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Hydrophobic Substituents on Isatin Derivatives Enhance Their Inhibition against Bacterial Peptidoglycan Glycosyltransferase Activity

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Abstract

Moenomycin A, the well-known natural product inhibitor of peptidoglycan glycosyltransferase (PGT), is a large amphiphilic molecule of molecular mass of 1583 g/mol and its bioavailablity as a drug is relatively poor. In searching for small-molecule ligands with high inhibition ability targeting the enzyme, we found that the addition of hydrophobic groups to an isatin-based inhibitor of bacterial PGT significantly improves its inhibition against the enzyme, as well as its antibacterial activity. The improvement in enzymatic inhibition can be attributed to a better binding of the small molecule inhibitor to the hydrophobic region of the membrane-bound bacterial cell wall synthesis enzyme and the plasma membrane. In the present study, a total of 20 new amphiphilic compounds were systematically designed and the relationship between molecular hydrophobicity and the antibacterial activity by targeting at PGT was demonstrated. The *in vitro* lipid II transglycosylation inhibitory effects (IC₅₀) against *E. coli* PBP1b and MICs of the compounds were investigated. Optimized results including MIC values of 6 μ g/mL for MSSA, MRSA, *B. subtilis* and 12 μ g/mL for *E. coli* were obtained with an isatin derivative **5m** which has a molecular mass of 335 g/mol.

1. Introduction

The World Health Organization's recent surveillance data on antibiotic resistance revealed high levels of resistance in a number of serious bacterial infections across many countries [1]. The development of antimicrobial resistance occurs so quickly that it was estimated by 2050 the number of death caused by antibiotic resistance will exceed that caused by cancer [2]. Thus, there

is an urgent need to develop the next generation antibiotics based on new drug targets or therapeutic mechanisms.

The peptidoglycan glycosyltransferase (PGT) that transfers the disaccharide-peptide of lipid II to the growing glycan chain in bacterial cell wall synthesis is regarded as a potential new drug target for new antibiotic development [3]. The PGT domain of penicillin-binding protein is anchored on the outer surface of the bacterial cell membrane by a transmembrane helix (Fig. 1) [4-6]. Lipid II, the substrate of PGT, is a large amphiphilic molecule containing a hydrophilic disaccharide-peptide head and a hydrophobic undecaprenyl diphosphate tail for its anchoring to the bacterial cell membrane during polymerization [7-9]. Moenomycin A (MoeA), the well-known inhibitor for PGT activity, is also an amphiphilic molecule consisting of a hydrophilic pentasaccharide head and a hydrophobic C_{25} -lipid moiety (Fig. 2) [10, 11]. However, the poor pharmacokinetic properties of MoeA prevents its direct utilization as antibiotic for human, and no PGT inhibitors are available for clinical trial or used currently as antimicrobial agents [12]. Nonetheless, previous understanding on the chemistry and biological functions of MoeA [10, 12] and its analogues provide helpful information for rational design of PGT inhibitors [13-15]. Recently, a small molecule-based inhibitor adopting tryptamine as the main molecular scaffold has been reported [16].



Fig. 1. The bacterial peptidoglycan biosynthesis. *E. coli* lipid II and PBP1b (PDB ID: 3VMA) were adopted in this figure.

The active site of PGT is hydrophilic in nature, which interacts with the disaccharidepeptide unit of lipid II or the pentasaccharide moiety of MoeA, while the adjacent surface is hydrophobic, allowing it to immerse in the membrane surface [5]. The structure of PGT also revealed an exposed hydrophobic region close to the active site, which most likely engages in the interaction with the lipid chains of the substrate while it enters the active site [5]. We therefore reason that an effective inhibitor of PGT is likely to be amphiphilic in nature and its hydrophobicity has to be designed in a well-balanced manner in order to maximize the interaction. As shown in Fig. 2, a small molecular fragment of guanidine-tagged isatin is selected to design an effective PGT inhibitor due to its known antibacterial and moderate PGT inhibitory activities [17-19], and a non-polar group (R) with adjustable length can be linked to the nitrogen atom of γ lactam to tune its hydrophobicity. In this study, a new series of isatin-based amphiphilic PGT inhibitors were synthesized and their Quantitative Structure-Activity Relationship with respect to the substituent (R group) of the molecules was studied.



Fig. 2. Chemical structures of *E. coli* lipid II, MoeA and a guanidine-tagged isatin amphiphilic derivative.

2. Results and discussions

2.1 Synthesis of inhibitors

Our previous study on isatin derivatives [19] as PGT inhibitors identified compound **3d** as a relatively potent inhibitor. We reasoned that the effectiveness of **3d** could be caused by a good balance of hydrophilicity and hydrophobicity betwwen the polar aminoguanidine group and the non-polar naphthyl group. In order to explore the molecular hydrophilicity influence in the structure-activity relationship of PGT inhibitors in-depth, twenty new amphiphilic isatin-based inhibitors were systematically designed by regulating the hydrophobicity of the molecules via R group modifications. These small-molecular based inhibitors were synthesized through two different synthetic routes as shown in Scheme 1. For inhibitor **3a-3c**, they were synthesized similar to **3d** as previous; y reported [19] by reacting the primary amines with chloroacetyl chloride in acetonitrile to yield **1**, followed by the reaction with isatin or 5-methylisatin in DMF with potassium carbonate to form **2**. Compounds **3a-3d** were obtained by reacting **2** with aminoguanidine HCl in acetic acid as the solvent under reflux conditions. The compounds were purified by flash column chromatography and isolated with 52-61 % yields.

