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A microplate assay for the coupled transglycosylase–transpeptidase activity of the penicillin binding proteins; a vancomycin-neutralizing tripeptide combination prevents penicillin inhibition of peptidoglycan synthesis

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ABSTRACT

A microplate, scintillation proximity assay to measure the coupled transglycosylase–transpeptidase activity of the penicillin binding proteins in *Escherichia coli* membranes was developed. Membranes were incubated with the two peptidoglycan sugar precursors UDP-N-acetyl muramylpentapeptide (UDP-MurNAc(pp)) and UDP-[^{3}H]N-acetylglucosamine in the presence of 40 μM vancomycin to allow *in situ* accumulation of lipid II. In a second step, vancomycin inhibition was relieved by addition of a tripeptide (Lys-D-ala-D-ala) or UDP-MurNAc(pp), resulting in conversion of lipid II to cross-linked peptidoglycan. Inhibitors of the transglycosylase or transpeptidase were added at step 2. Moenomycin, a transglycosylase inhibitor, had an IC_{50} of 8 nM. Vancomycin and nisin also inhibited the assay. Surprisingly, the transpeptidase inhibitors penicillin and ampicillin showed no inhibition. In a pathway assay of peptidoglycan synthesis, starting from the UDP linked sugar precursors, inhibition by penicillin was reversed by a 'neutral' combination of vancomycin plus tripeptide, suggesting an interaction thus far unreported.

1. Introduction

Peptidoglycan is the major structural component of the bacterial cell wall. It is a polymer of a repeating disaccharide–peptide unit, where the pentapeptide chains attached to adjacent sugar molecules are cross-linked. The disaccharide unit is synthesized on a lipid precursor from the sugar precursors UDP-N-acetyl glucosamine (UDP-GlcNAc) and UDP-N-acetyl-muramylpentapeptide (UDP-MurNAc(pp)). The MraY enzyme condenses UDP-MurNAc(pp) with undecaprenyl pyrophosphate present in the membrane to form lipid I. Subsequently, MurG catalyzes transfer of GlcNAc to lipid I to form lipid II. The final stage of peptidoglycan synthesis occurs in the periplasm: polymerization of the disaccharide unit (GlcNAc-MurNAc(pp)) by the transglycosylase (TG) to form peptidoglycan and simultaneous crosslinking of the peptide chains by the transpeptidase (TP) to form crosslinked peptidoglycan (Fig. 1A). The lipid carrier is released and recycled by the action of the lipid pyrophosphorylase. All enzymes and the lipid carrier are present in the membrane. The coupled TG and TP activities are catalyzed by penicillin binding proteins (PBP), e.g. PBP1a, 1b of *Escherichia coli* [1,2].

The TG and TP, being membrane-associated, and on the periplasmic surface are accessible to drug molecules and are attractive drug targets, especially since peptidoglycan is absent in eukaryotes. The TP is the target of the β-lactams, one of the most successful antibiotic classes to date. This target is being currently revisited, by the discovery of novel β-lactams and PBP inhibitors [3,4] as well as β-lactamase inhibitors [5] and combinations of the two [6] that overcome resistance to previous generations of β-lactams. However, both enzymes, and, in particular, the TG, are difficult to assay in a format amenable to high throughput screening.

Measuring the peptidoglycan TG activity is challenging, because the substrate, lipid II, is difficult to synthesize [7–9] and the TG

Abbreviations: UDP-GlcNAc, UDP-N-acetyl glucosamine; UDP-MurNAc(pp), UDP-N-acetyl-muramylpentapeptide; TG, transglycosylase; TP, transpeptidase; V + T, vancomycin + neutralizing tripeptide; PBP, penicillin binding protein; UDP-MurNAc-(pp), UDP-N-acetyl-muramylpentapeptide; SPA, scintillation proximity assay; WGA, wheat germ agglutinin.

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product, uncross-linked peptidoglycan, is not easily distinguished from the substrate; paper chromatography or HPLC may have to be used. Binding assays to detect inhibitors of the TG that compete with moenomycin have been reported [10]. Lipid II can be accumulated *in situ* in membranes using detergent [11] or a PBP1b mutant [12] and a high throughput, continuous assay has been described [13].

