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In vitro anti-hyperglycemic activity of 4-hydroxyisoleucine derivatives

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ABSTRACT

The nonproteinogenic amino acid, 4-hydroxyisoleucine (**1**) has been isolated in large quantities from the fenugreek (*T. foenum-graecum*) seeds. Few novel derivatives (**3**–**11** and **13**–**18**) were prepared from the naturally occurring 4-hydroxyisoleucine (**1**) and screened for their *in vitro* glucose uptake stimulatory effect in L-6 skeletal muscle cells. The derivatives **6**, **7**, **8**, **10** and **11** exhibited better glucose uptake stimulatory activity than parent compound, 4-hydroxyisoleucine at 5 and 10 μM concentrations and compounds **7** and **11** enhanced translocation of insulin sensitive glucose transporters-4 in skeletal muscle cells.

Introduction

Diabetes mellitus is a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both (Mitra, 2008). The chronic hyperglycemia of diabetes is associated with relatively specific long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD). It affects all age group worldwide. In 1985, an estimated 30 million people around the world were diagnosed with diabetes; in 2000, that figure rose to over 150 million; and in 2012, the International Diabetes Federation (IDF) estimated that 371 million people had diabetes (International Diabetes Federation, 2012, www.idf.org/diabetesatlas). That number is projected to rise up to 552 million (or 1 in 10 adults) by 2030, which equates to 3 new cases per second (International Diabetes Federation, 2012, www.idf.org/diabetesatlas).

The main pathophysiological feature of type 2 diabetes mellitus is insulin resistance, characterized by reduced ability of target tissues, such as the liver, skeletal muscle, and adipose, to respond to insulin (Reaven, 1995). This includes impairment in the insulin stimulated translocation of GLUT4 to cell surface and resulting defect in insulin-stimulated glucose uptake in peripheral tissues like skeletal muscle and fat. Hence, interventions with ability to stimulate glucose uptake might be important for the treatment of diabetes mellitus. Although numerous oral anti-hyperglycemic drugs exist along with insulin, there is no promising therapy for type 2 diabetes mellitus.

Natural products have played a key role in the discovery of antidiabetic drugs (Fig. 1) (Kanaujia et al., 2010) Metformin is currently used as antidiabetic agent in the treatment of type 2 diabetes. Metformin and its analogues (Shapiro et al., 1959) were synthesized on the basis of a natural product lead, that is, galegine (Bailey and Day, 1989). Acarbose, a complex of oligosaccharides isolated from *Actinoplanes* sp., was discovered by Bayer pharmaceuticals for a search of α-glucosidase inhibitors (Shu, 1998). Acarbose is an antidiabetic agent to treat type 2 diabetes. Few plant-derived unusual amino acids have been reported for their anti-hyperglycemic activity. Hypoglycin A and B isolated from the fruits of *Blighia sapida*, which are chemically related to lysine were reported for their anti-hyperglycemic activity (Hassall and Reyle, 1954; Feng and Patrick, 1958). Enormous amount of research work is going on 4-hydroxyisoleucine (4-HIL), due to its broad range of pharmaceutical activities as insulinotropic, anti-dyslipidemic, and anti-hyperglycemic agent (Sauvaire et al., 1998; Narender et al., 2006)

The 4-hydroxyisoleucine was first isolated as free acid from fenugreek (*T. foenum-graecum*) seeds (Fowden et al., 1973) and structure was validated by X-ray crystallography (Alcock et al., 1989), establishing the absolute stereochemistry as 2S, 3R, 4S. Further, the SAR studies indicated that the absolute configuration

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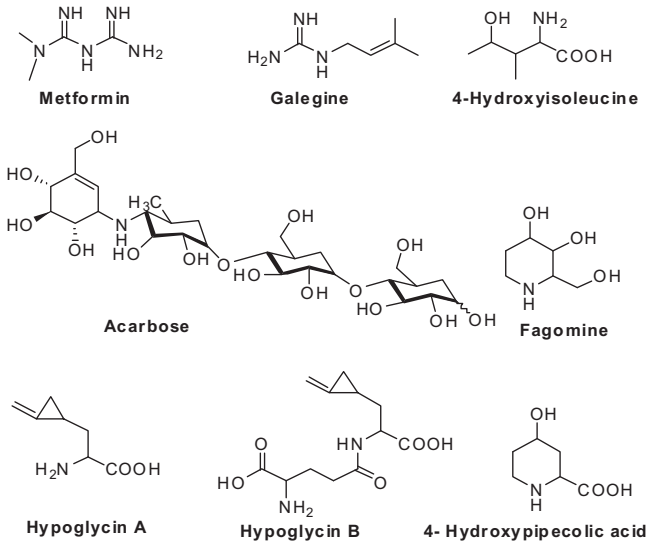


