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Rhein inhibits glucose uptake in Ehrlich ascites tumor cells by alteration of membrane-associated functions

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Rhein (RH), 4,5 dihydroxyanthraquinone-2-carboxylic acid, is known to inhibit the glycolysis of neoplastic cells by impairing glucose uptake. In order to establish whether this might be due to a selective interaction of the carrier with the drug or to functional modifications of the cell membrane, the effect of RH on glucose uptake in Ehrlich ascites tumor cells has been investigated. RH strongly inhibits the uptake of both 2-deoxyglucose and 3-*O*-methylglucose, so the reduced influx therefore cannot be ascribed to an effect on glucose phosphorylation. The inhibition of glucose transport does not depend on a reduction of the number of the carriers as indicated by the inability of the drug to interfere with the synthesis of the transporter. Moreover, the extent of total binding of cytochalasin B, as well as the fact that glucose specificity is not altered, indicate that the intrinsic activity of the glucose carrier is not affected. We therefore conclude that the inhibition of glucose uptake must be ascribed to an interaction of the drug with cell membranes that results in an alteration of membrane-associated functions.

Key words: Glucose transport, membranes, rhein, tumor cells.

Introduction

Cancer cells often exhibit an increased glycolytic capacity and the ability to convert glucose to lactic acid in the presence of oxygen.[1] The high rate of glucose utilization provides a selective advantage for tumor cell growth and survival, since it represents an alternate source of ATP production that is particularly important in low oxygen environments, such as in poorly vascularized tumors. To maintain their growth potential, the neoplastic cells, therefore, must possess a highly efficient system for sugar transport and metabolism. The transport of hexoses across the plasma membranes in a variety of mammalian cells has been demonstrated to be carrier-mediated.[2,3]

Because of the role of glycolysis in tumor growth, a selective inhibition of this energy-yielding pathway is of particular interest for a rational cancer chemotherapy as clearly established by Burk *et al.*[4] Indeed, in recent years, a novel approach to cancer treatment has focused on the aberrant energy metabolism of the neoplastic cells.[5–9]

Among the several agents that have been reported to act through a selective inhibition of the energy metabolism of the neoplastic cell, one of the most promising is rhein (RH), 4,5-dihydroxy-anthraquinone-2-carboxylic acid, an anti-inflammatory drug,[10] which lowers ATP availability by inhibiting respiration, aerobic and anaerobic glycolysis of tumor cell.[11] RH decreases the oxygen consumption by affecting the oxidation of NAD- and FAD-linked substrates at the dehydrogenase-coenzyme level,[12] whereas the inhibition of glycolysis must be ascribed to a reduced glucose uptake.[11]

The impairment of the energy-yielding pathways in tumor cells brought about by RH results in an inhibition of their biosynthetic processes[13] and growth,[14] thus raising the possibility of RH as a biochemical modulator to potentiate the effectiveness of other anticancer drugs[15,16] and treatments, such as hyperthermia,[17] as well as to reduce or to revert the multidrug resistance of tumor cells.

Although the effect of RH in reducing glucose uptake has been shown to be exerted at the level of the cell membrane,[11] it still remains to be established whether the drug affects the glucose carrier *per se* or whether it alters the functional properties of the cell membrane so as to inhibit glucose transport. The experiments reported in this communication

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were essentially designed to answer this question. We chose Erhlich ascites tumor as an experimental model because we previously employed this system to study the effect of RH on tumor energy metabolism.[11,13]

Materials and methods

Cells

Ehrlich ascites tumor cells were grown in 2-month-old male Swiss mice and harvested 7–10 days after inoculation. The cells were withdrawn from the killed animals and suspended in a medium containing, in final concentration, 105 mM NaCl, 5 mM KCl, 50 mM *N*-tris-[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), pH 7.4 (NKT). The cells were centrifuged at 300 *g* for 5 min at room temperature and washed three times with NKT medium. The packed cells were counted (Coulter Counter, model ZM) and resuspended in the same medium at a concentration of 2×10^8 cells/ml. Contamination with other cells, such as leukocytes, did not exceed 0.6%, according to the differential counting of smears stained by the May–Grünwald method. The viability of the cells was about 95–98% as indicated by phase-contrast microscopy in the presence of Trypan blue.

