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IN SILICO DESIGN, SYNTHESIS AND EVALUATION OF NOVEL BENZAMIDE DERIVATIVES AS NEWER GLUCOKINASE ACTIVATORS

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ABSTRACT

Background

Glucokinase activators (GKAs) are the new class of drugs which act on glucokinase (GK) enzyme and show their antihyperglycemic activity. Moreover, till date there is no single drug in market without any side effect as antidiabetic activity.

Objective

The present work was planned to design, synthesize and pharmacologically evaluate the antidiabetic activity of a series of newer benzamide derivatives as potential GKAs.

Method

This work involved synthesis of newer benzamide derivatives from benzoic acid and their evaluation by docking studies to determine the binding interactions for the best fit conformations in the binding site of GK enzyme. Based on the results of docking studies, the selected molecules were tested for in vitro GK enzyme assay and further tested for in vivo antidiabetic activity in animal models. Further the in vitro pharmacokinetic studies were analyzed for oral activity.

Results

Among all designed and synthesized derivatives the compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n showed excellent GK activation fold in the in vitro GK assay. Among these selected molecules the

compounds 5g and 5i possessed highest antihyperglycemic activity in oral glucose tolerance test during in vivo studies. In addition, the results of antihyperglycemic activity and in silico docking studies were found to support each other for all the synthesized molecules as glucokinase activators. The synthesized derivatives were further in vitro analyzed for pharmacokinetic properties. This series of compounds found appropriate for oral administration. These finding lead to the development of orally active glucokinase activators as antidiabetic agents.

Conclusion

The newer series of 3-substituted benzamide derivatives were designed, synthesized, characterized, and pharmacologically evaluated based on the structural requirements of the allosteric binding site of the GK protein and previously reported pharmacophoric requirements. Amongst the several synthesized derivatives, compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n showed appreciable GK activation profile in the in vitro enzymatic assay. In molecular docking studies the all the synthesized compounds showed the H-binding interactions with the Arg63 residue of the allosteric site of the GK protein. Amongst all the synthesized compounds tested in vivo for their antihyperglycemic activity, the compounds 5g and 5i have highest activity. This series of compounds found appropriate for oral administration. These findings lead to the development of safe, orally active and potent glucokinase activators as antidiabetic agents.

Keywords: Antihyperglycemic activity, Benzamide derivatives, Glucokinase enzyme, GK activators, In silico method, In vitro GK assay

INTRODUCTION

Type II Diabetes mellitus is a major and prevalent public health care problem associated with glucose homeostasis. The maintenance of glucose homeostasis is essential for mammalian life and is regulated by complex network of factors that control both glucose uptake and utilization. Imbalance in these factors leads to Type II Diabetes mellitus (T2DM). T2DM is characterized firstly by hyperglycemia which is associated firstly with attenuated insulin release from pancreatic beta cell; secondly with insulin resistance in skeletal muscle, liver and in adipose tissues; thirdly with increase hepatic glucose production **[1-4]**. The incidence of diabetes has increased dramatically in recent decades and has been closely associated with significant levels of morbidity and mortality including long term complication like incurable lifelong disease, involving multiple systems, and with devastating complications which ends up in severe disabilities and death. T2DM is the major devastating disease for the mankind and it is estimated that 366 million people had DM and by 2030, this would rise to 439 million worldwide. The treatment cost of T2DM is proved to the burden for the society. The treatment of the

disease aims to control and maintain the glucose level by various insulin preparations and oral hypoglycemic agents like sulphonylureas, meglitinide analogues, biguanides, alpha glucosidase inhibitors, inrcetin mimetics (GLP-1 analogs) and newly approved SLT2 inhibitors (dapagliflozin and canaglifozin) in conjuction with life style modification and appropriate diet **[5-9]**. But the monotherapy commonly results in treatment failure, leading to the need of combination therapy. In addition, these drugs exhibit side effect profile that includes the risk of hypoglycemia, GI disturbances, cardiovascular risks, pancreatitis risk, and urinary tract infections. Thus scientific communities focus on the development of safer medication for the treatment that includes a better glycemic control with cardiovascular safety, tolerability and ease of compliance **[10, 11]**. Recently, the activation of glucokinase has been searched and discussed exhaustively as novel strategy in T2DM treatment.

Glucokinase (GK also known as Hexokianse IV or Hexokianse D) is the member of hexokinase family, that catalyzes the rate limiting step of glycolysis and glycogen synthesis comprises of phosphorylation of glucose to glucose-6-phosphate in cellular metabolism. GK expression is limited to the major organssuch as pancreas, liver, brain and gastrointestinal tract **[12-14]**.

This enzyme exhibits positive co-operativity kinetics with respect to glucose and demonstrates half-maximal activity approximately at 8.0 mM glucose concentration with a Hill coefficient (nH) of approximately 1.7. Moreover it is not inhibited by its end product i.e. fructose-6-phophate unlike other isoforms of hexokinase. This enzyme kinetics manifests it ideal to function as “glucose sensor”. It regulates hepatic glucose metabolism to provide approximately 95% of hexokinase activity in liver cells and serves as a key controller of glucose dependent insulin secretion. As a sensor GK can initiates the cascade of transduction mechanisms for releasing insulin from pancreatic cells and stimulating hepatocytes for phosphorylation of glucose **[15-19]**. The pancreatic beta cells are summarized as typical glucose sensing cell that stimulated by glucose concentration and secrete insulin. In the beta cells GK act as pacemaker for glycolysis at physiological glucose levels. In hepatocytes GK activity is under short-term control by glucokinase regulatory protein (GKPR) that forms complex with GK and render it inactive **[20-24]**. The dissociated GK regulates glycolysis, pentose phosphate shunt, and oxidative phosphorylation with ATP production in hepatocytes and maintains

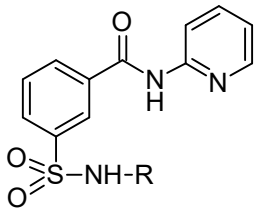
glucose homeostasis. The activation of GK by glucokinase activators (GKAs) have been identified that exhibit dual control of glucose homeostasis by increase insulin secretion from pancreatic beta cells and enhancing glucose uptake in liver cells any given concentration of glucose **[25-31]**. Therefore, GK act as attractive target for designing of novel antidiabetic therapy with least side effect profile.

In lieu of these finding it has been demonstrated by researchers that small molecules were capable of binding to GK at allosteric site distant (20 Å) and promote the activation of enzyme. These seminal findings initiated the efforts of identification of several small molecules including benzamide derivatives, acetamides, carboxamides, pyrimidines, urea derivatives, thiazoles, benzimidazoles that act as promising GKAs **[32-40]**.

A number of *in silico* studies have been performed for the maximum drug discovery and development of potent GK activators that includes QSAR studies, molecular docking, and virtual high throughput screening, comparative molecular field analysis **[41-48]**. Despite the several chemical moieties are being explored as GKAs by researchers, the maximum research efforts related to GKAs had mainly focused on small molecules such as substituted benzamide derivatives due to their binding patterns and interactions with that of enzyme. Various substituted benzamide derivatives having best GK activity reported by our research group through molecular docking studies *in silico*.

