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Assessment of the Antimicrobial and Antiproliferative Activities of Chloropyrazine-Tethered Pyrimidine Derivatives: In Vitro, Molecular Docking, and In-Silico Drug-Likeness Studies

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Abstract: Molecular hybridization (MH) of heterocyclic rings has enabled scientists to design and develop novel drugs and drug-like candidates. In our previous work, considering the importance of MH, we synthesized different kinds of chloropyrazine-tethered pyrimidine derivatives (**22–40**) containing either substituted phenyl or heteroaryl rings at position-6 of the pyrimidine ring and evaluated their antitubercular activity. Herein, we report the antimicrobial and antiproliferative activities of **22–40**. The antiproliferative activity of the target hybrids was superior to the antimicrobial activity. However, some compounds showed greater antimicrobial activity than the standard drugs. For instance, among the nineteen derivatives, compound **31** containing a 2'',4''-dichlorophenyl ring, showed the most potent antibacterial and antifungal activities (MIC 45.37 μ M), followed by compounds **25** and **30** bearing 4''-nitrophenyl and 2'',4''-difluorophenyl scaffolds with minimum inhibitory concentrations (MIC) values of 48.67 μ M and 50.04 μ M, respectively. Compound **35**, containing a bioisosteric 2''-pyridinyl ring, showed the most potent antiproliferative activity against the prostate cancer cell line (DU-145) with an IC₅₀ value of 5 $\pm$ 1 μ g/mL. Additional testing of compounds **22–40** on human normal liver cells (LO2) indicated that the compounds were more selective to cancer cell lines over normal cells. Further, molecular docking of the most potent compound **35** against dihydrofolate reductase (DHFR) (PDB ID: 1U72) had a good binding affinity with a docking score of -6.834 . The SwissADME program estimated the drug-likeness properties of compound **35**. Hybrid **35** is a potential lead molecule for the development of new anticancer drugs, whereas **31** is a promising antimicrobial lead candidate.

Keywords: molecular hybridization; heterocyclic rings; chloropyrazine; pyrimidine; antiproliferative activity; antimicrobial activity; cytotoxicity; molecular docking; drug-likeness

1. Introduction

Heterocyclic scaffolds are interesting chemical structures that are vital in the discovery of novel drugs for the diagnosis and treatment of diseases. Generally, medicinal chemists tether bioactive heterocyclic motifs in order to produce better drugs. Generally, medicinal chemists explore the strategy of combining the entire molecule or a part of the structure such as two or more heterocyclic rings, which can lead to the generation of new types of structures that have potential bioactivities, and this approach is called molecular hybridization (MH). The design, synthesis, and screening of different heterocyclic hybrids are interesting due to their excellent pharmacological properties and ease of preparation. Many heterocyclic rings are privileged structures due to their significant biological profiles.

Hybridization can provide these qualities that can further make the drugs with heterocyclic moieties to have better profiles in terms of their pharmacokinetics, pharmacodynamics, and toxicities [1]. The MH approach not only enhances the pharmacological activity of newly prepared compounds but also modulates the pharmacokinetic properties associated with each heterocyclic scaffold [2]. Additionally, heterocyclic hybrids have good interactions with the molecular drug target and they may have a dual or entirely new mode of action [3]. Nine of the 12 drugs approved in 2021 by the US FDA contain one or more privileged heterocyclic rings as their core elements that are crucial for their activity [4]. This state indicates the implication of utilizing heterocyclic scaffolds in drug discovery programs through the MH strategy.

