The data reported here demonstrate that TLR2 can bind PGN fragments derived from Gram-positive and -negative bacteria. In the case of lysine-containing PGN (derived from Gram-positive bacteria), the initial linear biosynthetic product that contains pentapeptides such as in compound **3a** and cross-linked PGN (e.g. dimer **4**) are poorly recognized by TLR2. However, fragments (**1**, **2a**, and **3b**) which can be formed by the remodeling of PGN by autolysins, complexed with moderate to high affinity. Gram-positive bacteria have a very thick lysine-containing PGN layer, which accounts for approximately half of the cell wall mass. Thus, TLR2 may have evolved in such a way

that it only recognizes a limited number of lysine-containing PGN motifs to avoid over-activation of innate immune responses.

Interestingly, a wider range of DAP-containing fragments (derived from Gram-negative bacteria) was recognized by TLR2 including the nascent muramyl pentapeptide **7** and PGN remodeling products such as muramyl tri- (**5**) and tetrapeptide **6a**. The relaxed recognition specificity is probably due to the fact that Gram-negative bacteria contain only a thin DAP-containing PGN layer in the periplasmic space and therefore are less likely to cause over-activation of the innate immune system.

The binding studies also reveal that modification of carboxylic acids of iso-Glu and DAP dramatically influences binding. The functional significance of this finding is that modification of the carboxylic acids may be a strategy used by bacteria to evade detection by TLR2. In this respect, the ability to evade host immune surveillance is a critical virulence determinant for any pathogenic microorganism. However, the host may have developed strategies to compensate for lack of recognition by TLR2 and for example it appears that NOD proteins respond differently to modification of the carboxylic acids of Iso-Glu and DAP¹⁰⁷.

The mechanism by which TLR2 discriminates between PGN of Gram-positive and -negative bacteria appears to be unique. In the case of the NOD proteins, two different receptors have evolved that either respond to DAP- (NOD1) or lysine- (NOD2) containing mucopeptides^{43b-d, 43f}. The four mammalian PGRPs (PGRP-L, PGRP-Ia, PGRP-Ib, and PGRP-S) employ dual strategies for PGN recognition: one based on lysine or DAP specificity and another that relies on sensing the PGN cross-bridge^{99d, 99f, 108}. In the case of TLR2, one receptor can recognize lysine as well as DAP-containing

muropeptides, yet it responds differently to these two classes of compounds due to different binding modes of lysine- and DAP-containing muropeptides.

To validate the biological significance of the binding studies, PGN from various bacterial strains were isolated and purified and examined over a wide concentration range for the ability to induce TNF- α production in a TLR2-dependent manner. The PGNs were selected in such a way that they contained either lysine or DAP as the third amino acids and modified differently at iso-Glu and DAP. Importantly, PGNs that contain muropeptides that bind with low affinity with TLR2, exhibited a low- or no potency for inducing the production of TNF- α . Furthermore, DAP containing PGNs from *B. licheniformis* and *B. subtilis* were significantly more potent than lysine-containing *S. aureus* PGN. Also, acetylation of the amino groups of *S. aureus* PGN, a modification that led to a reduced affinity of muropeptides for TLR2 (**2a** vs. **2b**), led to a reduction in potency of TNF- α production.

Collectively, the studies reported support the involvement of TLR2 is an innate immune sensing of polymeric PGN. A number of other reports support the notion that polymeric PGN possesses inflammatory properties. For example, *in vitro* and *in vivo* degradation of the glycan backbone of PGN by lysozyme abrogates arthrogenicity and cytokine formation in whole blood¹⁰⁹ as well as PGN-induced organ failure in rats¹¹⁰. The importance of PGN backbone integrity was also demonstrated in lysozyme-deficient mice, which are more susceptible to inflammation induced by PGN¹¹¹. The potency of cellular activation by NOD receptors is reduced when the saccharide backbone increases in length¹¹² and hence these proteins are probably not involved in inflammatory responses induced by polymeric PGN.

The potency of several PGNs to initiate inflammatory responses is lower than that of LPS and lipid A¹¹³. Bacterial autolysins such as carboxypeptidases and endopeptidases⁹⁶, which are involved in PGN remodeling can, however, solubilize and unmask glycopeptide moieties that can bind with high affinity with TLR2. Soluble polymeric PGN fragments that contain these peptide moieties should be able to induce the clustering of TLR2 receptors¹¹⁴ and initiate cellular activation. Hence, the functional significance of the binding data is that endotoxicity arises from these subcomponents rather than from naïve cell wall PGN.

It is to be expected that antagonists of TLR2 can prevent over-activation of innate immune responses and therefore play valuable roles in the treatment of Gram-positive and -negative sepsis. In this respect, potent antagonists of lipopolysaccharides, which induce inflammatory responses through TLR4, have already been developed and clinical studies indicate that these compounds are useful for the prevention or treatment of Gram-negative sepsis¹¹⁵.

CHAPTER 3

Selective inhibition of alpha-mannosidase by feedback pro-drug constructs of Mannostatin

3.1: Abstract

Alterations in cell-surface carbohydrates are one of the hallmarks of tumor progression and metastasis. Compounds that inhibit the biosynthesis of these oligosaccharides are thus attractive leads for drug development. Glycosidases like neuraminidases, mannosidase together with glycosyltransferases involved in catalytic synthesis and breakage of glycosic bonds are typically the targets of these inhibitors. However, most of these inhibitors lack specificity and may cause undesirable side effects in cancer treatment like lysosomal storage disorder (LSD). Employing a feedback mechanism, a three component pro-drug was design, synthesized and its biological activity vis-à-vis cellular uptake and toxicity were evaluated. The pro-drug consist of a α -mannosidase inhibitor mannostatin A, 4-hydroxyl benzyl carbamate, and an α -mannoside. Removal of the α -mannoside by the enzyme led to an unmasking cascade that resulted in the local and selective inhibition of mannosidases.

3.2. Introduction

Protein- and lipid-linked oligosaccharides at the surface of eukaryotic cells are responsible for a wide range of biological processes impacting health and disease.¹¹⁶ Examples of such processes include fertilization, embryogenesis, neuronal development, hormone activities, the proliferation of cells and their organization into specific tissues. Remarkable changes in cell-surface carbohydrates occur with tumor progression, which appears to be intimately associated with the dreaded state of metastasis¹¹⁷. Furthermore, carbohydrates of host cells are often employed by pathogens for cell entry. Not surprisingly, compounds that can interfere in the biosynthesis of oligosaccharides are regarded as attractive leads for drug discovery for wide range of diseases^{83, 118}. For example, inhibitors of pancreatic α -amylase, such as Arcarbose, Voglibose and Miglitol, have been introduced for the treatment of diabetes¹¹⁹. Furthermore, compounds such as Zanamivir and Oseltamivir (Tamiflu) have been developed as inhibitors of neuramidase for the treatment of flu¹²⁰. Despite these successes, it has been difficult to develop safe and efficacious glycosidase inhibitors for the treatment of many other diseases. A major problem of many natural and synthetic glycosidase inhibitors is that they inhibit other glycosidases, which may lead to serious side effects. More selective inhibitors may be obtained by carefully designed structure-activity relationship studies¹²¹ in combination with a better understanding of the mechanism of action of glycosidases. This approach is, however, complicated by the fact that of the approximately hundred mammalian glycosidases only a small number have been cloned and over-expressed.

We report here a general approach for the design and synthesis of more selective glycosidase inhibitors based on feedback inhibition. Feedback inhibition is a mechanism in which a biosynthetic pathway regulates itself through inhibition of the first committed step in the pathway by a down stream or final product¹²² Although this type of inhibition is widely used in nature, it has not been exploited for the design and synthesis of selective therapeutics. The feedback inhibition prodrugs described here are composed of an inhibitor that is modified by a linker that renders them inactive (Fig. 3.1a). The linker, in turn, is attached to a glycoside that can be hydrolyzed by a targeted glycosidase. The resulting intermediate is designed in such a way that it will undergo self-fragmentation to release the active inhibitor. Subsequently, the inhibitor will inhibit the enzyme that initiated its release. The attraction of this approach is that the inhibitor will only be released at the site of the enzyme. Selectivity is achieved due to the exquisite selectivity of glycosidases for their substrates. Furthermore, the release of the inhibitor is terminated when a sufficient quantity of the inhibitor is released. The remaining pool of pro-drug will, however, be activated when active enzyme reappears thereby approximating conditions of continuous infusion.

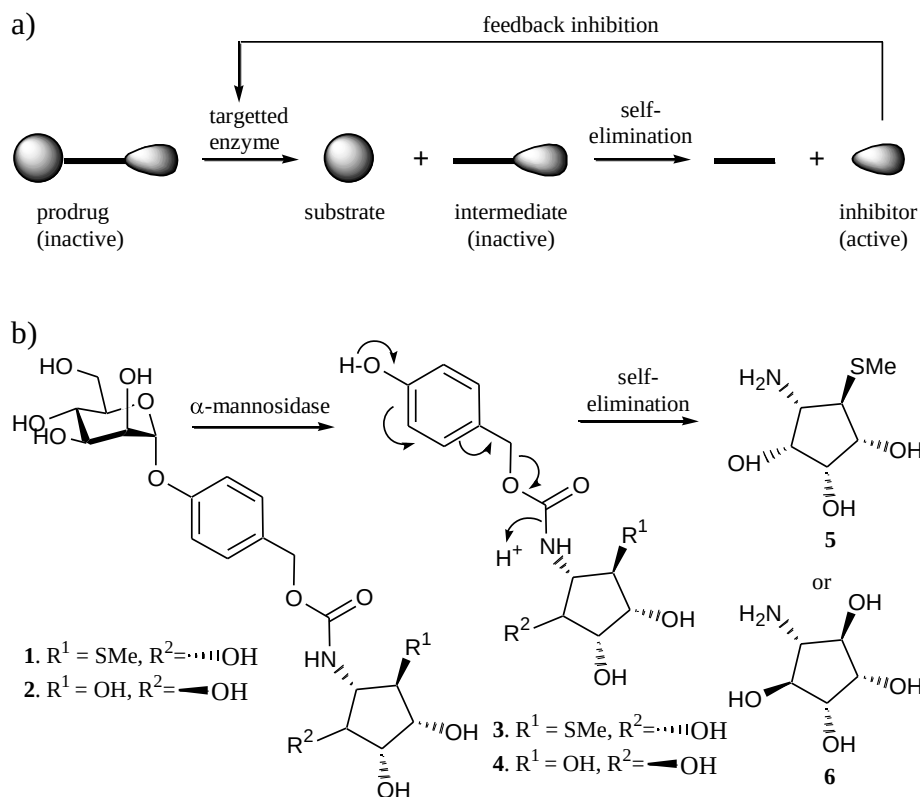


Fig. 3.1: a) General strategy for the inhibition of enzymes by a feedback mechanism. b) A feedback inhibitor for mannosidases.

3.3 Goals

The non-specific inhibition of Golgi and lysosomal mannosidases has led to the development of α -mannosidosis. This study seeks to address the following concerns:

- 1) Investigate the selective targeting of Golgi α -mannosidase using Mannostatin constructs.
 - i) Design and evaluate the efficacy of a pro-drug that can selectively inhibit Golgi α -mannosidase.
 - ii) Evaluate the efficacy of a pro-drug with improved cellular uptake

3.4 Experimental procedure

3.4.1: General Synthetic Procedure of Mannostatin constructs.

All compounds were synthesized by Dr. Jun Guo, a post doctoral fellow with the Boons' group. The reactions were carried out under a positive pressure of argon, unless otherwise noted. All chemicals were purchased from commercial suppliers and used without further purification, unless otherwise noted. All solvents employed were of reagent grade and dried by refluxing over appropriate drying agents. Column chromatography was performed on silica gel 60 (EM Science, 70-230 mesh). Reactions were monitored by TLC on Kieselgel 60 F254 (EM Science) and the compounds were detected by examination under UV light and visualized by dipping the plates in a cerium sulfate-ammonium molybdate solution followed by heating. ^1H NMR and ^{13}C NMR spectra were recorded with a Varian Inova 300 spectrometer and a Varian Inova 600 spectrometer equipped with Sun workstations. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Data are presented as follow: Chemical shift multiplicity: s = singlet, d = doublet, t = triplet, dd = double of doublet, m = multiplet and/or multiple resonances, b= broad, integration, coupling constant in Hertz (Hz), assignments: "m" for mannose, "a" for aromatic, and "d" for mannostatin A or cyclopentanetetrol. High resolution positive ion matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were recorded using an Applied Biosystems MALDI instrument by using gentisic acid as a matrix.

4-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranosyloxy)-benzaldehyde (9). A mixture of tetra-O-acetyl- α -D-mannopyranosyl bromide (2.40 g, 5.84 mmol), 4-hydroxybenzaldehyde (1.43 g, 11.7 mmol) and silver(I) oxide (1.76 g, 7.59 mmol) in acetonitrile (30 ml) in the dark was heated under reflux for 1 h. Then, the reaction mixture was filtered through celite, the filtration was concentrated *in vacuo*, and the residue was purified by flash column chromatography over silica (1:2 EtOAc/hexanes) to give **7** (1.08 g, 41%) as a colorless syrup. R_f = 0.55 (1:1 hexanes/EtOAc). ^1H NMR (CDCl_3 , 300 MHz) δ 2.00, 2.03, 2.04, 2.19 (4xs, 12H, 4x CH_3 , Ac), 3.98-4.12 (m, 2H, H-m5, m6 α), 4.25 (dd, 1H, J = 5.6, 12.2 Hz, H-m6 β), 5.36 (t, 1H, J = 10.1 Hz, H-m4), 5.44 (dd, 1H, J = 1.8, 3.4 Hz, H-m2), 5.53 (dd, 1H, J = 3.4, 10.1 Hz, H-m3), 5.62 (d, 1H, J = 1.8 Hz, H-m1), 7.21 (d, 2H, J = 8.8 Hz, H-a2), 7.85 (d, 2H, J = 8.8 Hz, H-a3), 9.91 (s, 1H, COH). ^{13}C NMR (CDCl_3 , 75 MHz) δ 20.80 (CH_3), 20.83 (CH_3), 20.85 (CH_3), 21.0 (CH_3), 62.2 (C-m2), 65.9 (C-m3), 68.8, 69.3, 69.8 (C-m6, m4, m5), 95.7 (C-m1), 116.8 (C-a2), 131.9 (C-a4), 132.0 (C-a3), 160.3 (C-a1), 169.8 (MeCO), 170.1 (2 carbons, MeCOx2), 170.6 (MeCO), 190.9 (PhCOH). MALDI-TOF MS (m/z) calcd for $\text{C}_{21}\text{H}_{24}\text{O}_{11}\text{Na}$: 475.1216; found 475.1298 $[\text{M}+\text{Na}]^+$.

[4-(hydroxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranoside) (10). To a colded suspension (0 °C) of **9** (0.407 g, 0.9 mmol) and silica gel (0.6 g) in CHCl_3 (8 ml) and *i*-PrOH (2 ml) was added sodium borohydride (0.151 g, 4.0 mmol). The reaction mixture was stirred at 0 °C for 1 h, and then allowed to warm to room temperature, after which it was filtered over a pad of celite which was washed with CH_2Cl_2 (20 ml). The combined filtrates were concentrated *in vacuo*, and the residue was purified by flash column chromatography over silica (1:1 EtOAc/hexanes) to give **10** (0.340 g, 82%) as a

colorless syrup. R_f = 0.25 (1:1 hexanes/EtOAc). ^1H NMR (CDCl_3 , 300 MHz) δ 2.00, 2.01, 2.03, 2.18 (4xs, 12H, 3x CH_3 , Ac), 2.85 (s, 1H, OH), 4.00-4.11 (m, 2H, H-m5, m6 α), 4.25 (dd, 1H, J = 5.5, 12.3 Hz, H-m6 β), 4.59 (s, 2H, PhCH_2), 5.34 (t, 1H, J = 10.1 Hz, H-m4), 5.42 (dd, 1H, J = 1.8, 3.4 Hz, H-m2), 5.49 (d, 1H, J = 1.6 Hz, H-m1), 5.53 (dd, 1H, J = 3.5, 10.1 Hz, H-m1), 7.04 (d, 2H, J = 8.7 Hz, H-a2), 7.27 (d, 2H, J = 8.7 Hz, H-a3). ^{13}C NMR (CDCl_3 , 75 MHz) δ 20.68 (CH_3), 20.71 (2x CH_3), 20.9 (CH_3), 62.2 (C-m2), 64.5 (PhCH_2), 66.0 (C-m3), 69.0, 69.2, 69.4 (C-m6, m4, m5), 95.9 (C-m1), 116.6 (C-a2), 128.5 (C-a3), 135.8 (C-a4), 155.0 (PhCH_2), 169.9 (MeCO), 170.08 (MeCO), 170.11 (MeCO), 170.7 (MeCO). MALDI-TOF MS (m/z) calcd for $\text{C}_{21}\text{H}_{26}\text{O}_{11}\text{Na}$: 477.1373; found 477.1275 $[\text{M}+\text{Na}]^+$.

[4-(4-nitro-phenoxy carbonyloxymethyl)phenyl]-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranoside) (11). To a solution of **10** (0.265 g, 0.583 mmol) and 4-nitrophenyl chloroformate (0.294 g, 1.458 mmol) in CH_2Cl_2 (8 ml) was added two drops of pyridine. The reaction mixture was stirred for 18 h at r.t., after which it was concentrated *in vacuo*, purified by flash column chromatography over silica (1:2 to 1:1 EtOAc/hexanes) to give **11** (0.325 g, 90%) as a white solid. R_f = 0.45 (1:1 hexanes/EtOAc). ^1H NMR (CDCl_3 , 300 MHz) δ 2.03, 2.04, 2.06, 2.21 (4xs, 12H, 4x CH_3 , Ac), 4.04-4.11 (m, 2H, H-m5, m6 α), 4.28 (dd, 1H, J = 5.7, 12.8 Hz, H-m6 β), 5.24 (s, 2H, PhCH_2), 5.38 (t, 1H, J = 10.0 Hz, H-m4), 5.44 (dd, 1H, J = 1.9, 3.5 Hz, H-m2), 5.55 (d, 1H, J = 1.9 Hz, H-m1), 5.56 (dd, 1H, J = 3.5, 10.0 Hz, H-m3), 7.12 (d, 2H, J = 8.7 Hz, H-a2), 7.37 (d, 2H, J = 9.2 Hz), 7.40 (d, 2H, J = 8.7 Hz, H-3a), 8.27 (d, 2H, J = 9.2 Hz, $\text{O}_2\text{N-Ph-H}$). ^{13}C NMR (CDCl_3 , 75 MHz) δ 20.88 (CH_3), 20.90 (CH_3), 20.91 (CH_3), 21.1 (CH_3), 62.3 (C-m2), 66.1 (C-m3), 69.0, 69.5 & 69.6, 70.7 (C-m6, m4, m5), 77.4 (PhCH_2), 96.0 (C-m1), 117.0

(C-a2), 122.0 (C-2 of PhNO₂), 125.5 (C-3 of PhNO₂), 129.0 (C-a3), 130.8 (C-a4), 145.6 (C-4 of PhNO₂), 152.6 (OCO₂), 155.7 (C-a1), 156.4 (C-1 of PhNO₂), 169.9 (MeCO), 170.18 (MeCO), 170.22 (MeCO), 170.7 (MeCO). MALDI-TOF MS (*m/z*) calcd for C₂₈H₂₉NO₁₅Na: 642.1435; found 642.1421 [M+Na]⁺.

