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Direct Inhibition of the Hexose Transporter GLUT1 by Tyrosine Kinase Inhibitors[†]

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ABSTRACT: The facilitative hexose transporter GLUT1 is a multifunctional protein that transports hexoses and dehydroascorbic acid, the oxidized form of vitamin C, and interacts with several molecules structurally unrelated to the transported substrates. Here we analyzed in detail the interaction of GLUT1 with a group of tyrosine kinase inhibitors that include natural products of the family of flavones and isoflavones and synthetic compounds such as the tyrphostins. These compounds inhibited, in a dose-dependent manner, the transport of hexoses and dehydroascorbic acid in human myeloid HL-60 cells, in transfected Chinese hamster ovary cells overexpressing GLUT1, and in normal human erythrocytes, and blocked the glucose-displaceable binding of cytochalasin B to GLUT1 in erythrocyte ghosts. Kinetic analysis of transport data indicated that only tyrosine kinase inhibitors with specificity for ATP binding sites inhibited the transport activity of GLUT1 in a competitive manner. In contrast, those inhibitors that are competitive with tyrosine but not with ATP failed to inhibit hexose uptake or did so in a noncompetitive manner. These results, together with recent evidence demonstrating that GLUT1 is a nucleotide binding protein, support the concept that the inhibitory effect on transport is related to the direct interaction of the inhibitors with GLUT1. We conclude that predicted nucleotide-binding motifs present in GLUT1 are important for the interaction of the tyrosine kinase inhibitors with the transporter and may participate directly in the binding transport of substrates by GLUT1.

GLUT1 is a member of a family of membrane proteins (the facilitative hexose transporter family, GLUTs) that transports hexoses down a concentration gradient and is widely expressed in cells and tissues (1–11). The hexose transporters participate in the cellular accumulation of substrates other than glucose. GLUT2 is a low-affinity transporter of glucose that also transports fructose although with even lower affinity (5, 12). GLUT5, the most divergent isoform in terms of primary sequence, is unable to transport glucose and is instead an efficient transporter of fructose (13, 14). The hexose transporters are also efficient transporters of the oxidized form of vitamin C, dehydroascorbic acid (15, 16). They are the main pathway mediating the transport of dehydroascorbic acid in normal human neutrophils (15, 17), human myeloid leukemia cells (17–20), and melanoma cells (21).

The isoflavone genistein (4',5,7-trihydroxyisoflavone) is a dietary-derived natural product present in a variety of plant

foods that potentially inhibits tyrosine kinases (22), protein-histidine kinase (23), and DNA topoisomerase II (24). Genistein inhibits the transport of hexoses and dehydroascorbic acid mediated by GLUT1 by directly interacting with the transporter (25). The isoflavone daidzein (4',7-dihydroxyisoflavone) failed to inhibit the activity of GLUT1 in a manner that suggested it was unable to directly interact with the transporter (25). Daidzein is also inactive as a tyrosine kinase inhibitor (22), suggesting that this property may relate importantly to the interaction of genistein with the glucose transporters. We tested this concept by performing a detailed analysis of the effect of flavonoids and well-characterized synthetic tyrosine kinase inhibitors, the tyrphostins, on the functional activity of GLUT1. We show here that the flavonoids and a subgroup of tyrosine kinase inhibitors potentially inhibit the functional activity of the glucose transporters in human myeloid HL-60 cells, Chinese hamster ovary (CHO)¹ cells overexpressing the glucose transporter GLUT1, and human erythrocytes. The characteristics and the specificity of the inhibition and the results of studies showing that the tyrosine kinase inhibitors affect the glucose-displaceable binding of cytochalasin B to GLUT1 in human erythrocytes, indicate that the inhibitory effect on transport is related to the direct interaction of the inhibitors with GLUT1. Only the tyrosine kinase inhibitors that compete with ATP binding to the tyrosine kinases inhibited transport through GLUT1 in a competitive manner, indicating

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¹ Abbreviations: CHO, Chinese hamster ovary; deoxyglucose, 2-deoxy-D-glucose; methylglucose, 3-O-methyl-D-glucose.

that predicted nucleotide binding sites present in GLUT1 may be important for the interaction of the tyrosine kinase inhibitors with the transporter. These results emphasize the ability of GLUT1 to interact with molecules structurally unrelated to glucose and have important implications for our understanding of the effects of protein tyrosine kinase inhibitors on cellular physiology in normal and malignant cells.

EXPERIMENTAL PROCEDURES

HL-60 cells were cultured in Iscove-modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. Cell viability was greater than 95%, as determined by trypan blue exclusion. CHO cells were cultured in IMDM supplemented with 10% fetal bovine serum and 0.25 mg/mL G418. We used CHO cells overexpressing the glucose transporter GLUT1 and CHO cells expressing the human placental insulin receptor (25). Human erythrocytes were purified from outdated blood samples obtained at the Hematology Service of the Hospital Regional in Valdivia, Chile. The flavonoids rhamnetin and isorhamnetin were purified from *Haplopappus baylahuen* leaves collected in Cordillera de Los Andes, near San Fernando, VII Region, Chile. The *H. baylahuen* leaves were extracted with chloroform, and the extract was chromatographed on a Silica Gel column followed by rechromatography on polyclar (polyamide) columns. The identity and purity of the isolated flavonoids was verified by UV, ^1H NMR, and mass spectroscopy (26). Other flavonoids were obtained from Sigma, while the tyrphostins and lavendustins were from Calbiochem.

For cytochalasin B binding, unsealed erythrocyte ghosts were prepared from washed red cells by hypotonic lysis (27). D-Glucose inhibitable binding of cytochalasin B to functional glucose transporters was calculated from the difference between cytochalasin B bound in the presence of 500 mM L-glucose and of 500 mM D-glucose. The incubation mixture (0.38 mg of erythrocyte membrane protein/mL equivalent to about 6×10^8 cells, 10 μM cytochalasin B, 500 mM D- or L-glucose, 0.07 μCi [4- ^3H]cytochalasin B (11.9 Ci/mmol, NEN-DuPont), and cold cytochalasin B for a final concentration of 0.01 μM) was incubated at room temperature for 10 min before collecting the membranes by centrifugation at 15000g for 10 min. Bound cytochalasin B was calculated from the amount of radioactive ligand associated with the membrane pellet and from the difference in the amount of soluble radioactivity before and after centrifugation (25).

For uptake assays, cells were incubated at room temperature in incubation buffer (19) containing 0.1–1 μCi L-[14C]-ascorbic acid (specific activity 4.7–6.7 mCi/mmol, NEN-DuPont), 2-[1,2- ^3H (N)]-deoxy-D-glucose (26.2 Ci/mmol, NEN-DuPont), [3H]-methylglucose (86.7 Ci/mmol, NEN-DuPont), or L-[3,4,5- ^3H (N)]-leucine (168 μCi /mmol, NEN-DuPont), and the respective unlabeled compounds at concentrations from 0.2 to 20 mM. The incubation times were varied from 10 s to 1 min as indicated in the respective figure legends. For dehydroascorbic acid uptake, 0.1–10 units of ascorbate oxidase (Sigma) were added to the incubation mix and incubated for 2 min prior to the uptake (19, 25).

GLUT1 is the main glucose transporter expressed by the HL-60 cells, and its functional characteristics are similar to those described for GLUT1 present in other cell types,

including the ability to transport hexoses and dehydroascorbic acid and its inhibition by cytochalasin B (19). We used 30-s uptake assays to determine the effect of the flavones on transport as distinct from their possible effects on accumulation. The initial rate of transport of dehydroascorbic acid, deoxyglucose, methylglucose, and leucine by the HL-60 cells is linear for the first 60 s of uptake, validating the assay for the determination of the kinetic constants of transport (19). We measured the transport of leucine, which is transported into cells by transport systems functionally unrelated to the glucose transporters (28, 29), to control for the specificity of the effect on the activity of the glucose transporters, as opposed to possible nonspecific effects on membrane transport. We demonstrated that the full effect of the isoflavone genistein on transport developed instantaneously without preincubation (25); therefore, the different test compounds were added at the beginning of the uptake assays simultaneously with the transported substrates from concentrated stock solutions freshly prepared (50 μM in dimethyl sulfoxide). Following uptake, cells were washed in cold phosphate-buffered saline that was free of Ca^{2+} and Mg^{2+} and lysed in 10 mM Tris-HCl (pH 8.0) containing 0.2% sodium dodecyl sulfate. The incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

Effect of Flavones that Compete for ATP Binding. The flavones quercetin and myricetin (22, 30, 31) inhibit the activity of tyrosine kinases by direct competition with the binding of ATP. Several flavones present in food are commercially available, but there is no information regarding their ability to act as tyrosine kinase inhibitors. Competition experiments revealed that the flavones quercetin, myricetin, morin, rhamnetin, and isorhamnetin inhibited the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in HL-60 cells in a dose-dependent manner (Figure 1, panels A–C). The order of potency of the flavones was isorhamnetin \geq rhamnetin $>$ quercetin \geq myricetin \geq morin, and the concentration of flavone that caused 50% transport inhibition ranged from as low as 10 μM for isorhamnetin to a maximum of 200 μM for morin (Figure 1, panels A–C). At higher concentrations, the flavones completely inhibited transport of deoxyglucose, with greater than 90% inhibition observed at concentrations that ranged from 30 μM for isorhamnetin to 500 μM for morin. On the other hand, less than 20% inhibition of the transport of leucine was observed at flavones concentrations as high as 500 μM (Figure 1, panel D), indicating that the inhibitory effect of the flavone was specific for the glucose transporters. Kinetic analyses of the effect of different concentrations of flavones on the substrate dependence of deoxyglucose transport indicated that the inhibitory effect of isorhamnetin, rhamnetin, quercetin, myricetin, and morin were of the competitive type (Figure 1, panels E–H), with a K_i in the range of 5–100 μM (Figure 2).

