

## Anti-hyperglycaemic activity of IND 01 and its interaction with glyburide and pioglitazone in alloxan induced diabetic mice

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### Abstract

The antihyperglycaemic activity of IND 01 and its interaction with glyburide and pioglitazone on serum glucose, body weight and oral glucose tolerance test (OGTT) was determined in alloxan-induced diabetic mice. IND 01 (100 mg/kg), glyburide (10 mg/kg), pioglitazone (10 mg/kg) and their concomitant administration were administered orally in alloxan (80 mg/kg, i.v.) induced diabetic mice. The study design consisted of estimation of serum glucose after acute, subacute and glucose load administration. Administration of IND 01 (100 mg/kg) alone significantly ( $p < 0.001$ ) reduced serum glucose level at 6 h after administration. The antihyperglycaemic effect of glyburide and their concomitant administration of IND 01 with glyburide were similar, that is, onset was 2 h; peak effect was 6 h but the effect waned at 24 h. The onset of concomitant administration of IND 01 with pioglitazone was 4 h; peak effect was at 6 h but the effect waned at 24 h. In the subacute study, reduction in serum glucose was observed on 28<sup>th</sup> day after withdrawal for 7 days. The effects of concomitant administration were more pronounced than single drug treatment. In mice treated with either IND 01 (100 mg/kg), glyburide, pioglitazone alone or their combination, the body weight was not reduced in contrast to that in the control group. In the oral glucose tolerance test (OGTT), increased glucose utilization was observed in animals after concomitant administration of IND 01 (100 mg/kg) and glyburide (10 mg/kg) as well as IND 01 (100 mg/kg) and pioglitazone (10 mg/kg). The concomitant administration of IND 01 with glyburide as well as pioglitazone produced synergistic antihyperglycaemic effect than either drug alone.

**Keywords:** Antihyperglycaemic, OGTT, IND 01, glyburide, pioglitazone.

### Introduction

Diabetes mellitus is a metabolic disorder treated by oral hypoglycemic agents such as sulphonylureas, biguanides, thiazolidinediones, meglitinide derivatives, and alpha glucosidase inhibitors.<sup>1</sup> IND 01 contains 40% 4-hydroxyisoleucine, 30% trigonelline and 30% galactomannan isolated from seeds of Fenugreek (*Trigonella foenum-graecum* L. Family: Leguminasae).

Fenugreek (*Trigonella foenum-graecum*) locally called as Methi; is one of the oldest medicinal plants, originating in India.<sup>2</sup> Fenugreek seed contain trigonelline, 4-hydroxyisoleucine, flavonoids, carotinoids, coumarins, proteins, saponins, lipids, galactomannan.<sup>2,3</sup> Galactomannan are polysaccharides consisting of mannose backbone with galactose side groups. The galactomannan in *Trigonella foenum-graecum* seed contain galactose and mannose in the ratio 48:52.

The antidiabetic effect of 4-hydroxyisoleucine<sup>4,7</sup> trigonelline<sup>8,9</sup> have been reported. In view of the antidiabetic effect of fenugreek seeds a formulation

containing three pure compounds isolated from seeds of fenugreek was prepared for first time. Therefore the present study was to study the antihyperglycaemic activity of this compound in diabetic mice, in order to access the suitability of this formulation. A study of the interaction of this compound with glyburide and pioglitazone was also undertaken. No study has yet been undertaken to evaluate the herb-drug interaction in terms of onset, rate and extent of effect, duration and effect on the dose of glyburide and pioglitazone with IND 01. The objective of the present investigation was to study the antihyperglycaemic activity of IND 01 2) and its interaction with glyburide and pioglitazone on serum glucose, body weight and oral glucose tolerance test (OGTT) in alloxan-induced diabetes in mice.

### Materials and Methods

#### Drugs and chemicals

Trigonelline and 4-hydroxyisoleucine were extracted as per method described by Shah et al.<sup>1,8</sup> Galactomannan was extracted by column chromatography. IND 01 was provided as a gift sample from Indus Biotech Pvt. Ltd (Pune, India). Glyburide (Ranbaxy Pvt. Ltd, India), pioglitazone (Ajanta Pharma Pvt. Ltd., India), glucose estimation kit (GOD/POD) (Accurex Biomedical Pvt. Ltd., India), alloxan monohydrate (Spectrochem, India), and D-glucose (S.D. Fine-Chem. Ltd, India) were purchased from the respective vendors.