Several assays have been described for the TP activity of the PBPs, but most are binding assays and are not truly reflective of the catalytic activity. The most commonly used is the binding of a β-lactam to the PBPs to screen for agents that compete with this binding [14,15]. Monitoring the enzyme activity of the TP is even more challenging than that of the TG, since its product, crosslinked peptidoglycan, is not easily distinguished from uncrosslinked peptidoglycan. An exception is the scintillation proximity assays (SPAs) reported from our research unit that can easily assay the transpeptidase, albeit in the environment of the membrane, either in isolation, [16] or coupled with enzymes in the pathway of peptidoglycan biosynthesis [12,17,18]. These showed that wheat germ agglutinin coated SPA (WGA-SPA) beads in the presence of a detergent, such as N-lauryl sarcosine (sarkosyl), can specifically capture and distinguish crosslinked peptidoglycan from uncrosslinked peptidoglycan or lipid II in membranes of *E. coli* [16–19]. The other two species can be captured by the same beads in the absence of detergent. Here a simple, coupled TG–TP assay is described.

2. Materials and methods

2.1. Materials

Wheat germ agglutinin-coated SPA (WGA-SPA) beads were from Amersham International plc. U.K. UDP-[^{3}H]-N-acetyl glucosamine was from NEN Dupont, USA. Other chemicals were from Sigma Chemical Co. USA. Moenomycin was a gift from Hoechst, India.

2.2. Enzyme and substrates

UDP-N-acetyl muramyl pentapeptide (UDP-MurNAc(pp)) was purified from *Bacillus cereus* [17]. Membranes were prepared from *E. coli* AMA1004 [17].

2.3. Enzyme assays

All enzyme reactions were performed in 96 well flexible plates (Wallac) in a final volume of 25 μl. Reactions were stopped by the addition of 5 μl of 90 mM EDTA. For the SPAs to measure cross-linked peptidoglycan 170 μl of WGA-SPA beads (0.5 mg) in 50 mM HEPES ammonia pH 7.3 was added along with sarkosyl to a final concentration of 0.2% [18]. For measurement of lipid II the sarkosyl was left out of the WGA-SPA bead mixture [17–19].

For paper chromatography analysis, a second set of reactions was run in parallel to the SPA and analyzed as described [17]. Peptidoglycan remains at the origin, whereas lipid II has an R_f of ~0.9 [17,20]. The chromatogram was cut into pieces and the radioactivity measured in a liquid scintillation counter.

2.4. Peptidoglycan pathway assay

The membrane steps of peptidoglycan synthesis, starting from the UDP-linked precursors, were performed similar to the method described [17]. *E. coli* membranes (4 μg protein) were incubated for 90 min at 37 °C with 15 μM UDP-MurNAc(pp), 2.5 μM UDP-[^{3}H]GlcNAc (0.2 μCi) in 50 mM HEPES ammonia pH 7.5, 10 mM $MgCl_2$, 8% DMSO. Reactions were carried out in triplicate, unless otherwise specified. The enzyme blank was a reaction without UDP-MurNAc(pp) and, for each type of capture condition, the cpm obtained in this reaction was subtracted from that of reactions containing both sugar precursors (complete or 100% reaction) as a measure of 'Activity'.

2.5. Transglycosylase–transpeptidase assay

The TG substrate, lipid II, was synthesized in *E. coli* membranes (4 μg) incubated for 120 min at 37 °C with 15 μM UDP-MurNAc(pp), 4.16 μM UDP-[^{3}H]GlcNAc (0.5 μCi), 40 μM vancomycin in 50 mM HEPES-ammonia pH 7.5, 10 mM $MgCl_2$, in 15 μl. Subsequently, in a second step, the TG–TP reaction was carried out by adding 10 μl of a solution containing UDP-GlcNAc (to 250 μM) to dilute out the radiolabel, DMSO (to 8%), Nα,Nε diacetyl Lysine-D-alanine-D-alanine (Lys-D-ala-D-ala) tripeptide (to 400 μM) to neutralize vancomycin and the reaction was incubated for 5 min at 37 °C. Inhibitors were added at this step. The reaction was stopped and analyzed by SPA or paper chromatography as described above; for experiments where products were analyzed in parallel by paper chromatography 1.2 μCi UDP-[^{3}H]GlcNAc was used. Reactions were carried out in triplicate, unless otherwise specified. For the enzyme blank, reactions were stopped and products measured at the end of step 1; an alternative blank used was a reaction containing no UDP-MurNAc(pp) at step 1.

