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Quercetin Inhibits Hexose Transport in a Human Diploid Fibroblast

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Summary. The flavonol quercetin, a phloretin analog, inhibits transport of 2-deoxyglucose and 3-O-methylglucose in a cultured human diploid fibroblast. This inhibition is related to transport itself and not to the reported effects of flavonoids on membrane-bound ATPases. From concentration-inhibition curves at several pH's we conclude that uncharged (acid) quercetin (pK = 7.65) is the inhibitory form of the molecule ($K_I = 10\,\mu M$). Quercetin, unlike phloretin, is rapidly degraded in 0.1 N NaOH; the degradation products are weakly inhibitory to hexose transport.

The flavonol quercetin has been observed in a number of systems to inhibit a variety of membrane ATPases (Carpenedo *et al.*, 1969; Fewtrell & Gomperts, 1977; Kuriki & Racker, 1976, and references cited therein). Suolinna and co-workers (1974, 1975) have also observed that quercetin inhibits glycolysis in several tumor cells; they ascribe the effect to the inhibition of one of several ATPases (depending on the cell) that generate ADP and P_i required for glycolysis. In addition, quercetin is an analog of phloretin (see below, Fig. 5), which also inhibits certain membrane ATPases (Robinson, 1969) and a number of other membrane-associated functions, notably hexose transport. Quercetin has been described as an inhibitor of glucose transport in human erythrocytes (Farley, Collins & Konigsberg, 1976).

We have assessed quercetin as an inhibitor of hexose transport in HSWP cells, a cultured human diploid fibroblast. For assay of hexose transport in cultured cells (reviewed in Plagemann & Richey, 1974), we and others commonly use 2-deoxyglucose (dGlc), since under appropriate conditions

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the sugar is rapidly phosphorylated as it enters the cell, but is otherwise not significantly metabolized; the transport step is then essentially rate-limiting in uptake. During such measurements, care must be taken that the cell's ATP supply is not depleted. Conversely, in the presence of a potential ATPase and/or glycolysis inhibitor like quercetin, care must also be taken that hexokinase activity is not blocked by backed-up glycolytic intermediates. In such cases it is useful to compare effects of the inhibitor on dGlc uptake with parallel effects on the efflux of 3-O-methylglucose (meGlc). Since the latter is not phosphorylated, its efflux into a large volume of external medium (from which back flux is inconsequential) gives a reasonably uncomplicated measure of hexose transport. Here we describe the effects of quercetin on such sugar fluxes and show, in addition, that it is the uncharged (acid) form of the flavonol that is the inhibitory species. Other effects of alkaline pH on the inhibitory capacity of quercetin and phloretin are also described.

Materials and Methods

Cells and Cell Growth

HSWP cells are a diploid cell strain derived from human foreskin. They were cultured in Eagle's minimal essential medium (EMEM; KC Biological, Inc.) with 10 % fetal calf serum (KC Biological, Inc.) and 25 μg/ml gentamicin (Schering). Cells were grown at 37 °C in a water-saturated atmosphere of 95 % air-5 % CO_2 and were used between the 10th and 25th passage. For each experiment, cells were removed from the stock cultures by trypsinization and seeded onto individual 11 × 25 × 1 mm glass slides in Petri dishes as originally described by Foster and Pardee (1969) and modified by Salter and Cook (1976). The cells were allowed to grow to confluency (more than 2 days) before use in an experiment. For all experiments described here, the cells had been maintained in a glucose-fed state prior to assay.

Uptake Measurements

The assay medium was glucose-free EMEM with Hanks' salts and 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffered at the desired pH as specified below. [^{3}H]hexose, with or without inhibitor, was added at concentrations and specific activity, also as specified below. Then 4 ml of this medium was placed in a 15 × 45 mm shell vial (Kimble Products) and equilibrated to 37 °C for experiments with dGlc. Experiments with meGlc were performed at 25 °C because of the rapid fluxes of this hexose. A glass slide with attached cells was removed from growth medium, rinsed in glucose-free EMEM at the temperature of the assay medium, and placed in the shell vial for the time required in the assay. The slide was then removed, rapidly rinsed several times in ice-cold Tris-buffered saline (0.15 M NaCl, 0.01 M Tris, pH 7.5), drained and placed in a glass 20-ml scintillation vial containing 1 ml 0.1 N NaOH to lyse the cells; the lysate was neutralized with 0.1 ml 1 N HCl. To this was added 10 ml counting solution made up in the ratio: 16.5 g Permeablend I (Packard), 1000 ml Triton X-100, and 2000 ml toluene. Vials were counted in a Nuclear-Chicago Mark I scintillation spectrometer.

