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# Identification of novel urea derivatives as PTP1B inhibitors: Synthesis, biological evaluation and structure-activity relationships

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5

## Electronic Supplementary Information (ESI)

10

## Contents:

**1. Experimental details for final compounds 4a-4k,5a-5i.**

**2. Biological methods.**

15

**3. Docking studies**

**4. Reference**

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**Experimental Section:**

***Experimental*:** Melting points were determined on an electrical heated m. p. apparatus /using silicon oil bath. Reactions were monitored by thin layer chromatography on self-made plates of silica gel G (Merck, India) or 0.25mm ready-made plates of silica gel 60F254, (E.Merck, Darmstadt, Germany). Column chromatography was performed on silica gel (Merck, 60 to 120mesh). Infrared spectra (IR) were recorded on Perkin- FTIR model PC spectrophotometer with frequency of absorptions reported in wave numbers. MS were recorded on JEOL spectrometer with fragmentation pattern reported as values. $^1$H NMR was recorded on Bruker spectrometer with a multinuclear inverse probe head with gradient at room temperature (298 K) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts were described in parts per million (ppm) relative to TMS (0.00 ppm) using scale, and coupling constants were reported in hertz (Hz).

**1-benzylurea: (3a)**

The water (60 ml) was added to the solution of benzylamine (12.0 g, 0.25 mol), urea (6.24 g, 1.0 mol) and conc. HCl (2 ml, 32 mmol) and the resulting mixture was refluxed for 3-5 hrs. cooled at rt (25-30$^o$C) to give the precipitate which was filtered, washed well with water and recrystallised by ethanol to give 3a as white crystals.[1]

Yield : 9.8 g (82.7 %) ; m.p.: 147-149 $^o$C; (lit.[1] m.p.: 149-150$^o$C)

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 4.30 (d, $J$ = 6.2 Hz, 2H), 5.66 (s, 2H), 6.22 (t, $J$ = 6.2 Hz, 1H), 7.62-8.09 (m, 5H); FTIR (KBr): cm$^{-1}$ 1346, 1585, 1652, 3319, 3469; ESI-Ms : m/z $(M+1)^+$ 151.

The other benzylurea 3b was also synthesized in the same manner.

**1-benzyl-3-(2-nitrobenyl)urea (4a)**

A mixture of 2-nitrobenzaldehyde (3.63 g, 1.0 mol), 1-benzylurea (4.40 g, 1.5 mol) and titanium (IV) isopropoxide (10 ml, 1.7 mol) was slurried in 100 ml of THF. This slurry was stirred at room temperature under nitrogen. After 5 hours, the reaction mixture was cooled to 0$^0$C and sodium borohydride (0.370 g, 0.5 mol) was added. The ice bath was removed and the resulting slurry was allowed to stir for 2-3 hours. The reaction mixture was again cooled to 0$^0$C and quenched by dropwise addition of 1.0 N HCl and water. The precipitated product during the acidic workup was filtered, washed with water and recrystallised from methanol.[2]

Anal. Calcd for $C_{15}H_{15}N_3O_3$ : C, 63.15; H, 5.30; N, 14.73; O, 16.82 %

Found: C, 60.08; H, 5.41; N, 13.84 %

Yield : 6.5 g (80.9 %) ; m.p.: 126 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 4.08 (s, 2H), 4.34 (s, 2H), 7.28-7.42 (m, 5H), 7.50 (d, $J$ = 1.5 Hz, 1H), 7.64 (d, $J$ = 7.8 Hz, 1H), 8.08 (d, $J$ = 8.1 Hz, 1H), 8.15 (s, 1H); FTIR (KBr): $cm^{-1}$ 697, 1349, 1529, 1626, 2928, 3338 ; ESI-Ms : m/z $(M+1)^+$286.

**1-benzyl-3(3-nitrobenzyl)urea (4b)**

Anal. Calcd for $C_{15}H_{15}N_3O_3$ : C, 63.15; H, 5.30; N, 14.73; O, 16.82 %

Found: C, 65.95; H, 6.03; N, 12.87 %

Yield : 82.9 % ; m.p.: 120 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 4.32 (s, 2H), 4.64 (s, 2H), 7.21-7.29 (m, 5H), 7.43-7.49 (m, 1H), 7.61-7.64 (m, 2H), 8.02 (d, $J$ = 7.8 Hz, 1H); FTIR (KBr): $cm^{-1}$ 695, 1063, 1341, 1523, 1626, 2326, 3325; ESI-Ms : m/z $(M+1)^+$286.

