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NEW CARBAMATES OF 1-(4-NITROPHENYL)PIPERAZINE AND *BIS*CARBAMATES OF 6-AMINOINDAZOLE: DESIGN, SYNTHESIS, CHARACTERIZATION, MOLECULAR DOCKING STUDIES AND BIOACTIVITY EVALUATION

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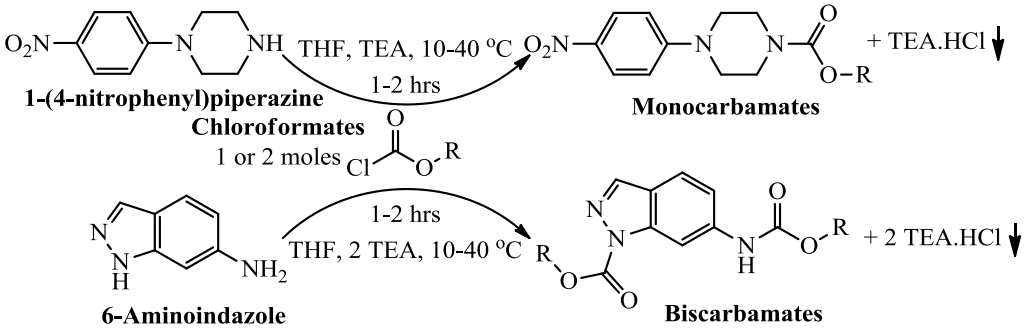
ABSTRACT
Two series of new carbamate derivatives of 1-(4-nitrophenyl)piperazine and *bis*carbamate derivatives of 6-aminoindazole were designed and synthesized in a simple method. The title compounds were characterized by IR, ^{1}H, ^{13}C-NMR, mass spectral data and elemental analysis. The molecules were docked with DNA gyrase A protein of *E. coli*. The dock scores of 06 compounds suggested that they befitted fast into the DNA gyrase A protein binding site which suggests they could find a place in the next generation antimicrobials. The compounds were screened for their *in vitro* antibacterial and antifungal activities against the selected pathogens. Two compounds showed very good antifungal activity against *C. neoformans* than the rest over antibacterial activity. The results also emphasized that the 1-(4-nitrophenyl)piperazine monocarbamates were better antifungals than the corresponding 6-aminoindazole *bis*carbamates.

KEYWORDS: 1-(4-nitrophenyl)piperazine, 6-aminoindazole, antibacterial activity, antifungal activity, molecular docking.

GRAPHICAL ABSTRACT



1. INTRODUCTION
Carbamates have gained recognition as a unique family of compounds that hold a wide spectrum of biological activities. Naturally the carbamate derived from the aminoimidazole is an intermediate in the biosynthesis of inosine, found in tRNAs and whose metabolism led to advances in immunotherapy. Interestingly a carbamate enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase fixes CO_2 molecule as phosphoglycerate in the Calvin Cycle which is essential for plant growth; thus nurtures the entire living world. A potent sodium ion channel blocker, guanidinium alkaloid, zetekitoxin AB (ZTX contains *N*-hydroxy carbamate group) extracted from the skin of Panamanian golden frog, *Atelopus zeteki* was shown to be very potent antagonist of Na_v, with potencies 60-500 fold more than those for saxitoxin (STX contains carbamate group) (fig. 1).[1]

Figure 1: Panamanian Golden Frog, *Atelopus zetiki* and its carbamate skin toxin.

Organic carbamates are derived from the less stable carbamic acid, H_2N–COOH by the substitution of amino and carboxyl moieties with aryl/alkyl or substituted alkyl/aryl or vice versa groups. Transformation of amines to carbamates imparts amazing physiological and therapeutic properties to the derivatives which find applications in agrochemicals such as pesticides - insecticides [aldicarb, carbofuran (Furadan), carbaryl (Sevin), ethienocarb, propaxur and methomyl kill insects by inactivating acetylcholine esterase] fungicides, rodenticides, herbicides, etc.,; pharmaceuticals – as drugs and prodrugs and in organic synthesis – as intermediates. Darunavir (**1**) (fig.2), a carbamate derivative is a protease inhibitor for HIV treatment.[2] Neostigmine (**2**) and revastigmine (structural analogues of natural alkaloid, physostigmine) act as cholinesterase inhibitors.[3] Carisoprodol (Soma) (**3**), meprobamate, etc., act as anxiolytic and muscle relaxant drugs.[4]

As a pharmacophore, carbamate has been extensively explored and exploited in natural, semi-synthetic and synthetic molecules against different clinical conditions and diseases such as potential dual adenosine A_1 and A_{2A} receptor antagonists for the treatment of Parkinson's disease;[5] as antibacterial and antifungal agents;[6-8] as DNA gyrase inhibitors;[9] as Resistance modifying agents against MRSA;[10] as tubulin modulating antifungal and antiproliferative agents;[11] as anti-mycobacterial and anti-*Propionibacterium acnes agents*;[12] as antimicrobial agents;[13] as Fatty Acid Amide Hydrolase (FAAH) inhibitors (URB597 and URB937 (**4**)) in the treatment of neuropathic pain, inflammation and cancer;[14] as antibacterial and antitubercular agents (pyridopyrazine analogs (**5**)) targetting the cell-division protein FtsZ;[15] as AChE and BChE inhibitors;[16] as antimicrobial, anti-mycobacterial and cytotoxic agents;[17] as gene delivery tools;[18] as antiproliferative agents against A549 human lung adenocarcinoma cells and PANC-1 human pancreatic carcinoma cells;[19] as antitumor agents (Mitomycin derivatives (**6**));[20] as NNRTIs in HIV-1 infection;[21] as inhibitor of human cytochrome P-450 in the treatment of HIV (Cobicistat (**7**));[22] as a drug (Riociguat (Adempas®) (**8**)) to treat chronic thromboembolic pulmonary hypertension (CTEPH).[23]