To test whether the effect of the flavones on transport is cell independent, we analyzed their effect in GLUT1-transfected CHO cells showing increased GLUT1 expression (25). The flavones caused a dose-dependent inhibition of deoxyglucose and dehydroascorbic acid uptake in the GLUT1-expressing as well as the control cells (Figure 3, panels A–F). The order of potency was isorhamnetin \geq rhamnetin $=$ quercetin $=$ myricetin $>$ morin. The concentra-

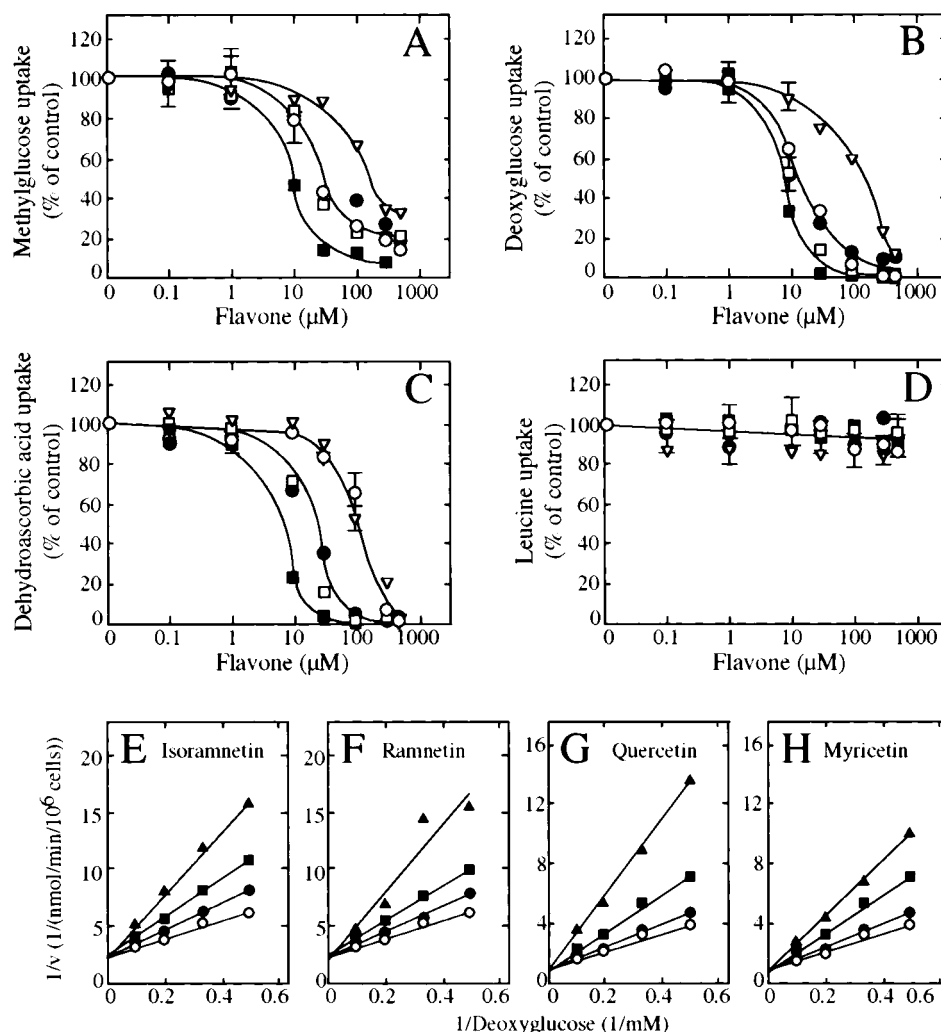


FIGURE 1: Effect of flavones on the transport of hexoses and dehydroascorbic acid in HL-60 cells. Panels A–D: Dose response of the effect of flavones on the transport of hexoses, dehydroascorbic acid, and leucine in HL-60 cells. Transport of methylglucose (A), deoxyglucose (B), dehydroascorbic acid (C), and leucine (D) was measured using a 30-s uptake assay in the presence of the indicated concentrations of myricetin (○), quercetin (●), morin (▽), ramnetin (□), or isoramnetin (■). Data are presented as percent of controls (samples without inhibitor) and represent the mean \pm standard deviation of four samples. Panels E–H: Lineweaver–Burk double-reciprocal plots of the effect of flavones on the substrate dependence for deoxyglucose transport. (E) Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 5 (●), 20 (■), and 30 μ M (▲) isoramnetin. (F) Transport of deoxyglucose in the absence (○) or in the presence of 3 (●), 10 (■), and 20 μ M (▲) ramnetin. (G) Transport of deoxyglucose in the absence (○) or in the presence of 5 (●), 20 (■), and 40 μ M (▲) quercetin. (H) Transport of deoxyglucose in the absence (○) or in the presence of 5 (●), 20 (■), and 30 μ M (▲) myricetin. Data represent the mean \pm standard deviation of one of two to three independent experiments performed in quadruplicate.

tion of flavone that caused 50% transport inhibition ranged from 8 μ M for isoramnetin to a maximum of 80 μ M for the inhibition of deoxyglucose uptake by morin (Figure 1, panels A–C). High concentrations of flavones completely inhibited transport, with greater than 90% inhibition observed at concentrations that ranged from 50 μ M for the inhibition of dehydroascorbic acid uptake by isoramnetin to 500 μ M for the inhibition of deoxyglucose uptake by morin (Figure 3, panels A–F).

GLUT1 is especially abundant in human erythrocytes, and most of the information on the structure and function of GLUT1 has been obtained using the erythrocyte transporter (1, 2). Confirming the data obtained in the HL-60 and CHO cell lines, the flavones myricetin, quercetin, morin, ramnetin, and isoramnetin inhibited the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in human erythrocytes in a dose-dependent manner (Figure 4, panels A–C). The erythrocytes were very sensitive to the inhibitory effect

of the flavones, with the result that 50% inhibition was observed at concentrations of flavones 1 order of magnitude lower than those effective in the HL-60 and CHO cells. Thus, 50% inhibition was observed at concentrations ranging from 1 μ M for the inhibition of methylglucose transport by myricetin to 30 μ M for the inhibition of methylglucose transport by morin. The order of potency of the flavones varied depending on the transported substrate. Myricetin was the most potent flavone for inhibiting the transport of both methylglucose and deoxyglucose, while isoramnetin was the most potent in inhibiting the transport of dehydroascorbic acid (Figure 4, panels A–C). Similar to the effect on the HL-60 and the CHO cells, morin was the weakest inhibitor of the transport of the three substrates; it was unable to inhibit the transport of methylglucose in erythrocytes by more than 60% (Figure 4, panel A). These results show that the effects of the flavones on the activity of GLUT1 are similar in the different cells tested.

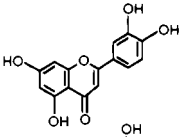
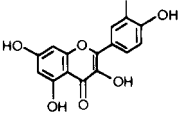
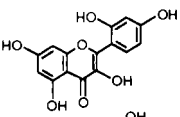
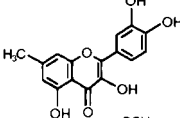
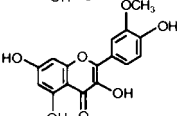
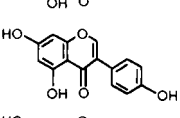
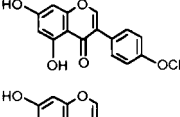
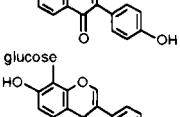
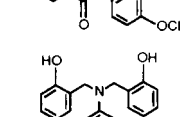
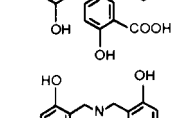
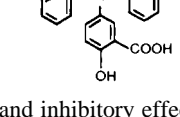
		Inhibitory effect
		Type (K_i, μM)
Myricetin		Competitive (23)
Quercetin		Competitive (8)
Morin		Competitive (105)
Ramnetin		Competitive (20)
Isoramnetin		Competitive (5)
Genistein		Competitive (15)
Biochanin A		Competitive (17)
Daidzein		No
Puerarin		No
Lavendustin A		Competitive (10)
Lavendustin B		Competitive (15)

FIGURE 2: Structure and inhibitory effect on the transport activity of GLUT1 of naturally occurring tyrosine kinase inhibitors with specificity for ATP binding sites. The K_i values and the characteristics of the inhibition were obtained from competition experiments measuring the effect of the inhibitors on the transport of deoxyglucose by HL-60 cells as described in Figures 1, 4, and 5. Data represent the average of two to three independent determinations performed in quadruplicate.

We next studied the effect of the flavones on the binding of cytochalasin B to GLUT1 present in purified human erythrocyte ghosts. Cytochalasin B binds to GLUT1 in a D-glucose displaceable manner, and the displacement assay is generally used to confirm the interaction with GLUT1 of compounds that compete for glucose transport (1). Increasing concentrations of myricetin, quercetin, morin, ramnetin, and isoramnetin efficiently competed for the glucose-sensitive cytochalasin B binding sites present in the erythrocyte membranes in a dose-dependent manner (Figure 4, panel D). The order of potency was isoramnetin = ramnetin =

myricetin = quercetin > morin, which in general parallels the order of potency for the inhibition of transport. Fifty percent inhibition of binding was observed at concentrations ranging from approximately 10 μM for isoramnetin, ramnetin, myricetin, and quercetin, to 100 μM for morin. Greater than 90% inhibition of binding was observed at 20–50 μM for isoramnetin, ramnetin, myricetin, and quercetin, while morin at 500 μM failed to inhibit the binding of cytochalasin B by more than 80%. These observations are consistent with the concept that the inhibitory flavones interact directly with the glucose transporter present in human erythrocytes.