3. Results

3.1. Assay principle

A coupled TG–TP assay was envisaged as a two step process with an inhibitor of the TG used in step 1 to allow accumulation of the TG substrate, lipid II, *in situ* in *E. coli* membranes (Fig. 1B and C). In a second step, the plan was to relieve inhibition of the TG to allow the coupled TG–TP to convert the lipid II to cross-linked peptidoglycan.

Experiments using moenomycin to inhibit the TG resulted in accumulation of lipid II, but the inhibitory effect of moenomycin could not be reversed by dilution. Vancomycin inhibits MraY, MurG, the TG and also the TP [21]. Experiments in our laboratory, suggested that the TG was more sensitive to inhibition by vancomycin than the MraY and MurG. If so, it was likely that vancomycin could be used at a concentration that inhibited the TG, but not MraY or MurG, so as to cause accumulation of lipid II *in situ* in *E. coli* membranes. The plan was to subsequently neutralize vancomycin by a peptide containing D-ala-D-ala or by the substrate UDP-MurNAc(pp) to allow catalysis by the TG and TP.

3.2. In situ synthesis of lipid II: titration of vancomycin concentration

The concentration of vancomycin was titrated in the peptidoglycan pathway assay with the aim of finding a condition where lipid II was formed, but not peptidoglycan (Table 1). This assay monitors the membrane steps of peptidoglycan synthesis starting from the UDP-linked sugar precursors [18]. One set of reactions was captured by WGA-SPA beads to monitor lipid II and a second set with WGA-SPA beads plus sarkosyl to monitor the quantity of cross-linked peptidoglycan synthesized [18].

At very low concentrations of vancomycin no inhibition of any of the enzymes occurred, resulting in synthesis of cross-linked peptidoglycan. However, at 30–60 μM vancomycin, the counts were highest in the SPA without detergent and minimal in that with detergent, indicating a significant quantity of lipid II and a minimal amount of crosslinked peptidoglycan were formed (Table 1). This was confirmed by paper chromatography analysis

