DR VINEY LATHER (Orcid ID : 0000-0002-7323-3142)

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N-Pyridin-2-yl Benzamide Analogues as Allosteric Activators of Glucokinase: Design, Synthesis, *In Vitro*, *In Silico* and *In Vivo* Evaluation

Ajmer Singh Grewal[1, 2], Rajeev Kharb[3], Deo Nandan Prasad[4], Jagdeep Singh Dua[4], Viney Lather[3,5*]

[1]*Chitkara College of Pharmacy, Chitkara University, Rajpura, 140401, Punjab, India;* [2]*I. K. Gujral Punjab Technical University, Jalandhar, 144601, Punjab, India;* [3]*Amity Institute of Pharmacy, Amity University, Noida, 201313, U.P., India;* [4]*Shivalik College of Pharmacy, Naya-Nangal, 140126, Punjab, India;* [5]*Jan Nayak Ch. Devi Lal Memorial College of Pharmacy, Sirsa, 125055, Haryana, India*

[*]**Corresponding author:** Prof. (Dr.) Viney Lather; Amity Institute of Pharmacy, Amity University, Noida, 201303, U.P., India; E-mail: vinnylather@gmail.com.

Abstract: Glucokinase (GK) is the key enzyme controlling levels of blood glucose under normal physiological range and GK activators are emerging class of drug candidates with promising hypoglycaemic activity. The current study was planned to design, synthesize and evaluate novel N-pyridin-2-yl benzamide analogues as allosteric activators of GK. A novel series of N-pyridin-2-yl benzamide analogues were synthesized starting from 3-nitrobenzoic acid and evaluated *in vitro* for GK activation followed by *in silico* studies to predict the binding interactions of the designed molecules with GK protein. The selected synthesized molecules (compounds **5b**, **5c**, **5e**, **5g**, **5h** and **6d**) which displayed excellent GK activity (GK fold activation around 2) in GK assay and appreciable binding interaction with GK in docking studies were further evaluated for their antihyperglycemic potential using oral glucose tolerance test (OGTT) in rats. Amongst the compounds tested in

vivo (OGTT assay) for antihyperglycemic potential, compounds **5c**, **5e** and **5g** displayed significant reduction in blood glucose levels. Compound **5e** displayed most significant antidiabetic activity and comparable to that of standard drug in animal studies. The N-pyridin-2-yl benzamide analogues discovered in current study can provide some lead molecules for the development of potent oral GK activators with minimum side effects for the management of type 2 diabetes.

Introduction

Type 2 diabetes (T2D) is a long-term disorder of food metabolism caused due to reduced action of insulin, characterized by hyperglycaemia and affects majority of the diabetic patients [1-2]. Despite the fact that various types of oral hypoglycaemic drugs are existing intended for the T2D management, in majority of T2D patients no single drug is helpful in achieving long-term management of blood glucose under normal physiological range. Owing to the above reason, currently physicians prescribe combination of antidiabetic drugs at an early stage for the therapeutics of T2D. Moreover, overdose of antihyperglycemic agents may lead to severe hypoglycaemia resulting in brutal toxic and side effects, and patients usually need urgent therapeutic treatment [1]. Currently, medicinal chemists are focusing on synthesizing novel potent antihyperglycemic drugs with biologically different mechanism of action which can be utilized as single drug therapy with better safety. Results from various latest studies together with promising clinical reports, have indicated that allosteric activators of glucokinase (GK) could be used as antidiabetic drugs with superior potency and safety [3-6].

GK is a cytoplasmic enzyme which accelerates the breakdown of glucose to glucose-6-phosphate in presence of ATP and helps in the maintenance of the normal blood glucose levels in humans. GK enzyme is expressed primarily in β-cells in pancreas and liver hepatocyte [4, 6-7]. In pancreatic β-cells, GK controls glucose-stimulated insulin release and in liver hepatocyte cells, it controls the metabolism of carbohydrates. GK is an emerging drug target for T2D therapeutics owing to its major role in the regulation of sugar metabolism. GK activators are the novel category of therapeutic agents which activate GK allosterically and demonstrate their hypoglycaemic potential [3, 8-10]. A broad variety of chemically different classes of compounds including substituted benzamide analogues [11-20], carboxamide derivatives like acetamides, butanamides and other [21-26], acrylamides [27], heterocyclic compounds like benzimidazole derivatives [28-29], quinazolines derivatives [30], thiazole derivatives [31], pyrimidine derivatives [32], and substituted urea compounds [33-34] were developed recently to act as potent GK activators with antidiabetic effects. Several GK activators had been

advanced to phase II clinical trials including Piragliatin, AZD6370, AZD1656, MK-0941, and AMG151; although potent reduction of blood glucose efficacy had been reported, possible adverse effects had also been observed, including hypoglycaemia and raised triglyceride levels. The maximum drug discovery and development programmes linked with GK activators were primarily cantered on the substituted benzamide derivatives possibly due to their complementary orientation pattern and binding interactions with the allosteric site of the GK enzyme [5]. Based on the significance of GK activators in T2D therapeutics and the potential of benzamide scaffold for GK activation, some newer N-pyridin-2-yl substituted sulfamoyl benzamide derivatives were designed as allosteric GK activators. General structure of the designed N-pyridin-2-yl benzamide derivatives and the possible interactions with residues in allosteric site of GK protein are presented in Figure **1**.

Materials and Methods

Chemicals and reagents were procured from SRL Pvt. Ltd., Spectrochem Pvt. Ltd., Sigma-Aldrich, Merck Pvt. Ltd., and Fisher Scientific etc., and used as such. Veego VMP-D melting point apparatus was used for determination of melting point of synthesized derivatives. Shimadzu IR affinity FTIR spectrophotometer (KBr pellet technique) was used for recording IR spectra. BrukerAvance II NMR spectrophotometer was used for recording ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra using DMSO-d_6 as solvent and presented in parts per million (δ, ppm) downfield from internal standard (tetramethylsilane).

