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Inhibition of glucose transport in human erythrocytes by 2,3-dioxoindole (*Isatin*)

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Abstract. 10 mM isatin (2,3-dioxoindole) inhibited glucose influx into human erythrocytes by over 30%. The inhibition is of the competitive type, where the affinity constant (K_t) was increased from 5.71 (control) to 11.11 mM in the presence of isatin with no change in V_{max} (130 nmol/min/ml packed cells). The observed inhibition of sugar transport by isatin was not mediated through membrane –SH groups accessible to iodoacetate, iodoacetamide, DTNB, DNP or sodium arsenite. Isatin inhibited sugar transport in the presence of 2 mM harmaline, an alkaloid inhibitor of Na^+, K^+-ATP_{ase} activity. The inhibition was non additive which suggests that these two compounds interact with the same or a similar site on the erythrocyte membrane.
Key words. Isatin; glucose transport; human erythrocytes; competitive inhibition.

Isatin (2,3-dioxoindole) is a heterocyclic compound with an indole nucleus. The drug is known as a pharmacological agent and its effects have been studied in a variety of tissues[1–4]. The compound has recently been detected in animal and human tissues[5]. Because of the presence of a highly reactive 3-oxo group in the molecule, the drug can bind to –SH or $-NH_2$ groups of proteins and modify their biological activity. We have previously shown that isatin is a strong inhibitor of the Na^+-dependent glucose transport in rat intestine, but it has no effect on the Na^+-independent uptake system[4]. The observed inhibition of the Na^+-glucose co-transporter was a consequence of isatin interaction with –SH groups of the transport protein[6]. In the present report, we have extended these observations to the glucose transport system in human erythrocytes and have shown that isatin is also an inhibitor of sugar transport in these cells. However, this inhibition is not mediated through surface –SH groups that are accessible to known –SH group-reacting agents under the experimental conditions.

Materials and methods

All chemicals used were of analytical grade, obtained from E. Merck Ltd. (India). Phloretin, iodoacetate, iodoacetamide, DTNB, DNP, and harmaline were purchased from Sigma Chemical Company, St. Louis (USA). Isatin was obtained from 'Riedel-de-Haen', Seelze-Hannover (Germany). [^{14}C]-U-D-glucose (55 mCi/m mole) was purchased from Radioisotope Division, Bhaba Atomic Research Centre, Trombay, Bombay (India).

Reagents. Isotonic saline (165 mM NaCl). Isotonic phosphate: 110 mM sodium hydrogen phosphate adjusted to pH 7.4 with 165 mM sodium dihydrogen phosphate. Phosphate buffered saline (PBS): 80 parts of isotonic saline and 20 parts of isotonic phosphate were mixed and the pH readjusted to 7.4. Stop solution: 1.2 mM KI, 2 mM $HgCl_2$, 0.1 mM phloretin in PBS.

Preparation of erythrocytes. Freshly collected blood from human volunteers was centrifuged at 3000 × *g* for 5 min. After removal of buffy coat, which contains the white cells, red blood cells were washed three times with 10 volumes of isotonic saline to remove both intra- and extracellular glucose.

Flux measurements. Glucose influx into erythrocytes was determined following the method of Lowe and Walmsley[7]. Typically, 1.5 ml PBS containing D-[^{14}C] glucose at the required concentration was added to 100 µl glucose-free red blood cells at about 50% haematocrit. After 5 min, influx of glucose was stopped by addition of 5 ml ice-cold stopping buffer. The cell suspension was immediately centrifuged at 3500 × *g* for 1 min. The supernatant was removed by aspiration, and the cells were washed by resuspension and recentrifugation in a further 5 ml stop solution. Sedimented cells were haemolyzed with 1 ml water and proteins were precipitated by addition of 0.1 ml (10%W/V) trichloroacetic acid (TCA) and sedimented by bench centrifugation. 0.6 ml of the supernatant was taken and the radioactivity was measured in a Packard scintillation counter model 1900 CA. The rate of glucose transport was calculated from the radioactivity incorporated into the cell extract and expressed as nmol glucose per min per ml packed cells. Statistical analysis of the data was done using Student's t-test.