Sugar uptake rates

For determination of 2-deoxy-D-glucose (2-DOG) uptake rates, 3×10^7 cells were suspended in 1.5 ml of NKT medium and incubated at 30°C for 15 min in the presence or absence of RH. Then 1 mM 2-deoxy-D-[^{3}H]glucose (1 μCi/μmol) was added to the tubes and mixed rapidly and the incubation was continued. Samples of 0.2 ml (4×10^6 cells) were removed at established intervals, placed into plastic tubes kept at 0°C containing 3 ml of NKT medium supplemented with 11 mM 2-DOG to chill cells rapidly and to dilute the specific activity of labeled sugar. The tubes were quickly mixed by inversion; the cells were collected by centrifugation at 780 *g* for 5 min at 4°C and washed three times with 3 ml of the above buffer. The supernatant was carefully removed by aspiration and discarded. The pellets were solubilized in 0.03 ml of 10% sodium dodecyl sulfate and 0.5 ml of 1N NaOH at 65°C for 2 h, neutralized with 0.5 ml 1N HCl and counted in 4.5 ml of Aquassure scintillation liquid in a LS-1800 Beckman liquid scintillation spectrometer.

Measurements of 3-*O*-methyl-D-glucose uptake into total cellular material was performed in a similar way. The assay medium (NKT) contained 5 mM 3-*O*-methyl-D-glucose (5 μCi/μmol).

'Zero-trans' influx of 2-DOG was measured using the oil-stop procedure according to Wright and Courtland White.[18]

Equilibrium binding of cytochalasin B

For the binding assays the cells were washed three times by centrifugation, resuspended in NKT medium at a concentration of 10^8 cells/ml and used immediately. [^{3}H]Cytochalasin B was used as a tracer. Equilibrium binding of cytochalasin B to Ehrlich ascites tumor cells was performed according to Cuppoletti *et al.*[19] The glucose carriers on Ehrlich ascites tumor cells were determined by Scatchard analysis[20] of the difference between cytochalasin bound in the presence of 100 mM D-glucose and in the absence of D-glucose.

Preparation of peptide and antisera production

The 14 residue fragment *Cys Glu Glu Leu Phe His Pro Leu Gly Ala Asp Ser Gln Val* corresponding to positions 480–493 of the glucose transport protein was synthesized by the solid-phase method,[21] using a Du Pont-Vega coupler, Model 1000. An additional cysteine was added at the N-terminal end of the peptide for binding purposes. The *N*-t-butoxycarbonyl protection method was used and trifunctional amino acids were protected as follows: *O*-benzyl [Ser, Glu, Asp]; *p*-methylbenzyl [Cys]; Tosyl [His]. At the end of the synthesis, the peptide was cleaved from the support and deprotected by treatment with trifluoromethansulfonic acid,[22] desalted on a 2.5 × 50 cm column of Sephadex G25 fine in 0.01 M ammonia and finally purified by gel filtration on a 2 × 80 cm column of AcA 202 high resolution resin (range: 1000–10 000) in the same solvent. The exact composition was assessed by amino acid analysis after complete hydrolysis with 6N HCl at 108°C for 24 h.

Antibodies were raised in a rabbit using as antigen a conjugate of the peptide with a carrier protein (keyhole limpet hemocyanin). The animal received a first subcutaneous injection of the complex (300 μg in 0.5 ml of Freund's complete

adjuvant), then successive immunizations with 200 μg at 10 day intervals. Animals were bled from the ears starting from the fourth injection. The sera were recovered by centrifugation and stored at -20°C until used. The detection of antibody binding to the peptide was routinely performed with an ELISA on microtiter plates as previously described.[23,24]