Synthesis of N-pyridin-2-yl benzamide derivatives (Scheme 1)

Dry 3-nitrobenzoic acid (0.01 mol) was taken in a round bottom flask fixed with a magnetic stirrer and the temperature was maintained between 10 and 15 ºC using cold water bath. Chlorosulphonic acid (8.0 mL) was introduced cautiously and checked to substantiate no leakage. After whole acid had been dissolved and the exothermic reaction had been over, the reaction flask was heated on water bath at 70-80 ℃ for 2 h to complete the reaction followed by cooling the flask. The contents of flask were added to 150 g crushed ice with stirring to break the lumps and precipitates of 3-(chlorosulphonyl)-5-nitrobenzoic acid were filtered under vacuum followed by washing with cold water and air dried. The product obtained above (0.01 mol) was refluxed with commercially available amines (0.01 mol) in chloroform until reaction completion as monitored by TLC on silica gel G followed by cooling and precipitates of respective sulphonamides were dried. The various

sulphonamides (0.01 mol) were refluxed with thionyl chloride (0.01 mol) for 3 h and excess $SOCl_2$ was distilled off to get the respective benzoyl chlorides. The different benzoyl chlorides (0.01 mol) and pyridin-2-amines (0.015 mol) were refluxed in chloroform and the final products were recrystallized using ethanol [18, 20, 35].

3-Nitro-5-(phenylsulfamoyl)-N-(pyridin-2-yl)benzamide (5a): Pale white solid; Yield- 72%; Mp (°C) 148-150; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3317.57 (NH str., CO-NH), 3157.46 (NH str., SO_2-NH), 3091.89 (CH str.), 1674.21 (C=O str.), 1620.21 (NH bend), 1608.43 (C=N str.), 1533.41 (NO_2 sym. str.), 1483.26 (C=C str.), 1392.61 (NO_2 asym. str.), 1348.24 (SO_2 asym. str.), 1155.36 (SO_2 sym. str.), 717.52 (CH bend).

3-[(2-Chlorophenyl)sulfamoyl]-5-nitro-N-(pyridin-2-yl)benzamide (5b): Pale white solid; Yield- 69%; Mp (°C) 168-169; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3319.49 (NH str., CO-NH), 3088.03 (NH str., SO_2-NH), 2926.01 (CH str.), 1674.81 (C=O str.), 1608.43 (C=N str.), 1529.55 (NO_2 sym. str.), 1483.26 (C=C str.), 1392.61 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1139.93 (SO_2 sym. str.), 717.52 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.70 (s, 1H, NH, CO-NH), 8.32-8.45 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.52-8.30 (m, 4H, CH, C_3, C_4, C_5 and C_6 of Pyridin-2-yl), 7.11-7.62 (m, 4H, CH of C_3, C_4, C_5 and C_6 of C_6H_4Cl), 2.52 (s, 1H, NH, SO_2NH); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 166.56 (C=O), 157.62 (C), 148.12 (C), 145.02 (CH), 138.92 (C), 135.52 (C), 133.38 (CH), 133.38 (CH), 130.78 (CH), 126.88 (CH), 126.78 (CH), 126.78 (CH), 126.68 (C), 126.56 (C), 125.58 (CH), 123.28 (CH), 123.26 (CH), 112.12 (CH), 110.10 (CH); HRMS (ESI TOF) m/z for $C_{18}H_{13}ClN_4O_5S$ $[M+H]^+$ Calcd 432.029, Found 432.067.

3-[(3-Chlorophenyl)sulfamoyl]-5-nitro-N-(pyridin-2-yl)benzamide (5c): Grayish black solid; Yield- 68%; Mp (°C) 174-179; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3352.28 (NH str., CO-NH), 3155.84 (NH str., SO_2-NH), 3097.68 (CH str.), 1666.50 (C=O str.), 1622.13 (NH bend), 1533.41 (NO_2 sym. str.), 1394.53 (NO_2 asym. str.), 1348.24 (SO_2 asym. str.), 1155.36 (SO_2 sym. str.), 719.45 (CH bend), 692.44 (C-Cl str.); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 9.89 (s, 1H, NH, CO-NH), 8.32-8.45 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.00-8.20 (m, 4H, CH, C_3, C_4, C_5 and C_6 of Pyridin-2-yl), 6.89-7.27 (m, 4H, CH of C_2, C_4, C_5 and C_6 of C_6H_4Cl), 4.81 (s, 1H, NH, SO_2NH); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 170.88 (C=O), 153.07 (C), 153.02 (C), 152.80 (CH), 140.92 (C), 140.62 (C), 137.99 (CH), 137.93 (C), 135.71 (C), 132.48 (CH), 128.94

(CH), 128.79 (CH), 123.96 (CH), 120.44 (CH), 119.18 (CH), 118.18 (CH), 117.58 (CH), 116.88 (CH); HRMS (ESI TOF) m/z for $C_{18}H_{13}ClN_4O_5S$ $[M+H]^+$ Calcd 432.031, Found 432.014.

3-[(4-Chlorophenyl)sulfamoyl]-5-nitro-N-(pyridin-2-yl)benzamide (5d): Light brown solid; Yield- 62%; Mp (°C) 169-171; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3323.45 (NH str., CO-NH), 3149.76 (NH str., SO_2-NH), 3088.03 (CH str.), 2968.45 (CH str.), 16770.35 (C=O str.), 1622.13 (NH bend), 1529.55 (NO_2 sym. str.), 1483.26 (C=C str.), 1390.68 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1138.00 (SO_2 sym. str.), 717.52 (CH bend), 690.52 (C-Cl str.).

3-Nitro-5-[(2-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5e): Pale yellow solid; Yield- 68%; Mp (°C) 162-164; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3691.91 (NH str., CO-NH), 3442.94 (NH str., SO_2-NH), 3095.75 (CH str.), 1674.21 (C=O str., CO-NH), 1624.06 (NH bend, Ar-NH), 1579.70 (C=N str.), 1529.55 (NO_2 sym. str.), 1384.89 (NO_2 asym. str.), 1348.24 (SO_2 asym. str.), 1172.72 (SO_2 sym. str.), 717.52 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.81 (s, 1H, NH, CO-NH), 8.32-8.45 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.58-8.44 (m, 4H, CH, C_3, C_4, C_5 and C_6 of Pyridin-2-yl), 6.64-7.88 (m, 4H, CH of C_3, C_4, C_5 and C_6 of $C_6H_4NO_2$), 2.51 (s, 1H, NH, SO_2NH); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 166.36 (C=O), 155.43 (C), 148.30 (C), 148.11 (CH), 144.04 (C), 135.81 (C), 137.54 (CH), 135.26 (CH), 134.41 (C), 126.74 (CH), 125.80 (CH), 115.91 (CH), 119.64 (CH), 114.31 (CH); HRMS (ESI TOF) m/z for $C_{18}H_{13}N_5O_7S$ $[M+H]^+$ Calcd 443.053, Found 444.016.

3-Nitro-5-[(3-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5f): Brownish yellow solid; Yield- 67%; Mp (°C) 161-162; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3332.99 (NH str., CO-NH), 3153.61 (NH str., SO_2-NH), 3093.82 (CH str.), 1670.35 (C=O str.), 1622.13 (NH bend), 1529.55 (NO_2 sym. str.), 1481.33 (C=N str.), 1388.75 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1143.79 (SO_2 sym. str.), 721.38 (CH bend).