The main objective of current investigation is the *in silico* evaluation of selected substituted benzamide derivatives in order to explore the binding modes of the selected compounds in allosteric site of GK protein and to establish the structural basis of their GK activity in order to design safe and effective GK activators using molecular docking.

The literature studies and previously reported pharmacophoric requirements focused us to develop newer benzamide derivatives as glucokinase activators. The lead is represented by compound (A) containing unsubstituted pyridine heterocyclic ring attached with amide functional group and substituted sulphonamide group with aliphatic, aryl/ heteroaryl moieties.



(A)

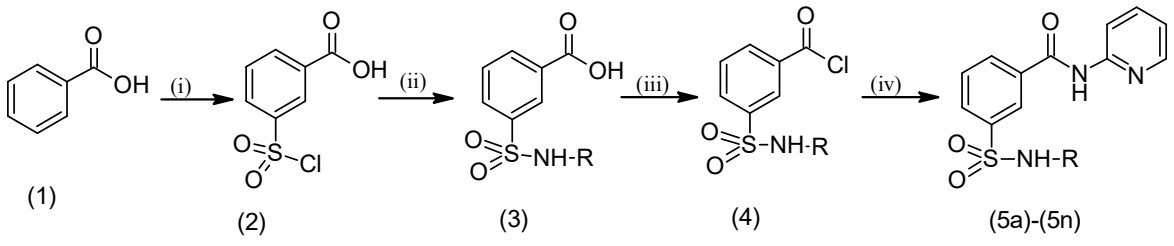
In this paper, we described the synthesis, molecular docking and pharmacological evaluation by in vitro and in vivo studies of newer benzamide derivatives.

MATERIALS AND METHODS:

All the chemicals which are used for the synthesis were procured from sigma Aldrich, Laboratories (Mumbai), SD Fine Chem. Ltd. (Mumbai), Otto Chem. Pvt. Ltd. (Mumbai), Merck Millipore *etc.* All the melting points were calculated by using open capillary tubes on a Veego VMP-D melting point apparatus and are uncorrected. The reaction completion was monitored by thin layer chromatography (TLC) on silica gel plates (Silica Gel-G) and the purity of the compounds was ascertained by single spot on TLC plate. 1H Nuclear magnetic resonance (1H NMR) spectra were taken on Bruker Avance II 400 MHz NMR spectrophotometer using appropriate deuterated solvents and are expressed in parts per million (δ, ppm) downfield from tetramethylsilane (internal standard). Infrared (IR) spectra were recorded on a Shimadzu IR affinity FTIR spectrophotometer using KBr pellet method.

Synthesis of disubstituted Benzamide derivatives

Synthesis of the designed derivatives was carried out as presented below in Scheme 1.The dried benzoic acid (0.01 mol) was placed in a flask fixed with a magnetic bead on magnetic stirrer and the temperature was kept constant between 10 and 15 ºC using cold water bath. Chlorosulphonic acid (8.0 mL) was added with precaution and further checked to confirm that no leakage is there. After whole acid was dissolved and the exothermic reaction ceased, the reaction flask was heated on water bath at 70-80 ℃ for 2 h to complete the reaction followed by cooling. Then the mixture was poured in the 150g of crushed ice and this will cause to form precipitates of 3-(chlorosulphonyl)benzoic acid which were further filtered under vacuum. Then obtained product is washed with cold water and air dried. The product obtained above (0.01 mol) was placed and refluxed with widely available amines (0.01 mol) after making its solution in acetone.



Scheme 1: Synthetic route followed for the synthesis of benzamide derivatives.
Reagents and conditions: i) Chlorosulphonic acid, reflux; ii) NH_2-R, acetone, reflux; iii) Thionyl chloride, acetone, reflux, 3hr; iv) 2-aminopyridine, acetone, reflux

The mixture was continuously refluxed until the completion of reaction which is monitored by TLC. Then this reaction mixture was allowed to cool and precipitates of respective sulphonamides which were dried further. The various

sulphonamides obtained (0.01 mol) were mixed and refluxed with thionyl chloride (0.01 mol) for 3 hr. The excess of thionyl chloride was collected by distillation and the respective benzoyl chlorides were obtained. Benzoyl chlorides (0.01 mol) obtained above were further refluxed with 2-aminopyrdine (0.015 mol) in acetone and reaction completion was monitored using single spot TLC on silica gel G plates. The final synthesized benzamides were refined by recrystallization using ethyl alcohol **[32, 58]**.

4.2 Spectral characterization

3-(methylsulfamoyl)-N-(pyridin-2-yl)benzamide(5a): Yield -71.11%, m.pt. 170-172 ºC, IR (KBr Pellets) ν cm-1: 3235.23 (NH str., CH3NH), 3078.39 (CH str., Aromatic), 2972.31 (CH str., Aliphatic), 1662.64 (C=O str., amide), 1325.10 (SO2 asym. str., sulphonamide), 1251.03 (CH bend., Aromatic), 1174.65 (SO2 sym. str. sulphonamide),690.29 (CH bend., Aromatic)

1H NMR (δ ppm, DMSO): 10.85 (s, 1H, NH of NH-C=O), 8.27-8.33 (m, 3H, CH of C4, C5 & C6 of C6H5-C=O), 6.63-6.66 (s, 1H, CH of C2 of C6H5-C=O), 1.02-1.18(d, 3H, CH3), 2.13 (q, 1H, NH of -SO2NH), 1.45-2.61 (m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 25.0 (CH_3 aliphatic), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O)

MS (ESI TOF) m/z for $C_{13}H_{13}N_3O_3S$ $[M+H]^+$ calcd 291.33, found 290.05.

3-(ethylsulfamoyl)-N-(pyridin-2-yl)benzamide (5b): Yield - 76.22% , m.pt. 165-168ºC, IR (KBr Pellets) ν cm-1: 3427.51(NH str., C2H5NH), 3062.96 (CH str., Aromatic), 2981.95 (CH str., Aliphatic), 1703.14 (C=O str., amide), 1325.10 (SO2 asym. str., sulphonamide), 1270.03 (CH bend., Aromatic), 1174.65 (SO2 sym. str., sulphonamide), 710.92 (CH bend., Aromatic)

1H NMR (δ ppm, DMSO): 10.05 (s, 1H, NH of NH-C=O), 7.85-8.00(m, 3H, CH of C4, C5 & C6 of C6H5-C=O), 6.40-6.56(s, 1H, CH of C2 of C6H5-C=O), 1.18(m, 2H, CH2), 2.18 (t, 1H, NH of -SO2NH), 2.01 (t, 3H, -CH3), 1.30-2.51(m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 14.0 (CH_3 aliphatic), 37.4 (CH_2aliphatic) 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O)

MS (ESI TOF) m/z for $C_{14}H_{15}N_3O_3S$ $[M+H]^+$ calcd 305.35, found 304.15.