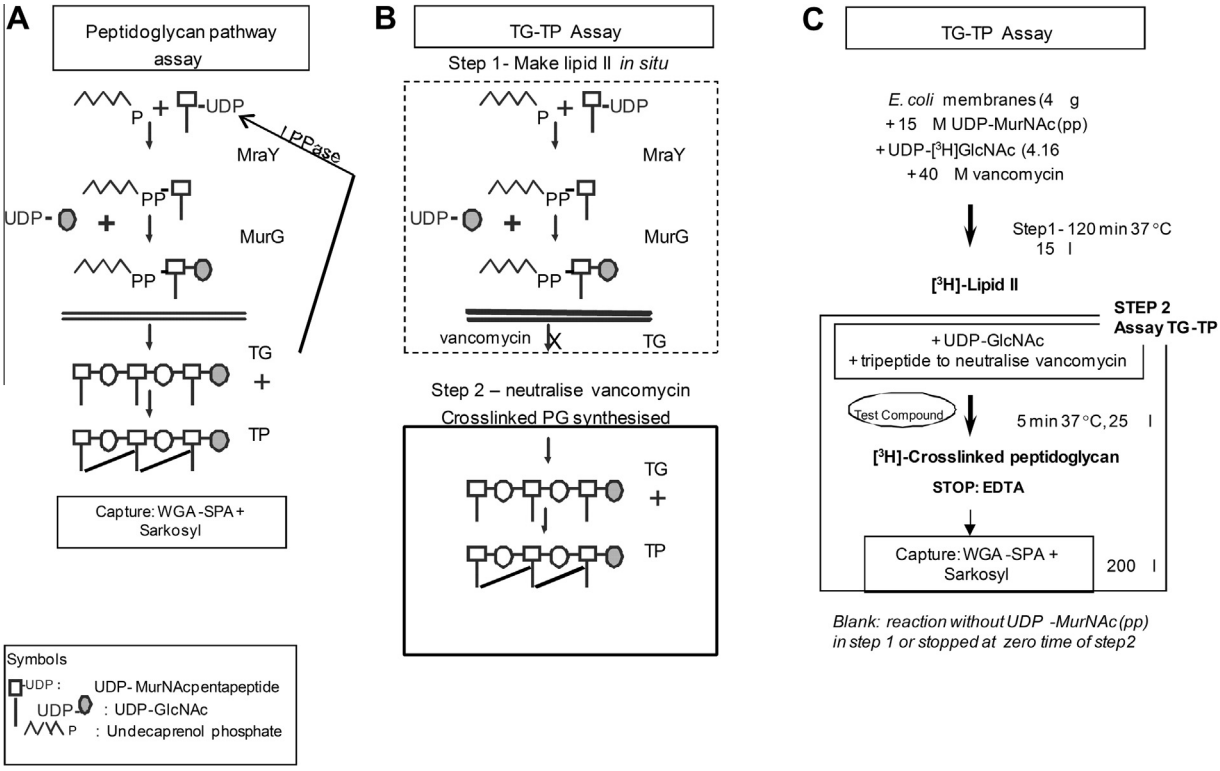

Fig. 1. Schematic of peptidoglycan pathway assay (A) and the coupled TG–TP assay (B) and (C). A: enzymatic reactions and molecular species formed during synthesis of crosslinked peptidoglycan in *E. coli* membranes incubated with UDP-[^{3}H]GlcNAc and UDP-MurNAc(pp). B: molecular species formed in the coupled TG–TP assay and C: steps taken to conduct the assay.

Table 1
Titration of vancomycin needed to inhibit synthesis of crosslinked peptidoglycan but allow accumulation of lipid II. The peptidoglycan pathway assay was set up and crosslinked peptidoglycan or lipid II were monitored. In the absence of vancomycin the peptidoglycan activity was 3988 ± 170 cpm (n = 2).

Vancomycin (μM)	Crosslinked peptidoglycan		Product captured without sarkosyl (lipid II)	
	Avg blank (cpm)	Activity (cpm) (avg ± S.D.)	Avg blank (cpm)	Activity (cpm) (avg ± S.D.)
15	698	1333 ± 28	1178	1869 ± 122
20	728	926 ± 69	1262	1622 ± 19
25	724	692 ± 9	1185	1668 ± 192
30	742	511 ± 51	1290	1320 ± 59
35	773	387 ± 33	1112	1052 ± 9
40	769	171 ± 36	1275	1583 ± 405
45	802	133 ± 23	1114	1392 ± 93
50	769	70 ± 22	1283	1477 ± 87

(data not shown). A concentration of 40 μM vancomycin was chosen for further assays.

3.3. Assay development

The feasibility of a two step assay for the coupled, endogenous TG–TP activity was studied. *E. coli* membranes were incubated with 15 μM UDP-MurNAc(pp), 4.2 μM UDP-[^{3}H]GlcNAc (0.5 μCi), 40 μM vancomycin for 90 min at 37 °C in 15 μl. In a second step, the reaction was diluted to 25 μl by addition of DMSO to 8%, UDP-GlcNAc (to 250 μM) plus a tripeptide or UDP-MurNAc(pp) and further incubated for 30 min. Reactions were analyzed for cross-linked peptidoglycan at the end of step 2. The quantity of crosslinked peptidoglycan formed at the end of step 1 was used as a blank for the step 2 TG–TP reaction analysis. The quantity of lipid II formed at the end of step 1 was also analyzed.

Under these conditions lipid II, but very little cross-linked peptidoglycan, was synthesized in the first step (Table 2). In the

Table 2
Feasibility of a two step SPA for TG–TP activity in *E. coli* membranes. In step 1 membranes (4 μg) were incubated with UDP-[^{3}H]GlcNAc (4.2 μM, 0.5 μCi), 15 μM UDP-MurNAc(pp) and 40 μM vancomycin for 90 min at 37 °C in 15 μl. In step 2 the reaction was diluted to 25 μl by addition of UDP-GlcNAc (to 250 μM) plus a tripeptide or UDP-MurNAc(pp) and incubated for 30 min at 37 °C. Crosslinked peptidoglycan was captured. The lipid II activity captured in step 1 was 2532 ± 132 cpm.

