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Does the cell wall of bacteria remain a viable source of targets for novel antibiotics?

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ARTICLE INFO

Article history:
Received 14 August 2005
Accepted 17 October 2005

Keywords:
Antibacterial agents
Antibacterial discovery
Antibiotics
Peptidoglycan synthesis
Mur pathway
Cell wall antibiotics

ABSTRACT

Whether the bacterial cell wall remains a viable source of novel antibacterials is addressed here by reviewing screen and design strategies for discovery of antibacterials with a focus on their output. Inhibitors for which antibacterial activity has been shown to be due to specific inhibition of a reaction (antibacterially validated inhibitors) are known for 8 of the 14 conserved essential steps of the pathway. Antibacterially validated enzyme inhibitors exist for six of these steps. The possible obstacles to finding validated inhibitors of the remaining enzymes are discussed and some strategies are suggested.

1. Introduction

It was through elucidation of the mechanism of action of penicillin that much of the biochemistry of bacterial peptidoglycan synthesis was determined. The bacterial cell wall was among the earliest targets of pathway-directed natural products screening, starting with a variety of phenotypic whole cell screens and continuing through various incarnations of whole cell target-specific screens to broken-cell assays, in vitro assays of purified enzymes, and ultimately, to biochemical screens of the reconstituted cytoplasmic portion of the pathway. While inhibitors have been found or designed for most of the pathway reactions, antibacterial activity due to inhibition of those steps is thus far limited to a subset of gene products, and fewer steps are the targets of clinically useful drugs.

Rather than discuss the enzymology of peptidoglycan synthesis and detailed mechanisms of inhibitor action, which has been done in many excellent reviews [1–9], this review will examine screening strategies and their output with a particular emphasis on the antibacterial activity of inhibitors and whether the targeted enzyme has been validated as the source of that activity.

2. The pathway

Through genetic and physiological studies and the discovery of inhibitors of many steps of the pathway, it is clear that the pathway reactions are essential, the genes generally conserved, with most lacking mammalian homologs and, in those regards, they are putative targets for antibacterial intervention. But screening for inhibitors of these targets has proceeded for decades in many screening programs at large and small pharmaceutical companies and academic institutions, yielding inhibitors of some steps and not of others.

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doi:10.1016/j.bcp.2005.10.029

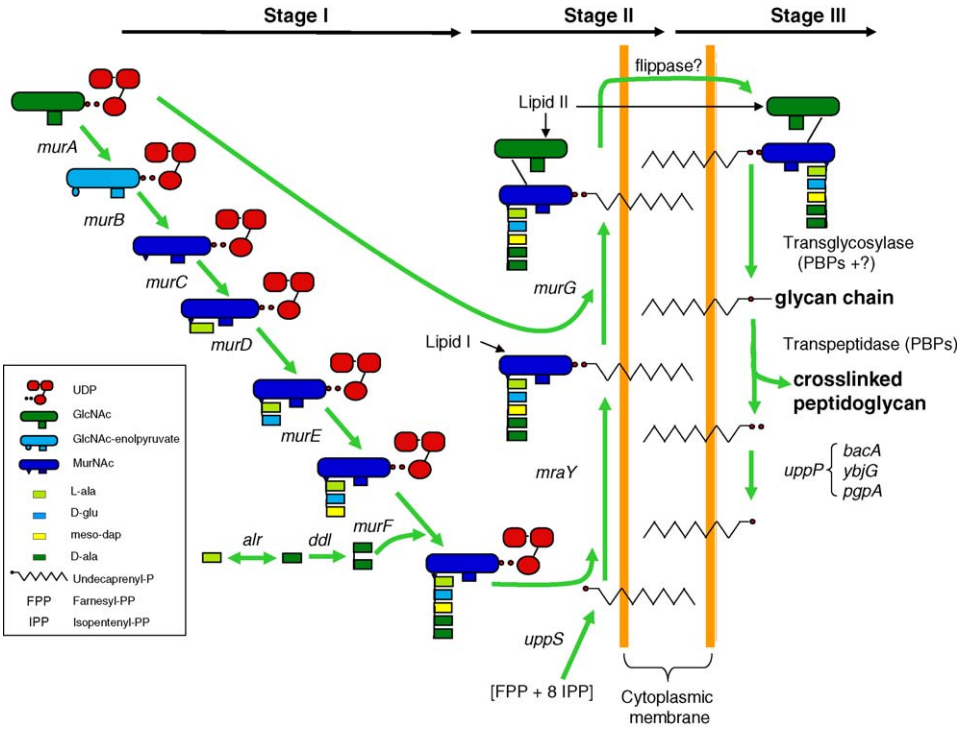

Fig. 1 – Schematic representation of the essential steps of peptidoglycan synthesis in *E. coli*.