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**Experimental animals and research protocol approval**

Swiss albino mice (25-30 g) of either sex were purchased from the National Toxicology Centre, Pune, India. They were maintained at a temperature of  $25 \pm 1^\circ\text{C}$  and relative humidity of 45 to 55% under 12-h light: 12-h dark cycle. The animals had free access to food pellets (Chakan Oil Mills, Pune, India) and water was available *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India.

**Preparation of IND 01, glyburide and pioglitazone**

Weighed quantities of IND 01, glyburide and pioglitazone were suspended in distilled water to prepare the suspension of concentration (100 mg/ml) used for pharmacological studies.

**Induction of experimental diabetes**

Diabetes was induced in Swiss albino mice by a single intravenous injection of aqueous alloxan monohydrate (80 mg/kg, i.v.) solution. After 48 h, animals showing serum glucose level above 300 mg/dl (diabetic) were selected for the study.

**Collection of blood and determination of serum glucose**

Blood samples from the experimental mice were collected by retro-orbital plexus technique using heparinised capillary glass tubes. The collected blood samples were placed in eppendorffs tube (1.5 ml). The serum was separated by centrifugation using Eppendorf Cryocentrifuge (Model no 5810, Germany), maintained at  $4^\circ\text{C}$  and run at speed of 7000 r.p.m. for 15 min. Ten microliters of serum and 1 ml of working reagent (GOD/POD) were mixed and incubated for 15 min at  $37^\circ\text{C}$ . The UV/Vis- spectrophotometer (Jasco V-530, Japan) reading was adjusted to 0 by measuring the absorbance of blank (distilled water). The absorbance of sample ( $A_s$ ) and standard  $A_{std}$  provided by manufacturer (Accurex Biomedical Pvt Ltd., Mumbai, India) were measured against blank at 505 nm. Glucose was estimated by using the formula:  $\text{Glucose (mg/dl)} = A_s/A_{std} * 100$ , Where,  $A_s$  = Sample reading;  $A_{std}$  = Standard reading

**Effect of IND 01, glyburide and pioglitazone on serum glucose in diabetic mice**

IND 01 was administered at various doses 25, 50, 100 and 200 mg/kg in diabetic mice. The dose of 100 mg/kg of IND 01 was selected for interaction study because it reduced blood sugar. Diabetic Swiss albino mice of either sex were divided into six groups ( $n = 6$ ) viz; Group I: vehicle (distilled water, 10 ml/kg), Group II: IND 01 (100 mg/kg), Group III: glyburide (10 mg/kg), Group IV: glyburide (10 mg/kg) + IND 01 (100 mg/kg), Group V: pioglitazone (10 mg/kg) and Group VI: pioglitazone (10 mg/kg) + IND 01 (100 mg/kg). IND 01, glyburide and pioglitazone were given orally.

Acute study involved the determination of serum glucose level at 0, 2, 4, 6 and 24 h after IND 01, glyburide,

pioglitazone and their concomitant administration. Animals had free access to feed and water during study. Subacute study involved repeated administration of IND 01, glyburide, pioglitazone and their concomitant administration for 28 days (once a day) at a predetermined time and serum glucose levels were determined in samples withdrawn after 6 h of IND 01, glyburide, pioglitazone and their concomitant administration on 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup> and 28<sup>th</sup> day. At the end of 28 days the drug administration was stopped followed by a rest period of 7 days. Serum glucose levels were determined on 35<sup>th</sup> day. The data was presented as mean serum glucose level  $\pm$  standard error of mean (S.E.M.).

**Effect of IND 01, glyburide and pioglitazone on body weight in diabetic mice**

During the study period of 35 days the mice were weighed daily and their body weights were recorded. From this data, mean change in body weight and S.E.M. were calculated.