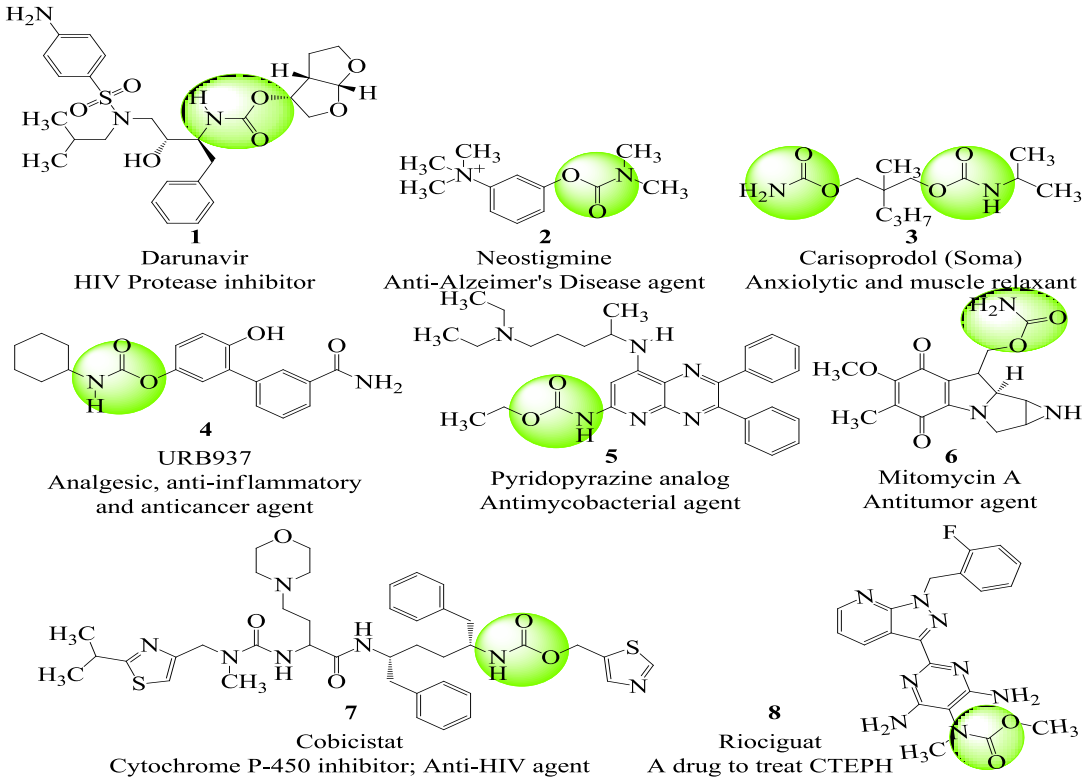


Figure 2: Carbamates of different Biological activities.

Interestingly the *bis*carbamates also find various applications in vivid pathologies. They have been found to be active against *Francisella tularensis* which causes an infectious disease, tularemia or rabbit fever (**9**) (fig. 3);[24] as novel cytotoxic agents;[25] as inhibitors of cholinesterases;[26] as inhibitors of NS5A- non-nucleoside polymerase in genotype 1 hepatitis C virus infection (ombitasvir hydrate (Viekira Pak™) (Technivie®) (**10**)) and as a number of anti-HCV agents viz., Asunaprevir (Sunvepra®), Vaniprevir (Vanihep®) (**11**), Daclatasvir dihydrochloride (Daklinza®) (**12**), Ledipasvir (Harvoni®).[27]

Another entity, piperazine moiety alone has been explored for cytotoxicity,[28] and piperazine derivatives for anticancer activity;[29] the inhibition of FGFR tyrosine kinase;[30] hypotensive and antihypertensive potential;[31] inhibition of phosphodiesterase;[32] cytotoxicity, anti-mycobacterial, anti-neuropathic activity;[33] antimicrobial, anti-biofilm, ROS accumulation and anticancer activities;[34] hCB1 receptor antagonist activity to find applications in the treatment of obesity, type 2 diabetes and metabolic syndromes;[35] HCV NS3 helicase inhibition in HCV replicon assay;[36] inhibition of HIV1 protease and integrase;[37] anti-mycobacterial activity;[38] anticancer activity;[39] antidepressant and antianxiety activity[40] and has been a part of 5-HT_6 receptor ligands to treat CNS diseases.[41] A number of piperazine derivatives have been extensively used in medicine: Lurasidone hydrochloride (*Latuda*®) (**13**) is used as an antipsychotic in the treatment of schizophrenia;[42] vilazodone hydrochloride (Viibryd®) and vortioxetine hydrobromide (Brintellix®) as an antidepressants; bosutinib hydrate (Bosulif®) (**14**) as Src/Abl kinase inhibitor (in blast phase Philadelphia chromosome-positive chronic myeloid leukemia);[43] nintedanib esylate (Ofev®) as an oral triple angiokinase inhibitor; olaparib (Lynparza®) (**15**) in the treatment of germline BRCA mediated advanced ovarian cancer and netupitant (Akynzeo®) is used to treat chemotherapy induced nausea and emisis.[44]

Presumably it was indazole motif and its derivatives which have been largely exploited for their therapeutic value such as anticonvulsant action;[45] as agonists of α_2-adrenoreceptors which find applications in hypotensive, analgesic, anxiolytic, sedative, hemodynamic-stabilizing, and organ-protective agents;[46] as antibacterial, antifungal, antipsychotic, analgesic, anticancer, anti-inflammatory activity, inhibitors of nitric oxide synthases, serotonin agonists and antagonists, NNRTIs, CCR4 antagonists, inhibitors of Factor Xa, aldosterone synthase inhibitors, Protein Tau Phosphorylation inhibitors, inhibitors of hormone sensitive lipase in addition to have been Rho kinase inhibitors;[47] as antiproliferative activity;[48] as antibacterial, antidepressant, anti-inflammatory, antihypertensive and anticancer activities;[49] as antiproliferative and apoptotic activities;[50] as c-Met kinase inhibition;[51] as α_2-adrenoreceptor agonist activity in hypotensive and bradycardic activities;[52] as multi-targeted anti-angiogenesis activity;[53] for modulation of *Cryptosporidium parvum* CpABC3 transporter;[54] and for histone deacetylase and fibroblast growth factor receptor inhibitors in cancer therapy;[55] as DNA gyrase inhibitors (Roche Indazole (**16**));[56] and as treatment for renal cell carcinoma (axitinib (Inlyta®) (**17**)).[57]

Above all carbamates containing a piperazine moiety were used as active Lck inhibitor which also indicated excellent selectivity against Hck, cSrc and KDR;[58] as anticancer agents;[59] against biofilm related MRSA infections;[60] as potent calcitonin gene-related peptide antagonists for migraine treatment;[61] as potent inhibitors of sphingosine kinase 1(an enzyme involved in the cancer and inflammatory diseases)[62] and as herbicides against dicotyledons.[63] Surprisingly indazole scaffolds with piperazine moieties have been exploited for their Rho kinase inhibition activity (DW1865 (**18**)) in cardiovascular diseases;[64] Factor Xa inhibition in cardiovascular diseases[65] and Carbamates, piperazine derivatives and Indazole analogues act as c-Met kinase inhibitors.[66]