Fewer of these steps have yielded marketable drugs. Is this indicative of poor target quality, poor screens, poor sample libraries? Will inhibitors of the ''unexploited targets'', those for which there are no marketed inhibitors, lead to viable drugs? Is it worthwhile to continue in the quest for new cell wall targeted antibacterials? To predict the future is not easy (!), but in this case, past performance may help to predict future events.

Fig. 1 is a schematic of the cell wall pathway based on the *Escherichia coli* system. Stages I and II include steps occurring in the cytoplasm, with Stage II reactions localized to the inner face of the cytoplasmic membrane. Stage III reactions occur on the extracytoplasmic side of the membrane. Differences from this scheme exist in other genera, as in staphylococci which have three additional essential genes (*femA*, *femB* and *femX*) involved in the synthesis of the interpeptide pentaglycine crossbridge which is added to Lipid II (post *murG*). All of the functions pictured are essential in *E. coli* and most other bacteria. The *murI* (glutamate racemase) and *dapF* (LL-diaminopimelate epimerase) genes have been excluded because their functions are not universally conserved. A number of steps of peptidoglycan synthesis have been shown, in various bacteria, to be carried out by duplicated or multiple genes. For example, Gram positive bacteria have two *murA* genes [10]. Many bacteria have two D-ala-D-ala-ligases (encoded by *ddlA* and *ddlB*) [11]. There are three genes for undecaprenyl pyrophosphate phosphatase (UppP) in *E. coli* (encoded by *bac*, *ybjG* and *pgpA*) [12]. In each of these cases, while deletion of a single gene

Table 1 – Validated antibacterial inhibitors of cell wall reactions

Target	Enzyme inhibition	Substrate sequestration
murA	Fosfomycin	
alr	Cycloserine, alaphosphin, O-carbamyl-D-serine, β-Cl-alanine, fluoro-D-alanine	
ddlA/B	Cycloserine	
mraY	Tunicamycin, pacidamycin, napsamycin, liposidomycin, muraymycin, mureidomycin, capuramycin	
murG		Ramoplanin, enduracidin, janiemycin
Transpeptidases	Penicillins, cephalosporins, cephamycins, carbapenems, monobactams	Glycopeptides (vancomycin, teicoplanin, etc)
Transglycosylases	Moenomycin, diumycin, prasinomycin, macarbomycin	Ramoplanin, glycopeptides, mersacidin-type lantibiotics, mannopeptimycin
UppP		Bacitracin

appears to indicate non-essentiality, the functions are essential, in that dual or triple deletion strains are nonviable. These are still useful antibacterial targets if a single compound can inhibit each of the analogs (or paralogs).

Antibacterial inhibitors of enzymes of many steps are known—though some only by substrate sequestration. Antibiotics for which antibacterial activity has been validated as due to inhibition of cell wall synthetic reactions are listed by target in Table 1. Many whole cell and enzymatic screens for inhibitors of cell wall synthesis have been published, with very little output disclosed. This reflects the tendency of large pharmaceutical companies to publish screening strategies and chemical optimization of screening leads after the program has been abandoned. Indeed, many of the screens and compounds mentioned herein are from groups or companies that have turned elsewhere and even abandoned antibacterial research. These programs or similar ones may rise again and wheels may be constantly reinvented.

