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Study of arginine mimetic benzamidine urea derivatives as PAD4 inhibitors

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Rheumatoid Arthritis (RA) is an autoimmune inflammatory disease, where antibodies are generated against citrullinated proteins. Citrullinated proteins are catalytic products of the Peptidyl Arginine Deiminase 4 (PAD4) enzyme. Since PAD4 acts on peptidyl arginine, a series of arginine mimetic compounds having 4-benzamidine urea scaffold were synthesized and evaluated for PAD4 inhibitory potential. These derivatives showed weak inhibitory activity. To understand the reason, docking studies were performed. Analysis of docking poses revealed that important binding interactions observed in the reported substrate were not exhibited by these synthesized compounds. These results suggest that 4-benzamidine urea derivatives known for their arginine mimetic property might not be useful as lead structures for development as PAD4 inhibitors.

Keywords: PAD4, Rheumatoid Arthritis, arginine mimetic, benzamidine urea.

Introduction

Rheumatoid Arthritis (RA) is an autoimmune systemic inflammatory disease with estimated 1% global prevalence[1]. It is characterized by destruction of cartilages and inflammation of the synovium and substantial disability[2]. In genetically susceptible individuals, citrullinated proteins are recognized by immature dendritic cells (DCs) as self antigen[3]. The DCs later mature into Antigen Presenting Cells (APCs) and activate T cells which, in turn, activate B cells and trigger autoimmunity against these antigens by producing antibodies such as rheumatoid factor or Anti Citrullinated Peptide Antibodies (ACPAs)[4]. These auto-antibodies bind to citrullinated proteins forming immune complex that trigger acute inflammation in synovium leading to chronic inflammation and joint destruction[5]. A high correlation was observed between erythrocyte sedimentation rate, IgM, IgG and the level of bone destruction[6,7]. Citrullinated proteins are formed by catalytic conversion of arginine residues to citrulline by Peptidyl Arginine Deiminase 4 (PAD4) (Fig. 1)[8]. Therefore, PAD4 serves as an important target to treat RA.

The PAD4 is a 663 amino acid 74 kDa protein belonging to the guanidino-group modifying enzyme superfamily (GMSF), where a basic catalytic motif Cys-His-Asp(Glu) is conserved[9]. The catalytic site present at C-terminal domain

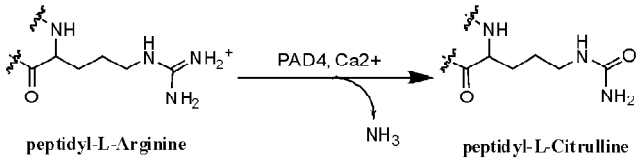


Fig. 1. Catalytic reaction of PAD4.

consists of charged and polar residues like Asp350, His471, Asp473, His644, and Cys645. Benzamidine derivatives are well known arginine mimetics. Substituted 4-amino benzamidines were found to inhibit factor VIIa[10]. Recently, sulfamoyl benzamidines have been reported as trypsin, thrombin, and matriptase-2 inhibitors[11,12]. Taking clue from these studies, a series of 4-benzamidine urea was explored to analyze the possibility of using the 4-benzamidine urea derivatives as PAD4 inhibitors.

Experimental

The reaction progress was monitored by thin layer chromatography (TLC) on silica gel coated aluminum plates (silica gel 60 F_{254}) obtained from Merck. The IR spectra were recorded with KBr on Shimadzu IR Prestige-21 Fourier Transform Infrared Spectroscopy and vibrational energy values are represented as wave number in cm^{-1} scale. The ^1H and

^{13}C NMR spectra were recorded on a Bruker (400 MHz) spectrometer with mixture of DMSO-d_6, $CDCl_3$ as solvent system using tetramethylsilane as the internal standard on a δ scale in ppm. Laboratory grade solvents and reagents obtained from Sigma Aldrich, SD Fine chemicals, Spectrochem and Merck were used without further purification for the synthesis.

