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Accumulation and Oriented Transport of Ampicillin in Caco-2 Cells from Its Pivaloyloxymethylester Prodrug, Pivampicillin

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Pivampicillin (PIVA), an acyloxymethylester of ampicillin, is thought to enhance the oral bioavailability of ampicillin because of its greater lipophilicity compared to that of ampicillin. The fate of PIVA in intestinal cells and the exact location of its conversion into ampicillin have, however, never been unambiguously established. Polarized Caco-2 cells have been used to examine the handling of PIVA and the release of ampicillin from PIVA by the intestinal epithelium. Experiments were limited to 3 h. Cells incubated with PIVA (apical pole) showed a fast accumulation of ampicillin and transport toward the basolateral medium, whereas PIVA itself was only poorly accumulated and transported. Cells incubated with free ampicillin accumulated and transported only minimal amounts of this drug. Release of ampicillin from cells incubated with PIVA was unaffected by PEPT1 and OCTN2 inhibitors but was sharply decreased after ATP depletion or addition of bis(4-nitrophenyl)-phosphate (BNPP; an esterase inhibitor). PIVA incubated with Caco-2 lysates released free ampicillin, and this release was inhibited by BNPP. Efflux studies showed that the ampicillin that accumulated in cells after incubation with PIVA was preferentially transported out of the cells through the basolateral pole. This efflux was decreased by multidrug resistance-associated protein (MRP) inhibitors (probenecid, MK-571) and by ATP depletion. A phthalimidomethylester of ampicillin that resists cellular esterases failed to cause any significant release (cell lysate) or transport (polarized Caco-2 cells) of ampicillin. These results show that when PIVA is given to Caco-2 cells from their apical pole, ampicillin is released intracellularly and that ampicillin is thereafter preferentially effluxed into the basolateral medium through an MRP-like transporter.

Pivampicillin (PIVA; the pivaloyloxymethylester of ampicillin) is one of the acyloxymethylesters of penicillins made originally to improve the oral bioavailability of ampicillin (46). The basic underlying concept was to obtain more lipophilic derivatives since lipid solubility was considered an essential determinant in the capacity of drugs to diffuse across biological membranes and through the intestinal barrier in particular. Among various potential candidates, PIVA was found (i) to give rise to elevated serum ampicillin concentrations after oral administration to rats with little or no circulating unhydrolyzed ester and (ii) to be quickly split off enzymatically by both serum and tissue esterases. It was concluded that PIVA diffuses through the intestinal cells. Neither its fate within intestinal cells nor the exact location (tissue or plasma) of its conversion into ampicillin, however, has been determined.

In the course of an ongoing program aimed at designing new prodrug esters of ampicillin, we reexamined the available evidence and found three elements that suggested to us that the delivery of ampicillin in serum from orally administered PIVA could rely on mechanisms more complex than those that were originally foreseen.

First, the discoverers of PIVA themselves noted that more lipophilic acyloxymethylesters of penicillins, such as those obtained with nonaminated penicillins (benzylpenicillin, methicillin, cloxacillin), had lower oral bioavailabilities than those obtained from ampicillin (45). Second, a simple diffusion model would predict that PIVA, as a weak organic base, would accumulate in cells and that once it was in the cells it would be sequestered in lysosomes and other acidic, membrane-bounded organelles (15), as is the case for other basic drugs such as chloroquine (50) and macrolide antibiotics (8, 9). When this property was specifically looked for in cultured macrophages, however, we observed that PIVA was merely bound to the pericellular membrane, with no evidence of true intracellular penetration and lysosomal accumulation (11). Third, studies with *Listeria*-infected macrophages showed that macrophages incubated with low concentrations of PIVA accumulated large amounts of free ampicillin (which was active against this intracellular organism), whereas no ampicillin (and no antibacterial activity) could be seen when cells were incubated with similar concentrations of ampicillin (10).