**1-benzyl-3-(4-nitrobenyl)urea (4c)**

Anal. Calcd for $C_{15}H_{15}N_3O_3$ : C, 63.15; H, 5.30; N, 14.73; O, 16.82 %

Found: C, 62.43; H, 5.17; N, 16.04 %

Yield : 82.5 % ; m.p.: 162 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 3.33 (s, 2H), 4.44 (s, 2H), 7.22-7.32 (m, 5H), 7.43 (d, $J$ = 1.26 Hz, 2H), 8.15 (d, $J$ = 6.45 Hz, 2H); FTIR (KBr): $cm^{-1}$ 698, 1243, 1346, 1523, 1619, 2879, 3323; ESI-Ms : m/z $(M+1)^+$286.

**1-benzyl-3-(2,6-dichloro-3,4-dimethoxybenzyl)urea (4d)**

Anal. Calcd for $C_{17}H_{18}Cl_2N_2O_3$ : C, 55.30; H, 4.91; Cl, 19.20; N, 7.59; O, 13.00 %

Found: C, 52.87; H, 4.97; N, 7.24 %

Yield : 81.9 % ; m.p.: 166 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 3.78 (d, $J$ = 10.3 Hz, 4H), 4.36 (s, 1H), 6.79 (s, 1H), 7.20-7.25 (m, 5H); FTIR (KBr): $cm^{-1}$ 691, 1044, 1239, 1621, 2363, 2944, 3328; ESI-Ms : m/z $(M^+)$ 369.

**1-benzyl-4-(hydroxy-3-methoxybenzyl)urea (4e)**

Anal. Calcd for $C_{16}H_{18}N_2O_3$ : C, 76.12; H, 6.34; N, 9.78; O, 16.76 %

Found: C, 67.08; H, 5.65; N, 9.87 %

Yield : 89.1 % ; m.p.: 188 °C;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.75, (s, 3H), 4.21 (d, $J$ = 2.16 Hz, 4H), 6.66 (d, $J$ = 2.1 Hz, 2H), 6.70 (s, 1H), 7.11-7.18 (m, 5H); FTIR (KBr): $cm^{-1}$ 699, 1076, 1272, 1592, 1631, 2326, 3303, 3370; ESI-Ms : m/z $(M+2)^+$287.

5 **1-benzyl-3-(3,5-di-tert-butyl-2-hydroxybenzyl)urea (4f)**

Anal. Calcd for $C_{23}H_{32}N_2O_2$ : C, 74.96; H, 8.75; N, 7.60; O, 8.68 %

Found: C, 73.46; H, 7.44; N, 7.83 %

Yield : 80.6 % ; m.p.: 164 °C;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 1.28 (d, $J$ = 5.4 Hz, 9H), 1.43 (d, $J$ = 5.4 Hz, 9H), 3.35 (bs, 1H), 4.27
10 (d, $J$ = 5.1 Hz, 4H), 6.95 (s, 1H), 7.22 (s, 1H), 7.24-7.28 (m, 5H); FTIR (KBr): $cm^{-1}$ 695, 1220, 1264, 1605, 2869, 2957, 3288; ESI-Ms : m/z $(M+1)^+$369.

**1-benzyl-3-(2,4,6-trimethoxybenyl)urea (4g)**

Anal. Calcd for $C_{18}H_{22}N_2O_4$ : C, 65.44; H, 6.71; N, 8.48; O, 19.37 %

Found: C, 65.46; H, 6.44; N, 8.83 %

15 Yield : 82.6 % ; m.p.: 154 °C;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.83 (s, 6H), 3.90 (s, 3H), 4.33 (d, $J$ = 3 Hz, 4H), 6.15 (s, 2H), 6.99-7.31 (m, 5H); FTIR (KBr): $cm^{-1}$ 697, 1122, 1222, 1638, 2841, 2941, 3310; ESI-Ms : m/z $(M)^+$330.

**1-benzyl-3-(thiophen-3-ylmethyl)urea (4h)**

Anal. Calcd for $C_{13}H_{14}N_2OS$ : C, 63.39; H, 5.73; N, 11.37; O, 6.50; S, 13.02 %

20 Found: C, 62.87; H, 6.21; N, 10.85 %

Yield : 87.2 % ; m.p.: 116 °C;

$^1$H NMR (300 MHz, $CD_3OH:D_2O$): $\delta$ 4.31 (s, 2H), 4.48 (s, 2H), 6.90 (t, $J$ = 4.9 Hz, 3H), 7.22-7.28 (m, 5H); IR (KBr) : 761, 1045, 1216, 1635, 2361, 3020, 3330 ; ESI-MS : m/z $(M+1)^+$247.