Additions to assay	Concentration (μM)	Activity (cpm)
Step 1		
Nil		139 ± 83
Step 2		
Nil		784 ± 99
UDP-MurNAc(pp)	150	2027 ± 139
Lys-D-Ala-D-Ala	40	1484 ± 130
	200	1949 ± 179
	400	2047 ± 180
	800	2295 ± 139
Lys-D-Ala-Lactate	200	763 ± 123
	800	921 ± 92

absence of any neutralizing agent, some cross-linked peptidoglycan was synthesized in step 2, possibly due to dilution of vancomycin below the concentration needed for complete inhibition of the TG. On addition of 150 μM UDP-MurNAc(pp) or 200 μM Lys-D-ala-D-ala tripeptide to neutralize vancomycin considerable cross-linked peptidoglycan was synthesized in step 2. As expected, a Lys-D-ala-D-lactate tripeptide had no effect, since this tripeptide cannot neutralize the inhibitory effect of vancomycin on peptidoglycan synthesis. Neutralization of vancomycin inhibition was better with 200 or 400 μM than with 40 μM tripeptide. For further experiments 40 μM vancomycin was used in step 1 and Lys-D-ala-D-ala tripeptide (400 μM) was used, rather than UDP-MurNAc(pp), for neutralization of vancomycin, since the tripeptide is commercially available.

The effect of varying the time of incubation in the first step was studied (Fig. 2A). The quantity of lipid II increases gradually and 120 min used for all further experiments. In the second step conversion of lipid II to cross-linked peptidoglycan was almost complete in 5 min (Fig. 2B). The formation of crosslinked peptidoglycan from lipid II was confirmed by paper chromatography (Fig. 2C). In step 1 very little peptidoglycan was formed (~1000 cpm, squares in Fig. 2C) and most of the radioactivity was in lipid II ($R_f \sim 0.9$). In step 2 there was partial conversion of the lipid II to peptidoglycan, as seen by the increase in cpm at the origin and decrease in cpm at the solvent front. However, in the chromatogram crosslinked peptidoglycan cannot be distinguished from uncrosslinked peptidoglycan, since both remain at the origin. Only the SPA data provides evidence that crosslinked peptidoglycan was formed [12,18]. (see Fig. 3)

3.4. Inhibitors

The effect of inhibitors on the coupled TG–TP SPA was studied. A parallel set of reactions was analyzed by paper chromatography looking for inhibition of the radioactive product at the origin. Since both cross-linked and uncross-linked peptidoglycan remain at the origin of the chromatogram, a TG inhibitor, which would result in no peptidoglycan at the origin, would show inhibition of both the SPA and the paper chromatography analysis. But a TP inhibitor would cause uncrosslinked peptidoglycan to accumulate, which would appear as radioactivity at the origin of the paper chromatogram; such inhibitors would only show inhibition in the SPA and not in the paper chromatography analysis of the same reactions.

As expected moenomycin, an inhibitor of the TG inhibited both the SPA and paper chromatography analysis with comparable IC_{50}s: 9 vs 8 nM, respectively. Nisin showed an IC_{50} of 29 vs 6 μg/ml on the SPA vs paper chromatography. Although nisin is reportedly a MurG inhibitor [19], given its binding to lipid II [22] it is not surprising that the TG was inhibited [12]. The reactions were also inhibited by vancomycin (IC_{50} of 32 μM in SPA vs 46 μM in paper chromatography analysis), despite the presence of the neutralizing tripeptide in the reaction. As expected, the IC_{50} of vancomycin increased when the quantity of neutralizing tripeptide in the assay was increased (data not shown). However, very surprisingly, penicillin and ampicillin failed to inhibit the reaction by both analyses. Several β-lactams were tested and the same phenomenon was observed for most. The exceptions were ceftriaxone, cefuroxime and cephalosporin C that showed marginal inhibition (40–60%) at 1 mM in the SPA but no inhibition whatsoever in the paper chromatography, suggesting inhibition of the transpeptidase. Other inhibitors of the peptidoglycan synthesis pathway e.g. bacitracin, tunicamycin had no effect.