**Effect of IND 01, glyburide and pioglitazone on oral glucose tolerance test (OGTT) in diabetic mice**

D-glucose (2.5 gm/kg, p.o.) was administered in diabetic mice in each of the six groups at 0, 2 and 4 h after pretreatment with IND 01, glyburide, pioglitazone and their concomitant administration. Serum glucose levels were estimated before and 2 h after glucose loading. The serum glucose was estimated immediately in the samples. The data was presented as mean serum glucose levels and standard error of mean (S.E.M.) were calculated.

**Statistical analysis**

Data was expressed as mean  $\pm$  S.E.M and statistical analysis was carried out by One-way ANOVA with post hoc Tuckey test performed using GraphPad InStat version 3.00 for Windows 98, GraphPad Software, San Diego, California USA. P value was considered significant when  $<0.05$ .

**Results****Effect of IND 01, glyburide and pioglitazone on serum glucose in diabetic mice**

Administration of IND 01 (100 mg/kg) alone significantly ( $p < 0.001$ ) reduced serum glucose level at 6 h after administration. The peak antihyperglycaemic effect of IND 01 was 6 h but effect waned at 24 h. The antihyperglycaemic effect of glyburide and their concomitant administration of IND 01 with glyburide were similar, that is. onset was 2 h; peak effect was 6 h but the effect waned at 24 h. The reduction in serum glucose from basal value (before) at 6 h after glyburide, IND 01 and their concomitant administration were 181.55, 98.39 and 267.50 mg/dl, respectively (Table 1).

The onset of antihyperglycaemic effect of pioglitazone was 2 h; the peak effect was 4 h but the effect waned at 6 h. The onset of antihyperglycaemic effect of concomitant administration of IND 01 and pioglitazone was 4 h; the peak effect was 6 h but the effect waned at 24 h. The reduction in serum glucose from basal value (before) at 4 h after

**Table 1:** Effect of IND 01, glyburide, pioglitazone alone and their concomitant administration on serum glucose (SG) in alloxan- induced diabetic mice

Groups	Treatment (mg/kg)	Acute study						Subacute Study				
		0 h	2 h	4 h	6 h	24 h	Day 7	Day 14	Day 21	Day 28	After 7 days rest period (Day 35)	
I	Vehicle (10 ml/kg)	434.52 ±10.79	455.10 ±14.93	464.56 ±14.36	465.09 ±10.14	473.68 ±7.73	470.97 ±10.49	509.01 ±16.53	501.67 ±15.66	513.04 ±14.41	500.22 ±18.27	
II	IND 01(100)	444.38 ±20.08	428.39 ±24.61	416.95 ±23.91	364.55 ±17.53** *	461.88 ±24.97	275.58 ±14.10 ***	267.91 ±13.32 ***	257.68 ±14.06 ***	251.82 ±15.06 ***	228.36 ±15.10***	
III	Glyburide (10)	459.38 ±13.14	350.92 ±18.45 **	300.92 ±18.45 ***	277.83 ±12.90** *	447.31 ±6.32	362.36 ±3.73* **	310.14 ±17.70 ***	224.61 ±9.49* **	194.99 ±5.51* **	179.94 ±8.36***	
IV	Glyb (10) + IND 01(100)	464.51 ±15.86	349.88 ±18.24 **	341.95 ±12.94 ***	197.02 ±9.61***	458.79 ±19.63	250.67 ±15.21 ***	189.06 ±9.63* **	160.02 ±4.64* **	132.85 ±4.64* **	111.43 ±5.59***	
V	Pioglitazone (10)	498.59 ±20.64	371.63 ±18.42 *	328.84 ±11.36 ***	459.70 ±17.93	515.68 ±13.70	408.80 ±18.69 *	373.17 ±15.10 ***	362.49 ±12.97 ***	307.91 ±10.41 ***	268.01 ±13.10***	
VI	Pio (10) + IND 01(100)	479.60 ±9.95	407.69 ±16.11	374.82 ±8.38* *	332.11 ±10.05** *	466.50 ±15.22	394.63 ±11.46 **	338.27 ±11.34 ***	314.39 ±6.34* **	239.94 ±8.16* **	172.20 ±8.12***	

Values are mean ± S.E.M., n=6 in each group; Statistical analysis by one-way ANOVA followed by post hoc Tuckey's test using Graphpad Instat software; P value \* $<0.05$ , \*\* $<0.01$ , \*\*\* $<0.001$  compared to vehicle treated group.