**1-benzyl-3-((5-(4-nitrophenyl)furan-2-yl)methyl)urea (4i)**

25 Anal. Calcd for $C_{18}H_{22}N_2O_4$ : C, 64.95; H, 4.88; N, 11.96; O, 18.21

Found: C, 64.87; H, 4.21; N, 11.85 %

Yield : 80.2 % ; m.p.: 126 °C;

$^1$H NMR (300 MHz, $CD_3OH:D_2O$): $\delta$ 4.39(d, J= 5.8 Hz, 2H), 4.67 (d, J= 3.6 Hz, 2H), 6.38 (d, $J$ = 3.4 Hz, 1H), 6.48( d, J= 3.4 Hz, 1H), 6.81 ( d, J= 3.36 Hz, 1H), 7.31-7.5 (m, 5H), 7.82( d, J= 8.8 Hz, 2H),

8.26( d, J= 8.9 Hz, 2H); IR (KBr) : 761, 1033, 1216, 1635, 2360, 3020, 3324 ; ESI-MS : m/z $(M+1)^+$352.

**1-benzyl-3-((5-chloro-1H-indol-3-yl)methyl)urea (4j)**

Anal. Calcd for $C_{17}H_{16}ClN_3O$ : C, 65.07; H, 5.14; Cl, 11.30; N, 13.39; O, 5.10 %

Found: C, 65.27; H, 5.74; N, 13.01 %

Yield : 82.3 % ; m.p.: 155 $^oC$;

$^1H$ NMR (300 MHz, $CD_3OH$): $\delta$ 1.19 (s, 2H), 3.268 (s, 2H), 7.13-7.25 (m, 9H), 9.81 (s, 1H); FTIR (KBr): $cm^{-1}$ 695, 1145, 1462, 1595, 2364, 3334; ESI-Ms : m/z $(M+1)^+$313.

**N-(4-((3-benzylureido)methyl)phenyl)acetamide (4k)**

Anal. Calcd for $C_{17}H_{19}N_3O_2$ : C, 68.67; H, 6.44; N, 14.13; O, 10.76 %

Found: C, 68.451; H, 6.36; N, 14.17 %

Yield : 78.5 % ; m.p.: 158 $^oC$;

$^1H$ NMR (300 MHz, $CD_3OH:D_2O$): $\delta$ 3.35 (s, 3H), 4.35 (d, J= 605 Hz, 2H), 4.59 (s,2H), 7.27 (d, J= 5.8 Hz, 4H), 7.32-7.37 (m, 5H), 7.53 (s,1H); FTIR (KBr): $cm^{-1}$ 695, 1023, 1321, 1568, 1601, 1653, 2364, 2930, 3330; ESI-Ms : m/z $(M+1)^+$ 298.

**1-(3-chlorobenzyl)-3-(3-nitrobenzyl)urea (5a)**

Anal. Calcd for $C_{15}H_{14}ClN_3O_3$ : C, 56.35; H, 4.41;Cl, 11.09; N, 13.14; O, 15.01 %

Found: C, 56.451; H, 4.86; N, 13.17 %

Yield : 68.5 % ; m.p.: 138 $^oC$;

$^1H$ NMR (300 MHz, $CD_3OH:D_2O$): $\delta$ 4.32 (s, 2H), 4.44 (s, 2H), 7.17-7.21 (m, 4H), 7.28 (s, 1H), 7.52 (t, $J$ = 7.9 Hz, 1H), 7.66 (d, $J$ = 7.6 Hz, 1H), 7.69 (s, 1H); FTIR (KBr): $cm^{-1}$ 722, 1080, 1348, 1627, 2362, 2925, 3322; ESI-Ms : m/z $(M+1)^+$ 320.

**1-(2-nitrobenzyl)-3-(3-nitrobenzyl)urea (5b)**

Anal. Calcd for $C_{15}H_{14}N_4O_5$ : C, 54.55; H, 4.27; N, 16.96; O, 24.22 %

Found: C, 54.08; H, 4.40; N, 16.86 %

Yield : (75.9 %) ; m.p.: 116 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 4.18 (s, 2H), 4.44 (s, 2H), 7.41-7.78 (m, 5H), 7.96 (m, 1H), 8.12-8.27 (m, 2H); FTIR (KBr): $cm^{-1}$ 696, 1350, 1530, 1636, 2930, 3330 ; ESI-Ms : m/z $(M+1)^+$331.