Inhibitors **5a-5p** were synthesized by the Chan-Evans-Lam coupling reaction [20-22]. The intermediates **4** with a desirable substituent (R_2 group) were prepared through the reaction of phenylboronic acids and isatin with Cu(OAc)₂ as the catalyst. Compound **4** was reacted with aminoguanidine in acetic acid at room temperature to give **5a-5p**. The yields of **5a-5c** were very low in the reaction with aminoguanidine because esterification of acetic acid with the hydroxyl groups of **4** also occurred under reflux condition. However, this side reaction can be reduced significantly when the reaction is taken place under room temperature for 4 h. The isolated yields for the compound were 29-36 %, which is comparable with their analogues **5d-5p** (35-56 % yields).

As shown in Fig. 2, the overall hydrophobicity of the isatin-based inhibitors can be tuned by varying the substituent (R group). The hydrophobicity of this series of 20 compounds were estimated by calculating the averaged LogP (cLogP) values with four different reported calculation methods [23-26]. The averaged cLogP values of the compounds ranged from -1.9 to 4.4. The molecular structure and cLogP values are listed in Table 1 and S2. In general, compounds with the substituent R containing a terminal hydroxyl group showed lower averaged cLogP values, while those compounds possessing a terminal alkoxyl group showed medium averaged cLogP values whereas higher averaged cLogP values of compounds were obtained with a terminal alkyl group of different chain lengths.





Scheme 1. Synthetic routes of isatin derivatives. (i) MeCN, 3h; (ii) DMF, K_2CO_3 , 80 °C, 12 h; (iii) acetic acid / reflux, 2 h; (iv) Cu(OAc)₂, Et₃N, CH₂Cl₂, 48 – 72 h; (v) acetic acid / room temperature, 4h.

2.2 Antimicrobial susceptible testing and their structure-activity relationship

To study the structure-activity relationship between the R group (Fig. 2) of the isatin derivatives and their antibacterial potencies, their antibacterial activities targeting PGT were examined with Gram-positive *S. aureus*, *B. subtilis* and Gram-negative *E. coli*, including the methicillin-resistant *S. aureus* strain (ATCC BAA-41). The MIC results and averaged cLogP values are listed in Table 1. Starting with compounds with methanediylamidyl linkage conserved (**3a-3d**, Scheme 1), **3a** with a terminal *m*-nitrobenzyl group and its R' group replaced from hydro group to methyl group did not further improve the antibacterial activities compared to its parent molecule (R' = H), suggested that the R' of **3a** may not be a promising position for further modifications. However, **3c** showed slight improvement to the antibacterial activity on the 3 strains (24 µg/mL on *S. aureus*; 12 µg/mL on *B. subtilis* and 48 µg/mL on *E. coli*) while **3d** showed even better potencies on *S. aureus* (12 µg/mL), the rigid non-polar naphthyl group of **3d** should play an important role on the enhanced activities.

In compounds 5a-5p, the substituent groups (R_2) can be further categorized as polar hydroxyl (5a - 5c), polar alkoxyl (5d - 5i) and non-polar (5j - 5p) groups (see Scheme 1), to offer a wide range of hydrophobicity for bioactivity comparison. MIC results (Table 1) showed that all compounds with polar hydroxyl and alkoxyl groups (except 5i) had no significant antibacterial activities against both Gram-positive and Gram-negative bacteria. These results may indicate that both the hydroxyl and alkoxyl groups in the substituent cannot achieve a well-balanced hydrophobicity for the amphiphilic inhibitors. Interestingly, the antibacterial potencies increased significantly when we adopted non-polar side groups (5j - 5p). Considering 5j and 5k, both with naphthyl substituents, but 5k (m, p-naphthyl) is 1-fold more potent than 5j (o, m-naphthyl) against the two Gram-positive bacteria (S. aureus and B. subtilis). This may suggest that the branch at *para*-position of the phenyl ring is more potent than that at *ortho*-position. For 5I - 5p, the compounds are analogues with different length of alkyl chains extended from the ortho-position of the phenyl ring of the substituent R₂. **5** with a 4-ethylphenyl substituent showed only moderate MICs, which are similar to that of **3a** and **5j**. However, when increasing the alkyl chain length (5m: n = 4), the antibacterial activities against Gram-positive bacteria were found to improve drastically, MICs against S. aureus and B. subtilis were both lowered to 6 µg/mL. Interestingly, **5m** also showed good antibacterial activity against *E. coli* (12 μ g/mL).

An attempt to further enhance the antibacterial activities by increasing the alkyl chain length from butyl to hexyl and octyl group, however, did not result in further improvement of the MIC against *S. aureus*. These compounds even showed weaker activities against *E. coli* (MIC =

48 µg/mL for **5n** and > 96 µg/mL for **5o** compared to 12 µg/mL for **5m**) and the trend is obviously different from that of *S. aureus*. Similar observation has also been found for MoeA, which shows excellent antibacterial activities against *S. aureus* and *B. subtilis* but no observable activity against *E. coli* because the high molecular mass MoeA cannot pass through the periplasmic region of the Gram negative bacteria [27]. We therefore postulated that the highly hydrophobic compounds **5n** and **5o** may behave in a similar manner as MoeA in that they stay mainly within the outer membrane rather than diffusing into the periplasmic space. On the other hand, the much smaller periplasmic space between the inner membrane and the peptidoglycan layer in Gram-positive bacteria (*S. aureus* and *B. subtilis*) represents a much lower hindrance to the entrance of hydrophobic compounds. Based on the isatin-based compounds examined in this report, a suitable cLogP range of 2.3 - 4.4 was found for the PGT inhibitors.

