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CONCISE ARTICLE

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

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The protein tyrosine phosphatase 1B (PTP1B) is an attractive target for the treatment of type 2 diabetes. A series of substituted 1, 3-benzyl urea has been synthesized and evaluated for PTP1B inhibitory, antidiabetic and antidyslipidemic activities. The most active compound of the series **5b** showed 79.4% PTP1B inhibition and 20.7% blood glucose lowering in STZ model. It also lowered the serum cholesterol level by 16.3 % and significantly increased the serum HDL-cholesterol by 46.8%. The high activity of the compound **5b**.has been explained by docking studies.

Introduction

The Type 2 diabetes is severe and growing disease in epidemic proportions world-wide. According to International Diabetes Federation (IDF), diabetes is the one of the major challenging health problems of 21st century.[1] India, China, and United States are expected to have the largest number of diabetic people by 2030, and India has been considered to be the diabetes time bomb.[2] Diabetes is caused due to the hepatic insulin resistance along with pancreatic cell defects which leads to the risk of premature death because of cardiovascular complications, nephropathy and retinopathy.[3]

The protein tyrosine phosphatase 1B (PTP1B) is the negative regulator of insulin and leptin receptor pathway and is considered as an attractive target for the treatment of diabetes and obesity. [5, 6] The PTP1B inhibition leads to the enhanced insulin action by prolonging the phosphorylation state of insulin receptors.[4] In a recent landmark study, PTP1B deficient mice showed increased insulin sensitivity and resistance to diet-induced obesity.[7,8]

Despite of the continuous efforts, no PTP1B inhibitor has successfully completed the clinical trials. It may be due to the low cell permeability and bioavailability of these inhibitors.[9] Efforts are made to discover orally active and selective PTP1B inhibitors which may also be useful for probing signal transduction pathways as well as for the treatment of diabetes and obesity.

In recent years we have been engaged in the design, synthesis and evaluation of novel PTP1B inhibitors of diverse classes.[10-12]

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We have previously reported the synthesis and antidiabetic activity of N-[2-(4-methoxy-phenyl)ethyl] acetamide derivatives(**1**).[10] Encouraged by the results of our previous work, and in order to further optimize the PTP1B inhibitory activity, herein we report novel urea derivatives as potent PTP1B inhibitors.

The structure-activity relationship (SAR) studies have been carried out by systemic molecular modulation on the hit molecule **1** (IC_{50}=69μM). The molecule **1** was divided into three regions, the substituted aromatic ring (region I); around acyl group (region II); and the naphthalene ring (Region III) (Fig.1). Initially considering the importance of NH group in increasing the PTP1B inhibitory activity probably due to hydrogen bonding with Gln 262, [10] the acetamide group was replaced by urea moiety to probe the possibility of additional hydrogen bonding with the side chains of Gln262. Further studies were centered on systemic substitution by different R_1 and R_2 groups around region I and III. It was observed by the docking pose of the compound **1** that the oxygen of 4-methoxyphenyl group forms only one hydrogen bonding interaction with the active site residue (Arg221). So it appeared of interest to insert nitro group which may show additional interactions and thus may enhance the PTP1B inhibitory activity. Therefore, in region I, the methoxy group was replaced by the hydrogen and nitro group to see the effect of hydrogen bonding on activity. In the third region different substituted aryl groups were introduced in place of naphthalene group because this group showed no interaction in the catalytic and non-catalytic sites thus making this transformation suitable to modulate the pharmacokinetic properties.

Chemistry

The strategy adopted for the synthesis of the title compounds has been outlined in scheme 1, where the key intermediates, namely, substituted 1-benzylureas (**3**) were synthesized using substituted benzylamine (**1**) and urea (**2**) according to the known reported methods.[13] The substituted 1-benzylureas derivatives (**3a-f**) were reacted with the substituted benzaldehydes in the presence of titanium isopropoxide and the resulting imine intermediates were reduced by sodium borohydride to give the corresponding substituted 1-benzyl-3-hetroarylureas (**4a-4k,5a-5i).**

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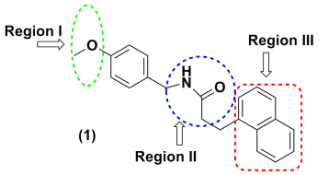
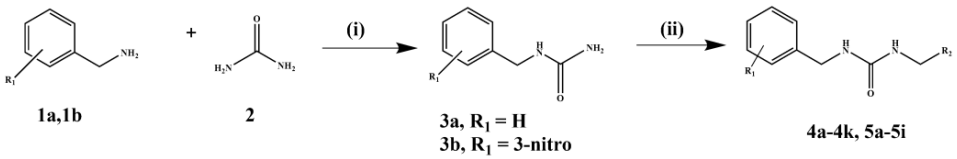


Fig.1



Scheme 1.