*General procedure for the synthesis of 1-{4-cyanophenyl}-3-substituted urea (**2a-n**)[12]:*

To the solution of 4-amino benzonitrile (0.77 g, 6.5 mM) in dichloromethane (15 ml) stirred at 0°C, was added substituted isocyanates (6 mM). The reaction mixture was stirred for 4–6 h. After completion of the reaction, the product was filtered and washed with 20 ml of diethyl ether. The formed urea derivatives (**2a**-**n**) were used without further purification.

*General procedure for the synthesis of 1-(4-amidine phenyl)-3-substituted urea derivatives (**3a**-**n**)[10]:*

The solution of benzonitrile derivative **2a**-**n** (1 mM) was heated under reflux with hydroxylamine hydrochloride (0.33 g, 10 mM) and triethyl amine (TEA) (1.40 ml, 10 mM) in 15 ml methanol for 4–5 h. After completion, zinc dust (0.65 g, 10 mM), ammonium formate (0.63 g, 20 mM) and 20 ml of methanol were added and the heating was continued for another 8–12 h. Methanol was distilled off after completion of the reaction. To the reaction mixture 100 ml of water was added and amidines formed were extracted with ethyl acetate (2×50 ml). Organic phase was separated and dried over anhydrous sodium sulphate followed by evaporation to yield corresponding amidines (**3a**-**n**).

Oral bioavailability and toxicity prediction:

Various physicochemical properties of ligands that influence oral bioavailability, like molecular weight (MW), hydrogen bond donor count (HBD) and hydrogen bond acceptor count (HBA), topological polar surface area (TPSA), and log partition coefficient (log P) were determined using Jchem for excel (Chem axon). Oral bioavailability of the synthesized compounds was predicted considering Lipinsky rule[13] and GSK rule (number of rotatable bonds <10, topological polar surface area <140 Å^2)[14]. Toxicity risk assessment was carried using OSIRIS property explorer[15].

In vitro PAD4 assay:

The assay kit was procured from Cayman Chemical's and experiments were performed as per protocol available in the kit (https://www.caymanchem.com/pdfs/700560.pdf). A 10 mM primary stock of inhibitors was prepared in DMSO and further diluted to get a final concentration of 100 μM in each well. After addition of the inhibitor, the enzyme reaction mixture was incubated at 37°C for 20 min, followed by addition of substrate in each well. The plates were covered and incubated for 30 min at 37°C. The reaction was stopped with calcium chelator. The amount of ammonia released after hydrolysis of substrate was estimated by adding ammonia detecting reagent. Fluorescence intensity was monitored after incubated at 37°C for 15 min. The percentage inhibition of the PAD4 enzyme was then calculated by the following equation.

$$\% \text{ Inhibition} = (\Delta \text{ RFU of PC} - \Delta \text{ RFU of EI})/(\Delta \text{ RFU of PC}) \times 100$$

where RFU = relative fluorescence units, PC = positive control or enzyme control, EI = enzyme inhibitor solution.

In silico binding energy and pose prediction:

The human PAD4 crystal structure 1WDA in complex with benzoyl-L-arginine amide (BAA) was used for docking study. The docking simulation was performed using Glide (SP, XP), FRED and Autodock. Using the Schrödinger Maestro (Schrödinger Suite 2009) program, Ala645 was mutated back to Cys645 and the protein was prepared for docking. The ligands were drawn with Marvin Sketch (http://www.chemaxon.com) and prepared with ligand preparation wizard of Maestro. For Autodock, the ligands were further processed by adding Gasteiger charges using MGL tools v1.5.4[16]. For glide docking, grid of 1WDA active site was created by using centroid of co-crystallised ligand (x, y, z coordinates: 24.11 Å, 49.33 Å, 22.86 Å). After validation of the docking protocol the docking of the ligands were carried in Glide SP and Glide XP mode[17]. The Glide XP poses were again subjected for binding energy estimation by Molecular mechanics with generalized Born and surface area solvation (MM-GBSA) method. All the protein atoms were kept frozen while ligand was made flexible. The minimization was carried out using Prime MM-GBSA (Schrödinger Suite 2013: Prime MM-GBSA) for 100 iterations. The ligands in unbound state were minimized with variable dielectric surface gener-