This eventually led us to directly reexamine the transport and ampicillin delivery capabilities of PIVA using as controls ampicillin itself and its phthalimidomethylester (PIMA; this compound is not an acyloxymethylester and, therefore, is less susceptible to hydrolysis by serum and cell esterases (36). Both PIVA and PIMA are weak bases and are therefore positively charged at neutral pH. In contrast, ampicillin, which carries a basic and an acidic function, is zwitterionic under the same conditions.

We elected to work with Caco-2 cells because (i) these cells have been extensively used to characterize the transintestinal absorption of many compounds (35, 47, 52) and (ii) they exhibit many morphological and functional similarities to the human small intestinal epithelial cells when they are grown as polarized cells (25) and express several drug transporters, the

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roles of which can be studied with inhibitors. On the basis of the structural determinants and general properties of ampicillin and the two prodrugs studied, we concentrated on two influx transporters, namely, the proton-coupled oligopeptide transporter PEPT1 (1), which recognizes beta-lactams due to their peptide-like structures (7, 19, 48, 49), and the organic cation/carnitine transporter OCTN2 (33). We also examined the ATP-dependent efflux transporters expressed at either the apical or the basolateral membrane, namely, the multidrug resistance protein (MDR1; P-glycoprotein) and the multidrug resistance-associated proteins (MRPs), which preferentially transport cationic and anionic amphiphiles respectively.

Our studies suggest that PIVA is modestly accumulated by Caco-2 cells from their apical side but that the accumulated PIVA quickly gives rise to large intracellular concentrations of ampicillin through the actions of cytosolic esterases. Cell-associated ampicillin is, thereafter, preferentially released from the basolateral pole through an MRP-like transporter.

MATERIALS AND METHODS

Preparation of products for experiments. Because of the intrinsic instabilities of both ester prodrugs in aqueous solutions, all samples were kept under a dry state at 4°C until they were needed for experiments. An aliquot was then weighed, dissolved in 100% ethanol, and diluted prior to use in ice-cold modified Krebs buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 10 mM glucose) containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0) and was then further diluted in the same medium to the desired concentration at the beginning of the experiment. To ensure that only intact prodrug was put into contact with the cells at the onset of our experiments, all these manipulations were performed in less than 5 min and all solutions were kept at 4°C until use.

Cell culture. Caco-2 cells, which originated from a human colonic carcinoma, undergo in vitro epithelial differentiation and, upon reaching confluence, express characteristics of enterocytic differentiation (54) and form continuous monolayers of polarized cells (21). They were obtained from the American Type Culture Collection (Manassas, Va.) as ATCC HTB-37 and were routinely cultured in 175-cm^2 culture flasks with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, and 1% L-glutamine in an atmosphere of 5% CO_2–95% air with 90% relative humidity at 37°C. For preparation of monolayers, the cells were trypsinized, centrifuged, resuspended in culture medium, and seeded on polyethylene membranes (pore size, 0.4 μm; 4.15 cm^2; Falcon; Difco-Becton Dickinson and Co., Sparks, Md.) at a density of 6×10^4 cells/cm^2. The culture medium was changed every 2 to 3 days, and the cells were maintained for 21 days to form a complete monolayer. The medium was then changed, and the transepithelial electrical resistance was measured 24 h later with a Millicell-ERS device (Millipore Corp., Bedford, Mass.) to check the integrity of the monolayer.

