Cell Handling, Membrane-Binding Properties, and Membrane-Penetration Modeling Approaches of Pivampicillin and Phthalimidomethylampicillin, Two Basic Esters of Ampicillin, in Comparison with Chloroquine and Azithromycin

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Purpose. The purpose of this work was to examine and understand the cellular pharmacokinetics of two basic esters of ampicillin, pivaloyloxymethyl (PIVA) and phthalimidomethyl (PIMA), in comparison with lysosomotropic drugs (chloroquine, azithromycin).
Methods. Cell culture studies (J774 macrophages) were undertaken to study uptake and release kinetics and to assess the influence of concentration, pH, proton ionophore (monensin), and MRP and P-gp inhibitors (probenecid, gemfibrozil, cyclosporin A, GF 120918). Equilibrium dialysis with liposomes were performed to directly asses the extent of drug binding to bilayers. Conformational analysis modeling of the drug penetration in bilayers was conducted to rationalize the experimental observations.
Results. PIVA and PIMA showed properties in almost complete contrast with those of chloroquine and azithromycin, i.e., fast apparent accumulation and fast release at 4°C as well as at 37°C, saturation of uptake (apparent K_d 40 μM), no influence of monensin, MRP, or P-gp inhibitors; tight binding to liposomes (K_d approx. 40 μM); and sharp increase in calculated free energy when forced in the hydrophobic domain.
Conclusions. Although they are weak organic bases, PIVA and PIMA show none of the properties of lysosomotropic agents. We hypothesize that they remain locked onto the pericellular membrane and may never penetrate cells as such in significant amounts.

KEY WORDS: pivampicillin; phthalimidomethylampicillin; azithromycin; chloroquine; membrane binding.

INTRODUCTION

Penetration and intracellular accumulation of antibiotics in target tissues has always been considered as an important determinant in their therapeutic activity. In this context, it has been known for long that penicillins, and, generally speaking, all β-lactam antibiotics do not accumulate in cells and tissues (1). All active compounds in this class of antibiotics display a free carboxylic function (or an equivalent proton-donor group) that is essential for binding to their microbial target (2,3). The presence of this acid function may actually explain why these drugs tend to be excluded from acid, membrane-bounded compartments, such as the cell, and still more the lysosomes and other intracellular acid vacuoles (4). We have shown that the masking of this function by a *N*-(3-dimethylaminopropyl) moiety in penicillin G allows the accumulation and the partial localization of the corresponding derivative in the lysosomes of macrophages (5). To be useful for intracellular chemotherapy, this type of basic derivative must, however, regenerate the free β-lactam. The pivaloyloxymethylester of ampicillin (PIVA; Ref. 6) and the phthalimidomethylester of ampicillin (PIMA; Ref. 7) have such property when exposed to aqueous media (8). These compounds are weak bases ($pK_a \sim 6.7$) and are therefore expected to penetrate in cells and to become sequestered in lysosomes by proton trapping, as is observed with many other basic, organic drugs (9). In preliminary experiments, PIVA and PIMA showed indeed a marked apparent cellular accumulation (7). These esters were therefore considered highly promising for tissue and cell-targeted therapy. We, accordingly, decided to examine in details the cell handling of these esters in comparison with two dicationic amphiphilic drugs, chloroquine (a well known antimalaria agent) and azithromycin (a macrolide antibiotic; Ref. 10). Both drugs are known for their large tissue and cellular accumulation. They distribute predominantly in lysosomes (11,12). Quite surprisingly, however, the data show that both esters behave very differently from the lysosomotropic drugs with respect to cell uptake and subcellular handling. Specifically, we observed that these esters remain located at the cell surface with no evidence of true intracellular penetration and accumulation. This unanticipated conclusion has been tentatively rationalized by computer-assisted conformational studies exploring and comparing the membrane-penetrating properties of these ampicillin esters with those of chloroquine and azithromycin.

MATERIALS AND METHODS

Biochemical and Cell Culture Studies

Ester Prodrugs of Ampicillin

Figure 1 shows the structure formulae of ampicillin, the two esters studied, and chloroquine and azithromycin, the two lysosomotropic drugs used for comparison. The synthesis and the main characteristics of PIMA have been described (7,8).

