Combating gram-negative resistance: targeting the cell envelope

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Chapter 4:

Evaluation of Globomycin-containing extracts in a biochemical LspA activity assay

Abstract

Exploring novel targets for antibiotic development is crucial in the fight against bacterial resistance. The lipoprotein signal peptidase LspA is essential in gram-negative bacteria and represents a promising target. LspA is inhibited by the natural products globomycin and myxovirescin, both of which exhibit antibacterial activity. In this chapter we evaluated a FRET-based LspA-activity assay for its capacity to detect inhibition from natural extracts derived from globomycin producing bacteria. Our results demonstrate that the assay is sufficiently sensitive to detect inhibition at low concentrations in both crude and fractionated extracts, supporting its potential application in high-throughput screening (HTS) campaigns aimed at discovering novel LspA-inhibiting natural products. Additionally, we show that the lipopolysaccharide (LPS) length influences the susceptibility of E. coli to globomycin and globomycin containing extracts, providing insight into the selections of strains suitable for phenotypic counter-screens during hit validation. These findings lay the groundwork for future HTS efforts to screen large libraries of natural products in the search for new antibiotics targeting LspA.

Introduction

To combat the rise of antibiotic resistance, exploring novel bacterial targets for antibiotics becomes increasingly important. This challenge is particularly pressing in gram-negative bacteria where the outer membrane and efflux mechanisms confer intrinsic resistance to many existing antibiotics.

One promising target for novel antibiotics is lipoprotein signal peptidase II (LspA), an essential enzyme in gram-negative bacteria[1]. There are two natural products, globomycin and myxovirescin, which are known LspA inhibitors with antimicrobial activity, but neither have a satisfactory efficacy *in-vivo*.

While LspA is not essential for gram-positive bacteria, it plays a major role in their virulence[2,3]. Inhibiting LspA has recently been shown to potentiate the activity of the lipopeptide antibiotic daptomycin and Human group IIA-secreted phospholipase A2 (hGIIA)[4]. On the other hand, it has been shown to increase the resistance of *S. aureus* to β-lactamases[5]. Importantly, LspA lacks homologues in eukaryotes, making it an attractive target for selective antibiotic development[6].

Lipoprotein processing

As illustrated in Figure 1, bacterial lipoproteins are processed by a conserved enzymatic pathway involving three enzymes: phosphatidylglycerol:prolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase (Lsp) and apolipoprotein N-acetlyltransferase (Lnt). Prolipoproteins possess a N-terminal signal sequence consisting of positively charged amino acids and a hydrophobic region of about 20 amino acids ending in the lipobox consensus sequence LA/SG/AC (reviewed[7]). Following the insertion of the prolipoprotein into the membrane by the Sec or Tat machinery, Lgt transfers a diacylglycerol (dag) onto the cysteine within the lipobox. The lipobox with the dagylated cysteine is recognized by LspA, an aspartyl peptidase, which cleaves the signal peptide at the position prior to the acylation of the cysteine. The final maturation step is catalyzed by Lnt which further acetylates the free N-terminus of the protein[8]. The mature lipoproteins are then transported to the outer membrane using the Lol (lipoprotein outer-membrane localization)

pathway. This system consists of the ABC-transporter LolBCDE, the chaperone LolA transporting the lipoproteins through the periplasm to the outer membrane, where LolB acts as the receptor, helping the lipoprotein insert into the membrane[7].

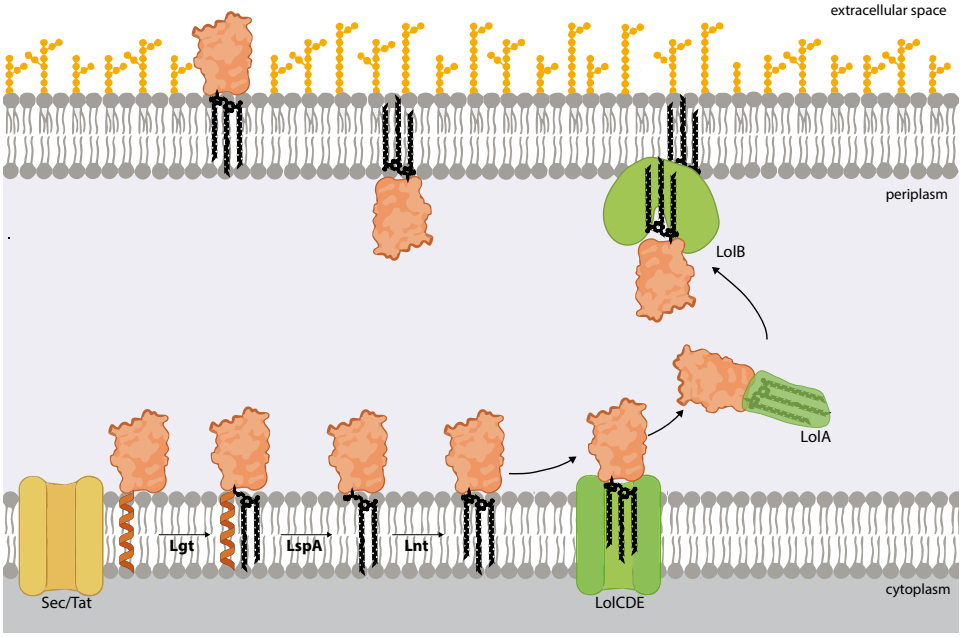

Figure 1: Schematic representation of the processing of lipoproteins (orange) in gram-negative bacteria: precursor lipoproteins are translocated across inner membrane via the SEC or TAT pathways (yellow). Upon insertion into the inner membrane Lgt catalyzes the transfer of a diacylglycerol from phosphatidylglycerol (PG) group to the conserved cysteine within. Subsequently, LspA cleaves the signal peptide and Lnt lipidates the free N-terminus of the apolipoprotein using phosphatidylethanolamine (PE). The mature lipoprotein is then transported to the outer membrane via the lipoprotein outer membrane localization (Lol) pathway (green), which involves the ABC-transporter LolCDE, the periplasmic chaperone LolA, and the outer membrane receptor LolB. In the outer membrane the lipoprotein is either inserted into the bilayer and or released into the extracellular space. Adapted from [7,9]

LspA inhibitors

To date, two natural product inhibitors of LspA have been identified: globomycin[10] and myxovirescin[11]. Both are macrocyclic molecules that inhibit LspA through the action of a specific hydroxy group (from serine in globomycin and at position 6 in myxovirescin) interacting with the catalytic aspartate residues[12].