alized Born (VSGB) solvation model and free energy was calculated. For docking with FRED (www.eyesopen.com, 2010), the protein prepared by Maestro was used and a grid box of volume 6935 Å^3 was created around co-crystallised ligand. After validating the docking protocol multi conformer database of the ligands prepared by OMEGA (OMEGA version 2.3.2) was docked using MMFF 94 force field. For docking with Autodock, Maestro processed file of 1WDA was used and Kollman charges were assigned. Grid box of of 54 Å –54 Å –54 Å with center at 24.11 Å, 49.33 Å and 22.86 Å (x, y, z) coordinate was created. The ligands were then docked on to the active site with genetic algorithm and lamarckian search (GA-LS) parameter producing 27000 generations in each run. 15 such runs were performed for each ligand. The docked poses were clustered and analyzed for interaction with the protein.

Table 1. Spectral data of synthesized compounds

Compound	Spectral data
3a	IR, $\tilde{\nu}$/cm^{-1}: 3329.07 (N-H stretching), 3165.19–3068.74 (aromatic C-H stretching), 2945.58 (aliphatic C-H stretching), 1645.28 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 2.38 (s, 3H), 3.54 (s, 2H), 6.63 (s, 1H), 6.92 (s, 1H), 7.45–7.98 (m, 8H), 9.29 (s, 1H); MS, *m/z* (I_r/%): 269.14 (16.5) (M+1)
3b	IR, $\tilde{\nu}$/cm^{-1}: 3350.12 (N-H stretching), 3117.03–3045.22 (aromatic C-H stretching), 3000.18 (aliphatic C-H stretching), 1638.78 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 2.35 (s, 3H), 3.48 (s, 2H), 6.37 (s, 1H), 6.99 (s, 1H), 7.32–7.87 (m, 8H), 9.31 (s, 1H); MS, *m/z* (I_r/%): 269.14 (16.5) (M+1)
3c	IR, $\tilde{\nu}$/cm^{-1}: 3371.78 (N-H stretching), 3166.08–3087.10 (aromatic C-H stretching), 2970.14 (aliphatic C-H stretching), 1655.08 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 3.65 (s, 3H), 6.21 (s, 1H), 6.40 (s, 1H), 7.41–8.03 (m, 8H), 9.94 (s, 1H); MS, *m/z* (I_r/%): 285.09 (17.8) (M+1)
3d	IR, $\tilde{\nu}$/cm^{-1}: 3369.10 (N-H stretching), 3172.43–3077.50 (aromatic C-H stretching), 2978.17 (aliphatic C-H stretching), 1639.09 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 3.84 (s, 3H), 6.25 (s, 1H), 6.63 (s, 1H), 7.47–8.45 (m, 8H), 9.91 (s, 1H); MS, *m/z* (I_r/%): 285.10 (19.5) (M+1)
3e	IR, $\tilde{\nu}$/cm^{-1}: 3361.78 (N-H stretching), 3176.73–3045.17 (aromatic C-H stretching), 2977.84 (aliphatic C-H stretching), 1645.78 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 3.87 (s, 3H), 6.53–6.66 (hump, 2H), 7.47–8.32 (m, 8H), 9.77 (s, 1H); MS, *m/z* (I_r/%): 285.12 (16.94) (M+1)
