**Synthesis, Pharmacological and Toxicological Screening of Penicillin-Triazole Conjugates (PNTCs)**

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Supporting Information

**ABSTRACT:** A series of hybrid antimicrobial compounds were prepared by carboxylic acid protection of 6-aminopenicillanic acid using benzyl alcohol and thionyl chloride succeeded by azide displacement using trifluoromethanesulfonyl azide in dichloromethane. The azide thus formed was reacted with substituted alkynes to furnish benzyl-protected penicillin-triazole conjugates. Benzyl deprotection of the conjugates resulted in furnishing PNTCs under water methanol mixture using Pd/C as a catalyst. The PNTCs (7a–j) formed were screened for in vitro antibacterial potency against pathogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes* and antifungal potency against *Candida albicans*, *Aspergillus niger*, and *Aspergillus clavatus*. Further antimicrobial evaluation revealed compounds 7c, 7d, 7e, 7g, and 7i to be the most compounds of the series with minimum inhibitory concentration value for antibacterial in the range 0.5–50 μg/mL and for antifungal in the range 9–300 μg/mL. Toxicological analysis documented for compounds 7c, 7d, 7e, 7g, and 7i revealed compound 7i to be the most promising member of the series with 1000 and 500 mg/kg LD50, and no-observed-adverse-effect level to facilitate future clinical studies of the same.

1. INTRODUCTION

Antimicrobials are certainly one of the most flourishing forms of chemotherapy in the history of medicine. With the serendipitous discovery of penicillin in 1929 emerged the golden era of antibiotics.† Armed with a high therapeutic window, the β-lactam antibiotics reigned as an elite class of antimicrobials‡ with penicillin enjoying the status of the most frequently prescribed drug for over a decade.§ To date, β-lactams account for approximately 58% of consumed packages of systemically administered antibacterial and approximately 63% of defined each day doses per 1000 inhabitants per day in the hospital sector.¶

Unfortunately in consequence to this widespread use coincident with increasing incidence of bacterial resistance, effectiveness of the safest antibacterial is severely compromised.† Furthermore, β-lactams are also limited because of poor acidic as well as alkaline stability. While the β-lactam ring degrades in acidic gaster, the N-acyl side chain of penicillin hydrolyzes to amine and acid derivatives in alkaline medium. Accordingly, penicillin G, the most effective member of the series,¶ is administered via im injection retarding both its usefulness and therapeutic compliance.© Penicillin V and some semisynthetic penicillins are modified for oral administration; however, they fail to attain the therapeutic prowess of penicillin G. Thus, with the aim of challenging the stated need of the hour, we have synthesized novel antimicrobial agent with increased efficacy, decreased toxicity, and stability in both acidic and alkaline media.

The rationale governing the design concept of the study was that replacing the amide bond in the β-lactam pharmacophore with 1,2,3-triazole would effectively address the problems associated with clinical penicillin. The therapeutic fame of triazoles is dictated by the profound pharmacological profile of the aromatic nucleus. In the same context, the class of 1,2,3-triazoles is antimicrobial of renowned efficacy.© Additionally, the triazole pharmacophore is an established class of antifungal agents.© Thus, we hypothesized that introduction of 1,2,3-triazole into penicillin’s would extend the pharmacological spectrum of the β-lactam moiety to antifungal activity. Also since the molecule is stable against both acidic and basic hydrolysis surrogates of amide bond in penicillin, which triazole would render conjugates with alkaline stability. As for the β-lactam instability in acidic gaster, incorporation of triazole at N-acyl side chain is known to decrease acid...
sensitivity of penicillin. Thus, for the purpose of exploiting the therapeutic advantage of both penicillin and triazoles in the current investigation, we have designed dual-acting heterodimeric penicillin-conjugated triazole analogues on the basis of combinatorial synthesis by molecular hybridization (Figure 1).

Figure 1. Representation of different active parts of newly synthesized compounds against bacterial and fungal infection.