**1-(2, 6-dichloro-3,4-dimethoxybenzyl)-3-(3-nitrobenzyl)urea (5c)**

Anal. Calcd for $C_{17}H_{17}Cl_2N_3O_5$ : C, 49.29; H, 4.14; Cl, 17.12; N, 10.14; O, 19.31 %

Found: C, 49.30; H, 4.51; N, 10.01 %

5 Yield : 74.5 % ; m.p.: 164 $^oC$;

$^1H$ NMR (300 MHz, DMSO): $\delta$ 2.08 (s, 3H), 2.61 (s, 3H), 3.10 (d, $J$ = 6.0 Hz, 2H), 3.18 (d, $J$ = 5.0 Hz, 2H), 5.03 (t, $J$ = 5.0 Hz, 1H), 5.24 (t, $J$ = 6.03 Hz, 1H), 5.95 (s, 1H), 6.39 (d, $J$ = 2.16 Hz, 1H), 6.46 (d, $J$ = 7.5 Hz, 1H), 6.84 (d, $J$ = 6.1 Hz, 2H); FTIR (KBr): $cm^{-1}$ 669, 1038, 1350, 1620, 2370, 2942, 3328; ESI-Ms : m/z $(M+2)^+$ 416.

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**1-(4-methoxybenzyl)-3-(3-nitrobenzyl)urea (5d)**

Anal. Calcd for $C_{16}H_{17}N_3O_4$ : C, 60.94; H, 5.43; N, 13.33; O, 20.30 %

Found: C,61.51; H, 4.86; N, 12.38 %

Yield : 88.2 % ; m.p.: 145 $^oC$;

15 $^1H$ NMR (300 MHz, $CD_3OH$:$CDCl_3$): $\delta$ 3.75 (s, 3H), 4.25 (d, $J$ = 3.18 Hz, 2H), 4.43 (d, $J$ = 3.1 Hz, 2H), 6.83 (t, $J$ = 1.8 Hz, 2H), 7.19 (t, $J$ = 2.0 Hz, 2H), 7.50 (t, $J$ = 7.9 Hz, 1H), 7.65 (d, $J$ = 9.2 Hz, 1H), 8.07 (d, $J$ = 8.2 Hz, 1H), 8.14 (s, 1H); FTIR (KBr): $cm^{-1}$ 760, 1041, 1216, 2358, 3020, 3435, 3613; ESI-MS : m/z $(M+1)^+$316.

**1-(3,5-dimethoxybenzyl)-3-(3-nitrobenzyl)urea (5e)**

20 Anal. Calcd for $C_{17}H_{19}N_3O_5$ : C, 59.12; H, 5.55; N, 12.17; O, 23.16 %

Found: C, 61.51; H, 5.85; N, 12.38 %

Yield : 45.7 % ; m.p.: 140 $^oC$;

$^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 3.36 (d, $J$ = 1.6 Hz, 4H), 4.45 (s, 6H), 6.38 (s, 1H), 6.54 (s, 1H), 7.57 (s, 1H), 7.77 (t, $J$ = 2.64 Hz, 2H), 8.14 (d, $J$ = 8.13 Hz, 2H), 8.20 (s, 2H); FTIR (KBr): $cm^{-1}$ 729, 1153,
25 1346, 1591, 1653, 2362, 3327, 3471; ESI-Ms : m/z $(M+1)^+$ 346.

**1-(3-nitrobenzyl)-3-(3,4,5-trimethoxybenzyl)urea (5f)**

Anal. Calcd for $C_{18}H_{21}N_3O_6$ : C, 57.59; H, 5.64; N, 11.19; O, 25.57 %

Found: C, 57.79; H, 5.82; N, 9.33 %

Yield : 43.3 % ; m.p.: 120 $^oC$;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.71 (s, 6H), 3.76 (s, 3H), 4.23 (s, 2H), 4.40 (s, 2H), 6.37 (s, 2H), 7.42 (t, $J$ = 7.8 Hz, 1H), 7.56 (d, $J$ = 7.5 Hz, 1H), 8.04 (d, $J$ = 8.1 Hz, 2H); FTIR (KBr): cm$^{-1}$ 726, 1009, 1127, 1348, 1423, 1617, 2364, 2941, 3323; ESI-Ms : m/z $(M+1)^+$ 375.

**1-(3-nitrobenzyl)-3-((6-(3-nitrophenyl)pyridin-2-yl)methyl)urea (5g)**

5 Yield : 84.1% ; m.p.: >220 $^o$C;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.36 (t, $J$ = 1.5 Hz, 4H), 7.41 (d, $J$ = 7.5 Hz, 1H), 7.52 (s, 2H), 7.67-7.78 (m, 3H), 7.89 (t, $J$ = 7.8 Hz, 1H), 8.09-8.27 (m, 3H), 8.29 (d, $J$ = 8.1 Hz, 1H), 8.91 (d, $J$ = 13.2 Hz, 2H); FTIR (KBr): cm$^{-1}$ 764, 1216, 1528, 2358, 3021, 3419, 3619; ESI-Ms : m/z $(M+1)^+$408.