Fractionation procedures and western blot analysis

Microsomes containing plasma membranes were prepared from cells (4×10^7) incubated for 1 h at 30°C in 3 ml of NKT with or without 150 μM RH. At the end of incubation the cells were collected by centrifugation at 650 *g* at 4°C and resuspended in 10 mM HEPES and 0.025 M sucrose, pH 7.3. After 10 min at 4°C, the cells were disrupted with a Dounce homogenizer in the presence of 1 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenate was centrifuged at 650 *g* for 10 min at 4°C; the supernatant was then centrifuged at 100 000 *g* at 4°C for 1 h in a Beckman ultracentrifuge. The membrane pellet was resuspended for 1 h at 4°C in the following solubilization medium: 1% deoxycholate, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF and 150 mM NaCl, pH 7.4. The mixture was centrifuged at 12 000 *g* for 10 min at 4°C and the protein concentration determined according to Bradford.[25] Western blot was performed essentially according to Towbin *et al.*[26] and glucose transport protein was visualized using a goat anti-rabbit peroxidase-conjugated second antibody and β-chloronaphtol. A buffer containing phosphate-buffered saline, 0.1% Tween 20 and 0.5% blotting grade gelatin was used in order to block non-specific sites on the nitrocellulose membrane and for the dilution of the rabbit antipeptide antibody (1/500–1/100).

Northern blot

Total RNA was isolated essentially as according to Chomczynski and Sacchi[27] from the cells incubated without or with 150 μM RH for the established time in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. It was subjected to electrophoresis on a 1.3% agarose gel containing formaldehyde[28] and transferred by capillary elution to a nylon membrane (Hybond-N, Amersham, UK). Membranes were hybridized at 42°C in the presence of 50% formamide with random priming labeled specific cDNA (clone pGT25L) using [α-^{32}P]dATP.[29] Bands were visualized by autoradiography at -70°C with Du Pont Cronex intensifying screens using Kodak XAR-5 film.

Chemicals

The following chemicals were purchased from the indicated sources: [^{3}H]cytochalasin B (specific activity: 20 Ci/mmol), [^{3}H]3-*O*-methyl-glucose (specific activity: 3.67 Ci/mmol), 2-deoxy-D-[^{3}H]-glucose (specific activity: 10 Ci/mmol) from Radiochemical Centre (Amersham, UK); Aquassure from New England Nuclear (Boston, MA, USA); TES, 3-*O*-methyl-glucose and silicone oil from Sigma (St Louis, MO); 2-deoxy-D-glucose from Calbiochem Corp (San Diego, CA, USA); cytochalasin B from Aldrich-Chemie (Steinheim, Germany). All other reagents were of analytical grade and were obtained from BDH Italia (Milan, Italy). RH was a gift of Dr Vittorio Behar, Proter Laboratories, Opera, Milan, Italy.

Results

Figure 1 illustrates the effect of RH on the time-course of uptake of 2-DOG by Ehrlich ascites tumor cells. In the control the uptake is linear during the first 30 s; then it declines, approaching to a plateau which nevertheless is not reached over the period examined (2 min).

In the cells incubated for 15 min with 150 μM RH the 2-DOG uptake is strongly reduced (-50%) although it is taken up in a qualitatively similar manner to that of the untreated cells.

2-DOG can be phosphorylated by hexokinase, but is not further metabolized by the cell, so this step represents its metabolic end-point. The observed effect of RH on 2-DOG uptake could therefore be the result of inhibition of transport or phosphorylation. Since RH does not affect hexokinase activity,[11] the reduced 2-DOG uptake should be ascribed to a transport-specific effect. The effect of RH on the transport component was examined by evaluating the uptake of a non-metabolizable glucose analog, 3-*O*-methylglucose (Figure 2), which is an efficient substrate for the hexose carrier, but not for hexokinase, so that it can only reach an intracellular concentration equal to that in the medium. In RH-treated cells the uptake of 3-*O*-methylglucose is also reduced by an extent