Effect of Isoflavones that Compete for ATP Binding. The isoflavones genistein and biochanin A inhibit the activity of tyrosine kinases by direct competition with the binding of ATP (22). We determined the dose dependence of the effect of genistein (25), biochanin A, daidzein, and puerarin on GLUT1-mediated transport in HL-60 cells. Biochanin A was a potent inhibitor of deoxyglucose and dehydroascorbic acid transport, with 50% inhibition observed at 30 μM biochanin A and greater than 90% inhibition at concentrations of 200 μM (Figure 5, panels B and C). Although genistein is a good inhibitor of methylglucose transport, biochanin A failed to completely block the transport of methylglucose in HL-60 cells; maximal inhibition of transport of approximately 40% was observed at 100 μM biochanin A, with no further inhibitory effect observed at concentrations from 100 to 500 μM (Figure 5, panel A). The isoflavone puerarin (7-hydroxy-3-[4-hydroxyphenyl]-1-benzopyran-4-one 8- β -D-glucopyranoside) did not affect the transport of methylglucose, deoxyglucose, or dehydroascorbic acid in HL-60 cells, with less than 10% inhibition observed at 500 μM (Figure 5, panels A–C). None of the isoflavones inhibited the transport of leucine in HL-60 cells, even at concentrations as high as 500 μM (Figure 5, panel D). Kinetic analysis of the effect of different concentrations of biochanin A on the substrate dependence of deoxyglucose transport indicated that biochanin A inhibited in a competitive manner the transport of deoxyglucose in HL-60 cells (Figure 5, panel E), with a K_i of 5 μM (Figure 2). Biochanin A also inhibited, in a dose-dependent manner, the uptake of deoxyglucose and dehydroascorbic acid in GLUT1-expressing as well as in control CHO cells (Figure 5, panels F and G). In both cell lines, 50% inhibition of deoxyglucose and dehydroascorbic acid uptake was observed at 15 and 30 μM biochanin A, respectively, and greater than 80% inhibition was observed at 300–500 μM biochanin A. Confirming the data in HL-60 cells, puerarin failed to inhibit the uptake of deoxyglucose and dehydroascorbic acid in the CHO cells; less than 20% inhibition was observed at 500 μM puerarin (Figure 5, panels F and G).

Biochanin A inhibited in a dose-dependent manner the uptake of deoxyglucose and dehydroascorbic acid in human erythrocytes; 50% inhibition of transport of deoxyglucose and dehydroascorbic acid was observed at 30 and 100 μM biochanin A, respectively, and greater than 70% inhibition of transport of both substrates was observed at 300 μM biochanin A (Figure 5, panels I and J). On the other hand, and similar to the results in HL-60 cells, biochanin A was a weak inhibitor of methylglucose transport, and at 500 μM inhibited the transport of methylglucose by the erythrocytes by less than 40% (Figure 5, panel H). Also confirming the results obtained with the HL-60 and the CHO cells, puerarin

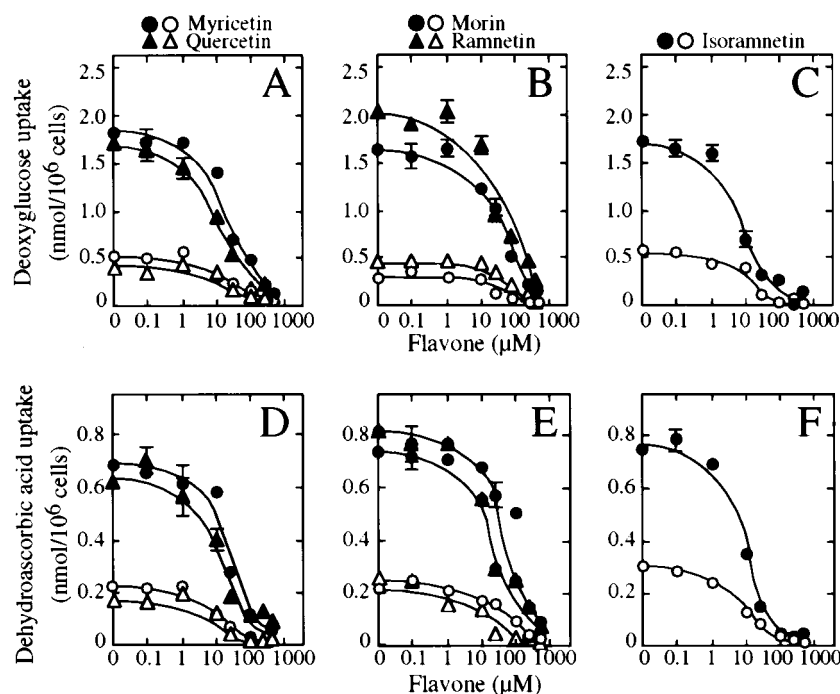


FIGURE 3: Effect of flavones on the uptake of deoxyglucose and dehydroascorbic acid in CHO cells overexpressing GLUT1. Dose dependence of the effect of (A, D) myricetin and quercetin, (B, E) morin and ramnetin, and (C, F) isoramnetin, on the uptake of deoxyglucose (A–C) and dehydroascorbic acid (D–F). Data represent the mean \pm standard deviation of one of two independent experiments performed in quadruplicate. Solid symbols (●, ▲) indicate transfected CHO cells overexpressing GLUT1. Open symbols (○, △) indicate control CHO cells.

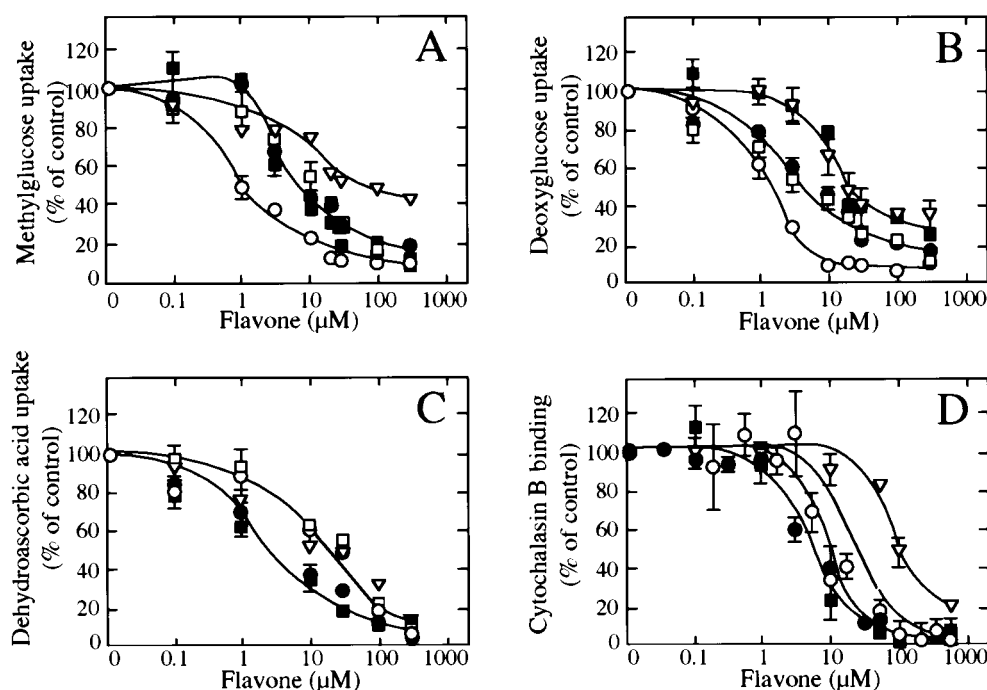


FIGURE 4: Dose dependence of the effect of flavones on the uptake of hexoses and dehydroascorbic acid and the binding of cytochalasin B in human erythrocytes. Panels A–C: transport of methylglucose (A), deoxyglucose (B), and dehydroascorbic acid (C) was measured using a 30-s uptake assay in the presence of the indicated concentrations of myricetin (○), quercetin (●), morin (▽), ramnetin (□), or isoramnetin (■). Data are presented as percent of control (samples without inhibitor) and represent the mean \pm standard deviation of four samples. (D) Dose dependence of the effect of myricetin (○), quercetin (●), morin (▽), ramnetin (□), and isoramnetin (■) on the binding of cytochalasin B to human erythrocyte membranes. Data represent the mean \pm standard deviation of one of two independent experiments performed in quadruplicate.

at 500 μ M inhibited by less than 10% the uptake of methylglucose, deoxyglucose, and dehydroascorbic acid in human erythrocytes (Figure 5, panels H and J).

Increasing concentrations of biochanin A efficiently competed for the glucose-sensitive cytochalasin B binding

sites present in human erythrocyte membranes (Figure 5, panel K). At 15 μ M, biochanin A inhibited cytochalasin B binding by 50%, with a maximal inhibition of 80% observed at 30 μ M biochanin A. Puerarin at 300 μ M failed to inhibit the binding of cytochalasin B to the erythrocyte membranes,