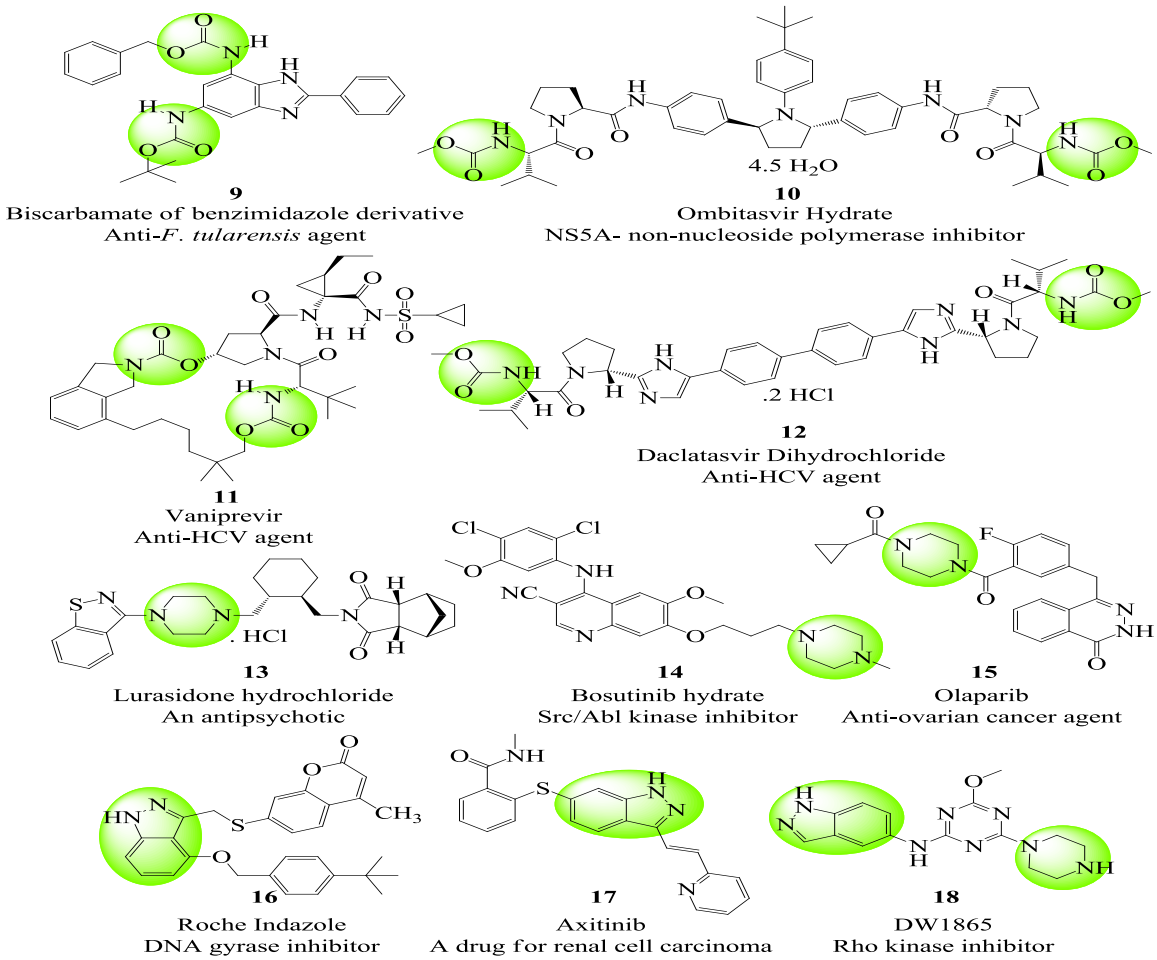


Figure 3: Biologically active biscarbamates, piperazine and indazole motifs.

Whenever the microbial resistance extends its arms there is always a unique reason for the exploration of new frame works to put an end to the microbial populations responsible for the human pathologies. In view of their versatile therapeutic nobility the authors have deliberately synthesized the carbamates of 1-(4-nitrophenyl)piperazine and *bis*carbamates of 6-aminoindazole and screened for their biological activity along with molecular docking studies.

2. MATERIALS AND METHODS

Experimental aspects

All the chemicals were purchased from Sigma-Aldrich and Merck and were used as such without further purification. The solvents used for spectroscopic and other physical studies were reagent grade and were purified further by literature methods. The melting points were determined on *EZ*-Melt Automated Melting Point Apparatus equipped with Digital Imaging Processing Technology of Stanford Research Systems using open capillary method and are uncorrected. The IR spectra were recorded on a Bruker Alpha-Eco ATR-FTIR (Attenuated Total Reflection - Fourier Transform Infrared) Interfero-meter with single reflection sampling module equipped with ZnSe Crystal and reported in reciprocal centimeters (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 and $CDCl_3$solvents on a Bruker FT-NMR 400 MHz spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The ^{1}H and ^{13}C chemical shifts were referenced to TMS and reported in delta (δ) values in ppm. Multiplicities were abbreviated as s (singlet), br s (broad singlet) d (doublet), br d (broad doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet). The mass spectra were recorded on LCMS-2010A Shimadzu (Method-ESI) mass spectrometer. The CHN analysis was carried on Flash EA 1112 Series Thermo Finnigan.

Synthesis of Carbamates of 1-(4-nitrophenyl)piperazine

To a mixture of 1-(4-nitrophenyl)piperazine (**19**) (0.001 mol, 0.207g) and methyl chloroformate (**20a**) (0.001 mol, 0.095 g, 0.0776 mL) in THF (6-10 mL) contained in a 50 mL RB flask, triethyl amine, TEA (0.001 mol, 0.101 g, 0.14 mL) was added and stirred for 3 hours at 10-40^{o}C. The progress of the reaction was monitored by TLC using *n*-hexane:ethylacetate (3:2) for every half an hour. After completion of the reaction, the reaction mixture was filtered to remove the TEA. HCl salt. The solvent in the filtrate was removed under reduced pressure to obtain crude pale yellow solid product. The crude product was purified by column chromatography using *n*-hexane:ethylacetate (7:3) to afford pure yellow product (**20a**). The same procedure was adapted for the

synthesis of remaining title compounds **21**(**b-f**) using other chloroformates (**20b-f**) (Scheme 1).

Synthesis of *Bis*carbamates of 6-aminoindazole

6-Aminoindazole (**22**) (0.001 mol, 0.133 g) and triethyl amine, TEA (0.002 mol, 0.202 g, 0.28 mL) in THF (10 mL) were taken in a 50 mL RB flask. Methyl chloroformate (**20a**) (0.002 mol, 0.190 g, 0.1552 mL) was added at 10^{o}C and stirred for 2 hours at 40^{o}C. The progress of the reaction was monitored by TLC using *n*-hexane:ethylacetate (1:1) for every half an hour. After completion of the reaction, the reaction mixture was filtered to remove the TEA. HCl salt. The solvent in the filtrate was removed under reduced pressure to obtain crude pale yellow solid product. The crude product was purified by column chromatography using *n*-hexane:ethylacetate (2:1) to afford pure colourless product (**23a**). The same procedure was adopted for the synthesis of remaining title compounds **23**(**b-f**) using other chloroformates (**20b-f**).

Spectral Data

Methyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21a): Yellow solid, yield: 96%, mp:154.2^{o}C; IR (cm^{-1}): 3077 (Ar C-H str); 2893 (methyl C-H str); 1695 (C=O str); 1590 (Ali. Ring C-C str); 1464 & 1327 (N-O str); 1221 (C-N str); 1107 (C-O str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm (*J, Hz*): 8.14 (2H, d, J = 8.4 Hz, H-9, 11); 6.83 (2H, d, J = 8.4 Hz, H-8, 12); 3.75 (3H, s, H-1', 1', 1'); 3.65 (4H, br s, H-3, 3, 5, 5); 3.42 (4H, br s, H-2, 2, 6, 6). ^{13}C NMR (100 MHz, CDCl3), δ, ppm: 155.79 (C-7); 154.59 (C=O); 138.93 (C-10); 126.08 (C-11); 125.98 (C-9); 113.01 (C-12); 112.29 (C-8); 52.92 (methyl C-1'); 46.89 (C-5); 46.09 (C-3); 45.85 (C-6); 43.06 (C-2). MW calcd: 265.27; Found LCMS: 266.25 $(M+H)^{+}$; Anal. calcd for $C_{12}H_{15}N_3O_4$: C, 54.33; H, 5.70; N, 15.84 Found: C, 54.26; H, 5.65; N, 15.72%.

Ethyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21b): Yellow solid, yield: 94%, mp:110.0^{o}C; IR (cm^{-1}): 3072 (Ar C-H str); 2989 (methyl C-H str); 2899 (methylene C-H str); 1679 (C=O str); 1588 (Ali. Ring C-C str); 1483 & 1327 (N-O str); 1442 (Ali. ring C-H str); 1216 (C-N str); 1112 & 1062 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.12 (2H, d, J = 8.4 Hz, H-9, 11); 6.83 (2H, d, J = 8.4 Hz, H-8, 12); 4.19 (2H, q, J = 6.8 Hz, H-1', 1'); 3.66 (4H, br s, H-2, 2, 6, 6); 3.44 (4H, br s, H-3, 3, 5, 5); 1.31 (3H, t, J = 6.6 Hz, H-2', 2', 2'). ^{13}C NMR (100 MHz, $CDCl_3$), δ, ppm: 155.38 (C=O); 154.60 (C-7); 138.75 (C-10); 125.93 (C-9, C-11); 112.91(C-8, C-12); 61.72 (C-1'); 46.84 (C-3, C-5); 46.07 (C-2, C-6); 14.66 (C-2').

2,2,2-trichloroethyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21c): Yellow solid, yield: 92%, mp:131.8 ^{o}C; IR (cm^{-1}): 3076 (Ar C-H str); 2850 (methylene C-H str); 1698 (C=O str); 1580 (Ali. Ring C-C str); 1482 & 1316 (N-O str); 1441 (Ali. ring C-H str); 1220 (C-N str); 1113 & 1045 (C-O-C str); 707 (C-Cl str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.15 (2H, d, J = 6.4 Hz, H-9, 11); 6.85 (2H, d, J = 5.6 Hz, H-8, 12); 4.79 (2H, s, H-1', 1'); 3.75 (4H, br s, H-2, 2, 6, 6); 3.48 (4H, br s, H-3, 3, 5, 5).

Isobutyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21d): Yellow solid, yield: 95%, mp: 90.4 ^{o}C; IR (cm^{-1}): 3078 (Ar C-H str); 2956 (methyl C-H str); 2890 (methyne C-H str); 1681 (C=O str); 1583 (Ali. Ring C-C str); 1470 (methylene C-H str); 1470 & 1314 (N-O str); 1218 (C-N str); 1111 & 1044 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.12 (2H, d, J = 9.2 Hz, H-9, 11); 6.83 (2H, d, J = 9.2 Hz, H-8, 12); 3.92 (2H, d, J = 6.8 Hz, H-1', 1'); 3.68 (4H, t, J = 4.8 Hz, H-2, 2, 6, 6); 3.46 (4H, t, J = 5.2 Hz, H-3, 3, 5, 5); 2.01 (1H, m, J = 6.8 Hz, H-2'); 0.96 (6H, d, J = 6.8 Hz, H-3', 3', 3', 3'', 3'', 3''). ^{13}C NMR (100 MHz, $CDCl_3$), δ, ppm: 155.44 (C-7); 153.98 (C=O); 138.76 (C-10); 126.24 (C-9, C-11); 112.91(C-8, C-12); 71.85 (C-1'); 46.83 (C-3, C-5); 42.99 (C-3, C-5); 14.66 (C-2'); 19.09 (C-3', C-3'').

4-nitrobenzyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21e): Yellow solid, yield: 97%, mp: 177.7 ^{o}C; IR (cm^{-1}): 3071 (Ar C-H str); 2919 (methylene C-H str); 1705 (C=O str); 1595 (Ali. Ring C-C str); 1505 & 1331 (N-O str); 1470 (Ar C-C str); 1223 (C-N str); 1114 & 1053 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.25 (2H, d, J = 8.4 Hz, 4', 6'); 8.16 (2H, d, J = 9.6 Hz, H-9, 11); 7.54 (2H, d, J = 8.4 Hz, H-3', 7'); 6.84 (2H, d, J = 9.6 Hz, H-8, 12); 5.27 (2H, s, H-1', 1'); 3.72 (4H, t, J = 5.2 Hz, H-3, 3, 5, 5); 3.47 (4H, t, J = 5.2 Hz, H-2, 2, 6, 6). ^{13}C NMR (100 MHz, $CDCl_3$), δ, ppm: 154.63 (C-7); 154.48 (C=O); 147.76 (C-5'); 143.68 (C-2'); 139.20 (C-10); 128.29 (C-3', C-7'); 125.98 (C-9, C-11); 123.88 (C-4', C-6'); 113.15 (C-8, C-12); 66.04 (C-1'); 46.92 (C-3, C-5); 45.82 (C-2, C-6).

4-nitrophenyl 4-(4-nitrophenyl)piperazine-1-carboxylate (21f): Yellow solid, yield: 93%, mp:145.1^{o}C; IR (cm^{-1}): 3089 (Ar C-H str); 2835 (methylene C-H str); 1702 (C=O str); 1585 (Ali. Ring C-C str); 1495 & 1314 (N-O str); 1196 (C-N str); 1098 & 1011 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.27 (2H, d, J = 7.6, H-3', 5'); 8.17 (2H, d, J = 8.0 Hz, H-9, 11); 7.33 (2H, d, J = 7.6 Hz, H-2', 6'); 6.88 (2H, d, J = 7.6 Hz, H-8, 12); 3.87 (4H, br d, J = 36.8 Hz, H-3, 3, 5, 5); 3.67 (4H, br d, J = 52.4 Hz, H-2, 2, 6, 6). ^{13}C NMR (100 MHz, CDCl3), δ, ppm: 155.90 (C-7); 154.41 (C=O); 152.25 (C-1'); 145.06 (C-4'); 139.20 (C-10); 126.25 (C-5', C-3'); 126.01 (C-11, C-9); 122.33 (C-6', C-2'); 113.22 (C-12, C-8); 46.83 (C-5); 46.02 (C-3); 44.00 (C-6); 43.33 (C-2). MW calcd: 372.33; Found LCMS: 374 $(M+2H)^{+}$; Anal. calcd for $C_{17}H_{16}N_4O_6$: C, 54.84; H, 4.33; N, 15.05 Found: C, 54.75; H, 4.26; N, 15.21%.

Methyl 6-((methoxycarbonyl)amino)-1H-indazole-1-carboxylate (23a): Dull white solid, yield: 91%, m.p: 138.1^{o}C; IR (cm^{-1}): 3305 (carbamate N-H str); 3137 (aro. C-H str); 2953 (methyl C-H str); 1717 (C=O str); 1622 (cyclic conjugated C=N str); 1441 (Ar. ring sideways str); 1223 & 1055 (C-O-C str); 858 (1,2,4 trisub. Benzene ring). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm:

8.29 (1H, s, N-H-8); 8.10 (1H, s, H-3); 7.99 (1H, s, H-7); 7.66 (1H, d, J = 8.0 Hz, H-4); 7.49 (1H, d, J = 6.8 Hz, H-5); 4.12 (3H, s, methyl H-10',10',10'); 3.82 (3H, s, methyl H-10,10,10).

Ethyl 6-((ethoxycarbonyl)amino)-1H-indazole-1-carboxylate (23b): Dull white solid, yield: 93%, m.p: 175.3 ^{o}C; IR (cm^{-1}): 3247 (carbamate N-H str); 3104 (Ar. C-H str); 2918 (methyl C-H str); 2856 (methylene C-H str); 1715 (C=O str); 1474 (Ar. ring sideways str); 1289 (Ar. Amine =C-N str); 1201 & 1043 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.33 (1H, s, N-H); 8.10 (1H, s, H-3); 7.65 (1H, d, J = 8.8 Hz, H-4); 7.46 (1H, d, J = 8.4 Hz, H-5); 7.01 (1H, s, H-7); 4.60 (2H, q, methylene C-H-9', 9'); 4.29 (2H, q, H-9, 9); 1.53 (3H, t, methyl H-10', 10', 10'); 1.34 (3H, t, methyl H-10, 10, 10). ^{13}C NMR (100 MHz, $CDCl_3$), δ, ppm: 153.60 (O=C-8); 150.68 (O=C-8'); 140.59 (C-4); 121.71 (C-1); 116.35 (C-2); 103.52 (C-7); 77.45 (C-3); 64.02 (C-9'); 61.45 (C-9); 14.48 (C-10, 10').