Pyrazine and pyrimidine are important six-membered nitrogenous rings belonging to a class of heterocyclic compounds known as diazines that are commonly distributed in many pharmacologically useful compounds. These rings are present as the fundamental pharmacophoric group in a variety of drug molecules that are used to treat different diseases. The pyrazine scaffold is present in antitubercular drugs, i.e., pyrazinamide and morinamide; bortezomib, an anti-cancer agent used in multiple myeloma; potassium channel blocker antidiabetic-glipizide; antihyperlipidemic triglyceride lipase inhibitor acipimox; and the potassium sparing diuretics, i.e., amiloride and benzamil [5]. On the other hand, pyrimidine is one of the routinely seen scaffold in drug molecules. This ring is particularly found in drugs used as chemotherapeutic agents because of its ability to interfere with the central metabolic pathways including folate and nucleic acid synthesis. Examples of drugs with a pyrimidine nucleus are antibacterial and antimalarial agents targeting the folate pathway: trimethoprim, iclaprim, sulfadimethoxine, pyrimethamine, and sulfadoxine; antimetabolite and ergosterol biosynthesis inhibitor antifungal drugs: flucytosine and voriconazole; antimetabolite anti-cancer agents: 5-fluorouracil and capecitabine; tyrosine kinase inhibitors: imatinib, dasatinib, and nilotinib; antivirals: ara-c, idoxuridine, zidovudine, stavudine, lamivudine, and emtricitabine; and the antitubercular drug capreomycin (Figure 1). Many substituted pyrazine derivatives were reported with potential antibacterial [6–11], antifungal [12–15], anticancer [16–21], antioxidant [22–25], and antitubercular [9,26–28] activities. Similarly, pyrimidine-based compounds also showed excellent antimicrobial (antibacterial and antifungal) [29–33], antimycobacterial [34–38], antioxidant [39–42], cytotoxic and anticancer [42–52], and antiviral [51–53] properties.

Figure 1. Chemical structures of selected drugs comprising pyrazine and pyrimidine scaffolds.

In our previous study, we reported the antimicrobial and antiproliferative activities of chloropyrazine-linked 1,5-benzothiazepine hybrids [54]. Considering the significant biological activities of pyrimidine derivatives, previously we synthesized and evaluated the antitubercular activity of chloropyrazine-tethered pyrimidine hybrids (22–40) [55]. These derivatives (22–40) are analogues of chloropyrazine-linked 1,5-benzothiazepine hybrids where the pyrimidine ring replaces the 1,5-benzothiazepine motif with the other parts of the compounds being the same (Figure 2). Here, we report the antimicrobial as well as the antiproliferative activities of chloropyrazine-tethered pyrimidine hybrids with the aim to develop potent antimicrobial and antiproliferative hybrids.

Figure 2. Design of chloropyrazine-tethered pyrimidine derivatives.

2. Results & Discussion

2.1. Chemistry

The different chloropyrazine-tethered pyrimidine derivatives (22–40) were obtained by the condensation of chloropyrazinyl chalcones (1–19) with guanidine hydrochloride (Scheme 1) using a base catalyst (KOH) in yields ranging from 56–92%. The molecular structures of the chloropyrazine-tethered pyrimidine hybrids were disentangled using FT-IR and ^1H NMR spectral data and are presented in our previous paper [55].

Scheme 1. Synthesis of target chloropyrazine-tethered pyrimidine hybrids.

2.2. Biological Studies

2.2.1. Antibacterial Activity

Compounds **22–40** were tested for antibacterial activity against four bacterial strains, namely, *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*, using ciprofloxacin as a standard drug, and the results are summarized in Table 1. The physicochemical properties of the chloropyrazine-tethered pyrimidine derivatives were modulated by incorporation of phenyl ring-bearing electron withdrawal and electron-donating groups along with bioisosteric heteroaryl substitution of the phenyl ring at the 6th position of the pyrimidine scaffold.

Table 1. Antibacterial activity of chloropyrazine-tethered pyrimidine hybrids (**22–40**) (MIC in μM).