Results

The effects of different concentrations of isatin on glucose influx into human red blood cells are presented in figure 1. There was 11% inhibition of glucose influx at

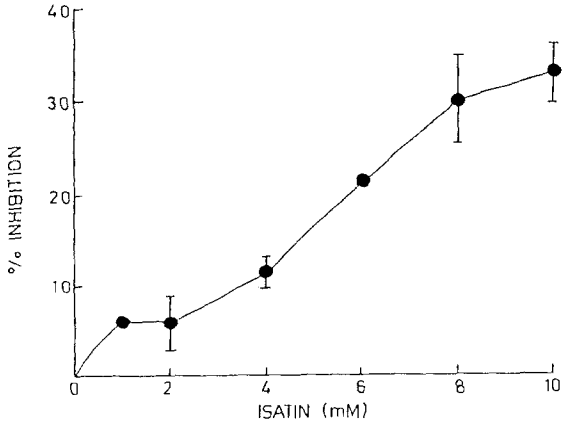

Figure 1. Effect of isatin on glucose uptake in human erythrocytes. 100 µl RBCs were incubated with C^{14}-D-glucose in 1.5 ml phosphate buffer saline pH 7.4 containing 0.0 to 10 mM isatin. Glucose influx was stopped after 5 min (n = 5). Bars are SD of the means.

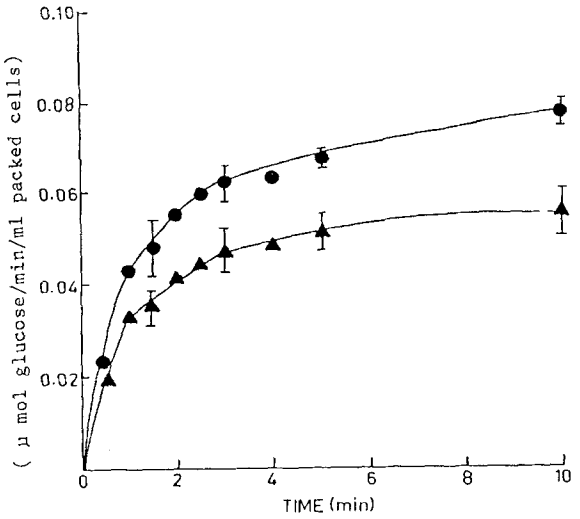

Figure 2. Time course of glucose uptake in human erythrocytes. Human erythrocytes were incubated with C^{14}-D-glucose in 1.5 ml phosphate buffer saline pH 7.4 in absence (●) or presence (▲) of 10 mM isatin (n = 5). Bars are SD of the means.

4 mM isatin, which was further increased at 10 mM isatin and was significantly ($p < 0.001$) different from the control. The observed inhibition of glucose uptake by isatin was time dependent. As shown in figure 2, after 30 s of incubation, glucose transport was reduced by 13% by 10 mM isatin. This inhibition reached 27% after 2 min of incubation. Further increase in incubation time did not influence the inhibitory activity of isatin towards glucose transport in RBCs.

Kinetics of glucose influx. Attempts were made to elucidate the kinetic mechanism responsible for the observed decrease in glucose uptake in human erythrocytes. Glucose transport across the erythrocyte membrane was studied at different sugar concentrations (2–10 mM) and the data were subjected to Lineweaver and Burke analysis to calculate the kinetic parameters. There was