**1-(furan-2-ylmethyl)-3-(3-nitrobenzyl)urea (5h)**

10 Anal. Calcd for $C_{13}H_{13}N_3O_4$ : C,56.72; H, 4.76; N, 15.27; O, 23.25 %

Found: C, 56.87; H, 5.78; N, 15.09 %

Yield : 77.7 % ; m.p.: 98 $^o$C;

$^1$H NMR (300 MHz, $CD_3OH{:}D_2O$): $\delta$ 4.22 (s, 2H), 4.33 (s, 2H), 6.11 (d, $J$ = 2.7 Hz, 1H), 6.22 (d, $J$ = 1.8 Hz, 1H), 7.30 (d, $J$ = 1.0 Hz, 1H), 7.45 (t, $J$ = 7.9 Hz, 1H), 7.58 (d, $J$ = 7.5 Hz, 1H), 8.00 (d, $J$ = 8.1
15 Hz, 1H), 8.07 (s, 1H); FTIR (KBr): cm$^{-1}$ 669, 772, 1130, 1402, 1638, 2362, 3420, 3877; ESI-Ms : m/z $(M+1)^+$276.

**1-(3-nitrobenzyl)-3-((5-4-nitrophenyl)furan-2-yl)methyl)urea (5i)**

Anal. Calcd for $C_{19}H_{16}N_4O_6$ : C, 57.58; H, 4.07; N, 14.14; O, 24.22 %

Found: C, 61.51; H, 4.86; N, 12.38 %

20 Yield : 54.5 % ; m.p.: 139 $^o$C;

$^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.34 (d, $J$ = 1.4 Hz, 4H), 6.40 (d, $J$ = 3.39 Hz, 1H), 6.89 (d, $J$ = 3.4 Hz, 1H), 7.53 (d, $J$ = 1.1 Hz, 2H), 7.67 (d, $J$ = 7.6 Hz, 1H), 7.79 (d, $J$ = 8.9 Hz, 1H), 8.23 (d, $J$ = 9.0 Hz, 4H); FTIR (KBr): cm$^{-1}$ 672, 773, 1344, 1518, 1635, 2326, 3327, 3470; ESI-Ms : m/z $(M+1)^+$397.

25 **Biological Testing.**

***In vitro***

PTP 1B enzyme inhibitory activity of different test compounds was determined by colorimetric, non-radioactive PTP 1B tyrosine phosphatise drug discovery kit -BML-AK 822 from Enzo Life Sciences, USA. Human recombinant PTP 1B enzyme is provided with the kit, PTP 1B inhibitory activity of test

compounds was tested by incubating them with enzyme. The reaction was carried out in 96 well flat bottomed microtiter plate by the addition of assay buffer, solution of test compounds and diluted PTP1B enzyme. Enzyme reaction was initiated by addition of 50µl of warmed 2x substrate then incubated the plate at $30^{0}$c for 30min. After incubation for 30 min. Reaction was terminated by addition of 25 µl of biomol red reagent and mixed thoroughly by repeating pipetting . Test compounds were dissolved in DMSO (dimethyl sulfoxide) different dilutions were made as required in the reactions, dilution of other component were accordingly as instructed in the kit. PTP1B phosphatase acting on the phosphopeptide substrate and release phosphate. The detection of free phosphate released is based on classic Malachite green assay.[3] After adding biomol red to reaction wells plate was kept for 20 min to develop the colour. Absorbance was recorded at $_{620nm}$ on a microplate reader. The percentage inhibition by test compounds on PTP1b enzyme was calculated based on activity in the control tube (without inhibitor) as 100 % from three independent set of experiments. The concentration of DMSO in the test well (1.0 %) had no demonstrable effect on PTP1b enzyme activity.

***In vivo* studies**

**Streptozotocin model (STZ)**

**Chemicals and Reagents**

Metformin and Streptozotocin were purchased from Sigma Aldrich Co., USA. One touch glucometer (Accu-Check sensor) and glucostrips was purchased from Roche Diagnostics India Ltd.

**Preparation of dosage of active drug and Synthetic compound:**

**Metformin:** Metformin was in microcrystalline form and freely soluble in water. The dosage was prepared in solution from using sterilized water that, each 0.1 ml of solution contained Metformin at dose of 100 mg/kg body weight since Metformin is effective in such dose.

