The Role of Passive Transbilayer Drug Movement in Multidrug Resistance and Its Modulation*

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The successful lowering of the intracellular concentration of multidrug resistance (MDR)-type drugs by P-glycoprotein (Pgp) relies on its ability to overcome the passive influx rate of each MDR-type drug. Thus, the aim of the present work was to study the effect of passive transbilayer drug movement on the multidrug resistance and its modulation. Fluorescence quenching studies indicated that whereas the Pgp substrate rhodamine 123 traverses an artificial lipid membrane with a lifetime of 3 min, the transbilayer movement rate of the MDR modulators, quinine and quinidine, was too fast to be detected with present methods. Transbilayer movement rates of drugs and modulators were estimated from their equilibration rate throughout artificial multilamellar vesicles. The equilibration rate of five selected modulators was faster than the equilibration rate of five representative MDR-type drugs tested, which was comparable with the rate of rhodamine 123 equilibration. Moreover, the carrier-type pepide ionophore, valinomycin, which is freely mobile in the membrane, inhibited Pgp-mediated efflux of rhodamine 123 from MDR cells. In contrast, the channel-forming ionophore gramicidin D, a Pgp substrate that flip-flops slowly across the membrane, did not modulate cellular Pgp activity. Pgp, with a turnover number of about 900 min⁻¹, can keep pace with the influx of an MDR-drug like rhodamine 123 exhibiting a transbilayer movement with a lifetime of minutes. On the other hand, Pgp would fail to protect MDR cells against cytotoxic drugs that are freely mobile through biological membranes and that re-enter cells faster than their Pgp-mediated active efflux rate. The relatively fast transbilayer movement exhibited by MDR modulators suggest that in contrast to MDR-type drugs, MDR modulators traverse the plasma membrane faster than the maximal expulsion rate of Pgp.

Inherent as well as acquired resistance to antineoplastic agents pose a major obstacle toward curative cancer chemotherapy (1, 2). Multidrug resistance (MDR) is characterized by the development of tumor cell resistance to diverse anticancer drugs. Mammalian cells with the typical MDR phenotype express increased levels of P-glycoprotein (Pgp), an integral component of the plasma membrane (3). Consequently, these cells display resistance to multiple cytotoxic hydrophobic agents, mostly of natural origin, including anthracyclines, Vinca alkaloids, epipodophyllotoxins, actinomycin D, taxoids, and hydrophobic peptides, such as gramicidin D, valinomycin, and dolo- statin 10. Pgp that possesses an ATPase activity functions as an energy-dependent extrusion pump that expels these hydrophobic cytotoxic agents from MDR cells (4). The resistance conferred by Pgp can be reversed by a group of structurally and chemically unrelated compounds referred to as modulators or alternatively as chemosensitizers, Pgp inhibitors, resistance modifiers, and reversing agents (5, 6).

Pgp extracted from plasma membranes of MDR cells and reconstituted into proteoliposomes displays an ATPase activity (5, 7–11) that is stimulated by certain MDR-type drugs and modulators. Recently, we (9) have demonstrated a competition of MDR-type drugs and modulators on a common Pgp pharmacophore, indicating that the interaction of both MDR-type drugs and modulators occurs at a common site on Pgp and ostensibly by a similar mechanism. Upon reconstitution, Pgp molecules located inside-out, with their ATPase site exposed to the medium, mediated an ATP-driven uptake of various substrates (7, 12–14). Recently, we have described an ATP-driven, valinomycin-dependent 86Rb⁺ uptake into proteoliposomes reconstituted with mammalian Pgp (12). Pgp mediated the ATP-dependent uptake of 86Rb⁺–ionophore complex into the proteoliposomes, where the radioactive cation was accumulated, thus circumventing the obstacle posed by the hydrophobicity of Pgp substrates in transport studies. Taking advantage of the high levels of Pgp expression in MDR Chinese hamster ovary cells and of this assay, we measured simultaneously both the ATPase and transport activities of Pgp under identical conditions and observed a stoichiometry of 0.5–0.8 ionophore molecules transported per ATP molecule hydrolyzed (13). The amount of 86Rb⁺ ions transported within one minute via the ATP- and valinomycin-dependent Pgp-mediated uptake was equivalent to an intravesicular cation concentration of 8 mM. Thus, this stoichiometry and transport capacity of Pgp resemble various potent ion-translocating ATPases that handle millimolar substrate concentrations.