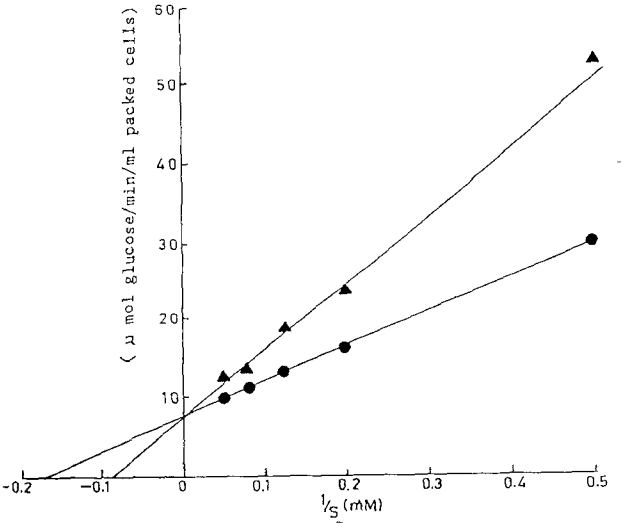

Figure 3. Double reciprocal plot for glucose uptake in human erythrocytes. Erythrocytes were incubated with different concentrations of C^{14}-D-glucose in absence (●) or presence (▲) of 10 mM isatin for 5 min (mean of two experiments).

essentially no change in maximum velocity (130 nmol/min/ml packed cells), but the affinity constant (K_t) for glucose influx was increased from 5.71 mM in the control to 11.11 mM in the presence of 10 mM isatin (fig. 3). The value of K_i was 4.12 mM under these conditions. Arrhenius analysis of glucose influx gave a biphasic plot (not shown) with a transition temperature (T_c) of 15 °C both in the presence and absence of isatin. The values of activation energy (Ea) in the presence of isatin above and below the T_c were 4.88 and 17.82 kcal/mol respectively. The corresponding values in the absence of isatin were 1.37 and 12.82 kcal/mol respectively (table 1).

Effect of various compounds on glucose influx. To examine whether the observed inhibition of glucose transport in erythrocytes by isatin results from its interaction with surface –SH groups, the effect of various sulfhydryl group-reacting agents (iodoacetate, iodoacetamide, DTNB, DNP and sodium arsenite) on glucose influx in the absence and presence of isatin was studied. The effect of harmaline on sugar influx was also investigated, since harmaline is known to impair the Na^+, K^+-ATP_{ase} activity of brain cells as well as in human erythrocytes, by interacting with Na^+-binding sites of the enzyme molecule[8]. Two sets of experiments were performed: a) Red cells were preincubated with various reagents and then washed with PBS, and glucose transport was determined in the presence and absence of isatin; b) The effect of various reagents on glucose transport was determined separately and together with isatin. As shown in table 2, addition of various –SH group-reacting agents to the incubation medium reduced glucose transport by 9–31% compared to controls. Inhibition of sugar uptake by DNP and harmaline

Table 1. Effect of isatin on kinetic parmeters of glucose uptake in human erythrocytes.

Inhibitor	r	V_{max} (nmol/min/ml packed cells)	K_t (mM)	K_i (mM)	Ea_1 (kcal/mol)	Ea_2	T_c
Control	0.997	130 ±10	5.31 ±0.53	-	1.37	12.82	15 °C
Isatin (10 mM)	0.995	130 ±10	11.11 ±2.34	4.12	4.88	17.10	15 °C

Values are mean ± SD of 4 observations.
Ea_1 and Ea_2 are the activation energies above and below transition temperature (T_c).
r = correlation coefficient.

Table 2. Effect of various reagents on glucose uptake by human erythrocytes in presence and absence of isatin.

Reagents added	Glucose uptake (nmol/min/ml packed cells)	
	Control	Isatin (10 mM)
Nil	80 ± 5	55 ± 6*
Iodoacetate 4 mM	65 ± 3	42 ± 3*
Iodoacetamide 4 mM	62 ± 3	41 ± 5*
DTNB 2 mM	61 ± 4	39 ± 6*
DNP 2 mM	63 ± 4	42 ± 2*
Sodium arsenite 2 mM	56 ± 5	33 ± 4*
Harmaline 2 mM	71 ± 1	69 ± 4

Values are mean ± SD of 4 observations. *($p < 0.001$) compared to the respective controls.

Table 3. Effect of isatin on glucose uptake by human erythrocytes pretreated with various reagents.