3f	IR, $\tilde{\nu}$/cm^{-1}: 3404.16 (N-H stretching), 3180.47–3027.86 (aromatic C-H stretching), 1640.85 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 3.82 (s, 2H), 6.21 (s, 1H), 6.35 (s, 1H), 7.15–8.54 (m, 8H), 9.07 (s, 1H); MS, *m/z* (I_r/%): 273.10 (18.2) (M+1)
3g	IR, $\tilde{\nu}$/cm^{-1}: 3398.78 (N-H stretching), 3146.33–3015.66 (aromatic C-H stretching), 1639.90 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 3.98 (s, 2H), 6.44 (hump, 1H), 6.98 (s, 1H, NH), 7.04–8.14 (m, 8H), 8.79 (s, 1H); MS, *m/z* (I_r/%): 273.12 (15.5) (M+1)
3h	IR, $\tilde{\nu}$/cm^{-1}: 3381.78 (N-H stretching), 3166.33–3062.15 (aromatic C-H stretching), 1641.08 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) : 4.01 (s, 2H), 6.18 (s, 1H), 6.54 (s, 1H), 6.98–7.84 (m, 8H), 9.88 (s, 1H); MS, *m/z* (I_r/%): 273.08 (18.8) (M+1)
3i	IR, $\tilde{\nu}$/cm^{-1}: 3358.74 (N-H stretching), 3147.22–3044.12 (aromatic C-H stretching), 1649.85 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 4.18 (s, 2H), 6.04 (s, 1H), 6.56 (s, 1H), 7.18 (d, 2H), 7.41 (d, 2H), 7.88 (d, 2H), 8.01 (d, 2H), 9.51 (s, 1H); MS, *m/z* (I_r/%): 289.08 (15.3%) (M+), 290.07 (32.0%) (M+1)
3j	IR, $\tilde{\nu}$/cm^{-1}: 3441.74 (N-H stretching), 3168.47–3014.95 (aromatic C-H stretching), 1646.15 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 4.23 (s, 2H), 6.06 (s, 1H), 6.64 (s, 1H), 7.18 (d, 2H), 7.36 (d, 2H), 7.71 (d, 2H), 8.13 (d, 2H), 9.68 (s, 1H); MS, *m/z* (I_r/%): 300.11 (15.6) (M+1)
3k	IR, $\tilde{\nu}$/cm^{-1}: 3438.47 (N-H stretching), 3285.19–3072.47 (aromatic C-H stretching), 2944.58 (C-H stretching), 1645.28 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 4.25–4.64 (hump, 4H), 6.05 (s, 1H), 6.52 (s, 1H), 7.35–8.10 (m, 8H), 9.05 (s, 1H); MS, *m/z* (I_r/%): 331.09 (19.0%) (M+1)
3l	IR, $\tilde{\nu}$/cm^{-1}: 3447.42 (N-H stretching), 3167.03–3064.41 (aromatic C-H stretching), 2984.54 (C-H stretching), 1638.78 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 1.25 (t, 3H), 4.15 (q, 2H), 6.53 (s, 2H), 7.35–8.27 (m, 8H), 9.18 (s, 1H); MS, *m/z* (I_r/%): 327.11 (16.1) (M+1)
3m	IR, $\tilde{\nu}$/cm^{-1}: 3350.12 (N-H stretching), 3117.03–3045.22 (aromatic C-H stretching), 2997.18 (C-H stretching), 1639.11 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 1.20 (t, 3H), 4.28 (q, 2H), 6.47 (s, 2H), 7.20–8.12 (m, 9H), 9.78 (s, 1H); MS, *m/z* (I_r/%): 327.14 (16.5) (M+1)
3n	IR, $\tilde{\nu}$/cm^{-1}: 3350.12 (N-H stretching), 3117.03–3045.22 (aromatic C-H stretching), 3025.10 (C-H stretching), 1636.66 (C=O stretching); ^{1}H NMR (mixture of DMSO-d_6, $CDCl_3$) δ: 1.22 (t, 3H), 4.35 (q, 2H), 6.76 (s, 2H), 7.07–7.98 (m, 9H), 9.95 (s, 1H); MS, *m/z* (I_r/%): 327.14 (15.9) (M+1)