MDR-type drugs and modulators enter cells by a passive diffusion through the plasma membrane. The resistance conferred upon MDR cells is the net result of the passive influx rate subtracted from the active efflux rate mediated by Pgp. Although the passive diffusion of doxorubicin in artificial vesicles has been demonstrated to be slow (14), no comparative study of various MDR-type drugs and modulators has been reported.

The aim of the present study was to estimate the transbilayer movement rate of MDR-type drugs and modulators. Fluorescence quenching and binding studies have indicated that the transbilayer movement of the MDR-type drugs examined is slow compared with the transbilayer mobility of various modulators tested. Modulation of MDR studies have indicated that
Transbilayer Movement and Multidrug Resistance

the mobile carrier valinomycin is readily capable of reversing MDR, whereas the channel-type ionophore, gramicidin D, fails to do so. These results suggest that the success of Pgp in lowering the intracellular concentration of an MDR-drug is determined also by a limited passive transbilayer movement of the drug. Moreover, a prerequisite for a successful modulator is the free passive transbilayer movement, allowing it to immediately re-enter into the cells and thus successfully occupy the Pgp pharmacophore.

EXPERIMENTAL PROCEDURES

Materials

Gramicidin D, valinomycin, progesterone, (+)-verapamil-HCl, reserpine, quinine-HCl, quinidine-HCl, rhodamine 123, trifluoperazine-2HCl, daunomycin-HCl, and doxorubicin-HCl were purchased from Sigma. (−)-(N-Methyl-3H]desmethoxyverapamil, [1,2,6,7-3H]progesterone, and [G-3H]vinblastine sulfate were purchased from Amersham Corp. [3H]Taxol was kindly provided by Dr. I. Ringel. Cardiolipin and egg phosphatidylcholine were products of Avanti Polar Lipids. Calcein-AM was purchased from Molecular Probes.

Methods

Liposome Preparation—Films obtained by evaporation of stock solutions containing 11 μmol lipid-P, of phosphatidylcholine and 2 μmol lipid-P, of cardiolipin were dried under vacuum for 30 min. Multilamellar vesicles were formed by gentle shaking in 1 ml of buffer A containing 25 mM Hepes-Tris (pH 7.5) and 0.2 mM NaCl for 4 h. The vesicles were centrifuged at 2 min for 5000 rpm in an Eppendorf 5415 C centrifuge. The pellets were resuspended in buffer A to a final concentration of 12.5 μM lipid P. The yield of lipid P was approximately 50%. Unilamellar vesicles were prepared by the reverse-phase evaporation technique (16) and were down-sized by extrusion twice through a 0.2-μm Nucleopore filter (17).

Estimation of Transbilayer Movement by Fluorescence Quenching Techniques—Rhodamine 123 (1 μM), quinidine (10 μM), or quinine (10 μM) solutions in buffer A solutions were preincubated and dialyzed at room temperature. The reaction was started by the addition of lipid vesicles to a final concentration of 0.5 μM lipid P. The fluorescence of rhodamine 123, quinidine, and quinidine (excitation 505, 336, and 336 nm; emission 594, 396, and 396 nm, respectively) was monitored continuously in a Perkin-Elmer LS-5 fluorimeter.

Estimation of Transbilayer Movement as the Equilibration Rate throughout Multilamellar Vesicles—The hydrophobic drugs tested here bind preferentially to phospholipid membranes. Upon the addition of a drug to vesicles, drug binding and equilibrium with the outer monolayer is rapidly reached. Further binding and equilibration with the inner layers of multilamellar vesicles depends on the individual rate of transbilayer movement of the drug. Thus, apart from the initial amount of drug bound, the binding rates actually reflect the transbilayer movement of drugs. The composition of the medium in and outside the vesicles was identical and, thus, concentric accumulation driven by electrochemical potential is not likely. Moreover, dissipation of a putative electrochemical potential by gramicidin D had no effect on drug association with the vesicles (data not shown). Because the encapsulated volume of free encapsulated drug was assumed to be negligible, the contribution of free encapsulated drug was therefore assumed to be practically all membrane-bound.