A series of hybrid antimicrobial compounds were prepared by carboxylic acid protection of 6-aminopenicillanic acid using benzyl alcohol and thionyl chloride succeeded by azide displacement using trifluoromethanesulfonyl azide in dichloromethane. The azide thus formed was reacted with substituted alkynes to furnish benzyl protected PNTCs. Benzyl deprotection of the conjugates resulted in furnishing PNTCs under water methanol mixture using Pd/C as a catalyst. The prevailing imperative of the investigation was to establish the efficacy and stability of the neoconjugates in the context of both bacterial and fungal pathogenic strains along with examining the onset and articulation of toxicity in vivo animal models. Relative potency of the predominant with the parent molecule as well as clinical standard was also evaluated with the aspiration to justify bench-to-bedside translation of the laboratory-created molecules as potent and safe antimicrobial agent.

2. RESULTS AND DISCUSSION

6-Azido penicillanic acid was used as an intermediate for the synthesis of PNTCs. The azide and alkynes 5a–j used for the purpose were synthesized using various protection and deprotection strategies. The benzyl-protected PNTCs 6a–j were synthesized in a Fritsch ball mill using copper sulfate and i.-sodium ascorbate as a catalyst at 300 rpm. Deprotection of the ester 6a–j will furnish the desired PNTCs 7a–j.

2.1. Synthesis of 6-Azidopenicillanlates 4 from 6-APA

For the conversion of benzyl (2S,SR,6R)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 2 to benzyl (2S,SR,6R)-6-azido-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 3, diazo transfer is a gentle and lofty yielding reaction. Initially, these reactions were unsuccessful on unprotected 6-APA 1 as azide group was added on the amine as well as carboxylic acid. Then, benzyl alcohol and thionyl chloride 2 were used to protect carboxylic acid 6-aminopenicillanic acid 1 (Scheme 1). The required carboxyl-protected 6-APA was synthesized adapting literature protocols. Using the benzyl-protected aminopenicillanlates, we synthesized protected 6-azidopenicillanlates using freshly prepared triflic azide under basic conditions in triethylamine, toluene solvent mixture. Desired azides 3 were obtained from carboxylic acid-protected amines in moderate to good yields of 60–65%.

2.2. Cu(I)-Catalyzed Cycloaddition Reaction between Alkynes 5a–j and 6-Azidopenicillanlates 4

To distinguish the optimum possible conditions for Cu(I)-catalyzed cycloaddition, reactions of azide 4 with 1-ethyl-4-methoxybenzene 5a, 1-ethyl-3-methoxybenzene 5b, (prop-2-yn-1-yl)benzene 5c and 1-ethynylcyclohexan-1-amine 5d, 1-ethynylcyclohexan-1-ol 5e, trimethyl(prop-2-yn-1-yl)silane 5f, N,N-dimethylprop-2-yn-1-amine 5g, N,N-diethylprop-2-yn-1-amine 5h, N,N-dipropylprop-2-yn-1-amine 5i, and N-butyl-N-(prop-2-yn-1-yl)butan-1-amine 5j were carried out in ball mill at 300 rpm. Our choice of terminal alkynes is informed by the possibility that cycloaddition between these azides 4 and alkynes 5a–j will, respectively, furnish triazolyl isosteres of penicillin V 7a, ciclacillin 7b, and eight other useful penicillin–triazole derivatives. Cu(I)-catalyzed reaction of azide 3 with alkylene 4a–j resulted in triazoles 6a–j in excellent yield. Benzyl ester deprotection of 6a–j results in furnishing the desired triazolyl compounds 7a–j in 70% overall yield (Scheme 2). We selected a subset of some terminal alkynes which were closely related to penicillin’s derivatives. Some of the alkynes were obtained from the commercial source, and the remaining were synthesized using reported literature protocols. Prop-2-yn-1-yloxybenzene 5c was synthesized after O-alkylation of phenol using propargyl bromide and sodium hydride in anhydrous N,N-dimethylformamide. Compounds 5a, 5b, and 5d–j were purchased from Sigma-Aldrich. Carboxyl-protected 6-azido penicillate was reacted with various alkynes to synthesize carboxyl-protected triazolyl compounds. Ester deprotection of these synthesized compounds furnishes the desired analogues of penicillin 7a–j.