1D-(1,2,3,4/5)-4-[4-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranosyloxy)-

benzyloxycarbonyl]amino-5-(methylsulfonyl)cyclopentane-1,2,3-triol (12). To a suspension of **11** (36.3 mg, 0.0585 mmol) and mannostatin A hydrochloride (12.6 mg, 0.0585 mmol) in DMF (4 ml) was added Et₃N (32.6 μ l, 0.234 mmol). After stirring the reaction mixture for 18 h at r.t., it was concentrated *in vacuo*. The residue was purified by flash chromatography over Iatrobeds (2-4% MeOH in CH₂Cl₂) to give **12** (32 mg, 83%) as white solid. *R_f* = 0.70 (1:9 MeOH/CH₂Cl₂). ¹H NMR (10% CD₃OD/CDCl₃, 300 MHz) δ 1.97 (s, 6H, 2xCH₃, Ac), 1.99 (s, 3H, Ac), 2.13 (s, 6H, CH₃, Ac, SCH₃), 2.81 (b, 1H, H-d5), 3.80 (b, 2H, H-d1, d4), 3.96-4.05 (m, 4H, H-d2, d3, m5, m6 α), 4.19 (dd, 1H, *J* = 5.0, 12.0 Hz, H-6 β), 4.98 (s, 2H, PhCH₂), 5.28 (t, 1H, *J* = 10.1 Hz, H-m4), 5.37 (m, 1H, H-m2), 5.45 (d, 1H, *J* = 2.2 Hz, H-m1), 5.49 (d, 1H, *J* = 3.4 Hz, H-m3), 7.00 (d, 2H, *J* = 8.4 Hz, H-a2), 7.25 (d, 2H, *J* = 8.4 Hz, H-a3). ¹³C NMR (10% CD₃OD/CDCl₃, 75 MHz) δ 14.0 (SCH₃), 20.6 (3 carbons, Acx3), 20.8 (Ac), 56.3 (C-d5), 62.2 (C-m2), 66.0 (C-m3), 66.4 (PhCH₂), 69.0, 69.2, 69.4 (C-m6, m4, m5), 71.2, 71.8, 75.8, 77.4 (C-d4, d3, d1, d2), 95.8 (C-m1), 116.5 (C-a2), 129.8 (C-a3), 131.2 (C-a4), 155.5 (OCONH), 156.5 (C-a1), 170.1 (MeCO), 170.3 (2 carbons, MeCOx2), 171.0 (MeCO). MALDI-TOF MS (*m/z*) calcd for C₂₈H₃₇NO₁₅SNa: 682.1782; found 682.1110 [M+Na]⁺.

(1,4/2,3,5)-5-[4-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranosyloxy)-

benzyloxycarbonyl]amino-1,2,3,4-cyclopentanetetrol (13). To a suspension of **11**

(209 mg, 0.338 mmol) and **6** (hydrochloride salt, 94 mg, 0.506 mmol) in DMF (4 ml) was added Et₃N (188 μ l, 1.35 mmol). The reaction mixture was stirred for 18 h, after which it was concentrated *in vacuo*. The residue was purified by flash chromatography over latrobeads (3-8% MeOH in CH₂Cl₂) to give **13** (193 mg, 91%) as white solid. R_f = 0.20 (1:9 MeOH/CH₂Cl₂). ¹H NMR (10% CD₃OD/CDCl₃, 300 MHz) δ 2.01, 2.02, 2.05, 2.19 (4xs, 12H, 4xCH₃, Ac), 3.61 (b, 1H, H-d5), 3.78 (b, 2H, H-d1, d4), 3.88 (b, 2H, H-d2, d3), 4.00-4.10 (m, 2H, H-m5, m6 α), 4.24 (dd, 1H, J = 4.9, 12.1 Hz, H-m6 β), 5.00 (s, 2H, PhCH₂), 5.34 (t, 1H, J = 10.2 Hz, H-m4), 5.42 (b, 1H, H-m2), 5.47-5.54 (m, 2H, H-m1, m3), 6.51 (d, 1H, J = 5.6 Hz, NH), 7.04 (d, 2H, J = 8.2 Hz, H-a2), 7.28 (d, 2H, J = 8.4 Hz, H-a3). ¹³C NMR (10% CD₃OD/CDCl₃, 75 MHz) δ 20.5 (CH₃), 20.6 (2 carbons, CH₃x2), 20.7 (CH₃), 61.8 (C-d5), 62.1 (C-m2), 65.9 (C-m3), 66.5 (PhCH₂), 69.0, 69.1, 69.3 (C-m6, m4, m5), 74.6 (C-d2, d3), 79.1 (C-d1, d4), 95.7 (C-m1), 116.5 (C-a2), 129.7 (C-a3), 130.9 (C-a4), 155.4 (OCONH), 158.0 (C-a1), 170.0 (MeCO), 170.2 (2 carbons, MeCOx2), 170.9 (MeCO). MALDI-TOF MS (m/z) calcd for C₂₇H₃₅NO₁₆Na: 652.1854; found 652.2129 [M+Na]⁺.

1D-(1,2,3,4/5)-4-[4-(α -D-mannopyranosyloxy)-benzyloxycarbonyl]amino-5-

(methylsulfanyl)cyclopentane-1,2,3-triol (1). Methanolic ammonia (7 N, 10 ml) was added to a cooled (0 °C) solution of compound **12** (32 mg, 0.0517 mmol) in MeOH (2 ml). The reaction mixture was stirred at 0 °C for 5 h, after which the solvents were concentrated *in vacuo*. The residue was purified over P2 size exclusion chromatography (1% *n*-butanol in water) to give **1** (24 mg, 94%) as white solid. ¹H NMR (D₂O, 300 MHz) δ 1.99 (s, 3H, SCH₃), 2.84 (t, 1H, J = 8.1 Hz, H-d5), 3.56-3.69 (m, 4H, H-m4, m5, m6), 3.72-3.84 (m, 2H, H-d4, d1), 3.89-4.00 (m, 3H, H-d2, d3, m3), 4.05 (dd, 1H, J = 1.9, 3.1

Hz, H-m2), 4.96 (s, 2H, PhCH₂), 5.50 (d, 1H, *J* = 1.5 Hz, H-m1), 7.05 (d, 2H, *J* = 8.5 Hz, H-a2), 7.28 (d, 2H, *J* = 8.5 Hz, H-a3). ¹³C NMR (D₂O, 75 MHz) δ 12.6 (SCH₃), 54.8, 56.1, 60.8, 66.8, 66.9, 70.0, 70.6, 70.8, 71.5, 73.5, 74.0, 98.3 (C-m1), 117.3 (C-a2), 129.8 (C-a3), 131.0 (C-a4), 155.5 (OCONH), 158.2 (C-a1). MALDI-TOF MS (*m/z*) calcd for C₂₀H₂₉NO₁₁SNa: 514.1359; found 514.1320 [M+Na]⁺.

(1,4/2,3,5)-5-[4-(α-D-mannopyranosyloxy)-benzyloxycarbonyl]amino-1,2,3,4-

cyclopentanetetrol (2). Hydrazine monohydrate (5 ml) was added to a colded (0 °C) solution of **13** (138 mg, 0.219 mmol) in MeOH (20 ml). After stirring the reaction mixture at 0 °C for 5 h, it was quenched by addition of acetone and the mixture was concentrated *in vacuo*. The residue was purified over P2 size exclusion chromatography (1% *n*-butanol in water) to give **2** (101 mg, 99%) as white solid. ¹H NMR (D₂O, 300 MHz) δ 3.44 (t, 1H, *J* = 8.1 Hz, H-d5), 3.50-3.67 (m, 6H, H-m4, m5, m6, d2, d3), 3.77 (b, 2H, H-d1, d4), 3.89 (dd, 1 H, *J* = 3.4, 9.1 Hz, H-m3), 3.99 (b, 1H, H-m2), 4.89 (s, 2H, PhCH₂), 5.43 (b, 1H, H-m1), 6.97 (d, 2H, *J* = 8.4 Hz, H-a2), 7.21 (d, 2H, *J* = 8.4 Hz, H-a3). ¹³C NMR (D₂O, 75 MHz) δ 60.3 (C-d5), 60.8, 66.7 (2 carbons), 70.1, 70.6, 73.5, 73.7, 78.2 (C-d2, d3), 98.2 (C-m1), 117.2 (C-a2), 129.8 (C-a3), 130.8 (C-a4), 155.5 (OCONH), 158.5 (C-a1). MALDI-TOF MS (*m/z*) calcd for C₁₉H₂₇NO₁₂Na: 484.1431; found 484.2137 [M+Na]⁺.

1D-(1,2,3,4/5)-4-[4-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyloxy)-

benzyloxycarbonyl]amino-1,2,3-tri-O-acetyl-5-(methylsulfanyl)cyclopentane-1,2,3-triol (14). Compound **1** (8 mg, 0.0163 mmol) was dissolved in pyridine (2 ml) and acetyl anhydride (0.5 ml). The reaction mixture was stirred for 5 h, after which the solvents were evaporated *in vacuo*. The residue was purified by flash chromatography over silica

(1:1 to 1:0 EtOAc/hexanes) to give **14** (10 mg, 78%) as white solid. $R_f = 0.15$ (1:1 hexanes/EtOAc). ^1H NMR (CDCl_3 , 300 MHz) δ 1.99-2.01 (m, 24H, 7xCH₃ Ac, SCH₃), 3.10 (t, 1H, $J = 7.2$ Hz, H-d5), 4.07 (m, 2H, H-m5, m6 α), 4.28 (m, 2H, H-m6 β , d4), 5.07 (s, 2H, PhCH₂), 5.14 (t, 1H, $J = 6.3$ Hz, H-d1), 5.27-5.34 (m, 2H, H-d2, d3), 5.36-5.44 (m, 2H, H-m4, m2), 5.52 (d, 1H, $J = 1.5$ Hz, H-m1), 5.57 (dd, 1 H, $J = 3.6, 10.0$ Hz, H-m3), 7.09 (d, 2H, $J = 8.6$ Hz, H-a2), 7.32 (d, 2H, $J = 8.6$ Hz, H-a3). MALDI-TOF MS (m/z) calcd for C₃₄H₄₃NO₁₈SNa: 808.2099; found 808.2131 [M+Na]⁺.

(1,4/2,3,5)-5-[4-(2,3,4,6-tetra-O-acetyl- α -D-mannopyranosyloxy)-

benzyloxycarbonyl]amino-1,2,3,4-tetra-O-acetyl-1,2,3,4-cyclopentanetetrol (15).

Compound **2** (22.6 mg, 0.0490 mmol) was dissolved in pyridine (2 ml) and acetyl anhydride (0.5 ml). The reaction mixture was stirred for 5 h, after which the solvents were evaporated *in vacuo*. The residue was purified by flash chromatography over silica (1:1 to 1:0 EtOAc/hexanes) to give **15** (39.1 mg, 92%) as white solid. $R_f = 0.50$ (1:2 hexanes/EtOAc). ^1H NMR (CDCl_3 , 300 MHz) δ 2.01-2.01 (m, 21H, 7xCH₃, Ac), 2.19 (s, 3H, CH₃, Ac), 4.01-4.09 (m, 2H, H-d5, m5), 4.11 (dd, 1H, $J = 7.2, 14.3$ Hz, H-6 β), 4.27 (dd, 1H, $J = 5.3, 12.1$ Hz, H-m6 β), 5.02 (s, 2H, PhCH₂), 5.17 (dd, 2H, $J = 4.3, 6.0$ Hz, H-d1, d4), 5.25 (d, 2H, $J = 4.1$ Hz, H-d2, d3), 5.36 (t, 1H, $J = 10.1$ Hz, H-m4), 5.43 (dd, 1 H, $J = 1.7, 3.4$ Hz, H-m2), 5.51 (d, 1H, $J = 1.5$ Hz, H-m1), 5.54 (dd, 1H, $J = 3.5, 10.1$ Hz, H-m3), 7.06 (d, 2H, $J = 8.6$ Hz, H-a2), 7.28 (d, 2H, $J = 8.6$ Hz, H-a3). MALDI-TOF MS (m/z) calcd for C₃₅H₄₃NO₂₀Na: 820.2276; found 820.2220 [M+Na]⁺.

3.4.2: Stability measurements of Mannostatin A compounds.

A solution of compound **1** or **2** (0.4 mM) in deuterated sodium acetate buffer (100 mM; pH 4.0; 0.2 ml) in a NMR tube was incubated at 37°C for 6 days. ¹H NMR spectra were recorded every day. No decomposition of product was observed.

3.4.3: Kinetic measurements of Mannostatin A compounds

. A solution of compound **1** or **2** (0.08 mmol) and human lysosomal mannosidase (HLM, 0.02 Unit) in deuterated sodium acetate buffer (0.1 M; pH 5.6; 0.2 ml) was incubated at 37°C, and ¹H NMR spectra (600 MHz) were recorded at different time intervals. Relevant signals were integrated to determine the rate of enzymatic hydrolysis, and the appearance of the intermediate and the final product.

3.4.4: Self-decomposition of Mannostatin A compounds: Half-time measurements.

A solution of compound **2** (0.08 mmol) and Jack Bean mannosidase (JBM) (1.4 Units) in deuterated 0.2 M phosphate/0.1 M citrate buffer (pH = 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5; 0.2 ml) was incubated at 37°C or 25°C, and ¹H NMR spectra (600 MHz) were recorded at different time intervals. Relevant signals were integrated to determine the appearance of the intermediate **4** and the final product **6**, and the decomposition rate.

3.4.5: Negative enzyme control experiments.

A solution of compound **1** or **2** (0.004 mmol) and α-glucosidase, α-galactosidase or α-L-fucosidase (0.1 Unit) in MES buffer (0.05 M; pH 6.8 for α-glucosidase, pH 6.5 for α-galactosidase or pH 5.5 for α-L-fucosidase; 1 ml) was incubated at 37°C for 20 h,

after which the reaction mixture was freezing-dried, and the solid residue was analyzed by ^1H NMR spectrometry and mass spectrometry. No decomposition of product was observed.

3.4.6: Cell maintenance.

MDAY-D2 lymphoma cells, provided by Dr. Kelly Moremen (CCRC, UGA, Athens, GA), were cultured in Eagle's Minimum Essential Medium (MEM) with 2 mM L-glutamine, Earle's Balanced Salt Solution, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate (ATCC) supplemented with penicillin (100 I.U./mL)/streptomycin (100 $\mu\text{g}/\text{mL}$; Mediatech) and heat-inactivated fetal bovine serum (h-iFBS; 10%; Hyclone). The cells were maintained in a humid 5% CO_2 atmosphere at 37°C . New batches of frozen cell stock were grown up every 2 months and growth morphology was evaluated.

3.4.7: Inhibition of cell surface poly lactosamine biosynthesis

MDAY-D2 cells were plated at 5×10^4 cells/well into 6-well cell culture plates (Costar) in the absence (control) or presence of compounds **14** or **15** (0.1 - 100 μM) and incubated for 48 h in culture medium containing 2% h-iFBS. On average 1×10^6 cells per well were obtained after incubation for 48 h. Cells were harvested by centrifugation (1,400 rpm for 5 min) and lysed in lysis buffer (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin) on ice. After centrifugation (14,000 rpm for 10 min at 4°C), the supernatants were stored at -80°C . Protein concentrations were determined using the BCA reagent (Pierce) and cellular

proteins (10 μ g) were separated by SDS-PAGE using 4-20% linear gradient polyacrylamide gels (Bio-Rad). Separated proteins were electrotransferred onto nitrocellulose membranes (Hybond-P; Amersham). The blots were incubated with 5 μ g/mL fluorescein labeled *Phaseolus vulgaris* Leucoagglutinin (PHA-L; Vector Laboratories) in PBS/0.1% BSA for 1 h at room temperature. Fluorescence bands were imaged at 495 nm (absorbance) / 520 nm (emission) and the intensity scanned by laser beam densitometry (Typhoon 9410 Imager; Amersham Biosciences).

3.5 Results

Compound **1** is a prototypic feedback inhibitor that is designed to selectively target α -mannosidases. It is composed of mannostatin A^{118b}, which is a potent inhibitor of α -mannosidases and has garnered attention as a lead compound for cancer drug development (Fig.3.1b). The amino group of mannostatin A is modified by a 4-hydroxyl benzyl carbamate, which in turn is linked to an α -mannoside. The acylation of the amino group of mannostatin A renders the compound inactive for inhibition of mannosidases¹²³. However, an α -mannosidase can hydrolyze the glycosidic linkage resulting in the formation of intermediate **3**, which will self fragment¹²⁴ to give mannostatin A (**5**). The active mannostatin A will then inhibit the α -mannosidase that initiated its release, terminating the enzymatic reaction. On the other hand, the enzymatic hydrolysis of compound **2** will release aminocyclopentitetrol **6**, which is not an inhibitor of an α -mannosidase¹²⁵ and therefore this reaction is expected to proceed to completion.

The synthesis of compounds **1** and **2** is summarized in figure 3.2. Coupling of bromo 2,3,4,6-tetra-*O*-acetyl- α -D-mannose (**7**) with *p*-hydroxybenzaldehyde (**8**) using silver(I)oxide as the promoter in refluxing acetonitrile gave **9** in a modest yield. The aldehyde moiety of **9** was reduced with NaBH₄ to give **10**, which was converted into activated intermediate **11** by reaction with 4-nitrophenyl chloroformate. This compound was immediately reacted with **5**¹²⁶ and **6**¹²⁷ to afford compounds **12** and **13**, respectively. The acetyl esters of **12** and **13** were cleaved by treatment with ammonia or hydrazine in methanol to give the targeted compounds **1** and **2**, respectively.