2,2,2-trichloroethyl 6-(((2,2,2-trichloroethoxy)carbonyl)amino)-1H-indazole-1-carboxyl-ate (23c): Dull white solid, yield: 95%, m.p: 180.7 ^{o}C; IR (cm^{-1}): 3250 (*N*-substituted amide N-H str); 3071 (Ar C-H str); 2821 (methylene C-H str); 1752 (C=O str); 1696 (cyclic conjugated C=N str); 1506 (Ar. ring asy. str); 1400 (CCl_3 umbrella (scissoring)); 1215 (aryl amine C-N str); 1215&1043 (C-O-C str); 876 (1,2,4-tri substituted benzene ring); 729 (C-Cl str). ^{1}H NMR (400 MHz, CDCl3), δ, ppm: 8.46 (1H, s, N-H or H-8); 8.22 (1H, s, H-3); 7.73 (1H, d, J = 8.4 Hz, H-4); 7.46 (1H, d, J = 8.0 Hz, H-5); 7.23 (1H, s, H-7); 5.16 (2H, s, H-9', 9'); 4.85 (2H, s, H-9,9). MW calcd: 483.95; Found LCMS: 485 $(M+H)^{+}$; Anal. Calcd for $C_{13}H_9Cl_6N_3O_4$: C, 32.26; H, 1.87; Cl, 43.95; N, 8.68%; O, 13.22 Found: C, 32.19; H, 1.82; N, 8.62%.

Isobutyl 6-((isobutoxycarbonyl)amino)-1H-indazole-1-carboxylate (23d): Dull white solid, yield: 93%, m.p: 128.3 ^{o}C; IR (cm^{-1}): 3235 (carbamate N-H str); 3119 (Ar. C-H str); 2955 (methyl C-H str); 2926 (methylene C-H str); 2891 (methyne C-H str); 1709 (C=O str); 1562 (cyclic conjugated C=N str); 1467 (CH_2 sci); 1377 (methyl umbrella); 1275 (CH_3 wag); 1201 (C-N str); 1141 & 1049 (C-O-C str). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.58 (1H, s, N-H); 7.80 (1H, s, H-3); 7.57 (1H, d, J = 9.2 Hz, H-4); 7.14 (1H, d, J = 8.8 Hz, H-5); 6.89 (1H, s, H-7); 4.34 (2H, d, J = 6.8 Hz, H-10,10); 3.98 (2H, d, J = 6.4 Hz, H-10', 10'); 2.25 (1H, m, H-11); 2.04 (1H, m, H-11'); 1.06 (6H, d, J = 6.4 Hz, methyl H-12, 12, 12, 13, 13, 13); 0.97 (6H, d, J = 6.4 Hz, methyl H-12', 12', 12', 13', 13', 13'). ^{13}C NMR (100 MHz, $CDCl_3$), δ, ppm: 153.50 (C=O); 151.64 (C=O); 150.11 (C-7); 138.49 (C-1); 124.62 (C-5); 121.97 (C-2); 120.05 (C-3); 119.28 (C-4); 104.31 (C-6); 77.34 (C-9'); 74.99 (C-9); 27.93 (C-10); 27.83 (C-10'); 19.07 (C-11, 12); 18.96 (C-11', 12'). MW calcd: 333.38; Found LCMS: 332 $(M-H)^{+}$; Anal. Calcd for $C_{17}H_{23}N_3O_4$: C, 61.25; H, 6.95; N, 12.60; O, 19.20%; Found: C, 61.32; H, 6.89; N, 12.52; O, 19.27%.

4-nitrobenzyl 6-((((4-nitrobenzyl)oxy)carbonyl)amino)-1H-indazole-1-carboxylate (23e): Pale brown solid, yield: 91%, m.p: 162.0 ^{o}C; IR (cm^{-1}): 3263 (carbamate N-H str); 3029 (Ar. C-H str); 2876 (methylene C-H str); 1714 (C=O str); 1600 (C-N str); 1518 & 1343 (nitro N-O str); 1243 & 1055 (C-O-C str); 835 (*p*-disubstituted benzene). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.42 (1H, s, N-H); 8.27 (4H, dd, J = 8.4 Hz, J = 8.4 Hz, H-11, 12, 11', 12'); 8.14 (1H, s, H-3); 7.72 (4H, dd, J = 13.2 Hz, J = 13.6 Hz, H-10, 13, 10', 13'); 7.59 (1H, d, J = 8.4 Hz, H-4); 7.36 (1H, d, J = 7.6 Hz, H-5); 7.03 (1H, s, H-7); 5.62 (2H, s, H-9', 9'); 5,33 (2H, s, H-9, 9). MW calcd: 491.41; Found LCMS: 490 $(M-H)^{+}$; Anal. Calcd for $C_{23}H_{17}N_5O_8$; C, 56.22; H, 3.49; N, 14.25; O, 26.05%; Found: C, 56.15; H, 3.45; N, 14.32; O, 25.88%.

4-nitrophenyl 6-(((4-nitrophenoxy)carbonyl)amino)-1H-indazole-1-carboxylate (23f): Pale pink solid, yield: 92%, m.p: 141.8 ^{o}C; IR (cm^{-1}): 3282 (carbamate N-H str); 3058 (Ar. C-H str); 1700 (C=O str); 1620 (C-N str); 1554 & 1357 (nitro N-O str); 1239 & 1055 (ester C-O-C str); 847 (wag *p*-disubstituted benzene). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 8.31 (1H, s, N-H), 7.99-6.78 (11H, m, Ar-H), 8.10 (1H, s, H-3).

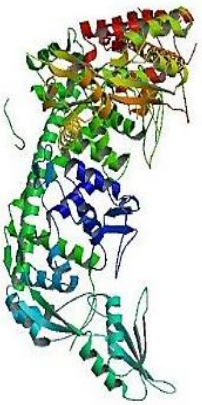
Molecular Docking Studies

DNA gyrase is an important bacterial enzyme which catalyzes the ATP-dependent negative super-coiling of double stranded closed-circular DNA. It belongs to topoisomerases which are involved in the topological transitions of DNA. The mechanism by which gyrase is able to influence the topological state of DNA molecules is of inherent interest from the perspective of enzymology. DNA gyrase is the intracellular target for a number of antibacterial agents and as a paradigm for other DNA topoisomerases.

Streptomycin is an aminoglycoside antibiotic derived from *Streptomyces griseus*. It binds to the 16S rRNA and S12 protein within the bacterial 30S ribosomal subunit. Consequently, this agent interferes with the assembly of initiation complex between mRNA and bacterial ribosome which ultimately results in the inhibition of protein synthesis. Above all, it also induces misreading of mRNA template which causes translational frame shift resulting in premature termination. This eventually leads to bacterial cell death.