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**Test Samples**: The synthetic compounds were dissolved in 1% gum acacia to prepare the solution according to the dose of 100 mg/kg body weight .

**Procurement and Selection of Animals-**

5 Male albino rats of SD strain (8 to 10 weeks of age: body weight range 160 ± 20 g) were procured from the animal colony of the Institute. Care and Research on animals was conducted in accordance with the guidelines of the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964. Rats were housed in groups of five in cages under controlled standard environmental conditions of temperature and 10 humidity-controlled environment with a 12 hr light-dark cycles. The animal had free access to pellet diet and tap water for the duration of the study . Prior to commencement of the experiment all the rats were acclimatized to the new environmental condition for a period of one week.

**Experimental design for streptozotocin-induced diabetic Rats**

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**Induction of diabetes**

Streptozotocin (STZ) is a broad-spectrum antibiotic and selected to induce experimental diabetes because of its greater selectivity of β-cells, lower mortality and relatively longer half life (15 min) of STZ in the body. A solution of STZ (60 mg/kg) in 100 mM citrate buffer, pH 4.5 was prepared and 20 calculated amount of the fresh solution was dosed to overnight fasted rats intraperitoneally. Two days later baseline blood glucose was drawn from tail vein and glucose levels were determined by glucostrips (Roche) to confirm the induction of diabetes.

**Animal Modelling, Grouping and Treatment**

**Assessment of antihyperglycemic effect by measuring fall in blood glucose level on 25 Streptozotocin treated diabetic rats**

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Rats having hyperglycaemia of the range of 270 and 450 mg/dl were considered as diabetic, selected and were divided into groups of five animals each. One Group were used for normal control receives only vehicle (gum-acacia) and this group was considered as diabetic control. The blood glucose measured at this time was termed the baseline (0 min) blood glucose. Rats of experimental groups were orally administered suspension of the desired test samples (made in 1.0% gum acacia) at desired dose levels and the biguanide derivative Metformin was used as standard anti-diabetic drug and was always given at a dose of 100 mg/kg body weight orally to the experimental group.After 30 minutes of drug treatment, blood glucose level was again measured with glucometer. The blood glucose assessment were collected from tail vein just prior administration of test sample i.e. 0, 30, 60, 90, 120, 180, 240, 300 and 1440 min post test sample administration. After 300 min the STZ treated animals were allowed to feed over night to overcome drug induced hypoglycaemia. The animals were fed ad lib during 5 to 24 hours of experiments.The average fall in AUC in experimental group compared to control group was termed as % antihyperglycaemic activity. Statistical analysis was done by Dunnett's test.

**Statistical Analysis-**

Quantitative glucose tolerance of each animal was calculated by Area under curve (AUC) method (Prism Software). The average fall in AUC of experimental group compared to control group was always termed as % antihyperglycemic activity. A response criterion of 15 % blood glucose lowering was considered effective. Percent antihyperglycemic effect of the test samples was determined according to the following formula:

$$\%\ \text{Antihyperglycemic effect} = \frac{\text{Mean blood glucose of treatment group x 100-100}}{\text{Mean blood glucose of control group}}$$

**Results-**

Table 1 showed the effects of test compounds (100 mg/kg b.w.) and standard drug Metformin (100 mg/kg b.w.) on Streptozotocin treated diabetic rats. The peak lowering in each case was observed at 5 h post-treatment.From the data shown in table-1, the compound **4g**,**5b** and **5i** showed significant higher lowering in around 16.1($p< 0.01$) , 20.4($p< 0.001$), and 18.4 ($p< 0.001$) % during 5 hr and 18.6($p< 0.01$),21.7($p< 0.001$),20.0($p< 0.001$), % during 24 hr respectively whereas compound **4i and 4k** showed mild activity in the tune of 12.5,14.2($p>0.05$), after 5 h and 18.8 and 14.8($p>0.05$) % activity after 24 intervals respectively. The standard anti-diabetic drug Metformin caused much more lowering effect on blood glucose level on STZ-treated diabetic rats even at 100 mg/kg body weight dose.The The anti-hyperglycemic activity of metformin was calculated to be around 21.0 % ($p < 0.001$) and 23.3 % ($p < 0.001$) after 5 h and 24h intervals respectively. Since no difference was found in food consumption between the sham treated control group compared to any of the experimental group during 24h, the observed blood glucose lowering effect is significant. The compound **5c** did not cause any significant fall lowering on the blood glucose level of Streptozotocin induced diabetic rats at 100 mg/kg dose level.