Uptake and transport studies. Modified Krebs buffer containing 25 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0; apical medium) or 25 mM HEPES (pH 7.4; basolateral medium) was used for all studies. Experiments were performed as follows. Each side of the monolayers was first washed twice with the appropriate medium, and the monolayers were then preincubated with the medium for 30 min at 37°C under gentle agitation by placing the culture plate on a controlled-environment shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) operated at 60 rpm. For the accumulation studies, 100 μl of stock solution of the prodrugs or ampicillin was added to the apical medium to initiate the experiment. At appropriate times, cells were collected by washing the monolayer three times with ice-cold phosphate-buffered saline, followed by scraping the cells into 250 μl of a mixture of methanol-water (70:30; vol/vol) at −20°C (2). We checked that no degradation of PIVA or PIMA occurred during this step of cell collection (recovery, >95%). A similar protocol was followed to initiate the transport studies. At appropriate times, 150-μl aliquots of the apical or basolateral medium were taken and replaced by fresh medium (this dilution factor was taken into account for the calculation of the actual drug concentrations). Each sample from the basolateral medium was immediately mixed with 15 μl of 0.1 N HCl to bring the pH to approximately 6 in order to avoid PIVA or PIMA degradation. All samples were then stored at 4°C before analysis by high-performance liquid chromatography (HPLC). The flux of mannitol through the Caco-2 cell monolayer (considered to represent paracellular leakage) was estimated by adding [^{3}H]D-mannitol (5 μM) to the apical side. Samples were taken from the basolateral side at appropriate times. Radioactivity was determined by liquid scintillation counting.

PIVA and PIMA degradation and ampicillin release at pH 7.4. Samples were prepared in the same buffer used for the transport studies and were either left as such (buffer alone) or mixed with the Caco-2 cell lysate (final protein concentration, 2 mg/ml). These lysates were obtained by harvesting monolayers in buffer and disrupting the cells by sonication (10 s at 80 W and 4°C). Samples collected at appropriate times were mixed with cold methanol-water (2:1) and kept at 4°C until analysis. Degradation of produgs was evaluated by measurement of the disappearance of the corresponding peak in HPLC recordings.

Analyses. Ampicillin, PIVA, and PIMA were assayed as described previously (11) by HPLC (Alliance 2690 separation module equipped with a 996 photodiode array detector [Waters Corp., Milford, Mass.]). All samples were subjected to centrifugation (14,000 rpm, 6 min, 4°C; Eppendorf 5415C centrifuge [Eppendorf AG, Hamburg, Germany]) prior to analysis. Typical retention times were 6.6, 10.3, and 10.1 min for ampicillin, PIVA, and PIMA, respectively. Assay parameters were as follows: lowest limits of quantification, 20 ng for ampicillin, 25 ng for PIVA, and 5 ng for PIMA; intraday coefficient of variation, <1%. Figure 1 shows an overlay chromatogram demonstrating the relationships between the peak area and the drug concentration for both ampicillin and PIVA (similar results were obtained with ampicillin and PIMA [data not shown]). There were no spurious peaks that interfered with those of ampicillin, PIVA, or PIMA in any of the samples analyzed. Spectral analyses of each peak were systematically made for all critical experiments, and the results did not show significant differences in comparison with the results for genuine samples or for ampicillin, PIVA, or PIMA. Total cell ATP was assayed as previously described for cultured fibroblasts (43) by using the ATP-dependent oxidation of D-luciferin by luciferase (Boehringer Manheim ATP-bioluminescence assay kit CLS II; Roche Diagnostics, F. Hoffmann-La Roche Ltd., Basel, Switzerland). Readings were made with a WALLAC type 1410 liquid scintillation counter (Perkin-Elmer Life Science, Boston, Mass.). Cell protein was assayed by the Folin-Ciocalteu biuret method (29) with serum albumin as the standard.

RT-PCR. Total RNA was isolated from Caco-2 cells with the TRIzol reagent, and 2 μg of total RNA was used for the reverse transcriptase reaction. Reverse transcription-PCR (RT-PCR) was performed with a portion of the reverse transcriptase-generated cDNA and primers specific for PEPT1 (sense primer, 5′-ATACTTTTAACGAGCTCAT-3′; antisense primer, 5′-TGCTGAACTGGCCTGCCCCT-3′) and OCTN2 (sense primer, 5′-CATCTTTGTGAACTGCTTCCTTTC-3′; antisense primer, 5′-TGACTCCAAACTTGCCCACCATCA-3′) (Invitrogen Corp. Carlsbad, Calif.). The PCR was performed by using optimized conditions (for PEPT1, denaturation at 94°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 30 s through a total of 35 cycles; for OCTN2, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s through a total of 35 cycles) with a thermocycler (Gene Cycler model; Bio-Rad Laboratories, Hercules, Calif.). The reaction products were electrophoretically separated in a 2% agarose gel. Ethidium-stained bands were detected with a Gel Doc 2000 apparatus (Bio-Rad Laboratories).