Accession of target protein

The 3D structure of DNA gyrase A (PDB: 3LPX) and the reference drugs such as streptomycin (Pub Chem ID 19649) was downloaded from the RCSB protein Data bank. The atomic coordinates of the protein was estranged and geometry optimization was done using Argus Lab 4.0.1. The enzyme 3LPX and the reference drug, streptomycin (Pub Chem ID 19649) were shown in fig. 4 (A & B).[67]



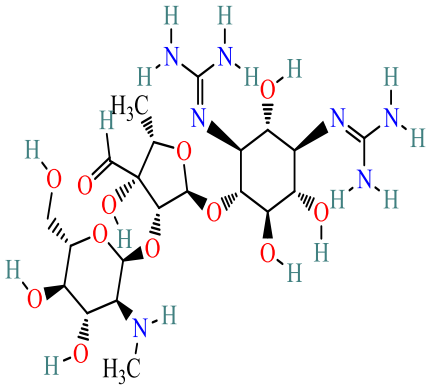


Figure 4: A. DNA gyrase A (*E. coli*) B. Streptomycin – PC ID 19649

Ligand Preparation

The chemical structures of compounds were drawn using Chem Bio Draw and converted all the ligands into Pdbqt file format and atomic coordinates were generated using Pyrx 2010.12.

Analysis of Target active binding sites

The active sites are the coordinates of the ligand in the original target protein grids and these active binding sites of target protein were analysed using the Drug Discovery Studio version 3.0 and 3D Ligand Site virtual tools.

Molecular docking analysis

Molecular docking studies were carried against DNA gyrase A protein with compounds **21a-f**, **23a-f** and the reference drug, streptomycin using the docking module implemented in Pyrx 2010.12. Initially the protein structures were protonated with the addition of polar hydrogens, followed by energy minimization with the MMFF94x force field in order to get the stable conformer of the protein. Flexible docking was employed, the inhibitor binding site residues were softened and highlighted through the "Site Finder" module implemented in the Pymol software. The grid dimensions were predicted as °X: 28.27, Y: 27.13, Z: 28.51 for aromatase respectively. The docking was carried out with the default parameters, i.e., placement: triangle matcher, recording 1: London dG, refinement: force field and a maximum of 10 conformations of each compound were allowed to be saved in a separate data base file in a .mdb format. After the docking process, the binding energy and binding affinity of the protein ligand complexes were calculated using Pymol viewer tool (www.pymol.org).[68]

Structure analysis and visualization:

Protein and ligand interactions were analysed and visualized through Pymol viewer tool (www.pymol.org).[69]

Biological activities

Study of Antimicrobial Activity

Primary antimicrobial screening study by whole cell growth inhibition assays, using the title compounds at a single concentration, in duplicate (n=2) was done. The inhibition of growth is measured against 5 bacterial *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853 and 2 fungal pathogens, *Candida albicans* ATCC 90028, *Cryptococcus neoformans var. grubii* H99: ATCC 208821. Colistin and Vancomycin were used as positive bacterial inhibitor standards for G-ve and G+ve bacteria respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*.

Sample Preparation

The samples were provided by the collaborator and stored frozen at -20 °C. Samples were prepared in DMSO and water to a final testing concentration of 32 µg/mL or 20 µM, in 384-well nonbinding surface plate (NBS) for each bacterial/fungal strain and in duplicate (n=2) and keeping the final DMSO concentration to a maximum of 1% DMSO. All the sample- preparation was done using liquid handling robots. Compounds that showed solubility issues during stock solution preparation are detailed in the data sheet.

Antimicrobial Assay

All bacteria were cultured in Cation-adjusted Mueller Hinton Broth (CAMHB) at 37°C overnight. A sample of each culture was then diluted 40-fold in fresh broth and incubated at 37°C for 1.5-3 h. the resultant mid-log phase cultures were diluted (CFU/mL measured by OD_{600}), then added to each well of the compound containing plates, giving a cell density of 5 x 10^5 CFU/mL and a total volume of 50 µL. All the plates were covered and incubated at 37°C for 18 h without shaking.

Antifungal Assay

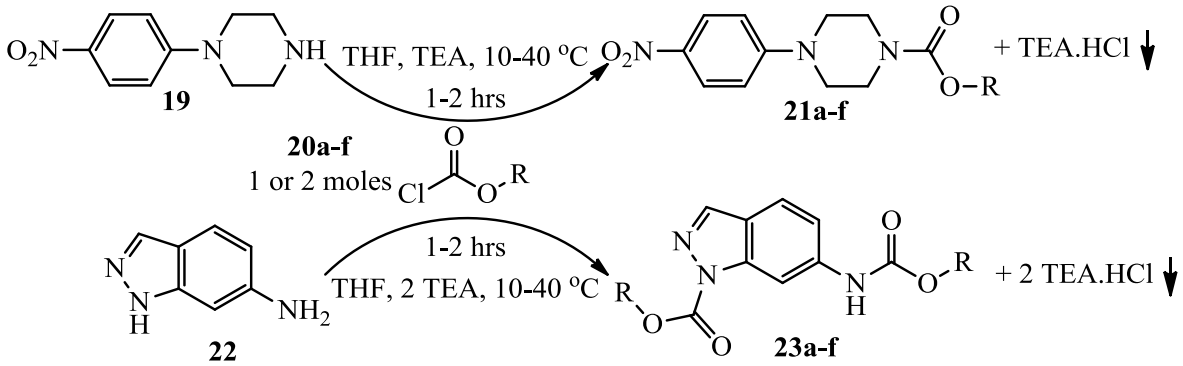
Fungal strains were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30°C. A yeast suspension of 1 x 10^6 to 5 x 10^6 CFU/mL (as determined by OD_{530}) was prepared from five colonies. The suspension was subsequently diluted and added to each well of the compound-containing plates giving a final cell density of fungi suspension of 2.5 x 10^3 CFU/mL and a total volume of 50 µL. All plates were covered and incubated at 35°C for 24 h without shaking.

3. RESULTS AND DISCUSSION

Chemistry

New series of carbamate derivatives of 1-(4-nitrophenyl)piperazine and *bis*carbamates of 6-aminoindazole were synthesized using different chloroformates by simple nucleophilic substitution in the presence of a base, TEA in THF as depicted in Scheme 1. The resulting TEA.HCl precipitate was filtered off and the crude products were further purified by column chromatography.

Scheme 1



Compound Code	20a	20b	20c	20d	20e	20f
R	$-CH_3$	CH_3	CCl_3	CH_3, CH_3	NO_2	NO_2

Scheme 1: Synthesis of carbamates of 1-(4-nitrophenyl)piperazine and *bis*carbamates of 6-Aminoindazole.

The structures of the title compounds were established by FT-IR, ^{1}H, ^{13}C, NMR, mass spectra and elemental analysis. In IR spectra, for compounds **21a-f** stretching bands between/at 3089-3071 (Ar C-H str), 2893-2989 (methyl C-H str), 2919-2835 (methylene C-H str), 2891 (methyne C-H str), 1705-1679 (C=O str), 1595-1580 (piperazine ring str), 1505-1464 & 1331-1314 (nitro N-O str), 1223-1196 (C-N str), 1114-1098 & 1062-1011 (carbamate C-O-C str) and 707 (C-Cl str); and for compounds **23a-f** stretching bands between/at 3305-3235 (carbamate N-H str), 3137-3029 (Ar C-H str), 2955-2918 (methyl C-H str), 2925-2821 (methylene C-H str), 2891 (methyne C-H str), 1752-1700 (C=O str), 1696-1600 (C-N str), 1554-1518 & 1357-1343 (nitro N-O str), 1400 (methylene scis), 1243-1141 & 1055-1043 (carbamate C-O-C str) and 729 (C-Cl str) are in good agreement with the characteristic corresponding functional groups.