3. Whole cell screens and output

3.1. *Whole cell "phenotypic" screens*

Initially, whole cell screens to detect inhibitors of cell wall synthesis were based on their expected phenotype. The spheroplasting screen [2] used at Merck (Rahway, NJ) from the 1960s to 1990s was based on the finding of Lederberg [13] that penicillin could induce spheroplast formation in Gram negative organisms in osmotically stabilized media. Inhibitors of many steps of the cell wall pathway were detected by this screen, including inhibitors of MurA (fosfomycin), Alr/Ddl (cycloserine), MraY (tunicamycin), transpeptidase (cephamycin C, thienamycin, penicillins, cephalosporins) and transglycosylase (moenomycin) [2]. Thus, as known from the work with penicillin and as expected from the work of Lugtenberg which showed that temperature-sensitive conditional mutants of the *murC* through *murF* genes lysed at non-permissive temperature [14–17], the screen was capable of detecting inhibitors throughout the pathway. The use of Gram negatives made this screen insensitive to many compounds excluded by the outer membrane (although later iterations used a permeable *E. coli* mutant). The L-form screen of Lepetit Research Center (Gerenzano, Italy) [18] identified compounds, which inhibited growth of wild type but not L-forms of bacteria, which lack cell walls. This screen should theoretically detect inhibitors of all steps of the pathway. Teicoplanin (a lipoglycopeptide), actagardine (a lantibiotic) and ramoplanin (a glycolipopeptide) were discovered via this screen [19–21].

The spheroplasting and L-form screens detected cell wall-specific activity at concentrations at or above the MIC. While the sensitivity of these assays was thus limited, they had the benefit of identifying activities, which were relatively specific for cell wall inhibition. If a given compound (or fermentation extract) had other activities, this would tend to counteract the cell wall-specific activity. That is, an off-target toxic, lytic, or, for example, antiprotein synthetic activity, prevents the formation of spheroplasts and obviates the L-form differential. The counterscreen was thus built in to the primary screen. These screens were used almost exclusively for screening natural products and were quite productive. But this screening took place in the golden age of natural product antibiotic discovery, when the resource was relatively unplumbed. As time went on, these screens had diminishing novel output from standard microbial sources and were eventually retired. It should be noted that the possibility of exploiting previously "unculturable" bacteria as a source may provide a renaissance for natural products antibiotic discovery. Methods for amplifying, cloning and expressing "metagenomic" DNA from soil have been proposed by Courtois [22], Martinez [23] and reviewed by Watts [24] and Handelsman [25]—with instances of cloned natural product operons reported by Donadio [26] and Seow [27]. This approach has been the basis for programs at a number of companies such as Ariad (Cambridge, MA), Diversa (San Diego, CA), Ecopia (Saint-Laurent, Quebec), Terragen (now Cubist [Lexington, MA]), and Biosearch Italia (Gerenzano, Italy). Novobiotic Pharmaceuticals (Cambridge, MA) is engaged in novel antibiotic discovery using methodology for culturing previously unculturable bacteria [28]. If these new sources prove productive, the simple whole cell phenotypic screens mentioned above could be profitably reactivated.

Although such screens were used over a period of nearly 40 years, none were reported to have detected specific inhibitors of the *mur* pathway enzymes MurB through MurF, even though many of them should have been theoretically sensitive to such inhibitors (see Section 6). Thus, since extracytoplasmic targets, such as transglycosylases and transpeptidases were physically accessible and targets in the later stages of cell wall synthesis were already validated by the existence of inhibitors, whole cell screens geared toward detecting inhibitors of the late stages of cell wall synthesis were deployed. A screen for glycopeptides using an agar diffusion assay was reported where the diffusion rate of glycopeptides was specifically retarded by the presence of di-*N*-acetyl-L-lys-D-ala-D-ala [29]. Similarly, the use of D-alanyl-D-alanine agarose columns [30] led to the discovery at Lepetit Research Center (Gerenzano, Italy) of A40926 [31] the parent of the semisynthetic glycopeptide dalbavancin, and at Smith Kline French (Philadelphia, PA) of the kibdelins [32].

Further phenotypic screens employed stress-inducible promoters driving various reporters which respond to inhibition of specific pathways. Transcriptome array analysis showed upregulation of various genes by inhibitors of late steps in cell wall synthesis in *Staphylococcus aureus* and *Bacillus subtilis* [33–36] and by cycloserine, oxacillin and bacitracin in *S. aureus* [37], indicating that stress-inducible reporter strains could be used in identifying cell wall mode of action of novel compounds, and, by extension, screening for them. In 2004, a stress-response assay for inhibitors of five pathways, including cell wall, in *B. subtilis* was described [38]. The extent of any stress-response screening for novel bacterial cell wall targeting antibacterials is unknown, and no inhibitors have been reported to have been discovered in that way. Stress-inducible screens and screens using other specifically inducible promoters driving reporters have the benefit of sensitivity over the spheroplasting and L-form screens in that the readout is evident (and even required) at sub-MIC levels.