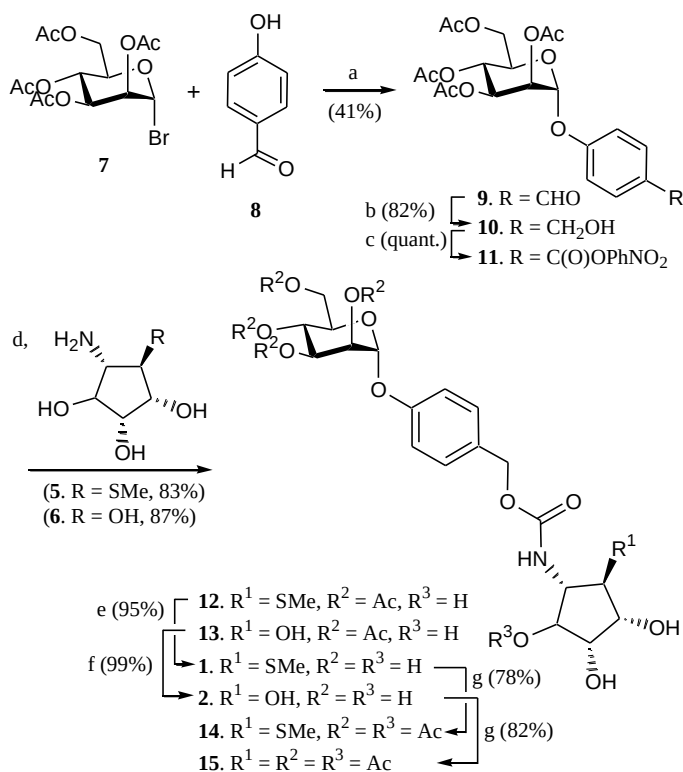


Fig. 3.2: Synthetic Scheme; Reagents and conditions: a) Ag₂O, CH₃CN, Δ , 1 hr; b) NaBH₄, silica, CHCl₃, *i*-PrOH; c) *p*-NO₂PhOCOC(=O)Cl, pyridine, DCM, 18 hrs; d) Et₃N, DMF, 18 hrs; e) NH₃ in MeOH; f) NH₂NH₂·H₂O, MeOH, 0°C, 5 hrs; g) Ac₂O, pyridine.

The enzymatic cleavage of **1** and **2** by human lysosomal mannosidase (HLM) was monitored by ^1H NMR (600 MHz)¹²⁸. Thus, HLM (20 mU) was added to a solution of **1** or **2** (80 nmol) dissolved in a deuterated sodium acetate buffer (100 mM, pH 5.6, 0.2 mL). The progress of the reaction was monitored by the integration of key signals of the starting material, the intermediate and final product. As can be seen in Figure 3.3a, HLM hydrolyzed **2** producing intermediate **4**, which self fragmented to aminocyclopentitetrol **6**. Compound **6** is not an inhibitor of HLM and as a result the enzymatic conversion progressed to completion. On the other hand, virtually no hydrolysis was observed when **1** was treated with HLM (Fig.3.3b). In this case, the released mannostatin A (**1**) inhibits HLM terminating further hydrolysis of **1**. Mannostatin A is a potent inhibitor of retaining α -mannosidases ($K_i = 90 \text{ nM}$, HLM)¹²⁹ and, thus the release of low concentrations of the active compound is sufficient to abolish enzyme activity. Indeed, the addition of 4-methylumbelliferyl α -D-mannopyranoside did not result in the release of fluorescence methylumbelliferone, demonstrating that the mannosidase was inhibited by the released mannostatin A. Compounds **1** and **2** were subjected to an α -glucosidase, α -fucosidase and α -galactosidase. As expected, these enzymes did not initiate the release of the compounds **5** and **6**, demonstrating that they have exquisite selectivity for α -mannosidases (data not shown).^[136]

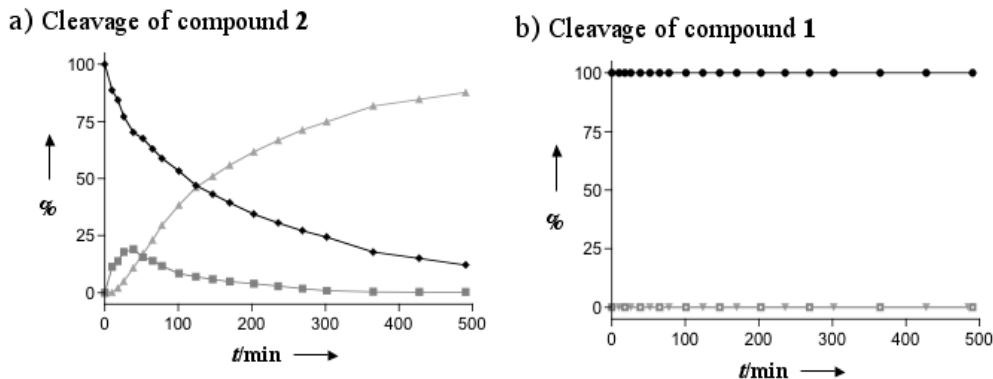


Fig. 3.3: Release of prodrugs **1** and **2** by human lysosomal mannosidase. HLM (20 mU) was added to a solution of **2** (a) and **1** (b) (80 nmol) in deuterated sodium acetate buffer (100 mM, pH 5.6, 0.2 mL). The enzymatic cleavage of **1** (●) and **2** (◆) and the formation of the intermediates **3** (□) and **4** (■) and the final products **5** (▼) and **6** (▲), respectively, were monitored by ^1H NMR (600 Mhz).

Next, the pH dependence of the self-fragmentation of intermediates **3** and **4** was investigated. It is well known that different organelles have different pH values. For example, lysosomes are significantly more acidic than the Golgi apparatus. Golgi mannosidase II has been identified as a target for anti-metastatic therapy^{83, 130}, whereas inhibition of lysosomal mannosidase leads to the phenotype of lysosomal storage disease. It was expected that intermediates **3** and **4** will fragment significantly faster in neutral as opposed to acidic conditions, providing a basis for some selectivity for Golgi-over the lysosomal mannosidase. Thus, compound **2** (0.4 mM) in deuterated 0.2 M phosphate/0.1 M citrate buffers of different pH was treated with a large quantity (7 Units/mL) of Jack Bean α -mannosidase (JBM) to completely convert the prodrug into intermediate **4**. Subsequently, the decomposition of **4** was monitored by ^1H NMR by integration of key signals of the intermediate and final product. As can be seen in Table 3.1, the decomposition of **4** is significantly faster at more basic pH and elevated temperatures. These results can easily be rationalized by assuming that at a higher pH

a large proportion of the intermediate is in the phenoxide form, which decomposes significantly faster than the analogues phenol.

Table 3.1. pH-dependence of half-lives of self-fragmentation of **2**.

pH	4.0	4.5	5.0	5.5	6.0	6.5
37°C (min)	480	270	100	45	16	9
25°C (min)	2800	--	--	--	--	45

Incubating of prodrug **2** (80 nmol) and Jack Bean mannosidase (1.4 U) in deuterated 0.2 M phosphate/0.1 M citrate buffer (0.2 mL) at 37°C or 25°C in a NMR tube and running ¹H NMR spectra over time on a 600 MHz spectrometer.

For many applications, compounds such as **1** need to be cell permeable to inhibit intracellular glycosidases. Analysis of fetal calf serum (FCS), which is often used for culturing cells, revealed that it contains a significant amount of mannosidase activity. Thus, these mannosidases can unmask mannostatin A, which can then pass through the cell membrane to inhibit intracellular mannosidases. It was anticipated that the *O*-acetylation of **1** and **2** to give **14** and **15**, respectively would considerably improve the metabolic stability of the prodrug. Furthermore, it has been shown that *O*-acetylation of saccharides can facilitate cellular uptake¹³¹ Subsequently, intra-cellular esterases can cleave the acetyl esters revealing the saccharide, which can then interfere in the biosynthesis of oligosaccharides. FCS exhibits only very low levels of esterase activity therefore improving extra-cellular stability of acetylated carbohydrates. Thus, it was anticipated that compound **14** would be easily taken-up by cells and subsequent deacetylation by intracellular esterases would reveal **1**, which can then be hydrolyzed by a α -mannosidase, initiating the release of mannostatin A. In order to investigate whether **14** possesses intracellular activity, it was subjected to MDAY-D2 cells and the

disappearance of polylactosamine monitored. It is known that the metastatic MDAY-D2 lymphoma cell line over-expresses the enzyme *N*-acetyl glucosaminyltransferase V (GnTV)⁸³. This enzyme adds an additional β 1-6-linked *N*-acetyl glucosamine moiety to Asn-linked oligosaccharides, which is subsequently extended by a polylactosamine chain. This increased branching has been observed in human breast, colon and skin carcinomas and has been correlated with invasive and metastatic potential. Inhibition of the mannose trimming enzyme, human Golgi α -mannosidase II (HGMII), which acts late in the *N*-glycan processing pathway, provides one route to blocking additional branching of *N*-linked oligosaccharides⁸³. In this case, the biosynthetic precursor for GnTV is not formed and hence the polylactosamine chain cannot be appended. Thus, in order to investigate whether the acetylated prodrugs can inhibit intra-cellular Golgi mannosidase II, MDAY-D2 lymphoma cells were cultured in the presence of different concentrations of **14** and **15** for two days. Subsequently, the presence of polylactosamine linked to proteins was determined by analyzing cell lysates by Western blotting using a fluorescein labeled PHA lectin¹³². Interestingly, at a concentration of 1 μ M of **14**, a 50% reduction in fluorescein labeling was observed, whereas compound **15** did not display any inhibitory activity (Fig. 3.4).

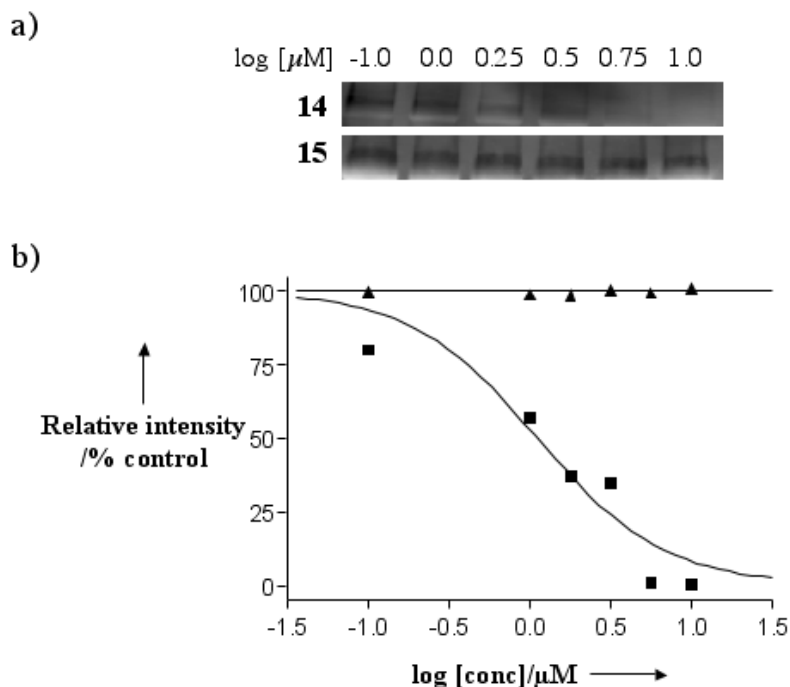


Fig.3.4: Inhibition of polylectosamine formation by compounds **14** and **15**. MDAY-D2 cells were cultured for 2 days in the absence (control) or presence of different concentrations of compounds **14** (■) or **15** (▲). (a) Cellular proteins (10 μ g) were subjected to SDS-PAGE and polylectosamine was visualized using a fluorescein labeled L-PHA lectin. (b) Fluorescence bands were scanned by laser beam densitometry, and the relative intensity depicted.

Furthermore, the effects of pro-drugs on MDAY-D2 cell viability was analyzed by staining an SDS PAGE protein lysate using a Coomassie dye (Fig. 3.5). The acetylated derivatives of both Mannosatin (**14**) and aminocyclopeptitretol (**15**) caused cell death at concentration greater than 10 μ M, i.e. at 100 μ M (lane A) and 30 μ M (lane A and B) respectively. The unacetylated derivatives of Mannosatin (**1**) and aminocyclopeptitretol (**2**) caused no cell death at 100 μ M. This indicated that though acetylation might make the drug more hydrophobic, improving cellular uptake, it can also have a toxic effect.

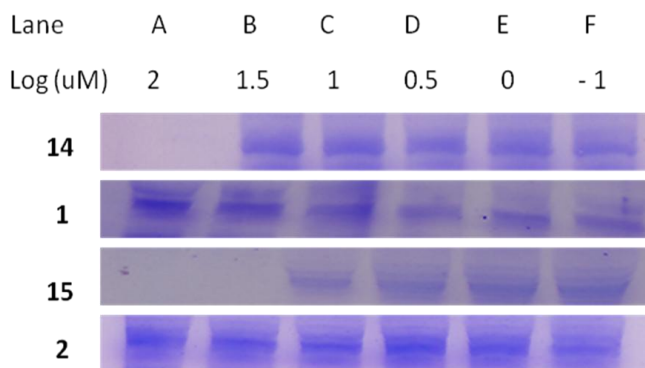


Fig.3.5: Effect of Pro-drug on MDAY-D2 cell viability. After 48 hour incubation, SDS-PAGE gel was stained with Coomassie dye. Cell death occurred in lane A of 14 and Lanes A and B of 15

3.6 Discussion

Changes in glycosylation pattern of cellular glycoproteins occur with tumor progression and metastasis^{88a, 117}. Thus compounds that can interfere in the biosynthesis of oligosaccharides are of critical importance in drug discovery^{83, 118}. Mannostatins A (**5**), originally isolated from the soil microorganism *Streptoverticillus*, is one of the most potent inhibitor of class II α -mannosidase. Earlier studies evaluating its structure activity relationship to dGMII indicate that basicity of the primary amine, the neighboring cis-diol and thiomethyl ether are essential for its inhibitory activity^{129, 133}. The 3,4-cis-diol of **5** complexes with an active site Zn²⁺ ion of dGMII resulting to a T6 coordination geometry, while the 2-hydroxyl forms hydrogen bonds with Asp472 and Tyr727. The thiomethyl moiety, structurally similar to the side chain of a methionine residue, is proposed to be involved in interactions important for protein stability¹³⁴. The thiomethyl moiety makes favorable hydrophobic interactions with Arg228 and Tyr727. On the other hand, aminocyclopentitetrol (**6**), lacking the thiomethyl ether is a poor inhibitor. This again showed that the thiomethyl moiety of **5** makes important interactions with the binding site of the enzyme.

The pro-drug derivatives of Mannosatin (**14**) and aminocyclopentitetrol (**15**) were hydrolyze by α -mannosidase resulting in the formation of intermediate **3**, which self fragment to give mannosatin A (**5**). As expected, the active mannosatin A inhibited the α -mannosidase that initiated its release, thus terminating the enzymatic reaction. On the other hand, the enzymatic hydrolysis of compound **2** released aminocyclopentitetrol **6**, a poor inhibitor of α -mannosidase. It was also observed that the acetylated pro-drugs of both **14** and **15** caused cell death at concentration greater than 100uM (lane A) and 30uM (lane A and B) respectively. This indicated that though acetylation might make the drug more hydrophobic, thereby improving cellular uptake, it also adds significant toxicity. Thus new approaches to pro-drug delivery into cells have to be considered such as liposomes and nanoparticles.

CHAPTER 4

Conclusions and Future Directions

The contributions of cellular oligosaccharides and glycoconjugates to critical biological processes such as bacterial and viral infection, leukocyte - endothelial cell adhesion, and immunological recognition of tumor cells continues to attract a lot of scientific inquiry and the molecular mechanisms of these processes are now understood in significant. In our study, we looked at the role of TLR2 in innate immunity as it serves as a receptor for bacterial glycoconjugate – peptidoglycan and how the information gleaned can be used to design compounds that are useful for the prevention or treatment of sepsis.

We demonstrate through surface plasmon resonance technology and cellular activation studies that TLR2 bind PGN fragments derived from both Gram- positive and -negative bacteria. The structural importance of each amino acid within the stem peptide was analyzed for TLR2 binding. The functional significance of the binding data was used to evaluate levels of endotoxicity that may arise from these bacterial PGN. The linear biosynthetic PGN from Gram-positive bacteria that contains pentapeptides (**3a**) and cross-linked PGN (e.g. dimer **4**) were poorly recognized by TLR2. However, remodeled Lysine containing fragments (**1**, **2a**, and **3b**) obtained as products of autolysins resulting from the bacteria cleaving its existing cell wall to introduce newly synthesized PGN subunits during replication, complexed with moderate to high affinity. On the other hand, all remodeled Dap containing negative Gram-products bound with

strong affinity. Furthermore, the binding studies also reveal that modification of carboxylic acids of iso-Glu and DAP dramatically influences binding, indicating sites where modification of the carboxylic acids may be a strategy used by bacteria to evade detection by TLR2.

The PGN structural information in combination with the crystal structure of TLR2 can serve as starting points in the design and synthesis of PGN mimetic. As a future project, an effective PGN mimetic must exploit the TLR2 binding features described by Jin et al (2007)¹³⁵. In this seminal work, the crystal structures of human TLR1-TLR2-lipopeptide complex were investigated. It was observed that TLR2 binds the tri-acylated lipopeptide, Pam3CSK4, and formed a heterodimer with TLR1 whereas the binding of the diacylated lipopeptide, Pam2CSK4, did not. Briefly, two of the three lipid chains of Pam3CSK4 are inserted into a hydrophobic pocket on the convex face of TLR2 at the TLR2/ TLR1junction. The other amide-bound lipid chain is inserted into a hydrophobic channel in TLR1 resulting to a stable heterodimer, critical to signal transduction. However, despite the lack of a third acyl group, diacylated Pam2CSK4 interacts with the lipid-binding pocket of TLR2 in virtually the same fashion as Pam3CSK4.

In this light, a good PGN mimetic would have to bind TLR2 strongly without initiating a signaling cascade; thereby mitigate the effects that come with overproduction of pro-inflammatory cytokines critical for the development of sepsis. Thus PGN remodeled fragments like the Tri- and Tetra- (Lys or Dap) containing PGN can be modified to bind TLR2. A diacyl group can be linked to the disaccharide of the PGN subunit while allowing the PGN peptide stem to still interact with the TLR2 protein and expectantly prevent its dimerization and signal transduction.

The next series of glycoconjugates studied were pro-drug constructs of Mannostatin A. Mannostatins A (**5**), originally isolated from the soil microorganism *Streptoverticillus*, is one of the most potent inhibitor of class II α -mannosidase known to interfere in the biosynthesis of oligosaccharides critical in tumor progression and metastasis. A feedback pro-drug inhibitor of mannostatin A (with aminocyclopetitretol as a control) was designed to selectively target α -mannosidases. Due to the non-selective inhibition of both golgi and lysosomal mannosidases, the inhibition of lysosomal mannosidase leads to the phenotype of lysosomal storage disease, a side effect during cancer treatment. The pro-drug **2** (aminocyclopetitretol) was enzymatically cleavage by human lysosomal mannosidase (HLM) to generate the final product which was non-inhibitory. However, no hydrolysis was observed when pro-drug **1**(mannostatin A) was use as the released of low amounts of mannostatin A immediately inhibited HLM, thus terminating further hydrolysis. Furthermore, because lysosomes are significantly more acidic than the Golgi apparatus, the effect of pH on the self-fragmentation of the intermediates **3** and **4** was investigated. It was expected that both intermediates will fragment significantly faster in neutral as opposed to acidic conditions, providing a basis for some selectivity for Golgi- over the lysosomal mannosidase. The analysis of ^1H NMR by integration of key signals of the intermediate and final product showed that the decomposition was significantly faster at more basic pH and elevated temperatures.