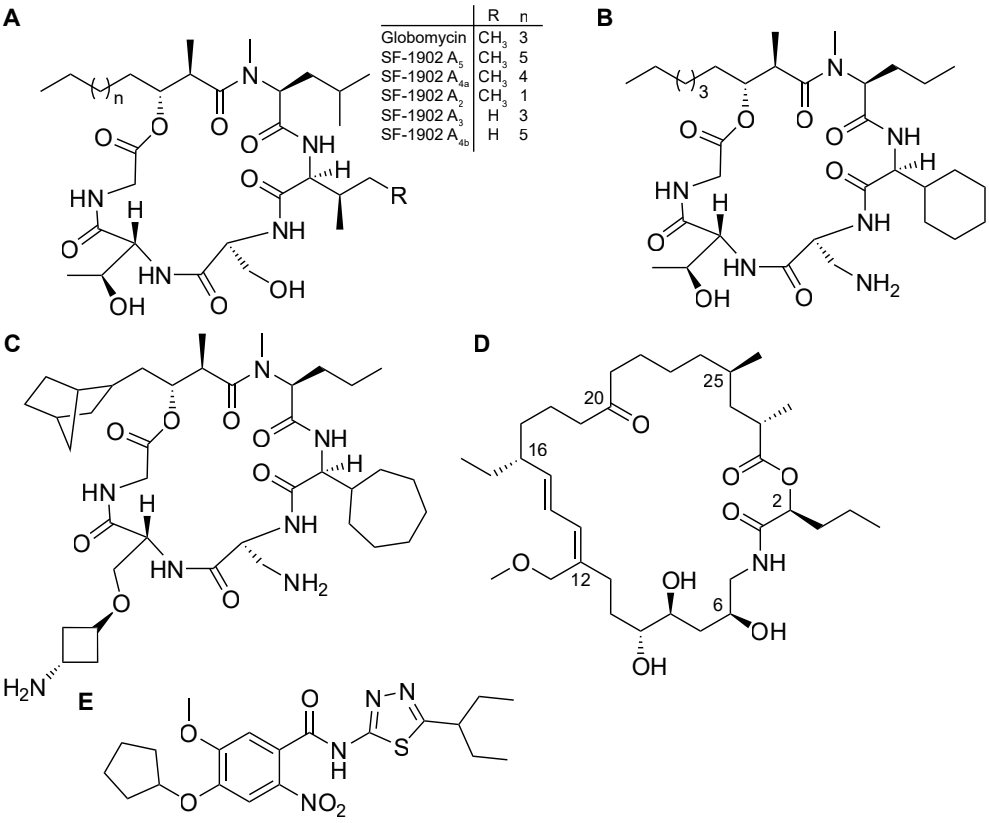

Figure 2: Structures of (A) globomycin and its congeners [13], (B) globomycin analogue G0790 [14], (C) globomycin analogue G5132 [15] (D) Myxovirescin A [11], and (E) small molecule LspA-Inhibitor Benzamidine 1j [16]

Globomycin

Globomycin (Figure 2A) is a macrocyclic peptide antibiotic produced by various *Streptomyces* species. It is composed of N-Me-L-Leu, L-*allo*-Ile, L-Ser, L-*allo*-Thr, Gly and a β-hydroxy-α-methyl carboxylic acid. Several naturally-occuring congeners have also been identified, differing in the length of the lipid tail and at the L-*allo*-Ile position, where valine can also be present (Figure 2A)[13]. Recent elucidation of the biosynthetic gene cluster (BGC) of globomycin revealed that module 5 appears capable of incorporating either valine or *allo*-isoleucine. Moreover, two genes upstream the BGC encode for homologues of known L-*allo*-isoleucine synthetases, suggesting a source

for this residue. The BGC also contains a nonribosomal peptide synthetase (NRPS) and an oxireductase, which are hypothesized to convert L-threonine to L-*allo*-threonine[17].

Globomycin binds to the active site of LspA primarily through the hydroxyl group on its serine residue, which anchors the inhibitor to one of the catalytic aspartate residues. Its polar backbone forms hydrogen bonds with LspA, while the hydrophobic residues interact with the inner membrane[9]. Globomycin shows moderate activity against gram-negative bacteria and has also been active against mycobacteria through an LspA-independent mechanism[18].

Recently reported SAR studies resulted in globomycin analogues with increased *in vivo* stability and *in vitro* activity against Enterobacteria as well as *P. aeruginosa* and *A. baumanii*. (Figure 2A. 2B) However, high plasma protein binding still limits their *in-vivo* efficacy[19,20]. Notable findings from these studies include:

- Increasing the lipid tail length significantly enhances activity, though higher lipophilicity may increase off-target effects such as general membrane disruption and unintended anti-gram-positive activity. Decreasing rigidity of the lipid tail through bicyclic aliphatic rings decreases the off-target effects, while keeping specific LspA activity.
- N-methyl leucine can be substituted by non-polar side chains with a length of three or more carbons and the branching is not needed.
- For L-*allo*-isoleucine, branching substituents are preferred, especially cyclohexyl and -cycloheptyl moieties.
- Replacing the hydroxyl group of the serine with an amino group increases the salt-bridge interactions to the aspartate and consequently the activity.
- Forming ethers between the threonine and amine substituted aliphatic chains or cycles increases the activity, especially in wild-type bacteria with an intact outer membrane.
- The ring size can be decreased to 18, especially if an (R)-methyl group is added.
- Amide bonds have good plasma stability and retain activity, especially if the ring size is decreased to 18. The lipid tail can be on the nitrogen or carbon or distributed between them.