Compound	D group	S. aureus	S. aureus	B. subtilis	E. coli	Averaged
	K group	(ATCC 29213)	(ATCC BAA-41)	168	(ATCC 25922)	cLogP
3 a	O P P NO ₂	48	48	24	96	-1.90
3b	Part N H	48	48	24	>96	1.53
3c	Prof. N CF3	24	24	12	48	1.94
3d	Part N H	12	12	12	> 96	1.85
5a	ŧ́—∕́—́ОН	> 96	> 96	96	> 96	1.08
5b	€OH	> 96	> 96	> 96	> 96	1.10
5c	ѯ −Он	> 96	> 96	> 96	> 96	1.76
5d	₹- O -	> 96	> 96	> 96	> 96	1.64
5e	ξ-√_−o	96	> 96	24	96	1.94
5f	₹- O -	> 96	> 96	96	> 96	2.00

Table 1. MIC values	$(\mu g/mL)$ and	averaged c	LogP v	values of the	small mole	ecular inhibitors.
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5g	ξ-√_o ^{−o}	> 96	> 96	96	> 96	1.59
5h	₹o	96	96	48	> 96	2.39
5i	₹ o	24	48	12	> 96	2.49
5j	ş	48	48	24	48	2.38
5k	ş	24	24	12	48	2.36
51	ξ-√∕	48	48	12	48	2.20
5m	ξ − √	6	6	6	12	2.97
5n		6	6	6	48	3.69
50	ş	6	6	6	> 96	4.40
5p		6	6	3	48	2.90
MoeA Ampicillin	Not available Not available	< 0.75 < 0.75	< 0.75 > 96	1.5 < 0.75	> 96 6	Not determined Not determined

2.3 Lipid II-based in vitro transglycosylation assay

To study the inhibitory effects of the compounds against PGT, fluorescent lipid II-based transglycosylation inhibition assays were conducted using purified *E. coli* PBP1b [28] as the targeting enzyme. The reaction rate of transglycosylation was fast in Triton X-100 buffered solution and about 35 % lipid II (substrate) was consumed within 5 min (Fig. S3). Five inhibitors (**5d**, **5e**, **5l**, **5m** and **5o**) were selected for the inhibitory assays (Table 2). Interestingly, the IC₅₀ of the compounds are highly correlated to their MIC values.

5d has an extraordinary high IC₅₀ of 2156.1 μ M, indicated that it is virtually not a PGT inhibitor. This result is also consistent to its MIC values of > 96 μ g/mL obtained against 3 selected bacteria species, meaning that it has no observable antibacterial activities. A similar analogue **5e**, however, showed a more than 10-fold decrease in IC₅₀ (203.4 μ M). The difference of *in vitro* activities is reflected in the MIC value of *B. subtilis*, which was lowered to 24 μ g/mL. **5e** is practically not effective against *S. aureus* and *E. coli*. **51**, **5m** and **5o** are analogues with non-polar substituents of different alkyl chain length (n = 2, 4 and 8, respectively). The IC₅₀ of **51** is 125.2 μ M which is slightly lower than that of **5e**, at the same time its MIC values showed a 1-fold decrease against the 3 selected bacteria species; the IC₅₀ of **5m** is reduced to 82.8 μ M and its MICs were also significantly lowered to 6 mg/mL for *S. aureus* and *B. subtilis* and 12 μ g/mL for *E. coli* respectively. These results support that the inhibition effect of the compounds on PGT is closely related to their antibacterial activities.

Increase in the alkyl chain length of the compounds in general enhances the PGT inhibitory activity. Although **50** has the lowest IC₅₀ (12.4 μ M) among the compounds, its antibacterial activities against Gram-positive bacteria did not further improve compared with that of **5m**. On the other hand, the MIC value against Gram-negative *E. coli* dropped significantly (> 96 μ g/mL). This inconsistency might be explained by its too high hydrophobicity (cLogP = 4.4). Its diffusion to the PGT target is hindered by the inefficiency to pass through the periplasmic region of the Gram-negative bacteria. As a result, the antibacterial activity against *E. coli* is compromised regardless of its stronger PGT inhibition ability. The cytotoxicity of two representative compounds **3d** and **5m** were also evaluated against human fibroblast cells (Detroit 551). Their IC₅₀ values were found to be 2.2 and 1.9 μ g/mL, respectively.

Compound	IC ₅₀ (µM)	<u>MIC (µg/mL)</u>				
		S. aureus	B. subtilis	E. coli		
		(ATCC BAA-41)	168	(ATCC 25922)		
5d	2156.1	> 96	> 96	> 96		
5e	203.4	96	24	96		
51	125.2	48	12	48		
5m	82.8	6	6	12		
50	12.4	6	6	> 96		
Vancomycin*	1.01	N.D.	N.D.	N.D.		

Table 2. *In-vitro* lipid II transglycosylation inhibitory constants (IC_{50}) against *E. coli* PBP1b of some compounds and their MICs.

*Positive control with a reported IC₅₀ value of 0.38 μ M using a modified lipid II substrate [29].