Compound No.	R	<i>B. subtilis</i> (MIC = μM)	<i>S. aureus</i> (MIC = μM)	<i>E. coli</i> (MIC in μM)	<i>P. aeruginosa</i> (MIC in μM)
22	Phenyl	>200 $\pm$ 0.92	>200 $\pm$ 0.88	>200 $\pm$ 0.53	>200 $\pm$ 0.43
23	4''-chlorophenyl	50.28 $\pm$ 0.09	50.28 $\pm$ 0.28	50.28 $\pm$ 0.19	50.28 $\pm$ 0.89
24	4''-fluorophenyl	53.03 $\pm$ 0.38	53.03 $\pm$ 0.56	53.03 $\pm$ 0.28	53.03 $\pm$ 0.62
25	4''-nitrophenyl	48.67 $\pm$ 0.66	48.67 $\pm$ 0.43	48.67 $\pm$ 0.22	48.67 $\pm$ 0.42
26	4''-hydroxyphenyl	>200 $\pm$ 0.58	>200 $\pm$ 0.15	>200 $\pm$ 0.19	>200 $\pm$ 0.22
27	4''-methylphenyl	>200 $\pm$ 0.23	>200 $\pm$ 0.62	>200 $\pm$ 0.38	>200 $\pm$ 0.73
28	4''-methoxyphenyl	>200 $\pm$ 0.91	>200 $\pm$ 0.45	>200 $\pm$ 0.38	>200 $\pm$ 0.56
29	4''-dimethylaminophenyl	195.84 $\pm$ 0.16	>200 $\pm$ 0.92	>200 $\pm$ 0.55	>200 $\pm$ 0.75
30	2'',4''-difluorophenyl	50.04 $\pm$ 0.31	50.04 $\pm$ 0.21	50.04 $\pm$ 0.81	50.04 $\pm$ 0.88
31	2'',4''-dichlorophenyl	45.37 $\pm$ 0.63	45.37 $\pm$ 0.33	45.37 $\pm$ 0.29	45.37 $\pm$ 0.54
32	3'',4''-dimethoxyphenyl	>200 $\pm$ 0.47	>200 $\pm$ 0.82	>200 $\pm$ 0.94	>200 $\pm$ 0.18
33	3''-methoxy-4''-hydroxyphenyl	>200 $\pm$ 0.52	>200 $\pm$ 0.71	>200 $\pm$ 0.79	>200 $\pm$ 0.95
34	3'',4'',5''-trimethoxyphenyl	85.60 $\pm$ 0.28	85.60 $\pm$ 0.66	85.60 $\pm$ 0.56	85.60 $\pm$ 0.56
35	2''-pyridinyl	>200 $\pm$ 0.62	>200 $\pm$ 0.59	>200 $\pm$ 0.22	>200 $\pm$ 0.28
36	3''-pyridinyl	>200 $\pm$ 0.93	>200 $\pm$ 0.83	>200 $\pm$ 0.38	>200 $\pm$ 0.33
37	4''-pyridinyl	>200 $\pm$ 0.99	>200 $\pm$ 0.77	>200 $\pm$ 0.76	>200 $\pm$ 0.39
38	2''-thienyl	>200 $\pm$ 0.91	>200 $\pm$ 0.32	>200 $\pm$ 0.43	>200 $\pm$ 0.53
39	2''-furfuryl	>200 $\pm$ 0.82	>200 $\pm$ 0.41	>200 $\pm$ 0.24	>200 $\pm$ 0.77
40	5''-pyrrolyl	>200 $\pm$ 0.65	>200 $\pm$ 0.59	>200 $\pm$ 0.61	>200 $\pm$ 0.82
Ciprofloxacin		145.71 $\pm$ 0.18	145.71 $\pm$ 0.18	72.85 $\pm$ 0.33	72.85 $\pm$ 0.33

The numbers in bold designate the activity of the compounds with more potency than the reference standard.

Nonsubstitution on the phenyl ring resulted in abolishment of the activity (**22** = MIC > 200 μM). Among the monosubstituted phenyl-based pyrimidine derivatives (**23–29**) containing different kinds of electron release and electron withdrawal groups at position-4'', the activity ranged from 48.67–200 μM . The compounds with electron withdrawal groups at position-4'' of the phenyl ring (**23–25**) exhibited activity with MIC in the range of 48.67–53.03 μM . These compounds were more potent than the reference drug ciprofloxacin (MIC = 145.71 μM). Among these three, the analogue **25** bearing a 4''-nitro group was most active with an MIC of 48.6 μM . Replacement of the nitro group at the para position by chlorine and fluorine atoms in compounds **23** and **24** decreased the antibacterial activity compared with **25**. On the other hand, the presence of electron-donating groups in position-4'' of the phenyl ring (**26–29**) appeared to be detrimental for antibacterial activity.

The derivatives with disubstitution on the phenyl ring at 2'',4''-positions showed good activity. Among them, the best activity was displayed by compound **31** with 2'',4''-dichlorophenyl substitution with an MIC of 45.37 μM . This derivative was the most active among all the compounds, with 3-fold more potency than the standard. Replacement of

2'',4''-di-Cl by 2'',4''-di-F (**30**) reduced the activity. Incorporation of the electron-donating groups at the meta and para positions was found to be deleterious (**32** & **33**, MIC = >200 µM) as there was a negative effect on the antibacterial activity. While 3'',4''-dimethoxyphenyl derivative **32** was inactive, introduction of a third methoxy group led to compound **34** with enhanced activity (MIC of 85.60 µM). The presence of heterocyclic substituents, independent of their position as well as nonsubstitution was detrimental (Figures 3 and 4). Among the bioisosteres (**35**–**40**), the activity was found to be >200 µM. Irrespective of the incorporation of various heterocycles, the activity was not found to be improved over standard ciprofloxacin. Overall, the most potent compound **31** was 3.2-times more potent against Gram-positive organisms and 1.6-fold more active against Gram-negative bacterial strains than Gram-positive strains. Thus, it can be concluded that activity of these group of compounds depends not only on the nature and position of the substituent on the phenyl ring but also on the number of substituents.