Another factor working against globomycin's use as an antibiotic is its susceptibility to tolC-mediated efflux[19]. In this regard, upregulation of efflux pumps and/or increasing LspA expression are associated with globomycin resistance[21]. Further, the downregulation of Brown's lipoprotein (Lpp), a protein critical in maintaining membrane integrity and permeability, confer resistance to globomycin analogue G0790 (Figure 2B)[14]. In *A. baumanii* the deletion of a previously uncharacterized protein LirL was also found to confer resistance to the globomycin analogue G5132 (Figure 2C)[15].

Myxovirescin

Myxovirescin is a polyketide macrocycle featuring a 28-atom ring (Figure 2D). It is synthesized by a PKS/NRPS megacomplex consisting of nine modules and several modifying enzymes[22]. Although mainly active against *E. coli*, myxovirescin also exhibits activity against *S. aureus* and *P. aeruginosa* at high concentrations[11].

While the total synthesis of myxoveriscin has been published[23], relatively few analogues have been described. Current SAR studies suggest the following modifications are tolerated without significant loss of activity [24,25]:

- Variations in alkyl chain length at position 2
- Substitution of moieties at position 12 and 16 with methyl groups
- Reduction of keto group at position 20
- Reduction of the $\Delta_{26,27}$ double bond

Aside from globomycin and myxoveriscin, Kitamura et al. identified a small molecule LspA inhibitor through high throughput screening (HTS). Optimization of the hit yielded benzamidine 1j, which exhibits an MIC of 25 μM (11 μg mL^{-1}) (Figure 2E)[16]. Additionally, LspA has also been reported to be inhibited by the general aspartyl protease inhibitor pepstatin[6].

LspA activity assays

LspA activity and inhibition can be observed using different methods. A low-throughput approach involves measuring the cleavage of the signal peptide from a prolipoprotein via SDS-PAGE[9]. In contrast, high throughput methods have also been reported employing FRET-based substrates composed of a diacylglycerylated (dag) lipobox sequence conjugated to both a fluoro-

phore and a quencher molecule. In these substrates, the quencher absorbs the emitted light of the fluorophore and upon enzymatic cleavage the fluorescence signal increases, providing a real-time, quantitative readout. To date, two FRET-based LspA substrates have been reported (Figure 3): Olatunji et al. designed a substrate based on the sequence LAGC*SS, where * denotes dagylation, using aminobenzoic acid and nitro-tyrosine as the FRET-pair[12]. Kitamura et al. developed a substrate using the peptide sequence VTGC*AK conjugated to the EDANS-Dabsyl FRET-pair[26].

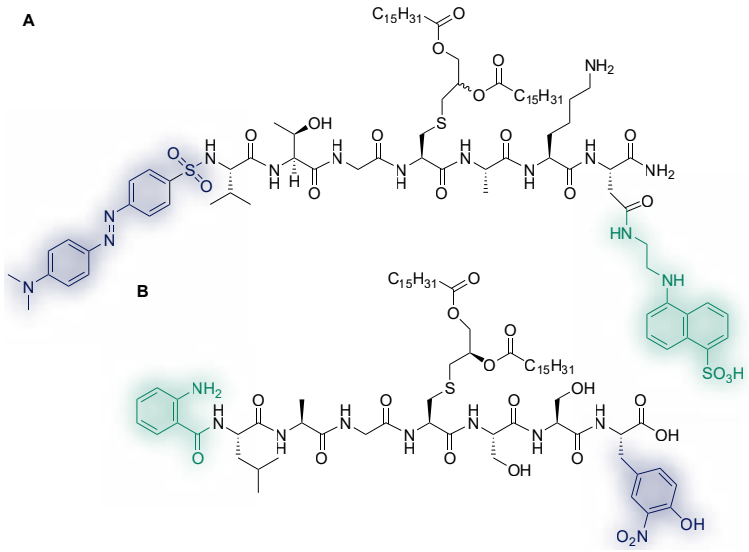

Figure 3: Structures of published LspA FRET-substrates: (A) the substrate published by Kitamura et al. [26] and (B) the substrate published by Olatunji et al.[12]. In each structure, the fluorophores are highlighted in green, and the quencher moieties in dark blue.

High throughput screening (HTS) is a powerful technique for identifying novel inhibitors from large libraries of molecules or natural extracts. While HTS using a FRET-based *in vitro* LspA inhibition assay has successfully been applied to a small molecule library, its application to natural product extracts remains unexplored. This chapter presents a proof-of-concept study evaluating the feasibility of using purified LspA and a FRET-based biochemical assay to detect LspA inhibitors in the context of complex natural extracts. To do so we specifically employed extracts from a *Streptomyces* strain known to produce globomycin.

Results

Initial Screen

Extracts from the known globomycin-producer *Streptomyces sp*. CA-278952, grown on a variety of media, were prepared by our collaborators at Fundación Medina (Granada, Spain) who also confirmed the globomycin content of the extracts using LC-MS techniques (Table S1).

Minimum Inhibitory Concentration (MIC) of globomycin containing extracts

To begin, we assessed the antibacterial activity of the globomycin-containing extracts provided to us by Fundación Medina. Table 1 reports the MIC values measured, expressed as the percentage of extract. Each extract was tested against a panel of bacterial strains: two *E. coli* WT strains (ATCC25922 and BW25113), one gram-positive strain *S. aureus* USA300, an efflux-pump deficient *E. coli* (*E. coli* BW25113 ΔtolC) and several *E. coli* strains with deletions in the LPS-synthesis pathway (ΔrfaC, ΔrfaP, ΔrfaY, ΔrfaG, ΔrfaI), corresponding to an increasing LPS length.