Counts in "zero time" controls (less than 5-sec exposure of the cells to the assay medium) were subtracted from the later time points. Duplicate coverslips were placed in 4 ml 0.2 % sodium dodecylsulfate and analyzed for protein by the spectrophotofluorometric method of Avruch and Wallach (1971) with bovine serum albumin as standard. A Perkin-Elmer model 204 fluorescence spectrophotometer was used, with excitation at 286 nm and emission read at 338 nm.

Controls on specificity of the transport system included assay for the uptake of [^{3}H]-L-glucose. In such controls, the coverslip-associated radioactivity did not significantly exceed that of the "zero-time" controls, did not detectably increase with time over a 10-min assay, and was not affected by the addition of 100 μM phloretin to the assay medium. We conclude that there is no significant nonspecific diffusion component to hexose transport in these cells.

Chemicals

All chemicals were of the purest available commercial grade. Quercetin (Sigma) was recrystallized from ethanol; the product had a melting point of 310–312 °C. Spectra were measured with a Cary recording spectrophotometer. Quercetin and phloretin were stored in ethanol at −20 °C at 10^{-2} M and were added as an ethanolic solution to the assay medium. Ethanol alone at equivalent concentrations did not affect hexose transport.

Radiochemicals used were 2-deoxy-D-[^{3}H(G)]glucose (20 Ci/mmole) from Amersham and 3-O-[methyl-^{3}H]methylglucose (3.6 Ci/mmole) from New England Nuclear.

Results

In a number of determinations (Salter, 1977) we have found in glucose-fed HSWP cells that the V_{max} for uptake of dGlc is approximately 23 nmoles (mg protein)$^{-1}$ min^{-1} and the transport *Km* is 4–6 mM. The *Km* for transport of meGlc is 13–15 mM. Since the uptake kinetics in these human cells, in contrast to rodent or chick cells, are independent of growth state, the present experiments were performed with confluent cells. Hexoses were assayed at concentrations much lower than their respective transport *Km*'s.

Figure 1 shows the influx of dGlc and the efflux of meGlc. The influx of dGlc (Fig. 1*A*) was assayed at 50 μM. The low dGlc concentration and short uptake times avoid rapid depletion of cellular ATP by dGlc phosphorylation and also keep intracellular free hexose concentrations low with respect to the capacity of the cells' hexokinase activity. In the experiment shown in Fig. 1*A*, which is typical for glucose-fed cells, the uptake of dGlc into the controls is approximately 35 μmoles (liter of cell water)$^{-1}$ min^{-1} from the 50 μM concentration in the medium. This rate can be maintained for at least 15 min (Salter & Cook, 1976) because of the rapid intracellular phosphorylation of the hexose. Under conditions for which phosphorylation capacity becomes limiting (e.g., ATP depletion) or excretion of metabolites becomes significant, a change in rate