Fig. 1. Naturally occurring and synthetic antidiabetic agents.

of 2S, 3R, 4S stereogenic centres has considerable influence on their pharmaceutical activity (Christophe et al., 2000; Broca et al., 1999). Recently we have reported the lipid lowering activity of 4-hydroxyisoleucine in high fat diet fed hamster model (Narender et al., 2006). We have also reported the glucose uptake stimulatory effect of 4-HIL mediated by enhanced translocation of insulin sensitive glucose transporters-4 in skeletal muscle cells (Jaiswal et al., 2012). Thus, 4-HIL seems a very promising dietary supplement in the treatment and management of diabetes mellitus. Therefore, we isolated a large quantity of 4-HIL (**1**) and prepared a series of 4-hydroxyisoleucine derivatives (**3**–**11** and **13**–**18**) and evaluated their *in vitro* glucose uptake stimulatory effect in skeletal muscle cells.

Materials and methods

Isolation of 4-hydroxyisoleucine

The seeds (20 kg) of *T. foenum-graecum* were collected from the local market of Lucknow and extracted with 16 l of ethyl alcohol four times in a percolator. The resultant alcoholic extracts were (64 l) combined and concentrated under reduced pressure to give 400 g of alcohol extract. This was fractionated with chloroform and n-butanol successively. The resultant aqueous fraction (40 g) was subjected to conventional silica gel column chromatography using ethyl acetate and methanol (90:10) solvent system to give an unusual amino acid (2 g). It was characterized as 4 hydroxyisoleucine by using ^{1}H NMR, ^{13}C NMR, IR and mass spectral data and comparing with literature data (Fowden et al., 1973; Alcock et al., 1989).

Cell culture

L6 skeletal muscle cells stably expressing rat GLUT4 with a *myc* epitope inserted in the first exofacial loop (L6-GLUT4myc), a kind gift of Dr Amira Klip, Program Cell Biology, The Hospital for Sick Children, Toronto, Canada, were maintained in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, 25 μg/ml amphotericin B) in a humidified atmosphere of air and 5% CO_2 at 37 °C. Differentiation was induced by switching confluent cells to medium supplemented with 2% FBS. Experiments were performed in differentiated myotubes 6–7 days after seeding.

Glucose uptake

The determination of 2-DG uptake in L6-GLUT4*myc* myotubes was performed as described previously (Tamrakar et al., 2010). Briefly myotubes were incubated with test compounds for 16 h under culture condition. Glucose uptake was assessed for 5 min in HEPES-buffered saline [140 mM NaCl, 20 mM HEPES, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$ (pH 7.4)] containing 10 μM 2-DG (0.5 μCi/ml 2-[^{3}H]-DG) at room temperature. Subsequently cells were rinsed with ice-cold saline solution. To quantify the radioactivity incorporated, cells were lysed with 0.05 N NaOH and lysates were counted with scintillation fluid in a β-counter. Nonspecific uptake was determined in the presence of cytochalasin B (50 μM) during the assay, and these values were subtracted from all other values. Glucose uptake measured in triplicate and normalized to total protein, was expressed as percent induction with respect to unstimulated cells.

GLUT4 translocation

GLUT4 level at the cell surface of non-permeabilized L6-GLUT4*myc* myotubes was measured by an antibody-coupled colorimetric assay as described previously (Tamrakar et al., 2011). Briefly, after the indicated treatments, cells were washed in ice-cold PBS (154 mM NaCl, 5.6 mM Na_2HPO_4, 1.1 mM KH_2PO_4) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4). Cells were then fixed in 3% paraformaldehyde for 30 min and quenched in 100 mM glycine for 10 min, all at 4 °C. Cells were blocked in 5% skimmed milk for 15 min and then incubated with anti-*myc* antibody solution (1.0 μg/ml in PBS with 3% skimmed milk) for 1 h at 4 °C. After labelling, excess antibodies were removed by extensive washing in ice-cold PBS. Cell surface GLUT4-bound antibodies were probed by HRP-conjugated secondary antibodies followed by detection of bound HRP by O-phenylenediamide assay. The fraction of GLUT4 at the cell surface, measured in triplicate, was expressed as fold induction with respect to unstimulated cells.