2.3. Biological Evaluation

The synthesized PNTCs were screened for in vitro antibacterial and antifungal assays. For antibacterial screening, ampicillin, chloramphenicol, norflo-
acin, and ciprofloxacin, while for antifungal activity, nystatin and griseofulvin were used as reference standard. Intensive in vitro antimicrobial evaluation revealed 7c, 7d, 7e, 7g, and 7i to be the most active compounds of the series with minimum inhibitory concentration (MIC) value for antibacterial and antifungal in the range of 2.5–50 μg/mL (Table 1) and for antifungal in the range of 100–300 μg/mL (Table 1). Out of the varied substituent lined to the parent penicillin–triazole scaffold, it was found that the tertiary amine-linked compounds 7g and 7i were relatively more potent than both the reference standards. On the basis of the biological investigation, it can be further deduced that on increasing the length of the N-alkyl chain (7g–i), the potency of the compounds also exhibited an increasing trend. This may be attributed to the increased
penetrating power rendered to the pharmacophoric lead due to long alkyl chain substitution of the parent scaffold (Table 1). Furthermore, it was observed that in comparison to aromatic substitution, the compounds with aliphatic substitution at the pharmacophore nucleus demonstrated enhanced biological activity. In lieu with the same compound, 7d with cyclohexylamine substitution was found to be the most potent compound of the series followed by that of compound 7g with N-dimethyl substitution.

Also, in contrast to the reference standard, the PNTCs 7b, 7c, 7d, 7g, and 7i exhibit relatively low in vitro cytotoxicity to human erythrocytes (Table 1). We then sought the in vivo toxicity profile of the PNTCs in Wistar albino rats. To manifest the lethal dose (LD₅₀) of the potent PNTCs, fixed-dose acute oral toxicity studies were performed wherein the animals were dosed in progressive procedure according to OECD TG 423 (Table 2).

From the study protocol, it was observed that although compounds 7c, 7d, 7e, and 7g exhibited slight epistasis at 300 mg/kg dose and mortality was observed on increasing the dose to 500 mg/kg, LD₅₀ could not be determined. On the other hand, 300 and 1000 mg/kg doses were computed as LD₅₀ for compounds 7d and 7i. Subsequent subacute repeated dose toxicity studies according to OECD TG 407 were performed to determine the no observed adverse effect level (NOAEL) of the potent PNTCs. The studies revealed that compound (7i) did not reveal any toxic expression at dose 500 mg/kg. From the explored biological and toxicological evaluation, it was observed that addition of triazole moiety to penicillin resulted in reduction of toxicity of the PNTCs.

3. EXPERIMENTAL PROTOCOLS

3.1. Material and Measurements. All reagents and chemicals were purchased from Alfa Aesar and Sigma-Aldrich and used without further purification. Thin layer chromatography analyses were performed on 0.2 mm Merck precoated silica gel 60 F254 aluminum sheets, and the spots were visualized under a UV lamp. Final purifications were performed using silica gel 60–120 mesh size. ¹H NMR and ¹³C NMR spectra were referenced to the internal standard tetramethylsilane, in the respective deuterated solvents. Coupling constants (J) are reported in hertz. Processing of the spectra was performed with Topspin software. High-resolution mass spectrometry was recorded on a Bruker Maxis spectrometer. Ultracentrifuge (Sigma-Aldrich, St. Louis, MO), autopipettes, and UV−vis spectrophotometer were used for cytotoxicological studies. Standard antibiotics such as ampicillin, chloramphenicol, Nystatin, and Griseofulvin were procured from Sigma-Aldrich.

3.2. Representative Procedure for Diazo Transfer Reaction. 3.2.1. Preparation of Catalyst Triflic Azide. Triflic anhydride (0.9 mL) was added dropwise to the solution of sodium azide in water at 0−5 °C. The resultant mixture was stirred for 4 h at room temperature. Finally, the triflic azide was extracted with CH₂Cl₂ (5 mL × 3) and washed with saturated sodium carbonate solution (10 mL). Dichloromethane solution containing catalyst is used as such for furnishing carboxyl-protected 6-azido penicillate.