Preparation of the Products for Experiments

Because of intrinsic instability of both ester prodrugs in aqueous solutions, all samples were kept under dry state at 4°C until needed for experiment. An aliquot was then weighted, dissolved in 100% ethanol, and diluted prior use in ice-cold 20 mM sodium acetate buffer (pH 5.4; this low pH is essential to protect the ester against degradation), and then diluted in culture medium to the desired concentration. Chloroquine was diluted from a water stock solution without other specific manipulation. Azithromycin was dissolved in 0.1 N

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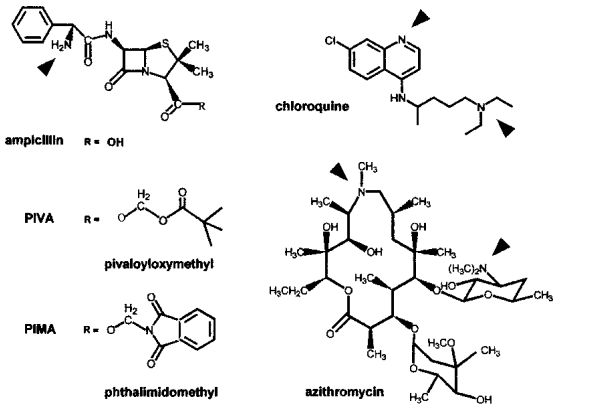

Fig. 1. Structural formulae of ampicillin, pivaloyloxymethylampicillin (pivampicillin), phthalimidomethylampicillin, chloroquine, and azithromycin. The arrows indicate the amino function conferring a basic character to the molecules (see Table I for pK_a values)

HCl at 30 mM (22.5 g/l) as stock solution and diluted at least 100 fold in cultured medium prior use.

Cell Culture, Incubations with Drugs, and Collection

All experiments were performed with J774 macrophages exactly as previously described (5). Experiments were started by gently sucking the cell culture medium and replacing it by a bicarbonate-free medium (buffered at pH 7.4 with 10 mM sodium dihydrogeno/disodium monohydrogenophosphate) containing 10% of fetal calf serum. Cells culture dishes were then transferred to adequate temperature for prewarming or precooling. Aliquots of the stock solution of each drug were then added to the cell culture medium to start the experiments. Efflux pump inhibitors were dissolved in 1 N NaOH or ethanol and thereafter diluted in the culture medium at the appropriate concentration and readjustment to pH 7.4 if necessary. After incubation with the cells, the medium was aspirated and the cell sheet was washed 3-fold with ice-cold phosphate buffered saline, scraped off with a Teflon® policeman, and resuspended quickly in the appropriate media.

Assay of Cell-Associated Drugs and Calculation of an Apparent Celullar to Extracellular Cellular Concentration Ratio

For the two esters, resuspension was made in ice-cold 20 mM sodium acetate buffer pH 5.4. Samples were immediately subjected to brief sonication (10 s, 80 W) at room temperature (Braun Labsonic, Braun Biotech International, Melsungen, Germany) followed by extraction in dichloromethane (dichloromethane/buffer, 4:1, vol:vol, 30 s with vigorous shaking). The aqueous phase was collected and reextracted a second time with dichloromethane, and the two organic phases analyzed separately (all this procedure was performed in less than 30 min). The prodrugs were then assayed by fluorimetry using a procedure that relies on the formation of a fluorescent derivative of β-lactams containing an amino group located in α position on the lateral chain (13). Final readings were made at λ_{exc} = 346 nm and λ_{em} = 422 nm. To correct for incomplete extraction of the esters, all assays included a set of standards prepared in lysates from control cells (i.e., not incubated with the esters). For azithromycin and chloroquine, resuspension was made in water and samples treated by sonication only. Chloroquine was assayed by fluorimetry (λ_{exc} = 335 nm λ_{em} = 378 nm) after precipitation of proteins with trichloroacetic acid and neutralization of the supernatant with concentrated NaOH to reach a pH > 10. Azithromycin was assayed by a microbiological method using *Micrococcus luteus* ATCC 9341 and alkalinized agar. Ciprofloxacin was assayed by radiochemical determination using [^{14}C]-labeled product. Proteins were assayed by the Lowry's assay. All cell drug contents were then expressed by reference to the sample protein content. We then computed an *apparent* cellular to extracellular concentration ratio. This was made by assuming that 1 mg of cell protein corresponded to a cell volume of 5 μL (this factor has been used in all our previous publications dealing J774 macrophages (5,7,12); it also roughly corresponds to what is observed in most cultured cells and tissues). This concentration ratio is only used to compare drugs and to give an idea of how much a given drug is concentrated by cells. It does not imply, as will be demonstrated here, that the drug is intracellular.