However, for these pro-drugs to be effective, they have to be cell permeable to inhibit intracellular glycosidases. It was anticipated that the *O*-acetylation of **1** and **2** to give **14** and **15**, respectively would considerably improve the metabolic stability of the pro-drug and improve uptake. Once in the cell, it was expected that intra-cellular

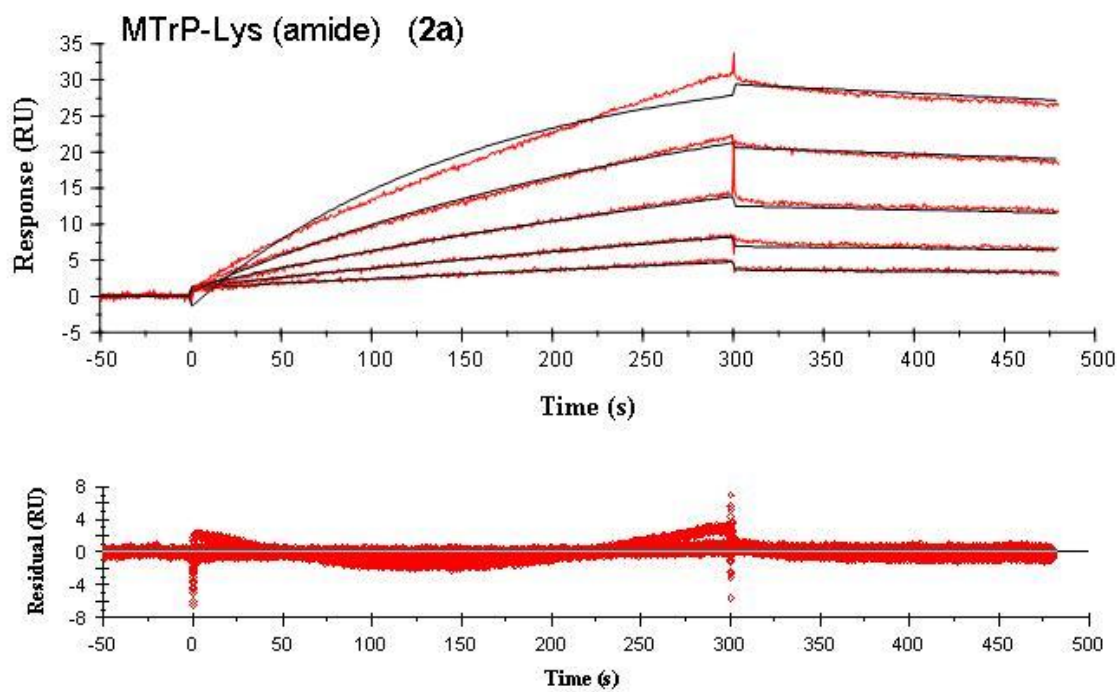
esterases would cleave the acetyl esters revealing the pro-drug which can then impede glycosylation of proteins in the golgi. The acetylated pro-drugs were incubated with MDAY-D2 cells and the disappearance of polylactosamine, a cancer metastatic biomarker monitored. Interestingly, at a concentration of 1 μ M of **14**, a 50% reduction in fluorescein labeling was observed, whereas compound **15** did not display any inhibitory activity. Unexpectedly, it was observed that the acetylated derivatives both caused cell death at concentration greater than 10 μ M. Thus in future studies, new delivery approaches such as the use of nanoparticles for pro-drug delivery is recommended.

APPENDICES

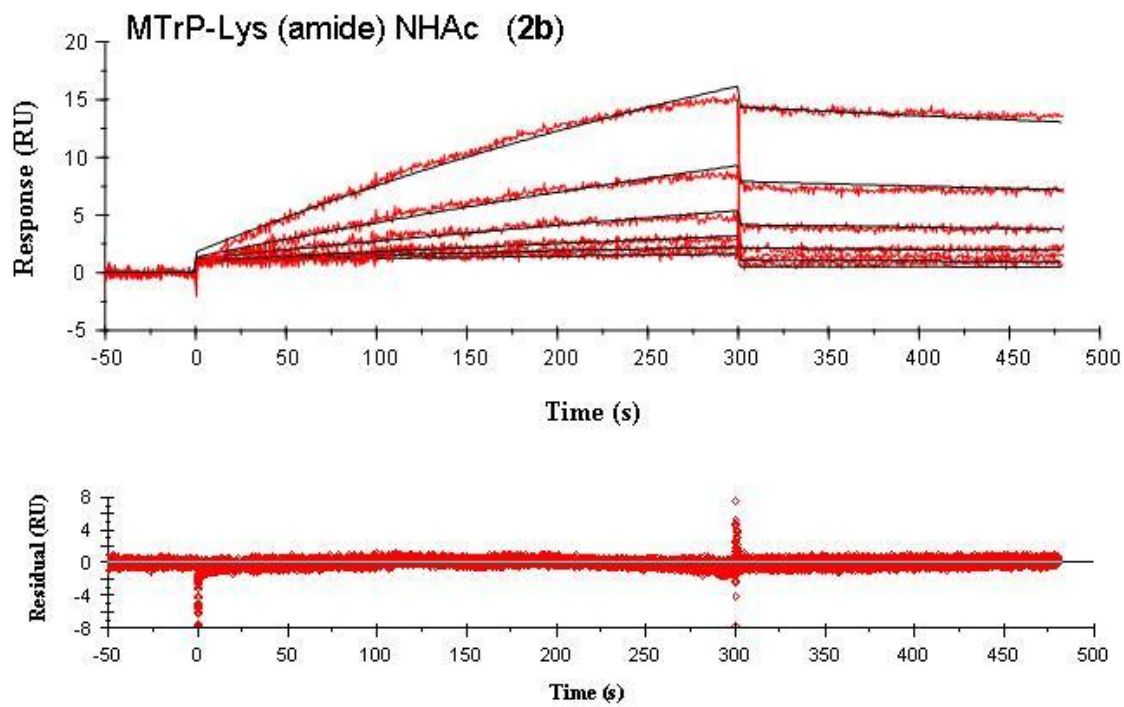
Appendix A

Sensorgrams representing the concentration-dependent kinetic analysis of lysine- and DAP-containing PGN part structures with immobilized sTLR2 (5,000 RU). Simultaneous kinetic analysis of two-fold serial dilutions of MTrP-Lys (amide) (**2a**; 250 to 15.6 μ M). At 500 μ M mass transport effects were observed with **2a**) (A), MTrP-Lys (amide) NHAc (**2b**; 500 to 15.6 μ M) (B), MTrP-Lys (Gly) (**2c**; 500 to 15.6 μ M) (C), MPP-Lys (D-Ala) (**3a**; 1000 to 31.3 μ M) (D), MPP-Lys (Gly) (**3b**; 500 to 15.6 μ M) (E), MTrP-(Gly)₅-MPP (**4**; 500 to 15.6 μ M) (F), MTrP-DAP (amide/acid) (**6a**; 100 to 1.6 μ M) (G), MTrP-DAP (acid/amide) (**6b**; 100 to 1.6 μ M) (H), MTrP-DAP (amide/amide) (**6c**; 100 to 1.6 μ M) (I), MTrP-DAP (acid/acid) (**6d**; 100 to 1.6 μ M) (J), and MPP-DAP (amide/acid) (**7**; 100 to 1.6 μ M) (K) are shown. Compounds **2a,b**, **3b**, **4**, and **6b** were fitted with a Langmuir 1:1 binding model and compounds **6a**, **6c**, and **7** with a two-state binding model (black lines). The corresponding residual values are plotted below the individual sensorgrams (except for **2c** and **6d**). Also shown are *E. coli* 055:B5 LPS (500 to 15.6 μ g/ml) (L) and Poly (I)·Poly (C) (2 to 0.125 mg/ml) (M) as negative controls for TLR2 binding and Pam₃CysSK₄ (5 to 0.08 nM) (N) as positive control.

A

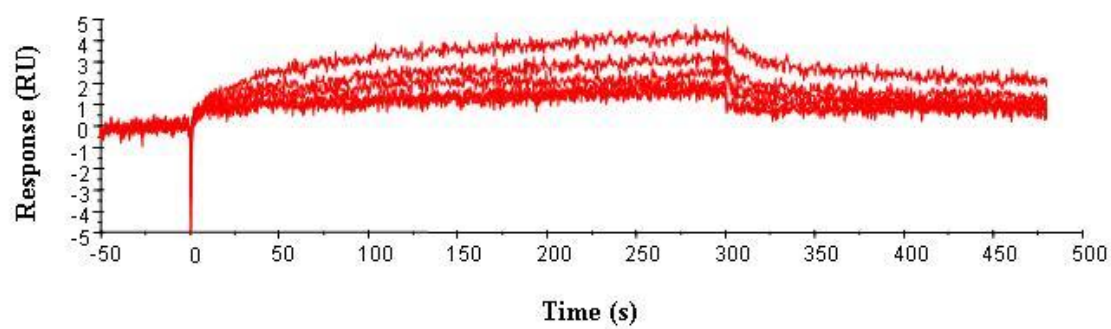


B



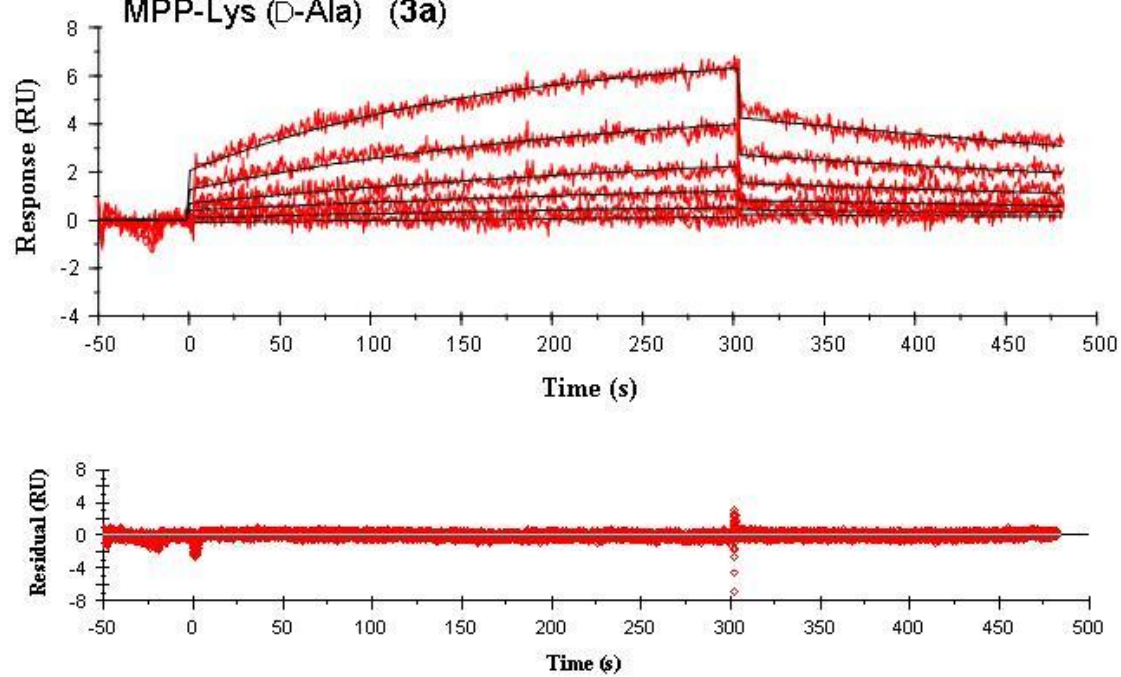
C

MTrP-Lys (Gly) (2c)

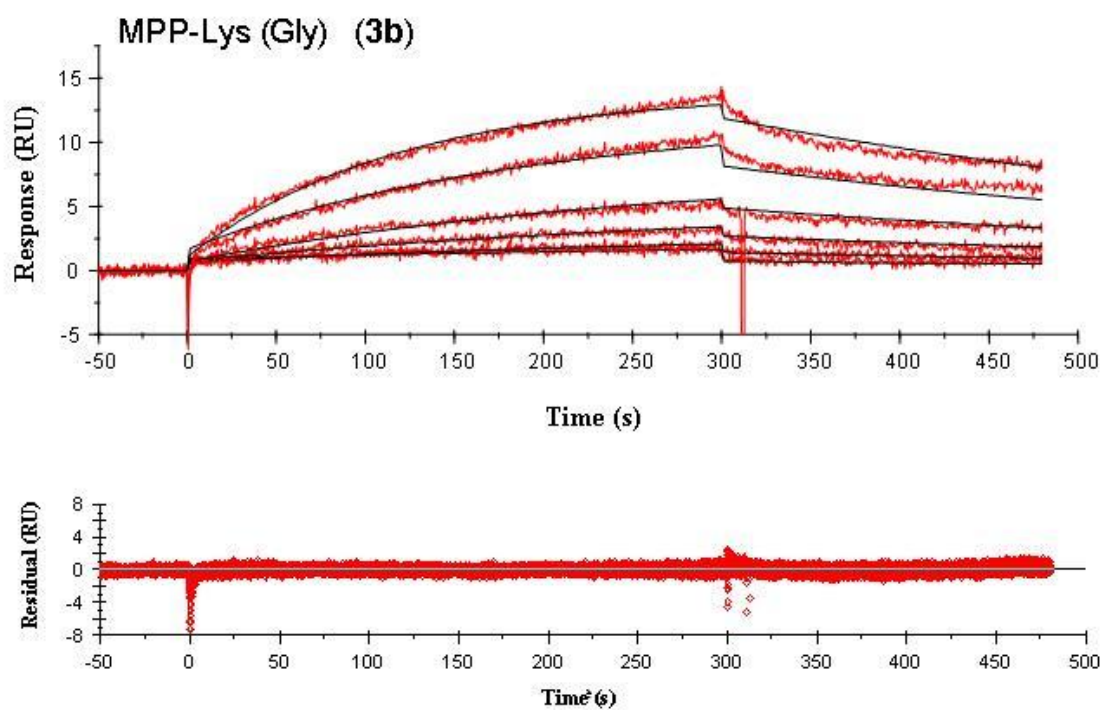


D

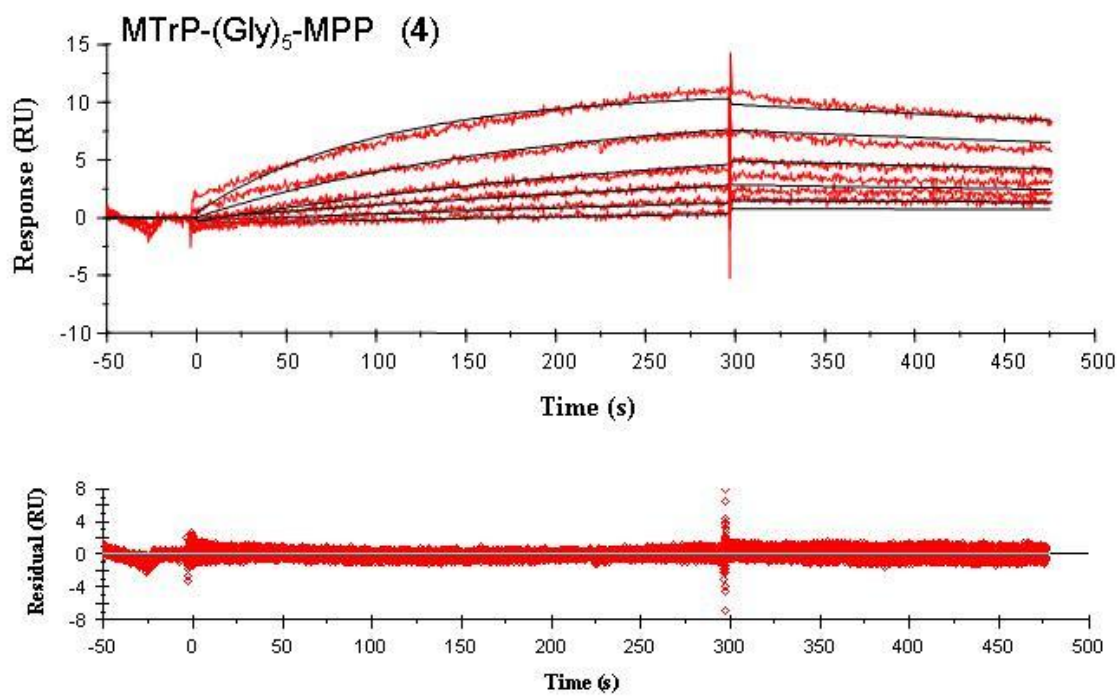
MPP-Lys (D-Ala) (3a)