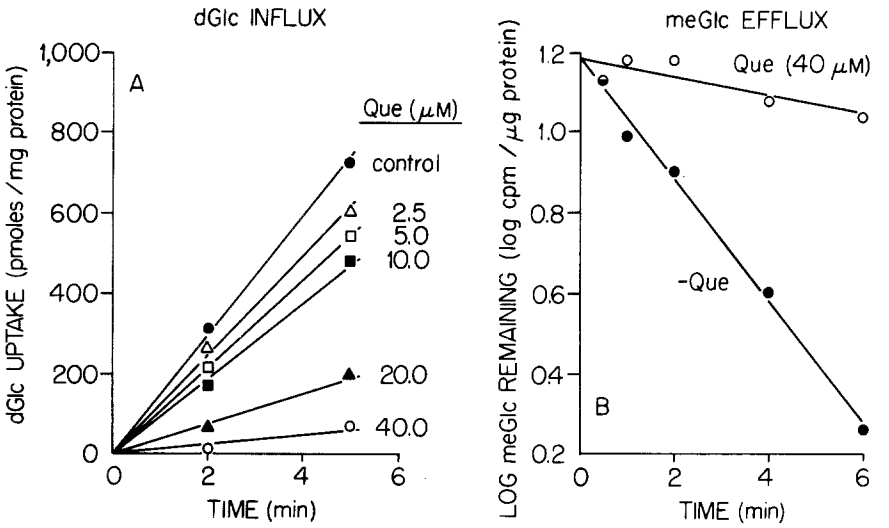


Fig. 1. Effect of quercetin on hexose fluxes in HSWP cells. (*A*): Uptake of dGlc at pH 7.0, 37 °C; [^{3}H] dGlc at 50 μM and 1 μCi/ml. Cells were preincubated with quercetin at the concentrations indicated for 5 min, and the assays were carried out at the same inhibitor concentrations. Each point is the mean of triplicate determinations with SE of ±5–10%; errors bars are omitted for clarity. (*B*): Efflux of meGlc at pH 7.2, 25 °C. Cells were preincubated in 1 mM [^{3}H]meGlc (5 μCi/ml) for 20 min, transferred to a medium with hexose at the same activity plus 40 μM quercetin for 10 min, and finally assayed for loss of [^{3}H]meGlc from cells into hexose-free medium containing 40 μM quercetin. Each point is the mean of triplicate determinations. Note logarithmic ordinate

may be expected, and the uptake curves change slope (Salter & Cook, 1976; Salter, 1977).

At pH 7.0 quercetin at 2.5 μM noticeably inhibits the dGlc uptake, and at 40 μM it reduces the flux to about 10% of the control value. At all concentrations of the inhibitor, the extent of inhibition is the same whether assayed at 2 or 5 min of hexose uptake; i.e., the effect appears to be on the rate of uptake and not on a limited uptake capacity in the presence of quercetin.

A second observation showing that the quercetin effect in hexose transport is on the transport itself is given in Fig. 1*B*. In this case the cells were loaded with 1 mM [^{3}H]meGlc for 20 min, more than sufficient time for this nonphosphorylable sugar to come to concentration equilibrium between cell water and medium. The cells were then transferred to a medium with the same [^{3}H] meGlc concentration but containing, in addition, 40 μM quercetin. After a further 10-min equilibration with hexose and inhibitor, the cells were placed in meGlc-free medium, but with the inhibitor still present, and the loss of [^{3}H] meGlc from the cells with time was followed. With the cells preloaded at meGlc concentrations substantially below its transport

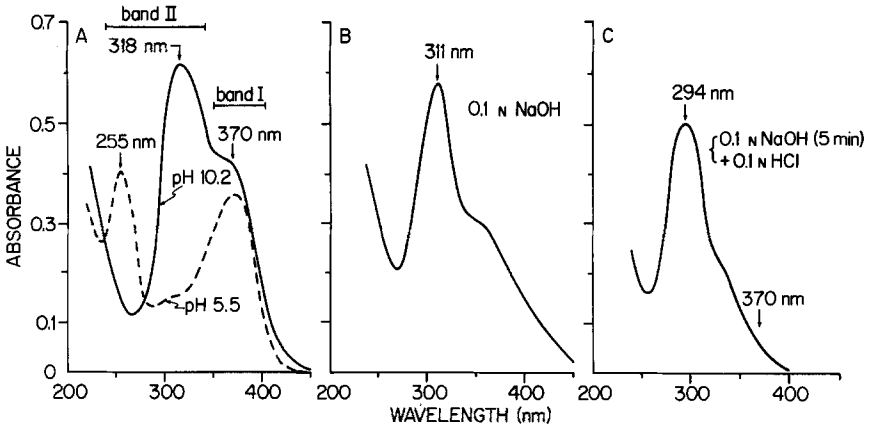


Fig. 2. Quercetin spectra in aqueous media at various pH's. (*A*): Spectra in 0.15 M NaCl at pH 5.5 (0.01 M phosphate buffer) and pH 10.2 (0.01 M Tris buffer) are reversible and were measured at >2 pH units below and above, respectively, the pK (7.65) of the peak at 318 nm. Quercetin concentration, 25 μM. (*B*): Spectrum immediately after dilution to 25 μM in 0.1 N NaOH. (*C*): As in *B*, following neutralization with 1 N HCl after 5 min in 0.1 N NaOH