2.4 Molecular modelling

From the results obtained, we reason that the small amphiphilic inhibitor **5m** is able to mimic certain properties of MoeA, in which the hydrophilic unit could bind to the active-site pocket of PGT whereas the alkyl chain could interact with the hydrophobic transmembrane region of the enzyme. The binding pose between *E. coli* PBP1b and **5m** was thus investigated by molecular docking using AutoDock Vina [30]. One of the interaction modes depicted in Fig. 3 showed that the hydrophilic guanidyl moiety (shown in blue-violet) is able to bind to the active site pocket of PGT, the same region occupied by the phosphate group of MoeA (shown in cyan), the well-known inhibitor of PGT. In addition, the hydrophobic unit of **5m** (4-butylphenyl group) points to the direction of the transmembrane domain of PBP1b. Detailed interaction between **5m** and individual residues of PGT are shown in Fig. S4.



Fig. 3. Binding pose between MoeA (skeleton shown in cyan), **5m** (skeleton shown in yellow) and *E. coli* PBP1b model. The hydrophobic regions and hydrophilic regions of the PBP1b model were indicated in red and grey, respectively. One of the best interaction modes of **5m** bound with the hydrophilic active-site pocket (indicated in grey) of PGT was calculated (see Fig. S4 for details). The guanidyl moiety of **5m** overlapped with the phosphate moiety of MoeA, suggested that they competed for the same active site pocket. The hydrophobic moiety of **5m** was pointing downward and in similar direction as transmembrane domain.

3. Conclusion

We demonstrated an amphiphilic approach to design potent inhibitors targeting at PGT. Our results showed that using guanidine-tagged isatin as the core scaffold accompanied by a hydrophobic group can lead to PGT inhibitors with broad-spectrum antibacterial activities against both Gram-positive and Gram-negative bacteria. The antibacterial activities of the amphiphilic inhibitors can be tuned by adjusting the overall hydrophobicity of the molecules. MICs of 6 μ g/mL for MSSA, MRSA and *B. subtilis* and 12 μ g/mL for *E. coli* (12 μ g/mL) were obtained with an inhibitor **5m** of averaged cLogP value 2.97. Our findings suggest that an effective inhibitor of PGT should be amphiphilic in nature consisting of a hydrophilic moiety to interact with the active site of the enzyme and a hydrophobic group to interact with the transmembrane region of the active-site pocket of PGT. Nevertheless, in order to maintain bioavailability, the hydrophobicity of the inhibitor molecule should not be too high to prevent it from entering the bacterial cell. The

molecular design of an effective PGT inhibitor should be a well balance of the hydrophilicity and hydrophobicity of the amphiphilic molecule.

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5. Experimental

5.1 Chemicals

Phenylboric acid derivatives, isatin, 5-methylisatin and aminoguanidine HCl were purchased from Aldrich. Dansyl-chloride was purchased from Sigma-Aldrich. Cell culture media were purchased from Oxoid; bacteria strains were purchased from American Type Culture Collection (ATCC). Moenomycin A (MoeA) was purified from flavomycin purchased from International Laboratory as described [31].

5.2 Synthetic procedures for the new isatin-based inhibitors

Compounds 3a - 3d were obtained by following the reported procedures [19].

General procedures for the synthesis of compound 5a - 5p: To a mixture of phenylboronic acid (0.30 mmol), isatin (0.33 mmol), copper(II) acetate (0.30 mmol) and triethylamine (125 µL) in dichloromethane (10 mL), dried molecular sieve (4Å) was added. The mixture was then allowed to react with stirring for 60 h at room temperature. Excessive isatin was removed by silica gel chromatography using a mobile phase of ethyl acetate: petroleum ether = 1:4. Some vacuum dried product (0.15 mmol) was further reacted with aminoguanidine HCl salt (0.27 mmol) with acetic acid (20 mL) as the solvent. The reaction mixture was allowed to stir for 2 h at room temperature. The crude products were precipitated by the addition of diethyl ether (200 mL) into the reaction mixture and were collected by filtration. The pure product was isolated as a yellowish solid after the purification with silica gel chromatography using a mobile phase of methanol: dichloromethane = 1: 4.

5.2.1 (E)-2-(3-(2-carbamimidoylhydrazineylidene)-5-methyl-2-oxoindolin-1-yl)-N-(3-

nitrophenyl)acetamide (3a): The reaction was taken place for 23 h. Isolated yield 52 %. ¹H NMR (400 MHz, CD₃OD) δ : 2.40 (s, 3H, Ar-CH₃), 4.73 (s, 2H), 6.97 (d, *J* = 8.1 Hz, 1H), 7.31 (d, *J* = 7.6 Hz, 1H), 7.59 (t, *J* = 8.2 Hz, 1H), 7.66 (s, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 8.02 (d, *J* = 8.1 Hz, 1H), 8.61 (s, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 21.01, 30.74, 44.00, 110.76, 115.50, 119.88, 120.40, 123.28, 126.63, 131.10, 134.02, 134.88, 138.66, 140.63, 142.83, 149.88, 162.79, 167.60. HRMS calculated for C₁₈H₁₈N₇O₄: m/z: 396.1415 [M+H]⁺, found 396.1405.