Table 1: Minimum Inhibitory concentrations (MICs) expressed as percentage of extract of selected natural extracts against a panel of *E. coli* BW25113 strains with varying lengths of polysaccharide in the LPS layer (ΔrfaC, ΔrfaP, ΔrfaY, ΔrfaI), an efflux pump deficient strain (ΔtolC), and the gram-positive *S. aureus* US300

		E. coli BW25113 LPS length →							
Extract	*E. coli* ATCC25922	ΔrfaC	ΔrfaP	ΔrfaY	ΔrfaG	ΔRfaI	WT	ΔtolC	*S. aureus* USA300
MO025	5 %	0.16 %	0.16 %	5 %	0.31 %	1.25 %	5 %	5 %	0.16 %
MO027	2.50 %	0.08 %	0.16 %	5 %	0.31 %	1.25 %	>5 %	>5 %	0.16 %
MO029	>5 %	>5 %	>5%	>5 %	>5 %	>5 %	>5 %	>5 %	>5 %
MO030	1.25 %	0.04 %	0.16 %	1.25 %	0.16 %	0.31 %	2.50 %	2.50 %	0.08 %
MO033	>5 %	1.25 %	2.50 %	>5 %	5.00 %	>5 %	>5 %	>5 %	1.25 %
MO035	1.25 %	0.04 %	0.31 %	1.25 %	0.16 %	0.63 %	2.50 %	2.5 %	0.08 %
MO037	>5 %	>5 %	>5 %	>5 %	>5 %	>5 %	>5 %	>5 %	>5 %
Globomycin [μM]	25	0.78	3.13	25	6.25	25	50	25	>100

As indicated in Table 1, extracts MO025, MO027, MO030 and MO035 inhibited the growth of all tested bacteria, including *S. aureus* USA300, which globomycin alone does not inhibit. In contrast, extracts MO029 and MO037 showed no antibacterial activity at the tested concentrations. Overall, the MIC values of the extracts decreased with shortening of the LPS layers, mirroring the trend observed with globomycin. An exception was observed with *E. coli* BW25113 ΔrfaY, where the MIC was comparable to *E. coli* BW25113 ΔrfaI or even the wild-type bacteria. Notably, the deletion of the efflux pump tolC had no impact on the MIC of the extracts, although it decreased the MIC of pure globomycin 2-fold.

Despite containing only 3 % of the globomycin by EIC peak area, extract MO025 and MO030 show a comparable MIC profile to extract MO027, suggesting the presence of additional globomycin congeners, which were not quantified in the LC-MC analyses of the extracts. Conversely, low globomycin extracts MO029 and MO037 (3 % and 18 % respectively) do not exhibit any antibacterial activity.

Hemolysis

Hemolytic activity was evaluated by incubating various extract concentrations for one hour with defibrinated sheep's blood using 0.1 % Triton-X100 as a positive control (Figure 4). Extracts MO027, MO030 and MO033 lysed blood cells at concentrations above 2 %, while extracts MO025, MO029, MO035 and MO037 showed no hemolytic activity. Notably, the hemolytic extracts also killed gram-positive bacteria, suggesting general membrane interaction may contribute to the antibacterial activity. However, non-hemolytic extracts MO025 and MO035 kill *S. aureus* USA300, indicating that alternative mechanisms of action may also be involved.

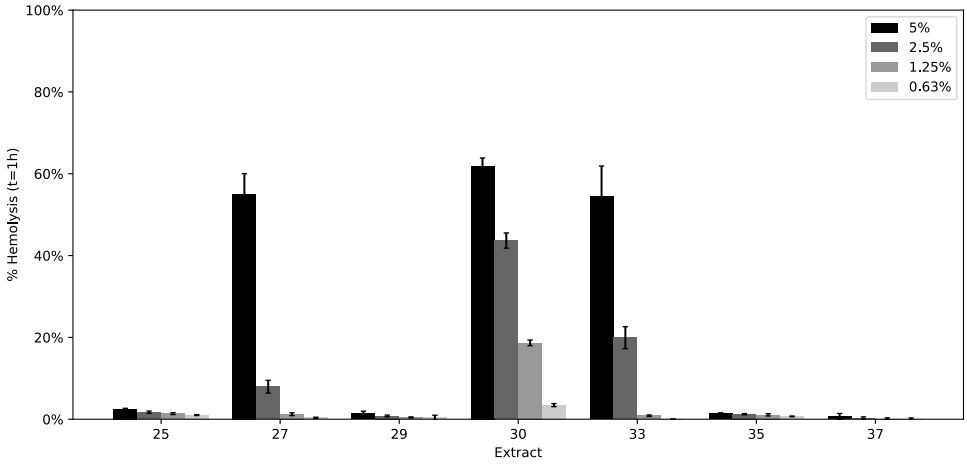

Figure 4: Hemolytic activity of globomycin containing extracts at 0.63-5 % after 1 hour of incubation. Values normalized to 100 % hemolysis with 0.1 % Triton-X100. Data represent the mean ± SD of three replicate experiments.

LspA Enzyme Inhibition

The extracts were further evaluated for their ability to inhibit purified LspA using a FRET-based biochemical assay employing a peptide substrate consisting of the LspA-recognition peptide linked to a fluorescent moiety and a quencher. Upon cleavage, the fluorophore is no longer quenched, and fluorescence can be measured[26]. Table 2 shows the measured IC_{50} values thus determined, expressed as the percentage of extract tested. For extracts produced using fermentation media FPY-6 and FR23, IC_{50} was determined for both replicate fermentations. The IC_{50} values range from 0.03 ± 0.004 % for MO030 to 0.45 ± 0.034 % for MO029.

Table 2: LspA inhibition measured using a FRET-based reporter substrate. IC_{50} values expressed as percentage of natural extract.

Extract	IC_{50}	Extract	IC_{50}
MO025	0.11 ± 0.006 %	MO031	0.16 ± 0.023 %
MO027	0.04 ± 0.003 %	MO033	0.17 ± 0.018 %
MO028	0.41 ± 0.028 %	MO035	0.09 ± 0.008 %
MO029	0.45 ± 0.034 %	MO037	0.21 ± 0.034 %
MO030	0.03 ± 0.004 %	Globomycin	<2nM

Fractionation

Based on the initial results, media DNPM, FR-23, and FRM were selected for refermentation at Fundación Medina, and the resulting extracts fractionated using reverse-phase chromatography and supplied to us in a 96-well format. In addition to the fractions, samples of the column-chromatography retentate (A2), flow through (B2) and crude extract (H11) were also supplied.