Figure 3. Structure–activity relationship (SAR) features of chloropyrazine-tethered pyrimidine hybrids. EWD = Electron withdrawal; ERG: Electron-releasing group.

2.2.2. Antifungal Activity

The target hybrids were screened for antifungal activity against the fungal strains *A. niger* and *C. tropicalis* using Fluconazole as a positive control (Table 2). Among the monosubstituted derivatives with substituents at the 4''-position of the phenyl ring, the MICs ranged from 97.34 to 214.94 µM and among them, good activity was exhibited by nitro derivative (**25**) with an MIC of 97.34 µM. In general, within the monosubstituted derivatives, compounds bearing electron-withdrawing substituents (F, Cl, & NO₂) showed 2–4-fold better activity over compounds containing electron-releasing groups such as hydroxy (OH), methoxy (OCH₃), methyl (CH₃), and dimethyl amino (N(CH₃)₂). The order of activity of the monosubstituted derivatives was 4''-NO₂ > 4''-Cl > 4''-F > 4''-N(CH₃)₂ >> 4''-OH = 4''-Me-4''-OMe.

Figure 4. Structures of the most potent chloropyrazine-tethered pyrimidine hybrids.

Among the disubstituted compounds, when the phenyl ring was substituted with F (**30**, MIC = 50.04 μ M) or Cl (**31**, MIC = 45.37 μ M) at the ortho and para positions of the phenyl ring, the activity was improved by twofold over compounds **23** (MIC = 100.57 μ M) & **24** (MIC = 106.06 μ M). Interestingly, compound **32**, containing 3'',4''-dimethoxysubstitution, had fourfold (MIC = 46.52 μ M) higher antifungal activity compared to its monosubstituted counter compound **28** (MIC = >200 μ M) against *A. niger*. However, the activity of **32** was not favorable against *C. tropicalis* (**32**, MIC = 186.17 μ M). The best activity among all compounds tested was observed for the 2'',4''-di-Cl-substituted compound **31** with an MIC of 45.37 μ M, followed by compound **32** (MIC = 46.52 μ M). Both compounds were found to be almost twice as potent as fluconazole against *A. niger* and around 1.4-times as potent against *C. tropicalis*. In this case, the order of activity was 2'',4''-di-Cl > 3'',4''-di-OMe > 2'',4''-di-F > 3''-OMe > 4''-OH.

Table 2. Antifungal (μM), antiproliferative and cytotoxic ($\mu\text{g/mL}$) activities of chloropyrazine-tethered pyrimidine hybrids (22–40).