3-(propylsulfamoyl)-N-(pyridin-2-yl)benzamide (5c):

IR (KBr Pellets) ν cm-1: 3371.67 (NH str., C3H7NH), 3054.96 (CH str., Aromatic), 2887.95 (CH str., Aliphatic), 1720.56 (C=O str., amide), 1350.40 (SO2 asym. str., sulphonamide), 1259.39 (CH bend., Aromatic), 1150.65 (SO2 sym. str., sulphonamide), 695.29 (CH bend., Aromatic).

1H NMR (δ ppm, DMSO): 10.13 (s, 1H, NH of NH-C=O), 8.18-8.35 (m, 3H, -CH of C4, C5 & C6 of C6H5-C=O), 5.83-5.99 (s, 1H, -CH of C2 of C6H5-C=O), 2.20 (s, 1H, -NH of -SO2NH), 1.43-1.54 (m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 45.6 (CH_2aliphatic), 22.3 (CH_2 aliphatic), 11.2 (CH_3aliphatic), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{15}H_{17}N_3O_3S$ $[M+H]^+$ calcd 319.38, found 318.05.

3-(butylsulfamoyl)-N-(pyridin-2-yl)benzamide (5d): Yield - 76.95%, m.pt. 170-174ºC, IR (KBr Pellets) ν cm-1:3446.51 (NH str., C4H9NH), 3037.89 (CH str., Aromatic), 2862.36 (CH str., Aliphatic), 1627.14 (C=O str., amide), 1406.11 (SO2 asym. str. sulphonamide), 1265.30 (SO2 sym. str. sulphonamide), 715.90 (CH bend, Aromatic)

1H NMR (δ ppm, DMSO): 9.58 (s, 1H, NH of NH-C=O), 7.89-8.82 (m, 3H, -CH of C4, C5 & C6 of C6H5-C=O), 5.99-6.11 (s, 1H, -CH of C2 of C6H5-C=O), 3.21 (s, 1H, -NH of -SO2NH), 1.45-1.51 (m, 4H, -CH of pyridine), 2.97 (d, 1H of CH2), 1.02 (s, 3H of CH_3).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 13.8 (CH_3 aliphatic), 31.3 (CH_2 aliphatic), 19.9 (CH_2 aliphatic), 41.6 (CH_2 aliphatic), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{16}H_{19}N_3O_3S$ $[M+H]^+$ calcd 333.14, found 332.67.

3-[(propan-2-yl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5e): Yield - 66.45%, m.pt. 200-203ºC,

IR (KBr Pellets) ν cm-1: 3425.58 (NH str., propan-2-yl), 3043.67 (CH str., Aromatic), 2980.00 (CH str., Aliphatic), 1693.14 (C=O str., amide), 1402.11 (SO2 asym. str., sulphonamide), 1178.51 (SO2 sym. str., sulphonamide), 718.45 (CH bend, Aromatic)

1H NMR (δ ppm, DMSO): 10..21 (s, 1H, NH of NH-C=O), 8.42-8.56 (m, 3H, -CH of C4, C5 & C6 of C6H5-C=O), 6.02-6.10 (s, 1H, -CH of C2 of C6H5-C=O), 2.25 (s, 1H, -NH of -SO2NH), 1.50-1.52 (m, 4H, -CH of pyridine), 2.97 (d, 1H of $CH(CH_3)_2$),1.02 (s, 3H of CH_3)

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl),

22.4(CH_3 aliphatic), 45.1 (CHaliphatic),158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{15}H_{17}N_3O_3S$ $[M+H]^+$ calcd 319.38, found 318.02.

3-(cyclohexylsulfamoyl)-N-(pyridin-2-yl)benzamide (5f): Yield - 68.50 %, m.pt. 168-171ºC,

IR (KBr Pellets) ν cm-1: 3269.34 (NH str., cyclohexyl-NH), 3109.03 (CH str., Aromatic), 2950.70 (CH str., cyclohexyl), 1631.78 (C=O str., amide), 1350.45 (SO2 asym. str., sulphonamide), 1182.36 (SO2 sym. str., sulphonamide), 685.71 (CH bend, Aromatic).

1H NMR (δ ppm, DMSO): 9.78(s, 1H, NH of NH-C=O), 7.99-8.15 (m, 11H, -CH of -NHC6H11) 8.37-8.43 (m, 3H, -CH of C4, C5 & C6 of C6H5-C=O), 5.99-6.12(s, 1H, -CH of C2 of C6H5-C=O), 2.25(s, 1H, -NH of -SO2NH), 1.50-1.52(m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 43.8 (CH-cyclohexane), 32.9 (CH_2-cyclohexane), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O), 22.9 (CH_2-cyclohexane), 28.8 (CH_2-cyclohexane).

MS (ESI TOF) m/z for $C_{18}H_{21}N_3O_3S$ $[M+H]^+$ calcd 359.44, found 358.23.

3-(benzylsulfamoyl)-N-(pyridin-2-yl)benzamide (5g): Yield - 91.70%, m.pt. 155-156ºC, IR (KBr Pellets) ν cm-1: 3271.27 (NH str., $CH2C_6H_5$), 2993.02 (CH str., Aromatic), 2881.65 (CH str., Aliphatic), 1618.78 (C=O str., amide), 1311.59 (SO2 asym. str., sulphonamide), 1172.36 (SO2 sym. str., sulphonamide), 692.71 (CH bend, Aromatic)

1H NMR (δ ppm, DMSO): 10.53(s, 1H, NH of NH-C=O), 8.20-8.30(m, 5H, -CH of –CH2C6H5) 8.00-8.25(m, 3H, -CH of C4, C5 & C6 of C6H5-C=O), 6.12-6.14(s, 1H, -CH of C2 of C6H5-C=O), 2.30(t, 1H, -NH of -SO2NH), 1.50-1.52(m, 4H, -CH of pyridine), 1.48(d, 2H, -CH2 of -CH2C6H5).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 46.2 (CH_2-benzyl), 141.7 (C-phenyl), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{19}H_{17}N_3O_3S$ $[M+H]^+$ calcd 367.42, found 366.13.

3-(phenylsulfamoyl)-N-(pyridin-2-yl)benzamide (5h): Yield - 74.00%, m.pt. 172-173ºC,

IR (KBr Pellets) ν cm-1: 3271.27 (NH str., ArNH), 2962.66 (CH str., Aromatic), 1691.57 (C=O str., amide), 1359.09 (SO2

asym. str., sulphonamide), 1172.72 (SO2 sym. str., sulphonamide), 680.90 (Cl-C_6H_5 str., Aromatic), 712.29 (CH bend, Aromatic)

1H NMR (δ ppm, DMSO): 8.59(s, 1H, NH of NH-C=O), 7.89-7.99 (m, 5H, -CH of –C6H5) 7.23-7.43 (m, 4H, -CH of C6H5-C=O), 2.25(s, 1H, -NH of -SO2NH), 1.45-2.16(m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 137.8.7 (C-phenyl), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{18}H_{15}N_3O_3S$ $[M+H]^+$ calcd 353.39s, found 352.19.

3-[(2-chlorophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5i): Yield - 67.12%, m.pt. 144-146ºC.