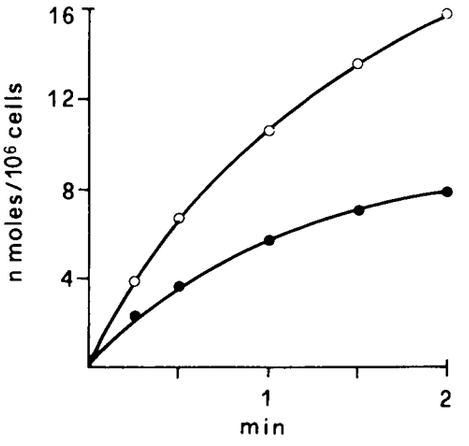

Figure 1. Time-course of 2-DOG uptake in Ehrlich ascites tumor cells. The cells (2×10^7 cells/ml) were pre-incubated at 30°C for 15 min without (○) or with (●) 150 μM RH. Then 1 mM radiolabeled 2-DOG (1 μCi/μmol) was added and the incubation was allowed to proceed. Samples of 0.2 ml (4×10^6 cells) were removed at established intervals and the uptake terminated by rapid dilution followed by centrifugation and washing. Each point was averaged from five different experiments, performed in duplicate, which yield reproducible results (±3%).

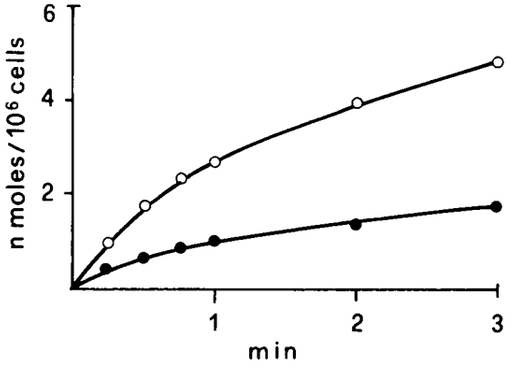

Figure 2. Time-course of 3-*O*-methylglucose uptake in Erhlich ascites tumor cells. The cells (2×10^7 cells/ml) were pre-incubated for 15 min at 22°C without (○) or with (●) 150 μM RH. Then 5 mM of radiolabeled 3-*O*-methylglucose (5 μCi/μmol) were added. Other experimental condition as in Figure 1. Each point was averaged from five different experiments, performed in duplicate, which yield reproducible results (±2%).

(−58%) essentially similar to that found for 2-DOG (Figure 1), indicating that the effect of RH on the glucose uptake is exerted at the level of transport.

To better define the effect of RH on transport components, the 'zero-trans' influx of 2-DOG was evaluated. 'Zero-trans' indicates the transport of a substrate from one side of a membrane to the other side where its concentration is initially zero. Graff *et al.*[30] have shown that the transport rate is 40% higher than that of phosphorylation so that transport would be strongly inhibited before any changes in the appearance of phosphorylated 2-DOG. Moreover, in order to slow the rate of approach to steady-state, a higher substrate concentration (2.5 mM) was used and the measurements were performed at 22°C. The reaction was terminated by centrifugation through silicone oil. Using this method, it has been shown that RH inhibits 2-DOG accumulation over the short period examined (Figure 3).

Although these data clearly demonstrate that RH reduces the uptake of hexoses by affecting the transport system, they do not allow us to establish whether the drug is carrier-specific or whether it induces functional perturbations of the membranes so as to inhibit hexose transport. To test a possible interaction of RH with the hexose transporter, its effect on the binding of cytochalasin B, a tight inhibitor of the hexose carrier, was examined. The binding of cytochalasin B to fresh Ehrlich ascites tumor cells reveals a non-linearity on the Scatchard plot (Figure 4A), indicating that there are at least two classes of cytochalasin binding sites in the cells with a high ($K_d = 0.94 \times 10^{-7}$ M) and a low affinity

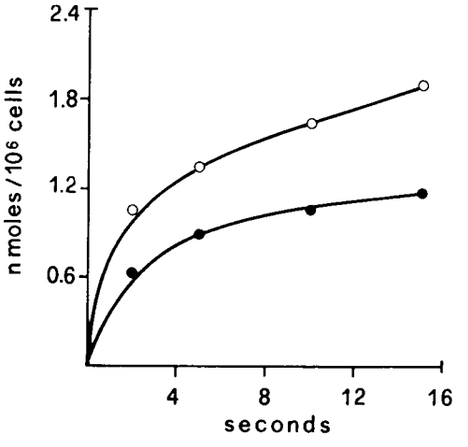

Figure 3. 'Zero-trans' influx of 2-DOG in Ehrlich ascites tumor cells. The cells (2×10^7 cells/ml) were incubated at 30°C for 15 min without (○) or with (●) 150 μM RH. The cells (4×10^6) were mixed at 22°C over an underlying layer oil with radiolabeled 2-DOG (1 μCi/μmol) at a concentration of 2.5 mM. Uptake was terminated by centrifugation through silicone oil. Each point was averaged from eight different experiments which yield reproducible results (±5%).