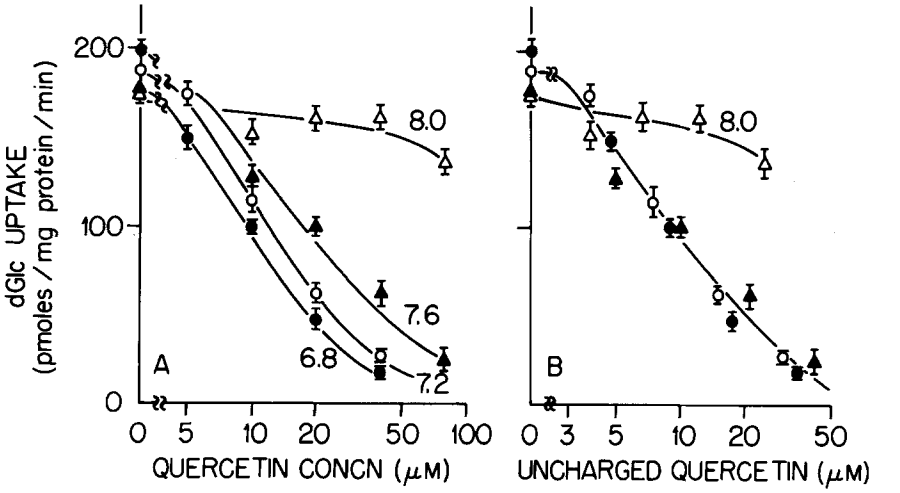


Fig. 3. Effect of pH on concentration dependence of quercetin in the inhibition of dGlc uptake. Cells were incubated in medium containing quercetin at the indicated concentration and pH for 5 min at 37 °C, then transferred to assay medium at the same quercetin concentration and pH. 5-min uptake was assayed in a medium with 50 μM [^{3}H]dGlc at 1 μCi/ml. (*A*): [^{3}H]dGlc uptake as a function of total quercetin concentration. Number next to each curve indicates pH of the medium. (*B*): Same data and symbols as in *A*, replotted as a function of the concentration of the uncharged (acid) form of quercetin (pK = 7.65). The data of pH 6.8, 7.2, and 7.6 are adequately described by a single curve for inhibition with $K_I = 10$ μM; the data at pH 8.0 do not fit this curve

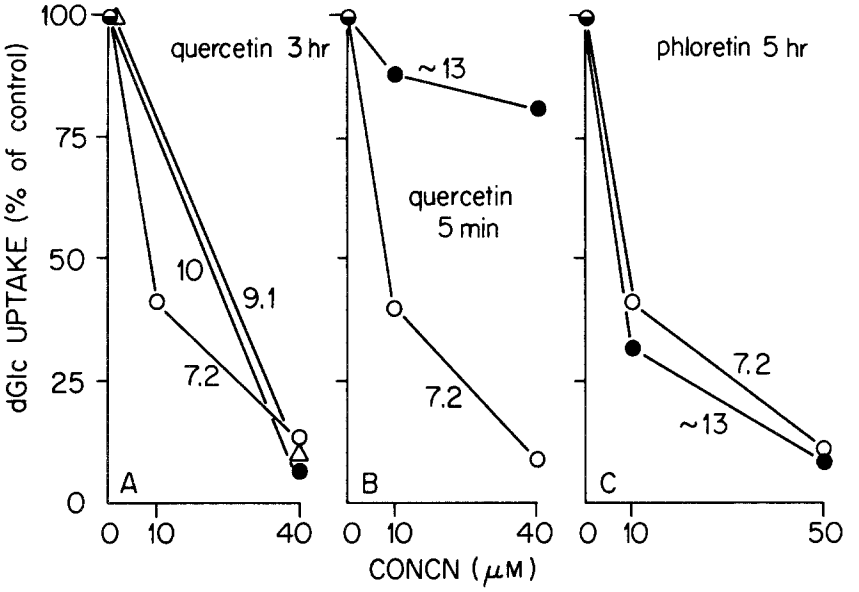


Fig. 4. Effect of incubation in alkaline media on the inhibitory capacity of quercetin and phloretin assayed at pH 7.2. Compounds were incubated in 0.15 M NaCl adjusted with Tris to pH 7.2, 9.1, or 10, as indicated on the curves, or incubated in 0.1 N NaOH (pH ~13). After incubation, samples were neutralized to pH 7.2 with 0.1 N HCl and diluted to the designated concentrations in the usual assay medium. dGlc uptake assayed as in Fig. 1. Controls incubated in pH 7.2 throughout. (*A*): Quercetin incubated 3 hr at pH 9.1 and 10. (*B*): Quercetin incubated 5 min in 0.1 N NaOH. (*C*): Phloretin incubated 5 hr in 0.1 N NaOH