Results and discussion

The benzamidine urea derivatives were synthesized by reacting various isocyanates with 4-amino benzonitrile. Amine acts as a nucleophile and attacks on carbonyl carbon of isocynate, followed by internal rearrangement leading to urea derivatives which get precipitated in the reaction mixture. Washing of the precipitate with ether yielded the pure product. Formation of the desired compounds was confirmed by IR spectroscopy. The compounds showed NH stretching at around 3321 to 3286 cm^{-1}, C-N stretching at 2227 to 2238 cm^{-1} and the carbonyl stretching peak corresponding to amide at 1716 to 1633 cm^{-1}. Benzonitriles were then condensed with hydroxylamine hydrochloride to form amidoxime. Amino group makes a nucleophilic attack on carbon of nitrile group and the intermediate amino alcohol rearranges to form amidoxime. After completion of the reaction, amidoxime was reduced using zinc and ammonium formate. Ammonium formate protonates the substrate and forms a complex with zinc. Zinc donates electron to carbon, followed by elimination of hydroxyl group as water. The amidines formed were extracted with ethyl acetate and purified by column chromatography. Formation of the desired compounds was confirmed by presence of N-H stretch at 3325 to 3479 cm^{-1}, disappearance of CN stretching peak in the IR and the presence of the characteristic peaks in the ^1H NMR spectra.

The physicochemical properties of the synthesized compounds (Table 2) were well within the criteria of Lipinski rule of five and GSK rule for oral bioavailability. *In silico* toxicity prediction studies showed that compounds with 4-methoxyphenyl (**3e**) and 4-methylphenyl (**3b**) substitution were predicted to have high risk of mutagenicity while 3-methoxyphenyl (**3d**) and 4-nitrophenyl (**3j**) substituted molecules were predicted to have medium risk of mutagenicity. The compound **3e** was predicted to possess mild risk of tumorigenicity and skin irritation. 3-Methoxyphenyl (**3d**) and 3-fluorophenyl (**3g**) substituted molecules were predicted to have high and medium risk of reproductive toxicity respectively. The compounds **3c** and **3i** showed good drug score and other molecules showed low to medium drug score.

The synthesized compounds were tested for PAD4 inhibitory potential at 100 μM concentration and activity is expressed as % inhibition. 4-Methylphenyl urea derivative **3b** was the most potent among the series with 36.22% inhibition, whereas the 3-methyl substitution rendered the compound **3a** inactive. Electron donating methoxy substitution at the 3- and 4-position (compounds **3d** and **3e**), rendered the compounds inactive towards PAD4 inhibition, while 2-methoxy substitution in **3c** caused moderate inhibition of PAD4 enzyme. The electron withdrawing groups such as nitro at 4 position made the compound **3j** inactive towards PAD4. The fluoro substitution at 2- and 3-position also rendered the corresponding compounds **3f** and **3g**, respectively,

Table 2. Physicochemical properties and *in silico* toxicity profile of the synthesized compounds

Code	MW (Da)	HBD	HBA	TPSA (Å^2)	Rot	JC log P	MUT	TUM	IRT	REP	Drug likeliness	Drug score
3a	268.31	5	5	91	3	1.86	–	–	–	–	1.21	0.78
3b	268.31	5	5	91	3	1.88	+	–	–	–	–0.22	0.38
3c	284.31	5	6	100.23	4	0.72	–	–	–	–	1.84	0.84
3d	284.31	5	6	100.23	4	1.15	+	–	–	++	1.39	0.39
3e	284.31	5	6	100.23	4	1.21	++	+	–	–	0.1	0.33
3f	272.28	5	5	91	3	0.19	–	–	–	–	0.26	0.69
3g	272.28	5	5	91	3	1.43	–	–	–	+	–1.25	0.43
3h	272.28	5	5	91	3	1.49	–	–	–	–	0	0.67
3i	288.73	5	5	91	3	1.94	–	–	–	–	2.23	0.8
3j	299.28	5	8	139.83	4	1.07	+	–	–	–	–12	0.35
3k	330.77	5	6	108.07	5	1.28	–	–	–	–	2.37	0.77
3l	326.35	5	7	117.3	6	1.84	–	–	+	–	–2.52	0.37
3m	326.35	5	7	117.3	6	1.66	–	–	–	–	–3.37	0.44
3n	326.35	5	7	117.3	6	1.5	–	–	–	–	–2.23	0.47