Binding of Drugs to Bilayers

We used an equilibrium dialysis approach with liposomes (small unilamellar vesicles) prepared by sonication in 40 mM Tris-maleate buffer pH 7.0. The composition of the liposomes was chosen to mimic the lipid content of plasma membrane (cholesterol:phosphatidylcholine:sphingomyelin:phosphatidylinositol:phosphatidylethanolamine; 5.5:4:1.7:3:2.3, molar ratio). Total phospholipids were assayed by phosphorus assay after complete mineralization, and the concentration of the liposomes adjusted to 10 mM (in phospholipids). Dialysis was performed at 4°C under constant rotation at 12 rpm during 5 h (esters) or overnight (chloroquine and azithromycin). The drug concentration in the chamber containing no vesicles (D_{free}) was measured at the end of the dialysis period in comparison with that of an undialyzed sample (D_{init}). The total concentration of drug in the chamber that contained vesicles (D_{tot}) was then calculated as $D_{init} - D_{free}$. Equilibrium was checked for each run by dialyzing the drugs against liposome-free buffer. The two esters were assayed by high-performance liquid chromatography (Waters Alliance® 2690 Separation module equipped with a Waters 996 Photodiode array detector, Waters Corp., Milford, MA, USA) using an Xterra® RP_{18} 5 μm 4.6 × 150 mm column with a guard column (Xterra® RP_{18} 5 μM 3.9 × 20 mm) with gradient elution (10 mM acetate buffer pH 5:acetonitrile; 9:1 for 3.5 min, linear variation from 9:1 to 2:8 in 6.5 min at a constant flow rate; 1 mL/min). Detection was performed at 220 nm (limit of detection: 25 ng [PIVA] and 5 ng [PIMA], linearity up to 1000 ng [r^2 = 0.999] for both prodrugs, and intraday coefficient of variation of 0.3%) and chloroquine and azithromycin were assayed as described previously for the cell samples.

Determination of Log P and Log D

The partition coefficient of PIVA and PIMA was determined by high-performance liquid chromatography. Reference compounds, chosen for their increasing log p value (benzamide [0.6], benzyl alcohol [1.1], phenol [1.5], nitrobenzene [1.9], benzene [2.1], toluene [2.7], chlorobenzene [2.8], bromobenzene [3], benzophenone [3.2], and naphthalene [3.6]; Ref. 14), were prepared in acetonitrile (1 mg/mL).

PIVA and PIMA were dissolved in ethanol (10 mg/mL) and then diluted in acetonitrile (1 mg/mL). Analysis was performed isocratically using acetonitrile:25 mM phosphate buffer pH 9 mixtures at decreasing volume ratios (50:50 to 35:65 v/v). Both esters ($pK_a \sim 6.7$; see Table I) were therefore under their non-ionized forms. Computation of the log p value was then made by intrapolation as described (15). The distribution coefficient (log D) was then calculated according to the formula $D = P\,(1/[1 + \{[H^+]/Ka_1\}])$ for the esters and $D = P\,(1/[1 + \{[H^+]/Ka_2\} + \{[H^+]^2/Ka_1.Ka_2\}])$ for chloroquine and azithromycin where Ka_1 and Ka_2 are the acidic constant of corresponding amino groups.

Materials

PIVA (99.5% purity and complying with the specifications of the Pharmacopée Européenne [3d ed. suppl. 2000, p 1076]) was obtained from Leo Laboratories Ltd, (Dublin, Ireland) on behalf of Leo Pharmaceuticals Product Ltd A/S (Ballerup, Denmark). PIMA was obtained as the chloride salt with a purity of > 95% (see details in Ref. 8). Azithromycin (dihydrate free base for microbiological standard; purity 94%) was the gift of Pfizer (Brussels, Belgium) on behalf of Pfizer Inc. (Groton, CT, USA). GF 120918 (*N*-[4-[2-(3,4-dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)ethyl]phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridinecarboxamide; Elacridar®) was donated by GlaxoWellcome Research and Development (Laboratoire GlaxoWellcome, Les Ulis, France). [^{14}C]-labeled ciprofloxacin (72.2 μCi/mmol; radiochemical purity, 85%, based on TLC analysis) and ciprofloxacin (microbiological standard; 85.5% potency) was obtained from Bayer AG (Leverkusen, Germany). Cyclosporin A was a product from Fluka Chemie (Buchs, Switzerland). Monensin, chloroquine, sphingomyelin, cholesterol, benzyl alcohol, phenol, probenecid, and gemfibrozil were purchased from Sigma-Aldrich Co (St Louis, MO, USA), and egg phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine were purchased from Lipid products (Redhill, Surrey, UK). Cell culture media and sera were from Life Technologies (Paisley, UK). All other reagents were from E. Merck (Darmstadt, Germany).

Conformational Analyses

We used a sequential procedure implying the 3D construction of the molecules and the modeling of the interactions between the drugs with a bilayer (IMPALA; Refs. 16,17). Ampicillin 3D models were obtained from its crystallographic structure (18) and the same data used to construct the 3D structures of PIVA and PIMA with the help of the Hyperchem 5.0 software. Chloroquine 3D model was constructed *de novo* using Hyperchem 5.0 software. Azithromycin was modeled from its crystallographic structure (19). All molecules were used under their ionized forms. Diagrams showing the restraint values as a function of the penetration of the mass center were drawn by plotting the lowest value obtained during the Monte Carlo simulation for each inward movement of 0.1 Å of the molecule within a bilayer. All points are then joined to generate a profile of the simulation.