3.5. Reversal of penicillin inhibition by vancomycin and tripeptide in the peptidoglycan pathway assay

The lack of inhibition by penicillin was investigated in the pathway assay (Table 3). As reported earlier [17,18], both penicillin (10 μM or 1 mM) and vancomycin (35 μM) inhibited peptidoglycan synthesis: 88–97% and 87% inhibition, respectively. The tripeptide Lys-D-ala-D-ala alone had no effect on peptidoglycan synthesis or on penicillin inhibition of the synthesis. The inhibition by vancomycin was neutralized by a Lys-D-ala-D-ala tripeptide, consistent with its mechanism of inhibition by binding the terminal D-ala-D-ala region of muramylpentapeptide (reduction of inhibition from 87% to 25%). However, when penicillin was added together with vancomycin and tripeptide, no inhibition by penicillin was observed (Table 3). The same phenomenon was observed with ampicillin, i.e. neutralization of its inhibitory effect by vancomycin plus tripeptide. Interestingly, the inhibition of 1 mM penicillin was prevented, or neutralized, by a concentration of vancomycin ~30-fold lower (35 μM).

In the above experiment vancomycin, Lys-D-ala-D-ala and penicillin were added at the same time, at the start of the reaction. The question was whether vancomycin + tripeptide (V + T) could reverse inhibition by penicillin – i.e. if V + T was added after penicillin, and during the course of the enzyme reaction. Accordingly, a peptidoglycan pathway synthesis reaction was set up with 1 mM penicillin and 0.7 mM Lys-D-ala-D-ala, which resulted in complete inhibition of the reaction (Fig. 4, triangles). To this reaction 40 μM vancomycin, or water as a control, was added at different times and at the end of 90 min the cross-linked peptidoglycan was captured (Fig. 4).

Penicillin caused close to 100% inhibition of the reaction and addition of tripeptide had no effect on this inhibition. Vancomycin was able to reverse the inhibition by penicillin such that there was very little inhibition seen if vancomycin was added at the start of the peptidoglycan synthesis reaction. The % inhibition by penicillin

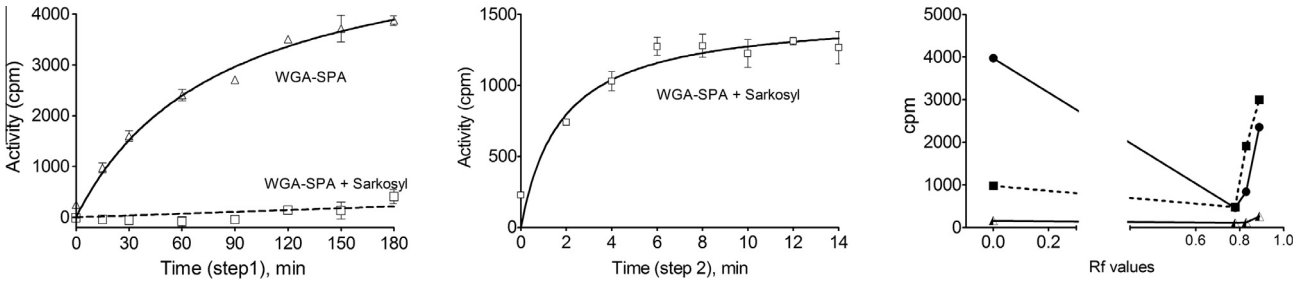

Fig. 2. Coupled TG–TP assay analyzed by SPA and paper chromatography. A: synthesis of lipid II (triangles) or crosslinked peptidoglycan (squares) in step 1. B: synthesis of crosslinked peptidoglycan (squares) in step 2; step 1 was performed for 120 min at 37 °C. C: paper chromatography analysis of products at step 1 and 2, showing no peptidoglycan or lipid II in the blank (triangles, no UDP-MurNAc(pp), lipid II but no peptidoglycan at step 1 (squares) and partial conversion of lipid II to peptidoglycan (circles) at step 2; lipid II $R_f \sim 0.9$, crosslinked and uncrosslinked peptidoglycan stays at the origin ($R_f \sim 0$). The quantity UDP-[^{3}H]GlcNAc used was 0.5 μCi (A) and (B) or 1.2 μCi (C).