| Groups | Blood glucose profile (mg/dl) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Pre-treatment* | | | | *Post-treatment* | | | | | *% reduction compared to control* | |
| | 0min | 30min | 60min | 90min | 120min | 180min | 240min | 300min | 1400min | 5 h | 24 h |
| Vehicle | 345.8 ±17.1 | 502.2 ±8.2 | 489.2 ±7.1 | 468.8 ±10.2 | 430.6 ±9.7 | 401.8 ±14.8 | 379.0 ±12.8 | 347.8 ±10.9 | 412.8 ±9.4 | -- | -- |
| 4g | 342.2 ±9.0 | 359.2 ±10.8 | 456.2 ±10.9 | 436.6 ±11.6 | 379.2 ±11.0 | 343.8 ±6.5 | 293.8 ±8.4 | 246.8 ±6.7 | 366.0 ±14.7 | 16.1** | 18.6** |
| 4k | 351.6 ±11.4 | 404.0 ±4.7 | 371.2 ±15.3 | 374.0 ±14.9 | 373.2 ±20.9 | 363.4 ±11.5 | 337.4 ±9.8 | 310.8 ±9.2 | 335.2 ±12.9 | 14.2* | 14.8* |
| 5b | 346.0 ±16.6 | 389.4 ±10.7 | 378.6 ±20.0 | 368.8 ±14.3 | 357.6 ±9.4 | 336.6 ±5.3 | 281.4 ±2.7 | 251.0 ±7.4 | 341.2 ±14.4 | 20.4*** | 21.7*** |
| 5c | 346.2 ±10.7 | 363.0 ±17.8 | 386.6 ±6.6 | 402.0 ±8.5 | 394.6 ±13.0 | 396.4 ±14.0 | 391.0 ±16.3 | 395.2 ±19.2 | 406.2 ±15.1 | 7.26 | 2.54 |
| 5i | 347.4 ±15.2 | 421.2 ±16.0 | 387.2 ±14.6 | 374.6 ±12.0 | 361.8 ±7.8 | 334.8 ±8.8 | 296.8 ±8.6 | 253.0 ±9.6 | 351.4 ±15.8 | 18.4*** | 20.0*** |
| Met | 341.4 ±3.5 | 450.6 ±10.1 | 448.8 ±16.8 | 395.6 ±16.5 | 355.0 ±10.8 | 298.0 ±14.6 | 252.0 ±9.4 | 209.0 ±1.8 | 369.2 ±11.1 | 21.0*** | 23.3*** |

## db/db mice model

### Experimental Animals

Male C57BL/Ks strain of mouse (db/db mouse) 10-12 weeks of age and around 40 ±3 g of body
5 weight were procured from the animal colony of the Institute. The work with these animals was cleared by institutional ethics committee for animal study and was conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) formed by the Government of India in 1964. The institute has taken permission from the animal ethical committee for the work (No. 129/07/Biochem/IAEC). The animals were housed four or
10 five in a polypropylene cage in the animal house. The following norms were always followed for animal room environment: temperature 23 ± 2°C;humidity 50-60%; light 300 lux at floor level with

regular 12 hr light cycle; noise level 50 decibel; ventilation 10-15 air changes per hour. After randomization into groups, the mice were acclimatized for 2-3 days in the new environment before initiation of experiment. Standard pellets were used as a basal diet during the experimental period.

**Antihyperglycaemic and Antidyslipidemic activity assessment in C57BL/Ks strain of mouse (db/db mouse)**

**Experimental Design**

The animals were allocated into groups of 5 animals in each. Prior to start of the sample feeding, a vehicle training period was followed from day -3 to day 0 during which all the animals were given vehicle ( 1% gum acacia) at a dose volume of 10 ml/kg body weight. At day 0 the animals having blood group level between 350 to 500 mg/dl were selected and divided into three groups containing 5 animals in each. One group was considered as control group while the other group one was treatment group. The experimental group was given suspensions of compound 5b and Pioglitazone at 30.0 and 10.0mg/kg body weight dose respectively. The control group was given an equal amount of vehicle. All the animals had free access to fresh water and normal diet. Random blood glucose of each mouse was checked daily at 10.00 pm. On day 10th and day 15th oral glucose tolerance (OGTT) test was performed to study the effect of compound on glucose tolerance. Blood has been withdrawn from the retro-orbital plexus of mice eye for the estimation of lipid profile on DIALAB DTN-410-K and insulin level by CALBIOTECH Insulin ELISA Kit. Body weight of each animal was measured on alternate day for studying the effect of test sample on body weight. The skeletal muscle from each mouse were quickly excised at the end of experiment under light anesthesia and frozen at -800 C until further use.