Fig.1 Structure of the previously reported acetamide analogue (1) as PTP1B inhibitor; **Scheme 1:** Reagents and Conditions (i) Water, HCl, 100ºC, 6-7 h (ii) Aromatic aldehydes, $Ti(OPr)_4$, $NaBH_4$, 24 h

Table:1 ***In vitro*** **PTP1B inhibitory and** ***in vivo*** **antihyperglycemic activity in STZ model for compound (4a-4k, 5a-5i)**

Compd.	R_1	R_2	% inhibition (IC_{50} value in µM)	% lowering on blood glucose level (100mg/kg)	
				0-5hr	0-24 hr
4a	H	2-NO_2Ph	2.10	-	-
4b	H	3-NO_2Ph	2.40	-	-
4c	H	4-NO_2Ph	0.90	-	-
4d	H	2,6-diCl-3,4-diOCH_3Ph	13.7	-	-
4e	H	3-methoxy-4-hydroxyPh	14.9	-	-
4f	H	3,5-di-tert-butyl-2-hydroxyPh	18.9	-	-
4g	H	2,4,6-trimethoxyPh	78.8 (7.6)	16.1**	18.6**
4h	H	2-thiophene	31.2	-	-
4i	H	5-(4-nitrophenyl)furan	21.4	-	-
4j	H	5-chloro-1H-indol-3-yl	19	-	-
4k	H	4-acetamidoPh	60.93 (9.39)	14.2*	14.8*
5a	3-Nitro	3-ChloroPh	32.9	-	-
5b	3-Nitro	2-NO_2Ph	79.4 (7.47)	20.4***	21.7***
5c	3-Nitro	2,6-diCl-3,4-diOCH_3Ph	56.6 (9.91)	7.26	2.54
5d	3-Nitro	4-MethoxyPh	2.85	-	-
5e	3-Nitro	3,5-dimethoxyPh	2.81	-	-
5f	3-Nitro	3,4,5-trimethoxyPh	7.50	-	-
5g	3-Nitro	6-(3-nitrophenyl)pyridine	40	-	-
5h	3-Nitro	2-furan	46.8	-	-
5i	3-Nitro	5-(4-nitrophenyl)furan	68.4 (8.06)	18.4***	20.0***
Suramin	-	-	60	-	-
Metformin	-	-	-	21***	23.3***

Values are mean ± SEM, n=6, p< 0.05 * p<0.01** and p<0.001***

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Therefore, a set of 21 substituted urea derivatives with different functionalities at $R_1$and R_2 were synthesized and evaluated against protein tyrosine phosphatase 1B (PTP1B). The effect of the synthesized compounds on PTP1B was studied using colorimetric, non-radioactive PTP1B drug discovery kit-BML-AK 822 from Enzo Life Sciences, USA. The assay was done according to the Kit protocol and first all the compounds were evaluated for PTP1B inhibition at 10 µM concentration. Further compounds showing more than 50% inhibition against PTP1B were evaluated at five different concentrations to calculate their IC_{50} value. Suramin was used as a reference standard.