RESULTS

Biochemical and Cell Culture Studies

We first validated our model by showing that ampicillin was not accumulated by J774 macrophages. For this purpose, cells were incubated for 5 h with ampicillin at increasing extracellular concentrations (10–100 mg/L; 27–270 μM). We observed an apparent cellular to extracellular drug concentration ratio of 0.4 ± 0.09 (n = 18). Kinetic studies revealed also that this ratio was reached after 4 h and remained stable thereafter. In contrast, both PIVA and PIMA showed a rapid and extensive uptake, which was then studied in comparison with chloroquine and azithromycin as, described below.

Accumulation and Release of PIVA and PIMA

Figure 2 shows the kinetics of uptake of PIVA, PIMA (both at 20 mg/L), chloroquine (13 mg/L), or azithromycin (25 mg/L) over time. Within 15 min, PIVA and PIMA reached an apparent cellular to extracellular concentration ratio of approximately 30- to 40-fold. Quite surprisingly, however, these ratios were essentially similar whether incubation was performed at 37°C or at 4°C. When the incubation time was

Table I. Physicochemical Parameters of Ampicillin, PIVA, PIMA, Chloroquine, and Azithromycin of Interest for the Present Study

	Ampicillin	PIVA	PIMA	Chloroquine	Azithromycin
log P (neutral form)	na	3.64[a]	3.22[a]	4.32[b]	4.04[c]
Log D (at pH 7.4)	−1.33[d]	3.56[e]	3.14[e]	0.12[e]	1.92[e]
H donors	4[f]	3[f]	3	1[f]	5[f]
H acceptors	7[f]	9[f]	11	3[f]	14[f]
Mw	349[f]	464[f]	509	320[f]	749[f]
pK_a	6.79[f]	6.72[f]	~6.72[g]	8.3[h]	8.1[c]
				10.48[h]	8.8[c]

Note: na, non applicable.
[a] Determined as described in the Materials and Methods section.
[b] From Ref. 33.
[c] Communicated by Pfizer Research Laboratories, Pfizer Inc, Groton, CT, USA.
[d] From Ref. 34.
[e] Calculated as described in the Materials and Methods section.
[f] Data from SciFinder Scholar version 2001, American Chemical Society, 2001.
[g] Assumed from data of PIVA.
[h] From Ref. 35.

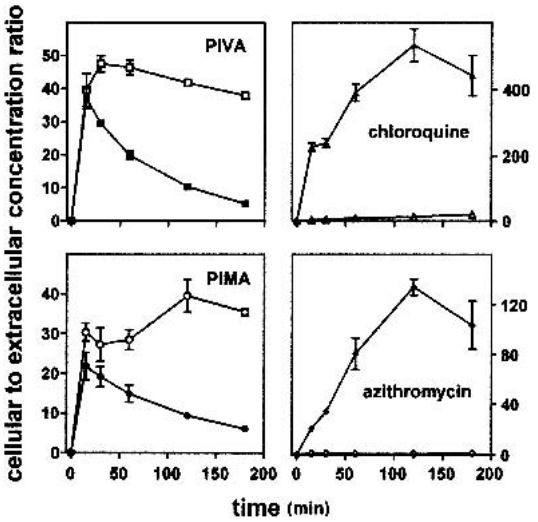

Fig. 2. Uptake of PIVA (20 mg/L, 43 μM), PIMA (20 mg/L, 39 μM), chloroquine (13 mg/L, 41 μM), and azithromycin (25 mg/L, 32 μM). Open symbols indicate incubation at 4°C; closed symbols indicate incubation at 37°C. The ordinate shows the apparent cellular to extracellular concentration ratio (accumulation factor) for each compound studied. Each value is the mean of three dishes (± SD; points without visible errors bars correspond to values for which this error bar is smaller than the symbol).