Binding rates were determined by two procedures. Multilamellar vesicles (1.25 mM lipid P) were preincubated in buffer A at room temperature. The drug was added and after further incubation for various times, samples were withdrawn and centrifuged for 2 min at 14,000 rpm in an Eppendorf centrifuge, and the drug amount left in the supernatant was determined. According to this procedure, the amount of drug bound to the vesicles was determined as the drug amount removed from the medium together with the vesicles.

An alternative procedure relies on a direct determination of the drug amount bound to the vesicles. Multilamellar vesicles (1.25 mM lipid P) were preincubated in 60% sucrose in buffer A. Drug was added, and after further various incubation periods, 0.2 ml samples were withdrawn, layered under 0.75 ml of 40% sucrose in buffer A and 0.5 ml of ether, and centrifuged for 2 min at 14,000 rpm in an Eppendorf centrifuge. After gentle shaking to render the ether fraction homogenous, 0.2-ml samples were withdrawn from the ether fraction for determination of the drug amount associated with the vesicles. Total recovery of the vesicle lipids was ascertained by inclusion of N-lysine rhodamine B-phosphatidylethanolamine (1% of total lipids, Avanti Polar lipids) in the vesicles and full recovery of the dye in the ether fraction. The drug amount present in the ether fraction in the absence of vesicles did not exceed 5% of the total and was subtracted as background values. Certain brands of tips absorbed large amounts of the hydrophobic drugs, leading to high background values, and were therefore avoided; glass tubes were used instead.

The amount of the various drugs was determined as follows: progesterone, verapamil, taxol, and vinblastine amount were determined by scintillation counting of [3H]radioactivity. Mitoxantrone was estimated as the absorbance at 620 nm of an ethanol/ether (8:2) solution. Quinine, quinidine, doxorubicin, daunorubicin, trifluoperazine, and rhodamine 123 were estimated as fluorescence (excitation 336, 336, 482, 482, 338, and 505 nm; emission 396, 396, 549, 549, 466, and 525 nm, respectively) of ethanol/ether (8:2) solutions.

Various Procedures—Cell cultures were maintained and emetine-resistant variants (Emt+) were derived from wild type Chinese hamster ovary cells using a stepwise selection protocol of increasing drug concentrations as described before (9). Modulation capacity of compounds was determined as described by Hollo et al. (20) using calcein-AM. Equilibrium dialysis of drugs was carried out at room temperature for 3 or 5 days with 100 volumes of 0 and 60% sucrose, respectively, in buffer A containing 1 mM sodium azide. Apparent equilibrium was attained under these conditions after 1 and 3 days, respectively. Drug concentrations were determined as detailed above.

RESULTS

The passive transbilayer movement of the chromophoric Pgp-substrate, rhodamine 123 (19), and quinine, an MDR modulator, was determined by essentially the method of Jain et al. (22), which follows transbilayer movement of a hydrophobic peptide. The transfer of rhodamine 123 from the aqueous phase to the bilayer of artificial lipid vesicles is accompanied by quenching of its fluorescence. As shown in Fig. 1, upon addition of low concentrations of the dye to unilamellar vesicles, quenching occurred in two steps. About 50% of the total fluorescence decrease occurred in the first step and was too fast to be recorded under our experimental conditions. The further decrease in fluorescence occurred slowly by a first order process that could be described by a linear semilog plot with a lifetime of about 3 min (data not shown). The 50:50 ratio for the change in the fluorescence intensity in the two steps was seen only in unilamellar vesicles. In multilamellar vesicles, which are composed of multiple concentric membranes, the fast decrease in fluorescence intensity comprised 10–15% of the total fluorescence decrease (Fig. 1). Compared with unilamellar vesicles, the decrease in the fluorescence intensity during the second phase observed with multilamellar vesicles was much slower. It could not be described by a single exponential, but the final fluorescence intensity reached at approximately 3 h was
similar to the final level of fluorescence intensity observed with unilamellar vesicles.

The initial rapid decline in fluorescence observed in the first phase represented binding of the dye to the outermost monolayer, which would have constituted 50% of the lipid in unilamellar vesicles and about 8–13% in multilamellar liposomes (21). The second slower phase was due to the transbilayer movement of the dye from the outer monolayer to the inner monolayer of unilamellar vesicles or movement from the outermost monolayer of multilamellar vesicles to the inner monolayers. Upon transfer of the drug to the inner monolayers, more drug could be bound to the outermost monolayer, thereby resulting in further quenching of the fluorescence. Thus, the rate of the slow binding phase actually reflects the transbilayer movement of the drugs.