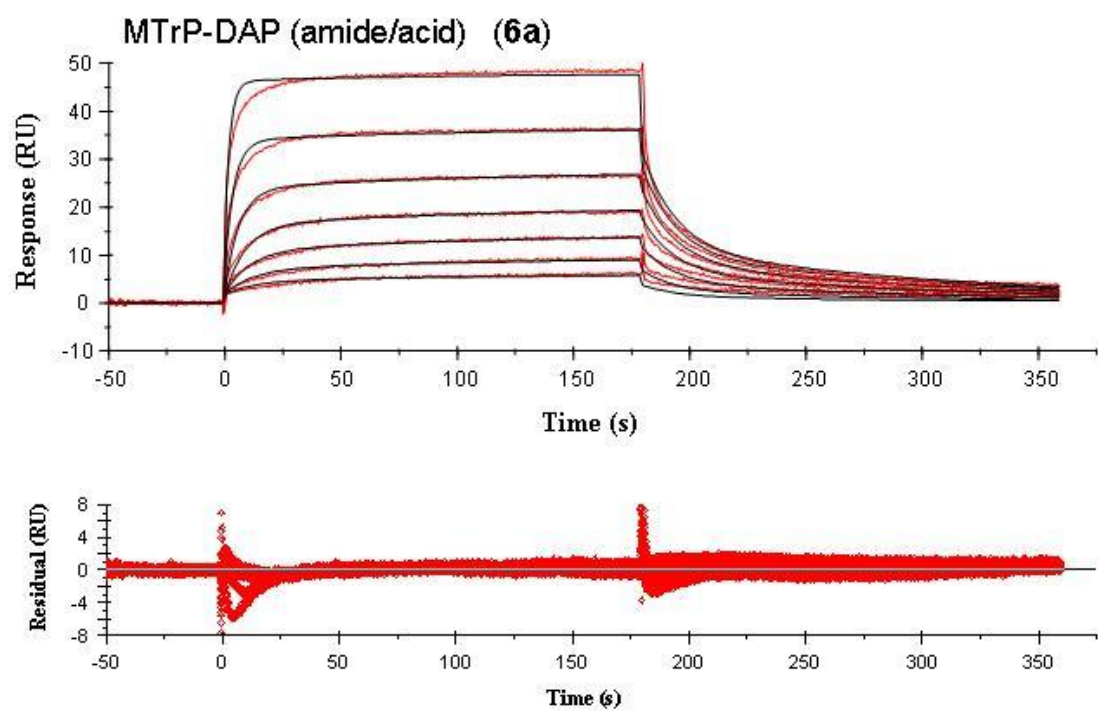
E



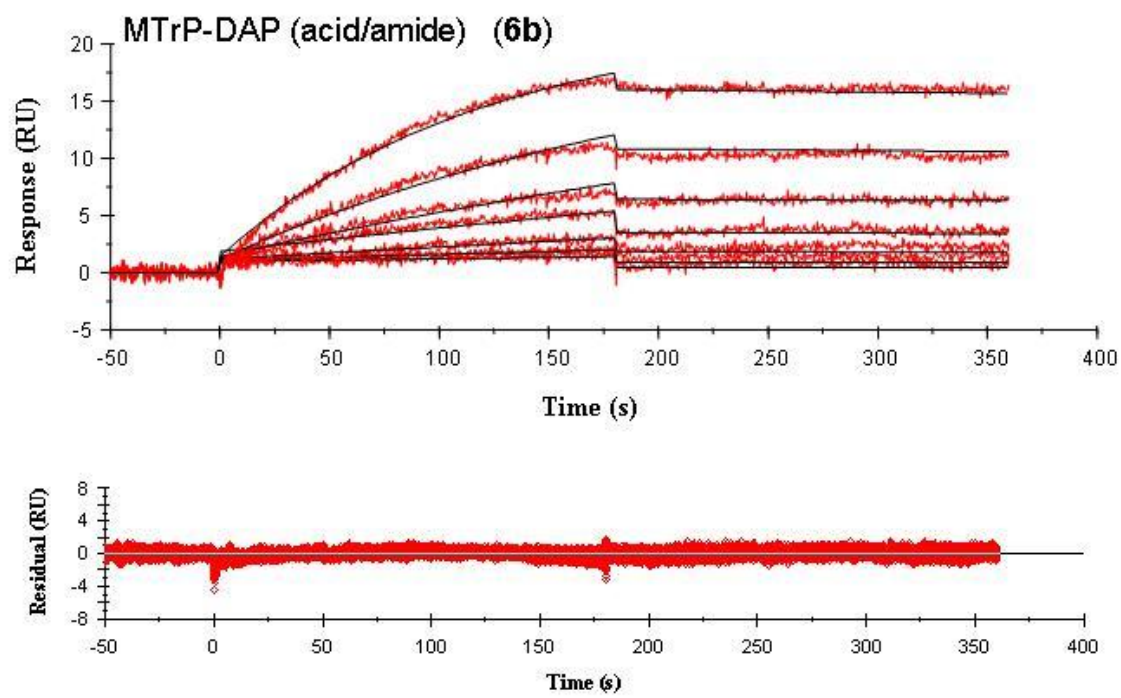
F



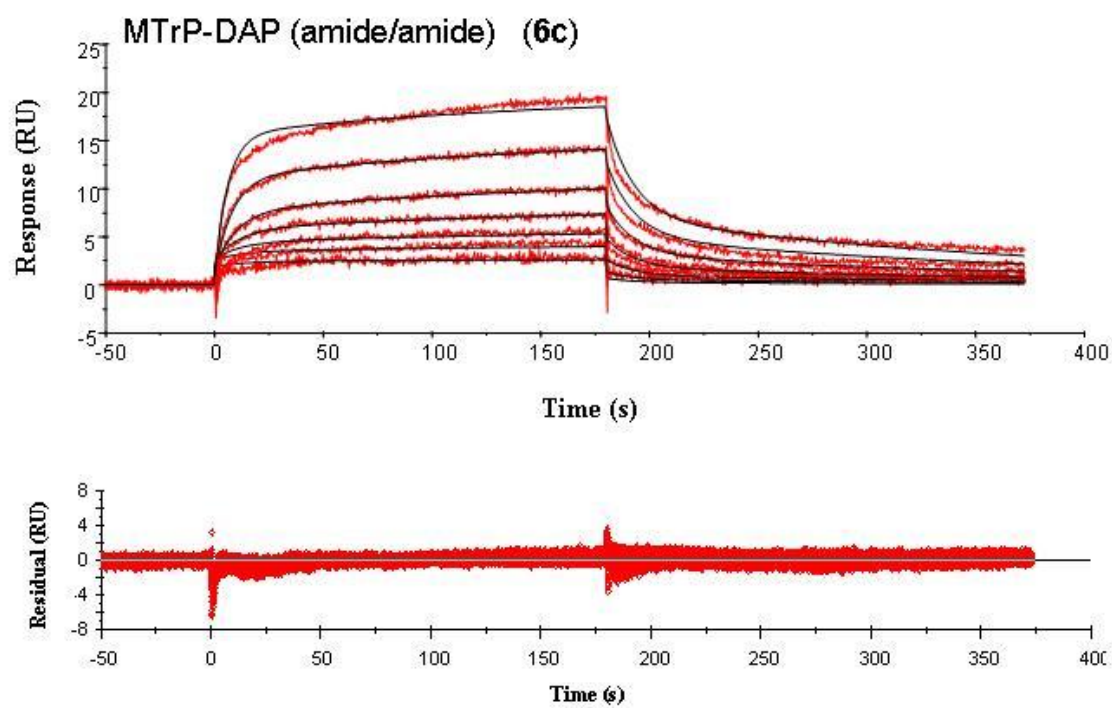
G



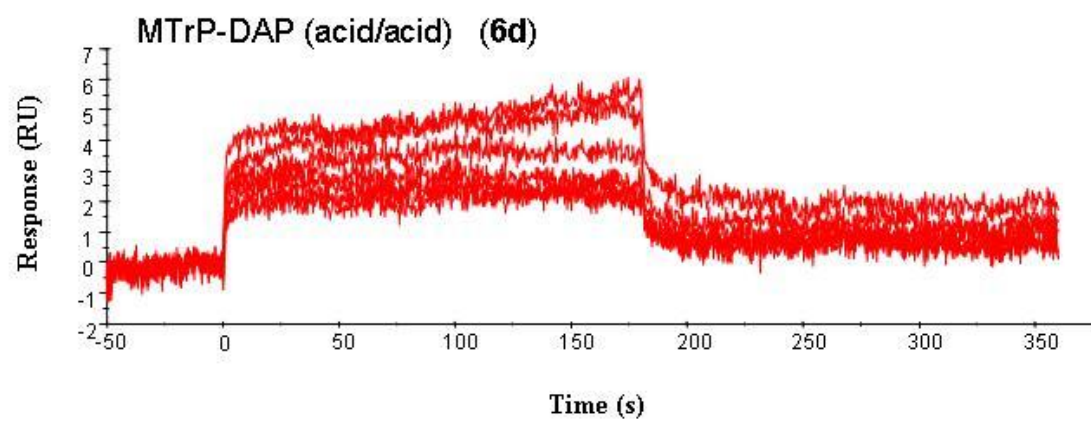
H



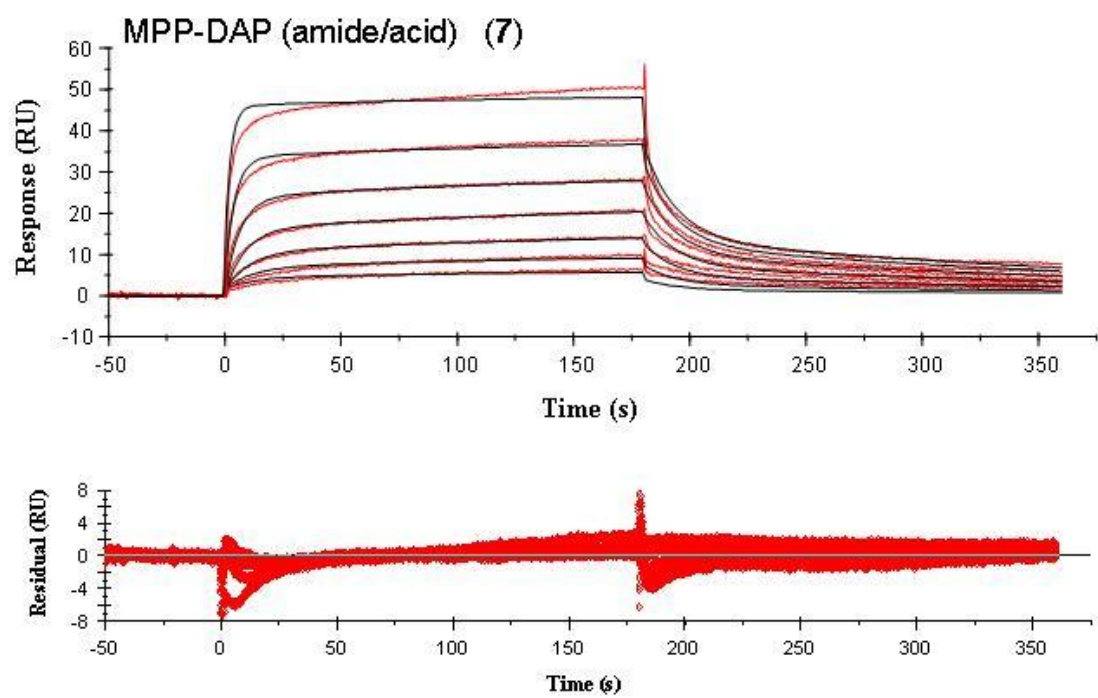
I



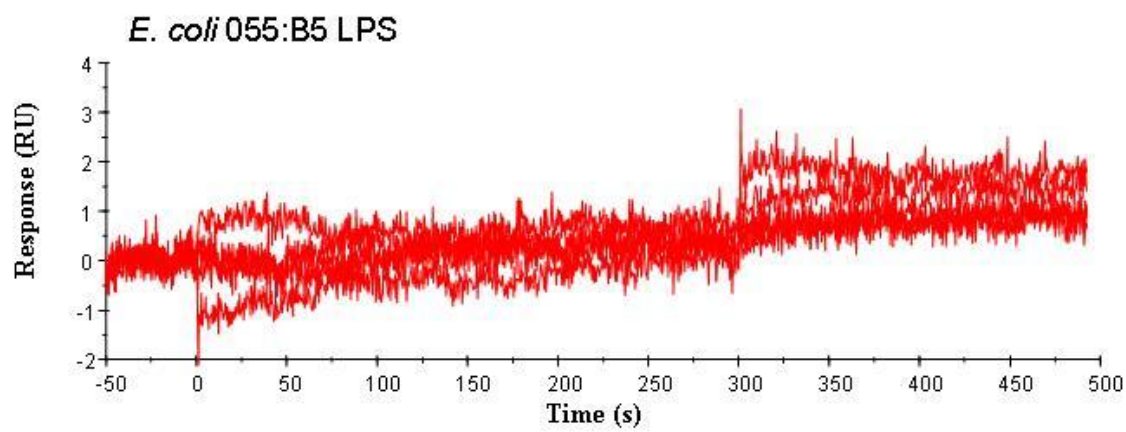
J



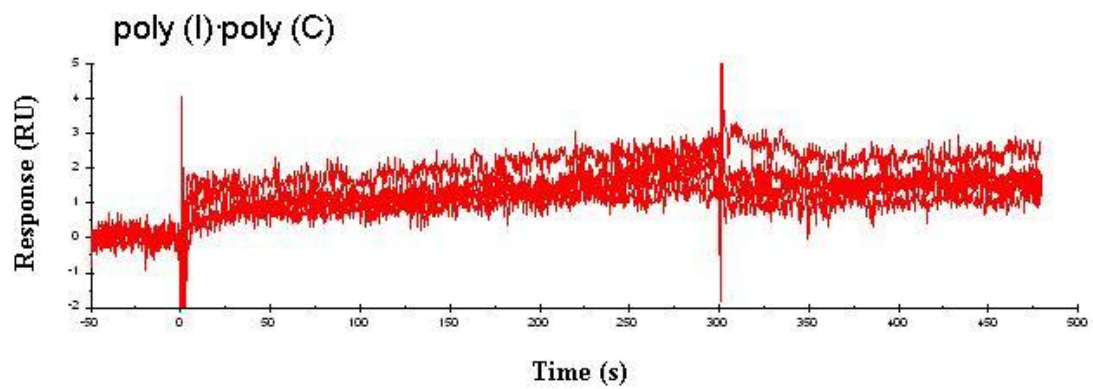
K



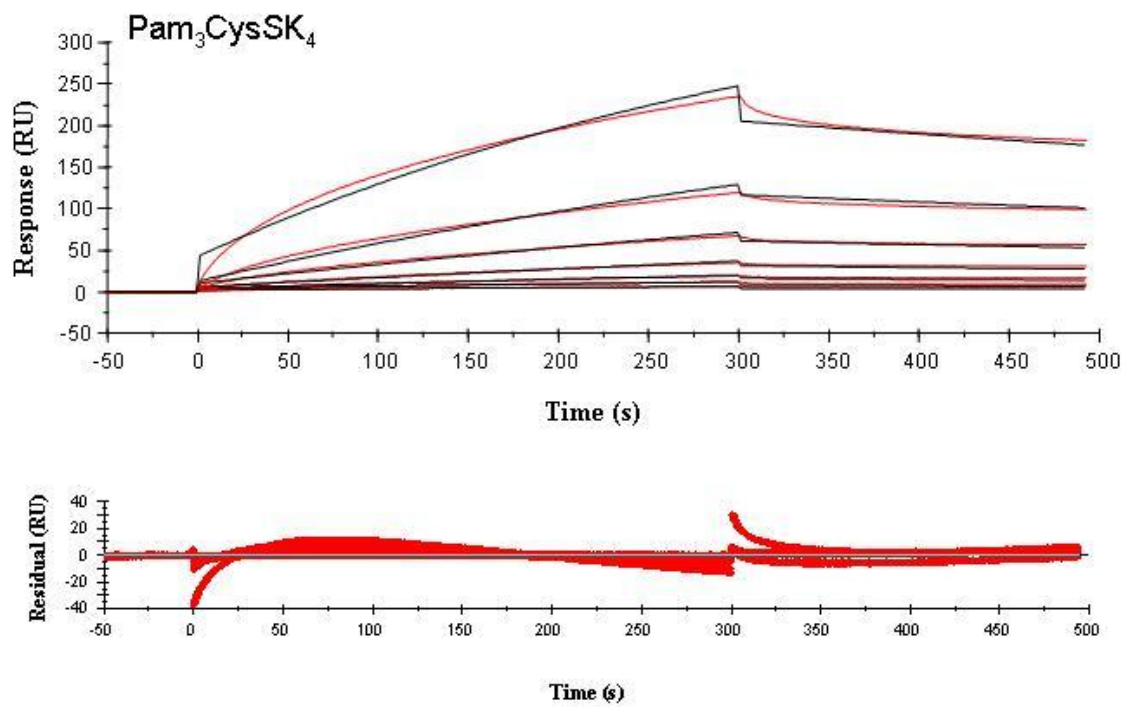
L



M

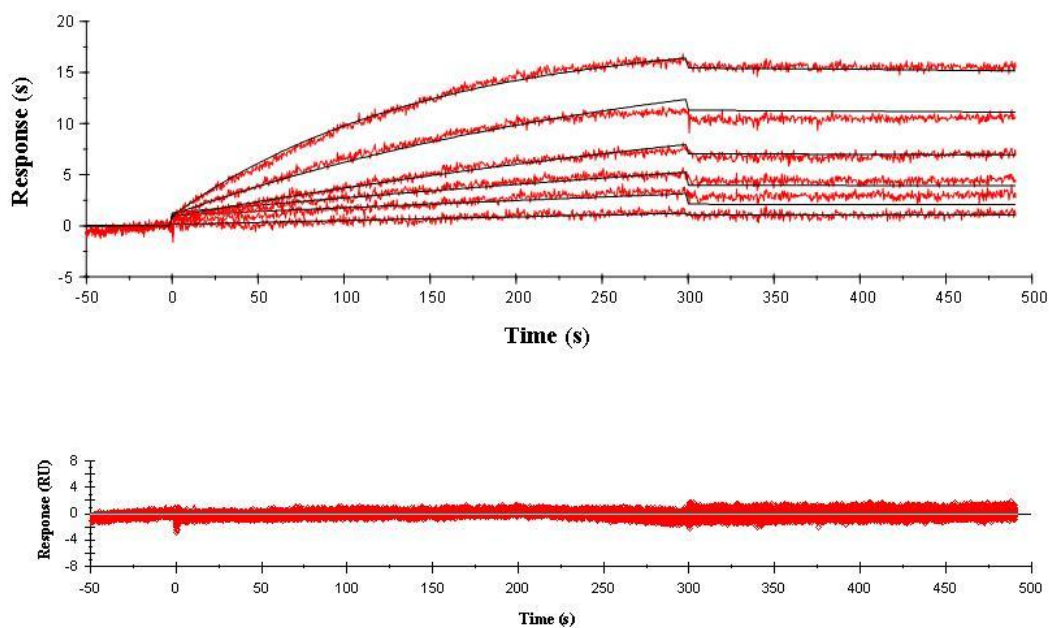


N



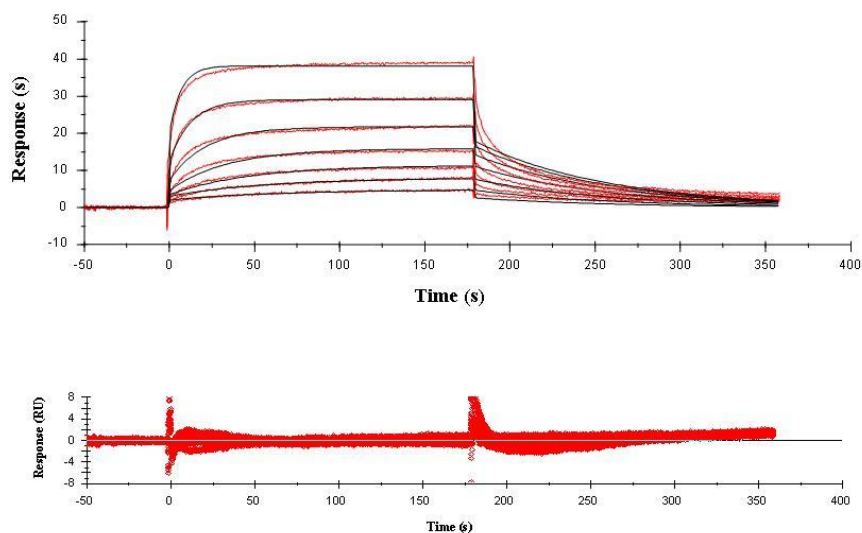
Appendix B

Sensorgrams representing the concentration-dependent kinetic analysis of MTP-Lys (amide) 1 with immobilized biotinylated sTLR2. Shown are sensorgrams representing simultaneous kinetic analysis of two-fold serial dilutions of MTP-Lys (amide) 1 with immobilized biotinylated sTLR2 (2,000 RU) at concentrations of 500 to 15.6 μ M, fitted with a Langmuir 1:1 binding model (black lines). The corresponding residual values are plotted below the individual sensorgrams. RU indicates resonance units.