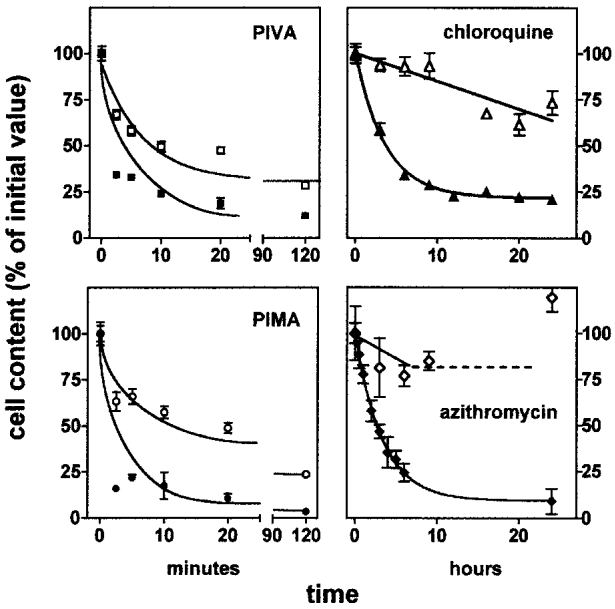

Fig. 3. Efflux of PIVA, PIMA, chloroquine, and azithromycin over time. Cells were incubated at 37°C with PIVA (20 mg/L, 43 μM) or PIMA (20 mg/L, 39 μM) for 15 min, or with chloroquine (13 mg/L, 41 μM) or azithromycin (25 mg/L, 32 μM) for 2 h. They were then transferred to drug-free medium for further incubation at 37°C (closed symbols) or at 4°C (open symbols). All values are given as percent of the amount of product accumulated after the first incubation period. Values are means of three dishes (± SD; points without visible errors bars correspond to values for which this error bar is smaller than the symbol).

prolonged, the apparent accumulation levels of both PIVA and PIMA declined markedly at 37°C to reach values lower than 10-fold at 3 h. In contrast, the apparent accumulation levels reached after 15 min at 4°C remained essentially unchanged upon prolonged incubation at this temperature. In contrast with the two ampicillin esters, chloroquine and azithromycin both showed a steady but slower kinetic of uptake at 37°C. A maximum was reached only after 2 h and at a considerably higher level than observed for the esters (apparent cellular to extracellular concentration ratios of 500 and 120 vs. 30–40). No or only minimal uptake took place at 4°C.

Both PIVA and PIMA were known to be unstable at 37°C in aqueous media (8). We reasoned that the lack of maintenance of a stable cellular level of both esters at 37°C could merely result from their extracellular hydrolysis, causing the ensuing displacement of cell-associated product. The stability of the esters in the culture medium at 37°C was therefore measured. We observed that both esters were quickly degraded at 37°C ($t_{1/2}$ of 36 and 38 min for PIVA and PIMA, respectively) but much more slowly at 4°C ($t_{1/2}$ ~ 13 h and ~40 h).

In the next series of experiments, we examined the release of PIVA and PIMA accumulated by cells at 37°C (15 min) upon subsequent reincubation at 37°C or at 4°C in ester-free medium. As shown in Fig. 3, the release of both PIVA and PIMA was very rapid not only at 37°C but also at 4°C (with about a 50% release within 20 min at either temperature). In contrast, the release of both chloroquine and azithromycin (2 h accumulation at 37°C) was considerably slower at 37°C (50% release in 2–4 h) and very slow (or even not observed for azithromycin) at 4°C.

Saturability of Uptake

The saturability of apparent accumulation of the esters in cells was examined in comparison with that of chloroquine and azithromycin over a wide range of extracellular concentrations (0–155 μM). Figure 4A shows that amount of PIVA or PIMA that could be found associated to cells (measured at 15 min) was saturable within that range but not for that of chloroquine or azithromycin (measured at 2 h).

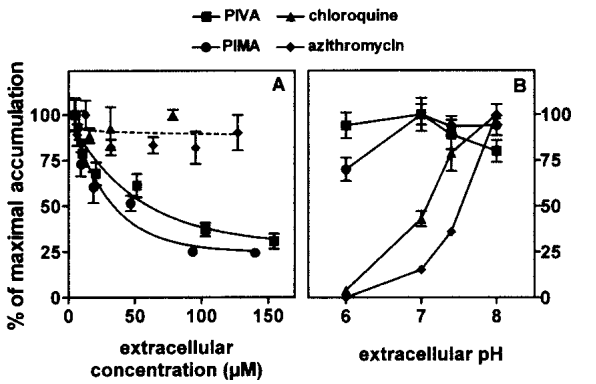

Fig. 4. (A) Apparent cellular to extracellular concentration ratios of PIVA, PIMA (15 min incubation each), or chloroquine, or azithromycin (2-h incubation each) at 37°C and at increasing extracellular concentrations (0–155 μM). The ordinate shows the value observed at each experimental points as percentage of the maximum. (B) Influence of pH on the uptake of PIVA (20 mg/L, 43 μM) or PIMA (20 mg/L, 39 μM; 15-min incubation for both), or chloroquine (13 mg/L, 41 μM) or azithromycin (25 mg/L, 32 μM; 2-h incubation for both) at 37°C. All values in both panels are means of 3 dishes (± SD; points without visible errors bars correspond to values for which this error bar is smaller than the symbol).

Modulation of Uptake by Acid pH

The amount of weak organic bases that cells are capable to accumulate is decreased if incubation is carried out at acid pH as was indeed demonstrated for azithromycin in J774 macrophages (12). We therefore compared PIVA and PIMA to chloroquine and azithromycin in this respect. Experiments were only conducted at 37°C in view of the lack of accumulation of chloroquine and azithromycin at 4°C. As shown in Fig. 4B, acid pH did not exert a marked effect on PIVA and PIMA apparent accumulation (15 min incubation time) whereas it completely abolished the apparent accumulation of both chloroquine and azithromycin.