Researchers at Millennium (Cambridge, MA) and Wyeth (Pearl River) collaborated for a number of years on development and deployment of whole cell phenotypic screens for cell wall inhibitors. One such screen monitored induction of a *C.*

freundii β-lactamase cloned in a permeable strain of *E. coli* [39]. The β-lactamase was induced by fosfomycin, cycloserine, ramoplanin, moenomycin, cefoxitin and vancomycin. While no inhibitors derived from this screen were described in this paper, it is clear that workers at Wyeth continued looking for cell wall inhibitors and have recently published a number of inhibitors based on leads from undisclosed biological screens against natural products and chemical libraries. These were phenyl thiazolyl urea and carbamate derivatives inhibiting both MurA and B [40] and 2-phenyl-5,6-dihydro-2*H*-thieno[3,2-c]pyrazol-3-ol derivatives inhibiting Mur B, C and D [41]. A MurAB inhibitor had antibacterial activity and did inhibit cell wall synthesis in whole cells and the antibacterial activity of the MurBCD inhibitors showed some correlation to enzyme inhibition, but it has not yet been shown that the MICs of these compounds are solely related to inhibition of the targeted enzymes. The β-lactamase induction screen [39] was used in conjunction with secondary assays to pinpoint inhibitors of late steps of the pathway [42] and detected inhibitors of transglycosylation and MraY, among them, the MraY inhibitor muraymycin~~s~~ [43]. Millennium also reported two other screening strategies for transglycosylase inhibitors [44]: a screen for compounds supporting the growth of vancomycin or moenomycin dependent *E. faecalis* strains and a screen using the *vanH* promoter fused to beta-galactosidase. Thielavin B, a fungal product was detected in these screens and was shown to inhibit peptidoglycan synthesis in an in vitro assay.

3.2. *Regulated expression screens*

Two groups have reported on genome-wide identification of essential bacterial genes based on regulatable expression by antisense RNA for all theoretical cistrons. This was done at Glaxo Smith Kline (Collegeville, PA) [45] and Elitra (La Jolla, CA) [46] in *S. aureus*. The latter paper demonstrates how these strains, in which an essential gene target is precisely down-regulated, can afford sensitization to inhibitors of that target gene product and can thus be used for screening. Both groups found a number of cell wall genes to be essential, as expected, and these targets may have been used in screening, but no inhibitors have been reported. Hypersensitization by insertion of a tightly regulated *ara* promoter upstream of selected targets genes, including *murA*, in *E. coli* was used for screening at Bristol-Myers Squibb (Wilmington, DE) [47]. The *murA* test strain was not sensitized to inhibitors of other steps in the pathway. Five MurA inhibitors were found whose antibacterial activity appeared to be due to MurA inhibition and which all inhibited the MurA enzyme, however these inhibitors were not described further.

Sensitization screens create a rate-limiting step and so detect compounds that might otherwise be missed. This could usefully be applied to the *murC–murF* genes.

4. In vitro screens

4.1. *Permeabilized/broken cell*

A number of permeabilized or broken cell screens have been reported. While most targeted inhibitors of Stages II and III, one such screen published by workers at Bristol-Myers Squibb (Wilmington, DE) [48] measured incorporation of [^{14}C]-GlcNAc from [^{14}C]-UDP-GlcNAc into SDS insoluble material in frozen and thawed *E. coli* cells and could detect inhibitors of the whole pathway. A time dependent inhibitor of MurA was found which had antibacterial activity against several bacterial strains and *C. albicans*. This group also reported on a screen for inhibitors of MurG and MraY monitoring incorporation of [^{14}C]-GlcNAc into Lipid II in membranes prepared from cells overexpressing MurG [49]. Many similar screens for MurG and/or MraY have been published that use membrane preparations and varying assay readouts [50–55]. A screen for transglycosylase inhibitors has been reported that is based on moenomycin competition for binding to labeled PBPs in crude membrane preparations [56]. No information has been published on the output of these Stage II and transglycosylase screens.