The antidiabetic activity including PTP1B inhibitory activities of the urea derivatives are presented in Table 1. It was observed from the results that the substitution at R_1 and R_2 positions influenced the biological activity of the synthesized analogues (**4a-4k**, **5a-5i**). These compounds in general showed enhanced PTP1B inhibitory activity in comparison to the compounds with acetamide groups reported earlier.[10] Initially the variation at R_1 position (region I, R_1= H , 3-nitro) were performed to derive structure activity relationship. Keeping R_1 as H, a systemic evaluation of R_2 (region III) with different groups that varied in size and polarity were explored (**4a-4k**). This series of compounds with simple phenyl ring in region I was less active than the series with 3-nitro group at R_1 position in region I. The compounds **4h**, **4i** and **4j** with heterocyclic rings at R_2 position and R_1 as H showed less activity with 31.2%, 21.4% and 19% PTP1B inhibition respectively. The compounds **4a** and **4d** having 2-nitro and 2,6-dichloro-3,4-dimethoxyphenyl at R_2 position showed highly reduced activity with 2.10% and 13.7% PTP1B inhibition. The compounds **4b**, **4c**, **4e** and **4f** also did not show PTP1B inhibitory activity. However, the compounds **4g** and **4k** having 2,4,6-trimethoxy phenyl group, and 4- acetamidophenyl at R_2 position showed good activity with 79.8% and 60.93% PTP1B inhibition respectively. Interestingly, majority of analogues with 3-nitro substituent at R_1(region I) showed higher inhibitory activity as compared to the corresponding analogue(s) with hydrogen. The compound **5b** with 3-nitro substituent at R_1 and 2-nitrophenyl at R_2 (region III) was the most active compound of the series with 79.4% inhibition (IC_{50}= 7.47 µM). The compounds **5g**, **5h**, **5i**, with heterocyclic rings substituted at R_2 position and 3-nitro group at R_1 position exhibited moderate activities (31- 68% inhibition; **5i**, IC_{50}= 8.06 µM) but better than the compounds with R_1 as H, while the presence of electron releasing group at R_2 position in compounds **5d**, **5e**, **5f** proved detrimental to the PTP1B inhibitory activity (2- 8% inhibition). However, the compound **5a** with 3-chlorophenyl group and the compound **5c** with 2,6-dichloro-3,4-dimethoxy phenyl group at R_2 position showed 33% and 56.9% PTP1B inhibition respectively.

Kinetics Measurements and Mechanism of Inhibition

Kinetics measurements and mechanism of inhibition was studied using the PTP1B tyrosine phosphatase drug discovery kit BML AK 822 from Enzo life science USA as used for the study of the percentage inhibition. For determination of type of inhibition we have performed the activity assay at different concentration of 0µM, 5 µM, 10µM and 20µM of the most active compound 5b with varying concentration of substrate IR5 (sequence from the insulin receptor ß subunit domain provided in the kit) from 10 to 100µM. The Lineweaver-Burk double reciprocal plot in Fig. 2 shows intercept of all lines (obtained with 0µM, 5 µM, 10µM and 20µM compound **5b** concentration) converging at y-axis ($1/V_{max}$), whereas slope and x-axis intercept ($-1/K_m$) vary with inhibitor concentration which is a characteristic of competitive inhibition, therefore the evaluated compound **5b** is a competitive inhibitor of PTP1B. The Km value for the IR5 substrate was determined from the lineweaver burk plot shown in the Fig.2 and was found 81.4 µM. The Ki value was determined by plotting the slope values *vs* [I]. The resulting secondary plot or "replot" have a Y-axis intercept of K_m/V_{max} and a slope of $K_m/V_{max}Ki$. The value of Ki was calculated from intercept on X-axis of this replot and found 7.88µM which is less than the IC_{50} of the substrate IR5 (85µM) indicating that the inhibitor binds the enzyme better than the substrate.

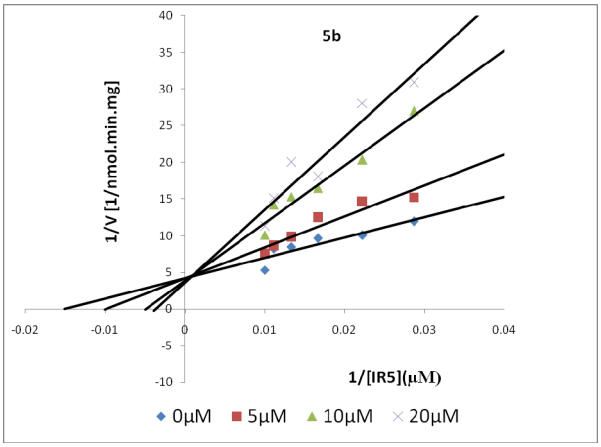

Fig. 2 Competitive inhibitory profile of compound 5b

Molecular modelling

In continuation of our earlier work where the docking studies was successfully applied to elucidate essential structural requirements for molecules acting on the same receptor/enzyme[10-12, 15] we herein report the docking studies of the most active compound **5b** with the PTP1B enzyme active site to gain an insight and understanding of the important interactions with the crucial amino acid residues using the GOLD docking software.[16]