Influence of Monensin and Inhibitors of Efflux Pumps

The proton ionophore monensin (20 μM) was thereafter used to explore the influence of transmembrane pH gradients on the handling of the esters. Monensin inhibited PIVA and PIMA uptake to 70 and 45% respectively (15 min incubation) but almost completely suppressed that of both chloroquine and azithromycin (> 95% inhibition, 2-h incubation). The handling of ampicillin esters was also examined in the presence of preferential inhibitors of the MRP (probenecid 5 mM; Ref. 20 and gemfibrozil 200 μM; Ref. 21) or of the P-gp (cyclosporine A 50 μM; Ref. 22 and GF 120918 200 nM; Ref. 23) efflux transporters. No influence of these inhibitors was observed. In parallel, we checked that probenecid and gemfibrozil increased the accumulation of the fluoroquinolone antibiotic ciprofloxacin, and cyclosporin and GF 120918 that of azithromycin (which, in J774 macrophages, are substrates of the MRP and of the P-gp efflux transporters, respectively; Refs. 24,25)

Biophysical and Conformational Studies

Determination of Log P and Log D Values

PIVA and PIMA showed log P values of 3.64 and 3.22, respectively. These values were then used to calculate the corresponding log D at pH 7.4 (3.56 and 3.14). A comparison with the corresponding data for chloroquine and azithromycin (Table I) indicates that all four drugs have globally a similar lipophilicity under their neutral form, but that chloroquine and azithromycin appeared considerably less lipophilic at pH 7.4.

Binding to Membrane Bilayers

Figure 5 and Table II show that both PIVA and PIMA bind tightly to lipid vesicles at pH 7.0 with similar kinetic parameters. Interestingly enough, half-saturation of binding was observed in a range of concentrations (approx. 40 μM) close to that observed for saturation of accumulation in the cell uptake studies described above. Variation of the vesicles content in phosphatidylinositol (from 4.5 to 18% of total phospholipids) did not markedly affected the dissociation constant but increased the binding capacity almost in direct proportion. Comparative binding studies of chloroquine and azithromycin, made at fixed drug concentrations (81 and 64 μM, respectively) revealed a considerably lower binding (Fig. 5; kinetics studies proved difficult to perform in details because of this low affinity).

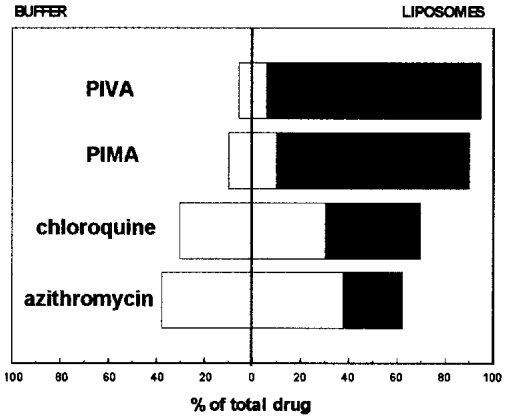

Fig. 5. Equilibrium dialysis of PIVA (40 mg/L, 86 μM), PIMA (40 mg/L, 78 μM), chloroquine (26 mg/L, 81 μM), and azithromycin (50 mg/L, 64 μM) against liposomes (10 mM total phospholipids, see composition in Materials and Method). Open bars, free drug (determined at equilibrium); closed bar, bound drug (calculated from the difference between measured free drug in the liposome-free compartment and initial drug concentration).

Conformational Studies

The most probable conformers of the ionized forms of ampicillin, PIVA, PIMA, chloroquine, and azithromycin, as equilibrated within an imaginary layer of phosphatidylcholine, are shown in Fig. 6 together with the profile of the minimal restraint values vs. the penetration of each compound into a bilayer. Ampicillin, PIVA and PIMA were clearly located at or close to the hydrophilic-hydrophobic interface with the phenylacetamido moiety (for all three drugs molecules, and the phthalimidomethyl or the pivaloyloxymethyl moiety for the esters) oriented toward the hydrophobic domain. The uncharged forms of the same molecules displayed almost the same conformation and disposition with respect to the interface, indicating that the charge itself was not critical for this behavior. In contrast, chloroquine was entirely equilibrated within the hydrophobic domain, and azithromycin, although spanning the hydrophilic/hydrophobic interface, was also equilibrated deeply within the hydrophobic domain (as previously described for phosphatidylinositol monolayers; Ref. 26). The Impala procedure was then used to explore in a dynamic way the change in energy behavior of the five compounds when moving from the hydrophilic toward the hydrophobic domain. As shown in the graphs adjacent to each conformational model, striking differences were seen between 1) ampicillin, 2) the two esters, and 3) chloroquine and azithromycin, respectively. Ampicillin showed a minimum of energy in the hydrophilic region but the level of energy rose mark-