Km, the efflux is first-order, with a half-time in the controls of about 2 min. As in the case of dGlc influx, meGlc efflux is inhibited about 90% by 40 μM quercetin (Fig. 1*B*) and about 60% by 20 μM quercetin (not shown). This result supports the proposition that hexose transport itself is the affected step. Inhibition of ATPases or of glycolysis, directly or indirectly, should have no effect on meGlc efflux. The meGlc data further suggest that quercetin acts on both membrane surfaces; because of its hydrophobic character, quercetin probably penetrates these cells readily.

Like phloretin (LeFevre & Marshall, 1959), quercetin may be titrated in the physiological pH range. Spectra for quercetin in 0.15 M NaCl buffered to various pH's are shown in Fig. 2. The peak at 318 nm (Band *II*, Fig. 2*A*) has a pK = 7.65. Further details of these spectra are discussed below. The data of Fig. 3 show that it is the uncharged (acid) form of quercetin that is the effective transport inhibitor. In Fig. 3*A* are displayed the concentration-inhibition curves for quercetin at several pH's. While the transport of dGlc in the controls is pH-independent between pH 6.8 and 8.0, quercetin is a more effective inhibitor at the more acid pH. More quantitatively, if the data are replotted as

a function of concentration of quercetin in the uncharged (acid) form (Fig. 3*B*), all of the data between pH 6.8 and pH 7.6 are satisfactorily described by a single curve with $K_I = 10\,\mu M$. Above pH 7.7, quercetin inhibits more weakly than can be accounted for either by ionization of the group with pK = 7.65 or by a direct effect on the transport system. Possible reasons for this will be considered in the Discussion.

The pH effects shown in Fig. 3 are reversible, including the unexpected loss of inhibitor activity at pH 8. Following incubation in aqueous solution at pH 10 (or lower) for several hours, quercetin regains its full activity when neutralized (Fig. 4*A*). However, after as little as 5 min at pH ~ 13 (0.1 N NaOH), most of the inhibitory activity is irreversibly lost (Fig. 4*B*). In contrast, even after 5 hr in 0.1 N NaOH, phloretin regains its full inhibitory activity when neutralized (Fig. 4*C*). The quercetin spectra recorded at these pH's are given in Fig. 2*B* and *C*.

Discussion

The results presented above show that quercetin, like phloretin (Fig. 5), inhibits hexose transport directly and independently of its additional effects on membrane-bound ATPases. This action complicates the interpretation of data on quercetin inhibition of glycolysis. Suolinna and co-workers (1974, 1975) showed that quercetin at 40 and 65 μM inhibited lactic acid production in several tumor cells, as did other inhibitors of membrane-bound ATPases. At least part of the quercetin effect could well be due to inhibition of glucose uptake as the first step in carbohydrate flow through the cells' metabolic machinery.

The pH data in the present work suggest that the uncharged form of quercetin is the active form in transport inhibition. The ultraviolet absorption

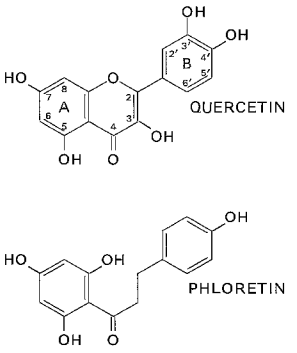
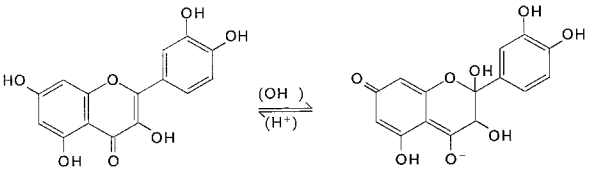