MW: molecular weight; HBD: hydrogen bond donor count; HBA: hydrogen bond acceptor count; TPSA: topological polar surface area; Rot: number of rotatable bonds; JC log P: Jchem log partition coefficient. MUT = mutagenesicity, TUM = tumorigenic, REP = reproductive effect, IRT = skin irritation. Values in column MUT, TUM, Irritant and REP represents –: no indication, +: medium and ++ = high risk.

Table 3. Biological assay and docking analysis

Code	% inhibition[a]	Autodock	Glide SP	Glide XP	MMGBSA	FRED
3a	23.96	–6.68	–7.7	–1.98	–39.73	–41.01
3b	36.22	–6.58	–7.64	–2.14	–27.22	–48.99
3c	31.18	–6.06	–8.24	–2.01	–40	–42.35
3d	IA	–6.22	–6.41	ND	ND	–39.26
3e	IA	–6.37	–5.01	–1.69	–25.45	–38.35
3f	IA	–6.2	–7.77	–2.32	–35.1	–45.38
3g	IA	–6.38	–7.8	–2.15	–23.87	–39.32
3h	19.9	–6.29	–7.79	–1.64	–27.21	–39.03
3i	33.07	–5.52	–7.38	-2.11	–31.13	–47.4
3j	IA	–6.36	–7.58	ND	ND	–35.17
3k	30.5	–5.53	–7.4	–1.44	–35.99	–31.37
3l	16.68	–6.66	–8.06	–2.07	–40.59	–38.16
3m	25.78	–6.2	–6.82	ND	ND	–46.06
3n	18.79	–5.16	–7.21	–0.88	–44.62	–32.98

[a]% inhibition at 100 μM concentration (SEM <10%); Docking scores are expressed in kcal/mol; IA: inactive; ND: not docked.

as inactive but the substitution at 4-position made the compounds **3h** moderately active with 19.9% inhibition. Activity was further enhanced to 33.07% in compound **3i**, when 4-fluoro substituent was replaced with the chloro group. Activity was decreased slightly from 33.07 to 30.5% when the 4-chloro substituent was replaced with the CO-CH_2Cl (chloro acetyl) group in compound **3k**. The activity decreased to 18.79% by replacing the chloroacetyl group with the $COOCH_2CH_3$ (ethoxy carbonyl) group in the compound **3n**. Substitution of the ethoxy carbonyl group at 2-position as in **3l** and at 3-position as in **3m** showed 16.68 and 26.78% inhibition, respectively.

Since the benzamidine urea series displayed poor activity, the *in silico* studies were performed to assess the interaction pattern of these compounds. It was observed that in the docking poses of the compound **3l**, **3c**, **3m**, and **3n** the amidine moiety was outside the cavity in Autodock as well as in Glide SP and XP, though these compounds maintained hydrophobic and hydrogen bond interaction with Arg374 or Arg639 (Fig. 2). In FRED docking, all the poses maintained hydrogen bond interaction with Asp350 and hydrophobic interaction but failed to interact with Arg374 or Arg639 (Fig. 3).