IR (KBr Pellets) ν cm-1: 3273.27 (NH str., Ar-NH), 2962.66 (CH str., Aromatic), 1697.57 (C=O str., amide), 1310.45 (SO2 asym. str., sulphonamide), 1170.72 (SO2 sym. str., sulphonamide), 770.89 (Cl-C_6H_5 str., Aromatic), 707.92 (CH bend, Aromatic).

1H NMR (δ ppm, DMSO): 10.32 (s, 1H, NH of NH-C=O), 7.99-8.10 (m, 4H, -CH of –C6H5Cl) 7.06-7.13 (m, 4H, -CH of C6H5), 2.56 (s, 1H, -NH of -SO2NH), 1.36-1.41 (m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 127.8 (C-phenyl), 120.2.8 (CH-phenyl), 125.8 (C-phenyl) 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{18}H_{14}ClN_3O_3S$ $[M+H]^+$ calcd 387.84, found 386.23.

3-[(2-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5j): Yield - 86.14%, m.pt. 132-134ºC.

IR (KBr Pellets) ν cm-1: 3371.72 (NH str., C5H5N), 3097.66 (NH str., Ar-NH), 2962.66 (CH str., Aromatic), 1630.33 (C=O str., amide), 1550.45 (NO2 asym. str., aromatic), 1321.24 (SO2 asym. str., sulphonamide), 1290.80 (NO2 sym. str., aromatic) 1130.72 (SO2 sym. str., sulphonamide), 729.45 (CH bend, Aromatic).

1H NMR (δ ppm, DMSO): 9.43(s, 1H, NH of NH-C=O), 8.02-8.15 (m, 4H, -CH of –C6H5NO2) 7.20-7.33(m, 4H, -CH of C6H5), 2.75 (s, 1H, -NH of -SO2NH), 1.31-1.39 (m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 141.8 (C-phenyl), 135.7 (C-phenyl), 121.9 (CH-phenyl), 117.8 (CH-phenyl),158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{18}H_{14}N_4O_5S$ $[M+H]^+$ calcd 398.39, found 397.13.

3-[(4-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5k): Yield - 71.45%, m.pt. 138-139ºC, IR (KBr Pellets) ν cm-1: 3369.64 (NH str., Ar-NH), 3037.89 (CH str., Aromatic), 1600.33 (C=O str., amide), 1500.62 (NO2 asym. str., Aromatic), 1340.52 (NO2 sym. str., Aromatic), 1309.67 (SO2 asym. str., sulphonamide), 1112.93 (SO2 sym. str., sulphonamide), 715.56 (CH bend, aromatic).

1H NMR (δ ppm, DMSO): 8.53(s, 1H, NH of NH-C=O), 8.25-8.35 (m, 4H, -CH of –C6H5NO2) 7.09-7.13(m, 4H, -CH of C6H5), 2.30(s, 1H, -NH of -SO2NH), 1.29-1.49(m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 144.9 (C-phenyl), 138.4 (C-phenyl), 121.9 (CH-phenyl), 117.8 (CH-phenyl), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{18}H_{14}N_4O_5S$ $[M+H]^+$ calcd 398.39, found 397.09.

3-[(2-chloro-4-nitrophenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5l): Yield - 92.15%, m.pt. 144-146ºC.

IR (KBr Pellets) ν cm-1: 3271.64 (NH str., Ar-NH), 2962.89 (CH str., Aromatic), 1660.33 (C=O str., amide), 1520.12 (NO2 asym. str., Aromatic), 1310.52 (NO2 sym. str., Aromatic), 1320.67 (SO2 asym. str., sulphonamide), 1168.86 (SO2 sym. str., sulphonamide), 740.65 (Cl-C6H5, str., aromatic), 700. 89 (CH bend, aromatic).

1H NMR (δ ppm, DMSO): 8.53(s, 1H, NH of NH-C=O), 7.65-7.75(m, 4H, -CH of –C6H5) 6.99-7.23 (m, 3H, -CH of C6H5), 2.24 (s, 1H, -NH of -SO2NH), 1.67-1.78 (m, 4H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 133.9 (C-phenyl), 139.3 (C-phenyl), 126.4 (C-phenyl), 117.8 (CH-phenyl), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{18}H_{13}ClN_4O_5S$ $[M+H]^+$ calcd 432.84, found 431.45.

3-[(2-methylphenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5m): Yield - 73.52%, m.pt. 155-157ºC, IR (KBr Pellets) ν cm-1: 3271.27 (NH str., Ar-NH), 3109.03 (CH str., Aromatic), 2910.56 (CH str., aliphatic), 1631.78 (C=O str., amide), 1330.67 (SO2 asym. str., sulphonamide), 1170.86 (SO2 sym. str., sulphonamide), 725.45 (CH bend, aromatic).

1H NMR (δ ppm, DMSO): 8.34(s, 1H, NH of NH-C=O), 7.55-7.59(m, 4H, -CH of –C6H5) 7.00-7.13 (m, 4H, -CH of C6H5), 2.52 (s, 1H, -NH of -SO2NH), 1.25-1.37 (m, 4H, -CH of pyridine), 3.62 (s, 3H, -CH_3)

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl),

136.9 (C-phenyl), 126.6 (CH-phenyl), 129.5 (C-phenyl), 15.4 (CH3-aliphatic), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{19}H_{17}N_3O_3S$ $[M+H]^+$ calcd 367.42, found 366.12.

3-[(4-methoxyphenyl)sulfamoyl]-N-(pyridin-2-yl)benzamide (5n): Yield - 75.94%, m.pt. 155-158ºC.

IR (KBr Pellets) ν cm-1: 3250.27 (NH str., Ar-NH), 2877.89 (CH str., Aromatic), 1680.33 (C=O str., amide), 1330.67 (SO2 asym. str., sulphonamide), 1170.86 (SO2 sym. str., sulphonamide),1070.67 (C-O-C str., aromatic), 692. 76 (CH bend, aromatic).

1H NMR (δ ppm, DMSO): 8.53(s, 1H, NH of NH-C=O), 7.65-7.75(m, 4H, -CH of –C6H5) 6.99-7.23(m, 4H, -CH of C6H5), 2.30(s, 1H, -NH of -SO2NH), 1.29-1.49(m, 5H, -CH of pyridine).

^{13}C NMR (δ ppm, DMSO): 113.3 (CH-pyridin-2-yl), 148.2(CH-pyridin-2-yl), 136.9 (C-phenyl), 117.8 (CH-phenyl), 150.7 (C-phenyl), 55.7 (CH3-aliphatic), 158.6 (C-pyridin-2-yl), 138.3 (CH-pyridin-2-yl), 120.4 (CH-phenyl), 139.8 (CH-phenyl), 134.7 (C-phenyl), 130.5 (CH-phenyl), 168.5 (C=O).

MS (ESI TOF) m/z for $C_{19}H_{17}N_3O_4S$ $[M+H]^+$ calcd 383.42, found 382.22.