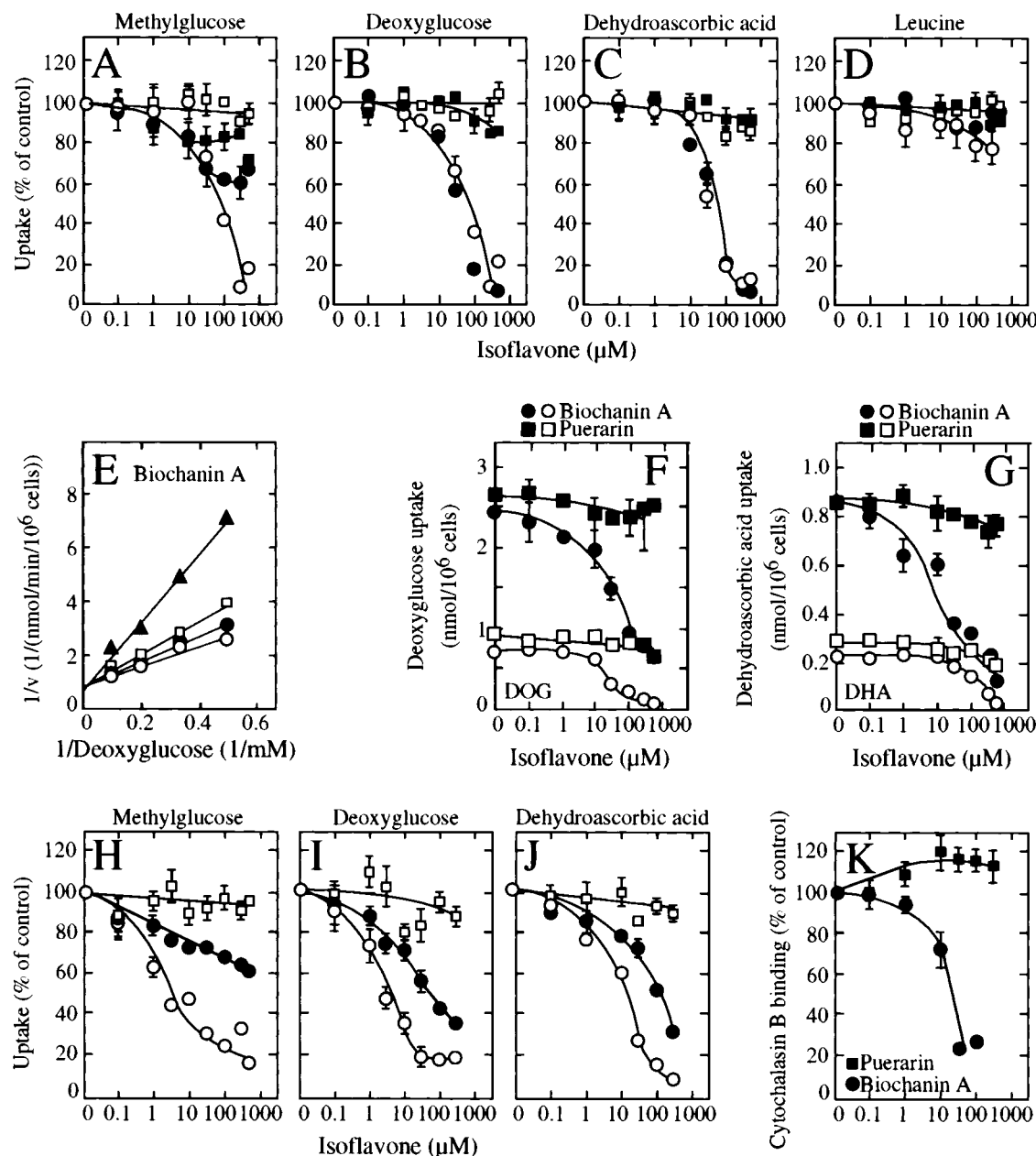


FIGURE 5: Effect of isoflavones on the activity of glucose transporters in HL-60 cells, GLUT1-expressing CHO cells, and human erythrocytes. Panels A–D: Dose response of the effect of isoflavones on the transport of methylglucose (A), deoxyglucose (B), dehydroascorbic acid (C), and leucine (D) in HL-60 cells. Transport was measured using a 30-s uptake assay in the presence of the indicated concentrations of genistein (○), biochanin A (●), daidzein (□), or puerarin (■). Data are presented as percent of control (samples without inhibitor) and represent the mean \pm standard deviation of four samples. (E) Lineweaver–Burk double-reciprocal plot of the effect of biochanin A on the substrate dependence for deoxyglucose transport in HL-60 cells. Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 10 (●), 20 (■), and 40 μ M (▲) biochanin A. Data represent the mean \pm standard deviation of four samples. Panels F–G: Dose dependence of the effect of biochanin A and puerarin on the uptake of deoxyglucose (F) and dehydroascorbic acid (G) in CHO cells overexpressing GLUT1 (●, ■) and in control CHO cells (○, □). Data represent the mean \pm standard deviation of four samples. Panels H–J: Dose response of the effect of isoflavones on the transport of (H) methylglucose, (I) deoxyglucose, and (J) dehydroascorbic acid in human erythrocytes. Transport was measured in the presence of the indicated concentrations of genistein (○), biochanin A (●), or puerarin (■). Data are presented as percent of control (samples without inhibitor) and represent the mean \pm standard deviation of four samples. (K) Dose-dependence of the effect of biochanin A (●) and puerarin (■) on the binding of cytochalasin B to human erythrocyte membranes. Data represent the mean \pm standard deviation of one of two independent experiments performed in quadruplicate.

which confirms the transport data and speaks of the specificity of the effect of biochanin A. These observations are consistent with the concept that biochanin A inhibits the transport of methylglucose, deoxyglucose, and dehydroascorbic acid by directly interacting with GLUT1.

Effect of Lavendustins that Compete for ATP Binding. The lavendustins are natural compounds isolated from *Streptomyces* and are potent tyrosine kinase inhibitors. Lavendustin

A is a competitive inhibitor with respect to ATP and a noncompetitive inhibitor with respect to a peptide substrate (32). Lavendustins A and B inhibited the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in HL-60 cells with a similar dose dependence (Figure 6, panels A–C). Fifty percent inhibition was observed at less than 15 μ M, and greater than 90% inhibition of transport was observed at lavendustin concentrations of about 100 μ M. The

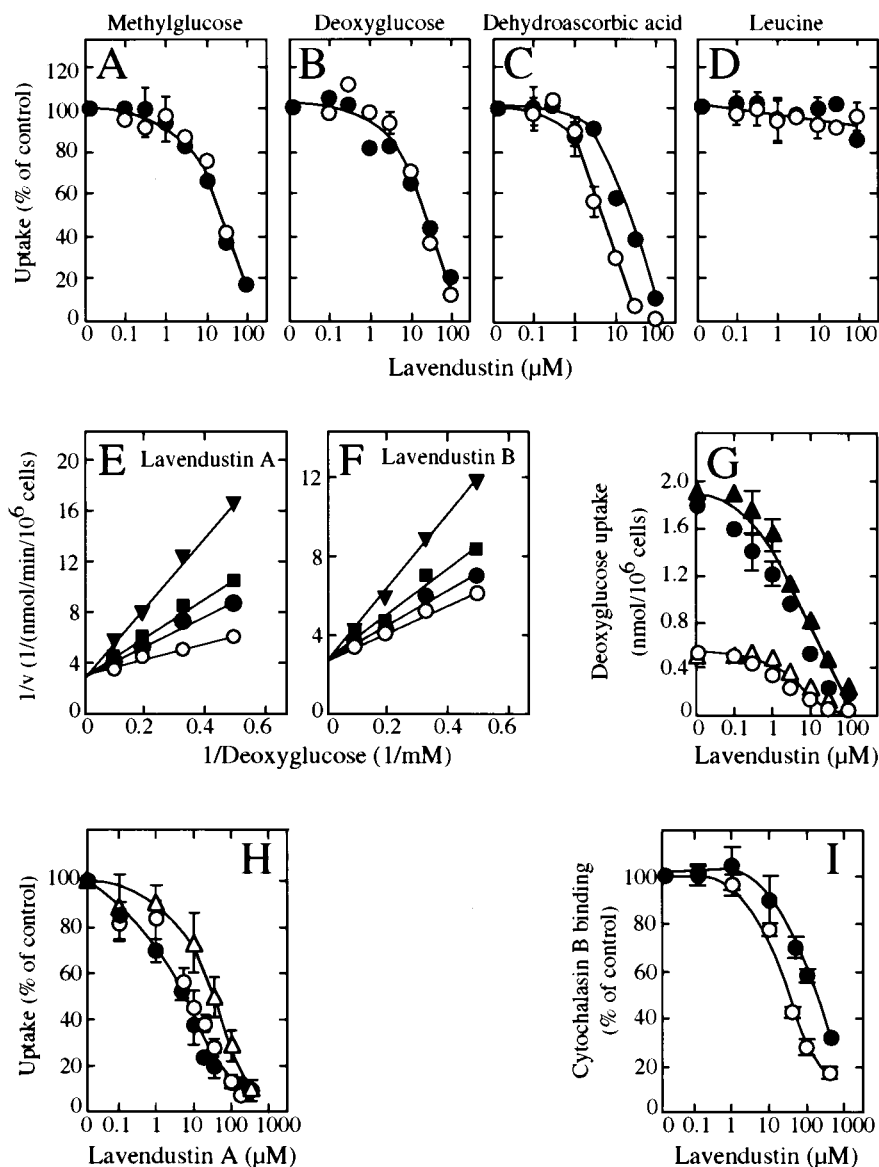


FIGURE 6: Effect of lavendustin A and B on the activity of glucose transporters in HL-60 cells, GLUT1-expressing CHO cells, and human erythrocytes. Panels A–D: Dose response of the effect of lavendustins A (○) and B (●) on the transport of (A) methylglucose, (B) deoxyglucose, (C) dehydroascorbic acid, and (D) leucine in HL-60 cells. Transport was measured in the presence of the indicated concentrations of the lavendustins. Data are presented as percent of control (samples without inhibitor) and represent the mean \pm standard deviation of four samples. Panels E–F: Lineweaver–Burk double-reciprocal plots of the effect of the lavendustins A and B on the substrate dependence for deoxyglucose transport in HL-60 cells. Data represent the mean of four samples. (E) Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 5 (●), 10 (■), and 30 μ M (▲) lavendustin A. (F) Transport of deoxyglucose in the absence (○) or in the presence of 3 (●), 10 (■), and 20 μ M (▲) lavendustin B. (G) Dose dependence of the effect of the lavendustins A (●, ○) and B (▲, △) on the uptake of deoxyglucose in CHO cells overexpressing GLUT1 (●, ▲) and in control CHO cells (○, △). Data represent the mean \pm standard deviation of four samples. (H) Dose response of the effect of lavendustin A (●, ○) and B (▲, △) on the transport of methylglucose (●, ○), deoxyglucose (○, △), and dehydroascorbic acid (▲, △) in human erythrocytes. Data represent the mean \pm standard deviation of one of two independent experiments performed in quadruplicate. (I) Dose-dependence of the effect of lavendustins A (○) and B (●) on the binding of cytochalasin B to human erythrocyte membranes.