The transbilayer movement of rhodamine 123, an established Pgp substrate, occurred in unilamellar vesicles with a lifetime of ~3 min, and equilibration of the dye throughout multilamellar vesicles required ~1 h (Fig. 1). As shown in Fig. 1, the comparable movement of the MDR modulator, quinine, measured under similar conditions, was much faster. The equilibration of quinidine with both monolayers of large unilamellar vesicles was too fast to be observed under our experimental conditions. The equilibration of quinidine throughout all the monolayers of multilamellar vesicles was also relatively fast and was complete within 10 min. Practically identical results were obtained with the analogue modulator, quinine (data not shown).

Determination of solute transbilayer movement by the fluorescence quenching technique is limited to drugs whose fluorescence is altered upon transfer from the aqueous phase into the membrane bilayer. However, the data obtained with the multilamellar vesicles suggest an alternative approach to determine transbilayer movement of solutes. Upon incubation with multilamellar vesicles, drugs initially bind very quickly to the outermost monolayer and subsequently equilibrate more slowly throughout the vesicles by transbilayer movement. An estimation of the transbilayer movement rate of drugs can be obtained by determination of the binding rates to the vesicles. The binding kinetics of two MDR-type drugs, doxorubicin and mitoxantrone, and three MDR modulators, verapamil, quinidine, and quinine, are presented in Fig. 2. The drug amount determined to be associated with the vesicles after prolonged incubation was similar to the amount predicted from equilibrium dialysis studies (Table I). After the initial fast binding of MDR-type drugs, further equilibration throughout the multilamellar vesicles proceeded slowly with equilibrium reached after more than 0.5 and 2 h with doxorubicin and mitoxantrone, respectively. In contradistinction to MDR-type drugs, the equilibration rates of the modulators were much faster, and most of the equilibration was essentially complete within 1 min. The transbilayer equilibration rate of the three modulators was too fast to be estimated by this procedure, yet the results clearly demonstrate that the modulators traverse the vesicle bilayers much faster than the MDR-type drugs.

Estimation of binding kinetics described above was achieved without washing the vesicles, and therefore, it could be applied only to MDR-type drugs and modulators with high affinity toward the vesicles. An alternative, more sensitive approach was to determine directly the amount left in the vesicles after a wash by centrifugation. The latter procedure results in loss of drug bound to the outer monolayer during the wash step, which lasted for about 1 min. The drug amount lost would have included also the drug fraction capable of diffusing outward from the inner monolayers during the wash. Thus, the size of the fraction lost during the wash step constitutes an additional indirect measure of transbilayer movement rate.

Binding rates of six classical MDR-type drugs, doxorubicin, daunomycin, rhodamine 123, taxol, vinblastine, and mitoxantrone, were analyzed (Figs. 3A and 4A). Of the MDR-type drugs tested, mitoxantrone proved the slowest and taxol the fastest. The equilibration period was relatively long and varied between 1 and 3 h; as expected from slow moving drugs, the amount of drug lost during the wash step from vesicles at equilibrium was ~10%. In contrast, the equilibration within the multilamellar vesicles of the four modulators tested, verapamil, quinidine, trifluoperazine, and progesterone, was much faster and was practically completed within 5 min (Figs. 3B and 4B). The rapid transbilayer mobility of progesterone, quin-
idine, or quinine caused a large loss of bound modulator during the wash step. Even the slowest moving MDR modulator tested, verapamil, reached equilibrium with the vesicles much faster than the fastest MDR drug, taxol.

Thus, the rate of transbilayer movement of MDR-type drugs was shown by three different experimental approaches to be markedly slower than the rate of modulators. No clear correlation was observed among the following three characteristics of the various drugs: (a) partition coefficient into octanol (Table I), (b) binding to multilamellar vesicles (Table I), and (c) the transbilayer movement rate in multilamellar vesicles. Thus, the slowest moving MDR-type drug, mitoxantrone, exhibited moderate partition coefficient but was tightly bound to multilamellar vesicles. The well established MDR modulator, verapamil, exhibited a similar partition coefficient but moved much faster across membrane bilayers and displayed the highest affinity toward multilamellar vesicles.