Compound No.	R	<i>A. niger</i> (MIC = $\mu\text{M} \pm \text{SD}$)	<i>C. tropicalis</i> (MIC = $\mu\text{M} \pm \text{SD}$)	* DU-145 (IC ₅₀ = $\mu\text{g/mL} \pm \text{SD}$)	* Human Normal Liver Cells (LO2; $\mu\text{g/mL}$)
22	Phenyl	>200 $\pm$ 0.91	>200 $\pm$ 0.58	40 $\pm$ 1	>40
23	4''-chlorophenyl	100.57 $\pm$ 0.52	100.57 $\pm$ 0.16	18 $\pm$ 2	>40
24	4''-fluorophenyl	106.06 $\pm$ 0.19	106.06 $\pm$ 0.88	47 $\pm$ 2	>40
25	4''-nitrophenyl	97.34 $\pm$ 0.28	97.34 $\pm$ 0.52	112 $\pm$ 2	>40
26	4''-hydroxyphenyl	>200	>200 $\pm$ 0.66	121 $\pm$ 2	>40
27	4''-methylphenyl	>200	>200 $\pm$ 0.73	132 $\pm$ 2	>40
28	4''-methoxyphenyl	>200	>200 $\pm$ 0.22	88 $\pm$ 2	>40
29	4''-dimethylaminophenyl	195.84 $\pm$ 0.98	>200 $\pm$ 0.81	24 $\pm$ 2	>40
30	2'',4''-difluorophenyl	50.04 $\pm$ 1	50.04 $\pm$ 0.22	10 $\pm$ 2	>40
31	2'',4''-dichlorophenyl	45.37 $\pm$ 0.66	45.37 $\pm$ 0.18	12 $\pm$ 2	>40
32	3'',4''-dimethoxyphenyl	46.52 $\pm$ 0.99	186.17 $\pm$ 0.63	72 $\pm$ 2	>40
33	3''-methoxy-4''-hydroxyphenyl	>200 $\pm$ 0.87	>200 $\pm$ 0.29	110 $\pm$ 1	>40
34	3'',4'',5''-trimethoxyphenyl	85.60 $\pm$ 0.55	85.60 $\pm$ 0.90	56 $\pm$ 2	>40
35	2''-pyridinyl	112.39 $\pm$ 0.77	56.19 $\pm$ 0.34	5 $\pm$ 1	>40
36	3''-pyridinyl	112.39 $\pm$ 0.23	112.39 $\pm$ 0.30	78 $\pm$ 2	>40
37	4''-pyridinyl	>200 $\pm$ 0.58	112.39 $\pm$ 0.61	52 $\pm$ 2	>40
38	2''-thienyl	110.44 $\pm$ 0.71	110.44 $\pm$ 0.38	82 $\pm$ 2	>40
39	2''-furfuryl	116.92 $\pm$ 0.82	>200 $\pm$ 0.72	96 $\pm$ 2	>40
40	5''-pyrrolyl	>200 $\pm$ 0.85	>200 $\pm$ 0.53	105 $\pm$ 1	>40
Fluconazole	-	84.14 $\pm$ 0.19	63.10 $\pm$ 0.44	-	-
Methotrexate	-	-	-	11 $\pm$ 1	>75

The numbers in bold designate the activity of the compounds with more potency. * Data are the mean $\pm$ SD ($n = 3$); IC₅₀ in $\mu\text{g/mL}$. The target hybrids and the standard were dissolved in DMSO and diluted with culture medium containing DMSO (0.1%). The control cells were treated with culture medium containing DMSO (0.1%).

Derivatives with bioisosteric heterocyclic substituents showed moderate activity, with MIC in the range of 110.44–200 μM . The order of activity of these compounds was 2''-thienyl > 2''-pyridinyl = 3''-pyridinyl > 2''-furfuryl > 4''-pyridinyl = 5''-pyrrolyl. The six-membered heterocycle-based compounds (35–37) fared better over the five-membered ring containing compounds (38–40), and the best activity was seen for compound 35 (MIC = 56.19 μM) with MIC 1.2-fold higher than that of Fluconazole against *C. tropicalis*. None of them exceeded the activity of the reference drug. Thus, the structure–activity relationship study showed the same behavior for monosubstituted derivatives as in the case of antibacterial activity. In the case of disubstitution, the presence of 2'',4''-di Cl (31) was favorable for antifungal activity followed by 3'',4''-OMe (32). Introduction of 2'',4''-di-F (30) slightly decreased the activity compared to 32, while the presence of 3''-OMe, 4''-OH substitution (33) was detrimental. In the case of heterocyclic substitution, 2''-thienyl was found to have a positive effect on antifungal activity, while 4''-pyridinyl and 5''-pyrrolyl had very negative influences on antifungal activity (Figure 4)

2.2.3. Antiproliferative Activity

Next, the target chloropyrazine-tethered pyrimidine hybrids (22–40) were screened for antiproliferative activity against prostate cancer cell lines (DU-145) and normal human liver cell lines (LO2) using methotrexate as a standard (Table 2). In the monosubstituted phenyl-based pyrimidine series, the prostate cancer IC₅₀'s ranged from 18 $\pm$ 2 to 132 $\pm$ 2 $\mu\text{g/mL}$. Compound 23, containing the electron-withdrawing 4''-Cl group, played a key role in improving the activity, with an IC₅₀ value of 18 $\pm$ 2 $\mu\text{g/mL}$. According to the structure–activity relationships for monosubstituted derivatives, the most favorable was 2'',4''-di-Cl substitution (23), replacement of which by electron donating 4''-N(CH₃)₂ (29) slightly decreased the activity. Introduction of the 2'',4''-di-F substituent (24) decreased the activity more, while the presence of the 4''-NO₂ group lowered activity. Finally, the presence of 4''-Me was detrimental (Figure 4). The order of the antiproliferative activity of these derivatives was 4''-Cl > 4''-N(CH₃)₂ > 4''-F > 4''-NO₂ > 4''-OMe > 4''-OH > 4''-Me.