5.2.2 (E)-2-(3-(2-carbamimidoylhydrazineylidene)-2-oxoindolin-1-yl)-N-(3-

ethylphenyl)acetamide (*3b*): The reaction was taken place for 23 h. Isolated yield 56 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.23 (t, *J* = 7.6 Hz, 3H), 2.64 (q, *J* = 7.6 Hz, 2H), 4.71 (s, 2 H), 7.00 (d, *J* = 8.1 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 7.23 (q, *J* = 7.5 Hz, 2H), 7.38 - 7.43 (m, 2H), 7.51 (t, *J* = 7.9, 1H), 7.82 (d, *J* = 7.5 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 16.05, 29.86, 43.60, 111.01, 112.31, 118.62, 120.64, 122.97, 124.12, 124.82, 125.28, 129.90, 133.76, 139.21, 144.57, 146.49, 157.67, 162.80, 166.82. HRMS calculated for C₁₉H₂₁N₆O₂: m/z: 365.1721 [M+H]⁺; found 365.1732.

5.2.3 (E)-2-(3-(2-carbamimidoylhydrazineylidene)-2-oxoindolin-1-yl)-N-(3-

(*trifluoromethyl*)*phenyl*)*acetamide* (*3c*): The reaction was taken place for 23 h. Isolated yield 61 %. ¹H NMR (400 MHz, CD₃OD) δ : 4.75 (s, 2H), 7.10 (d, *J* = 8.0 Hz, 1H), 7.23 (t, *J* = 7.6 Hz, 1H), 7.43 – 7.45 (m, 1H), 7.49 - 7.57 (m, 2H), 7.78-7.84 (m, 2H), 8.01 (s, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 43.65, 111.02, 117.49, 120.37, 121.90, 122.90, 124.24, 124.88, 130.93, 132.14, 132.40, 133.73, 138.64, 140.18, 145.19, 157.67, 162.80, 167.27. HRMS calculated for C₁₈H₁₆F₃N₆O₂: m/z: 405.1281 [M+H]⁺, found 405.1289.

5.2.4 (E)-2-(3-(2-carbamimidoylhydrazineylidene)-2-oxoindolin-1-yl)-N-(naphthalen-2-

yl)acetamide (*3d*): The reaction was taken place for 23 h. Isolated yield 62 %. ¹H NMR (400 MHz, CD₃OD) δ : 4.78 (s, 2H), 7.13 (d, *J* = 7.9 Hz, 1H), 7.24 (t, *J* = 7.6 Hz, 1H), 7.41 - 7.54 (m, 3H), 7.59 (d, *J* = 8.9, 1H), 7.77-7.87 (m, 4H), 8.20 (s, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 43.68, 111.06, 118.01, 120.40, 121.00, 122.88, 124.84, 126.28, 127.63, 128.57, 128.63, 129.76, 132.29, 133.72, 135.18, 136.74, 139.86, 145.29, 147.29, 162.83, 167.07. HRMS calculated for C₂₁H₁₉N₆O₂: m/z: 387.1564 [M+H]⁺, found 387.1573.

5.2.5 (E)-2-(1-(4-hydroxyphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (5a):

The reaction was taken place for 64 h. Isolated yield 32 %. ¹H NMR (400 MHz, CD₃OD) δ : 6.83 (d, *J* = 8.0 Hz, 1H), 7.00 (d, *J* = 8.8 Hz, 2H), 7.21 (t, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 2H), 7.44 (t, *J* = 7.6 Hz, 1H), 7.84 (d, *J* = 7.2 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 111.54, 117.40, 120.27, 122.87, 124.89, 125.45, 129.17, 133.57, 138.84, 146.60, 157.68, 159.30, 162.17. HRMS calculated for C₁₅H₁₄N₅O₂: m/z: 296.1142 [M+H]⁺, found 296.1161.

5.2.5 (E)-2-(1-(4-(hydroxymethyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-

carboximidamide (*5b*): The reaction was taken place for 64 h. Isolated yield 29 %. ¹H NMR (400 MHz, CD₃OD) δ : 4.73 (s, 2H), 6.90 (d, *J* = 8.0 Hz, 1H,), 7.24 (t, *J* = 7.5 Hz, 1H), 7.43 - 7.50 (m, 3H), 7.58 - 7.62 (m, 2H), 7.86 (d, *J* = 7.4 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 64.52, 111.49, 120.53, 122.92, 125.03, 127.63, 129.18, 133.22, 133.42, 138.52, 143.88, 145.92, 157.95, 161.85. HRMS calculated for C₁₆H₁₆N₅O₂: m/z: 310.1299 [M+H]⁺, found 310.1301.

5.2.7 (E)-2-(1-(4-(3-hydroxypropyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-

carboximidamide (*5c*): The reaction was taken place for 64 h. Isolated yield 36 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.92 (quin, *J* = 7.1 Hz, 2H), 2.82 (t, *J* = 7.6 Hz, 2H), 3.64 (t, *J* = 6.2 Hz, 2H), 6.90 (d, *J* = 8.0 Hz, 1H), 7.24 (t, *J* = 7.6 Hz, 1H), 7.40 - 7.49 (m, 5H), 7.86 (d, *J* = 7.2 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 32.80, 35.35, 62.10, 111.53, 120.35, 123.02, 125.03, 127.60, 130.91, 131.95, 133.54, 138.65, 144.65, 146.07, 157.62, 161.92. HRMS calculated for C₁₈H₂₀N₅O₂: m/z: 338.1612 [M+H]⁺, found 338.1614.

5.2.8 (E)-2-(1-(4-(methoxymethyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-

carboximidamide (*5d*): The reaction was taken place for 64 h. Isolated yield 41 %. ¹H NMR (400 MHz, CD₃OD) δ : 3.46 (s, 3H), 4.58 (s, 2H), 6.91 (d, *J* = 8.4 Hz, 1H), 7.24 (t, *J* = 7.6, 1H), 7.43 - 7.51 (m, 3H), 7.59 - 7.61 (m, 2H), 7.86 (d, *J* = 7.2, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 58.59, 74.89, 111.51, 120.52, 122.96, 125.06, 127.62, 130.03, 133.44, 133.63, 138.50, 140.63, 145.84, 157.84, 161.82. HRMS calculated for C₁₇H₁₈N₅O₂: m/z: 324.1455 [M+H]⁺, found 324.1453.