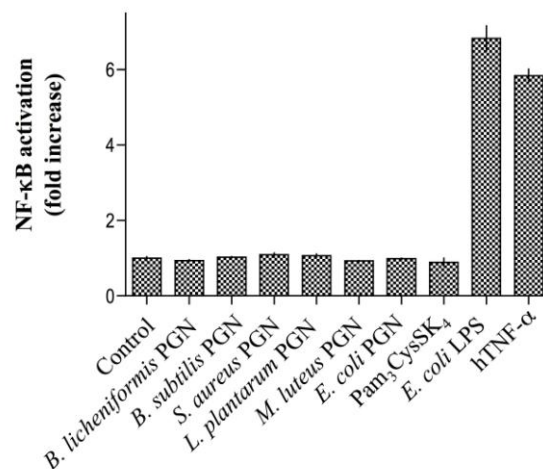
Appendix C

Sensorgrams representing the concentration-dependent kinetic analysis of MTP-DAP (amide/acid) **5 with immobilized sTLR2 fitted with a Langmuir 1:1 binding model.** Shown are sensorgrams representing kinetic analysis of two-fold serial dilutions of MTP-DAP (amide/acid) **5** with immobilized sTLR2 (5,000 RU) at concentrations of 100 to 1.6 μM , fitted with a Langmuir 1:1 binding model (black lines). The corresponding residual values are plotted below the individual sensorgrams. RU indicates resonance units. The 1:1 binding model is unsatisfactory due to nonrandom distribution of residuals during the dissociation phase. Furthermore, an initial fast binding is followed by slow and gradual increase in binding and the results of a contact time experiment support that binding proceeds through a two-state model. Indeed, a two-state binding model provided a good fit and a K_D of 11 μM was established



Appendix D

Response of HEK293T cells expressing human TLR4/MD2/CD14 to different PGNs. Induction of NF- κ B activation was determined in cultures of HEK293T cells stably transfected with human TLR4/MD2/CD14 and transiently transfected with pELAM-Luc and pRL-TK plasmids. Forty-four h post-transfection, cells were treated with purified PGNs (100 μ g/ml each) or were left untreated (control). Forty-eight h post-transfection, NF- κ B activation, as firefly luciferase activity relative to *Renilla* luciferase activity, was determined. As positive controls *E. coli* LPS (1 ng/ml) and human TNF- α (10 ng/ml) and as negative control Pam₃CysSK₄ (1 μ g/ml) were used. Forty-eight h post-transfection, NF- κ B activation was determined. Fold change is fold increase over control (cells exposed to medium only is arbitrarily set at 1). Data represent mean values $\pm$ s.d. ($n = 3$).



REFERENCES

1. (a) Tort, L., Balasch, J.C., Mackenzie, S., , FISH IMMUNE SYSTEM. A CROSSROADS BETWEEN INNATE AND ADAPTIVE RESPONSES”, *Inmunología*, 22 (3): 277-286. *Inmunología* **2003**, 22 (3), 277-286; (b) Fearon, D. T.; Locksley, R. M., Elements of immunity - The instructive role of innate immunity in the acquired immune response. *Science* **1996**, 272 (5258), 50-54.
2. Kvell, K.; Cooper, E.; Engelmann, P.; Bovari, J.; Nemeth, P., Blurring borders: Innate immunity with adaptive features. *Clinical & Developmental Immunology* **2007**, 1-10.
3. Shinkai, K.; Mohrs, M.; Locksley, R. M., Helper T cells regulate type-2 innate immunity in vivo. *Nature* **2002**, 420 (6917), 825-829.
4. (a) Martin, G. S.; Mannino, D. M.; Eaton, S.; Moss, M., The epidemiology of sepsis in the United States from 1979 through 2000. *New England Journal of Medicine* **2003**, 348 (16), 1546-1554; (b) Akira, S.; Uematsu, S.; Takeuchi, O., Pathogen recognition and innate immunity. *Cell* **2006**, 124 (4), 783-801.
5. (a) Kawai, T.; Akira, S., Signaling to NF-kappa B by Toll-like receptors. *Trends in Molecular Medicine* **2007**, 13 (11), 460-469; (b) Takeda, K.; Akira, S., Toll-like receptors in innate immunity. *International Immunology* **2005**, 17 (1), 1-14.
6. Huysamen, C.; Brown, G. D., The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors. *FEMS Microbiol Lett* **2009**, 290 (2), 121-8.
7. Yoneyama, M.; Fujita, T., RIG-I family RNA helicases: Cytoplasmic sensor for antiviral innate immunity. *Cytokine & Growth Factor Reviews* **2007**, 18 (5-6), 545-551.

8. Takaoka, A.; Taniguchi, T., Cytosolic DNA recognition for triggering innate immune responses. *Advanced Drug Delivery Reviews* **2008**, *60* (7), 847-857.
9. (a) Akira, S.; Yamamoto, M.; Takeda, K., Role of adapters in Toll-like receptor signalling. *Biochemical Society Transactions* **2003**, *31*, 637-642; (b) Akira, S.; Takeda, K., Toll-like receptor signalling. *Nature Reviews Immunology* **2004**, *4* (7), 499-511; (c) Carpenter, S.; O'Neill, L. A. J., Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. *Biochemical Journal* **2009**, *422*, 1-10.
10. (a) Hoshino, K.; Takeuchi, O.; Kawai, T.; Sanjo, H.; Ogawa, T.; Takeda, Y.; Takeda, K.; Akira, S., Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: Evidence for TLR4 as the Lps gene product. *Journal of Immunology* **1999**, *162* (7), 3749-3752; (b) Poltorak, A.; He, X. L.; Smirnova, I.; Liu, M. Y.; Van Huffel, C.; Du, X.; Birdwell, D.; Alejos, E.; Silva, M.; Galanos, C.; Freudenberg, M.; Ricciardi-Castagnoli, P.; Layton, B.; Beutler, B., Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science* **1998**, *282* (5396), 2085-2088.
11. (a) Takeuchi, O.; Sato, S.; Horiuchi, T.; Hoshino, K.; Takeda, K.; Dong, Z. Y.; Modlin, R. L.; Akira, S., Cutting edge: Role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of Immunology* **2002**, *169* (1), 10-14; (b) Takeuchi, O.; Kawai, T.; Muhlradt, P. F.; Morr, M.; Radolf, J. D.; Zychlinsky, A.; Takeda, K.; Akira, S., Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International Immunology* **2001**, *13* (7), 933-940.
12. Alexopoulou, L.; Holt, A. C.; Medzhitov, R.; Flavell, R. A., Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. *Nature* **2001**, *413* (6857), 732-738.

13. Gewirtz, A. T.; Navas, T. A.; Lyons, S.; Godowski, P. J.; Madara, J. L., Cutting edge: Bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *Journal of Immunology* **2001**, *167* (4), 1882-1885.
14. (a) Heil, F.; Hemmi, H.; Hochrein, H.; Ampenberger, F.; Kirschning, C.; Akira, S.; Lipford, G.; Wagner, H.; Bauer, S., Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **2004**, *303* (5663), 1526-1529; (b) Diebold, S. S.; Kaisho, T.; Hemmi, H.; Akira, S.; Sousa, C. R. E., Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **2004**, *303* (5663), 1529-1531.
15. Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S., A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408* (6813), 740-745.
16. Mitchell, J. A.; Paul-Clark, M. J.; Clarke, G. W.; McMaster, S. K.; Cartwright, N., Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. *Journal of Endocrinology* **2007**, *193* (3), 323-330.
17. Doyle, S. L.; O'Neill, L. A. J., Toll-like receptors: From the discovery of NFkB to new insights into transcriptional regulations in innate immunity. *Biochemical Pharmacology* **2006**, *72* (9), 1102-1113.
18. (a) Chuang, T. H.; Ulevitch, R. J., Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors (vol 5, pg 495, 2004). *Nature Immunology* **2004**, *5* (9), 968-968; (b) Sun, L. J.; Deng, L.; Ea, C. K.; Xia, Z. P.; Chen, Z. J. J., The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Molecular Cell* **2004**, *14* (3), 289-301; (c) Ea, C. K.; Sun, L.; Inoue, J.; Chen, Z. J. J., TIFA activates I kappa B kinase (IKK)

by promoting oligomerization and ubiquitination of TRAF6. *Proceedings of the National Academy of Sciences of the United States of America* **2004**, 101 (43), 15318-15323.

19. Arancibia, S. A.; Beltran, C. J.; Aguirre, I. M.; Silva, P.; Peralta, A. L.; Malinarich, F.; Hermoso, M. A., Toll-like receptors are key participants in innate immune responses. *Biological Research* **2007**, 40 (2), 97-112.

20. Sato, S.; Sanjo, H.; Takeda, K.; Ninomiya-Tsuji, J.; Yamamoto, M.; Kawai, T.; Matsumoto, K.; Takeuchi, O.; Akira, S., Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nature Immunology* **2005**, 6 (11), 1087-1095.

21. Oganessian, G.; Saha, S. K.; Guo, B. C.; He, J. Q.; Shahangian, A.; Zarnegar, B.; Perry, A.; Cheng, G. H., Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* **2006**, 439 (7073), 208-211.

22. (a) Rangel-Frausto, M. S., Sepsis: Still going strong. *Archives of Medical Research* **2005**, 36 (6), 672-681; (b) Cohen, J., The immunopathogenesis of sepsis. *Nature* **2002**, 420 (6917), 885-891; (c) Bernard, G. R.; Vincent, J. L.; Laterre, P.; LaRosa, S. P.; Dhainaut, J. F.; Lopez-Rodriguez, A.; Steingrub, J. S.; Garber, G. E.; Helterbrand, J. D.; Ely, E. W.; Fisher, C. J.; Protei, R. H. A., Efficacy and safety of recombinant human activated protein C for severe sepsis. *New England Journal of Medicine* **2001**, 344 (10), 699-709; (d) Angus, D. C.; Wax, R. S., Epidemiology of sepsis: An update. *Critical Care Medicine* **2001**, 29 (7), S109-S116; (e) Myhre, A. E.; Stuestol, J. F.; Dahle, M. K.; Overland, G.; Thiemermann, C.; Foster, S. J.; Lilleaasen, P.; Aasen, A. O.; Wang, J. E., Organ injury and cytokine release caused by peptidoglycan are dependent on the structural integrity of the glycan chain. *Infect Immun* **2004**, 72 (3), 1311-7.

23. Saturnino, S. F.; Andrade, M. V., Toll-like receptors, new horizons in sepsis. *Current Molecular Medicine* **2007**, 7 (5), 522-531.

24. (a) Scheffers, D. J.; Pinho, M. G., Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* **2005**, 69 (4), 585-607; (b) Schleifer, K. H.; Kandler, O., Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **1972**, 36 (4), 407-77; (c) Holtje, J. V., Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* **1998**, 62 (1), 181-+.
25. (a) Ghuysen, J. M., Use of Bacteriolytic Enzymes in Determination of Wall Structure and Their Role in Cell Metabolism. *Bacteriological Reviews* **1968**, 32 (4p2), 425-&; (b) Walsh, T. R.; Howe, R. A., The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. *Annu Rev Microbiol* **2002**, 56, 657-75; (c) Simelyte, E.; Rimpilainen, M.; Zhang, X.; Toivanen, P., Role of peptidoglycan subtypes in the pathogenesis of bacterial cell wall arthritis. *Annals of the Rheumatic Diseases* **2003**, 62 (10), 976-982; (d) Consaul, S. A.; Jacobs, W. R., Jr.; Pavelka, M. S., Jr., Extragenic suppression of the requirement for diaminopimelate in diaminopimelate auxotrophs of *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **2003**, 225 (1), 131-5; (e) Barrett, J. F.; Shockman, G. D., Isolation and characterization of soluble peptidoglycan from several strains of *Streptococcus faecium*. *J Bacteriol* **1984**, 159 (2), 511-9.
26. Hayhurst, E. J.; Kailas, L.; Hobbs, J. K.; Foster, S. J., Cell wall peptidoglycan architecture in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America* **2008**, 105 (38), 14603-14608.
27. (a) Harz, H.; Burgdorf, K.; Holtje, J. V., Isolation and Separation of the Glycan Strands from Murein of *Escherichia-Coli* by Reversed-Phase High-Performance Liquid-Chromatography. *Analytical Biochemistry* **1990**, 190 (1), 120-128; (b) Ward, J. B., Chain-Length of Glycans in Bacterial Cell-Walls. *Biochemical Journal* **1973**, 133 (2), 395-398; (c) Boneca, I.

- G.; Huang, Z. H.; Gage, D. A.; Tomasz, A., Characterization of *Staphylococcus aureus* cell wall glycan strands, evidence for a new beta-N-acetylglucosaminidase activity. *Journal of Biological Chemistry* **2000**, 275 (14), 9910-9918.
28. Labischinski, H.; Barnickel, G.; Naumann, D.; Keller, P., Conformational and Topological Aspects of the 3-Dimensional Architecture of Bacterial Peptidoglycan. *Annales De L Institut Pasteur-Microbiologie* **1985**, 136A (1), 45-50.
29. Stroming, J.; Izaki, K.; Matsuhara, M.; Tipper, D. J., Peptidoglycan Transpeptidase and D-Alanine Carboxypeptidase - Penicillin-Sensitive Enzymatic Reactions. *Federation Proceedings* **1967**, 26 (1), 9-&.
30. (a) Dejonge, B. L. M.; Chang, Y. S.; Gage, D.; Tomasz, A., Peptidoglycan Composition of a Highly Methicillin-Resistant *Staphylococcus-Aureus* Strain - the Role of Penicillin Binding Protein-2a. *Journal of Biological Chemistry* **1992**, 267 (16), 11248-11254; (b) Iketani, M.; Nishimura, H.; Akayama, K.; Yamano, Y.; Morishima, I., Minimum structure of peptidoglycan required for induction of antibacterial protein synthesis in the silkworm, *Bombyx mori*. *Insect Biochemistry and Molecular Biology* **1999**, 29 (1), 19-24.
31. (a) Vollmer, W.; Blanot, D.; de Pedro, M. A., Peptidoglycan structure and architecture. *Fems Microbiology Reviews* **2008**, 32 (2), 149-167; (b) Atrih, A.; Bacher, G.; Allmaier, G.; Williamson, M. P.; Foster, S. J., Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *J Bacteriol* **1999**, 181 (13), 3956-66.
32. Barreteau, H.; Kovac, A.; Boniface, A.; Sova, M.; Gobec, S.; Blanot, D., Cytoplasmic steps of peptidoglycan biosynthesis. *Fems Microbiology Reviews* **2008**, 32 (2), 168-207.

33. Bouhss, A.; Trunkfield, A. E.; Bugg, T. D. H.; Mengin-Lecreulx, D., The biosynthesis of peptidoglycan lipid-linked intermediates. *Fems Microbiology Reviews* **2008**, 32 (2), 208-233.
34. Healy, V. L.; Lessard, I. A. D.; Roper, D. I.; Knox, J. R.; Walsh, C. T., Vancomycin resistance in enterococci: reprogramming of the D-Ala-D-Ala ligases in bacterial peptidoglycan biosynthesis. *Chemistry & Biology* **2000**, 7 (5), R109-R119.
35. Glauner, B., Separation and quantification of mucopeptides with high-performance liquid chromatography. *Anal Biochem* **1988**, 172 (2), 451-64.
36. Markiewicz, Z.; Glauner, B.; Schwarz, U., Murein Structure and Lack of Dd-Carboxypeptidase and Ld-Carboxypeptidase Activities in *Caulobacter-Crescentus*. *Journal of Bacteriology* **1983**, 156 (2), 649-655.
37. Schleife, K.; Plapp, R.; Kandler, O., Identification of Threo-3-Hydroxyglutamic Acid in Cell Wall of *Microbacterium Lacticum*. *Biochemical and Biophysical Research Communications* **1967**, 28 (4), 566-&.
38. (a) Wyke, A. W.; Perkins, H. R., The specificity of enzymes adding amino acids in the synthesis of the peptidoglycan precursors of *Corynebacterium poinsettiae* and *Corynebacterium insidiosum*. *J Gen Microbiol* **1975**, 88 (1), 159-68; (b) Perkins, H. R., Homoserine and diaminobutyric acid in the mucopeptide-precursor-nucleotides and cell walls of some plant-pathogenic corynebacteria. *Biochem J* **1971**, 121 (3), 417-23.
39. Kamio, Y.; Itoh, Y.; Terawaki, Y., Chemical-Structure of Peptidoglycan in *Selenomonas-Ruminantium* - Cadaverine Links Covalently to the D-Glutamic Acid Residue of Peptidoglycan. *Journal of Bacteriology* **1981**, 146 (1), 49-53.
40. (a) Holtje, J. V., From Growth to Autolysis - the Murein Hydrolases in *Escherichia-Coli*. *Archives of Microbiology* **1995**, 164 (4), 243-254; (b) Park, J. T., Why Does *Escherichia-Coli*

- Recycle Its Cell-Wall Peptides. *Molecular Microbiology* **1995**, 17 (3), 421-426; (c) Goodell, E. W., Recycling of murein by Escherichia coli. *J Bacteriol* **1985**, 163 (1), 305-10.
41. Park, J. T.; Uehara, T., How bacteria consume their own exoskeletons (Turnover and recycling of cell wall peptidoglycan). *Microbiology and Molecular Biology Reviews* **2008**, 72 (2), 211-227.
42. Vollmer, W.; Joris, B.; Charlier, P.; Foster, S., Bacterial peptidoglycan (murein) hydrolases. *Fems Microbiology Reviews* **2008**, 32 (2), 259-286.
43. (a) Doyle, R. J.; Dziarski, R., The bacterial cell: peptidoglycan. In *Molecular Medical Microbiology*, Sussman, M., Ed. Academic Press, London, England: 2001; Vol. 1, pp 137-154; (b) Chamaillard, M.; Girardin, S. E.; Viala, J.; Philpott, D. J., Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell. Microbiol.* **2003**, 5 (9), 581-592; (c) Dziarski, R., Recognition of bacterial peptidoglycan by the innate immune system. *Cell. Mol. Life Sci.* **2003**, 60 (9), 1793-1804; (d) Inohara, N.; Nunez, G., NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* **2003**, 3 (5), 371-382; (e) Dziarski, R., Peptidoglycan recognition proteins (PGRPs). *Mol. Immunol.* **2004**, 40 (12), 877-886; (f) Girardin, S. E.; Philpott, D. J., The role of peptidoglycan recognition in innate immunity. *Eur. J. Immunol.* **2004**, 34 (7), 1777-1782; (g) Steiner, H., Peptidoglycan recognition proteins: on and off switches for innate immunity. *Immunol. Rev.* **2004**, 198, 83-96; (h) Boneca, I. G., The role of peptidoglycan in pathogenesis. *Curr. Opin. Microbiol.* **2005**, 8 (1), 46-53; (i) Cloud-Hansen, K. A.; Peterson, S. B.; Stabb, E. V.; Goldman, W. E.; McFall-Ngai, M. J.; Handelsman, J., Breaching the great wall: peptidoglycan and microbial interactions. *Nat. Rev. Microbiol.* **2006**, 4 (9), 710-716; (j) Myhre, A. E.; Aasen, A. O.; Thiemermann, C.; Wang, J. E., Peptidoglycan - an endotoxin in its own right? *Shock* **2006**, 25 (3), 227-235.