All fractions were tested in the biochemical LspA FRET assay at an initial concentration of 0.1 % (Figure 5). Fractions that exhibited >80 % inhibition at 0.1 % extract were analyzed by LCMS to confirm the masses for globomycin and its congeners (Table 3; EICs in Figure S4). All highly active fractions were conformed to contain globomycin or one of its congeners. Notably, by overlay of the fractionation chromatograms (Figure S1-Figure S3), one other fraction stands out: Fraction H5 from the fermentation in DNPM medium, which was found to inhibit LspA by 47 %, despite not having detectable amounts of globomycin congeners, with the dominant mass observed at 601.2 Da ($M+H^+$) (Figure S7). Whether this represents a lower MW globomycin congener or a novel LspA inhibitor remains to be established.

To compare the sensitivity of the FRET assay with that of phenotypic antibacterial assays, the MICs of fractions B8, D8, E8, C11, and D11 from the fermentation in FRM-medium were determined using the LPS-deficient *E. coli* BW25113 ΔrfaC and *S. aureus* USA300 up to a maximum concentration of 1.25 %. At this concentration no growth inhibition was observed.

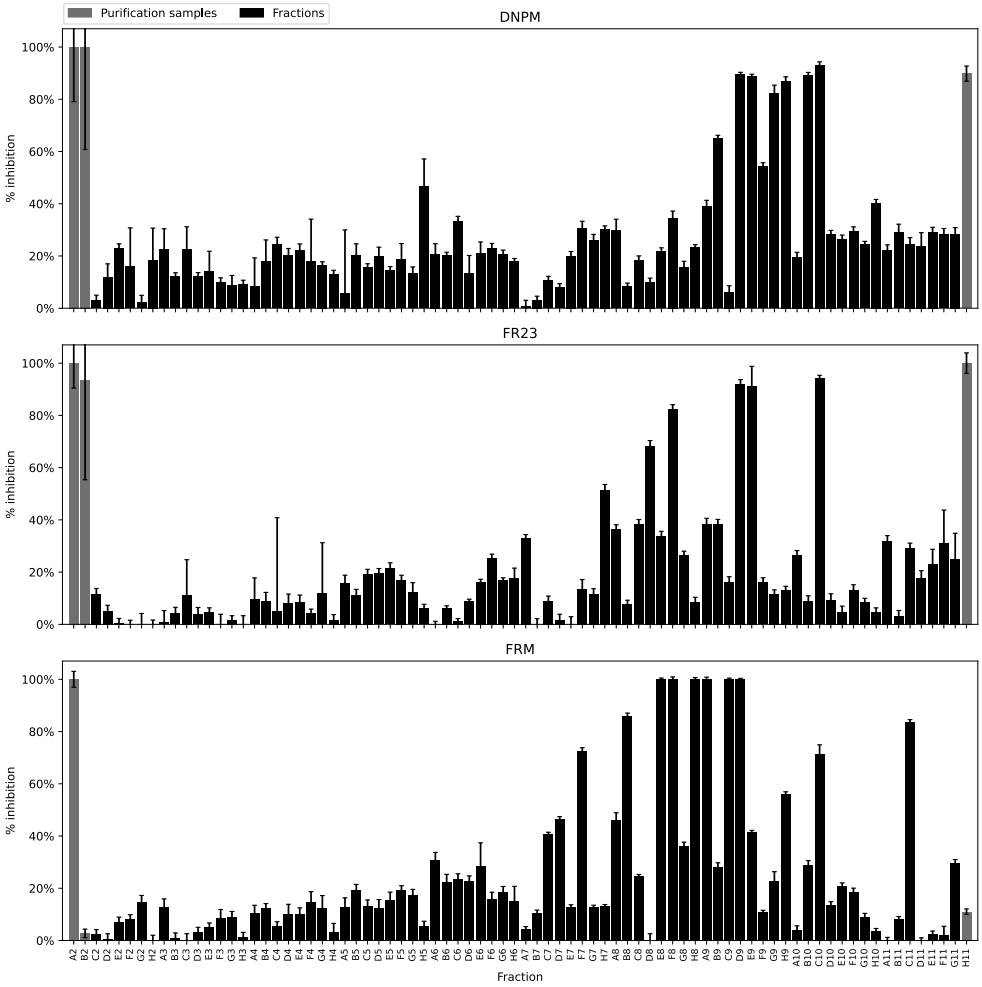

Figure 5: Overview of the LspA inhibition of each fraction at 0.1 % extract concentration from the fractionation of crude extract obtained from fermentation in different media (DNPM, FR23, FRM) Grey bars indicate purification samples: A2 (column chromatography retentate), B2 (column chromatography flowthrough), H11 (crude extract). The bars represent the mean ± SD of two replicate experiments.

Table 3: Identified congeners of globomycin in fractions from cultivations in various media (DNPM, FR23, FRM) with inhibition above 80 %

DNPM		FR23		FRM	
fraction	congener	fraction	congener	fraction	congener
D9	Globomycin	F8	SF-1902 A_2	B8	SF-1902 A_{4ab}
E9	Globomycin	D9	Globomycin	E8	SF-1902 A4 (traces)
G9	SF-1902 A_{4a-b}	E9	Globomycin	F8	SF-1902 A2
H9	SF-1902 A_{4a-b}	C10	SF-1902 A_5	H8	SF-1902 A3
B10	SF-1902 A_5			C9	SF-1902 A3
C10	SF-1902 A_5			D9	Globomycin
				H9	SF-1902 A3
				C11	traces of globomycin, SF-1902 A_4, SF-1902 A_5

Discussion

We evaluated both the crude and fractionated extracts from a globomycin-producing *Streptomyces* strains using a FRET-based *in-vitro* LspA activity assay. Previously, this assay was successfully applied in identifying LspA inhibitors via high throughput screening (HTS) of a small molecule library[16]. Our results demonstrate that this approach is also compatible with microbial extracts.