3-Nitro-5-[(4-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5g): Yellow solid; Yield- 73%; Mp (°C) 158-160; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3346.50 (NH str., CO-NH), 3172.90 (NH str., SO_2-NH), 2972.31 (CH str.), 1668.43 (C=O str.), 1625.99 (NH bend), 1533.41 (NO_2 sym. str.), 1404.18 (NO_2 asym. str.), 1344.38 (SO_2 asym. str.), 1170.79 (SO_2 sym. str.), 717.52 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.63 (s, 1H, NH, CO-NH), 8.32-8.60 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.59-8.44 (m, 4H, CH, C_3, C_4, C_5 and C_6 of Pyridin-2-yl), 6.79-7.94 (m, 4H, CH of C_2, C_3, C_5 and C_6 of $C_6H_4NO_2$), 2.50 (s, 1H, NH, SO_2NH); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 166.04 (C=O), 156.18 (C), 155.02 (C), 148.28 (CH), 143.89 (C), 136.87 (CH), 136.03 (CH), 135.73 (C), 130.79 (CH), 127.65 (CH), 126.78 (CH), 124.10 (CH), 113.54 (CH), 112.82 (CH), 112.42 (CH); HRMS (ESI TOF) m/z for $C_{18}H_{13}N_5O_7S$ $[M+H]^+$ Calcd 443.042, Found 443.064.

3-(Benzylsulfamoyl)-5-nitro-N-(pyridin-2-yl)benzamide (5h): Light brown solid; Yield- 62%; Mp (°C) 144-146; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3348.42 (NH str., CO-NH), 3149.76 (NH str., SO_2-NH), 3026.26 (CH str.), 2962.66 (CH str., Alkyl), 1670.35 (C=O str., CO-NH), 1622.13 (NH bend), 1533.41 (NO_2 sym. str.), 1479.40 (CH bend), 1390.68 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1153.43 (SO_2 sym. str.), 717.52 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 9.89 (s, 1H, NH, CO-NH), 7.27-8.20 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.89-8.18 (m, 4H, CH, C_3, C_4, C_5 and C_6 of Pyridin-2-yl), 6.00-7.27 (m, 5H, CH of C_2, C_3, C_4, C_5 and C_6 of $C_6H_4NO_2$), 7.25 (s, 1H, NH, SO_2NH), 4.81 (d, 1H, CH, CH_2); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 170.92 (C=O), 160.93 (C), 153.08 (C), 147.28 (CH), 143.95 (C), 140.57 (C), 138.12 (CH), 135.70 (C), 128.93 (CH), 128.77 (CH), 117.25 (CH), 117.12 (CH), 45.36 (CH_2); HRMS (ESI TOF) m/z for $C_{19}H_{16}N_4O_5S$ $[M+H]^+$ Calcd 412.084, Found 412.039.

3-(Ethylsulfamoyl)-5-nitro-N-(pyridin-2-yl)benzamide (5i): Pale white solid; Yield- 69%; Mp (°C) 151-153; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3687.90 (NH str., CO-NH), 3350.35 (NH str., SO_2-NH), 3095.75 (CH str.), 2926.01 (CH str.), 1674.21 (C=O str.), 1624.06 (NH bend), 1533.41 (NO_2 sym. str.), 1442.75 (CH bend), 1394.53 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1138.00 (SO_2 sym. str.), 719.45 (CH bend).

3-Nitro-5-(phenylsulfamoyl)-N-(4-methylpyridin-2-yl)benzamide (6a): Red solid; Yield- 58%; Mp (°C) 149-150; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3361.93 (NH str., CO-NH), 3188.33 (NH str., SO_2-NH), 3088.03 (CH str.), 2922.16 (CH str.), 1664.57 (C=O str.), 1612.49 (NH bend), 1527.62 (NO_2 sym. str.), 1481.33 (CH bend), 1352.10 (NO_2 asym. str.), 1311.59 (SO_2 asym. str.), 1151.50 (SO_2 sym. str.), 711.73 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.76 (s, 1H, NH, CO-NH), 8.32-8.45 (s, 3H, CH, C_2, C_4

and C_6 of C_6H_3CO), 6.52-8.30 (m, 3H, CH, C_3, C_5 and C_6 of Pyridin-2-yl), 7.11-7.62 (m, 4H, CH of C_2, C_3, C_4, C_5 and C_6 of C_6H_5), 2.52 (s, 1H, NH, SO_2NH), 2.36 (s, 3H, CH, CH_3).

3-[(2-Chlorophenyl)sulfamoyl]-5-nitro-N-(4-methylpyridin-2-yl)benzamide (6b): Pale white solid; Yield- 78%; Mp (°C) 144-145; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3361.93 (NH str., CO-NH), 3188.33 (NH str., SO_2-NH), 3088.03 (CH str.), 2922.16 (CH str.), 1726.29 (C=O str.), 1676.14 (NH bend), 1525.69 (NO_2 sym. str.), 1481.33 (CH bend), 1386.82 (NO_2 asym. str.), 1348.24 (SO_2 asym. str.), 1232.51 (SO_2 sym. str.).

3-[(3-Chlorophenyl)sulfamoyl]-5-nitro-N-(4-methylpyridin-2-yl)benzamide (6c): Dark brown solid; Yield- 58%; Mp (°C) 178-180; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3329.00 (NH str., CO-NH), 3319.49 (NH str., SO_2-NH), 3089.96 (CH str.), 2924.09 (CH str.), 1662.64 (C=O str., CO-NH), 1624.06 (NH bend), 1533.41 (NO_2 sym. str.), 1533.41 (C=N str.), 1481.33 (C=C str.), 1440.33 (CH bend), 1417.68 (NO_2 asym. str.), 1352.10 (SO_2 asym. str.), 1141.86 (SO_2 sym. str.), 715.59 (CH bend), 690.52 (C-Cl str.).