44. Boneca, I. G., The role of peptidoglycan in pathogenesis. *Current Opinion in Microbiology* **2005**, 8 (1), 46-53.
45. (a) Leclerc, C.; Juy, D.; Bourgeois, E.; Chedid, L., Invivo Regulation of Humoral and Cellular Immune-Responses of Mice by a Synthetic Adjuvant, N-Acetyl-Muramyl-L-Alanyl-D-Isoglutamine, Muramyl Dipeptide for Mdp. *Cellular Immunology* **1979**, 45 (1), 199-206; (b) Martin, S. A.; Karnovsky, M. L.; Krueger, J. M.; Pappenheimer, J. R.; Biemann, K., Peptidoglycans as Promoters of Slow-Wave Sleep .1. Structure of the Sleep-Promoting Factor Isolated from Human-Urine. *Journal of Biological Chemistry* **1984**, 259 (20), 2652-2658; (c) Traub, S.; von Aulock, S.; Hartung, T.; Hermann, C., MDP and other muropeptides - direct and synergistic effects on the immune system. *Journal of Endotoxin Research* **2006**, 12 (2), 69-85.
46. Dziarski, R.; Gupta, D., Staphylococcus aureus peptidoglycan is a toll-like receptor 2 activator: a reevaluation. *Infect Immun* **2005**, 73 (8), 5212-6.
47. Travassos, L. H.; Girardin, S. E.; Philpott, D. J.; Blanot, D.; Nahori, M. A.; Werts, C.; Boneca, I. G., Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep* **2004**, 5 (10), 1000-6.
48. (a) Girardin, S. E.; Philpott, D. J., Mini-review: the role of peptidoglycan recognition in innate immunity. *Eur J Immunol* **2004**, 34 (7), 1777-82; (b) Chamaillard, M.; Girardin, S. E.; Viala, J.; Philpott, D. J., Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol* **2003**, 5 (9), 581-92; (c) Wolfert, M. A.; Roychowdhury, A.; Boons, G. J., Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. *Infection and Immunity* **2007**, 75 (2), 706-713.

49. Dziarski, R., Recognition of bacterial peptidoglycan by the innate immune system. *Cell Mol Life Sci* **2003**, 60 (9), 1793-804.
50. Liu, C.; Xu, Z. J.; Gupta, D.; Dziarski, R., Peptidoglycan recognition proteins - A novel family of four human innate immunity pattern recognition molecules. *Journal of Biological Chemistry* **2001**, 276 (37), 34686-34694.
51. Yoshida, H.; Kinoshita, K.; Ashida, M., Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *Journal of Biological Chemistry* **1996**, 271 (23), 13854-13860.
52. Choe, K. M.; Werner, T.; Stoven, S.; Hultmark, D.; Anderson, K. V., Requirement for a peptidoglycan recognition protein (PGRP) in relish activation and antibacterial immune responses in *Drosophila*. *Science* **2002**, 296 (5566), 359-362.
53. Dziarski, R.; Platt, K. A.; Gelius, E.; Steiner, H.; Gupta, D., Defect in neutrophil killing and increased susceptibility to infection with-nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. *Blood* **2003**, 102 (2), 689-697.
54. Nau, R.; Eiffert, H., Minimizing the release of proinflammatory and toxic bacterial products within the host: a promising approach to improve outcome in life-threatening infections. *FEMS Immunol Med Microbiol* **2005**, 44 (1), 1-16.
55. Tipper, D. J.; Strominger, J. L., Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proc Natl Acad Sci U S A* **1965**, 54 (4), 1133-41.
56. Stone, K. J.; Strominger, J. L., Mechanism of action of bacitracin: complexation with metal ion and C 55 -isoprenyl pyrophosphate. *Proc Natl Acad Sci U S A* **1971**, 68 (12), 3223-7.

57. Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D., Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. *Science* **1999**, *284* (5413), 507-11.
58. (a) Vila, J., Mechanisms of antimicrobial resistance in *Acinetobacter baumannii*. *Reviews in Medical Microbiology* **1998**, *9* (2), 87-97; (b) Epstein, B. J.; Gums, J. G.; Drlica, K., The changing face of antibiotic prescribing: The mutant selection window. *Annals of Pharmacotherapy* **2004**, *38* (10), 1675-1682; (c) Hawkey, P. M.; Jones, A. M., The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy* **2009**, *64* (4), 3-10.
59. (a) Andersson, I.; van Scheltinga, A. C.; Vøllestad, K., Towards new beta-lactam antibiotics. *Cell Mol Life Sci* **2001**, *58* (12-13), 1897-906; (b) Pootoolal, J.; Neu, J.; Wright, G. D., Glycopeptide antibiotic resistance. *Annual Review of Pharmacology and Toxicology* **2002**, *42*, 381-408.
60. (a) Myszka, D. G.; He, X.; Dembo, M.; Morton, T. A.; Goldstein, B., Extending the range of rate constants available from BIACORE: Interpreting mass transport-influenced binding data. *Biophysical Journal* **1998**, *75* (2), 583-594; (b) Boozer, C.; Kim, G.; Cong, S. X.; Guan, H. W.; Londergan, T., Looking towards label-free biomolecular interaction analysis in a high-throughput format: a review of new surface plasmon resonance technologies. *Current Opinion in Biotechnology* **2006**, *17* (4), 400-405.
61. (a) Shliom, O.; Huang, M.; Sachais, B.; Kuo, A.; Weisel, J. W.; Nagaswami, C.; Nassar, T.; Bdeir, K.; Hiss, E.; Gawlak, S.; Harris, S.; Mazar, A.; Higazi, A. A., Novel interactions between urokinase and its receptor. *Journal of Biological Chemistry* **2000**, *275* (32), 24304-24312; (b) Mischiati, C.; Borgatti, M.; Bianchi, N.; Rutigliano, C.; Tomassetti, M.; Feriotto, G.; Gambari, R., Interaction of the human NF-kappa B p52 transcription factor with DNA-PNA

hybrids mimicking the NF-kappa B binding sites of the human immunodeficiency virus type 1 promoter. *Journal of Biological Chemistry* **1999**, 274 (46), 33114-33122; (c) Quinn, J. G.; O'Neill, S.; Doyle, A.; McAtamney, C.; Diamond, D.; MacCraith, B. D.; O'Kennedy, R., Development and application of surface plasmon resonance-based biosensors for the detection of cell-ligand interactions. *Analytical Biochemistry* **2000**, 281 (2), 135-143.

62. McDonnell, J. M., Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. *Current Opinion in Chemical Biology* **2001**, 5 (5), 572-577.

63. Hahnefeld, C.; Drewianka, S.; Herberg, F. W., Determination of kinetic data using surface plasmon resonance biosensors. *Methods Mol Med* **2004**, 94, 299-320.

64. Day, Y. S. N.; Baird, C. L.; Rich, R. L.; Myszka, D. G., Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods. *Protein Science* **2002**, 11 (5), 1017-1025.

65. Myszka, D. G.; Jonsen, M. D.; Graves, B. J., Equilibrium analysis of high affinity interactions using BIACORE. *Analytical Biochemistry* **1998**, 265 (2), 326-330.

66. Myszka, D. G., Improving biosensor analysis. *J Mol Recognit* **1999**, 12 (5), 279-84.

67. Lofas, S.; Johnsson, B.; Edstrom, A.; Hansson, A.; Lindquist, G.; Hillgren, R. M. M.; Stigh, L., Methods for Site Controlled Coupling to Carboxymethyldextran Surfaces in Surface-Plasmon Resonance Sensors. *Biosensors & Bioelectronics* **1995**, 10 (9-10), 813-822.

68. Johnsson, B.; Lofas, S.; Lindquist, G.; Edstrom, A.; Muller Hillgren, R. M.; Hansson, A., Comparison of methods for immobilization to carboxymethyl dextran sensor surfaces by analysis of the specific activity of monoclonal antibodies. *J Mol Recognit* **1995**, 8 (1-2), 125-31.

69. Pearson, J. E.; Kane, J. W.; Petraki-Kallioti, I.; Gill, A.; Vadgama, P., Surface plasmon resonance: a study of the effect of biotinylation on the selection of antibodies for use in immunoassays. *J Immunol Methods* **1998**, *221* (1-2), 87-94.
70. (a) Futamura, M.; Dhanasekaran, P.; Handa, T.; Phillips, M. C.; Lund-Katz, S.; Saito, H., Two-step mechanism of binding of apolipoprotein E to heparin: implications for the kinetics of apolipoprotein E-heparan sulfate proteoglycan complex formation on cell surfaces. *J Biol Chem* **2005**, *280* (7), 5414-22; (b) Karlsson, R., Affinity analysis of non-steady-state data obtained under mass transport limited conditions using BIAcore technology. *Journal of Molecular Recognition* **1999**, *12* (5), 285-292.
71. O'Shannessy, D. J.; Brigham-Burke, M.; Soneson, K. K.; Hensley, P.; Brooks, I., Determination of rate and equilibrium binding constants for macromolecular interactions using surface plasmon resonance: use of nonlinear least squares analysis methods. *Anal Biochem* **1993**, *212* (2), 457-68.
72. Karlsson, R.; Falt, A., Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *Journal of Immunological Methods* **1997**, *200* (1-2), 121-133.
73. Lipschultz, C. A.; Li, Y. L.; Smith-Gill, S., Experimental design for analysis of complex kinetics using surface plasmon resonance. *Methods* **2000**, *20* (3), 310-318.
74. Yong, K. T.; Roy, I.; Swihart, M. T.; Prasad, P. N., Multifunctional nanoparticles as biocompatible targeted probes for human cancer diagnosis and therapy. *Journal of Materials Chemistry* **2009**, *19* (27), 4655-4672.

75. Arnold, J. N.; Saldova, R.; Hamid, U. M. A.; Rudd, P. M., Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics* **2008**, 8 (16), 3284-3293.
76. Orntoft, T. F.; Vestergaard, E. M., Clinical aspects of altered glycosylation of glycoproteins in cancer. *Electrophoresis* **1999**, 20 (2), 362-71.
77. Sears, P.; Wong, C. H., Enzyme action in glycoprotein synthesis. *Cell Mol Life Sci* **1998**, 54 (3), 223-52.
78. (a) Schachter, H., Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. *Biochem Cell Biol* **1986**, 64 (3), 163-81; (b) Trombetta, E. S., The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. *Glycobiology* **2003**, 13 (9), 77r-91r.
79. van den Eijnden, D. H.; Koenderman, A. H.; Schiphorst, W. E., Biosynthesis of blood group i-active polylactosaminoglycans. Partial purification and properties of an UDP-GlcNAc:N-acetyllactosaminide beta 1----3-N-acetylglucosaminyltransferase from Novikoff tumor cell ascites fluid. *J Biol Chem* **1988**, 263 (25), 12461-71.
80. Herscovics, A., Structure and function of Class I alpha 1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control. *Biochimie* **2001**, 83 (8), 757-762.
81. Lau, K. S.; Partridge, E. A.; Grigorian, A.; Silvescu, C. I.; Reinhold, V. N.; Demetriou, M.; Dennis, J. W., Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* **2007**, 129 (1), 123-134.
82. (a) Dennis, J. W.; Granovsky, M.; Warren, C. E., Glycoprotein glycosylation and cancer progression. *Biochimica Et Biophysica Acta-General Subjects* **1999**, 1473 (1), 21-34; (b) Goss,

P. E.; Baker, M. A.; Carver, J. P.; Dennis, J. W., Inhibitors of carbohydrate processing: A new class of anticancer agents. *Clinical Cancer Research* **1995**, 1 (9), 935-944; (c) Yousefi, S.; Higgins, E.; Zhuang, D. L.; Pollexkruger, A.; Hindsgaul, O.; Dennis, J. W., Increased Udp-Glcnaac-Gal-Beta-1-3galnac-R (Glcnaac to Galnac)Beta-1,6-N-Acetylglucosaminyltransferase Activity in Metastatic Murine Tumor-Cell Lines - Control of Polylactosamine Synthesis. *Journal of Biological Chemistry* **1991**, 266 (3), 1772-1782.

83. Goss, P. E.; Baker, M. A.; Carver, J. P.; Dennis, J. W., Inhibitors of carbohydrate processing: A new class of anticancer agents. *Clinical Cancer Research* **1995**, 1, 935-944.

84. Moremen, K. W., Golgi alpha-mannosidase II deficiency in vertebrate systems: implications for asparagine-linked oligosaccharide processing in mammals. *Biochimica Et Biophysica Acta-General Subjects* **2002**, 1573 (3), 225-235.

85. (a) Henrissat, B., A Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. *Biochemical Journal* **1991**, 280, 309-316; (b) Park, C.; Meng, L.; Stanton, L. H.; Collins, R. E.; Mast, S. W.; Yi, X. B.; Strachan, H.; Moremen, K. W., Characterization of a human core-specific lysosomal alpha 1,6-mannosidase involved in N-glycan catabolism. *Journal of Biological Chemistry* **2005**, 280 (44), 37204-37216.

86. Karaveg, K.; Siriwardena, A.; Tempel, W.; Liu, Z. J.; Glushka, J.; Wang, B. C.; Moremen, K. W., Mechanism of class 1 (Glycosylhydrolase family 47) alpha-mannosidases involved in N-glycan processing and endoplasmic reticulum quality control. *Journal of Biological Chemistry* **2005**, 280 (16), 16197-16207.

87. Kavar, Z.; Karaveg, K.; Moremen, K. W.; Jarvis, D. L., Insect cells encode a class II alpha-mannosidase with unique properties. *Journal of Biological Chemistry* **2001**, 276 (19), 16335-16340.

88. (a) Fiaux, H.; Kuntz, D. A.; Hoffman, D.; Janzer, R. C.; Gerber-Lemaire, S.; Rose, D. R.; Juillerat-Jeanneret, L., Functionalized pyrrolidine inhibitors of human type II alpha-mannosidases as anti-cancer agents: Optimizing the fit to the active site. *Bioorganic & Medicinal Chemistry* **2008**, *16* (15), 7337-7346; (b) Wielgat, P.; Walczuk, U.; Szajda, S.; Bien, M.; Zimnoch, L.; Mariak, Z.; Zwierz, K., Activity of lysosomal exoglycosidases in human gliomas. *J Neurooncol* **2006**, *80* (3), 243-9.
89. (a) van den Elsen, J. M.; Kuntz, D. A.; Rose, D. R., Structure of Golgi alpha-mannosidase II: a target for inhibition of growth and metastasis of cancer cells. *EMBO J* **2001**, *20* (12), 3008-17; (b) Rabouille, C.; Kuntz, D. A.; Lockyer, A.; Watson, R.; Signorelli, T.; Rose, D. R.; van den Heuvel, M.; Roberts, D. B., The Drosophila GMII gene encodes a Golgi alpha-mannosidase II. *Journal of Cell Science* **1999**, *112* (19), 3319-3330.
90. Robinson, A. J.; Crawley, A. C.; Auclair, D.; Weston, P. F.; Hirte, C.; Hemsley, K. M.; Hopwood, J. J., Behavioural characterisation of the alpha-mannosidosis guinea pig. *Behavioural Brain Research* **2008**, *186* (2), 176-184.
91. Will, A.; Cooper, A.; Hatton, C.; Sardharwalla, I. B.; Evans, D. I. K.; Stevens, R. F., Bone-Marrow Transplantation in the Treatment of Alpha-Mannosidosis. *Archives of Disease in Childhood* **1987**, *62* (10), 1044-1049.
92. (a) Janeway, C. A.; Medzhitov, R., Innate immune recognition. *Annu. Rev. Immunol.* **2002**, *20*, 197-216; (b) Medzhitov, R.; Janeway, C. A., Decoding the patterns of self and nonself by the innate immune system. *Science* **2002**, *296* (5566), 298-300.
93. (a) Pittet, D.; Tarara, D.; Wenzel, R. P., Nosocomial blood-stream infection in critically ill patients - Excess length of stay, extra costs, and attributable mortality. *JAMA, J. Am. Med.*

Assoc. **1994**, 271 (20), 1598-1601; (b) Rice, T. W.; Bernard, G. R., Therapeutic intervention and targets for sepsis. *Annu. Rev. Med.* **2005**, 56, 225-248.

94. (a) Darveau, R. P., Lipid A diversity and the innate host response to bacterial infection. *Curr. Opin. Microbiol.* **1998**, 1 (1), 36-42; (b) Ghuysen, J. M., Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. *Bacteriol. Rev.* **1968**, 32 (4 Pt 2), 425-464; (c) Schleifer, K. H.; Kandler, O., Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **1972**, 36 (4), 407-477.

95. Tipper, D. J.; Katz, W.; Strominger, J. L.; Ghuysen, J. M., Substituents on the alpha-carboxyl group of D-glutamic acid in the peptidoglycan of several bacterial cell walls. *Biochemistry* **1967**, 6 (3), 921-929.

96. (a) Strominger, J. L.; Ghuysen, J. M., Mechanisms of enzymatic bacteriolysis. Cell walls of bacteria are solubilized by action of either specific carbohydrases or specific peptidases. *Science* **1967**, 156 (772), 213-221; (b) Smith, T. J.; Blackman, S. A.; Foster, S. J., Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology* **2000**, 146, 249-262; (c) Scheffers, D. J.; Pinho, M. G., Bacterial cell wall synthesis: new insights from localization studies. *Microbiol. Mol. Biol. Rev.* **2005**, 69 (4), 585-607.

97. (a) Yoshimura, A.; Lien, E.; Ingalls, R. R.; Tuomanen, E.; Dziarski, R.; Golenbock, D., Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. Lett.* **1999**, 163 (1), 1-5; (b) Travassos, L. H.; Girardin, S. E.; Philpott, D. J.; Blanot, D.; Nahori, M. A.; Werts, C.; Boneca, I. G., Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. *EMBO Rep.* **2004**, 5 (10), 1000-1006; (c) Dziarski, R.; Gupta, D., *Staphylococcus aureus* peptidoglycan is a Toll-like receptor 2 activator: a reevaluation. *Infect. Immun.* **2005**, 73 (8), 5212-5216.