Fig. 5. Structures of quercetin and phloretin in their uncharged forms

peaks in aqueous medium at pH 5.5 correspond to those observed in nonaqueous methanol [255 (band II), 269 (shoulder), 301 (shoulder), and 370 nm (band I); Markham & Mabry, 1975]. Band I is principally affected by chemistry of the B-ring, while band II reflects chemistry of the A-ring. On alkaline titration, band I is little affected, while band II undergoes a 63-nm red shift. This suggests that the 2, 3 double bond becomes saturated, possibly by the addition of water, so that the B-ring is no longer conjugated with the rest of the molecule and remains unmodified by the mild alkaline conditions. At the same time the A-ring, analogous to the titration of phloretin (Owen, Steggall & Eyring, 1974) takes on the resonance form:



As shown in Fig. 3, the concentration dependence of quercetin as an inhibitor of hexose transport, when assayed at several pH's in the physiological range, strongly supports the suggestion that the uncharged (acid) form of the flavonol is the effective molecule.

The data between pH 7.8 and 10 are more difficult to understand. We have observed (Salter, 1977, and *unpublished*) that there is no detectable difference in dGlc uptake into glucose-fed cells between pH 5.0 and 8.4. In the titration of quercetin, no new peaks are observed other than the titration of band II at $pK = 7.65$. Even after 3-hr incubation at pH 10 at room temperature and ambient oxygen tensions, quercetin regains its full inhibitory activity when neutralized (Fig. 4), indicating that no significant degradation of the molecule has occurred. Nevertheless, above pH 7.8 the inhibitory activity of the flavonol decreases far more markedly than can be quantitatively accounted for by the ionization of the uncharged form. At pH 8.4 there is no detectable inhibition by quercetin even at 120 μM (data not shown). Although the data have not been developed in detail, quercetin appears to be a noncompetitive inhibitor of hexose transport (Salter, 1977, and *unpublished*). It is therefore probable that quercetin's site of action in the transport system is different from the hexose binding site. Effects of pH on the quercetin-reactive site would not be observed in the quercetin-free controls.

Above pH ~ 13 (0.1 N NaOH) there is a major change in the quercetin spectrum that is not reversed by neutralization (Fig. 2*B*, *C*). Flavonols are well known to be sensitive both to alkaline degradation (cleavage of the B-ring and opening of the γ-pyrone) and to oxidation in alkaline solution (Gottlieb,

1975). The virtually complete loss of band I shows that the molecule is rapidly degraded in 0.1 N NaOH, and the small hexose-transport inhibitory activity remaining after this treatment (Fig. 4*B*) is probably due to the reaction product(s) rather than to residual quercetin.

This alkaline sensitivity is due to the presence of the 3-OH in the γ-pyrone ring (for details, *see* Gottlieb, 1975). Phloretin, lacking this $-OH$, is not alkaline-sensitive; its characteristic pH 6 spectrum is fully restored after 5-hr incubation in 0.1 N NaOH (not shown), as is its hexose-transport inhibitory capacity (Fig. 4*C*). These observations have a practical consequence in transport studies. Because both phloretin and quercetin (and other analogs) are very sparingly soluble in aqueous media, the preparation of reasonably concentrated stock solutions is not possible in aqueous media at physiological pH. Consequently, the many investigators using phloretin have dissolved the inhibitor in either ethanol, dimethylsulfoxide, or 0.1 N NaOH. The former two solvents are inconvenient because they require appropriate controls on the effects of solvent alone; therefore the latter solvent has frequently been adopted. With flavonols, however, the use of such alkaline media is clearly not feasible.

Finally, the question remains as to the mechanism of action of quercetin on membrane transport systems, including (Na^+, K^+) ATPase (Kuriki & Racker, 1976). We have observed (Salter, 1977, and data *to be published*) that quercetin is an inhibitor of [^{3}H]phloretin binding to HSWP cells in the concentration ranges where these compounds are effective against transport. Both compounds are most effective in their nonpolar form against transport systems that carry highly polar ions or hexoses. It is thus likely either that these inhibitors affect the hydrophobic portion of the transporters responsible for anchoring them in the membrane bilayer, or that the inhibitors perturb the membrane bilayer itself and thereby alter the environment in which the transporters must function.

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