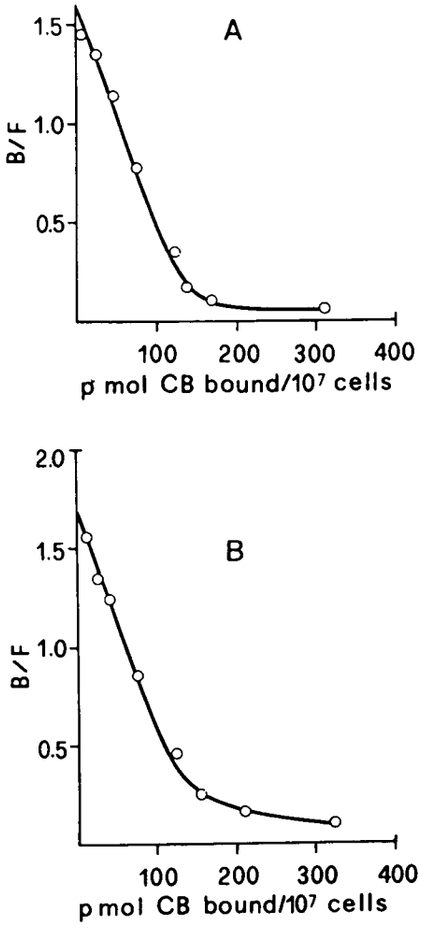

Figure 4. (A) Scatchard plot of the cytochalasin B binding to Ehrlich ascites cells. Cells were equilibrated for 30 min with graded concentration (10^{-8} to 10^{-5} M) cytochalasin B containing 0.1 μCi of radiolabeled compound. The K_d values for high affinity and low affinity binding sites are 0.94×10^{-7} and 2.3×10^{-6} M, respectively. $B_o = 466$ pmol/10^7 cells. (B) Scatchard plot of the cytochalasin B binding to Ehrlich cells in the presence of 150 μM RH. Other experimental conditions as in (A). The K_d values for high and low affinity binding sites are 0.94×10^{-7} and 2.3×10^{-6} M, respectively. $B_o = 420$ pmol/10^7 cells. Each point in (A) and (B) was averaged from five different experiments, performed in duplicate, which yield reproducible results ($\pm$4%).

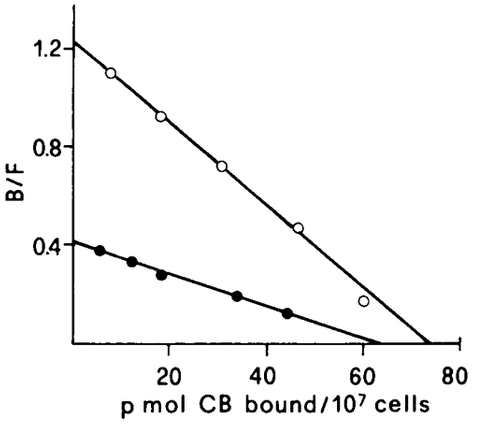

Figure 5. Scatchard plot of the specific binding of cytochalasin B to hexose carrier. The specific binding was determined by subtraction from total binding of residual binding in the presence of 100 mM D-glucose. Other experimental conditions as in Figure 5. (○) Control: $K_d = 0.6 \times 10^{-7}$ M; $B_o = 74 \pm 6$ pmol/10^7 cells. (●) With 150 μM RH: $K_d = 1.5 \times 10^{-7}$ M; $B_o = 64 \pm 9$ pmol/10^7 cells. Each point was averaged from four different experiments, performed in duplicate, which yield reproducible results ($\pm$3%).