3-[(4-Chlorophenyl)sulfamoyl]-5-nitro-N-(4-methylpyridin-2-yl)benzamide (6d): Brown solid; Yield- 64%; Mp (°C) 167-168; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3446.79 (NH str., CO-NH), 3321.42 (NH str., SO_2-NH), 3080.32 (CH str.), 2926.01 (CH str.), 1670.35 (C=O str.), 1624.06 (NH bend), 1525.69 (NO_2 sym. str.), 1487.12 (C=C str.), 1436.97 (CH bend), 1488.75 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1138.00 (SO_2 sym. str.), 717.52 (CH bend); 638.44 (C-Cl str.); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 9.89 (s, 1H, NH, CO-NH), 7.21-8.20 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.18-8.18 (m, 3H, CH, C_3, C_5 and C_6 of Pyridin-2-yl), 6.89-7.27 (m, 4H, CH of C_2, C_3, C_5 and C_6 of C_6H_4Cl), 4.81 (s, 1H, NH, SO_2NH); ^{13}C-NMR (δ ppm, 100 MHz, DMSO-d_6): 170.85 (C), 160.76 (C), 159.39 (C), 143.02 (C), 123.96 (CH), 153.07 (C), 152.80 (CH), 140.92 (C), 135.71 (C), 128.94 (CH), 120.44 (CH), 120.11 (CH), 116.88 (CH), 26.56 (CH_3); HRMS (ESI TOF) m/z for $C_{19}H_{15}ClN_4O_5S$ $[M+H]^+$ Calcd 446.053; Found 446.032.

3-Nitro-5-[(2-nitrophenyl)sulfamoyl]-N-(4-methylpyridin-2-yl)benzamide (6e): Yellow solid; Yield- 70%; Mp (°C) 157-159; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3477.66 (NH str., CO-NH), 3360.00 (NH str., SO_2-NH), 3089.96 (CH str.), 2926.01 (CH str.), 1674.21 (C=O str.), 1625.99 (NH bend), 1525.69 (NO_2 sym. str.), 1388.75 (NO_2 asym. str.), 1350.17 (SO_2 asym. str.), 1251.80 (SO_2 sym. str.), 719.45 (CH bend).

3-Nitro-5-[(3-nitrophenyl)sulfamoyl]-N-(4-methylpyridin-2-yl)benzamide (6f): Yellow solid; Yield- 71%; Mp (°C) 153-155; R_{f-} 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3325.28 (NH str., CO-NH), 3167.12 (NH str., SO_2-NH), 3093.82 (CH str.), 2924.09 (CH str.), 1664.57 (C=O str.), 1624.06 (NH bend), 1531.48 (NO_2

sym. str.), 1483.26 (C=C str.), 1442.74 (NO_2 asym. str.), 1352.10 (SO_2 asym. str.), 1141.86 (SO_2 sym. str.), 723.31 (CH bend); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.74 (s, 1H, NH, CO-NH), 7.76-8.22 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.59-7.89 (m, 3H, CH, C_3, C_5 and C_6 of Pyridin-2-yl), 6.56-7.86 (m, 4H, CH of C_2, C_4, C_5 and C_6 of $C_6H_4NO_2$), 2.50 (s, 1H, NH, SO_2NH), 2.32 (s, 3H, CH, CH_3).

3-Nitro-5-[(4-nitrophenyl)sulfamoyl]-N-(4-methylpyridin-2-yl)benzamide (6g): Yellow solid; Yield- 74%; Mp (°C) 162-164; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3444.87 (NH str., CO-NH), 3323.35 (NH str., SO_2-NH), 2929.87 (CH str.), 2856.58 (CH str.), 1662.64 (C=O str.), 1627.92 (NH bend), 1531.48 (NO_2 sym. str.), 1348.24 (NO_2 asym. str.), 1305.81 (SO_2 asym. str.), 1111.00 (SO_2 sym. str.).

3-(Benzylsulfamoyl)-5-nitro-N-(4-methylpyridin-2-yl)benzamide (6h): Light brown solid; Yield- 63%; Mp (°C) 148-150; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3327.21 (NH str., CO-NH), 3165.19 (NH str., SO_2-NH), 3088.03 (CH str.), 2960.73 (CH str.), 1624.06 (NH bend), 1533.41 (NO_2 sym. str.), 1481.33 (C=C str.), 1448.54 (NO_2 asym. str.), 1352.10 (SO_2 asym. str.), 1141.86 (SO_2 sym. str.), 715.59 (CH bend).

3-(Ethylsulfamoyl)-N-(4-methylpyridin-2-yl)-5-nitrobenzamide (6i): Orange solid; Yield- 52%; Mp (°C) 153-154; R_f- 0.71 (Benzene: Ethyl acetate); IR (KBr Pellets) ν cm^{-1}: 3446.79 (NH str., CO-NH), 3323.35 (NH str., SO_2-NH), 3165.19 (CH str.), 2926.01 (CH str.), 1670.35 (C=O str.), 1627.92 (NH bend), 1529.55 (NO_2 sym. str.), 1350.17 (NO_2 asym. str.), 1230.58 (SO_2 asym. str.), 1138.00 (SO_2 sym. str.); ^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6): 8.74 (s, 1H, NH, CO-NH), 7.76-8.22 (s, 3H, CH, C_2, C_4 and C_6 of C_6H_3CO), 6.52-7.88 (m, 3H, CH, C_3, C_5 and C_6 of Pyridin-2-yl), 7.68 (s, 1H, NH, SO_2NH), 3.36 (m, 2H, CH of CH_2), 2.32 (s, 3H, CH, CH_3), 1.08 (t, 3H, CH of CH_3).

In vitro enzyme assay

The GK activity of the synthesized compounds was evaluated using a coupled reaction with glucose-6-phosphate dehydrogenase (G-6-PDH) spectrometrically. All the compounds were prepared in dimethyl sulfoxide (DMSO) and the assay was performed in a final volume of 2000 µL containing 2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (25 mM, pH 7.4), glucose (10 mM), KCl (25 mM), $MgCl_2$ (1 mM), dithiothreitol (1 mM), adenosine triphosphate (1 mM), NAD (1 mM), G-6-PDH (2.5 U/mL), GK (0.5 µg), and test compounds (10 µM). Absorbance was measured at 340 nm after 3 min incubation period and GK activation fold by the synthesized compounds and GK fold activation was calculated compared with control (GK activation by DMSO only was considered as 100%) [20, 36-38].

Docking studies

Docking simulations were performed in the allosteric site of GK protein with Glide 5.8 module of Schrödinger Suite 2012 with extra precision mode [39-41]. The X-ray crystallographic information of GK protein with the allosteric activator was obtained from protein data bank. After studying a numbers of entries, the best entry (PDB code: 3IMX) was selected based on resolution and was used as the docking model. The 2D structures for the designed ligands were drawn in MarvinSketch and transformed to 3D with LigPrep 2.5 with OPLS2005 force field [42]. A similar docking methodology was used for the molecular docking of the synthesized derivatives as described in detail in earlier publications using Glide and the ligand poses with most favorable docking score were selected [18-19]. The binding interactions were analysed further for the docked poses of the ligands using PyMOL [20].