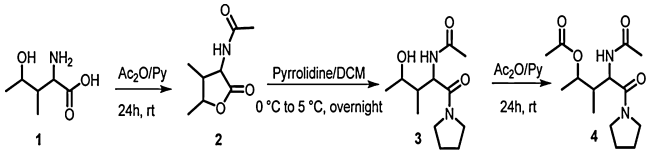
Results and discussion

Chemistry

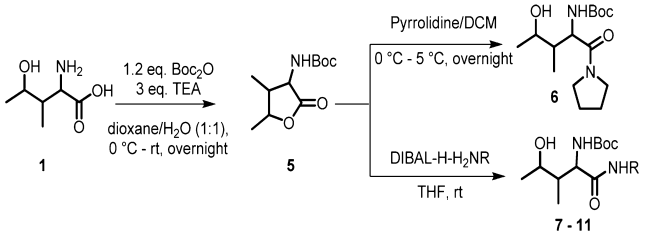
The synthesis of derivatives of pure 4-HIL (**1**) was accomplished by subjecting the acetylation of 4-hydroxyisoleucine by using acetic anhydride in pyridine to afford the N-acetylated lactone (**2**). The resultant compound **2** was treated with pyrrolidine in DCM at 0 °C for overnight to afford the 4-HIL amide (Nicolas et al., 2006) (**3**). Further this amide **3** was treated with acetic anhydride in presence of pyridine at room temperature to afford corresponding *O*-acetylated derivative (**4**) (Scheme 1).

Furthermore the synthesis of N-Boc protected lactone (**5**) was accomplished by subjecting the 4-HIL to the anhydride Boc_2O in the presence of base to obtain the compound **5**. The resultant lactone (**5**) was converted in to amides (Huang et al., 2001) by using DIBAL–H–H_2NR complexes in THF or pyrrolidine in DCM system to afford the compounds **6**–**11** in good yields (Scheme 2).

Furthermore N-tosylated amides of 4-HIL (**14**–**18**) were synthesized from 4-HIL as shown in Scheme 3. Treatment of lactone (**12**),



Scheme 1. Synthesis of 4-HIL derivatives.



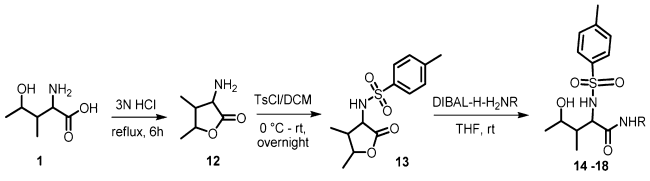
Scheme 2. Synthesis of N-Boc protected 4-HIL derivatives.

which was obtained from 4-HIL in the presence of 3 N HCl, with tosylchloride in dry DCM at 0 °C to room temperature for overnight afforded the N-tosylated lactone (Nicolas et al., 2006) (**13**). Then the resultant lactone was subjected to aminolysis (Nicolas et al., 2006) by treating with DIBAL–H–H_2NR complex in THF to afford the amides **14**–**18** in good yields.

Biological evaluation Glucose uptake in L6 skeletal muscle cell lines

To check the *in vitro* antidiabetic effect, all the compounds were evaluated for their effect on glucose uptake in L6-GLUT4*myc* skeletal muscle cell lines. Skeletal muscle is the major insulin-target tissues responsible for maintenance of whole body glucose homeostasis. Insulin stimulates cellular glucose uptake in muscle tissues by inducing the translocation of glucose transporter-4 (GLUT4) from an intracellular pool to the plasma membrane. In the diabetic state, insulin stimulated translocation of GLUT4 gets impaired leading to decreased glucose uptake by muscle cells, which significantly contribute to the elevated blood glucose levels. Cells were differentiated to myotube stage and treated overnight with indicated concentration of compounds under culture conditions followed by determination of glucose uptake according to Hwang et al. (2008) with some minor modifications. The results of the glucose uptake stimulatory effect of compounds are summarized in Table 1

The naturally occurring compound 4-HIL (**1**) enhanced the glucose uptake by 38.67% in L6 muscle cells at 10 μM concentration (Table 1). From the tested compounds **3**–**11** and **13**–**18,** compound **7** and **11** showed maximal stimulation of glucose uptake 109.5% (2.10 folds vs control) and 105.25% (2.05 folds vs control) respectively at 10 μM concentration (Table 1). Further these two compounds were evaluated for their dose-dependent effect using different concentrations of compounds (Fig. 2). In L6 cells compound **7** enhanced the glucose uptake 70.5% (1.71 folds vs control basal) at 5 μM concentration and 122.5% (2.22 folds vs control basal) at 25 μM concentrations where as compound **11** enhanced the glucose uptake by 62.7% (1.63 folds vs control basal) and 143.0 (2.43 folds vs control basal) at 5 μM and 25 μM concentrations respectively (Table 1; Fig. 2). From the remaining compounds, **6**, **8** and **10** also significantly increased the glucose uptake by 55.4% (1.55 folds vs control basal), 66.0% (1.66 folds vs control basal) and 50.25% (1.55 folds vs control basal) at 10 μM concentrations respectively (Table 1). Compounds **6**, **7**, **8**, **10** and **11** also exhibited better glucose uptake activity than it's naturally occurring 4-HIL (**1**). All other synthesized



Scheme 3. Synthesis of N-Ts protected 4-HIL derivatives.