lavendustins had no effect on the transport of leucine by the HL-60 cells, with less than 15% inhibition observed at 100 μ M lavendustin A or B (Figure 6, panel D). Kinetic analysis of the effect of different concentrations of the lavendustins on the substrate dependence of deoxyglucose transport by the HL-60 cells indicated that they inhibited in a competitive manner the transport of deoxyglucose (Figure 6, panels E and F), with similar K_i values in the range of 10–15 μ M (Figure 2). Both compounds also caused a dose-dependent inhibition of the uptake of deoxyglucose and dehydroascorbic acid in GLUT1-expressing and in control CHO cells (Figure 6, panel G, and data not shown). Fifty percent inhibition was

observed at approximately 5–10 μ M, and complete inhibition was observed at concentrations of 100 μ M. Further studies revealed that the lavendustins A and B inhibited the uptake of methylglucose, deoxyglucose, and dehydroascorbic acid in human erythrocytes in a dose-dependent manner, with 50% inhibition observed at approximately 10–30 μ M (Figure 6, panel H). Moreover, increasing concentrations of lavendustin A and B inhibited, in a dose-dependent manner, the binding of cytochalasin B to human erythrocyte membranes (Figure 6, panel I). Fifty percent inhibition was observed at approximately 20 μ M, and greater than 80% inhibition of binding was observed at 300 μ M. The data are therefore

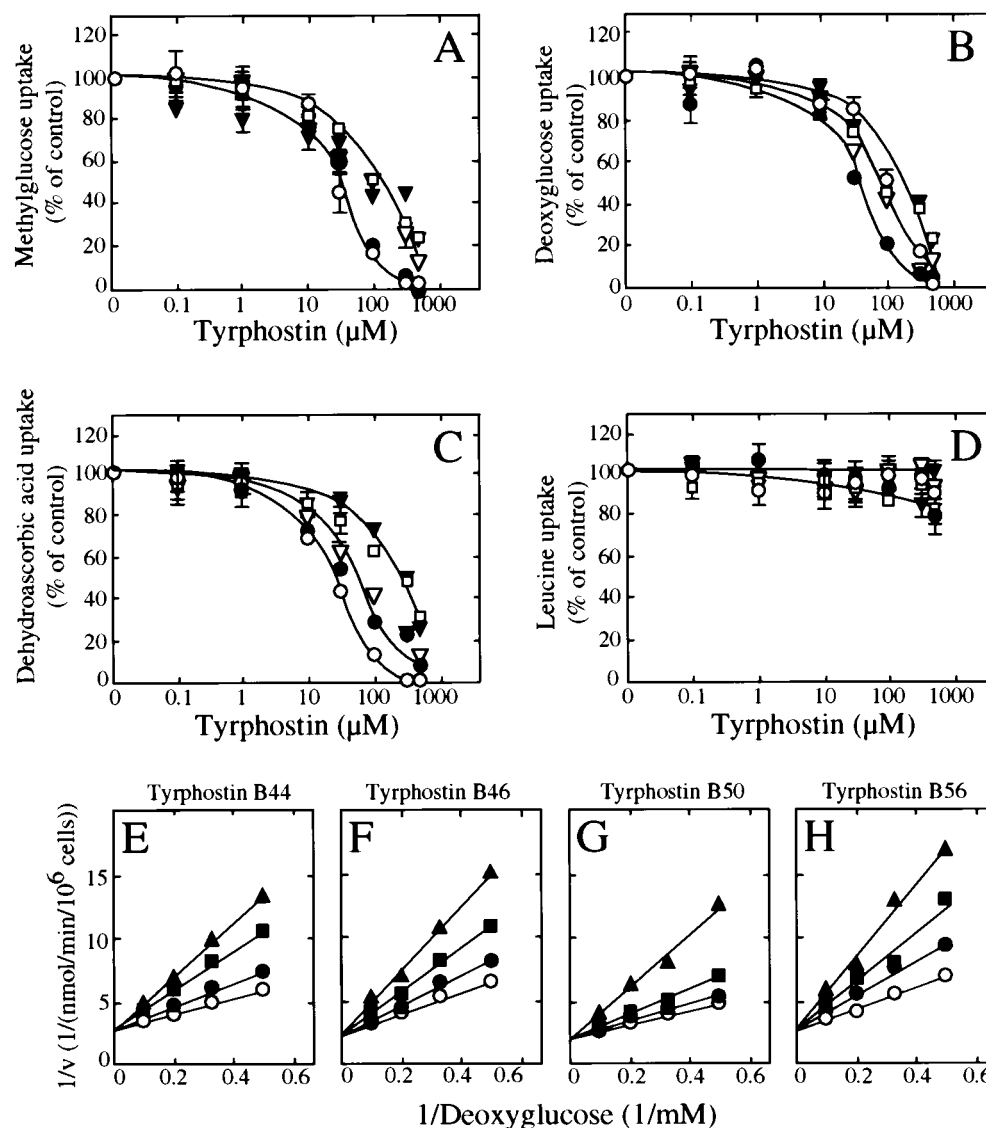


FIGURE 7: Effect of aromatic α -amido tyrphostins and tyrphostin AG879 on the transport of hexoses and dehydroascorbic acid in HL-60 cells. Panels A–D, dose response of the effect of tyrphostins on the transport of hexoses, dehydroascorbic acid, and leucine in HL-60 cells. Transport of (A) methylglucose, (B) deoxyglucose, (C) dehydroascorbic acid, and (D) leucine was measured in the presence of the indicated concentrations of the tyrphostins B44 (○), B46 (●), B50 (▽), B56 (▼), or AG879 (□). Data are presented as percent of control (samples without inhibitor) and represent the mean \pm standard deviation of one of two to three independent experiments performed in quadruplicate. Panels E–H: Lineweaver–Burk double-reciprocal plots of the effect of the different tyrphostins on the substrate dependence for deoxyglucose transport. Data represent the mean of four samples. (E) Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 30 (●), 100 (■), and 200 μM (▲) tyrphostin B44. (F) Transport of deoxyglucose in the absence (○) or in the presence of 30 (●), 50 (■), and 100 μM (▲) tyrphostin B46. (G) Transport of deoxyglucose in the absence (○) or in the presence of 30 (●), 100 (■), and 300 μM (▲) tyrphostin B50. (H) Transport of deoxyglucose in the absence (○) or in the presence of 30 (●), 100 (■), and 300 μM (▲) tyrphostin B56.

consistent with the lavendustins acting as competitive inhibitors of transport as a result of their direct interaction with GLUT1.

Effect of Tyrphostins that Compete for ATP Binding. The tyrphostins are synthetic, low molecular weight inhibitors of protein tyrosine kinases (31, 33, 34). We tested a group of tyrphostins (tyrphostins B44, B46, B48, B50, and B56) that correspond to substituted α -amido tyrphostins in which the substituent is an aromatic amide (34). In these tyrphostins, the amido aromatic ring at the α position is removed from the amide nitrogen by the gradual addition of methylene groups (34). The α -amido tyrphostins inhibit the activity of protein tyrosine kinases by competing with the binding of ATP (34, 35). We also analyzed the effect of the tyrphostin AG879, a potent inhibitor of the phosphorylating activity of

epidermal growth factor and HER2–Neu kinases (36). These tyrphostins inhibited, in a dose-dependent manner, the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in HL-60 cells (Figure 7, panels A–C). The order of potency was tyrphostin B44 \approx tyrphostin B46 \geq tyrphostin B50 \geq tyrphostin B56. Fifty percent inhibition was observed at concentrations that ranged from 40 μM for the inhibition of methylglucose and dehydroascorbic acid transport by the tyrphostin B44, to a maximum of 200 μM for the inhibition of dehydroascorbic acid by the tyrphostin B56 (Figure 7, panels A–C). Greater than 90% inhibition of transport was observed at concentrations that ranged from 100 μM for tyrphostin B44 to 300 μM for tyrphostin B56. No effect of the tyrphostins on the transport of leucine in HL-60 cells was observed in these studies; less than 20%

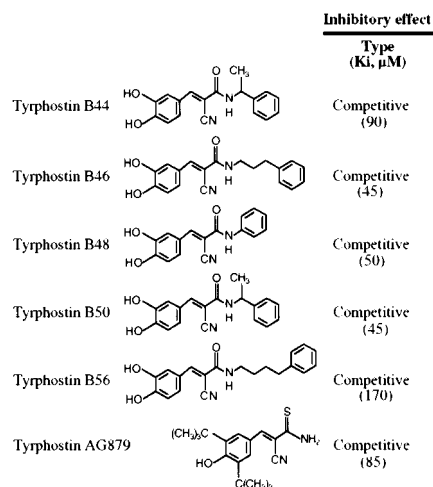


FIGURE 8: Structure and inhibitory effect on the transport activity of GLUT1 of synthetic tyrosine kinase inhibitors (tyrphostins) with specificity for ATP binding sites. The K_i values and the characteristics of the inhibition were obtained from competition experiments measuring the effect of the inhibitors on the transport of deoxyglucose by HL-60 cells as described in Figure 7. Data represent the average of two to three independent determinations performed in quadruplicate.

inhibition was observed with tyrphostins B46 or B44 at 500 μM , the tyrphostins that were the most potent inhibitors of the transport of methylglucose, deoxyglucose, and dehydroascorbic acid by HL-60 cells (Figure 7, panel D). Kinetic analysis of the effect of different concentrations of tyrphostins on the substrate dependence of deoxyglucose transport indicated that they inhibited in a competitive manner the transport of deoxyglucose in HL-60 cells (Figure 7, panels E–H, and data not shown), with K_i values in the range of 30–180 μM (Figure 8).