The transbilayer movement rate of the various drugs was measured at different drug concentrations, and it was interesting to note the effect of drug concentration on the affinity of the drug toward the vesicles and on the rate of transbilayer movement. As is shown in Fig. 5 for verapamil, at the MDR modulation-relevant concentrations of up to 10 μM, the drug concentration had no effect on either the apparent affinity of the drug for the vesicles or on the rate of transbilayer movement. At higher verapamil concentrations, the total amount of bound drug was reduced, but the equilibration rate was not altered. The passive transport data presented here indicate that modulators traverse the membrane faster than MDR-type drugs. Among the well established substrates of Pgp are two peptide ionophores, valinomycin and gramicidin D (9, 12). The transbilayer movement rate of these substrates had been determined by transport studies taking advantage of their function as ionophores, and thus we examined their ability to modulate Pgp-mediated drug efflux. Valinomycin is a mobile carrier that is freely soluble in the membrane (22), whereas gramicidin D is a channel-forming ionophore with a slow transbilayer flip-flop (23). The ability of these ionophores to modulate Pgp function was tested using the assay of calcein-AM efflux inhibition in EmtR1 cells, essentially as described by Holló et al. (20). The nonfluorescent hydrophobic dye, calcein-AM, diffusing into cells is hydrolyzed to its hydrophilic cognate dye, calcein, which is trapped in the cytoplasm. Calcein-AM extrusion by Pgp from MDR cells prevents hydrolysis and accumulation of its fluorescent hydrophilic product. Modulators that inhibit the Pgp-mediated efflux allow intracellular accumulation of calcein, and thus cellular fluorescence of calcein can serve to monitor Pgp modulation. As shown in Fig. 6, although the modulator, verapamil, inhibits Pgp-mediated efflux in EmtR1 cells, the MDR-type drugs, doxorubicin and taxol, had no effect. The carrier-type ionophore, valinomycin, served as an efficient modulator, whereas the relatively immobile channel-forming ionophore, gramicidin D, behaved as an MDR-drug and had no effect on Pgp-mediated efflux. These results support the rapid transbilayer movement of modulators, unlike MDR-type drugs.
Figure 5. Effect of drug concentration on equilibration kinetics of verapamil with multilamellar vesicles. The experimental conditions were similar to those described in the legend to Fig. 3 except that the vesicles were incubated with the following verapamil concentrations: 20 nM (squares), 1 µM (circles), 10 µM (triangles), 100 µM (inverted triangles), and 1 mM (diamonds).

The transport assays in defined artificial vesicles are not complicated by accumulation in intracellular compartments as is the case in whole cells. Moreover, the conclusions obtained from the in vitro data suggest answers to various puzzling aspects of Pgp function in MDR cells. The wide variability in membrane permeability of MDR-type drugs and modulators suggests a possible role of passive membrane permeability in Pgp-mediated multidrug resistance. The success of Pgp in extruding drugs from cells is determined by its ability to overcome the passive influx rate of the drugs. Pgp, with an estimated turnover number of about 900 min$^{-1}$ (8, 9), can efficiently handle an MDR-drug like rhodamine 123 exhibiting a transbilayer movement with a lifetime of minutes. On the other hand, Pgp is inefficient in protecting MDR cells against molecules freely mobile through biological membranes, like modulators and the carrier-type ionophore, valinomycin, which traverse membranes within microseconds. These relatively fast moving compounds are expected to re-enter and traverse cell membranes faster than their active efflux rate by Pgp.

The Pgp substrate rhodamine 123 traverses artificial lipid membranes slowly with a lifetime of about 3 min. The limiting step in this process is interpreted to be the flip-flop of the dye from one monolayer to the other. The long time periods required for equilibration of MDR-type drugs in multilamellar vesicles indicate that all these drugs exhibit slow transbilayer movement. In contrast to the MDR-type drugs, the MDR-modulators, quinidine and quinine, traverse the membrane with a sub-second lifetime, too fast to be followed by the various methods used here. In this respect, the MDR-type drugs and the modulators resemble the two peptide-ionophore substrates of Pgp, Gramicidin D and valinomycin, respectively (12). Gramicidin D is a hydrophobic peptide that functions as a channel-forming ionophore (24, 25). Taking advantage of its electrical conductance capacity, it has been shown that gramicidin D flip-flops across the membrane with a lifetime of minutes (23). Presumably, the hydrophobic gramicidin D is anchored at the membrane-surface by its relatively hydrophilic hydroxyl group, which prevents it from moving freely across the membrane. On the other hand, valinomycin is a cyclic carrier-type peptide ionophore with a uniform hydrophobic surface and is freely soluble in biological membranes. Although both valinomycin and gramicidin D are soluble in organic solvents, valinomycin traverses the membrane at least $25 \times 10^4$ times s$^{-1}$, i.e., with a lifetime of microseconds (22), whereas gramicidin D flip-flops across the membrane with a lifetime of minutes (23).