In vitro enzyme assay

The GK activity of the synthesized benzamides was tested spectrometrically using a coupled reaction with glucose-6-phosphapte dehydrogenase enzyme (G-6-PDH) (26, 49-51). All the derivatives were prepared as solution in dimethyl sulfoxide (DMSO)and in vitro enzyme assay was performed by making a final volume of 2000 µL having glucose (25mM), magnesium chloride (1mM), dithiothreitol (1mM), 2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid, ATP (1 mM), NAD (1 mM), G-6-PDH (2.5 U/mL), GK enzyme (0.5 µg), and synthesized compounds (10 µM). The solutions were kept under incubation period of 3 minutes and then absorbance was measured at 340nm. The GK activation fold was calculated for the synthesized compounds and compared to control (i.e., DMSO only) which was considered as 100%).

Docking Studies

In silico docking studies were performed for the designed and synthesized benzamides in allosteric site of GK protein using AutoDock Vina **[52]** and AutoDock Tools **[53]**. The 2D chemical structures of all the ligands were prepared by MarvinSketch (MarvinSketch 18.5.0, 2018, ChemAxon) followed by conversion to 3D by Frog2 server **[55]**. The ligands were converted to “pdbqt” files using AutoDock Tools. After assessing a number of co-crystallized structures for GK enzyme

available in the protein data bank; the best ligand bound complexes (PDB entry: 3IMX) were selected based on higher resolution and key binding interactions between ligands and GK protein. The PDB file of 3IMX was edited using PyMOL (PyMOL Molecular Graphics System, Schrödinger, LLC.) by removing the co-crystallized activator, all the water molecules as well as all non-interacting ions. The "pdbqt" files of GK proteins were generated from the PDB files using AutoDock Tools. The grid parameters were calculated using "Grid" tool of AutoDock Tools and all the data regarding target protein, ligand, grid size and geometry were saved in "txt" file. Docking was performed using command line on Windows 10 computer. The reference ligand was docked in the active site of GK protein and compared with that of co-crystallized activator of GK i.e. 3IMX ligands for determining accuracy of docking protocol. The 3D optimized ligands were further docked in the allosteric site of the refined GK models and scored by scoring function. The binding free energy (ΔG, kcal/mol) for each ligand was reported in log file and the binding interactions of the ligands in allosteric site of GK were analyzed using PyMOL **[54, 55]**.

Oral Glucose Tolerance Test (OGTT)

Healthy Sprague-Dawley rats (150-200g) were used for carrying out oral glucose tolerance test (OGTT) which is purchased from National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, Mohali. The rats were kept on the normal plain diet, water ad libitum at controlled temperature. The Institutional Animal Ethics Committee has given the consent to conduct this study (1355/PO/RE/L/10/CPCSEA). The results of in vitro GK enzyme assay and molecular docking studies were the basis for carrying out OGTT. The best selected compounds 5i, 5g, 5n, and 5j were further evaluated for OGTT model. During OGTT the rats were divided into different six groups and six animals were present in each group and these rats were fasted overnight for 8 h before experimentation. Three groups were made that include control, standard, test group which was administered vehicle (5% DMSO, p.o.) only, metformin (30 mg/kg, p.o.), and selected compounds 5i, 5g, 5n, and 5j (50 mg/kg, p.o.). All the animals were fed with glucose (3 kg/kg, p.o.) at 30 mins after drug administration. The collection of the blood sample is done before drug administration and 0, 30, 60, 90, and 120 mins after oral glucose administration. The glucose area under the curve (AUC) was calculated after measuring the glucose concentration in blood and the data is recorded. The OGTT

results were statistically calculated by two-way ANOVA **[56-58]** and further analyzed.

RESULT AND DISCUSSION

Chemistry

The synthetic pathway used for the preparation of benzamide derivatives is presented in **Scheme 1**. 3-chlorosulphonylbenzoic acid (2) was obtained by chlorosulphonation of ortho benzoic acid (1) and the obtained product (2) was further refluxed with widely available aromatic and aliphatic amines to procure desired sulphonamides (3). These obtained sulphonamides was further refluxed with thionyl chloride to obtain respective benzoyl chloride (4), which is further reacted with 2-aminopyridine heterocyclic moiety to synthesize designed benzamide derivatives (5a-5n). Theyield obtained of the synthesized compounds was appreciable. The physicochemical properties of the synthesized compounds are presented in **Table 1**. The synthesis and purity of the synthesized derivatives were ensured by single spot TLC and was further established by their consistent FTIR, ^{1}H, ^{13}C NMR and mass spectra.

The IR spectra of the synthesized derivatives showed the amide –NH stretching vibrations at around 3300-3200 cm^{-1} which confirmed the presence of amide group in the compounds. The aromatic nature of the compounds was confirmed by the presence of aromatic CH-stretching vibrations above 3000 cm^{-1}; the asymmetric and symmetric stretching vibrations of SO2 was observed around 1400-1300 cm^{-1} and 1200-1100 cm^{-1}respectively. The FT-IR spectra of the synthesized compounds showed the stretching vibrations of sulphonamide NH in the range of 3400-3100 cm^{-1}. The stretching vibrations at 1700-1600cm^{-1} indicated the presence of amide carbonyl (C=O) functionality in the compounds. The –NH bending vibrations are observed at around 1600 cm^{-1} which confirmed the presence of aromatic –NH. The –CH bending vibrations at around 800-700cm^{-1} indicated presence of the aromatic ring in these compounds. Thus the spectra confirmed the presence of both sulphonamide and amide group in the structure of synthesized molecules. In the IR spectra of the compounds the presence of C=N stretching vibrations at around 1700-1600cm^{-1} depicted the occurrence of pyridine ring these compounds. The IR spectra of the compounds (5a-5g) showed the aliphatic CH-stretching vibrations in the range of 3000-2800cm^{-1} confirming the presence of aliphatic group (e.g., ethyl or benzyl) in the structure of these compounds.

The H1 NMR spectra of synthesized singlet benzamide showed the singlet signal equivalent to one proton of –CONH functional group at around δ 9–10 ppm

confirming the formation of amide linkage and presence of single singlet for one –NH proton of SO2NH functional group at around δ 2.5 ppm confirming the formation of sulphonamides in the synthesized benzamide derivatives. The proton at carbon 2 of the phenyl ring showed the single singlet at δ 6.63-6.66 ppm indicating the substitution at meta (3^{rd}) position of the phenyl ring, which is sulphonamide group. The presence of multiplet around δ 1.45 -2.61 ppm confirmed that the pyridine heterocyclic ring is present in synthesized compounds. The different peak values were observed in the range of 1.83 -2.83 due to presence of aliphatic carbon chain substitutions in the compounds (5a-5e).

In the 13C-NMR spectra of the synthesized compounds, singlet signal equivalent to carbonyl (C=O) carbon was observed at around δ 160-170 ppm indicating the presence of amide linkage (i.e. CO-NH) in the structure of synthesized derivatives. The signal around δ 158 ppm indicating the presence of pyridine-2-yl ring in the compounds attached with the -CONH group.

The C13-NMR spectra showed the signals corresponding to CH2, CH3 and aliphatic group around 0.85- 3.18 indicated the presence of ethyl, methyl and other aliphatic group in the synthesized molecules.