The tyrphostins B44, B46, B48, B50, B56, and AG879 also inhibited, in a dose-dependent manner, the uptake of deoxyglucose and dehydroascorbic acid in GLUT1-expressing as well as in control CHO cells (Figure 9, panels A and B, and data not shown). The order of potency followed approximately that observed in the HL-60 cells; fifty percent inhibition of uptake was observed at approximately 40–100 μM , and complete inhibition was observed at concentrations of 300 μM or higher. Increasing concentrations of the tyrphostins B44, B46, B48, B50, and B56 inhibited the binding of cytochalasin B to human erythrocyte membranes in a dose-dependent manner (Figure 9, panel C). The concentration necessary for inhibiting cytochalasin B binding by 50% depended on the particular tyrphostin tested and ranged from about 15 μM for tyrphostin B46 to about 100 μM for tyrphostin B44. Greater than 90% inhibition of binding was observed at 300 μM . On the other hand, tyrphostin AG879 showed only a minor effect on binding of cytochalasin B to erythrocyte membranes; at 500 μM it inhibited cytochalasin B binding by less than 20% (Figure 9, panel C). Overall, the data indicate that the aromatic α -amido tyrphostins inhibit the activity of GLUT1 by directly interacting with the transporter.

Effect of Tyrphostins that Compete with the Substrate (Tyrosine) Site. The tyrphostins A1, A23, A25, A46, A47, A51, and A63 are derived from the benzylidene malononitrile nucleus which contains structural elements of both the amino acid tyrosine and the natural protein tyrosine kinase inhibitor

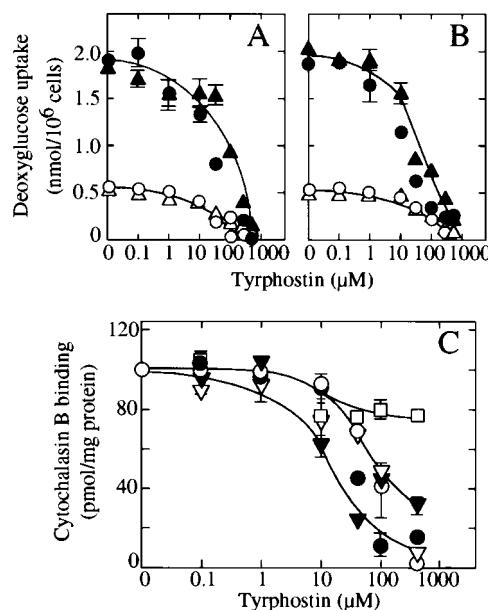


FIGURE 9: Effect of aromatic α -amido tyrphostins and tyrphostin AG879 on the uptake of deoxyglucose in GLUT1-expressing CHO cells and the binding of cytochalasin B in human erythrocytes. Panels A and B: Dose dependence of the effect of (A) tyrphostins B44 (\circ , \bullet) and B46 (Δ , \blacktriangle) and (B) tyrphostins B50 (\circ , \bullet) and B56 (Δ , \blacktriangle) on the uptake of deoxyglucose. Data represent the mean \pm standard deviation of four samples. Symbols: (\bullet , \blacktriangle) transfected CHO cells expressing the glucose transporter GLUT1; (\circ , Δ) control CHO cells. Panel C: dose-dependence of the effect of the tyrphostins B44 (\circ), B46 (\bullet), B50 (∇), B56 (\square), and AG879 (\square) on the binding of cytochalasin B to human erythrocyte membranes. Data represent the mean \pm standard deviation of one of two independent experiments performed in quadruplicate.

erbstatin (33). These compounds were synthesized to be tyrosine kinase inhibitors capable of interacting with the substrate binding site of the protein tyrosine kinases without influencing ATP binding. They are competitive inhibitors of the substrate (tyrosine) site of the epidermal growth factor receptor tyrosine kinase (33). From the 7 different tyrphostins belonging to this group, only tyrphostin A47 inhibited the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in HL-60 cells (Figure 10, panels A–C). Fifty percent inhibition was observed at approximately 120 μM for deoxyglucose and dehydroascorbic acid, while 50% inhibition of methylglucose transport was observed at 250 μM tyrphostin A47. Greater than 80% inhibition of transport was observed at concentrations of tyrphostin A47 of 500 μM or higher. Control experiments revealed no effect of tyrphostin A47 on the transport of leucine, with less than 10% inhibition at 1 mM tyrphostin (Figure 10, panel D). On the other hand, less than 30% inhibition of transport of methylglucose, deoxyglucose, or dehydroascorbic acid was observed when using the tyrphostins A1, A23, A25, A46, A51, or A63 at concentrations as high as 1 mM (Figure 10, panel A, and data not shown). Kinetic analysis of the effect of different concentrations of tyrphostin A47 on the substrate dependence of deoxyglucose transport indicated that it inhibited in a noncompetitive manner the transport of deoxyglucose in HL-60 cells (Figure 10, panel E), with a K_i of 160 μM (Figure 11).

Tyrphostin A47 inhibited, in a dose-dependent manner, the uptake of deoxyglucose and dehydroascorbic acid in GLUT1-expressing as well as in control CHO cells (data

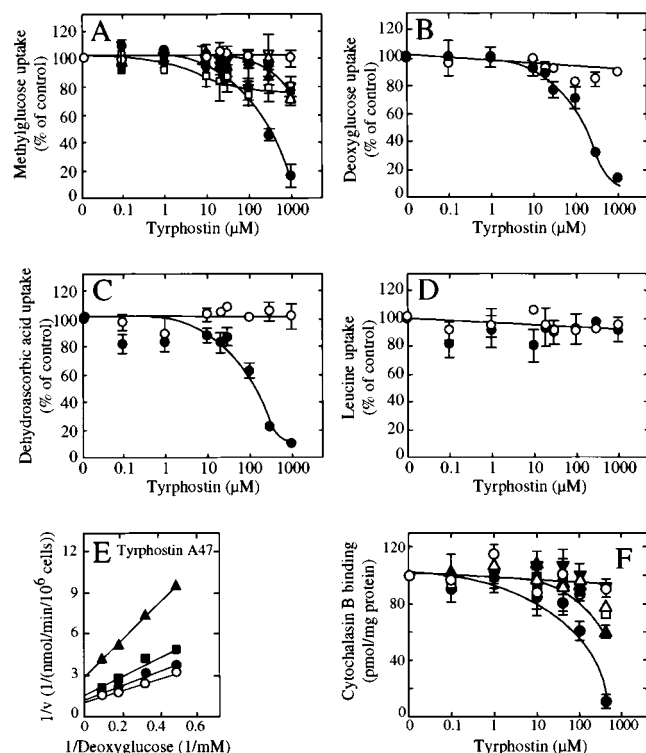


FIGURE 10: Effect of tyrphostins on the activity of glucose transporters in HL-60 cells, GLUT1-expressing CHO cells, and human erythrocytes. Panels A–D: dose response of the effect of tyrphostins on the transport of (A) methylglucose, (B) deoxyglucose, (C) dehydroascorbic acid, and (D) leucine in HL-60 cells. Transport was measured using a 30-s uptake assay in the presence of the indicated concentrations of the tyrphostins A1 (○), A23 (□), A25 (▼), A46 (▲), A47 (●), A51 (■), or A63 (△). Data are presented as percent of controls (samples without inhibitor) and represent the mean \pm standard deviation of four samples. (E) Lineweaver–Burk double-reciprocal plot of the effect of tyrphostin A47 on the substrate dependence for deoxyglucose transport in HL-60 cells. Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 100 (●), 200 (■), and 400 μ M tyrphostin A47. Data represent the mean of four samples. (F) Dose-dependence of the effect of the tyrphostins A1 (○), A23 (□), A25 (▼), A46 (▲), A47 (●), A51 (■), or A63 (△) on the binding of cytochalasin B to human erythrocyte membranes. Data represent the mean \pm standard deviation of one of two to three independent experiments performed in quadruplicate.

not shown). Fifty percent inhibition was observed at 200–300 μ M, and complete inhibition was observed at concentrations of 500 μ M or higher. Confirming the results obtained with the HL-60 cells, the tyrphostins A1, A23, A25, A46, A51, and A63 failed to affect transport of deoxyglucose or dehydroascorbic acid in the CHO cells (data not shown). Further experiments showed that increasing concentrations of tyrphostin A47 inhibited the binding of cytochalasin B to human erythrocyte membranes in a dose-dependent manner, with 50% inhibition observed at approximately 100 μ M, while greater than 90% inhibition of binding was observed at 400 μ M (Figure 10, panel F). On the other hand, the tyrphostins A1, A23, A25, A51, and A63 had only a minor effect on the binding of cytochalasin B to the erythrocyte membranes, with less than 20% inhibition observed when these tyrphostins were used at concentrations as high as 500 μ M. In addition to tyrphostin A47, only tyrphostin A46 had a noticeable effect on binding of cytochalasin B to the erythrocyte membranes, with a maximum of about 40% inhibition of binding observed at 500 μ M. Overall, these