Oral glucose tolerance test (OGTT)

Sprague-Dawley rats (150-200 g) were procured from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar and kept at controlled room temperature (22 ± 2 ºC) and humidity (55 ± 5%). All the rats were fed with the normal pellet diet and water ad libitum, prior to the dietary manipulation. Consent was taken from Institutional Animal Ethics Committee to conduct this study (Approval No. JCDMCOP/IAEC/07/15/30). Based on the results of *in vitro* and *in silico* studies, selected synthesized derivatives (**5b**, **5c**, **5e**, **5g**, **5h** and **6d**) were evaluated in rat OGTT. Rats were divided into different groups containing six animals in each group and all animals were fasted overnight for at least 8 h before treatment. Control group was administered vehicle only (5% DMSO, p.o.), standard group was administered metformin (30 mg/kg, p.o.), and test groups were administered compounds **5b**, **5c**, **5e**, **5g**, **5h** and **6d** (50 mg/kg, p.o.). All the animals were administered with glucose (3 g/kg, p.o.) 30 min after drug administration. Blood samples were collected by puncturing the retro orbital sinus just prior to drug administration, and 0, 30, 60, 90, 120 min after glucose administration. Serum glucose level was measured immediately by using glucose estimation kit. Glucose area under curve (AUC) was calculated from the data (from 0 h to 120 h). The OGTT assay results were statistically analyzed by two-way ANOVA [20, 43].

Results and discussion

Chemistry

The general synthetic pathway followed for the preparation of pyridin-2-yl benzamide derivatives is given in Scheme **1**. 3-nitrobenzoic acid (**1**) was chlorosulphonated to obtain 3-(chlorosulphonyl)-5-nitrobenzoic acid (**2**) followed by reacting with amines to obtain respective sulphonamides (**3**). The sulphonamides were reacted with $SOCl_2$ to get respective benzoyl chlorides (**4**) which were then reacted with 2-aminopyridine and 2-amino-4-methylpyridine to obtain the final products (**5a-5i** and **6a-6i**, respectively). The synthesis as well as purity of synthesized derivatives was ensured by single spot TLC and was further established by their consistent FTIR, ^{1}H and ^{13}C NMR spectra.

The ^{1}H-NMR spectra of the synthesized benzamide derivatives contained a singlet signal corresponding to one proton of CO-NH around δ 9-10 ppm verifying the amide linkage formation and occurrence of singlet signal equivalent to one NH proton of SO_2NH around δ 2.5 ppm established the sulphonamides formation in the synthesized benzamide derivatives. The presence of three singlet signals around δ 8 ppm belonging to the protons at C_2, C_4 and C_6 of the aryl ring derived from 3-nitrobenzoic acid (*meta*-nitro benzoic acid) indicated that the CONH bond, SO_2NH bond and NO_2 group were placed *meta* to each other. In the ^{1}H-NMR spectra of compounds **5a-5i**, two doublet signals and two triplet signals corresponding to four aromatic CH protons were observed in the range δ 7-8 ppm which confirmed the presence of pyridin-2-yl ring. In the ^{1}H-NMR spectra of compounds **6a-6i**, two doublet signals and one singlet signal corresponding to three aromatic CH protons were observed around δ 7-8 ppm which confirmed the presence of 4-methylpyridin-2-yl. The presence of methyl group attached to C_4 of pyridine ring in compounds **6a-6i** was further confirmed by the presence of a singlet signal corresponding to methyl protons in the ^{1}H-NMR spectra of these compounds. In the ^{13}C-NMR spectra of synthesized compounds, singlet signal equivalent to carbonyl carbon was observed around δ 165-170 ppm indicating the presence of amide linkage in the structure of the synthesized benzamide derivatives. In the ^{13}C-NMR spectra of synthesized compounds singlet signal for carbon around δ 152 ppm confirming the presence of nitro group. The ^{13}C-NMR spectra of synthesized compounds showed singlet signal around δ 151 ppm corresponding to C_2 carbon of pyridine-2-yl ring indicating the presence of pyridine ring attached to CONH group in the structure of these compounds. The signal for C_4 of pyridine ring around δ 158 ppm and signal corresponding to methyl carbon around δ 26 ppm in the ^{13}C-NMR spectrum of compound **6d** indicated the presence of methyl group attached to pyridine ring. The IR spectra of the synthesized benzamide derivatives

showed the presence of amide NH- stretching vibrations around 3300-3200 cm^{-1}; aromatic CH stretching vibrations above 3000 cm^{-1}; SO_2 asymmetric and symmetric stretching vibrations around 1400-1300 cm^{-1} and 1200-1100 cm^{-1} respectively; and sulphonamide NH- stretching vibrations in the range 3400-3100 cm^{-1}, thus supporting the fact that an amide linkage and a sulphonamide functional group were present in the structure of the synthesized molecules. The NH- bending vibrations around 1600 cm^{-1} were present in the IR spectra of the synthesized molecules confirming the presence of aromatic NH functional group. The IR spectra of the synthesized benzamide derivatives showed presence of NO_2 symmetric and asymmetric stretching vibrations around 1600-1500 cm^{-1} and 1400-1300 cm^{-1} respectively supporting the presence of nitro functional group in the structure of all the synthesized molecules. In the IR spectra of the synthesized compounds, presence of C=N stretching vibrations around 1700-1600 cm^{-1} depicted the occurrence of pyridine ring in these compounds.

In vitro GK activity

The results of the *in vitro* GK assay (fold activation) are presented in Table 1. Amongst the synthesized derivatives, compounds **5b**, **5c**, **5e**, **5g**, **5h** and **6d** showed maximum GK activation in the *in vitro* GK assay (fold activation around 2 compared to control). Compounds **5a, 5d, 6a, 6g** and **6h** showed moderate fold activation (around 1.5 compared to control) of GK enzyme. Compounds **5f, 6b, 6c, 6e** and **6f** showed lower GK activation (around 1.25) compared to that of control. Compounds **5i** and **6i** were found to be inactive in the GK assay. The results of *in vitro* GK assay depicted that substitution of the pyridine-2-yl ring attached to CONH with 4-methyl group resulted in decreased GK activity compared to unsubstituted pyridine-2-yl ring. Amongst the compounds bearing 4-methylpyridin-2-yl ring only compound **6d** showed good GK activation (fold activation of 1.92) compared to compounds bearing unsubstituted pyridine-2-yl ring attached to benzamide nucleus. The N-pyridin-2-yl substituted benzamide derivative bearing N-2-nitrophenyl sulphonamide group (compound **5e**) displayed highest GK fold activation of 2.07. The N-pyridin-2-yl substituted benzamide derivatives bearing N-3-clorophenyl and N-benzyl substituted sulphonamide group (compounds **5c** and **5h**) displayed potent GK fold activation of 2.02 and 2.01, respectively. The N-pyridin-2-yl substituted benzamide derivatives bearing N-2-clorophenyl and N-4-nitrophenyl substituted sulphonamide group (compounds **5b** and **5g**) displayed good GK fold activation of 1.96 and 1.95, respectively compared to control. The results of *in vitro* GK assay demonstrated that replacement of the aromatic ring attached to sulphonamide NH with alkyl group (compounds **5i** and **6i**) led to decreased GK activity compared to compounds bearing aromatic ring attached to sulphonamide NH.