**Table 2:** Effect of IND 01, glyburide, pioglitazone alone and their concomitant administration on body weight in alloxan-induced diabetic mice

Groups	Treatment	Mean body weight (g ± SEM)					
		Day 0	Day 7	Day 14	Day 21	Day 28	After 7 days rest period (Day 35)
I	Vehicle (10 ml/kg)	28.33±0.67	26.50±0.43	24.33±0.56	22.50±1.14	20.83±1.01	18.33±0.92
II	IND 01(100)	28.00±0.73	28.17±0.83	29.33±0.42***	31.00±0.45***	31.67±0.62***	31.67±0.92***
III	Glyburide (10)	27.67±0.71	26.00±1.00	27.33±1.18*	28.00±0.93***	28.50±0.85***	25.83±0.31***
IV	Glyb (10) + IND 01(100)	26.67±0.67	27.67±0.67	28.00±0.51**	29.33±0.67***	30.17±0.95***	30.83±0.60***
V	Pioglitazone (10)	26.67±0.42	27.17±0.40	28.33±0.49**	29.17±0.48***	29.83±0.79***	30.50±0.72***
VI	Pio (10) + IND 01(100)	27.67±0.34	27.83±0.40	28.83±0.31***	30.33±0.21***	31.00±0.36***	31.67±0.34***

Values are mean ± S.E.M., n=6 in each group; Statistical analysis by one-way ANOVA followed by post hoc Tuckey's test using Graphpad Instat software; P value \* $<0.05$ , \*\* $<0.01$ , \*\*\* $<0.001$  compared to vehicle treated group

pioglitazone and 6 h after concomitant administration IND 01 with pioglitazone administration was 169.75, 98.39 and 147.49 mg/dl, respectively (Table 1).

In the subacute study, repeated administration (once a day for 28 days) of IND 01, glyburide, pioglitazone and their concomitant administration caused significant ( $p<0.001$ ) reduction in the serum glucose level as compared to vehicle treated group. The reduction in serum glucose level of IND 01, glyburide, pioglitazone alone and their concomitant administration (IND 01 with glyburide and pioglitazone) were 216.58, 279.44, 230.58, 344.75 and 307.40 mg/dl,

respectively on the 35<sup>th</sup> day, that is, after 7 days rest period. The effects of concomitant administration were more pronounced than single drug treatment (Table 1).

#### **Effect of IND 01, glyburide and pioglitazone on body weight in diabetic mice**

The body weight of vehicle- treated diabetic mice decreased during study period. IND 01, glyburide, pioglitazone and their concomitant administration prevented a decrease in body weight of diabetic mice. In fact, an increase in body weight was seen which indicated a beneficial effect of the concomitant administration (Table 2).

**Table 3:** Effect of IND 01, glyburide, pioglitazone alone and their after concomitant administration on oral glucose tolerance test (OGTT) in alloxan-induced diabetic mice

Groups	Treatment	Mean fasting serum glucose level (mg/dl ± SEM)					
		Before glucose	2h	Before glucose	4h	Before glucose	6h
I	Vehicle (10 ml/kg)	419.13±4.98	532.60±10.13	431.13±5.15	529.26±8.43	420.86±5.36	523.00±5.84
II	IND 01(100)	440.22±12.75	426.40±14.99 ***	418.93±7.94	377.93±10.48 ***	437.06±5.21	352.09±6.58* **
III	Glyburide (10)	417.44±9.45	386.89±15.06 ***	429.18±4.84	372.62±14.48 ***	424.23±10.94	357.19±13.02 ***
IV	Glyb (10) + IND 01(100)	445.22±6.63	416.56±11.91 ***	430.48±6.82	356.30±10.63 ***	445.89±6.19	321.92±5.38* **
V	Pioglitazone (10)	439.23±10.93	479.12±9.85*	450.79±9.85	347.24±13.02 ***	436.35±16.95	427.05±10.43 ***
VI	Pio (10) + IND 01(100)	456.78±12.10	418.48±6.50* **	427.05±10.43	396.68±8.62* **	445.31±12.55	303.35±11.9* **