All tested extracts fully inhibit LspA at a concentration of 1 %, with 50 % inhibition reached at 0.02-0.45 %. In a potential HTS setup, extract concentrations in the assay could be up to 50 %, as the extracts contain 20 % DMSO, and LspA activity is optimal at 10 % DMSO. Conversely, in phenotypic antibacterial screening the DMSO percentage should be kept below 1 %, so the maximum testable extract concentration is 5 %. The MICs against wild-type *E coli* ranged from 1.25 % to >5 %, which means that extracts containing the potent LspA inhibitor globomycin would not have been picked up in a phenotypic screen. Our results also reveal that the sensitivity of bacteria increases with decreasing lipopolysaccharide (LPS) length, likely due to increased membrane permeability [27]. The deletion of efflux component tolC does not seem to influence the bacteria's susceptibility to the tested extracts.

After fractionation, we identified globomycin and its congeners in the most active fractions, even though the fractions did not exhibit antimicrobial activity at 1.25 % against LPS-deficient *E. coli*. This finding confirms that the *in-vitro* assay is compatible with the fractionation and can be used to identify active fractions for subsequent elucidation of the active inhibitors.

The FRET assay here investigated offers several advantages over phenotypic screening, including high sensitivity, as well as the potential for automatization and miniaturization. Notably, the concentrations of extracts needed to achieve detectable inhibition were significantly lower than those necessary to observe antibacterial activity. Moreover, extracts exhibiting hemolytic or anti-gram-positive activity, which would possibly be disregarded in phenotypic screens, still produced robust inhibition in the assay.

Our data revealed that MICs did not always predict the efficacy of an extract in the *in-vitro* assay. For example, some extracts with limited antibacterial activity (MIC >5 %), still exhibited low IC_{50} values (e.g. MO027), which may be attributed to poor membrane permeability of the LspA inhibitor(s) present in the extract. Furthermore, one can imagine a scenario wherein a microbial extract might contain multiple classes of antibiotics that, if reliant on MIC assays alone, would obscure the detection of an LspA inhibitor.

Autofluorescence of some extracts poses a challenge for HTS, particularly if a single point measurement was chosen. Auto-fluorescent extracts may either saturate the detector or overlap with the signal from the cleaved substrate. This issue could be mitigated by multiple time-point measurements and an initial assessment of autofluorescence prior to the inhibition assay.

Incorporating counter-screens into HTS is crucial for verifying hits and eliminating false-positives. For the FRET-based LspA assay, a complementary phenotypic screen could prove especially valuable. The use of hypersensitive gram-negative bacteria mitigates potential discarding of active compounds caused by low membrane permeability, which can be optimized during subsequent SAR studies.

Similarly, a hyperpermeable *E. coli* strain harboring a mutation in the LPS assembly protein LptD has been used both in counter-screens of small molecules against a LPS transporter protein[28] and in assessing the activity of globomycin analogues[19]. Additionally, the hypersensitive strain *E. coli* BW25113

ΔbamBΔtolC, characterized by a compromised outer membrane and lack of efflux, has been used to evaluate βlactams that typically cannot cross the outer membrane (Chapter 5), and in HTS of natural products targeting metallo-β-lactamases[29]. Engineered strains reporting on bacterial stress responses may also aid in the identification of potent LspA inhibitors[30,31].

The MIC values for globomycin against both wild-type and LPS-deficient *E. coli* strains in our study are consistent with previously reported values [19–21,32]. Similarly, the IC_{50} values from our *in-vitro* assay align with previous reports[16,33]. However, given that the measured IC_{50} values are close to the enzyme concentration used in the assay, the values should be interpreted with caution.

Conclusion

In summary, our studies demonstrate that the FRET based *in-vitro* LspA inhibition assay is a robust and sensitive method suitable for high throughput screening (HTS) of natural product extracts to detect LspA inhibitors. This assay offers significant advantages over phenotypic screens based on bacterial killing, as it can detect active compounds at much lower extract concentrations and depends primarily on the inhibitor's affinity for the enzyme. These findings pave the way for screening large libraries of natural extracts at HTS-facilities, such as those at Fundación Medina, and provide valuable insight into selecting appropriate bacterial strains for subsequent phenotypic counter-screening to further narrow down potential hits.

Materials and Methods

Unless otherwise specified chemicals were obtained from Merck. The LPS disrupted *E. coli* strains from the Keio collection were obtained from Horizon discovery.

Globomycin extracts

Globomycin containing extracts were prepared by Fundación Medina (Malaga, Spain) as previously described[17]. In short, *Streptomyces sp*. CA-278952 was grown in duplicate in various media, and the supernatant was mixed with an equal volume of acetone. After removal of acetone, the aqueous phase was loaded on a HP-20 resin chromatography column and eluted with an acetone/methanol mixture.

The acetone/methanol fraction was further fractionated using reverse-phase chromatography in acetonitrile. The resulting fractions were dried and dissolved in 20 % DMSO/Water.

LspA inhibition assay

LspA was purified as previously described [9]. Experiments were carried out in 100 mM MES/NaOH pH 5.4, 150 mM NaCl, 0.05% Lauryl maltose neopentyl glycol (LMNG), 10% DMSO.

The FRET-based LspA substrate was synthesized as previously described[12].

Serial dilutions of the extracts were prepared in black half-area polystyrene 96-well microplates (Greiner) and preincubated with LspA (50 nM final) at 37 °C for 5 min. Crude extracts were tested in triplicate, while the fractions were tested in duplicate. After addition of the substrate (final concentration 7 μM), the increase of fluorescence (ex. 320 nm, em. 420 nm) was measured using a microplate reader (Tecan) and the IC_{50} calculated using Prism 10 (Graphpad).

Growth inhibition assay

Growth inhibition assays were carried out according to the CLSI guideline. In brief, bacteria were grown to OD_{600} 0.5 in TSB, diluted 1000-fold into MHB and added to triplicate serial dilutions of natural extracts in sterile round bottom 96-well plates (Corning). After overnight incubation at 37 °C, the wells were inspected for growth.