A significantly lower Glide XP score compared to Glide SP score clearly indicates that the synthesized benzamidines interact very weakly. The FRED docking pose indicates that due to linear nature of the urea linker, phenyl ring derived from isocyanates tends to have greater solvent exposure

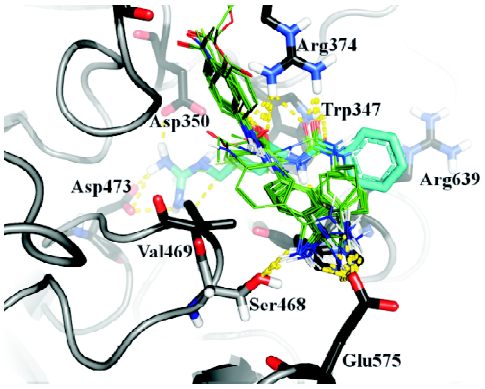


Fig. 2. Glide SP docking pose of synthesized compounds (green wire) along with cocrystallized ligand BAA (cyan color).

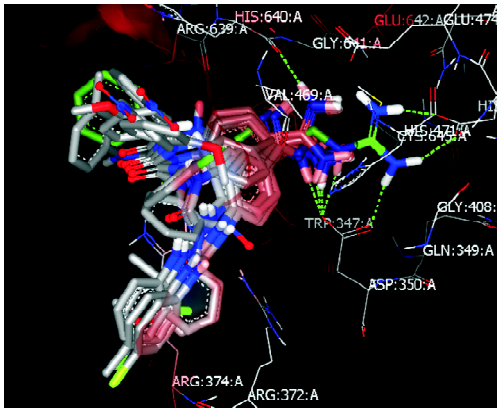
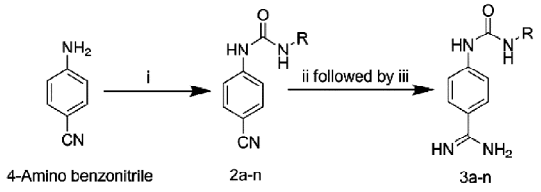


Fig. 3. FRED docking pose of synthesized compounds (gray) along with cocrystallized ligand BAA (green).



	R		R
3a	$-C_6H_4$-3-CH_3	**3h**	$-C_6H_4$-4-F
3b	$-C_6H_4$-4-CH_3	**3i**	$-C_6H_4$-4-Cl
3c	$-C_6H_4$-2-OCH_3	**3j**	$-C_6H_4$-4-NO_2
3d	$-C_6H_4$-3-OCH_3	**3k**	$-C_6H_4$-4-$COCH_2Cl$
3e	$-C_6H_4$-4-OCH_3	**3l**	$-C_6H_4$-2-$COOC_2H_5$
3f	$-C_6H_4$-2-F	**3m**	$-C_6H_4$-3-$COOC_2H_5$
3g	$-C_6H_4$-3-F	**3n**	$-C_6H_4$-4-$COOC_2H_5$

Scheme 1. Reagents and conditions: (i) substituted isocyanates, CH_2Cl_2, 0°C, 4–6 h; (ii) $NH_2OH.HCl$, triethyl amine, CH_3OH, reflux, 4–5 h; (iii) Zn, HCO_2NH_4, CH_3OH, reflux, 8–12 h.

which is unfavorable. Therefore, to minimize the exposure of nonpolar group to solvent side, docked poses showed either amidine moiety outside the cavity or the molecule was tilted resulting reduced interaction with the Asp350 and Asp473 which are necessary for binding. Hence, failure of these compounds to interact with the desired amino acid residues may be a probable reason for low PAD4 inhibitory activity. Similar benzamidine urea derivatives act as arginine mimetic and are reported to have sub-micromolar activity against human thrombin[18]. However, these derivatives did not inhibit PAD4 at 100 μM concentration. This may serve as an indication about the selectivity of these compounds towards thrombin, trypsin, and other serine proteases and rules out the possibility that benzamidines may act as lead for PAD4.

Conclusions

The synthesized arginine mimetic compounds displayed weak activity against PAD4. The docking simulation study revealed that the benzamidine derivatives failed to display the desired interactions with the active site of PAD4 enzyme. The docking study was performed using different softwares and the synthesized compounds scored low in all of these. Hence, it may be concluded that the benzamidine urea derivatives may not serve as lead for future design of PAD4 inhibitors.

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