The binding pose analysis of the most active compound **5b** clearly provided rational explanation to its higher potency as compared to the corresponding acetamide derivatives as envisaged by us where the additional hydrogen bonding of both the NH groups in the urea moiety in compound **5b** with the amidic oxygen of Gln262 was observed. The nitro group at 3-position of phenyl ring in region I was also involved in hydrogen bonding interaction with the active site residues Arg221, Asp181 and Ser216 providing the anchorage of this molecule into the active site of PTP1B. These catalytic residues, Arg221 and Asp181, also play a key role in the binding mode of the co-crystallized ligand.[17] (PDB ID-2F70; Fig.3) It is known that the site B of PTP1B differs from that of TCPTP by a few amino acids

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and thus offers an opportunity to improve selectivity over TCPTP.[18, 19] The 2-nitrophenyl ring in the region III of the compound 5b showed potential to make hydrogen bond interactions with the secondary binding site (site B) of PTP1B, characterized by Arg24 and Arg 254. As mentioned earlier interaction with the site B is essential for achieving selectivity over TCPTP. Hence, the favorable interactions of compound **5b** with the sites A and site B of PTP1B enzyme suggest that the compound may be selective over TCPTP.

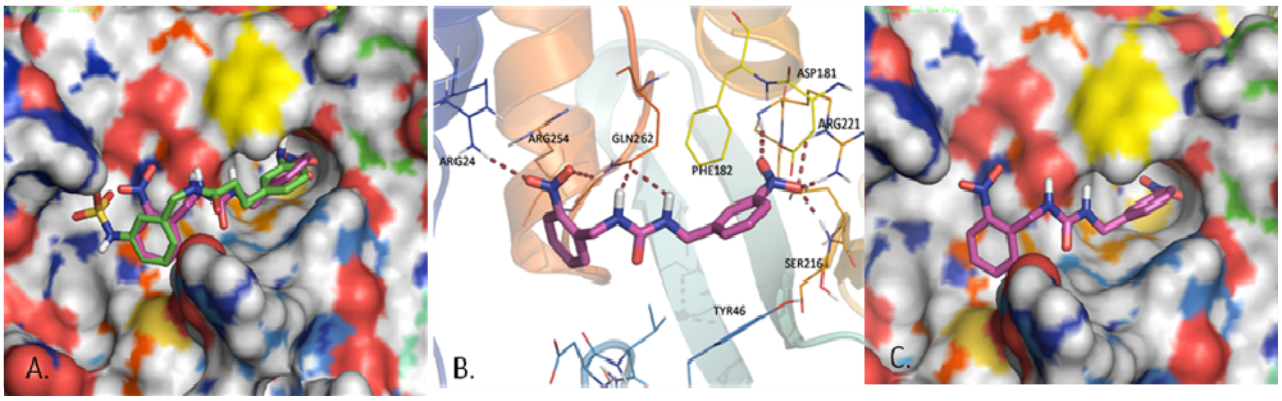


Fig. 3. A) Pose attained by compound 5b (pink) and co-crystallized ligand of PDB Id: 2f70 (green) in the active site of PTP1B ; **B)** Predicted conformation of compound 5b in the binding site cavity of PTP1B; **C)** Analysis of predicted binding pose of 5b with PTP1B

The 3-nitrophenyl ring of the compound 5b also showed pi-pi interaction with Tyr46 and Phe 182 similar to the co-crystallized ligand. However, the higher activity of the compound **5b** than the co-crystallized ligand (PDB ID: 2F70, IC_{50}= 33.5 µM) may be due to the additional hydrogen bonding interaction by the urea group of **5b** with Gln 262.

In vivo biological activity

Based on the high PTP1B inhibitory activity *in vitro* a limited number of compounds with more than 50% PTP1B inhibition (Table 1) were evaluated *in vivo* in Streptozotocin-induced rats model (STZ). Among these, the compounds **4g**, **5b** and **5i** showed lowering of blood glucose level by 16.1 %, 20.4 % and 18.4% during 5h and 18.6 %, 21.7 % and 20.0 %, during 24h, respectively (Fig. 4).

Inspired by the findings the most potent compound 5b was further evaluated for anti-hyperglycemic and antidyslipidemic activities in C57BL/KsJ-db/db mice.(Table 2). The compound **5b** at the dose of 30 mg/kg showed significant blood glucose lowering from day 8 till the end of the experiment as compared to the reference drug pioglitazone which showed significant lowering in blood glucose from day 6 till the end of the experiment at the dose of 10mg/kg (Fig. 5).