Cells pretreated with	Glucose uptake (nmol/min/ml packed cells)	
	Control	Isatin (10 mM)
Nil	85 ± 4	62 ± 2*
Iodoacetate 4 mM	80 ± 3	62 ± 4*
Iodoacetamide 4 mM	78 ± 4	62 ± 5*
DTNB 2 mM	74 ± 2	54 ± 2*
DNP 2 mM	79 ± 5	49 ± 5*
Sodium arsenite 2 mM	77 ± 5	58 ± 2*
Harmaline 2 mM	68 ± 6	66 ± 4

Values are mean ± SD of 4 observations *($p < 0.001$) compared to the respective controls.

was 11% under these conditions. Addition of isatin together with various -SH-reacting reagents increased the inhibition of glucose uptake significantly ($p < 0.001$) to 47–59%. Similar results were obtained when the cells were preincubated with -SH group-reacting reagents and were washed, following which glucose influx was measured in the presence of 10 mM isatin (table 3).

Discussion

The results described here indicate that isatin depresses the facilitated glucose transport in human erythrocytes. These observations are in contrast to the findings with rat intestine, where Na^+-independent glucose uptake is unaffected by this drug[4]. Since the inhibition of sugar transport by isatin was observed to occur rapidly (30 s after addition of the drug), it probably interacts at the external location of the glucose transport carrier in RBCs.

Kinetic analysis revealed that the inhibition of glucose influx by isatin was competitive. The value of K_t was increased with no change in V_{max}. This effect of isatin is similar to that of tetrathionate on glucose influx in erythrocytes[9]. Krupka and Deves[10] showed that steroids inhibit glucose transport by binding to carrier sites which are exposed on both outer and inner surfaces of the cell membrane. The inhibition was competitive with respect to substrate and it was suggested that if the binding is exclusively intracellular, the inhibition should be non-competitive; if exclusively outside, then purely competitive[11]. However, when the inhibitor binds on both sides of the membrane, a mixture of competitive and non-competitive inhibition is seen. There is no structural similarity between isatin and glucose, so the competitive nature of isatin inhibition of glucose transport further indicates that the interaction of the drug is through certain critical residues at the outer membrane surface. It is also possible that the drug may bind to a site close to the transporter, and being bulky, may overlap with the carrier binding site, thus giving the apparent kinetics of fully competitive type[12].

Arrhenius analysis of glucose influx in the presence of isatin indicated an increase in activation energy of the transport system. This may suggest that the binding of isatin to the membrane induces conformational changes in the transport carrier, resulting in the modification of lipid-protein interactions. Although the underlying

mechanism of isatin effects on glucose transport in response to temperature is unknown, two possibilities may be considered: a) isatin binds to the membrane through –SH or $-NH_2$ groups involved in the function of the transport carrier, and b) the hydrophobic indole nucleus in the isatin molecule may cause it to bind to the lipid-protein matrix of the membrane and thus modify the transport activity.

The present findings also suggest that the site of isatin binding on erythrocyte membranes is distinct from that of iodoacetate, iodoacetamide, DTNB, DNP and sodium arsenite. Harmaline, a known inhibitor of Na^+-binding sites in glucose and amino acid transport systems in the intestine[13], produced only a mild suppression of the glucose influx in human erythrocytes. This indicates that the Na^+-independent transport system is unaffected by harmaline. Interestingly, addition of isatin together with harmaline did not inhibit glucose uptake in erythrocytes which suggests that harmaline and isatin bind to similar/identical sites in human erythrocytes. Once the membrane sites are blocked by harmaline binding, they become inaccessible to isatin interaction. Thus, harmaline and the various –SH-reacting reagents tested affect the inhibition of glucose influx by isatin in different ways. Harmaline and isatin have no structural similarity except that both are weak amines and contain ring structures in their molecules. Therefore the interaction of isatin with harmaline binding to RBCs membrane must be through steric hindrance.

In conclusion, these results indicate that isatin inhibits glucose uptake in human erythrocytes by modifying the affinity of the transport carrier for glucose without affecting V_{max}. The precise mechanism of inhibition of glucose uptake by isatin remains unknown, but its interactions with –SH and $-NH_2$ groups of membrane proteins essential for glucose transport may be responsible for the inhibitory action of the drug. The finding that inhibition of glucose influx by isatin is not mediated through membrane –SH groups accessible to known sulfhydryl-reacting agents makes isatin a useful compound to examine the differential role of various –SH groups in membrane functions.

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