Among the disubstituted analogues, the best activity was observed with the 2'',4''-di-F substituent (30) followed by 2'',4''-di-Cl (31), which were almost equipotent as methotrexate.

Neither disubstitution nor trisubstitution of the phenyl ring with electron-donating groups brought any significant change in activity. Among the compounds bearing bioisosteric scaffolds, the activity ranged from 5 ± 1 to 105 ± 1 $\mu\text{g/mL}$. The best activity was observed for compound **35**, with an IC_{50} value of 5 ± 1 $\mu\text{g/mL}$, which was twofold more active than the standard drug, i.e., methotrexate (11 ± 1 $\mu\text{g/mL}$). The order of activity of this group of derivatives was 2''-pyridinyl > 4''-pyridinyl > 3''-pyridinyl > 2''-thienyl > 2''-furyl > 5''-pyrrolyl.

The cytotoxicity against normal human liver cell lines (LO2) of compounds **22–40** and methotrexate was found to be greater than 40 $\mu\text{g/mL}$ and 75 $\mu\text{g/mL}$ respectively, indicating less selectivity of compounds towards the normal human liver cells over the prostate cancer cell lines, indicating the safety of the target compounds.

Comparison of the antibacterial, antifungal, and antiproliferative activities between benzothiazepines and pyrimidine derivatives indicated that both benzothiazepines and pyrimidine derivatives showed comparable antibacterial activities; however, the antifungal activity was improved in benzothiazepines compared to pyrimidine derivatives (**21**, MIC 19.01 μM vs. **31** MIC 45.37 μM). On the other hand, the pyrimidine derivative **35** ($\text{IC}_{50} = 5 \pm 1$ $\mu\text{g/mL}$) displayed excellent antiproliferative activity in the series. The benzothiazepines and chloropyrazine-tethered pyrimidine hybrids displayed excellent safety profiles against the LO2 cell line.

Overall, the replacement of the 1,5-benzothiazepine ring with the pyrimidine scaffold improved the antiproliferative activity and reduced the antimicrobial activity in comparison to the benzothiazepine series. The SAR features of the target compounds are displayed in Figure 3. A summary of the potential compounds from this study with significant antibacterial, antifungal, and antiproliferative activities among **22–40** is depicted in Figure 4.

2.3. In Silico Studies

2.3.1. Molecular Docking

The scaffolds similar to the target compounds were reported earlier with respect to their potent anticancer activities [56]. This prompted us to perform a computational molecular docking study to understand the interaction between the most active antiproliferative compound **35** and dihydrofolate reductase (DHFR) by using the GLIDE docking module of Schrödinger suite 2014-3 [57]. The docking study was carried out on compound **35**, where the structure of the complex of DHFR and methotrexate was selected as the docking model (PDB ID: 1U72) [58]. Table 3 depicts the results of docking along with the major interactions for compound **35** and methotrexate with DHFR. More detailed analysis of compound **35** with the DHFR receptor is presented in Figure 5. Compound **35** showed a two-hydrogen bond interaction with the active residues Glu30 and Thr56. The amino part of pyrimidin-2-amine of compound **35** acts as a hydrogen bond donor and established a H-bond interaction with the active site residue Thr56 ($d = 3.57$ Å), and the nitrogen atom of 5-chloropyrazin-2-yl group acts as a hydrogen bond acceptor and formed a H-bond with the active residue Glu30 ($d = 2.02$ Å). Further, the pyrimidin-2-amine group of **35** formed an arene–arene (π – π) interaction with the Phe34 residue. Additionally, several hydrophobic interactions were observed between compound **35** and the active site residues of DHFR i.e., Ile7, Val8, Ala9, Leu22, Phe31, Tyr33, Phe34, Ile60, Leu67, Val115, and Tyr121, which stabilized the binding of compound **35** in the active site of DHFR.

Table 3. GLIDE docking results for compounds **35**, **27** and methotrexate at the active site of human dihydrofolate reductase.

S. No.	Ligand Name	Docking Score	Interactions		
			H-Bonds	π - π	Hydrophobic
1	Compound 35	−6.834	Glu30, Thr56	Phe34	Ile7, Val8, Ala9, Leu22, Phe31, Tyr33, Phe34, Ile60, Leu67, Val115, Tyr121
2	Compound 27	−5.211	Glu30	Phe34	Val8, Ala9, Leu22, Phe34, Ile60, Leu67, Tyr121
3	Methotrexate	−11.808	Ile7, Glu30, Gln35, Arg70, Val115	-	Ile7, Val8, Ala9, Leu22, Phe31, Tyr33, Phe34, Ile60, Pro61, Leu67, Val115, Tyr121

Figure 5. Docking model of compound **35** in the active site of the human dihydrofolate reductase receptor. The red dashed line represents a hydrogen bond. The dark green color residue (Phe34) is involved in the π - π interaction.