98. Iwaki, D.; Mitsuzawa, H.; Murakami, S.; Sano, H.; Konishi, M.; Akino, T.; Kuroki, Y., The extracellular Toll-like receptor 2 domain directly binds peptidoglycan derived from *Staphylococcus aureus*. *J. Biol. Chem.* **2002**, 277 (27), 24315-24320.
99. (a) Chowdhury, A. R.; Siriwardena, A.; Boons, G. J., A highly convergent approach for the synthesis of disaccharide repeating units of peptidoglycan. *Tetrahedron Lett.* **2002**, 43 (43), 7805-7807; (b) Chowdhury, A. R.; Boons, G. J., The synthesis of diaminopimelic acid containing peptidoglycan fragments using metathesis cross coupling. *Tetrahedron Lett.* **2005**, 46 (10), 1675-1678; (c) Roychowdhury, A.; Wolfert, M. A.; Boons, G. J., Synthesis and proinflammatory properties of muramyl tripeptides containing lysine and diaminopimelic acid moieties. *Chembiochem* **2005**, 6 (11), 2088-97; (d) Kumar, S.; Roychowdhury, A.; Ember, B.; Wang, Q.; Guan, R.; Mariuzza, R. A.; Boons, G. J., Selective recognition of synthetic lysine and meso-diaminopimelic acid-type peptidoglycan fragments by human peptidoglycan recognition proteins Ialpha and S. *J. Biol. Chem.* **2005**, 280 (44), 37005-37012; (e) Guan, R.; Roychowdhury, A.; Ember, B.; Kumar, S.; Boons, G. J.; Mariuzza, R. A., Structural basis for peptidoglycan binding by peptidoglycan recognition proteins. *Proceedings of the National Academy of Sciences of the United States of America* **2004**, 101 (49), 17168-17173; (f) Swaminathan, C. P.; Brown, P. H.; Roychowdhury, A.; Wang, Q.; Guan, R.; Silverman, N.; Goldman, W. E.; Boons, G. J.; Mariuzza, R. A., Dual strategies for peptidoglycan discrimination by peptidoglycan recognition proteins (PGRPs). *Proceedings of the National Academy of Sciences of the United States of America* **2006**, 103 (3), 684-689.
100. Hancock, M. A.; Spencer, C. A.; Koschinsky, M. L., Definition of the structural elements in plasminogen required for high-affinity binding to apolipoprotein(a): A study utilizing surface plasmon resonance. *Biochemistry* **2004**, 43 (38), 12237-12248.

101. (a) Karlsson, R.; Falt, A., Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *J. Immunol. Methods* **1997**, *200* (1-2), 121-133; (b) Lipschultz, C. A.; Li, Y.; Smith-Gill, S., Experimental design for analysis of complex kinetics using surface plasmon resonance. *Methods* **2000**, *20* (3), 310-318.
102. (a) Selvi, P. T.; Ashish, B.; Murthy, G. S., Determination of thermodynamic parameters of antigen-antibody interaction from real-time kinetic studies. *Current Science* **2002**, *82* (12), 1442-1448; (b) Boniface, J. J.; Reich, Z.; Lyons, D. S.; Davis, M. M., Thermodynamics of T cell receptor binding to peptide-MHC: Evidence for a general mechanism of molecular scanning. *Proceedings of the National Academy of Sciences of the United States of America* **1999**, *96* (20), 11446-11451.
103. Hayashi, K., Rapid-Determination of Sodium Dodecyl-Sulfate with Methylene-Blue. *Analytical Biochemistry* **1975**, *67* (2), 503-506.
104. Jandik, P.; Cheng, J.; Avdalovic, N., Analysis of amino acid-carbohydrate mixtures by anion exchange chromatography and integrated pulsed amperometric detection. *Journal of Biochemical and Biophysical Methods* **2004**, *60* (3), 191-203.
105. Jonsson, U.; Fagerstam, L.; Ivarsson, B.; Johnsson, B.; Karlsson, R.; Lundh, K.; Lofas, S.; Persson, B.; Roos, H.; Ronnberg, I.; et al., Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques* **1991**, *11* (5), 620-627.
106. Thomas, C. J.; Surolia, N.; Surolia, A., Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B. *J. Biol. Chem.* **1999**, *274* (42), 29624-29627.

107. Wolfert, M. A.; Roychowdhury, A.; Boons, G. J., Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. *Infect. Immun.* **2007**, *75* (2), 706-713.
108. Guan, R.; Mariuzza, R. A., Peptidoglycan recognition proteins of the innate immune system. *Trends Microbiol* **2007**, *15* (3), 127-134.
109. (a) Janusz, M. J.; Esser, R. E.; Schwab, J. H., *In vivo* degradation of bacterial cell wall by the muralytic enzyme mutanolysin. *Infect. Immun.* **1986**, *52* (2), 459-467; (b) Hoijs, M. A.; Melief, M. J.; Debets, R.; Hazenberg, M. P., Inflammatory properties of peptidoglycan are decreased after degradation by human *N*-acetylmuramyl-L-alanine amidase. *Eur. Cytokine Netw.* **1997**, *8* (4), 375-381.
110. Myhre, A. E.; Stuestol, J. F.; Dahle, M. K.; Overland, G.; Thiemermann, C.; Foster, S. J.; Lilleaasen, P.; Aasen, A. O.; Wang, J. E., Organ injury and cytokine release caused by peptidoglycan are dependent on the structural integrity of the glycan chain. *Infect. Immun.* **2004**, *72* (3), 1311-1317.
111. Ganz, T.; Gabayan, V.; Liao, H. I.; Liu, L.; Oren, A.; Graf, T.; Cole, A. M., Increased inflammation in lysozyme M-deficient mice in response to *Micrococcus luteus* and its peptidoglycan. *Blood* **2003**, *101* (6), 2388-2392.
112. Inamura, S.; Fujimoto, Y.; Kawasaki, A.; Shiokawa, Z.; Woelk, E.; Heine, H.; Lindner, B.; Inohara, N.; Kusumoto, S.; Fukase, K., Synthesis of peptidoglycan fragments and evaluation of their biological activity. *Org. Biomol. Chem.* **2006**, *4* (2), 232-242.
113. Zhang, Y.; Gaekwad, J.; Wolfert, M. A.; Boons, G. J., Modulation of innate immune responses with synthetic lipid A derivatives. *J. Am. Chem. Soc.* **2007**, *129*, 5200-5216.

114. Ozinsky, A.; Underhill, D. M.; Fontenot, J. D.; Hajjar, A. M.; Smith, K. D.; Wilson, C. B.; Schroeder, L.; Aderem, A., The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America* **2000**, 97 (25), 13766-13771.
115. Hawkins, L. D.; Christ, W. J.; Rossignol, D. P., Inhibition of endotoxin response by synthetic TLR4 antagonists. *Current topics in medicinal chemistry* **2004**, 4 (11), 1147-1171.
116. (a) Varki, A., Biological Roles of Oligosaccharides - All of the Theories Are Correct. *Glycobiology* **1993**, 3 (2), 97-130; (b) Dwek, R. A., Glycobiology: Toward understanding the function of sugars. *Chem. Rev.* **1996**, 96, 683-720; (c) Kolter, T.; Sandhoff, K., Sphingolipids--their metabolic pathways and the pathobiochemistry of neurodegenerative diseases. *Angew. Chem., Int. Ed.* **1999**, 38, 1532-1568; (d) Dennis, J. W.; Granovsky, M.; Warren, C. E., Protein glycosylation in development and disease. *Bioessays* **1999**, 21 (5), 412-421.
117. (a) Fuster, M. M.; Esko, J. D., The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nature Reviews Cancer* **2005**, 5 (7), 526-542; (b) Dube, D. H.; Bertozzi, C. R., Glycans in cancer and inflammation. Potential for therapeutics and diagnostics. *Nature Reviews Drug Discovery* **2005**, 4 (6), 477-488; (c) Dennis, J. W.; Granovsky, M.; Warren, C. E., Glycoprotein glycosylation and cancer progression. *Biochim. Biophys. Acta* **1999**, 1473 (1), 21-34.
118. (a) Lillelund, V. H.; Jensen, H. H.; Liang, X. F.; Bols, M., Recent developments of transition-state analogue glycosidase inhibitors of non-natural product origin. *Chemical Reviews* **2002**, 102 (2), 515-553; (b) Berecibar, A.; Grandjean, C.; Siriwardena, A., Synthesis and biological activity of natural aminocyclopentitol glycosidase inhibitors: Mannostatins, trehazolin, allosamidins, and their analogues. *Chemical Reviews* **1999**, 99 (3), 779-844; (c) Asano, N.; Nash,

R. J.; Molyneux, R. J.; Fleet, G. W. J., Sugar-mimic glycosidase inhibitors: natural occurrence, biological activity and prospects for therapeutic application. *Tetrahedron-Asymmetry* **2000**, *11* (8), 1645-1680; (d) Heightman, T. D.; Vasella, A. T., Recent insights into inhibition, structure, and mechanism of configuration-retaining glycosidases. *Angew. Chem., Int. Ed.* **1999**, *38* (6), 750-770; (e) vonItzstein, M.; Thomson, R. J., Sialic acids and sialic acid-recognising proteins: Drug discovery targets and potential glycopharmaceuticals. *Current Medicinal Chemistry* **1997**, *4* (3), 185-210; (f) Asano, N., Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology* **2003**, *13* (10), 93R-104R.

119. Perfetti, R.; Barnett, P. S.; Mathur, R.; Egan, J. M., Novel therapeutic strategies for the treatment of type 2 diabetes. *Diabetes-Metabolism Reviews* **1998**, *14* (3), 207-225.

120. (a) Von Itzstein, M.; Wu, W. Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Phan, T. V.; Smythe, M. L.; White, H. F.; Oliver, S. W.; Colman, P. M.; Varghese, J. N.; Ryan, D. M.; Woods, J. M.; Bethell, R. C.; Hotham, V. J.; Cameron, J. M.; Penn, C. R., Rational design of potent sialidase-based inhibitors of influenza-virus replication. *Nature* **1993**, *363* (6428), 418-423; (b) vonItzstein, M.; Dyason, J. C.; Oliver, S. W.; White, H. F.; Wu, W. Y.; Kok, G. B.; Pegg, M. S., A study of the active site of influenza virus sialidase: An approach to the rational design of novel anti-influenza drugs. *Journal of Medicinal Chemistry* **1996**, *39* (2), 388-391; (c) Kim, C. U.; Lew, W.; Williams, M. A.; Liu, H. T.; Zhang, L. J.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Mendel, D. B.; Tai, C. Y.; Laver, W. G.; Stevens, R. C., Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: Design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *Journal of the American Chemical Society* **1997**, *119* (4), 681-690; (d) Li, W. X.; Escarpe, P. A.; Eisenberg, E. J.; Cundy, K. C.; Sweet, C.; Jakeman, K. J.; Merson,

J.; Lew, W.; Williams, M.; Zhang, L. J.; Kim, C. U.; Bischofberger, N.; Chen, M. S.; Mendel, D. B., Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. *Antimicrobial Agents and Chemotherapy* **1998**, 42 (3), 647-653; (e) Chand, P.; Kotian, P. L.; Dehghani, A.; El-Kattan, Y.; Lin, T. H.; Hutchison, T. L.; Babu, Y. S.; Bantia, S.; Elliott, A. J.; Montgomery, J. A., Systematic structure-based design and stereoselective synthesis of novel multisubstituted cyclopentane derivatives with potent antiinfluenza activity. *Journal of Medicinal Chemistry* **2001**, 44 (25), 4379-4392.

121. (a) Godin, G.; Compain, P.; Martin, O. R.; Ikeda, K.; Yu, L.; Asano, N., alpha-1-C-alkyl-1-deoxynojirimycin derivatives as potent and selective inhibitors of intestinal isomaltase: remarkable effect of the alkyl chain length on glycosidase inhibitory profile. *Bioorganic & Medicinal Chemistry Letters* **2004**, 14 (24), 5991-5995; (b) Platt, F. M.; Neises, G. R.; Dwek, R. A.; Butters, T. D., N-Butyldeoxynojirimycin Is a Novel Inhibitor of Glycolipid Biosynthesis. *Journal of Biological Chemistry* **1994**, 269 (11), 8362-8365; (c) Platt, F. M.; Neises, G. R.; Karlsson, G. B.; Dwek, R. A.; Butters, T. D., N-Butyldeoxygalactonojirimycin Inhibits Glycolipid Biosynthesis but Does Not Affect N-Linked Oligosaccharide Processing. *Journal of Biological Chemistry* **1994**, 269 (43), 27108-27114; (d) Overkleeft, H. S.; Renkema, G. H.; Neele, J.; Vianello, P.; Hung, I. O.; Strijland, A.; van der Burg, A. M.; Koomen, G. J.; Pandit, U. K.; Aerts, J., Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase. *Journal of Biological Chemistry* **1998**, 273 (41), 26522-26527; (e) Wu, C. Y.; Chang, C. F.; Chen, J. S. Y.; Wong, C. H.; Lin, C. H., Rapid diversity-oriented synthesis in microtiter plates for in situ screening: Discovery of potent and selective alpha-fucosidase inhibitors. *Angewandte Chemie-International Edition* **2003**, 42 (38), 4661-4664; (f) Johnson, L. L.; Houston, T. A., A drug targeting motif for glycosidase inhibitors: an iminosugar-boronate

shows unexpectedly selective beta-galactosidase inhibition. *Tetrahedron Letters* **2002**, 43 (49), 8905-8908; (g) Robina, I.; Moreno-Vargas, A. J.; Fernandez-Bolanos, J. G.; Fuentes, J.; Demange, R.; Vogel, P., New leads for selective inhibitors of alpha-L-fucosidases. Synthesis and glycosidase inhibitory activities of (2R,3S,4R)-3,4-dihydroxypyrrolidin-2-yl furan derivatives. *Bioorganic & Medicinal Chemistry Letters* **2001**, 11 (18), 2555-2559; (h) Lysek, R.; Schutz, C.; Vogel, P., (1S,2S,3R,6R)-6-aminocyclohex-4-ene-1,2,3-triol (=(-)-conduramine B-1) is a selective inhibitor of alpha-mannosidases. Its inhibitory activity is enhanced by N-benylation. *Helvetica Chimica Acta* **2005**, 88 (10), 2788-2811; (i) Butters, T. D.; Dwek, R. A.; Platt, F. M., Therapeutic applications of imino sugars in lysosomal storage disorders. *Current Topics in Medicinal Chemistry* **2003**, 3 (5), 561-574.

122. Pardee, A. B.; Reddy, G. P. V., Beginnings of feedback inhibition, allostery and multi-protein complexes. *Gene* **2003**, 321, 17-23.

123. King, S. B.; Ganem, B., Synthetic Studies on Mannostatin-a and Its Derivatives - a New Family of Glycoprotein Processing Inhibitors. *J. Am. Chem. Soc.* **1994**, 116 (2), 562-570.

124. de Groot, F. M. H.; Loos, W. J.; Koekkoek, R.; van Berkom, L. W. A.; Busscher, G. F.; Seelen, A. E.; Albrecht, C.; de Bruijn, P.; Scheeren, H. W., Elongated multiple electronic cascade and cyclization spacer systems in activatable anticancer prodrugs for enhanced drug release. *Journal of Organic Chemistry* **2001**, 66 (26), 8815-8830.

125. Ogawa, S.; Morikawa, T., Synthesis of a potent aminocyclitol alpha-mannosidase inhibitor, 1L-(1,2,3,5/4)-5-amino-4-O-methyl-1,2,3,4- cyclopentanetetrol. *Bioorg. Med. Chem. Lett.* **2000**, 10 (10), 1047-1050.

126. Ogawa, S.; Yu, Y. M., Synthesis of an Alpha-Mannosidase Inhibitor, (+/-)-Mannostatin-a and Its Isomer. *Journal of the Chemical Society-Chemical Communications* **1991**, (13), 890-891.

127. Suami, T.; Sakota, Y.; Tadano, K.; Nishiyama, S., Aminocyclitols .26. Synthesis of Aminocyclopentanetetrols. *Bulletin of the Chemical Society of Japan* **1971**, *44* (8), 2222-&.
128. Wilson, J. C.; Kiefel, M. J.; Angus, D. I.; von Itzstein, M., Investigation of the stability of thiosialosides toward hydrolysis by sialidases using NMR spectroscopy. *Organic Letters* **1999**, *1* (3), 443-446.
129. Li, B.; Kawatkar, S. P.; George, S.; Strachan, H.; Woods, R. J.; Siriwardena, A.; Moremen, K. W.; Boons, G. J., Inhibition of Golgi mannosidase II with mannostatin analogues: Synthesis, biological evaluation, and structure-activity relationship studies. *Chembiochem* **2004**, *5* (9), 1220-1227.
130. Elbein, A. D., Glycosidase Inhibitors - Inhibitors of N-Linked Oligosaccharide Processing. *Faseb Journal* **1991**, *5* (15), 3055-3063.
131. Sarkar, A. K.; Fritz, T. A.; Taylor, W. H.; Esko, J. D., Disaccharide Uptake and Priming in Animal-Cells - Inhibition of Sialyl-Lewis-X by Acetylated Gal-Beta-1-]4glcnac-Beta-O-Naphthalenemethanol. *Proc. Natl. Acad. Sci. USA* **1995**, *92* (8), 3323-3327.
132. Dennis, J. W.; Koch, K.; Yousefi, S.; Vanderelst, I., Growth-Inhibition of Human-Melanoma Tumor Xenografts in Athymic Nude-Mice by Swainsonine. *Cancer Res.* **1990**, *50* (6), 1867-1872.
133. (a) Ogawa, S.; Washida, K., Synthesis and glycosidase inhibitory activity of five stereoisomers of 5-amino-5-C-methyl-1,2,3,4-cyclopentanetetrol. *European Journal of Organic Chemistry* **1998**, (9), 1929-1934; (b) Kawatkar, S. P.; Kuntz, D. A.; Woods, R. J.; Rose, D. R.; Boons, G. J., Structural basis of the inhibition of Golgi alpha-mannosidase II by mannostatin A and the role of the thiomethyl moiety in ligand-protein interactions. *Journal of the American Chemical Society* **2006**, *128* (25), 8310-8319.

134. Iwaoka, M.; Takemoto, S.; Okada, M.; Tomoda, S., Weak nonbonded S center dot center dot center dot X (X=O, N, and S) interactions in proteins. Statistical and theoretical studies. *Bulletin of the Chemical Society of Japan* **2002**, 75 (7), 1611-1625.

135. Jin, M. S.; Kim, S. E.; Heo, J. Y.; Lee, M. E.; Kim, H. M.; Paik, S. G.; Lee, H. Y.; Lee, J. O., Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* **2007**, 130 (6), 1071-1082.

[136] Treatment of compound **1** with cell lysate in the presence of the mannosidase inhibitors swainsonine and kifunensine did not lead to significant decomposition indicating that proteases or other enzymes do not decompose the pro-drug.