Hemolysis assay

Defibrinated sheep's blood (Fisher) was centrifuged at 500 x g, 10°C for 10 minutes and washed using phosphate-buffered saline with 0.05 % Tween-20 (PBS-T). Washing was repeated until the supernatant's color remained consistent over two washes. To normalize the blood cell count, samples of blood cells were lysed with 0.1% Triton-X100, diluting 5-fold with PBS-T and the OD_{415} was measured. The final blood cell suspension adjusted to positive control with 0.1 % Triton-X100 reaching OD_{415} 2.5

To assess hemolytic activity, dilutions of the extracts were tested in triplicate in 96-wellplates (Greiner). The extracts were incubated with the resuspended blood cells at 37°C shaking at 200rpm for one hour. Intact blood cells were pelleted by centrifuging at 800 x g for 10 min and a 5-fold diluted sample of the supernatant was used to measure OD_{415}. The results were normalized to the 0.1 % Triton-X-100-treatment as the 100% hemolysis control

LCMS Analysis of Fractions

Extract fractions were diluted 1:5 and 2 µL was injected into LC-MS system (LCMS-8040 Shimadzu) using a C18 column and a gradient of 5-100 % acetonitrile against 0.1 % formic acid. For each congener, the extracted ion chromatograms (EIC) with $M+H^+$ at 628.3916 ± 0.013 for SF1902-A_2, 670.4386 ± 0.013 for SF1902-A_4, 642.4073 ± 0.013 for SF1902-A_3, 656.4229 ± 0.013 for globomycin and 684.4542 ± 0.013 for SF1902 A_5 were obtained as previously described[17].

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Supplementary Information

Table S1: Names, fermentation medium and Globomycin content of the studied extracts shown as peak area of EIC and relative to the extract containing the most globomycin

Extract	Fermentation Medium	EIC 656.423±0.005	Relative Globomycin
MO024	CLA	739617	0.30
MO025	CLA	797541	0.32
MO026	DNPM	2597649	1.06
MO027	DNPM	2456497	1.00
MO028	FPY-6	75058	0.03
MO029	FPY-6	70452	0.03
MO030	FR23	78405	0.03
MO031	FR23	74777	0.03
MO032	FRM	507156	0.21
MO033	FRM	571580	0.23
MO034	MO016	361471	0.15
MO035	MO016	972747	0.40
MO036	NOC-2	562478	0.23
MO037	NOC-2	448078	0.18

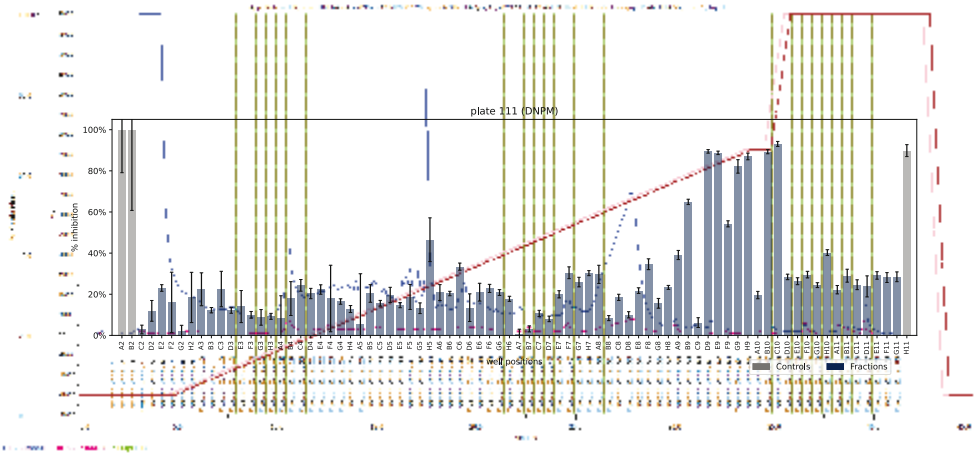

Figure S1: Overlay of Chromatogram and LspA inhibition of fractions resulting from fermentation in DNPM medium

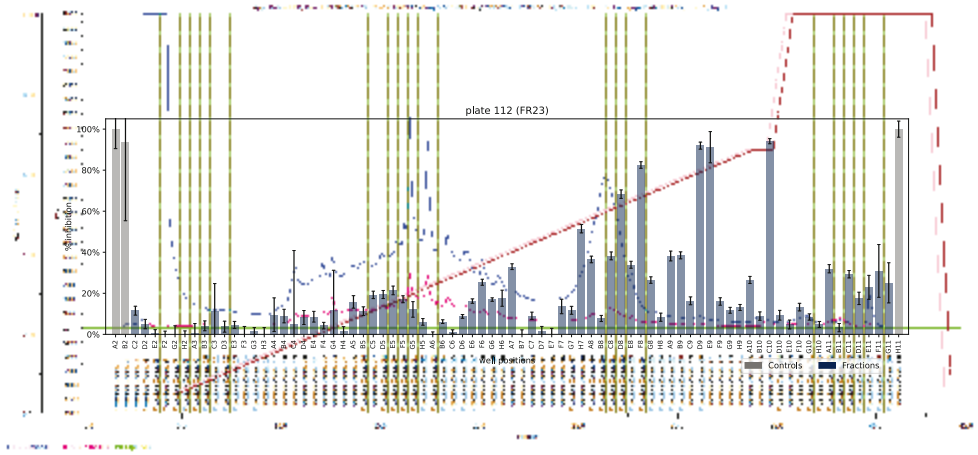

Figure S2: Overlay of chromatogram and LspA inhibition of fraction resulting from fermentation in FR23 medium