4.2. *Biochemical—cytoplasmic steps*

Two reviews [3,4] discussing the application of biochemical methodology to screening for inhibitors of the cytoplasmic steps of the mur pathway have been published by groups that have reported on the establishments of such screens [57,58]. As the intermediate substrates of these enzymes (except for MurA) are not commercially available and often hard to isolate in quantity, these screens were constructed with purified MurA through MurF proteins, the initial substrates and cofactors (UDP-GlcNAc, phosphoenolpyruvate, ATP, NADH, L-ala, D-glu, m-dap, D-ala) and run as a "one pot" reaction, one enzyme providing substrate for the next. In both cases, screens were set up so that no step would be rate limiting, which is a theoretical benefit in that it is generally thought that inhibitors of rate-limiting steps will be preferentially detected in whole cell screens. As for many screens described herein, no compound output was indicated.

Workers at Université Laval (Quebec, Canada) screened a combinatorial library of 432 compounds designed around a D-amino acid for inhibition of the MurA, B and C enzymes of *Pseudomonas aeruginosa* in a one-pot assay [59]. Several low potency inhibitors were found which inhibited MurC (IC_{50}s of 30–50 mM) and had activity against *E. coli* and/or *S. aureus*. No evidence connecting antibacterial activity to MurC inhibition was shown. The same group reported screening of a phage display library to find peptides inhibiting MurC [60]. They found two peptides with IC_{50}s of 1.5 and 5 mM. These had no activity against *P. aeruginosa* or *E. coli* but exhibited weak activity against *S. aureus*. The Astra-Zeneca (Waltham, MA) group has reported using a MurC screen employing purified enzyme and substrate [61] which identified a benzofuran acyl-sulfonamide inhibitor that had time-dependent, partially reversible activity competitive with ATP (K_i of 4.5 mM) and UDP-MurNAc (K_i 6.3 mM) but no antibacterial activity was noted.

Three MurA inhibitors were reported by R.W. Johnson PRI (Raritan, NJ) [62] that were found through chemical screening, presumably with a MurA enzyme assay. These inhibitors are reversible (unlike fosfomycin) and are competitive with fosfomycin for enzyme binding. They have antibacterial activity, but that activity is not specifically due to MurA

enzyme inhibition. Interesting MurA inhibitors were recently reported by workers from the University of Kansas (Lawrence, KA), the Max Planck Institute (Dortmund, Germany), Aventis Pharma (Vitry sur Seine, France and Tucson, AZ) and Novexel (Romainville, France) [63]. These 5-sulfonoxy-anthranilic acid derivatives were discovered through high throughput screening and are competitive with UDP-GlcNAc, but no test of antibacterial activity was reported.

5. Inhibitors by design and directed synthesis

Several groups have attempted rational design of inhibitors of cytoplasmic enzymes of the pathway. Inhibitors of MurB were designed based on the UDP-GlcNAc-enolpyruvate substrate by researchers at Bristol Myers Squibb (Wallingford, CT). The initial thialozolidinone leads were low micromolar inhibitors of the enzyme but had no antibacterial activity [64]. Imidazolinones were later described, among them an inhibitor with an IC_{50} of 16 μM and an MIC of 2 μg/ml against *S. aureus*. Although there was a correlation of variation of IC_{50}s and MICs with these compounds, no independent support for antibacterial activity due to MurB inhibition was demonstrated. The pyrazolidinedione scaffold was identified as a potential inhibitor of MurB through screening of a focused chemical library at Wyeth (Pearl River, NY) [65]. Synthesis of alkyl substituted bis(Cl-phenyl)pyrazolidinediones yielded inhibitors of MurB, some with activity against MurA. Several compounds showed reasonable anti-Gram positive activity, though all were highly serum protein bound.

Phosphinate transition state analogs have been designed as specific inhibitors of MurC [66], MurD [67], MurE [68] and MurF [69]. Some are exceedingly potent inhibitors; for example, a MurD inhibitor [67] has a K_i of <1 nM. None of these show antibacterial activity, presumably due to lack of cell entry.

Workers at Merck (Rahway, NJ) reported on phosphinic acid dipeptides [70] and aminooxoalkyl phosphonic [71] inhibitors of D-alanine:D-alanine ligase (Ddl). Some of the phosphinic acid dipeptides showed antibacterial activity, synergized fluoro-D-alanine (an inhibitor of alanine racemase) and prevented fluoro-D-alanine self reversal seen at higher concentrations, which is consistent with the inhibitors having an effect in whole cells on Ddl. The phosphonates had no significant antibacterial activity.