5.2.9 (*E*)-2-(1-(4-ethoxyphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (5e): The reaction was taken place for 64 h. Isolated yield 42 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.45 (t, *J* = 7.0 Hz, 3H), 4.14 (q, *J* = 6.9 Hz, 2H), 6.84 (d, *J* = 8.0 Hz, 1H), 7.12 (d, *J* = 8.4, 2H), 7.22 (t, *J* = 7.6 Hz, 1H), 7.38 - 7.45 (m, 3H), 7.84 (d, *J* = 7.2, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 15.08, 64.94, 111.51, 116.58, 120.34, 122.88, 124.93, 126.56, 129.11, 133.54, 138.77, 146.44, 157.73, 160.69, 162.11. HRMS calculated for $C_{17}H_{18}N_5O_2$: m/z: 324.1455 [M+H]⁺, found 324.1454.

5.2.10 (*E*)-2-(1-(4-(ethoxymethyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (*5f*): The reaction was taken place for 64 h. Isolated yield 41 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.28 (t, *J* = 7.0 Hz, 3H), 3.64 (q, *J* = 7.0 Hz, 2H), 4.63 (s, 2H), 6.91 (d, *J* = 8.0 Hz, 1H), 7.24 (t, *J* = 7.6, 1H), 7.43 - 7.51 (m, 3H), 7.59 - 7.61 (m, 2H), 7.86 (d, *J* = 7.8, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 15.48, 67.09, 72.93, 111.57, 120.42, 123.02, 125.08, 127.61, 130.00, 133.52, 133.58, 138.67, 141.04, 145.98, 157.64, 161.91. HRMS calculated for C₁₈H₂₀N₅O₂: m/z: 338.1612 [M+H]⁺, found 338.1614.

5.2.11 (E)-2-(1-(4-(methoxymethoxy)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-

carboximidamide (*5g*): The reaction was taken place for 64 h. Isolated yield 35 %. ¹H NMR (400 MHz, CD₃OD) δ : 3.52 (s, 3H), 5.29 (s, 2H), 6.86 (d, *J* = 8.0 Hz, 1H), 7.20 - 7.28 (m, 3H), 7.40 - 7.46 (m, 3H), 7.84 (d, *J* = 7.6, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 56.37, 95.54, 114.48, 118.39, 120.37, 122.90, 124.97, 127.78, 129.08, 133.50, 138.61, 146.21, 157.79, 158.85, 162.02. HRMS calculated for C₁₇H₁₈N₅O₃: m/z: 340.1404 [M+H]⁺, found 340.1407.

5.2.12 (E)-2-(1-(4-(tert-butoxymethyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-

carboximidamide (*5h*): The reaction was taken place for 64 h. Isolated yield 42 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.35 (s, 9H), 4.60 (s, 2H), 6.90 (d, *J* = 8.0 Hz, 1H), 7.24 (t, *J* = 7.6 Hz, 1H), 7.43 - 7.48 (m, 3H), 7.59 - 7.61 (m, 2H), 7.86 (d, *J* = 7.6 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 26.61, 63.29, 73.82, 110.23, 119.04, 121.68, 123.74, 126.16, 128.52, 131.81, 132.25, 137.34, 140.94, 144.69, 156.28, 160.57. HRMS calculated for C₂₀H₂₄N₅O₂: m/z: 366.1925 [M+H]⁺, found 366.1926.

5.2.13 (*E*)-2-(1-(2-isobutoxyphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (**5i**): The reaction was taken place for 64 h. Isolated yield 48 %. ¹H NMR (400 MHz, CD₃OD) δ : 0.79 [m, 6H], 1.85 [m, 1H], 3.79 [m, 2H], 6.59 (d, *J* = 8.0 Hz, 1H), 7.14 - 7.26 (m, 3H), 7.40 - 7.41 (m, 2H), 7.54 (t, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 7.2 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 19.25, 19.27, 29.36, 75.81, 111.97, 114.57, 120.09, 122.13, 122.54, 122.75, 124.81, 130.66, 132.29, 133.60, 138.58, 146.58, 156.250, 157.69, 162.30. HRMS calculated for C₁₉H₂₂N₅O₂: m/z: 352.1768 [M+H]⁺, found 352.1768.

5.2.14 (*E*)-2-(1-(naphthalen-1-yl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (**5***j*): The reaction was taken place for 62 h. Isolated yield 55 %. ¹H NMR (400 MHz, CD₃OD) δ : 6.42 (d, *J* = 7.9 Hz, 1H), 7.23 (t, *J* = 7.5 Hz, 1H), 7.34 (t, *J* = 7.4 Hz, 1H,), 7.50 - 7.71 (m, 5H), 7.92 (d, *J* = 7.4 Hz, 1H), 8.06 (d, *J* = 8.2, 1H), 8.11 (d, *J* = 8.2, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 118.83, 120.46, 123.02, 123.58, 125.07, 126.97, 127.64, 128.05, 128.50, 129.86, 130.66, 131.01, 131.37, 133.67, 136.24, 138.63, 146.88, 157.74, 162.54. HRMS calculated for C₁₉H₁₆N₅O: m/z: 330.1349 [M+H]⁺, found 330.1362.