In ^{1}H NMR spectra of the monocarbamate derivatives **21a-f** the protons of 4-nitrophenyl group of the piperazine resonated between 8.17-8.12 & 6.88-6.83 ppm and that of chloroformate moiety between 8.27-8.25 & 7.54-7.33 ppm. The piperazinyl ring protons resonated between 3.87-3.65 & 3.67-3.42 ppm. The methyl protons existed between 3.75-0.96 ppm, methylene protons between 5.27-3.92 ppm and methyne proton at 2.01 ppm; and for the *bis*carbamates of 6-aminoindazole **23a-f** the N-H proton of carbamate group resonated between 8.58-8.29 ppm. The protons of five membered indazole moiety resonated between 8.22-8.10 ppm. The aromatic protons resonated between 8.27-6.78 ppm. Benzylic protons resonated at 5.62 and 5.33 ppm; methyl protons between 4.12-097 ppm; methylene protons between 5.16-3.98 ppm and methyne protons at 2.25 and 2.04 ppm. In ^{13}C NMR spectral data obtained for the compounds **21a, 21b, 21d, 21e, 21f, 23b** and **23d** stood as additional support for the title compounds.

The LCMS and CHN analysis data obtained for compounds **21a, 21f, 23c, 23d** and **23e** were in good agreement with the calculated values, and thus provided the additional support for the title compounds.

Molecular Docking Studies

In order to provide strength to the newly synthesized compounds, docking analysis was carried out for compounds **21a-f** and **23a-f** with selective pharmacological target such as DNA gyrase A protein of *E. coli* which is a suitable target for antibacterial activity. The structure of DNA gyrase A (PDB ID: 3LPX) was retrieved from the protein data bank.

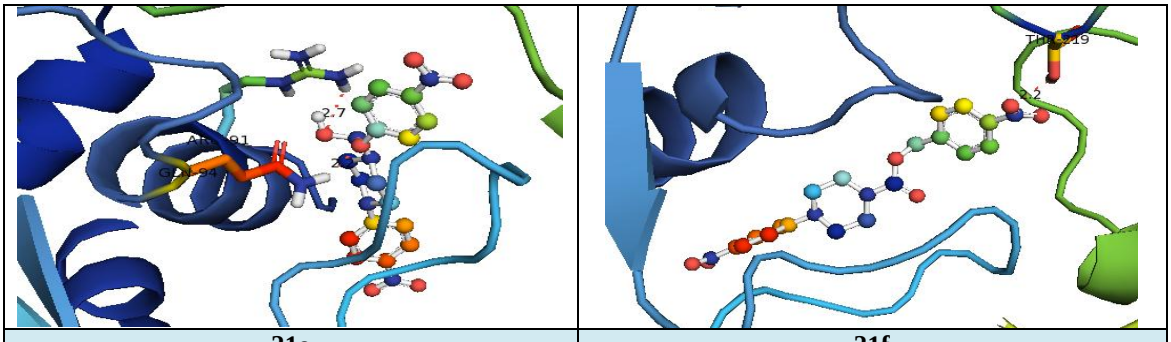
The docking results of DNA gyrase A showed that compounds **23f, 23e, 21f, 21e, 23c** and **23d** have higher binding energies with dock scores of -8.9, -8.8, -8.2, -7.8, -7.1 and -7.0 respectively when compared to the control drug streptomycin (-6.9) while those of **21c** and **21d** are equal to streptomycin. The H-bonds, binding affinities and energy profiles of compounds **21a-f** and **23a-f** along with reference drug towards the active site amino acids of the enzyme are summarized in Table 1.

Table 1: Bonding characterization of synthesized compounds 21A-F, 23A-F and the reference drug, Streptomycin (STM) against *E. coli* DNA gyrase A protein.

Sl. No.	Compound	Rank	Binding Energy (kcal/mol)	Binding Interaction	Bond Length (Å)	Bond Angle (°)	Bond Type
1.	**Streptomycin**	R	—6.9	Asg 139 CG … HN Leu 135 CD … HN His 132 CB … OH Asp 53 CG … OC Asp 53 OC … OC Asp 58 OD … OH Asp 58 OD … HN His 132 ND … OC His 132 ND … OC His 132 OC … OH	2.2 2.7 2.5 3.4 2.9 2.0 2.5 2.8 2.7 2.5	124.4 125.7 125.0 116.7 118.9 118.6 116.4 126.2 120.0 119.8	H-don* H-don H-acc* H-acc H-acc H-acc H-don H-acc H-acc H-acc
2.	**21a**	11	—6.3	Asp 113 CG … HO	2.0	126.1	H-don
3.	**21b**	12	—6.2	Leu 264 CA … ON Leu 264 CA … ON	2.3 2.4	122.2 98.5	H-acc H-acc
4.	**21c**	7	—6.9	Arg 91 CG … OC Thr 219 CB … ON	2.5 2.1	74.6 109.6	H-acc H-acc
5.	**21d**	8	—6.9	Leu 264 HN … ON Leu 264 HN … ON	2.3 2.3	114.7 122.2	H-acc H-acc
6.	**21e**	4	—7.5	Gln 94 CD … ON Arg 91 NH … OH	2.2 2.7	118.4 120.8	H-acc H-acc
7.	**21f**	3	—8.2	Thr 219 CB … ON	2.2	109.6	H-acc
8.	**23a**	10	—6.5	Thr 171 CZ … HN Asp 113 CG … HO	2.1 2.4	122.2 118.4	H-don H-don
9.	**23b**	9	—6.6	Thr 171 CA … HN Arg 91 CZ … OC Thr 119 CB … ON	2.1 2.3 2.7	114.6 120.8 118.6	H-don H-acc H-acc
10.	**23c**	5	—7.1	Gln 94 CD … OC	2.1	118.4	H-acc
11.	**23d**	6	—7.0	Asp 115 CB … HN Met 120 CA … HN	2.8 2.2	119.4 122.3	H-don H-don
12.	**23e**	2	—8.8	His 45 CB … ON Lys 42 CZ … ON Arg 91 CA … OC Arg 91 CA … OC Arg 91 CA … ON Gln 267 CB … ON	2.2 2.0 2.3 1.9 2.5 2.3	122.4 122.2 116.5 126.1 109.8 116.2	H-acc H-acc H-acc H-acc H-acc H-acc
13.	**23f**	1	—8.9	Arg 91 CZ … OC Arg 91 CZ … OC Thr 219 CB … ON	2.1 2.8 2.2	118.1 119.0 109.6	H-acc H-acc H-acc

* don= donor; acc= acceptor; (Row lines are shown to prevent confusion.)

The binding modes of compounds **23f, 23e, 21f, 21e, 23c** and **23d** suggested that they fitted more stably in to the DNA gyrase binding pocket as depicted in Figure 5. Hence, the present investigation demonstrates that the synthesized compounds could be the promising next generation antimicrobials.