Materials. PIVA (of 99.5% purity and in compliance with the specifications of the *Pharmacopée Européenne* [3rd ed., suppl. 2000, p. 1076]) was obtained from Leo Laboratories Ltd. (Dublin, Ireland) on behalf of Leo Pharmaceutical Products Ltd. A/S (Ballerup, Denmark). PIMA was synthesized at the Unité de Chimie Pharmaceutique of our university (18) and was obtained as the chloride salt (purity, >95%). Strawberry and banana extracts were kindly donated by P. Augustijns (Laboratory for Pharmaceutical Technology and Biopharmacy, Catholic University of Louvain, Louvain, Belgium). Gly-sarcosine (Sar), Gly-Leu, captopril, tetraethylammonium, quinidine, L-carnitine, sodium azide, monensin, ampicillin, and the TRIzol reagent were purchased from Sigma-Aldrich Co. (St. Louis, Mo.); verapamil and 2-D-deoxy-glucose were from Fluka Chemie (Buchs, Switzerland); and [^{3}H]D-mannitol were from NEN (Perkin-Elmer Life Sciences, Boston, Mass.). ^{3}H-labeled Gly-Sar was obtained from Amersham BioScience (Piscataway, N.J.). Rabbit polyclonal anti-OCTN2 antibody (raised against a 17-amino-acid sequence in the cytoplasmic C-terminal region of OCTN2) was purchased from Alpha Diagnostic International (San Antonio, Tex.). Cell culture media and sera were from Life Technologies (Paisley, United Kingdom). All other reagents were from E. Merck AG (Darmstadt, Germany).

RESULTS

Characterization of Caco-2 cells. Confluent Caco-2 cell monolayers were obtained within 21 days of culture with a resistivity