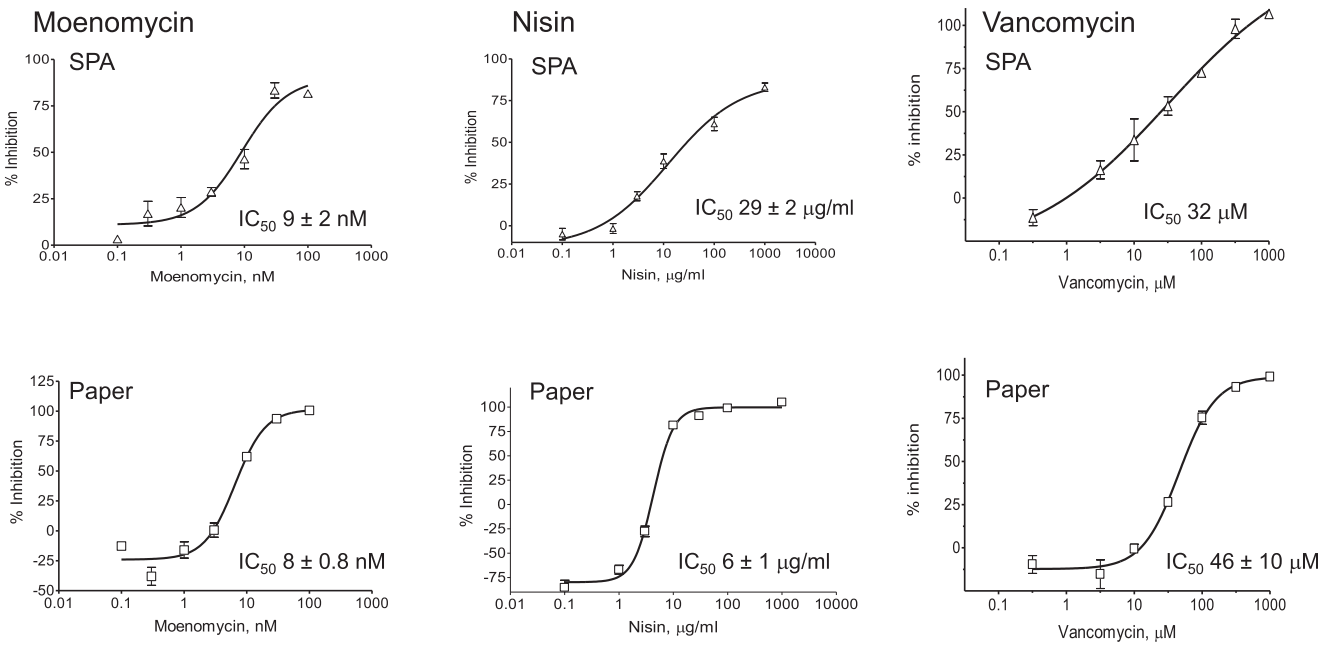

Fig. 3. The effect of moenomycin nisin and vancomycin on the coupled TG–TP assay. Inhibitors were added at the start of step 2 and crosslinked peptidoglycan was captured by WGA-SPA beads + sarkosyl (top panel) or total peptidoglycan was analyzed by paper chromatography (lower panel). Mean % inhibition ± S.D. (n = 2) were plotted and IC_{50} ± 95% confidence limits of the value were calculated by fitting to the 4 parametric equation in ExcelFit.

Table 3
Vancomycin + tripeptide neutralizes inhibitory effect of penicillin in a peptidoglycan pathway assay. Vancomycin inhibition was neutralized by Lys-D-ala-D-ala. Penicillin was ineffective in the presence of a neutral combination of "vancomycin + tripeptide". The concentrations used were penicillin 10 μM or 1 mM, vancomycin 35 μM, tripeptide (Lys-D-ala-D-ala) 350 μM. The average blank was 327 cpm (n = 3).