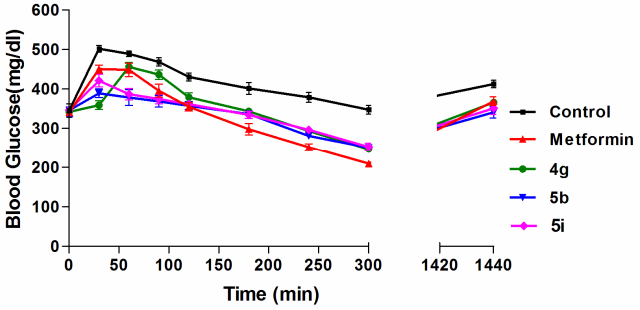
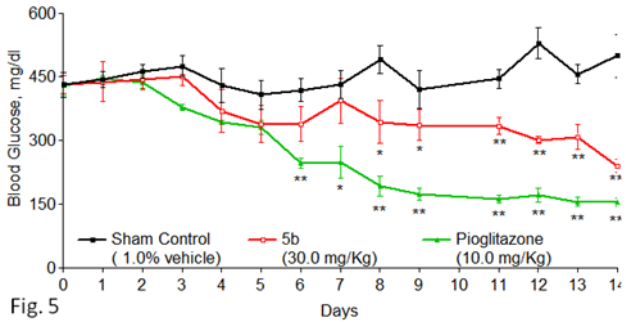


Fig.4 Antihyperglycemic activity of test compounds in low dosed Streptozotocin-induced diabetic rats. Statistical analysis was made by Dunnet test (Prism software 3)

To observe the effect of drug on glucose tolerance an OGTT was conducted on day 10th and 15th during the course of dosing in overnight fasted mice. Compound 5b significantly improves the glucose tolerance by 24.2 and 35.9 % ($p<0.01$) at dose level 30.0 mg/kg body weight on day 10 and 15 respectively whereas the standard drug pioglitazone showed a significant improvement of 40.8 and 52.9% ($p<0.01$) at dose level 10.0 mg/kg body weight on day 10 and 15 respectively as compared to vehicle treated sham control group (Fig. 6).

Table 2: Antihyperglycemic and antidyslipidemic activity in db/db mice(% efficacy, 10 and 15 days)

Compd No.	Antihyperglycemic		Antidyslipidemic			Insulin resistant reversal		
	10 days	15 days	TG	CHOL	HDL	Fasting blood glucose	Serum insulin level	HOMA-index
5b	24.1	35.9	8.9	16.3	46.8	52.3	34.5	69.7
Poiglitazone	40.8	52.9	12.6	9.17	11.1	64.7	46.1	70.3



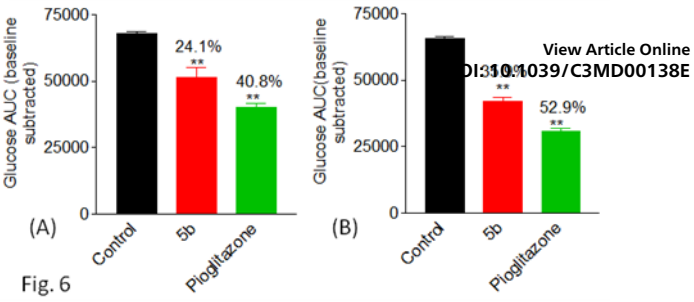


Fig 5. Effect of compound 5b and Pioglitazone on blood glucose of db/db mice; **Fig 6.** Effect of compound 5b and pioglitazone on OGTT on day 10 (A) and day15 (B) posttreatment

Treatment of compound **5b** also restores the altered serum lipid profile as evident from the Fig. 7. The compound **5b** significantly lowers the serum cholesterol level by 16.3 % ($p<0.05$) with significant increase in serum HDL-cholesterol by 46.8% ($p<0.05$). Treatment with the standard drug pioglitazone significantly declines the serum triglyceride level by 12.6% ($p<0.05$). Insulin resistance is one of the characteristic features of db/db mice. Decreased insulin sensitivity leads to hyperglycemia, hyperinsulinemea. Treatment with compound **5b**, near normalizes the fasting blood glucose by 52.3% ($p<0.01$) and restores the altered insulin level by 34.5% ($p<0.01$) in treated diabetic mice as compared to vehicle treated control group as shown in Fig 8A and 8B. Homeostatic model assessment (HOMA) is a method used to quantify insulin resistance on the basis of fasting blood glucose and fasting serum insulin level. Treatment with compound **5b** significantly improves the insulin resistance state by improving the HOMA-index by 69.7% ($p<0.01$) (Fig 8C).