**Western blot analysis**

Collected tissues and cells were homogenized into PBS  containing 1% NP40, 5 mM EDTA, phosphatase inhibitors  and protease inhibitors cocktail ( Ripalysis buffer). Samples were homogenized and incubated on ice for 15 min. Sample is then store at – 80 ºC and thawed at 37 ºC in water bath.Sample was then centrifuged at 16000 rpm at 4ºC. Then supernatant was taken and quantified by

Bradford assay. 40 μg protein (supernatant) of each incubation was resolved on SDS-PAGE, transferred to nitrocellulose membranes and probed with p-IRS2 (Cell Signaling, MA, USA) and IRS1, (Santa Cruz, CA, USA) antibodies. β-actin (Santa Cruz) was taken as the loading control. Immunoreactive bands will be visualized by Enhanced Chemiluminescence according to manufacturer's instructions (GE Healthcare, UK).

**Densitometry analysis**

Protein expression was evaluated by densitometric analysis performed with Alpha DigiDoc 1201 software (Alpha Innotech Corporation, CA, USA). The same size rectangle box was drawn surrounding each band and the intensity of each was analyzed by the program after subtraction of the background intensity.

**Statistical analysis**

The homeostatic model assessment (HOMA) was used to calculate relative insulin resistance as follows: Fasting blood glucose (mg/dl) × Fasting serum insulin (μIU/ml). Statistical analysis was carried out by Students t test. Data was expressed as mean +SE. The criterion for statistical analysis was significant (*$p<0.05$), more significant (**$p<0.01$), highly significant (**$p<0.01$) and not significant (ns).

**Results**

**Effect on hyperglycemia**

Figure 1 depicts the effect of compound 5b on blood glucose profile from day 1 to 14$^{th}$ post treatment on db/db mice. It is evident from the results that treatment compound 5b at dose 30.0 and Pioglitazone at dose 10.0 mg/kg body weight lowered the blood glucose profile of db/db mice when compared to the vehicle treated control group. The compound 5b significantly improves the blood glucose from day 8 till the end of the experiment, where in pioglitazone treated group the lowering was significant from day 6$^{th}$ onwards and persisted till the last day of experiment.

**Effect on oral glucose tolerance**

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Figure 2 represents the effect of compound 5b at dose of 30.0 mg/kg on oral glucose tolerance test in overnight fasted *db/db* mice on day $10^{th}$ and 15th post treatment. The overnight fasted *db/db* mice were subjected to an oral glucose tolerance test post 3.0 goral glucose load on these days. Compound 5b shows a significant improvement of 24.1 and 35.9 % ($p<0.01$) whereas standard drug pioglitazone improves the glucose tolerance 0-120 min (AUC) by 40.8 and 52.9% ($p<0.01$) at 10.0 mg/kg dose level.

**Effect on serum lipid profile**

Figure 3 represents the effect of compound 5b and pioglitazone at dose 30.0 and 10.0 mg/kg respectively on serum lipid profiles level of *db/db* mice. The treatment of compound 5b significantly lowers the serum cholesterol level by 16.3% ($p<0.05$) and increases the serum HDL-c by 46.8% ($p<0.05$) whereas as pioglitazone shows significant reduction in serum triglyceride level by 12.6% ($p<0.05$).

**Effect on fasting blood glucose, serum insulin level and HOMA-index**

Treatment of compound 5b significantly improves the fasting blood glucose by 52.3% ($p<0.01$). Compound 5b also significantly improves the hyperinsulinimic condition by 34.5% ($p<0.01$). HOMA-index is a measure of insulin resistance, compound 5b significantly improves the insulin resistance state by improving the HOMA-index by 69.7% ($p<0.01$).

**Docking:**

The crystallographic structure was obtained from the Protein Data Bank (PDB), accession code 2F70.[ref] Initially, the protein was considered without ligand for docking simulations. The CHARMm force fields available in the software Discovery studio 2.0[15] were applied and the protein was minimized up to a gradient of 0.01 kcal/ (mol Å).The energy minimized protein structure was used for further docking analysis using the GOLD docking software/program with the default setting of the parameters: population size (100); selection-pressure (1.1); number of operations (10,000); number of

islands (1); niche size (2); and operator weights for migrate (0), mutate (100) and crossover (100). The active site was defined within 10 Å and the ligand binding sites were considered. The docked poses were scored using Goldscore (GS) to find the required docking pose

5 **Reference:**

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