Additions	Activity (cpm)	% Inhibition
Nil (control)	4961 ± 111	0
Vancomycin	660 ± 140	87
Tripeptide (Lys-D-ala-D-ala)	5286 ± 139	−7
Vancomycin + tripeptide (Lys-D-ala-D-ala)	3743 ± 191	25
Penicillin G (10 μM)	604 ± 57	88
Penicillin G (1 mM)	160 ± 19	97
Penicillin G (1 mM) + vancomycin	619 ± 56	88
Penicillin G (1 mM) + vancomycin + tripeptide	5339 ± 91	−8
Penicillin G (1 mM) + tripeptide (Lys-D-ala-D-ala)	166 ± 21	97

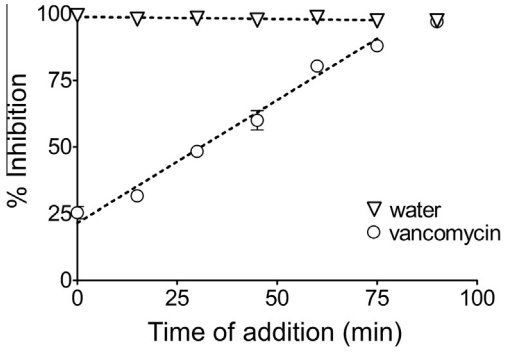

Fig. 4. Reversal of penicillin inhibition by vancomycin + tripeptide. The peptidoglycan pathway assay was set up with 1 mM penicillin and 0.7 mM Lys-D-ala-D-ala tripeptide. At various times during the 90 min incubation vancomycin (to 35 μM) (circles) or water (triangles) was added. Data is presented as % inhibition compared to a reaction with no penicillin or tripeptide added. Values represent the avg ± S.D. (n = 3).

was higher if vancomycin was added at a later time, resulting in a linear relationship between % inhibition and time of addition of vancomycin.

4. Discussion

The evidence that transpeptidation was not inhibited in this TG–TP coupled assay is indirect and based on previous large body of work that indicates crosslinked peptidoglycan is synthesized *in vitro* under these conditions and that capture by WGA-SPA beads + sarkosyl measures crosslinked peptidoglycan [12,16–18,20]. The lack of inhibition of this TG–TP assay by β lactams is very difficult to explain in the context of what is understood of the mechanism of action of both penicillin and vancomycin. Penicillin covalently binds to the proteins (PBPs) involved in peptidoglycan synthesis whereas vancomycin is believed to bind to the peptide stem of peptidoglycan. There is no published data showing an interaction between the two drugs. Since vancomycin could neutralize inhibition by a 30-fold molar excess penicillin, it is unlikely that the neutralization is due to sequestration of penicillin by vancomycin. However, there are examples of analogs of vancomycin that inhibit peptidoglycan synthesis without binding to the tripeptide and these have been shown to bind to the PBPs [21,23]. One possibility is that vancomycin + tripeptide enhances the rate of dissociation of the β-lactam–PBP complex. This hypothesis could be tested and also the question asked whether the tripeptide is necessary for this interaction; although reversal of the inhibition by penicillin was shown by a vancomycin–tripeptide combination, it is likely vancomycin alone would reverse the inhibition. However, in a system such as used here, neutralization of the inhibitory effect of penicillin would be masked by inhibition due to vancomycin.

In our hands the combination of vancomycin + tripeptide could not reverse inhibition by penicillin of the growth of *Bacillus subtilis* or *Staphylococcus aureus*. The neutralization observed in the enzyme assay may be peculiar to *E. coli*, or to PBP1b. Even if the

inhibition of PBP1b was reversed by vancomycin, inhibition of other PBPs would cause growth inhibition. Preliminary experiments indicated the vancomycin–tripeptide combination did not inhibit binding of labeled penicillin to the PBPs in *E. coli* membranes, or to purified PBP1b.

In summary, a high throughput screening compatible, scintillation proximity assay for the coupled TG–TP activity of peptidoglycan synthesis was developed that could be used to discover novel inhibitors of the TG. Surprisingly, the assay did not measure inhibition by ampicillin and penicillin, so it is questionable whether it would detect other classes of PBP inhibitor. This work led to an interesting observation: the reversal of penicillin inhibition by a neutral combination of vancomycin plus a D-ala-D-ala containing tripeptide, although the mechanism by which this occurs remains to be understood.

Acknowledgments

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