Docking studies

Lead optimization of the synthesized derivatives was done via calculation of drug-likeness properties (log P, mol. wt., hydrogen bond acceptors (HBA), and hydrogen bond donors (HBD). Almost all the synthesized derivatives showed drug like properties as established using Lipinski's rule of five (Table **1**). The docking studies were performed using Glide in the allosteric site of GK protein (PDB entry: 3IMX) and validated by docking of 3IMX ligand in the allosteric site. The designed GK activators were docked in the allosteric binding site comprising of Arg63, Tyr215, Met210, Tyr214, Val452 and Val455 residues. Glide score and Glide energy of the synthesized derivatives are presented in Table **1**. The docking studies of these molecules suggested a complimentary fit in the allosteric site of GK protein. On the basis of their Glide score, lowest Glide energy (kcal/mol) and docking interactions, compounds **5c**, **5e** and **5h** were further analyzed in details using PyMOL to explore the binding mode and docking interactions of the designed molecules with the amino acid residues in the allosteric site of GK protein.

A superimpose of the docked poses of compounds **5c, 5e** and **5h** with that of 3IMX ligand showed that these compounds had the similar orientation and binding pattern in the allosteric site of GK protein as that of reference ligand (Figure **2a**). The pyridin-2-yl group of compounds **5c, 5e** and **5h** projected in the hydrophobic pocket demonstrating interactions with Val455, Ala456, and Lys459 of the R13 helix, as well as Pro66 of connecting region I and Ile159 of the large domain, phenyl ring of benzamide scaffold protruded between Tyr214, Met210 and Val455 whereas the substituted aryl group of SO_2NH projected into the hydrophobic pocket consisting of residues Leu451, Tyr215, and Trp99 (Figure **2a**). The docked pose of the selected compounds **(5c, 5e** and **5h)** displayed the H-bond interaction between the amide NH and 'N' of pyridin-2-yl with backbone carbonyl (C=O) and NH of Arg63 residue on GK protein with H-bond distance of 2.9 Å and 2.9 Å, 3.0 Å and 2.9 Å, and 3.0 Å and 2.9 Å respectively for compounds **5c, 5e** and **5h** (Figures **2b, 2c and 2d**). The molecular docking of the designed N-pyridin-2-yl benzamide derivatives in the allosteric binding site of GK protein helped in predicting that the designed N-pyridin-2-yl benzamide derivatives could act as potent activators of GK enzyme.

In vivo antihyperglycemic activity

Based on screening by *in vitro* GK assay and docking studies selected compounds (**5b, 5c, 5e, 5g, 5h** and **6d**) were further evaluated for their glucose lowering effects by means of rat OGTT assay using metformin as standard antidiabetic drug. The results of antihyperglycemic activity were measured as blood glucose levels (mg/dL) at different time intervals and glucose AUC represented in Figure **3**. The results of antihyperglycemic activity assay depicted that amongst compounds tested for OGTT assay, compounds **5c**, **5e** and **5h** were found to be highly active with compound **5e** having superior efficacy than compounds **5c** and **5h** in OGTT assay. Compound **5e** was almost equipotent to standard drug at 30 and 60 min and decreased blood glucose levels equivalent to that of standard at 120 min interval. Compound **5e** was found to reduce significantly glucose AUC compared to control and analogues to that of standard. Compounds **5c** and **5h** displayed appreciable reduction in blood glucose levels compared to that of standard drug in OGTT assay. The results of antihyperglycemic activity assay indicated that the compounds **5c**, **5e** and **5h** followed the similar pattern in blood glucose lowering as that of metformin. Compounds **5b**, **5g** and **6d** were found to be fairly effective in the *in vivo* assay compared to standard drug metformin. All the compounds tested for antihyperglycemic activity reduced blood glucose in safe range at time interval of 120 during OGTT assay (i.e., no hypoglycaemic effect was observed during assay period). The results of antihyperglycemic activity assay showed that substitution with electron withdrawing groups like chloro and nitro at phenyl ring attached to sulphonamide led to better antihyperglycemic activity which can be seen from *in vivo* results of compounds **5c** and **5e**. These antihyperglycemic activity results were in accordance to the results reported for acrylamide derivatives [27]. Further the results of GK activity assay indicated that replacement of substituted phenyl ring at sulphonamide NH with alkyl groups such as ethyl resulted in decreased activity as observed for compounds **5i** and **6i**. The results of antihyperglycemic activity assay indicated that substitution of pyridine-2-yl ring attached to benzamide nucleus with electron donating groups such as methyl resulted in reduced antihyperglycemic activity as observed for compound **6d**.

Conclusion

Based on the pharmacophoric characteristics necessary for binding of GK activators with GK protein, benzamide moiety was selected for the design of novel N-pyridin-2-yl benzamide analogues via substitution at CONH linker and introduction of SO_2NH group at the aromatic ring. Amongst the synthesized derivatives, compounds **5b, 5c, 5e, 5g, 5h** and **6d** displayed appreciable GK activity in the *in vitro* enzymatic assay and

appreciable binding interaction with Arg63 of allosteric site of GK protein in docking studies. Amongst the derivatives tested *in vivo* for their antihyperglycemic activity (OGTT), compounds **5c, 5e** and **5h** showed better activity in antihyperglycemic studies. The results of the *in vivo* antihyperglycemic activity assay were in parallel to that of the enzyme assay and docking studies. These newly discovered molecules can serve as the starting hits for the development of safe, potent and orally active GK activators for diabetic disorders.

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Conflict of Interest

The authors declare no conflict of interest.

Figure 1: Pharmacophoric requirements and general structure of the designed N-pyridin-2-yl benzamide GK activators.

Scheme 1: Synthetic route for the N-pyridin-2-yl benzamide derivatives. **Reagents and Conditions: (**a) $HClO_3$, stir, 10-15 ºC, heat, 80 ºC, 2 h; (b) NH_2-R, chloroform, reflux; (c) $SOCl_2$, chloroform, reflux, 3 h; (d) 2-Aminopyridines, chloroform, reflux.