Values are mean ± S.E.M., n=6 in each group; Statistical analysis by one-way ANOVA followed by post hoc Tuckey's test using Graphpad Instat software; P value \*<0.05, \*\*\*<0.01 compared to vehicle treated group

#### Effect of IND 01, glyburide and pioglitazone on oral glucose tolerance test (OGTT) in diabetic mice

In oral glucose tolerance test, IND 01 (100 mg/kg), glyburide (10 mg/kg), pioglitazone (10 mg/kg) and their concomitant administration produced significant ( $p < 0.001$ ) increase in glucose threshold at 6<sup>th</sup> h in diabetic mice (Table 3).

#### Discussion

Several reports are available on the hypoglycaemic effects of the seed of *Trigonella foenum-graecum* extract<sup>10, 11, 12</sup> and their alkaloids; trigonelline<sup>8, 13</sup> and amino acid; 4-hydroxyisoleucine.<sup>5, 6, 7</sup> Several mechanisms of action for *Trigonelline foenum-graecum* hypoglycaemic action were proposed. It antagonized the hyperglycaemia caused by alloxan or cadmium in rats. Cadmium has been shown to cause hyperglycaemia by increasing the release of epinephrine in intact rats and by decreasing release of insulin in isolated perfused rat pancreas.<sup>14</sup> A higher level of antioxidants in animals on a *Triognella foenum-graecum* supplemented diet as compared with animals on a control diet leads to the assumption that its seed used as a supplement in the diet may normalize the disrupted free radical metabolism<sup>15</sup>. *Trigonella foenum-gracum* brought the high glucose-6-phosphatase and fructose 1,6-phosphatase activities in the kidney and liver back to the normal level in diabetic rats. These enzymes are involved in increased production of glucose and fructose.<sup>16</sup> *Triognella foenum-graecum* powder supplementation also led to normalization of creatinine kinase activity in diabetic rats and restored normoglycaemia comparable with insulin and vanadate (an antidiabetic agent).<sup>17</sup> It was also shown that *Trigonella foenum-gracum* seed powder increased the glycolysis and decreased gluconeogenesis in the liver and kidney in diabetic rats.<sup>18</sup>

IND 01 (100 mg/kg) showed peak antihyperglycaemic effect at 6 h indicating a lag period of 5 to 6 h before the

peak effect was reached and effect waned at 24 h. The subacute study indicated that a period of seven days is required for attaining a steady state concentration of IND 01 in the blood to reveal its antihyperglycaemic effect and the effect was sustained even after withdrawal of drug for seven days.

Glyburide is a potent, second generation, oral sulfonylureas used for the treatment of diabetes. The hypoglycaemic action of glyburide is due to stimulation of pancreatic islets cells, which results in an increase in insulin secretion.<sup>19</sup>

Our result showed that the onset of action of glyburide is short and the duration of action is about 6 h. Glyburide appears to be a more effective antihyperglycaemic agent than IND 01. The subacute treatment with glyburide was effective in reducing blood glucose after 7 days of treatment and thereafter. Glyburide withdrawal after 28 days did not affect the reduction in blood sugar. To obtain the advantage of the short onset of action glyburide and to determine whether it has synergistic effect with IND 01, both glyburide and IND 01 were administered one after other within an interval of 5 min. The results obtained in acute study indicated that the onset was rapid, which may be due to the glyburide and peak reduction in blood sugar was more than either drug alone. The results thus indicated that concomitant administration of IND 01 and glyburide produced synergistic antihyperglycaemic effect with advantage of rapid onset of action.

In the present investigation a second oral hypoglycaemic agent pioglitazone was used. Pioglitazone is member of thiazolidinedione derivatives, a group of insulin sensitizing agent that enhances sensitivity to insulin in the liver, fatty tissue and striated muscle. They decrease hepatic glucose production and increase its peripheral use.<sup>20-23</sup>

Pioglitazone (10 mg/kg) showed a quick onset of antihyperglycaemic effect at 2 h with peak at 4 h. In the

subacute study 7 days treatment of pioglitazone did not significantly decrease blood sugar level compared to subsequent days, indicating that more than one week treatment is required for a significant antihyperglycaemic effect. Pioglitazone maintained a decrease in blood glucose during the rest period of seven days. Concomitant administration of IND 01 and pioglitazone did not significantly decrease serum glucose at 2 h as compared to that of pioglitazone alone at 2 h. On the other hand at 6 h more reduction in glucose level was observed compared to that of pioglitazone at 6 h. The results thus indicated that concomitant administration of IND 01 and pioglitazone resulted in synergistic antihyperglycaemic effect.