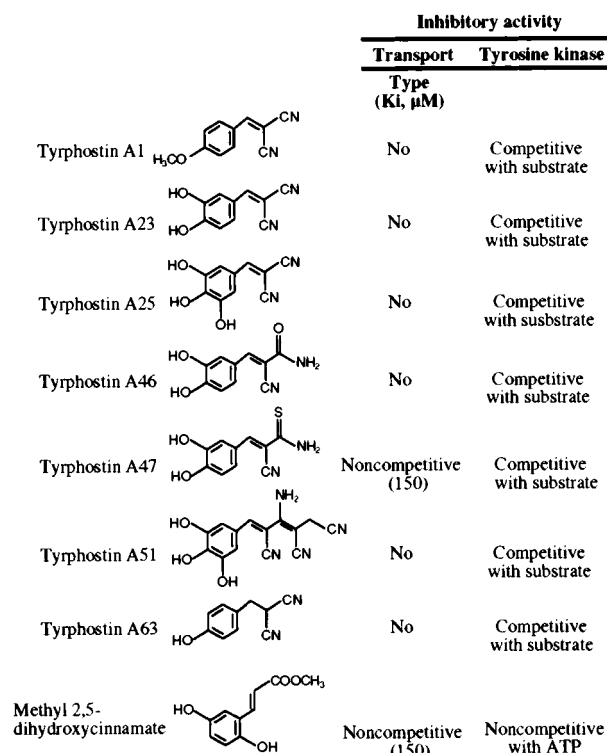


FIGURE 11: Structure and inhibitory effect on the transport activity of GLUT1 of synthetic tyrosine kinase inhibitors (tyrphostins and 2,5-dihydroxycinnamate) with specificity for the substrate (tyrosine) site. The K_i values and the characteristics of the inhibition were obtained from competition experiments measuring the effect of the inhibitors on the transport of deoxyglucose by HL-60 cells as described in Figures 10 and 11. Data represent the average of two to three independent determinations performed in quadruplicate.

observations indicate that, with the exception of tyrphostin A47 which inhibited transport in a noncompetitive manner, tyrosine kinase inhibitors that interact with the substrate site in the protein tyrosine kinases were unable to interact with GLUT1 and failed to inhibit its transport activity.

Effect of Methyl 2,5-dihydroxycinnamate, a Competitor of the Substrate Site. The compound erbstatin is a natural product that was isolated from *Actinomycetes* and is a potent inhibitor of the epidermal growth factor receptor-associated tyrosine kinase (37). Erbstatin is, however, an unstable compound that is rapidly inactivated in serum. Methyl 2,5-dihydroxycinnamate is a synthetic, stable erbstatin analogue that inhibits the activity of epidermal growth factor receptor in a manner competitive with the substrate and noncompetitive with ATP (38). Methyl 2,5-dihydroxycinnamate inhibited the transport of methylglucose, deoxyglucose, and dehydroascorbic acid in HL-60 cells with a similar dose dependence (Figure 12, panel A). Fifty percent inhibition of transport was observed at approximately 100 μ M methyl 2,5-dihydroxycinnamate, with greater than 90% inhibition observed at 300 μ M. Parallel studies revealed that methyl 2,5-dihydroxycinnamate had no effect on the transport of leucine by the HL-60 cells, with less than 5% inhibition at 1 mM inhibitor, a concentration that completely inhibited the activity of GLUT1 (Figure 12, panel A). Kinetic analysis of the effect of different concentrations of methyl 2,5-dihydroxycinnamate on the substrate dependence of deoxyglucose transport by the HL-60 cells indicated that it inhibited in a noncompetitive manner the transport of deoxyglucose (Figure 12, panel B), with a K_i of 190 μ M (Figure 11). Methyl 2,5-

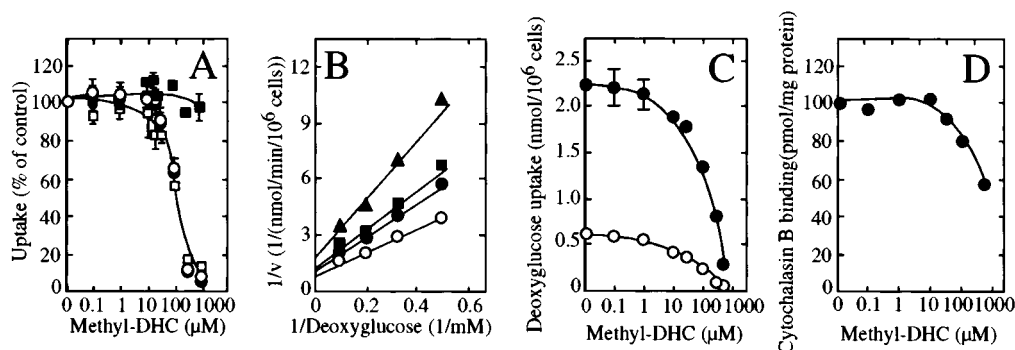


FIGURE 12: Effect of methyl 2,5-dihydroxycinnamate on the activity of glucose transporters in HL-60 cells, GLUT1-expressing CHO cells, and human erythrocytes. (A) Dose response of the effect of methyl 2,5-dihydroxycinnamate on the transport of methylglucose (●), deoxyglucose (○), dehydroascorbic acid (□), or leucine (■) in HL-60 cells. Transport was measured using a 30-s uptake assay in the presence of the indicated concentrations of methyl 2,5-dihydroxycinnamate. Data are presented as percent of controls (samples without inhibitor) and represent the mean \pm standard deviation of four samples. (B) Double-reciprocal plots of the effects of methyl 2,5-dihydroxycinnamate on the substrate dependence for deoxyglucose transport in HL-60 cells. Transport of deoxyglucose at 2, 3, 5, and 10 mM was measured for 30 s in the absence (○) or in the presence of 200 (●), 300 (■), and 400 μ M (▲) methyl 2,5-dihydroxycinnamate. Data represent the mean of four samples. (C) Dose dependence of the effect of methyl 2,5-dihydroxycinnamate on the uptake of deoxyglucose in CHO cells overexpressing GLUT1 (●) and in control CHO cells (○). Data represent the mean \pm standard deviation of four samples. (D) Dose-dependence of the effect of methyl 2,5-dihydroxycinnamate on the binding of cytochalasin B to human erythrocyte membranes. Data represent the mean \pm standard deviation of one of two to three independent experiments performed in quadruplicate.

dihydroxycinnamate also caused a dose-dependent inhibition of deoxyglucose and dehydroascorbic acid uptake in GLUT1-expressing and in control CHO cells (Figure 12, panel C, and data not shown). Fifty percent inhibition of uptake was observed at approximately 200 μ M, and greater than 90% inhibition was observed at concentrations of 500 μ M. On the other hand, increasing concentrations of methyl 2,5-dihydroxycinnamate inhibited only partially the binding of cytochalasin B to human erythrocyte membranes, with about 40% inhibition observed at approximately 500 μ M 2,5-dihydroxycinnamate (Figure 12, panel D). Overall, the data are consistent with the direct interaction of methyl 2,5-dihydroxycinnamate with GLUT1 with the result that the transport activity is inhibited in a noncompetitive manner. Similar to the effect of the lavendustins, the effect of methyl 2,5-dihydroxycinnamate on the transport activity of GLUT1 correlates better with its effect on the binding of ATP to the tyrosine kinases than with its effect on the substrate (tyrosine) binding site.

DISCUSSION

We show here that tyrosine kinase inhibitors inhibit the cellular uptake of methylglucose, deoxyglucose, and dehydroascorbic acid mediated by GLUT1. We used as experimental systems HL-60 cells and human erythrocytes, cells that express the facilitative hexose transporter GLUT1. Additionally, we used stably transfected CHO cells overexpressing GLUT1. Overall, our data are compatible with the concept that only those tyrosine kinase inhibitors that directly interact with ATP binding sites competitively inhibit the transport activity of GLUT1. Our results also indicate that the effect on transport is due to the direct interaction of the inhibitors with GLUT1 and does not involve the participation of cellular phosphorylation–dephosphorylation events.

These conclusions are supported by the following findings: (i) No preincubation step was necessary to observe the effect of the tyrosine kinase inhibitors on the uptake of methylglucose, deoxyglucose, or dehydroascorbic acid. The effect was instantaneous and maximum when the tyrosine kinase inhibitors were added to the uptake assay simulta-

neously with the test substrate at time zero. This instantaneous effect is different from that of the tyrosine kinase inhibitors on protein tyrosine phosphorylation, which is a time-dependent phenomenon that usually requires a preincubation step with the inhibitor to fully develop. (ii) Tyrosine kinase inhibitors competed with GLUT1 substrates in a cell-independent manner. The inhibitors showed similar dose–effect curves for the inhibition of transport in HL-60 cells, in control and GLUT1-transfected CHO cells, and in human erythrocytes, suggesting that they likely acted through common mechanisms in the different cell types analyzed. This result is consistent with the expression of GLUT1 in the three cellular systems examined. (iii) The effect of the tyrosine kinase inhibitors was generally independent of the transported substrate. Similar dose–response curves were observed for inhibition of the uptake of methylglucose, deoxyglucose, and dehydroascorbic acid. The data suggest that the tyrosine kinase inhibitors inhibit uptake of the three substrates at the common transport step prior to the intracellular trapping of deoxyglucose (by phosphorylation) and dehydroascorbic acid (by reduction). Methylglucose is a substrate of the glucose transporters that is not metabolized and therefore is not trapped (or accumulated) intracellularly. (iv) The tyrosine kinase inhibitors that compete for the binding of ATP to the tyrosine kinases inhibited the transport of deoxyglucose in HL-60 cells in a competitive manner. The simplest interpretation of the data is that these tyrosine kinase inhibitors exert their effect on transport by directly interacting with GLUT1 at a site (or sites) related to the binding or the transport of deoxyglucose. (v) The tyrosine kinase inhibitors blocked the glucose-sensitive binding of cytochalasin B to GLUT1 present in human erythrocytes. As above, this observation is consistent with the direct interaction of the tyrosine kinase inhibitors with GLUT1.