A recent paper [72] describes new, non-β-lactam, inhibitors of penicillin binding proteins (PBPs), the arylalkylidene rhodanines and arylalkylidene iminothiazolidin-4-ones. The antibacterial and PBP inhibiting activities of these compounds show good correlations and they have been shown to inhibit peptidoglycan synthesis in broken and whole cells. Nonspecific activities have not been ruled out in contributing to their antibacterial activity.

5.1. *Modification of Stages II and III inhibitors*

Liposidomycin, a natural product inhibitor of MraY, has been subjected to extensive deconstruction and modification by workers at Aventis (Romainville, France) (for example [73]) yielding simplified submicromolar inhibitors of MraY with good antibacterial activity.

The potent transglycosylase inhibitor, moenomycin, has been similarly deconstructed and derivatized by a group at Advanced Medicine East Inc. (Cranbury, NJ) to yield di- and trisaccharide moenomycin analogs with improved spectrum and bactericidal activity [74,75]. Neither of these approaches has thus far led to development candidates; however, derivatization of glycopeptides to improve their spectrum and pharmacokinetic properties has been pursued widely. The late stage development compounds telavancin (Theravance, South San Francisco, CA) [76] and dalbavancin (Vicuron, now Pfizer) [77] arose from such studies.

The mannopeptimycins [78], discovered at Wyeth (Pearl River, NJ) bind to Lipid II [79] and inhibit transglycosylation. Derivatization of mannopeptimycin has led to a compound, AC98-6446, with greatly improved pharmacokinetics and in vivo efficacy [80].

6. Why have there been so few antibacterial inhibitors of the Stage I mur enzymes?

Table 2 lists published inhibitors of MurA through MurF, which have been described in the above sections. Most have been obtained by biochemical screens or design, rather than phenotypic screens for anticell wall activity. In fact, there has been a marked lack of detection of MurB-MurF inhibitors (except for the multi-target inhibitors found in undisclosed "biological screens" [40,41]) from whole cell screening. Does this lack indicate the inappropriateness of these steps as antibacterial targets? While some of the *mur* inhibitors shown in Table 2 have antibacterial activity, which might be taken as validating these steps as targets, this activity has not been shown to be solely due to *mur* enzyme inhibition for any of these, except for fosfomycin (MurA inhibitor). Antibacterial activity due to enzyme inhibition has not been excluded in many cases, but it should not be assumed. While it does not actually matter what the target of a good antibacterial is, it matters in principle, if the *mur* pathway is to be demonstrated a useful target.

Several obstacles to discovery of antibacterial inhibitors with validated intracellular MurB-MurF targets can be envisioned. For compounds discovered and optimized as enzyme inhibitors, the lack of cell penetration is a most likely barrier. It has been noted [81] that passage of antibacterials across the cytoplasmic membrane is often via utilization of existing transport mechanisms. In the cell wall pathway, this is true, for fosfomycin [82] D-cycloserine [83] and pacidamycin [84] (and likely for other uridyl-peptide MraY inhibitors). Resistance to these inhibitors has been shown to arise with high frequency (in vitro) due to loss of these transport mechanisms [82–84]. However, some studies have shown that certain transport mechanisms are required for virulence [85]; thus inhibitors using these uptake mechanisms (naturally or by design) could be clinically useful. Tautologically speaking, essential metabolic genes are essential because their products are not available, transportable or diffusible from the environment. To generalize wildly, transition state analog inhibitors of essential enzymes (such as the MurC, D, E and F