Figure 6 illustrates the superimposition of compound **35** and co-crystal (Methotrexate) at the active site of the human dihydrofolate reductase receptor. Compound **35** overlapped well with the native ligand Methotrexate at the active site of DHFR and formed key interactions with the active site residues of DHFR.

A bioisosteric 6-membered nitrogenous heterocyclic ring such as 2''-pyridinyl ring containing compound **35** has very good antiproliferative activity and binding affinity compared to compounds of chloropyrazine-tethered pyrimidine hybrids with a simple phenyl ring. This observation can be verified in the case of compound **27** as it has low antiproliferative activity as well as a low docking score and interaction with active site residues compared to compound **35**. Figure 7 represents the receptor–ligand interaction diagram (2D view) of compounds **27** at the active site of the human dihydrofolate reductase receptor.

Figure 6. Superimposition of compound 35 (grey) with the co-crystal structure (green) at the active site of the human dihydrofolate reductase receptor (PDB code: 1U72).

Figure 7. Receptor–ligand interaction diagram (2D view) of compound 27 at the active site of the human dihydrofolate reductase receptor. The maroon-colored arrow and green-colored lines represent hydrogen bonding and π – π interactions, respectively.

2.3.2. In Silico Drug-Likeness Studies

Compound 35, which showed the best potency in the antiproliferative study, was characterized for certain properties using web-based SwissADME software (Table 4). It can be observed that compound 35 passed the Lipinski rule and had high GI absorption and no inhibition effect on CYP2C19. However, it showed an inhibitory effect against CYP2D6. Hence, compound 35 shows good drug-like properties and is considered a potential lead molecule for further in vivo investigation.

Table 4. Computed properties of compound **35** using SWISSADME.

Compound #	GI Absorption	Lipinski #Violations	CYP2D6 Inhibitor	CYP2C19 Inhibitor
35	High	0	Yes	No

3. Materials and Methods

3.1. Biological Studies

3.1.1. Antibacterial and Antifungal Activities

The strains used for testing antifungal activity were procured from the Institute of Microbial Technology, Chandigarh, India. Ciprofloxacin and fluconazole were used as standard drugs for antibacterial and antifungal assays, respectively. The organisms used in the study included the bacterial strains *Bacillus subtilis* (ATCC-60511) and *Staphylococcus aureus* (ATCC-11632) [Gram-positive], *Escherichia coli* (ATCC-10536), and *Pseudomonas aeruginosa* (ATCC-10145) [Gram-negative], as well as fungi: *Aspergillus niger* (ATCC-6275) and *Candida tropicalis* (ATCC-1369). The antibacterial activity was executed using nutrient agar whereas the antifungal activity testing was done using potato–dextrose–agar medium. The target compounds with a concentration of 1.024 mg/mL were prepared by dissolving 2.048 mg/mL of individual test compounds in different vials separately. The experiments were done three times independently, and the values are reported as the mean of three measurements. The microbes were cultured in their corresponding medium at 37 °C and prepared as a suspension comprising 10⁷ cells/mL by dilution in nutrient broth medium (sterile). The suspension was utilized as the inoculum. The test tubes used for the experiments were incubated for a period of 18 h at 37 °C. Analogous testing was carried out without compound, but with other components including the medium, inoculum, and methanol to check there was no influence of methanol for making the dilutions. Initial indication of growth in the test tube was recorded using a spectrophotometer. The MIC values were determined for the target compounds by taking the concentration used in the test tube just before the first indication of growth [59]. The same protocol was done three times to derive the MIC values of the 19 target compounds, and the results reported are the average of three determinations. Due to the structural difference of ciprofloxacin and fluconazole compared to the target hybrids (**22–40**), the MIC values recorded in µg/mL were transformed into µM.