### Table 1. In Vitro Antibacterial, Antifungal, and Hemolytic Activity (Human Erythrocytes) of the PNTCs 7a−j

| compound code | in vitro antibacterial activity MIC(μg/mL) | in vitro antifungal activity MFC(μg/mL) | hemolytic dose (HD₅₀) (μg/mL) |
|---------------|------------------------------------------|----------------------------------------|------------------------------|
|               | EC⁺  | PA⁺  | SA⁺  | SP⁺  | CA⁺  | AN⁺  | AC⁺  |                |
| 7a            | >200 | 109.4 ± 5.19 | 62.5 ± 3.40 | 256.9 ± 1.03 | 950.4 ± 16.5 | 480.6 ± 20.3 | 928.3 ± 18.90 | 7.26 ± 0.24 |
| 7b            | >200 | 190.4 ± 5.19 | 62.5 ± 3.40 | 256.9 ± 1.03 | 950.4 ± 16.5 | 480.6 ± 20.3 | 928.3 ± 18.90 | 7.26 ± 0.24 |
| 7c            | 54.5 ± 3.78 | 62.5 ± 3.40 | 250.4 ± 0.40 | 193.3 ± 0.30 | 159.4 ± 1.60 | 28.7 ± 2.20 | 51.3 ± 1.39 | 16.26 ± 1.20 |
| 7d            | 50 ± 2.35 | 75 ± 2.10 | 50 ± 1.24 | 4.5 ± 0.16 | 120.5 ± 2.45 | 80 ± 3.90 | >500 | 13.65 ± 0.23 |
| 7e            | 50 ± 1.32 | 100 ± 5.29 | 50 ± 1.02 | 15 ± 0.53 | 100.4 ± 1.29 | 500 ± 2.38 | 300 ± 1.34 | 7.36 ± 0.58 |
| 7f            | >1000 | 200 | 62.5 ± 1.48 | >1000 | 1000 ± 8.73 | 500 ± 2.38 | 1000 ± 7.91 | 3.34 ± 0.30 |
| 7g            | 10 ± 0.12 | 0.5 ± 0.02 | 2.5 ± 0.14 | 10 ± 0.45 | 10 ± 1.95 | 1.2 ± 0.25 | >1000 | 19.32 ± 0.26 |
| 7h            | 62.5 ± 3.75 | 32.5 ± 2.01 | >100 | >100 | 1006 ± 2.95 | 507.5 ± 3.04 | 1021.7 ± 13.78 | 12.54 ± 0.31 |
| 7i            | 52.19 ± 1.38 | 25.30 ± 4.26 | 10.27 ± 1.04 | 50.30 ± 2.03 | 62.5 ± 5.06 | 9.3 ± 0.48 | 1000 ± 15.45 | 16.34 ± 0.19 |
| 7j            | 10.5 ± 0.93 | 50.4 ± 0.87 | 10.9 ± 1.38 | 100.3 ± 12.7 | >1000 | >1000 | >1000 | 0.65 ± 0.57 |
| ampicillin    | 100 ± 5.28 | 250 ± 15.7 | 100 ± 3.45 | 9.87 ± 1.04 | 100.5 ± 3.78 | 95.7 ± 3.78 | 100 ± 6.34 | 16.21 ± 0.17 |
| nystatin      | 100 ± 5.28 | 250 ± 15.7 | 100 ± 3.45 | 9.87 ± 1.04 | 100.5 ± 3.78 | 95.7 ± 3.78 | 100 ± 6.34 | 16.21 ± 0.17 |

**MIC** = minimum inhibitory concentration. **MFC** = minimum fungicidal concentration. **EC** = *Escherichia coli* MTCC 443. **PA** = *Pseudomonas aeruginosa* MTCC 1688. **SA** = *Staphylococcus aureus* MTCC 96. **SP** = *Streptococcus pyogenes* MTCC 442. **CA** = *Candida albicans* MTCC 227. **AN** = *Aspergillus niger* MTCC 282. **AC** = *Aspergillus clavatus* MTCC 1323. Data are represented as mean ± standard error of the mean (SEM). All of the experiments were performed in triplicate.