5.2.15 (*E*)-2-(1-(naphthalen-2-yl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (5k): The reaction was taken place for 62 h. Isolated yield 50 %. ¹H NMR (400 MHz, CD₃OD) δ : 6.90 (d, *J* = 7.9 Hz, 1H), 7.22 (t, *J* = 7.5 Hz, 1H), 7.40 (t, *J* = 7.7 Hz, 1H), 7.51 - 7.58 (m, 3H), 7.85 - 8.04 (m, 5H). ¹³C NMR (125 MHz, CD₃OD) δ : 111.68, 120.51, 123.03, 124.99, 125.15, 126.66, 128.11, 128.24, 128.99, 129.09, 130.89, 131.69, 133.60, 134.42, 135.02, 138.70, 146.06, 157.70, 162.06. HRMS calculated for C₁₉H₁₆N₅O: m/z: 330.1349 [M+H]⁺, found 330.1364.

5.2.16 (*E*)-2-(1-(4-ethylphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (51): The reaction was taken place for 62 h. Isolated yield 55 %. ¹H NMR (400 MHz, CD₃OD) δ : 1.29 (t, 3H, *J* = 7.6 Hz), 2.74 (q, 2H, *J* = 7.6 Hz), 6.84 (d, *J* = 8.0 Hz, 1H), 7.20 (t, *J* = 7.5 Hz, 1H), 7.35 - 7.43 (m, 5H), 7.84 (d, *J* = 7.5, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 16.08, 29.60, 111.58, 120.39, 122.95, 125.00, 127.64, 130.30, 131.85, 133.55, 138.73, 146.17, 146.69, 157.69, 161.96. HRMS calculated for C₁₇H₁₈N₅O: m/z: 308.1506 [M+H]⁺, found 308.1521.

5.2.17 (*E*)-2-(1-(4-butylphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (**5m**): The reaction was taken place for 62 h. Isolated yield 54 %. ¹H NMR (400 MHz, CD₃OD) δ : 0.97 (t, 3H, *J* = 7.4 Hz), 1.40 (m, 2H), 1.65 (m, 2H), 2.70 (t, *J* = 7.5 Hz, 2H), 6.83 (d, *J* = 8.0 Hz, 1H), 7.20 (t, *J* = 7.6 Hz, 1H), 7.33 - 7.41 (m, 5H), 7.83 (d, *J* = 7.5 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 14.10, 23.16, 34.59, 36.10, 111.31, 120.18, 122.79, 124.83, 127.22, 130.57, 131.57, 133.20, 138.14, 144.90, 145.65, 157.53, 161.55. HRMS calculated for C₁₉H₂₂N₅O: m/z: 336.1819 [M+H]⁺, found 336.1830.

5.2.18 (*E*)-2-(1-(4-hexylphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (5n): The reaction was taken place for 62 h. Isolated yield 52 %. ¹H NMR (400 MHz, CD₃OD) δ : 0.94 (t, 3H, *J* = 6.9 Hz), 1.38 (m, 6H), 1.70 (m, 2H), 2.74 (t, *J* = 7.7 Hz, 2H), 6.89 (d, *J* = 8.0 Hz, 1H), 7.23 (t, *J* = 7.5 Hz, 1H), 7.38-7.46 (m, 5H), 7.85 (d, *J* = 7.5, 1H). ¹³C NMR (125 MHz, CD₃OD) δ: 14.42, 23.69, 30.04, 32.62, 32.88, 36.62, 111.36, 122.54, 124.02, 124.80, 127.59, 130.81, 132.10, 133.48, 138.12, 143.41, 144.45, 145.13, 161.63. HRMS calculated for C₂₁H₂₆N₅O: m/z: 364.2132 [M+H]⁺, found 364.2145.

5.2.19 (*E*)-2-(1-(4-octylphenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide (**5o**): The reaction was taken place for 62 h. Isolated yield 46 %. ¹H NMR (400 MHz, CD₃OD) δ : 0.90 (t, 3H, *J* = 6.7 Hz), 1.30 - 1.36 (m, 10H), 1.68 (m, 2H), 2.71 (t, *J* = 7.6, 2H), 6.85 (d, *J* = 8.0 Hz, 1H), 7.20 (t, *J* = 7.5 Hz, 1H), 7.38 - 7.42 (m, 5H), 7.83 (d, *J* = 7.4, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 14.44, 23.72, 30.35, 30.41, 30.58, 32.64, 33.04, 36.61, 111.59, 120.36, 122.97, 125.01, 127.52, 130.85, 131.83, 133.57, 138.74, 145.29, 146.16, 157.63, 161.96. HRMS calculated for C₂₃H₃₀N₅O: m/z: 392.2445 [M+H]⁺, found 392.2465.

5.2.20 (E)-2-(1-(4-(sec-butyl)phenyl)-2-oxoindolin-3-ylidene)hydrazine-1-carboximidamide

(*5p*): The reaction was taken place for 62 h. Isolated yield 56 %. ¹H NMR (400 MHz, CD₃OD) δ : 0.90 (t, *J* = 7.4 Hz, 3H), 1.32 (d, *J* = 7.2 Hz, 3H), 1.70 (m, 2H), 2.74 (m, 1H), 6.90 (d, *J* = 8.0 Hz, 1H), 7.23 (t, *J* = 7.6 Hz, 1H), 7.41 - 7.47 (m, 5H), 7.86 (d, *J* = 7.2 Hz, 1H). ¹³C NMR (125 MHz, CD₃OD) δ : 12.58, 22.32, 32.16, 42.84, 111.55, 120.44, 122.92, 124.98, 127.55, 129.49, 131.98, 133.46, 138.59, 146.04, 149.94, 157.80, 161.89. HRMS calculated for C₁₉H₂₂N₅O: m/z: 366.1819 [M+H]⁺, found 336.1814.