Molecular Docking Studies

The lead of synthesized derivatives were further optimized for finding the types of drug interactions involved and the residues of the GK protein involved in these interactions. In silico molecular docking studies were carried out for exhaustive studies of affinity and binding interactions of the designed moleculesin the allosteric site of GK protein using AutoDock Vina (PDB ID: 3IMX). The allosteric site of the GK enzyme was surrounded by the β1 strand and α5 helix of the large domain, the C-terminal α13 helix of the small domain, and the GK specific connecting region I (Ser64-Gly72). The reference ligand of PDB 3IMX was docked into the allosteric site of GK; and the docked reference GK activator produced a similar binding pattern and superposition on the binding mode of co-crystallized GK activator with ΔG of -9.0 kcal/mol validating accuracy of docking methodology. Most of the docked ligands/ synthesized derivatives showed appreciable binding interactions in the allosteric site of GK enzyme as confirmed by analyzing their binding interactions and ΔG of the best docked confirmations **(Table 2)**.

On the basis of their lowest binding free energy (ΔG) and docking interactions in the allosteric site, compounds5g, 5i, 5n, 5h, 5j, 5l and 5mwere further analyzed in details using PyMOL for exploring binding interactions of these molecules with

allosteric site residues of GK protein. The docked pose showing H-bond interaction of the selected compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n in the allosteric binding .site of GK protein in **Figure 1**. The selected benzamides displayed similar H-bonding interactions with amino acid residues in the allosteric binding site of GK protein as that of the co-crystallized ligand (PDB ID: 3IMX).

Super-positioning of the docked poses of the selected compounds with that of PDB Ligand 3IMX in the allosteric site of GK protein showed that the selected molecules had the similar binding and orientation pattern in the allosteric site of enzyme as that of co-crystallized ligand **(Figure 2)** supporting the in vitro GK activity of these compounds.

An overlay of the docked poses of the selected compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n with that of 3IMX ligand showed that these compounds had the similar orientation and binding pattern in the allosteric binding site of GK enzyme as that of co-crystallized ligand **(Figure 3)**. All the selected molecules were found to bind to an allosteric pocket of GK protein, which is about 20 Å remote from the glucose binding site.

The compound 5g showed H-bond interactions between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 3.0 Å; and 3.0 Å, respectively. Compound 5h showed H-bond interaction between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 3.0Å; and 3.9Å, respectively.

The docked pose of compound 5i showed H-bond interactions between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 2.9 Å; and 3.2 Å, respectively. Compound 5j interacted with amide 'NH' of Arg63 residue on GK protein via 'N' of pyridine-2-yl ring with distance of 2.9Å and benzamide –NH- and backbone 'carbonyl' interacts by H bonding with the distance of 3.1Å. Compound 5l showed H-bond interaction between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 3.3Å; and 3.9Å, respectively. Compound 5m showed H-bond interaction between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 3.1Å; and 4.1Å, respectively. Compound 5n showed H-

bond interaction between 'N' of pyridine-2-yl ring and amide 'NH' of Arg63 residue; and benzamide 'NH' and backbone 'carbonyl' of Arg63 residue on GK protein with H-bond distance of 3.2Å; and 3.6Å, respectively. Overall, the substituted heteroaryl group attached to the benzamide 'NH' of the selected compounds protruded in the hydrophobic pocket showing the 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 packs between Tyr214, Met210 and Val455 **(Figure 4)**.

Thus, the molecular docking of the designed benzamide derivatives in the allosteric site of GK protein helped in understanding the mechanism of GK activation by these newly designed molecules and also helped in predicting that the designed benzamide derivatives could act as potent allosteric activators of GK enzyme.

In vitro enzyme activity

The results of in vitro GK assay (fold activation of GK enzyme by test compounds compared to control i.e., DMSO only) are presented in table 1. Amongst the synthesized derivatives tested *in vitro*, the compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n showed appreciable GK activation in the in vitro GK assay (fold activation in the range of 1.8 to 2.1 compared to control i.e., DMSO only). The other synthesized compounds demonstrated lower GK activation (fold activation around 1.20) as compared to that of control.

The results of in vitro GK assay depicted that the aryl moiety containing compounds at –NH of sulphonamide group shown the improved binding energy as shown by 5g, 5i, 5n, 5j, 5h, 5l and 5m compounds as compared to aliphatic/ alicyclic side chain containing compounds (5a-5f). The ortho or para substitution on aryl moiety make the derivatives more active as shown by the 5g, 5i, 5n, 5l, 5m and 5j compounds as compared to unsubstituted aryl containing compounds (5h).

In vivo Antihyperglycemic activity

In vivo antihyperglycemic activity based on screening by in vitro GK assay and in silico docking studies the most active compounds (5j, 5g, 5i, and 5n) were further evaluated for their glucose lowering effect in animal models by means of OGTT assay. The results of antihyperglycemic activity (i.e. OGTT assay) were measured as blood glucose levels (mg/dL) at different time interval (0, 30, 60, 90, and 120 mins after oral glucose administration) and glucose AUC represented in **Figure 3 and 4** respectively. The results of antihyperglycemic activity assay depicted that amongst compounds evaluated in OGTT assay, compound 5g and 5i were found to be highly active and shown better potency than other compounds in OGTT

assay. These compounds were almost equipotent to standard antidiabetic drug metformin at 30 and 60 mins and decreased the blood glucose levels equivalent to that of standard antidiabetic drug metformin at 120 mins intervals.

The other selected compounds 5j and 5n were found to fairly reduce glucose AUC compared to control. These compounds displayed significant reduction in blood glucose concentration as compared to that of standard drug (metformin) in OGTT assay.

All the compounds tested for in vivo antihyperglycemic activity in rat OGTT assay reduced blood glucose in safe range at time interval of 120 mins during OGTT assay (i.e., no hypoglycemic effect was observed during assay period, 0- 120 min after glucose administration.

Prediction of ADME properties

ADME properties including molecular weight (MW), partition coefficient (log P), distribution coefficient (log D), water solubility (log Sw), topological polar surface area (tPSA), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), solubility (mg/mL) and number of rotatable bonds (NRB) were predicted for initial in vitro determination of the pharmacokinetic parameters that are helpful in finding oral bioavailability of the drugs. The compounds were selected on the basis of results obtained after in vitro enzyme assay. All the selected derivatives were found to have good results of analysis and can lead to the development of newer orally active antidiabetic agents.