Figure 2: (a) Superimpose of the docked poses for compounds **5c** (red stick), **5e** (yellow stick) and **5h** (blue stick) with that of PDB Ligand 3IMX (black stick); (b) Docked pose showing H-bond interactions for compound **5c;** (c) compound **5e;** (d) compound **5h** in the allosteric binding site of GK protein.

Figure 3: (a) Effect of selected compounds on blood glucose levels at specified time intervals in rat OGTT; (b) Glucose AUC reduction exhibited by the selected compounds in rat OGTT model. All the values are mean of six measurements ± SD. *Data were significantly different compared to control group ($p < 0.05$), **Data were not significantly different from control group.

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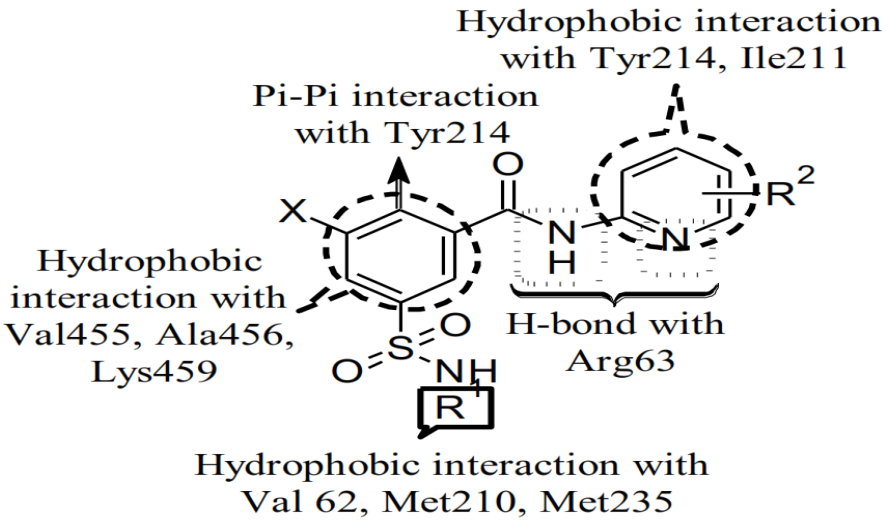
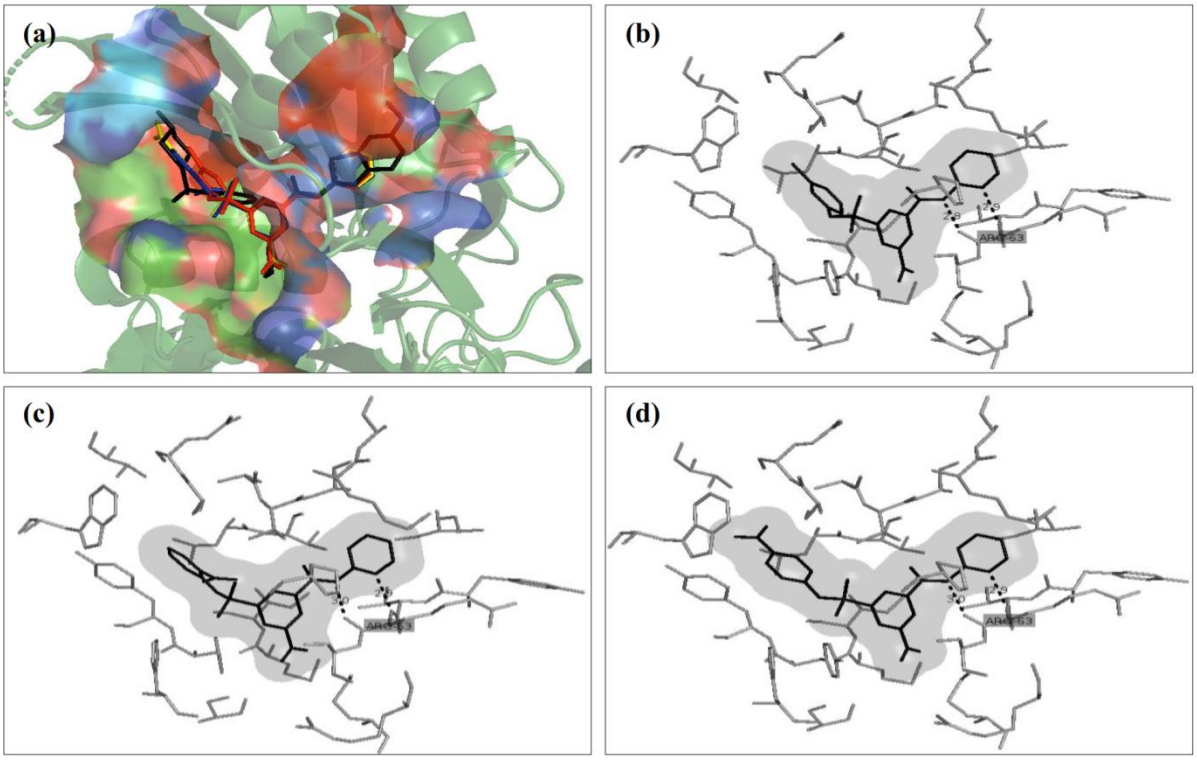
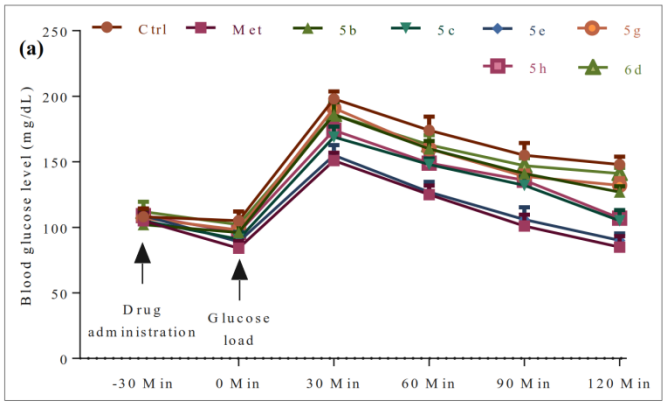
Table 1: **Molecular properties, GK activity, docking score and Glide energy of the synthesized molecules.**