The inhibitors analyzed in this study segregate into four categories when analyzed with respect to the mechanism by which they affect the transport activity of GLUT1, and members within each category share a similar mechanism by which they affect the activity of the tyrosine kinases. (i) The first group comprises those compounds that inhibited

the activity of GLUT1 in a competitive manner. The compounds in this group inhibit the activity of tyrosine kinase by competing with the binding of ATP (22, 30, 31, 34–36). The tyrosine kinase inhibitors belonging to this group are structurally heterogeneous and comprise natural products (the flavones quercetin and myricetin, the isoflavones genistein and biochanin A, and the lavendustins A and B) as well as synthetic compounds (the tyrphostins B44, B46, B48, B50, B56, and AG879). The structural heterogeneity was reflected in their respective inhibition constants (K_i) that varied from 8 μ M for quercetin to 170 μ M for tyrphostin B56. We also established that three other flavones (morin, rhamnetin, and isorhamnetin) for which there is no information regarding their capacity to inhibit the activity of tyrosine kinases were potent inhibitors of glucose transporter function and did so in a competitive manner. The K_i values for inhibition of transport by these flavones varied from 5 to 105 μ M. On the basis of the above considerations, we propose that the flavones morin, rhamnetin, and isorhamnetin may function as potent tyrosine kinase inhibitors. For the tyrphostin AG879, there is no direct data on the mechanism by which it inhibits the activity of tyrosine kinases; because it inhibited the transport activity of GLUT1 in a competitive manner, we suggest that it may be a competitive inhibitor of protein tyrosine kinases. (ii) A second group of potent tyrosine kinase inhibitors was unable to inhibit the functional activity of GLUT1. This group comprised several compounds that were synthesized to mimic the spatial structure of tyrosine. Accordingly, these compounds block the activity of tyrosine kinases by competitively inhibiting the binding of tyrosine (31, 33). The tyrphostins belonging to this category (A1, A23, A25, A46, A51, and A63) failed to inhibit the activity of GLUT1 in the three cellular systems analyzed, HL-60 cells, CHO cells, and human erythrocytes. (iii) A third group of tyrosine kinase inhibitors, consisting of the tyrphostin A47 and the erbstatin analogue methyl 2,5-dihydroxycinnamate, inhibited transport mediated by GLUT1 in a noncompetitive manner. The tyrphostin A47 inhibits the activity of tyrosine kinases by competitively blocking the binding of tyrosine to the tyrosine kinases (31, 33) and is structurally similar to the second group of tyrosine kinase inhibitors (tyrphostins A1, A23, A25, A46, A51, and A63) that failed to inhibit the transport activity of GLUT1. Similar to tyrphostin 47, the tyrosine kinase inhibitor methyl 2,5-dihydroxycinnamate also inhibited the activity of GLUT1 in a noncompetitive manner. Methyl 2,5-dihydroxycinnamate inhibits the activity of tyrosine kinases in a manner noncompetitive with ATP (37, 38), which is therefore consistent with the transport inhibition data. (iv) Two additional compounds failed to inhibit the activity of GLUT1, the isoflavones daidzein and puerarin. The isoflavone daidzein lacks activity of tyrosine kinase, which parallels its lack of glucose transporter inhibitory activity, while no information is available in this regard for puerarin (22). Taken together, the data establish that only those compounds that competitively inhibit the binding of ATP to the tyrosine kinases were able to inhibit in a competitive manner the transport activity of GLUT1. The data also indicate that the inhibitory effect on the glucose transporter was not mediated through inhibition of tyrosine phosphorylation and is instead the result of the direct interaction of the tyrosine kinase inhibitors with GLUT1.

Although the precise nature of the interaction between the tyrosine kinase inhibitors and GLUT1 cannot be deduced from the experiments reported here, the characteristics of the inhibition and the results of the cytochalasin B-binding experiments suggest that the tyrosine kinase inhibitors interact with the glucose transporters at a site (or sites) directly involved in the binding of the transported substrates or with sites located within the transmembrane channel of GLUT1 involved in transport. In this regard, detailed studies of the structural–functional properties of the erythrocyte glucose transporter have established the concept that a reversible inhibitor of transport that acts at the sugar import domain functions as a competitive inhibitor of sugar entry and as a noncompetitive inhibitor of sugar exit (39, 40). On the other hand, a reversible inhibitor that acts at the sugar exit domain functions as a competitive inhibitor of sugar exit and as a noncompetitive inhibitor of sugar entry. Thus, the results of the competition studies may be interpreted as revealing the existence of two classes of glucose transporter inhibitors, each group acting preferentially at sites accessible extracellularly or intracellularly. The first group (class 1 inhibitors) includes the flavones quercetin, myricetin, morin, rhamnetin, and isorhamnetin, the isoflavones genistein and biochanin A, the lavendustins A and B, and the tyrphostins B44, B46, B48, B50, B56, and AG879. These compounds inhibited GLUT1-mediated uptake in a competitive manner and presumably block transport by binding to a site (or sites) in GLUT1 accessible externally. The second group (class 2 inhibitors) includes the tyrphostin A47 and methyl 2,5-dihydroxycinnamate, both of which inhibited GLUT1-mediated uptake in a noncompetitive manner and therefore may block transport by binding to a site (or sites) in GLUT1 accessible internally. On the other hand, it is also possible that the effect of tyrphostin 47 on transport may be related to the presence of a double-bonded sulfur in position 7. GLUT1 possesses an exofacial SH group that appears to be sensitive to the presence of externally applied sulfhydryl reagents that inhibit glucose transport in a noncompetitive manner (41).

The interaction of GLUT1 with compounds functionally defined by their specific interaction with protein tyrosine kinases poses a number of questions regarding both the identity of the amino acid residues forming part of the binding site(s) and the functional characteristics of these sites as they relate to the overall regulation of glucose transporter function. Directly relevant to this issue is the evidence showing that the tyrosine kinase inhibitors interact with other cellular targets besides the tyrosine kinases and the interaction with GLUT1 described here. For example, several tyrphostins and the tyrosine kinase inhibitors genistein and erbstatin are potent inhibitors of DNA topoisomerases (24, 30), and genistein is also an inhibitor of protein histidine kinase (23). Similar to the tyrosine kinases, these proteins have nucleotide binding sites and their function requires ATP hydrolysis. Thus, our data point to the existence of discrete structural motifs in GLUT1 with the functional properties expected for nucleotide binding sites, motifs that may be responsible for the effect of the tyrosine kinase inhibitors on transport. GLUT1 is, however, a transporter of the facilitative type that transports its substrates (hexoses and dehydroascorbic acid) down a concentration gradient. A detailed analysis of the transport mechanism of GLUT1 in reconstituted systems

consisting of highly purified GLUT1 reconstituted in lipid vesicles has revealed that the transport cycle occurs in the absence of ATP (1). Sugar transport mediated by GLUT1 is, however, regulated by intracellular ATP in a manner that suggests that the glucose transporter is sensitive to the cellular content of ATP, although no hydrolysis of ATP is required for this regulation of GLUT1 function (42–48). Although the exact nature of the regulatory action of ATP on transport is presently unknown, we can envision at least two different modes of regulation: indirect regulation through the nucleotide-dependent interaction of the transporter with accessory proteins and a direct regulatory effect of the nucleotide on the glucose transporters. Evidence is available indicating that GLUT1 can bind accessory proteins in an ATP-dependent manner and that the interaction may have a regulatory role on transporter function (49–51). On the other hand, experiments utilizing purified GLUT1 reconstituted in lipid vesicles or using red cell ghosts have revealed that ATP regulation of the transporter occurs through its direct interaction with GLUT1 (44, 45, 52–54). These data and the data presented here strongly support the concept that GLUT1 possesses nucleotide binding sites that are important for the activity of the transporter. Direct evidence for the presence of these binding sites in glucose transporters has been obtained from experiments showing that purified GLUT1 can be photolabeled with azido ATP and that labeling is selectively and specifically competed by glucose and cytochalasin B (52, 54). Further support for the existence of nucleotide binding sites in the glucose transporters is provided by experiments that showed that 8-azido adenosine is a highly specific photoaffinity labeling reagent for the erythrocyte glucose transporter (55). Although 8-azido adenosine is transported into human erythrocytes by a transport system that is functionally unrelated to the glucose transporter, photolabeling of GLUT1 by 8-azido adenosine was specifically competed by glucose and cytochalasin B (55, 56). Moreover, 8-azido adenosine blocked the binding of cytochalasin B to the erythrocyte glucose transporter in a competitive manner (56).

A detailed analysis of the primary structure of GLUT1 reveals the presence of three short sequence segments that are highly homologous to consensus nucleotide binding sequences present in protein segments that are discontinuous in the primary sequence and form the ATP binding sites in several ATPases and other nucleotide-binding proteins (57, 58). In GLUT1, the three consensus sequences correspond to amino acid residues 111–118 (GFSKLGKS, consensus sequence I), 225–229 (KSVLK, consensus sequence II), and 332–343 (GRRTLHLIAG, consensus sequence III) (52). In the 12-helix model (3), consensus sequence I is located within the predicted transmembrane domain 3 or it may be exposed in the short extracellular loop between predicted transmembrane domains 3 and 4. Consensus sequence II is located in the large intracellular loop located between predicted transmembrane domains 6 and 7, and consensus sequence III is located in the short intracellular loop between predicted transmembrane domains 8 and 9. A recent photolabeling study followed by detailed peptide mapping strongly points to consensus sequence III as part of an ATP-binding site in GLUT1 (54). The equivalent sequence in adenylate cyclase corresponds to a hydrophobic strand of a parallel β -pleated sheet flanking the triphosphate binding site

of MgATP (58). There are data indicating that the transport activity of purified GLUT1 reconstituted in liposomes is affected by extracellularly applied ATP, an observation that is consistent with the presence of nucleotide binding sites in GLUT1 that are accessible from the extracellular milieu (53).

The most notable difference between the present study and previous studies showing that ATP regulates the activity of GLUT1 is that the tyrosine kinase inhibitors completely blocked transport through GLUT1. The competitive nature of the inhibition strongly suggests that, while the putative nucleotide binding sites in GLUT1 may be important as regulatory sites, it seems likely they are in fact an integral part of the substrate binding site(s) or the transmembrane transport channel in the transporter.

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