Table 1: Physicochemical properties and GK activity of synthesized benzamide derivatives

Comp.	R	Mol. Wt.	Mol. Formula	M. Pt. (°C)	R_f*	% Yield	GK activity[b]
5a	$-CH_3$	C13H13N3O3S	291.33	170-172	0.69	67.56	1.40 ± 0.06
5b	$-CH_2CH_3$	C14H15N3O3S	305.35	165-168	0.84	78.13	1.42 ± 0.05
5c	$-CH_2CH_2CH_3$	C15H17N3O3S	319.38	170-173	0.76	92.12	1.47 ± 0.08
5d	$-CH_2CH_2CH_2CH_3$	C16H19N3O3S	333.41	152-154	0.89	78.56	1.52 ± 0.06
5e	$-CH(CH_3)_2$	C15H17N3O3S	319.38	138-139	0.51	80.12	1.35 ± 0.04
5f	$-C_6H_{11}$	C18H21N3O3S	359.44	167-169	0.78	67.45	1.60 ± 0.03
5g	$-CH_2C_6H_5$	C19H17N3O3S	367.42	113-115	0.38	45.87	2.18 ± 0.06
5h	$-C_6H_5$	C18H15N3O3S	353.39	142-144	0.57	65.69	2.01 ± 0.07
5i	2-Cl-C_6H_4	C18H14ClN3O3S	387.84	138-142	0.84	66.12	2.15 ± 0.05
5j	2-NO_2-C_6H_4	C18H14N4O5S	398.39	181-183	0.40	87.13	1.95 ± 0.03
5k	4-NO_2-C_6H_4	C18H14N4O5S	398.39	129-131	0.66	78.43	1.85 ± 0.02
5l	2-Cl-4-NO_2-C_6H_4	C18H13ClN4O5S	432.84	200-203	0.80	67.88	1.90 ± 0.05
5m	2-CH_3-C_6H_4	C19H17N3O3S	367.42	131-133	0.75	30.56	1.90 ± 0.06
5n	2-CH_3O-C_6H_4	C19H17N3O4S	383.42	186-188	0.43	55.67	2.11 ± 0.08

aTLC mobile phase: Benzene: Ethyl Acetate (7:3)

b All the values are mean of three measurements± SD (as GK fold activation at 10 µM concentration compared to control i.e, DMSO only)

Table 2: Binding interactions and docking score (ΔG) of the docked benzamide derivatives.

Ligand	H-bond interactions		Residues involved in hydrophobic interactions	ΔG
	Residues	Distance (Å)		
5a	Arg63	3.9, 3.0	Ser69, Ile159, Ile211, Met235, Lys459	-8.1
5b	Arg63	3.9, 3.0	Ser69, Ile159, Ile211, Val455, Lys459	-8.0
5c	Arg63	3.9, 3.0	Ser69, Ile159, Ile211, Met235, Val455, Lys459	-8.3
5d	Arg63	4.0, 3.1	Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-8.0
5e	Ser69	3.2	Ser69, Tyr214, Val455	-6.8
5f	Arg63	4.4, 3.3	Ser69, Ile159, Ile211, Met235, Val455, Lys459	-8.6
5g	Arg63	3.2, 3.0	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-10.1
5h	Arg63	3.9, 3.0	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-9.6
5i	Arg63	3.2, 2.9	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-10.2
5j	Arg63	3.1, 2.9	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-9.3
5k	Arg63	3.9, 3.0	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-9.1
5l	Arg63	3.9, 3.3	Ser69, Ile211, Met235, Val455, Lys459	-9.2
5m	Arg63	4.1, 3.1	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-9.2
5n	Arg63	3.6, 3.2	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-9.9
3IMX ligand	Arg63 Ser69	3.5, 3.2 2.8	Ser69, Ile159, Ile211, Tyr214, Met235, Val455, Lys459	-10.8

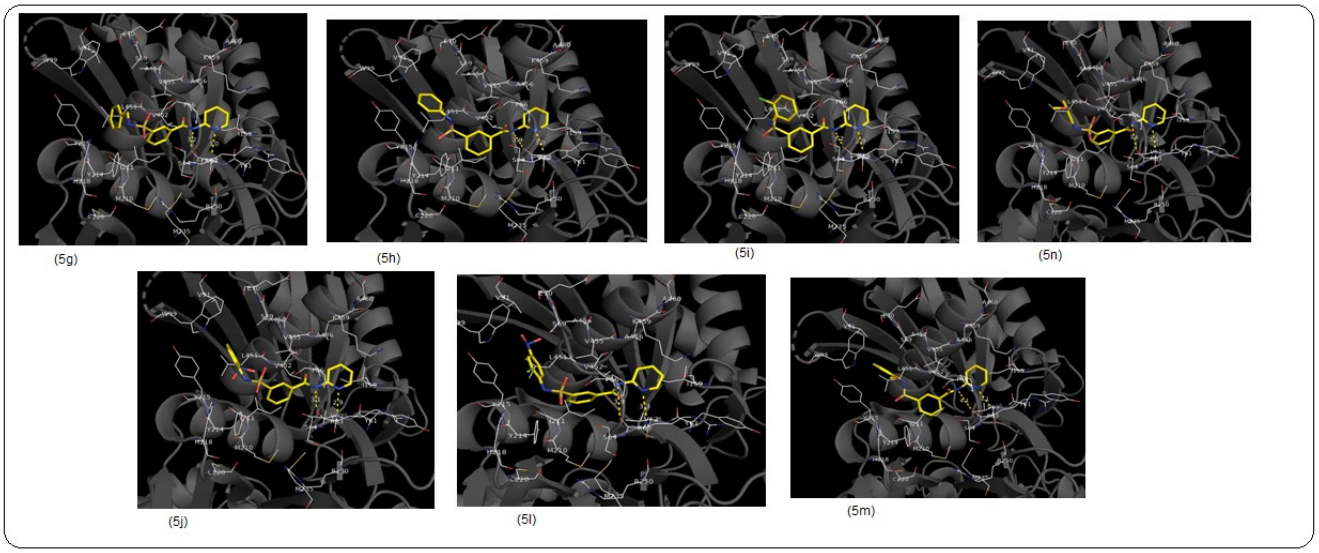


Figure: 1 Docked pose showing H-bond interactions for compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n in the allosteric binding site of GK protein

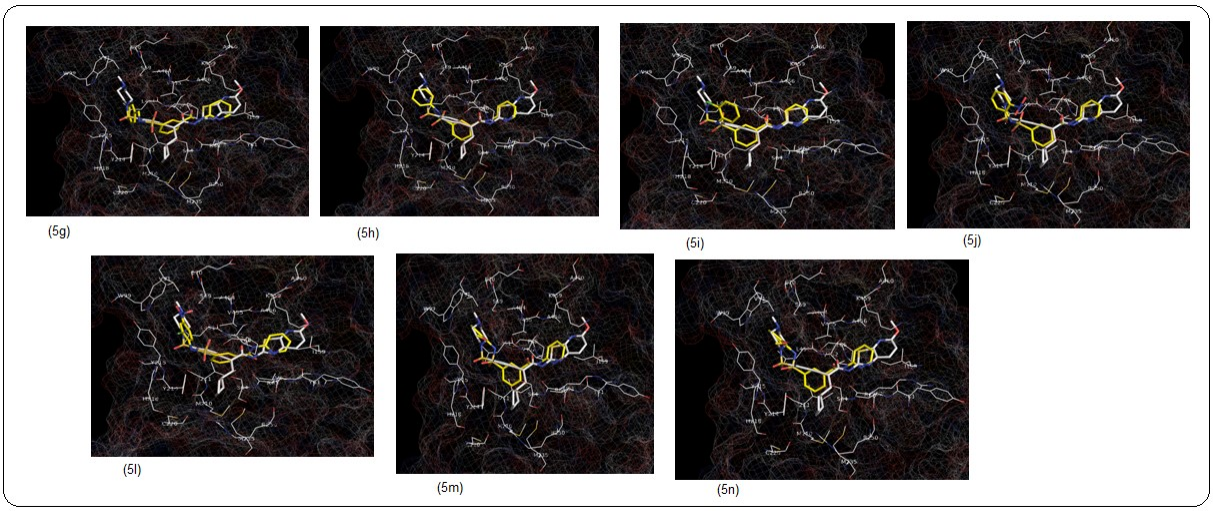


Figure 2: Superimpose of the docked pose of compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n with that of co-crystallized ligand (PDB ID: 3IMX) in the allosteric binding site of GK protein