### Table 2. Toxicity Profile of Promising PNTCs

| compound code | LD₅₀ (mg/kg) | NOAEL (mg/kg) |
|---------------|--------------|---------------|
| 7c            | 200          | 200           |
| 7d            | 300          | 200           |
| 7e            | 200          | 200           |
| 7g            | 200          | 200           |
| 7i            | 1000         | 500           |
| ampicillin    | 200          | 100           |
| nystatin      | 300          | 200           |

**LD₅₀** is the lethal dose (in mg/kg) of the most potent members of the PNTCs (7i) was found to be 1000 mg/kg and no observed adverse effect level (NOAEL), 500 mg/kg.
3.2.2. Synthesis of Carboxyl-Protected 6-Azido penicillate. Benzyl ester of 6-aminopenicillanate (3 mmol) was dissolved in dichloromethane (6 mL) in a round-bottom flask. Triethylamine (8 mmol) and a solution of copper sulfate (0.4 equiv) were added to a container successively. Freshly prepared dichloromethane solution of trilic azide was then added, and the solution was brought to homogeneity by adding methanol (4 mL). The resulting solution was stirred at room temperature for 2 h. The reaction mixture was then poured into saturated aqueous sodium bicarbonate (30 mL) and methanol (4 mL). The resulting solution was stirred at room temperature prepared dichloromethane solution of triethylamine (8 mmol) and a solution of copper sulfate into the mixture. The crude product was purified by silica gel chromatography (0.49 g, 90%).16,17

3.2.3. Procedure for the Synthesis of Penicillin–Triazole Conjugates by Planetary Ball Milling. Carboxyl-protected 6-azido penicillate (1 mmol) and alkynes 5a–s (1.1 mmol) were taken in a stainless steel (SS) jar (50 mL capacity) containing 10 SS balls (10 mmol), and sodium ascorbate (0.4 mmol) was further added to it followed by the addition of copper sulfate (0.2 mol mmol). The resulting mixture was subsequently ground in a planetary ball mill (Retech PM-100, Retech GmbH, Germany) at 300 rpm. After completion of the reaction, the mixture was dissolved in ethyl acetate (EA) and isolated by column chromatography (EA/hexane) to furnish analytically pure product.18

3.2.4. Procedure for the Deprotection of Benzyl Ester. Benzyl-protected 6-triazolypenicillanic derivatives (6a–j, 5 mmol) were dissolved in a mixed solvent of ethyl acetate and methanol. Pd/C (0.25 g) was added, and the solution was stirred under hydrogen atmosphere (reaction time, 3 h; hydrogen pressure, 5 bar) at room temperature. The reaction mixture was filtered and evaporated in vacuum to furnish the analytically pure product.19

3.2.5. Biological Screening. 3.2.5.1. Antimicrobial Assay. All of the synthesized PNTCs were inspected for antimicrobial activity against two Gram-positive bacterial strains, two Gram-negative bacterial strains, and three fungal strains using agar dilution method.20 The strains procured from Institute of Microbial Technology, Chandigarh, are as follows: *E. coli* (MTCC 443), *P. aeruginosa* (MTCC 1688), *S. aureus* (MTCC 96), *S. pyogenes* (MTCC 442), *C. albicans* (MTCC 227), *A. niger* (MTCC 282), and *A. clavatus* (MTCC 1323). Ampicillin, chloramphenicol, ciprofloxacin, and norfloxacin were used as standard control drugs for antibacterial activity, whereas griseofulvin and nystatin were used as standard control drugs for antifungal activity. The results are reported in Table 1 in the form of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).