Table II. Binding Parameters of the Prodrugs to Liposomes of Increasing Phosphatidylinositol Content

Phosphatidylinositol content[a]	K_d (μM)		B_{max} (μM)	
	PIVA	PIMA	PIVA	PIMA
4.5	27.8 ± 9.8	22.2 ± 10.4	108.0 ± 11.4	89.9 ± 11.7
9	37.8 ± 5.6	40.4 ± 9.4	259.7 ± 18.4	196.8 ± 20.7
18	34.4 ± 9.5	55.8 ± 7.4	568.3 ± 71.3	552.0 ± 37.5

[a] % of total phospholipids.

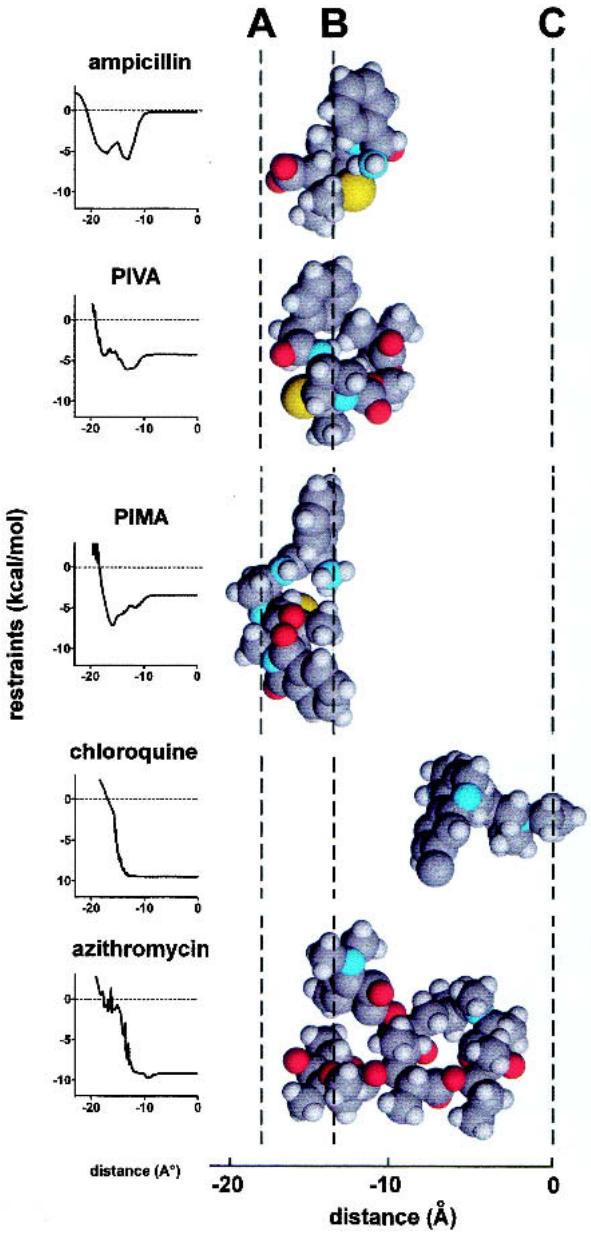

Fig. 6. Left panel: Profile of the minimal restraints values vs. the penetration of the molecule inside the bilayer (The abscissa values between –18 Å and –13.5 Å correspond to the hydrophilic domain whereas the hydrophobic domain spreads from –13.5 Å to 0 Å. Right panel: Most probable conformers of ampicillin, PIVA, PIMA, chloroquine, and azithromycin under their protonated forms at a hydrophilic-hydrophobic interface mimicking a phospholipid bilayer of a global neutral charge. The hydrophilic domain is comprised between the two dotted lines marqued A and B (at –18 Å and –13.5 Å). The dotted line marked C (at 0 Å) is the center of the bilayer. (A larger-sized, full-color version of this figure can be downloaded from <http://www.md.ucl.ac.be/facm/Chanteux-Pharm-Res-2003>).

edly when the molecule was forced into the hydrophobic domain (increase of 5.82 kcal/mol from its minimal). PIVA and PIMA showed also a low level of energy in the hydrophilic domain and a rise in energy when forced in the hydrophobic domain, although this rise was less marked than that of ampicillin (increases of 1.84 and 3.6 kcal/mol for PIVA and PIMA, respectively). In sharp contrast, however, the energy of both chloroquine and azithromycin decreased steadily when moving from the hydrophilic to the hydrophobic domain, reaching a minimum when entering the latter domain and remaining almost stable thereafter (with only a modest increase of 0.56 kcal/mol for azithromycin). Thus, both chloroquine and azithromycin explored a considerably wider conformational space during the simulation.