5.3 Antimicrobial susceptible testing

To prepare fresh bacteria media, frozen stock bacterial strains were streaked on a TSA plate and incubated at 37 °C overnight. A single colony was picked and added to corresponding media and grew at 37 °C for 5 h at 250 rpm. Working bacterial culture solutions were prepared by further diluting 2 µL of the grown media into 5 mL fresh media. The working bacterial culture solutions were used immediately. Minimum inhibitory concentrations (MICs) were conducted using broth micro-dilution method as described [19]. Three bacterial strains were used in this experiment: MSSA (ATCC 29213), MRSA (ATCC BAA-41), *B. subtilis* 168 and *E. coli* (ATCC 25922). MHB was the medium for *B. subtilis* and *E. coli*, CA-MHB was the medium for *S. aureus*.

5.4 Over-expression and purification of E. coli PBP1b

E. coli PBP1b (residues 58 - 604) was expressed and purified as described [5] without the second purification step (size exclusion). The (His)₆-tag was retained after purification. The final protein concentration was measured by Bradford assay using bovine serum albumin as reference protein.

5.5 Lipid II-based in vitro transglycosylation assay

Lipid II was prepared as described [32]. Dansyl fluorophore was attached to lipid II for spectroscopic detection (see structure and mass spectrum of dansyl-lipid II in Fig. S5) [33]. E. coli PBP1b and compounds were pre-incubated at 30 °C for 15 min in reaction buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM CaCl₂) with additional 0.04 % Triton X-100, 25 % DMSO and 1 U/µL muramidase. Dansyl-lipid II (working concentration 2 µM) was then added into reaction mixture and mixed well to initiate the reaction. The reaction was allowed to stand for 5 min at 30 °C, and MoeA (working concentration 15 µM) was added to quench the reaction. The mixtures were stored at -80 °C before HPLC analysis as described [33, 34]. 20 µL of samples were injected into an Agilent 1200 series equipped with a Supelco SAX1 anion-exchange column and eluted using a gradient of NH₄OAc/MeOH (20 mM to 500 mM). Half maximal inhibitory concentrations (IC₅₀) of selected compounds were calculated by GraphPad Prism 7 with the model: $Y=1/(1+10^{(LogIC_{50}-X)*h)})$ (Y, normalized response; X, log[inhibitor]; h, hillslope). The Doseresponse curve for in vitro transglycosylation inhibition assays were shown in Fig. S2. The isatinbased compounds were measured in triplicate while vancomycin (positive control) was measured in duplicate, and half maximal inhibitory concentrations (IC_{50}) were calculated as described [16]. HPLC chromatograms of *in vitro* transglycosylation assay of 5m were shown in Fig. S1.

5.6 Molecular modelling

The docking study was using a *E. coli* PBP1b model built based on a protein crystal structure of *E. coli* PBP1b (PBP ID: 3VMA [5]) with the missing residues 241-246 and 252-266 added by Modeller [35] and Coot with reference to other PGT crystal structures (PBP ID: 3FWL [5], 3NB6 [36], 2OQO [37] and 6FTB [38]). The missing tail of MoeA in 3VMA was replenished based on the MoeA ligand from 6FTB, and was adjusted to point into the membrane (cyan, Fig. 3 of the main text).

Molecular model of the PGT domain of *E. coli* PBP1b in the presence of **5m** was performed using AutoDock Vina [30]. The structure of **5m** was drawn and 3D coordinates were generated by ChemDraw Ultra 8.0. Energy of **5m** was minimized by Avogadro with Merck Molecular Force Field 94 (MMFF94) [39]. PBP1b and **5m** were treated by reported procedures [40], the interaction between the hydrocarbon moiety and membrane did not count. The best mode with **5m** bound to active-site of PGT domain was selected for reporting. Results were shown in Fig. 3 and Fig. S4.

5.7 Cytotoxicity

Human fibroblast cells (Detroit 551) (American Type Culture Collection) were grown in Eagle's Minimum Essential Medium with 10% fetal bovine serum at 37° C in a humidified atmosphere of 5% CO₂ in the air. Cells were seeded at ~5000 cells per well in 96-well plates and allowed to settle overnight. The compounds were diluted into the growth medium with stated concentrations and the plates were incubated for 3 days. Quantitative cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent according to the instructions provided by the manufacturer. All experiments were performed in triplicate and the results are the average of three independent experiments.

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Highlights

- New amphiphilic isatin-based inhibitors targeting bacterial peptidoglycan glycosyltransferase were synthesized and investigated.
- The hydrophobicity of the inhibitors showed significant influence on the inhibition potency to the enzyme and antibacterial activity.
- The structure-activity relationship of the new isatin-based inhibitors was evaluated.
- The isatin-based inhibitors showed high potential to be developed as a broad-spectrum antibiotic.

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IC₅₀ (*E. coli* PBP1b) = 82.8 μ M MIC (*S. aureus*) = 6 μ M MIC (*B. subtilis*) = 6 μ M MIC (*E. coli*) = 12 μ M

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