3.1.2. Antiproliferative Activity

The in vitro antiproliferative activity of pyrimidine derivatives (**22–40**) was screened against DU-145 (prostate cancer cell lines) by the renowned Mosmann's MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay method [54]. The antiproliferative activity of **22–40** was measured to determine their IC₅₀ values by comparison against reference standard Methotrexate (Mtx; positive control). In MTT assay, the principal is based on the fact that in living cells there is a reduction in MTT (soluble, 0.5 mg/mL, 100 µL) due to the enzymatic activity of mitochondrial reductase to blue formazan product.

DU-145 cell lines were cultured at 37 °C and humidified at 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM); 0.1% DMSO was employed to prepare the stock solutions of target compounds (**22–40**). Later, the required concentrations were obtained by dilution using sterile water. Temporarily, cells were placed on 96-well plates at 100 µL total volume, with a density of 1 × 10⁴ cells per well, and allowed to adhere for a duration of 24 h. Further, the medium was substituted with fresh medium containing different dilutions of target compounds and incubated at 37 °C for an additional 48 h in DMEM with FBS (Fetal Bovine Serum, 10%). Then, the medium was substituted by freshly prepared 90 µL of DMEM without any FBS. The above wells were incubated for 3–4 h at 37 °C after treatment with 10 µL MTT reagent (5 mg/mL of stock solution in DMEM without FBS). This resulted in the formation of formazan crystals (blue color). These crystals were solubilized in DMSO (200 µL), and the optical density (OD) was assessed using a micro plate

reader at a wavelength of 570 nm. The assay was done three times as three independent experiments. A similar study was performed in order to check that there was no effect of DMSO on activity. The reproducibility of the results was good, and the standard errors were less than 10%. The results of antiproliferative activity are reported as IC₅₀ (µg/mL) values. Different inhibitor concentrations were added for the calculation of the IC₅₀ values.

3.2. *In Silico Studies*

3.2.1. Molecular Docking

The crystal structures of DHFR and the methotrexate complex were obtained from the Protein Data Bank (PDB ID: 1U72). The protein preparation tool was used for the preparation of the DHFR. The grid was generated by selecting the active site where the co-crystal was located, with a grid box of 10 × 10 × 10 Å (Schrödinger 2014-3). With the use of the 2D sketcher, the potent compound **35** was sketched and energy minimized, and ligand preparation was performed for the generation of different conformers (Schrödinger 2014-3). The various conformers obtained were subjected to molecular docking with XP Glide (Schrödinger 2014-3). The poses generated were assessed, and the best one was reported.

3.2.2. *In Silico* Drug Likeness Prediction

SwissADME, a web tool, was utilized to evaluate the properties of the most potent compound **35** using in-silico parameters such as GI absorption, Lipinski rule of five, and CYP2C19 and CYP2D6 inhibition, in order to meet the requirements of the drug-likeness. (<http://www.swissadme.ch/> (accessed on 17 September 2021)) [60].

4. Conclusions

Here, we report the antimicrobial (antibacterial and antifungal) and antiproliferative activities of 19 chloropyrazine-tethered pyrimidine hybrids (**22–40**) synthesized previously by replacing the 1,5-benzothiazepine scaffold of chloropyrazine-linked 1,5-benzothiazepine derivatives. The tested analogues (**22–40**) showed more antiproliferative activity and less antimicrobial activity than the corresponding chloropyrazine-linked 1,5-benzothiazepine derivatives. The results indicated that the electron-withdrawing atoms/groups on the phenyl ring of the chloropyrazine-tethered pyrimidine hybrids are vital for improving the antimicrobial, whereas the bioisosteric heteroaryl scaffold 2''-pyridinyl substitutions are vital for antiproliferative activity. Compound **31**, containing a 2'',4''-dichlorophenyl ring, showed the most potent antibacterial and antifungal activities with an MIC value of 45.37 µM, while compounds **25** and **30**, containing 4''-nitrophenyl and 2'',4''-difluorophenyl moieties, had MIC values of 48.67 µM and 50.04 µM, respectively. Compound **35**, containing a 2''-pyridinyl ring, showed the most potent IC₅₀ value of 5 ± 1 µg/mL against DU-145. All compounds can be considered safe as they had less selectivity to LO2 normal human liver cells compared to the cancerous cells. Molecular docking results for compound **35** showed major binding interactions at the binding site, correlating with the in vitro antiproliferative activity as well as the SwissADME results of **35** for drug-likeness, suggesting the usefulness of computational studies in the development of new drug-like candidates. The potential lead compounds identified through this work will be useful in the further design and optimization of drug candidates against antimicrobial activities, and compound **35** is a potential lead compound for the development of new anticancer agents.

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