DISCUSSION

PIVA has been classically viewed as a lipophilic and therefore as a highly diffusible prodrug of ampicillin, which is converted into ampicillin upon absorption and transport in the body (6). PIVA indeed provides earlier and higher peak concentrations of ampicillin than administration of ampicillin it-self (27,28). As a weak organic base, PIVA was also expected to accumulate in cells and to become sequestered in lysosomes. This behavior is indeed observed with many other compounds with similar biophysical properties that are collectively regrouped under the name of lysosomotropic agents (9). These include chloroquine (11) and azithromycin (12). The same behavior was also observed for a basic derivative of penicillin G (5). We now show, however, that PIVA, and the closely related PIMA, behave, in almost all respects, in complete contrast with chloroquine and azithromycin. Key differences bear upon 1) the rate, extent and reversibility of uptake at 4°C and 37°C; 2) the saturation of uptake at low concentrations (~40 μM); 3) the influence of a variation of the extracellular pH and of monensin; and 4) the binding to lipid bilayers at neutral pH. These data strongly suggest that PIVA and PIMA merely bind to the cell membrane and never, actually, penetrate cells (we know that azithromycin and chloroquine cross the pericellular membrane to reach the cytosol and to be subsequently accumulated in lysosomes and other acidic vacuoles). We, unfortunately, could not, in the present study, examine directly the subcellular distribution of PIVA and PIMA by cell fractionation techniques (an approach widely used to identify the storage site(s) of drugs in cells) because of the fragility of the esters in aqueous media and their fast release from cells even at 4°C.

Two successive steps are usually involved in the interaction of a cationic amphiphilic drug with bilayers, namely 1) an electrostatic interaction between the positively charged amino group of the drug and the negatively charged phospho groups in the phospholipids, and 2) an hydrophobic interaction of the lipophilic moieties of the drug with the hydrocarbon chains of the fatty acids. Translocation of molecules from the outer to the inner part of the monolayer appears to be a consequence of the stress induced by the asymmetric uptake of solutes into membranes (29). It is dictated by critical physicochemical parameters, and most notably by the lipophilicity, the charge, the molecular size, and the H-bonding capacity. The values of these parameters, for each drugs studied here, are shown in Table I. Molecular size and H-bonding capacity can be ruled out since no consistent ranking among the four compounds studied can be made on this basis. Lipophilicity, based on log P determination (isotropic model) seems also ruled out because ranking of drugs on this basis is inconsistent with our results. However, drug interactions with the biological membrane are complex and are difficult to be mimicked by an isotropic model (30). Correction for ionization (log D values [isotropic model]) actually shows that PIVA and PIMA are

slightly more lipophilic than chloroquine and azithromycin at pH 7.4. Data from the equilibrium dialysis experiments (which mimic an anisotropic model) strongly support this conclusion. Our results, therefore, are in agreement with the general conclusion drawn from the pioneering work of Herbette's group (30), who showed that anisotropic models are more adequate to predict interaction between drugs and biological membranes because the pK of an ionizable drug (and therefore its log D value) could be quite different following its partitioning between a membrane hydrocarbon core (where the dielectric constant is ~3) and water (dielectric constant of 80). Actually, the computer-aided modeling of the dynamics of the penetration of the drugs in bilayers shows that the ampicillin esters, in contrast to chloroquine and azithromycin, are largely unable to enter in the hydrophobic domain. We interpret this as indicating that stereochemical factors could have a considerable influence on the interaction of solutes with phospholipid membranes.

Examining our data globally, we suggest first that ampicillin esters do not accumulate within cells (although we cannot, strictly speaking, exclude that the esters eventually penetrate cells but are then quickly degraded, thereby escaping detection). Yet, in any event, these esters are not lysosomotropic agents. This leads us to our first conclusion, which is that other properties than those deriving directly from a weak organic base character must be taken into account to correctly predict the cellular handling and intracellular disposition of drugs. We need also to explain how PIVA, as a prodrug of ampicillin, gives rise to higher serum and tissue concentrations of ampicillin than ampicillin it-self after oral administration if it does not diffuse across epithelial barriers as originally suggested. Our results would indeed suggest that PIVA (and probably other similar esters, including PIMA) would mainly bind to cell surface of enterocytes. Yet, being particularly unstable at neutral and alkaline pH, these esters may release and create large local concentrations of ampicillin. The latter may then be efficiently transported through H^+/peptide symporter known to be present in these cells (31) and of which ampicillin is a substrate (32). This leads us to our second conclusion which is the fate of drugs *in vivo* must be examined and analyzed much beyond conventional lipophilicity/hydrophilicity considerations. Progresses in this area may help in a better understanding of the pharmacokinetic profile of drugs, including tissular and intracellular penetration, subcellular distribution and, globally speaking, bioavailability.

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