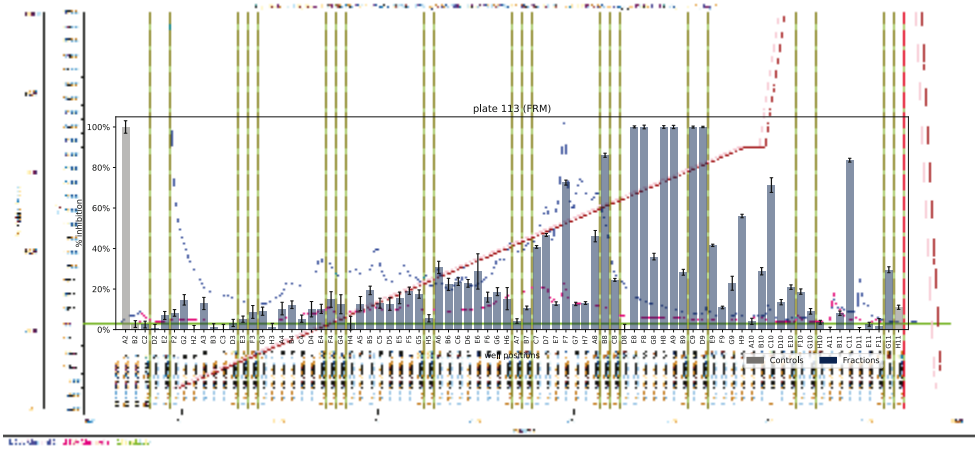

Figure S3: Overlay of chromatogram and LspA inhibition of fraction resulting from fermentation in FRM medium

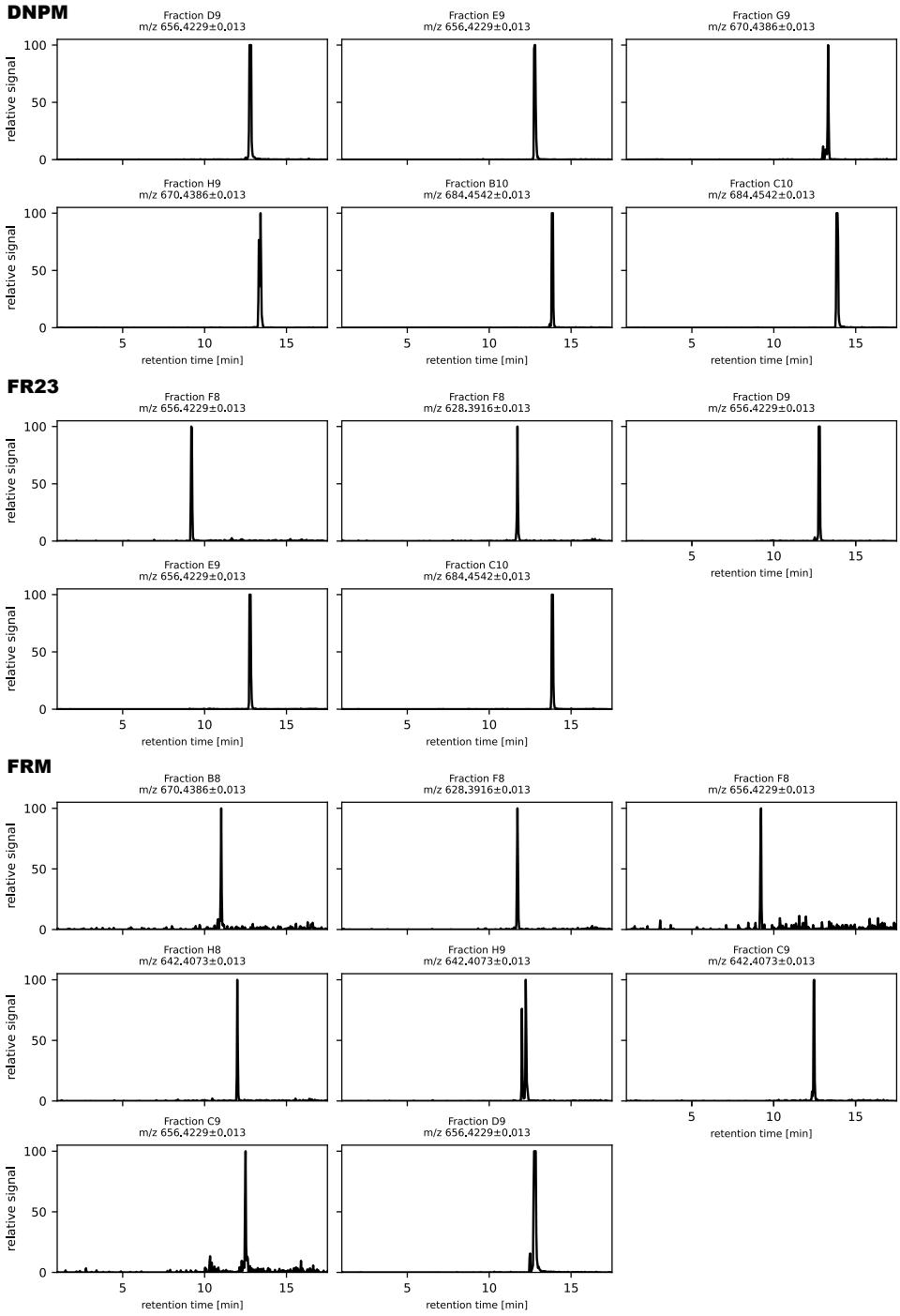

Figure S4: Extracted ion chromatograms (EICs) for selected m/z-ranges for fractions with an LspA-Inhibition >80 %

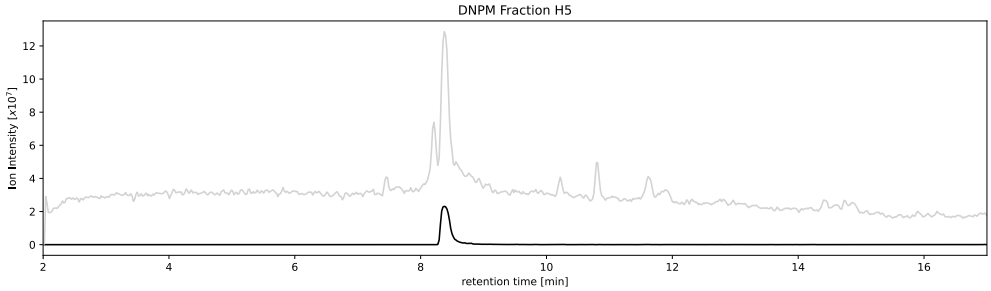

Figure S5: Total positive Ion Chromatogram (TIC(+)) and Extracted Ion Chromatogram at 601±0.3 of DNPM Fraction H5