Table 2 – Inhibitors of Mur A-MurF

Targets	Refs.	Screen/design	Inhibitors	Antibacterial activity	MIC due to target inhibition
murA	[82,103]	Spheroplast assay	Fosfomycin	Yes	Yes
murA	[47]	Underexpression of *murA*	Five undisclosed compounds	Yes	Yes
murA	[48]	Precursor incorporation into frozen-and-thawed *E. coli*	1,1-Diphenyl-1-allyloxy-4-dimethylamino-2-butanone	Yes	Not tested
murA	[62]	Screening chemical library (with MurA enzyme assay?)	Cyclic disulfide, purine analog, pyrazolopyrimidine	Yes	Non-specific inhibition
murA	[63]	High throughput screen (for MurA inhibition?)	5-Sulfonoxy-anthranilic acid derivatives	Not tested	
murA, B	[40]	Optimization based on unknown biological screen	Phenyl thiazolyl urea and carbamate derivatives	Yes	Inhibition of cell wall synthesis; probable other target in respiration
murB	[104]	Design	Imidazolinones	No	
murB	[64]	Design	Thiazolidinones	Yes	Unknown; correlation of MIC and IC_{50}
murB	[65]	Design based on screening of a focused set of compounds	Pyrazolidinedione derivatives	Yes	Unknown
murBCD	[41]	Optimization based on unknown biological screen	2-Phenyl-5,6-dihydro-2*H*-thieno[3,2-c]pyrazol-3-ol derivatives	Yes	Unknown; correlation of MIC and IC_{50}s
murC	[61]	MurC enzyme assay	Benzofuran acyl-sulfonamide	No	
murC	[59]	MurABC screen of combinatorial library	substituted D-amino acids	No	
murC	[60]	MurC screen of phage display library	DHRNPNYSWLS, CQDTPYRNC	Weak vs. *S. aureus*	Not tested
murC	[66]	Design	Phosphinate transition state analog	No	
murD	[67]	Design	Phosphinate transition state analog	No	
murE	[68]	Design	Phosphinate transition state analog	No	
murF	[69]	Design	Phosphinate transition state analog	No	

inhibitors [66–69]) may be poor transport candidates. As with all antibacterial discovery programs, there is a need for exploration of the chemical parameters which allow passive diffusion through membranes without membrane disturbance.

But it is possible that the non-antibacterial enzyme inhibitors can enter bacteria at some rate and yet do not exert strong growth inhibition. In describing and recommending biochemical screens of the entire mur pathway engineered to run with no rate limitation [3,4], the authors of two reviews note that with whole cell screens, inhibitors of rate-limiting steps will be preferentially selected and high enzyme levels and intermediate pools will require high potency inhibitors of the pathway. Possible upregulation of pathway enzymes that would tend to lead to insensitivity to inhibitors is also mentioned as an obstacle [3].

It is not clear which steps are rate limiting for the cell wall pathway. Many of the *mur* enzymes are expressed more or less constitutively [5] but the overall regulation of the pathway is not yet elucidated. Pool sizes in the pathway (in *E. coli*) are well above the K_m for amino acids and close to the K_m for the pathway intermediates [86] thus arguing against high substrate levels (for the intermediates) in the uninhibited cell as a bar to detection of inhibitors. It is more likely that a rise in intermediate levels would occur after inhibitor action. There appears to be feedback inhibition of MurA (by UDP-MurNAc [87], UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide [88]) in some organisms, and so inhibitor action downstream of MurA would decrease this feedback, leading to greater flux through the pathway which would tend to overcome inhibition by substrate competitors. For this reason, non-competitive or uncompetitive inhibitors might be sought. For the Stage II enzymes, it appears that the level of MurG is rate limiting [49]. A posteriori, it might be argued that if inhibitors of rate-limiting steps are preferentially detected in whole cell screens, then the *murA* and *mraY* steps are the rate-limiting steps in the cytoplasm. In any case, it may well be that for significant antibacterial activity, MurB through MurF must be very strongly inhibited (say >99%) in the whole cell, which would require a combination of specific potency and good cell penetration. As noted in Section 3.2, whole cell screens in which targets are underexpressed should lead to the creation of synthetic rate-limiting steps and thus could be very useful in finding inhibitors of the *mur* pathway. While such underexpression might lead to increased pathway flux, it would likely favor non- or uncompetitive inhibitors.

Another route is the design or selection of inhibitors of multiple steps in the *mur* pathway, which might require less complete inhibition of each step (synergy) and would lead to lower rates of resistance selection. It may be that the multi-step inhibitors described by the Wyeth group [40,41] exemplify this.

7. Speculations on target viability of other pathway steps

Aside from the inability of potential inhibitors to enter cells, there is no obvious reason why inhibitors of MurG or UppS

have not been found in screening. Perhaps some inhibitors of MurG also inhibit MraY and are not distinguished in many of the assays described (although the MurG assay [54] would do this). UppP activity, which is extracyctoplasmic but mediated by three enzymes in *E. coli* [12], would require good inhibition of these multiple enzymes.