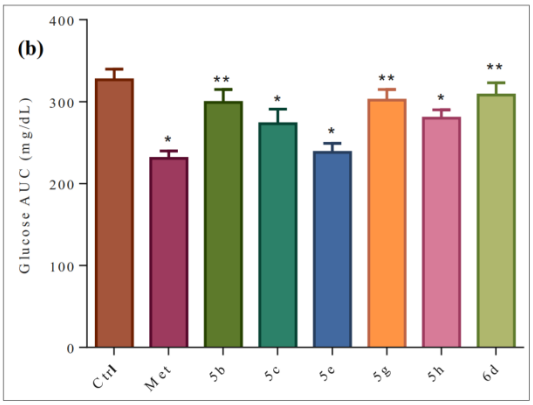
Compound	Mol. Formula	M. Wt.*	log P*	HBA*	HBD*	GK activity#	Glide score	Glide energy
5a	$C_{18}H_{14}N_4O_5S$	398.39	3.00	6	2	1.42 ± 0.03	-9.57	-48.21
5b	$C_{18}H_{13}ClN_4O_5S$	432.84	3.52	6	2	1.96 ± 0.06	-8.75	-47.21
5c	$C_{18}H_{13}ClN_4O_5S$	432.84	3.52	6	2	2.02 ± 0.07	-11.16	-52.69
5d	$C_{18}H_{13}ClN_4O_5S$	432.84	3.52	6	2	1.49 ± 0.05	-10.54	-52.85
5e	$C_{18}H_{13}N_5O_7S$	443.39	2.96	8	2	2.07 ± 0.04	-11.26	-49.70
5f	$C_{18}H_{13}N_5O_7S$	443.39	2.96	8	2	1.33 ± 0.04	-7.51	-55.93
5g	$C_{18}H_{13}N_5O_7S$	443.39	2.96	8	2	1.95 ± 0.07	-7.75	-48.37
5h	$C_{19}H_{16}N_4O_5S$	412.12	3.10	6	2	2.01 ± 0.08	-11.12	-55.01
5i	$C_{14}H_{14}N_4O_5S$	350.35	1.67	6	2	1.04 ± 0.10	-11.14	-56.34
6a	$C_{19}H_{16}N_4O_5S$	412.42	3.47	6	2	1.35 ± 0.07	-9.92	-56.47
6b	$C_{19}H_{15}ClN_4O_5S$	446.86	3.99	6	2	1.26 ± 0.04	-10.05	-56.48
6c	$C_{19}H_{15}ClN_4O_5S$	446.86	3.99	6	2	1.14 ± 0.06	-11.11	-55.66
6d	$C_{19}H_{15}ClN_4O_5S$	446.86	3.99	6	2	1.92 ± 0.08	-9.48	-57.17
6e	$C_{19}H_{15}N_5O_7S$	457.42	3.43	8	2	1.11 ± 0.07	-11.79	-50.74
6f	$C_{19}H_{15}N_5O_7S$	457.42	3.43	8	2	1.19 ± 0.04	-10.12	-51.10
6g	$C_{19}H_{15}N_5O_7S$	457.42	3.43	8	2	1.30 ± 0.06	-10.12	-52.49
6h	$C_{20}H_{18}N_4O_5S$	426.45	3.57	6	2	1.51 ± 0.10	-7.63	-50.04
6i	$C_{15}H_{16}N_4O_5S$	364.38	2.13	6	2	1.02 ± 0.08	-9.43	-49.99

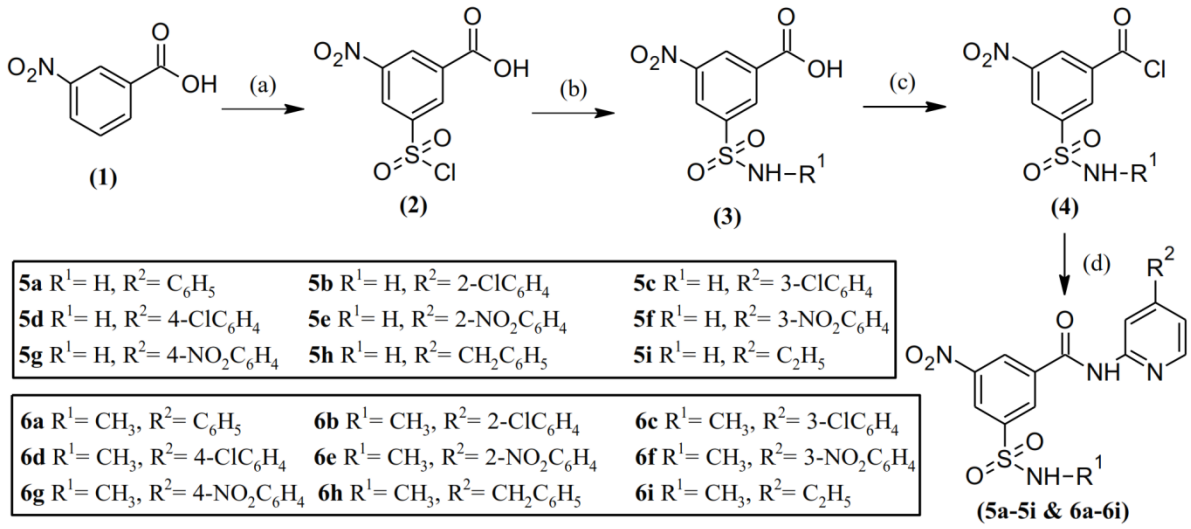
*Mol. Wt., Log P, HBA, and HBD were calculated using MarvinSketch (2015); #All the values are mean of three measurements ± SD (as GK fold activation at 10 µM concentration).











5a R^1= H, R^2= C_6H_5	**5b** R^1= H, R^2= 2-ClC_6H_4	**5c** R^1= H, R^2= 3-ClC_6H_4
5d R^1= H, R^2= 4-ClC_6H_4	**5e** R^1= H, R^2= 2-$NO_2C_6H_4$	**5f** R^1= H, R^2= 3-$NO_2C_6H_4$
5g R^1= H, R^2= 4-$NO_2C_6H_4$	**5h** R^1= H, R^2= $CH_2C_6H_5$	**5i** R^1= H, R^2= C_2H_5

6a R^1= CH_3, R^2= C_6H_5	**6b** R^1= CH_3, R^2= 2-ClC_6H_4	**6c** R^1= CH_3, R^2= 3-ClC_6H_4
6d R^1= CH_3, R^2= 4-ClC_6H_4	**6e** R^1= CH_3, R^2= 2-$NO_2C_6H_4$	**6f** R^1= CH_3, R^2= 3-$NO_2C_6H_4$
6g R^1= CH_3, R^2= 4-$NO_2C_6H_4$	**6h** R^1= CH_3, R^2= $CH_2C_6H_5$	**6i** R^1= CH_3, R^2= C_2H_5