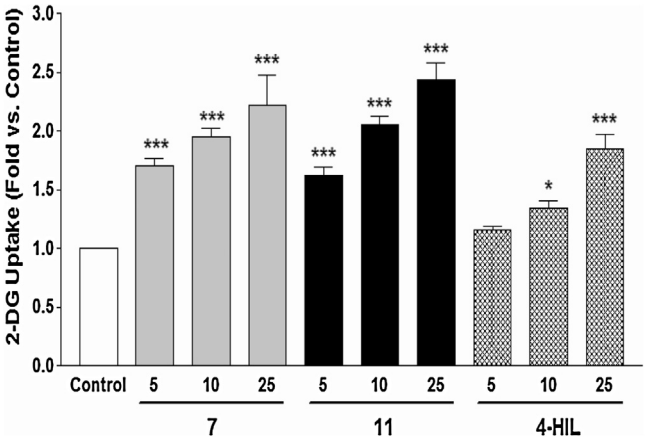


Fig. 2. Dose dependent effect of 4-HIL (**1**) and its derivatives (**7** and **11**) on glucose uptake in L6-GLUT4*myc* skeletal muscle cell lines. Results are expressed as fold stimulation over control. $^{*}p < 0.05$, $^{***}p < 0.001$ relative to control.

compound showed poor to no effect on glucose uptake in L-6 muscle cells at 10 μM concentration.

It is further assessed whether the ability of **7** and **11** to stimulate glucose uptake was mediated by potentiation of insulin action or activation of cellular processes independent of the hormone. Cells were treated with **7** and **11** (10 μM) for 16 h with final three hour in serum-deprived medium and a subset of cells was stimulated with insulin (100 nM) for 20 min before the measurement of glucose uptake. As shown in Fig. 3, insulin alone caused 2.2-fold ($p < 0.001$ vs. control basal) stimulation of glucose uptake in L6 myotubes over basal state. Pretreatment with **7** and **11** (16 h) caused further increase in insulin signal, suggesting that the effect of **7** or **11** and insulin on glucose transport is additive. But the gain in transport was lower than that calculated from the independent effect of compounds (**7** and **11**) and the insulin. These observations suggest that compounds **7**, **11** and insulin activate glucose transport by different sub cellular pathways, but some elements common to the action of both agents may be finite.

Effect of 4-HIL derivatives on GLUT4 translocation to cell surface in L-6 GLUT4myc skeletal muscle cell lines

To check whether the increase in glucose uptake by the compounds is attributable to the translocation of GLUT4 from intercellular compartment to plasma membrane, surface level

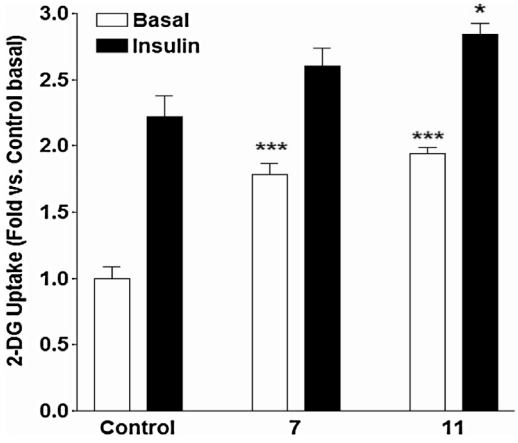
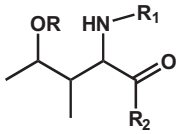
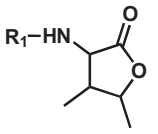
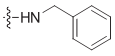
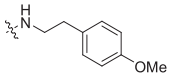
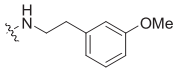
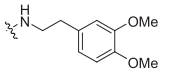
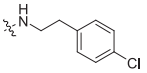
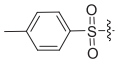
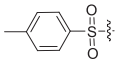
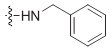
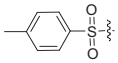
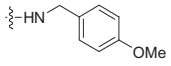
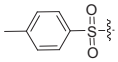
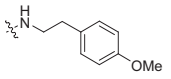
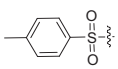
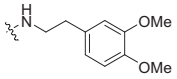
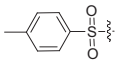
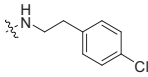


Fig. 3. Effect of 4-HIL – derivatives (**7** and **11**) on insulin stimulated glucose uptake in L6-GLUT4*myc* skeletal muscle cell lines. Results are expressed as fold stimulation over control basal. $^{*}p < 0.05$, $^{***}p < 0.001$ relative to control.