Given the observed potency of **5b**, we proceeded to evaluate the ability of **5b** to inhibit the PTP1B inside the cell. Previous studies have demonstrated that PTP1B serves as a negative regulator of the insulin activated signaling pathways by catalyzing the dephosphorylation of the insulin receptor β (IRβ) subunit. It is evident from the western blot analysis that inhibition of PTP1B with compound **5b** at the oral dose of 30 mg/kg dose significantly improved the insulin signaling and sensitivity which is observed with the enhanced expression of phosphorylated-IRS1 protein in skeletal muscle of db/db mice. The compound 5b resulted in enhancing the expression of p-IRS1 by 1.25 fold whereas reference drug pioglitazone showed an increase of 1.37 fold in expression of p-IRS1 as showed in Fig. 8D. Thus, the inhibition of PTP1B by compound **5b** stimulated the phosphorylation of IRS1 protein which ultimately provides the docking site for the intermediate receptors in the downstream insulin signaling.

Conclusions

In summary, a series of novel benzylurea derivatives was designed, synthesized and evaluated against PTP1B enzyme. Among the 20 synthesized compounds five compounds showed good PTP1B inhibitory activity namely **4g** (7.6 μM), **4k** (9.39 μM), **5b** (7.47 μM), **5c** (9.91 μM) and **5i** (8.06 μM). The docking study performed on the most active compound **5b** provided rational explanation to its higher potency as compared to the corresponding acetamide derivatives as envisaged by us. The compound **5b** interacts well with the active site residues Arg221, Asp181 and Gln262. It also showed good interaction with secondary binding site residues Arg24 and Arg 254 which may attribute to its selectivity over highly homologous phosphtase TCPTP. The docking studies also explained higher activity of compound **5b** than the co-crystallized ligand (PDB Id: 2F70). Further, the compound **5b** also showed promising antihyperglycemic, antidyslipidemic and insulin resistant reversal activities *in vivo* in STZ model and db/db mice model. Thus these studies may be helpful in developing novel PTP1B inhibitors with improved pharmacological properties.

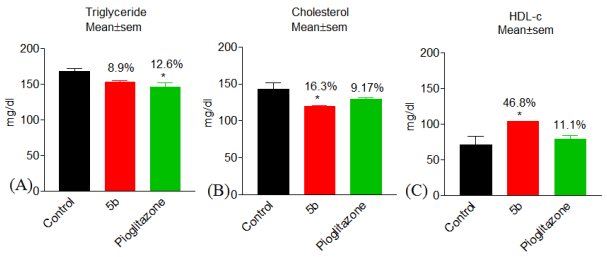


Fig 7. Effect of compound 5b on serum lipid profile

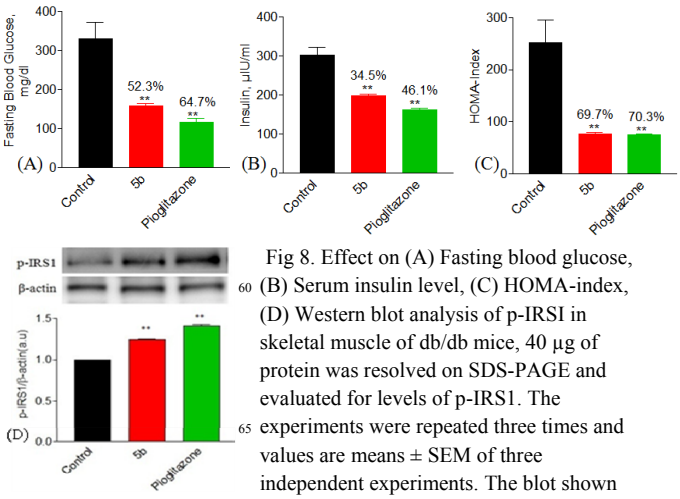


Fig 8. Effect on (A) Fasting blood glucose, (B) Serum insulin level, (C) HOMA-index, (D) Western blot analysis of p-IRSI in skeletal muscle of db/db mice, 40 μg of protein was resolved on SDS-PAGE and evaluated for levels of p-IRS1. The experiments were repeated three times and values are means ± SEM of three independent experiments. The blot shown were representatives of the indicated groups and the densitometric analyses of the same are given below. *p< 0.05, **p < 0.01

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