3.2.5.2. Ex Vivo Cytotoxicity. Hemolytic Assay Erythrocyte suspension was prepared by centrifuging 10 mL of whole human blood with isotonic buffer for 10 min at 3000 rpm. The supernatant was separated, and packed cells were suspended with equal volume of normal saline and re-centrifuged. The process was repeated until a clear supernatant was obtained. From the resultant, 10% erythrocyte suspension was prepared. To 2 mL of saline solution, 1 mL of PBS and 1 mL sample solution (7a–j, ampicillin, Nystatin) and 0.5 mL were added and incubated at 37 °C for 30 min. After incubation the reaction vessel was allowed to cool, and absorbance was measured spectrophotometrically at 560 nm. Percentage hemolysis and HD50 of the compound under study were calculated.21

3.2.6. Toxicological Evaluation. 3.2.6.1. Animals Used. For acute toxicological analysis as well as determination of LD50, female, while for subacute toxicological, either sex Wistar albino rats were used. All the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC). The experimental animals were housed at constant temperature (21 ± 2 °C) and RH 55 ± 5% with 12 h alternate light and dark cycles and free access to food and water. The protocol used in context of the study was pre-approved by the Institutional Animal Ethical Committee (Reg No: 379/CPCSEA/IAEC-2018/030).

3.2.6.2. Single-Dose Acute Toxicity Studies. Acute toxicity studies of the 7c, 7d, 7e, 7g, and 7i were performed as per OECD TG 420. Animals were dosed in stepwise method using set dose levels of 5, 50, 200, 300, and 2000 mg/Kg bw. A total of five animals per dose level were used. Former to dosing, the animals were subjected to overnight fasting. The test substances were administered orally. Sighting studies were performed to find the starting dose of the study. Based on the absence and presence of signs of toxicity or mortality, further dose levels were evaluated. A period of 24 h was allowed between administrations of each subsequent dose level. All the animals were observed for 14 days.22

3.2.6.3. Determination of LD50. To determine the LD50 of the synthesized conjugates, OECD test guideline 425 (up and down procedure) was followed with slight modification. As per the described study design, animals were segregated into control and treatment groups and fasted overnight prior to the study. The test compounds were orally administered in a single ordered test progression (175, 550, 1000, and 2000 mg/Kg), one at a time at 48 h intervals. Subsequent animals were administered a lower or higher dose on the basis of appearance of either morbidity or mortality. Dosing was discontinued if three consecutive animals survived at the upper bound dose. Following this, an estimate of LD50 was calculated using the maximum likelihood method (Table 2).23

3.2.6.4. Subacute Toxicity Studies. Subacute toxicity studies were performed using the 28-days repeated dose protocol prescribed by OECD TG 407 with a slight modification. Compounds 7c, 7d, 7e, 7g, and 7i were daily administered orally at a dose of 300 mg/kg bw and 500 mg/kg bw for a period of 28 days. The animals were routinely checked for signs for morbidity and mortality. From the data obtained, no observed adverse effect level (NOAEL) was reported.24–26

3.2.7. Statistical Analysis. The *in vitro* antibacterial and antifungal activities were performed in triplicate. The results of *in vivo* acute, median lethal dose and subacute studies were presented as mean ± standard error of the mean (SEM). Variation in mean values was analyzed by one-way analysis of variance, followed by Dunnett’s analysis by means of GraphPad InStat 3 software.

4. CONCLUSIONS

In conclusion, we synthesized a series of PNTCs which are bioisostere of penicillin V, ciclacin, and some relatively close bioisostere of the penicillin family. Compounds 7b, 7d, 7e, 7g, and 7i showed highest antibacterial activity, whereas 7b showed excellent antifungal activity. Further, 7a, 7b, and 7f proved good candidates as antifungal agents. The studies revealed that some of the compounds exhibited activities more than that of reference drugs. Compounds containing
substituted benzene and cyclic ring displayed superior activity to the standard, thereby showing the role of 1,2,3-triazole moiety on the antimicrobial potential of the target compounds. Compound 7g was found to be the most vigorous against *P. aeruginosa*, *S. pyogenes*, and *S. aureus* with MIC values of 0.5, 1.0, and 2.5 μM/mL, respectively. Thus, from the studies, it could be accomplished that conjoining triazole moiety with the β-lactam pharmacophore would yield compounds successful in addressing the issue of drug resistance along with enhanced antibacterial potency and reduced toxicity.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01724.

1H and 13C NMR; high-resolution mass spectra of PNTCs; analysis reports (PDF)

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Notes

The authors declare no competing financial interest.

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