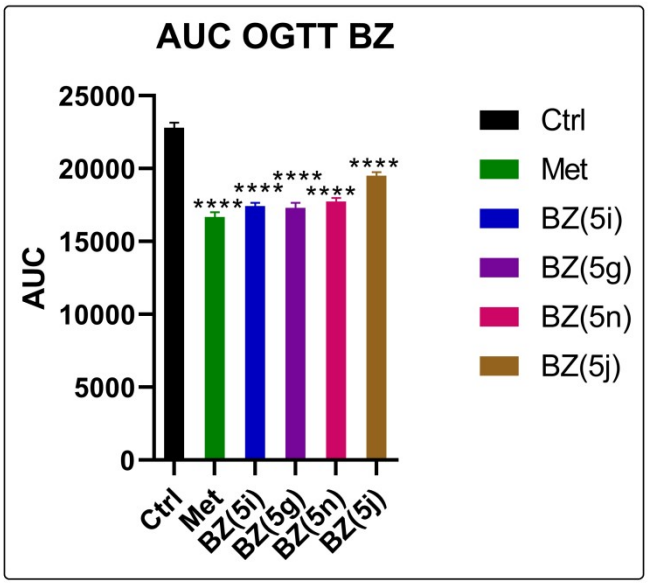


Figure 3: Effect of selected compounds (5g, 5i, 5j and 5n) on blood glucose levels at specified time intervals in OGTT. Ct = control and St = standard. All the values are mean of six measurements ± SD. The antihyperglycemic activity data of metformin treated group and test groups were significantly different from the control group ($p< 0.05$); and data of all the groups was also significantly different in various time intervals compared to 0 min interval ($p< 0.05$)

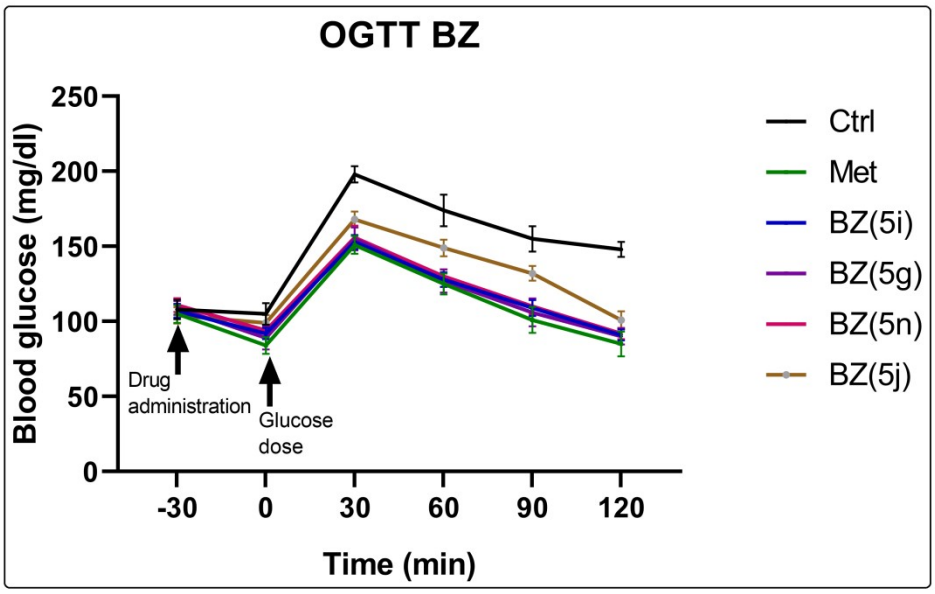


Figure 4: Glucose AUC reduction exhibited by the selected compounds (5g, 5i, 5j and 5n) in rat OGTT model. Ct = Control and St = standard. All the values are mean of six measurements ± SD. * Data were significantly different from that of control group ($p< 0.05$) ** Data were not significantly different from that of control group as analyzed statistically by one-way ANOVA

Table 3: Predicted ADME properties of selected benzamide derivatives

Ligand	MW	Log P	Log D	logSw	tPSA	HBA	HBD	Solubility	NRB
5g	367.42	3.00	2.92	-3.79	88.16	4	2	8999.43	5
5i	387.84	3.53	3.05	-4.06	88.16	4	2	5310.18	4
5n	383.42	2.77	2.95	-3.88	97.39	5	2	7447.82	5
5h	353.39	2.93	3.07	-4.70	88.16	4	2	6909.11	4
5j	398.39	2.87	3.22	-3.67	133.98	6	2	9140.40	5
5l	432.84	3.47	3.03	-4.70	133.98	6	2	9140.40	5
5m	367.42	3.44	3.23	-4.18	88.16	4	2	4936.26	4

CONCLUSION

The newer series of 3, 5-disubstituted benzamide derivatives were designed, synthesized, characterized, and pharmacologically evaluated based on the structural requirements of the allosteric binding site of the GK protein and previously reported pharmacophoric requirements. Amongst the several synthesized derivatives, compounds 5g, 5h, 5i, 5j, 5l, 5m and 5n showed appreciable GK activation profile in the in vitro enzymatic assay. In molecular docking studies the all the synthesized compounds showed the H-binding interactions with the Arg63 residue of the allosteric site of the GK protein. Amongst all the synthesized compounds tested in vivo for their antihyperglycemic activity (OGTT assay), compounds 5g and 5i have highest activity. The results of the in vitro enzyme assay and in vivo antihyperglycemic activity are parallel to each other. This series of compounds found appropriate for oral administration. These findings lead to the development of safe, orally active and potent glucokinase activators as antidiabetic agents.

List of Abbreviations

GK	**Glucokinase enzyme**
T2DM	**Type 2 Diabetes Mellitus**
SLT2 inhibitors	**Sodium-glucose co-transporter-2**
GKPR	**Glucokinase regulatory protein**
GKAs	**Glucokinase activators**
QSAR	**Quantitative Structure Activity Relationship**
OGTT	**Oral Glucose Tolerance Test**

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Availability of data and material

"NONE" No data is used from database

Ethics approval and consent to participate

The approval for the animal studies was given by Institutional Animal Ethics Committee with approval no: 1355/PO/RE/L/10/CPCSEA

Under the *Committee for the Purpose of Control And Supervision of Experiments on Animals, India (*CPCSEA) which is a statutory Committee, which is established under Chapter 4, Section 15(1) of the Prevention of Cruelty to Animals Act 1960.

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