($K_d = 2.3 \times 10^{-6}$ M). The total binding (B_o) was about 460 pmol/10^7 cells with 30% of high affinity binding sites. The treatment with 150 μM RH does not modify the total cytochalasin binding (Figure 4B), as indicated by the values of K_d and B_o which are similar to those found for the control.

Since cytochalasin B also associates with other cellular structures, its specific binding to the hexose carrier was evaluated following the subtraction of D-glucose-insensitive binding from the total binding. This glucose-sensitive portion of cytochalasin B binding showed an apparent linearity on the Scatchard plot (Figure 5) with a K_d of 0.6×10^{-7} M and a B_o of 73 pmol/10^7 cells. The addition of RH does not induce any modification in the amount of the glucose-sensitive binding sites (64 pmol/10^7 cells), but slightly increases the K_d value (1.5×10^{-7} M).

The inability of RH to modify the amount of binding sites indicates that the inhibition of glucose uptake cannot be ascribed to reduced carrier numbers, as further confirmed by the data of Figures 6 and 7.

Figure 6 shows the Western blot analysis performed on plasma membrane proteins prepared from cells incubated for 30 min in the absence (lane A) and in the presence of 150 μM RH (lane B). The antibody against residues 480–493 of the glucose transporter protein was utilized to detect the glucose transporter.[51] A narrow band of approximately M_r 55 000 is heavily and almost exclusively shown by the antibody in both detergent extracts of membranes isolated from control and RH-treated cells.

Figure 7 shows the Northern blot analysis performed on cells treated with 150 μM RH for 15, 30, 45 and 60 min, respectively. Using the nearly full-length pGT25L glucose transporter cDNA

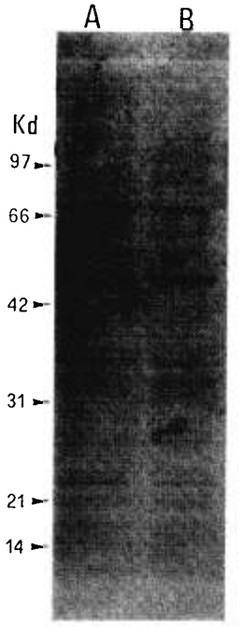

Figure 6. Western blotting of the membrane protein isolated from control (lane A) and RH-treated cells (lane B) Ehrlich ascites tumor cells. The concentration of RH was 150 μM and the time of treatment 1 h. Further experimental details under Materials and methods.

clone as probe the specific glucose transporter mRNA was demonstrated. The results were normalized using β-actin cDNA as a probe in order to better quantitate the mRNA loaded for each lane. No differences in the amount of glucose transporter specific mRNA can be demonstrated even after 2 h of treatment.

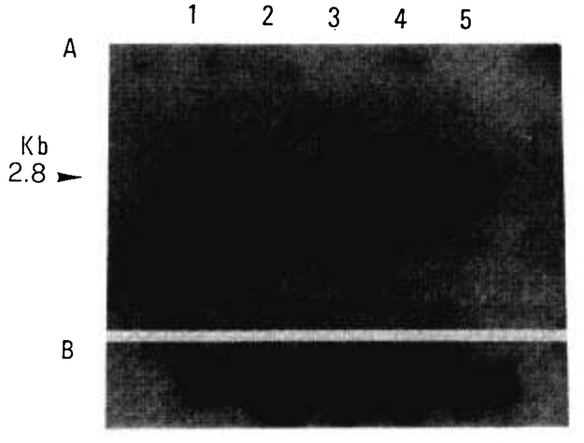

Figure 7. (A) Detection of glucose transporter mRNA in control (lane 1) and RH-treated (lanes 2–5) Ehrlich ascites tumor cells. The final concentration of RH was 150 μM whereas the time of treatment was: 15 min (lane 2); 30 min (lane 3); 60 min (lane 4) and 120 min (lane 5). (B) Detection of β-actin mRNA in control (lane 1) and RH-treated Ehrlich ascites tumor cells. The lanes as in (A).