21e **21f**

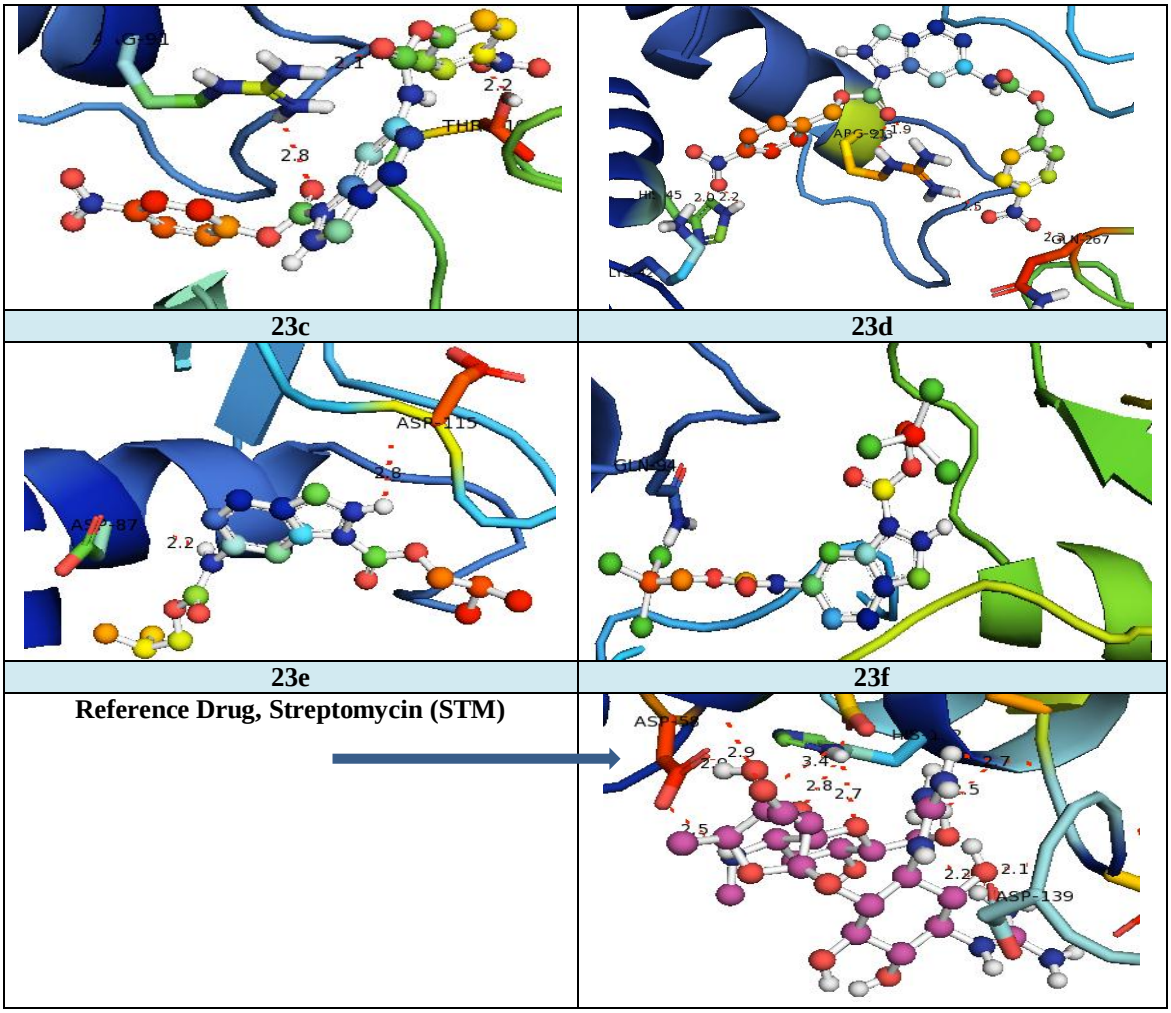


Figure 5: Graphical representation of ligand binding modes of compounds 23f, 23e, 21f, 21e, 23c and 23d with DNA gyrase A protein of *E. coli.*

Biological Activity

Antibacterial Data Collection

Inhibition of bacterial growth was determined measuring absorbance at 600 nm (OD_{600}) while a Tecan M1000 Pro monochromator plate reader. The % of growth inhibition was calculated for each well, using the negative control (media only) and positive control (bacteria without inhibitors) on the same plate as references.

Table 2: Antibacterial and antifungal activity of 21a-f and 23a-f compounds (inhibition %).

Sl. No.	Compound code	Sa	Ec	Kp	Pa	Ab	Ca	Cn	Conc.
1.	**21a**	19.36	11.42	25.75	11.13	6.81	0.45	-17	32 ug/mL
2.	**21b**	21.91	8.22	23.06	11.07	3.81	43.16	2.09	32 ug/mL
3.	**21c**	50.88	46.07	66.24	70.80	39.28	49.22	-27.23	32 ug/mL
4.	**21d**	38.08	38.88	20.37	13.28	2.67	1.94	91.28	32 ug/mL
5.	**21e**	44.08	51.39	54.88	69.16	51.32	58.22	109.9	32 ug/mL
6.	**21f**	54.03	61.86	56.87	80.14	82.63	49.14	68.22	32 ug/mL
7.	**23a**	13.99	1.9	15.79	10.31	12.02	3.99	-8.27	32 ug/mL
8.	**23b**	12.92	2.84	24.66	7.48	6.87	8.44	-3.61	32 ug/mL
9.	**23c**	60.66	49.18	38.36	40.02	58.18	60.36	76.99	32 ug/mL
10.	**23d**	38.12	51.38	57.94	56.97	60.19	71.12	68.22	32 ug/mL
11.	**23e**	60.04	64.87	43.78	71.81	59.24	44.56	73.82	32 ug/mL
12.	**23f**	63.16	67.07	51.18	69.04	60.16	54.37	86.66	32 ug/mL

Note: 1. Negative inhibition values indicate that the growth rate (or OD_{600}) is higher compared to the Negative Control (bacteria/fungi only, set to 0% inhibition).

2. The growth rates for all bacteria and fungi have a variation of -/+ 10%, which is within the reported normal distribution of bacteria/fungal growth.

Antifungal Data Collection

Growth inhibition of *C. albicans* was determined measuring absorbance at 530 nm (OD_{530}) while the growth inhibition of *C. neoformans* was determined measuring the difference in absorbance between 600 and 570 nm ($OD_{600-570}$), after the addition of resazurin (0.001% final concentration) and incubation at 35 ^{o}C for additional 2 h. the absorbance was measured using a Biotek Synergy HTX plate reader. The percentage of growth inhibition was calculated for each well, using the Negative Control (media only) and Positive Control (bacteria without inhibitors) on the same plate as references.

Inhibition

Percentage growth inhibition of an individual sample is calculated based on Negative Controls (media only) and Positive Controls (bacteria/fungal media without inhibitors).

4. CONCLUSION

In the present study the dock scores of compounds **23f, 23e, 21f, 21e, 23c** and **23d** suggested that they befitted fast into the DNA gyrase A protein binding site throwing light on that they could be the optimistic next generation antimicrobials. However, the antimicrobial assays revealed that the compounds **23f, 23e** and **21f** were good antibacterials and compounds **21e** and **21d** were more active against *C. neoformans* which suggest that they could find a place in the next generation antifungals. The studies also showed that the carbamates of piperazine derivatives were potent antifungals than the *bis*carbamates of 6-aminoindazole.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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