The demonstration that MDR-type drugs do not move freely across lipid membranes raises the possibility that the composition of the cell plasma membrane can modulate the transbilayer movement rate of MDR-type drugs across the membrane and consequently affect the "competition" between the active efflux of drugs and the passive uptake. Thus, a conceivable atypical non-Pgp-mediated mechanism of MDR is based on alteration of the lipid composition of plasma membrane leading to reduced passive influx of MDR-type drugs. Changes in lipid order associated with doxorubicin resistance have been described for murine leukemia cells (32). This modality will con-
fer resistance against all MDR-type drugs whose transblayer movement is not free. Recently we (33) have shown that various MDR modulators fluidize artificial membranes as well as biological membranes, a change in membrane structure that could accelerate the transblayer movement of MDR-type drugs. Indeed, we have shown that Pgp modulators increase the sensitivity to MDR-type drugs in both sensitive and MDR cells, presumably through a change in the lipid matrix of the plasma membranes. Changes in lipid structure could confer an extra measure of resistance to MDR cells, in addition to the main effect of modulators, which is the direct inhibition of Pgp.

Fast membrane permeability could be a decisive characteristic of modulators facilitating the overcoming of the cellular defense against xenobiotics mediated by Pgp. The multidrug efflux transporter offers protection of MDR cells against a variety of cytotoxic agents of the MDR group but fails to lower the intracellular concentration of modulators and thus “protect” the cells against inhibition by modulators. Fast influx rates of the modulators can foil the defense efforts of Pgp. A logical conclusion of these possibilities is that the level of Pgp protection against anti-cancer drugs, in addition to other factors, will be inversely correlated with the mobility rate of drugs across membranes. Fast passive influx rate of modulators can explain the seemingly puzzling observation that the modulator, verapamil, and not MDR-type drugs increases cellular ATP consumption by 10% (34). Our data suggest that Pgp extrudes both verapamil and the MDR-type drugs by a similar mechanism and efficiency, but the modulator, verapamil, diffuses back into the cells faster than the MDR-type drugs. Although a similar Pgp-mediated ATP expenditure is required for active transport of drugs across membranes, the amount of cellular ATP consumed in the effort to reduce the level of intracellular verapamil concentration is high compared with MDR-type drugs. At high modulator concentrations the Pgp ATPase functions close to its $V_{\text{max}}$ whereas comparable concentrations of MDR-type drugs are efficiently removed, and the Pgp ATPase functions below its $K_{\text{m}}$. Thus, the modulators seem to act as uncouplers. They stimulate the Pgp ATPase activity, yet there is no net transport, whereas ATP consumption is accelerated by the futile recycling of the modulators.

An important lesson from the results presented here, applicable to all hydrophobic drugs, is that the membrane permeability properties of a drug are not determined solely by its partition coefficient into organic solvents (e.g., n-octanol). The direct correlation of passive transblayer diffusion and partition coefficient was determined with a variety of small molecules (35, 36). Anti-cancer drugs are larger, some of which clearly possess an amphipathic nature that could favor location at the membrane surface and interfere with transblayer movement. Thus, a single hydrophilic domain can hinder transblayer movement of a large hydrophobic molecule, e.g., gramicidin D. Similarly, a single hydroxyl group has been shown to slow the transblayer movement of protonated bile acids by a factor of 20-fold (26).

The wide variability in membrane permeability of seemingly hydrophobic drugs is not limited to the MDR field and could play a major role in a wide variety of physiological and pathological situations. The intracellular concentration of a drug will be determined largely by its membrane permeability rate, where its cellular concentration is a product of a competition between its passive transport and either an active transport process or metabolic processing. The easily performed methods described here for estimation of transblayer movement rate should aid in both defining the role played by passive drug permeability and possible construction of better drugs that efficiently penetrate lipid bilayers.

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