Table 1
In vitro glucose uptake in L6-GLUT4*myc* skeletal muscle cells of 4-HIL derivatives.





Compound	R	R_1	R_2	Concen-tration (μM)	% Glucose uptake	No. folds vs basal
1	H	H	OH	5 10 25	16.0 38.67* 116.0***	1.16 1.38 2.16
3	H			10	32.50	1.33
4				10	10.50	1.11
5	–		–	10	9.00	1.09
6	H			10	55.4*	1.55
7	H			5 10 25	70.5* 109.50*** 122.0***	1.71 2.10 2.22
8	H			10	66.00*	1.66
9	H			10	−12.00	0.88
10	H			10	50.25*	1.50
11	H			5 10 25	62.7* 105.25*** 143.0***	1.63 2.05 2.43
13	–		–	10	−1.00	0.99
14	H			10	8.50	1.09
15	H			10	2.23	1.02
16	H			10	−14.00	0.86
17	H			10	−7.50	0.93
18	H			10	10.50	1.11
		Rosiglitazone		10	96.5**	1.96

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ relative to control.

of GLUT4*myc* was measured by an antibody-coupled assay (Tamrakar et al., 2011). Prolonged exposure (16 h) of L6-GLUT4*myc* myotubes to compounds **7** and **11** caused a substantial increase in the level of GLUT4 molecules at the plasma membrane in a concentration-dependent fashion (Fig. 4). A significant augmentation in surface level of GLUT4 was observed at 10 μM ($p < 0.01$) concentration, reaching to the maximal level at 25 μM ($p < 0.01$). Insulin alone (100 nM), when added 20 min before

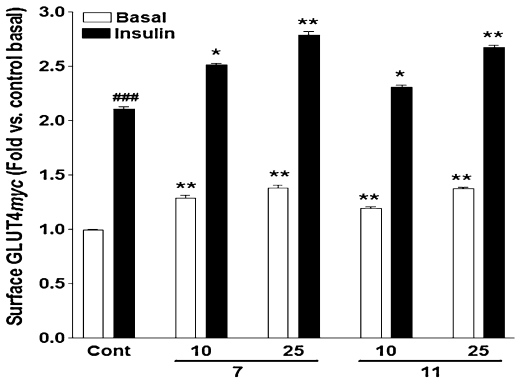


Fig. 4. Effect of 4-HIL derivatives on GLUT4 translocation to cell surface in L-6 GLUT4*myc* skeletal muscle cell lines. Results are expressed as fold stimulation over control basal. $^{*}p < 0.05$, $^{**}p < 0.01$ relative to respective control condition, $^{###}p < 0.001$ relative to control basal.

the measurement of GLUT4 translocation resulted in 2.2-fold ($p < 0.001$) increase in surface level of GLUT4 compared to basal state in L6-GLUT4*myc* (Fig. 4). Treatment with **7** and **11** caused significant enhancement of insulin stimulated translocation of GLUT4*myc* to cell surface ($p < 0.01$) compared to control insulin treated (Fig. 4).

Conclusion

In conclusion, we isolated the nonproteinogenic amino acid (2S, 3R, 4S)-4-hydroxyisoleucine from seeds of fenugreek in large quantity and prepared a series of amides (**3**–**11** and **13–18**) related to natural product 4-hydroxyisoleucine and screened their glucose uptake activity in L6 rat muscle cells. Some of the compounds of this series (**6**, **7**, **8**, **10** and **11**) are more potent to the natural product 4-HIL (**1**) in *in vitro* glucose uptake activity. Our preliminary structure activity relationship data indicated that N-Boc protected amides are more active than N-tosylated and N-acetylated amides (**7** vs **13** and **8** vs **3**).

Conflict of interest

The authors have no conflict of interest.

Disclosure statement

The authors have nothing to disclose.

Acknowledgments

Authors are thankful to Director, CSIR-CDRI for constant encouragement on the program on natural products' synthesis, SAIF for spectral data, ICMR for ado project (No. 59/06/2010/BMS/TRM) and fellowship to Mr. Rohit. This is CDRI communication No. 8817.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2014.09.007.

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