Discussion

The observations recorded in this paper show that the inhibition of glucose uptake brought about by RH may be mainly attributed to an alteration of the functional properties of plasma membrane.

Hexose transport and metabolism in mammalian cells are generally considered as a tandem process where hexose enters the cytosol before phosphorylation by hexokinase. However, every modification of the phosphorylation process should influence the transport. Nevertheless, an effect on glucose phosphorylation by RH must be excluded because of the inhibited uptake of 3-*O*-methylglucose, which is not a substrate for hexokinase. Similarly, in the uptake of 2-DOG, which enters the cell, it is phosphorylated and not metabolized further, such an effect must be excluded because the inability of RH to affect the hexokinase activity.[11] However, it has been reported that the hexose transport rate exceeds the phosphorylation rates by up to 40%, so that the rate of transport does not represent a rate-limiting step in its metabolism.[30] Therefore, a dramatic decrease of the transport would be observed before any modification in the appearance of phosphorylated substrate. The 'zero-trans' influx was evaluated under conditions in which the phosphorylation rate was strongly reduced (22°C).

A decrease in glucose uptake observed in RH-treated cells due to a reduced number of transporters should also be excluded because, in spite of its capacity to inhibit protein synthesis,[13] RH does not interfere at all with that of the glucose transporter (Figures 6 and 7). However, the extent of total cytochalasin B binding (Figure 4), as well as glucose-specific binding to intact cells (Figure 5), is not altered, i.e. the drug does not mask or expose membrane proteins such as transport carriers. It remains, however, to be established whether the impairment of carrier-mediated transport by RH might be related to an alteration of the transporter *per se* or that it might be due to changes of the plasma membrane which, in turn, affect the carrier functionality.

A modification of the intrinsic activity of the hexose carrier as a result of its interaction with the drug is excluded since the only modification that can be demonstrated is the slight increase in the K_d

value (Figure 5). On the contrary, an action of RH at the level of the cell membrane explains well the inhibitory effect on carrier-mediated transport.

The mode of action of the carrier involves at least one step of mechanical movement, which in most cases is rate limiting. Since the carrier presumably spans the membrane, its motion and therefore its rate of operation are determined to a large extent by the viscosity of the membrane lipid bilayer.[31] Biophysical analyses by electron paramagnetic resonance (EPR) spectroscopy have demonstrated that RH induces a remarkable increase in the membrane order, i.e. in membrane 'rigidity'.[32] This increase may be due to a direct effect of the drug on the membrane structure or related to a perturbation of ion balance inducing indirect changes in membrane functionality that, in turn, modifies the permeability and/or carrier activity.[33–35] Membrane fluidity is an important factor in regulating membrane activity and biological function since small changes in the fluid state of the membrane result in large changes in the activity of membrane-bound proteins.[36,37]

The membrane fluidity can be modulated by many different drugs, including antineoplastic ones.[38–42] These antitumor drugs have no common mechanism of action other than of a membrane perturbant. In addition, most of them, e.g. VM-26, nitrogen mustard derivatives, adriamycin and chlorambucil, have a cyclic moiety in common. In this respect, it is particularly relevant that the quinonic structures of adriamycin and RH are so similar that polyclonal antibodies raised against adriamycin generally cross-react with RH.[43] Since a general property of anticancer agents containing quinone is the ability to trigger formation of oxygen radicals,[44–47] it cannot be excluded that the increase in membrane order brought about by RH might be due, at least partially, to lipid peroxidation. This might occur, although this drug, in spite of its quinonic moiety, does not seem to inhibit the transplasma membrane redox system through the generation of free radicals.[16]

Because the plasma membrane represents a sensitive target for anticancer agents,[48] the membrane perturbing action of RH, which is reflected in an impairment of the synthetic and energy-yielding processes[11–13] as well as of the growth,[14] may be important for cancer chemotherapy. Since almost all antineoplastic drugs enter the cell by diffusion or carrier-mediated mechanisms, the membrane-related effects of RH may increase the cytotoxicity of the co-administered drugs.[15,16] Alternatively RH might potentiate the effectiveness of other anticancer treatments, such as hyperthermia.[17]

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