In the subacute study concomitant administration of both drugs resulted in antihyperglycaemic effect at 28 days, which was maintained during rest period. The antihyperglycaemic effect of concomitant administration of IND 01 and pioglitazone was more compared to that of IND 01 and glyburide. The results indicated that long-term treatment with pioglitazone and IND 01 was more effective than either drug alone.

IND 01, metformin, repaglinide alone and their concomitant administration increase the body weight of diabetic mice compared to vehicle-treated group. The increase in the body weight may also be due to effective control of blood sugar level.

Diabetic animals have impaired glucose tolerance. Additional load of glucose impaired the tolerance further as evident from 0, 2 and 4 h readings in vehicle-treated animals. The earlier experiment showed the peak effect of IND 01 at 6 h therefore OGTT experiments were designed to study the effect of glucose load at 2, 4 and 6 h after pretreatment with IND 01. The reduction in serum glucose after 2 h pretreatment with IND 01 was less compared to 4 h pretreatment. Glyburide showed a more effective antihyperglycaemic effect at 2, 4 and 6 h, which correlates with the short onset of action of glyburide. The glucose tolerance in diabetic animals increased. Administration of IND 01 and glyburide was more effective than either drug alone in lowering blood glucose after glucose load.

In the group of animals in which glucose load was given after pretreatment with pioglitazone for 2 h, significant ( $p < 0.001$ ) reduction in serum glucose level was observed at 4 h compared to the vehicle group. After pioglitazone treatment, the activation of PPRA- $\gamma$  (peroxisome proliferator-activated receptor- $\gamma$ ) receptor involved in sensitization of insulin required the effective activation period of 2h. Concomitant administration of IND 01 with pioglitazone in OGTT resulted in reduction of serum glucose after glucose load in diabetic animals indicating that pioglitazone potentiated antihyperglycaemic effect of IND 01 in OGTT. Concomitant administration of IND 01 with pioglitazone appears to be more effective than concomitant administration of IND 01 and glyburide.

Earlier experiments indicated that trigonelline and 4-hydroxyisoleucine acted by pancreatic regeneration and the

insulin produced by regenerated beta cells exhibited antihyperglycaemic response due to increased sensitization of the insulin receptors by pioglitazone.<sup>1,8</sup> 4-hydroxyisoleucine is one of the most potent insulinotropic (insulin sensitizing) agents.<sup>5,6,7</sup> 4-Hydroxyisoleucine increased glucose induced insulin release (ranging from 100  $\mu$ mol/to 1mmol/L) through a direct effect on the isolated islets of Langerhans in both rats and humans. This pattern of insulin secretion was biphasic, glucose-dependent, occurred in the absence of any change in pancreatic alpha and delta cells activity and without interaction with other agonists of insulin secretion (such as leucine, arginine, tolbutamide, glyceraldehydes).<sup>24</sup> Trigonelline may exert hypoglycaemic effect in healthy non-diabetic volunteers.<sup>25</sup>

It is apparent that from the results that concomitant administration of IND 01(100 mg/kg) with either glyburide or pioglitazone resulted in synergistic antihyperglycaemic effect. Increased insulin secretion or increased glucose threshold or regeneration of pancreatic beta cells may be involved in the antihyperglycaemic effect. Careful designing of timing of administration of each antidiabetic drug is important in obtaining the synergistic effect with IND 01.

### Conclusions

The results thus indicated that IND 01 has antihyperglycaemic activity in alloxan-induced diabetic mice. The peak antihyperglycaemic effect was at 6 h. The metabolism of drug takes place after the peak and the effect waned at 24 h. The concomitant administration of synthetic oral hypoglycaemic drugs like glyburide and pioglitazone with IND 01 showed synergistic antihyperglycaemic effect.

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