The mechanism of translocation of Lipid II from the inner to the outer face of the cytoplasmic membrane is unknown and a ''flippase'' activity has been theorized. Such an activity has been proposed for the *rodA* and *ftsW* gene products [89]. The *ftsW* gene is conserved [90] and essential [91] among wall producing bacteria and the *rodA* gene is essential in rod-shaped bacteria [92]. The genes are related in sequence, are required for determination of cell shape [93] and are each transcribed with, physically associated with and necessary for the functioning of PBPs (FtsW with PBP3 [94] and RodA with PBP2 [95]). These integral membrane proteins are founding members of the SEDS (shape, elongation, division, sporulation) family of proteins [92]. While both *rodA* and *ftsW* are essential, it appears that peptidoglycan synthesis can continue at non-permissive temperature in double mutants containing temperature sensitive alleles of both *ftsW* and *rodA* [96,97] and in the absence of the functioning of PBP2 and PBP3, presumably through the functioning of PBP1a or 1b and/or PBP1c [98]. No such SEDS proteins have been so far identified for the PBP1 proteins. Thus, RodA and FtsW cannot be solely responsible for flippase activity unless such flipping is a passive function of these integral membrane proteins due to physical proximity to a peptidoglycan synthetic complex [99]. Such a passive role in flipping of phospholipids across membranes was hypothesized for integral membrane proteins and shown to occur for certain membrane spanning proteins but not for others [100]. For the present, although RodA and FtsW have a role in peptidoglycan synthesis and could be antibacterial targets, it seems unprofitable to focus on any flippase activity they may have. Whole cell screens for inhibition of cell wall synthesis should be able to detect inhibitors of such activity, if it does exist.

8. Substrate sequestration

Several peptidoglycan synthesis inhibitors are active by dint of their sequestration of substrates of extracellular targets (Table 1). The glycopeptides, which bind to the terminal D-ala-D-ala of Lipid II, are a notable example, as is bacitracin which binds to undecaprenyl-PP and prevents its dephosphorylation. A number of inhibitors have other recognition sites in Lipid II. The target of ramoplanin (which also binds to Lipid I) is the minimal PP-MurNAc-L-ala-D-glu [101]. Mersacidin which is specific for Lipid II, seems to require the disaccharide MurNAc-GlcNAc and at least some of the lipid moiety [102] (possibly just the pyrophosphate linkage) but no peptide is required. Mannopeptimycin is Lipid II-specific and seems to have the same binding requirements as mersacidin but the lack of competition by mersacidin for mannopeptimycin binding indicates yet another specificity. Lipid II is a viable target for continued screening.

9. Conclusion

The problem of endowing inhibitors of cytoplasmic enzymes with the chemical characteristics necessary for cell entry and avoidance of efflux remains a barrier to the development of new antibacterial agents of previously unexploited targets. This is a rate-limiting step for antibacterial discovery and must be addressed for antibacterial inhibitors of all cytoplasmic targets including those of the peptidoglycan synthetic pathway. Studies are needed of the chemical characteristics required to promote diffusion through the cytoplasmic membrane without membrane perturbation, since such perturbation is liable to add unwanted cytotoxicity. Alternatively, inhibitors may be modified by addition of moieties that promote uptake via active transport mechanisms, provided that these transport systems are not dispensable for virulence and that the chemical moieties are not subject to degradation in serum.

The lack of antibacterially validated inhibitors of most of the *mur* enzymes, even though they have been screened for over decades, argues that these targets are particularly refractory. It is likely that very potent inhibitors and/or almost complete enzyme inhibition are required for a strong effect on bacterial growth and survival. Leads for such inhibitors might be obtained by underexpression screens which create synthetic rate-limiting steps in the pathway. Design or directed biochemical screening for inhibitors of multiple steps of the *mur* pathway could yield synergistic effects with relatively low potency inhibitors; such multiple inhibitors would be less susceptible to resistance development. Natural products have been a good source of antibacterial agents and efforts to produce secondary metabolites from previously ''unculturable'' bacteria may be productively applied to finding cell wall inhibitors. A variety of antibiotics have been found which sequester extracellular substrates of cell wall synthesis and might be screened for more directly by binding or competition assays. The cell wall remains a source of targets for novel antibacterials, but the particular obstacles to finding antibacterial inhibitors of those targets must be more directly addressed.

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