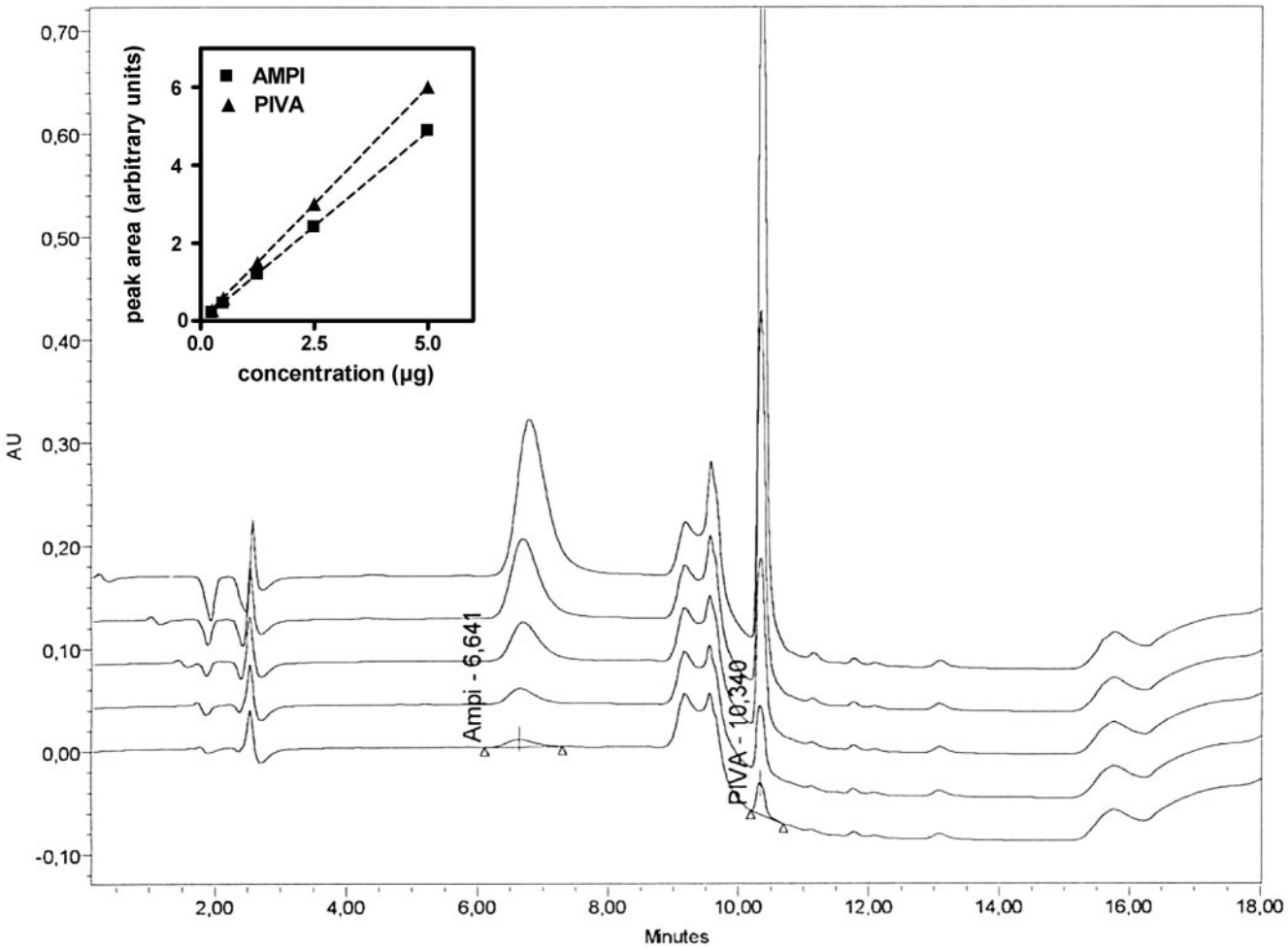

FIG. 1. Overlay of five chromatograms of ampicillin (AMPI) and PIVA standards at increasing concentrations (from 250 ng to 5,000 ng). Typical retention times: ampicillin, 6.64 min; PIVA, 10.34 min. The inset shows the correlation between the peak area and the concentration (R^2 = 0.999). AU, arbitrary units.

of $\geq$300 $\Omega \cdot cm^2$ and a permeability to [^{3}H]mannitol of less than 0.25% $\cdot$ $cm^{-2} \cdot h^{-1}$, as in previous studies (26). mRNAs of PEPT1 and OCTN2 transporters were clearly detected in our cells by RT-PCR analysis. Uptake experiments with ^{3}H-labeled Gly-Sar, a known substrate of PEPT1, showed a saturable process, with K_m values (1.38 $\pm$ 0.24 mM) similar to those reported by others (32, 43). Competitive inhibition studies also revealed that an excess of unlabeled Gly-Sar or that the addition of Gly-Leu and ampicillin inhibit the uptake of ^{3}H-labeled Gly-Sar by Caco-2 cells (data not shown). This analysis revealed a strong band compatible with the known molecular weight of this transporter (data not shown), as recently found by others (16). As a negative control, the expression of OCTN2 at the protein level was also checked by Western blot analysis with THP-1 cells (a human macrophage cell line; as far as is known, OCTN2 is expressed only in epithelial cells). Caco-2 cells have been screened and found to be positive for MRP1 and MRP2 mRNAs (5) and for MRP3 mRNA (A. F. Gabriel and Y. J. Schneider, personal communication; sense primer, 5′-CTCCAAGGACATCTATGTCG-3′; antisense primer, 5′-GTAGCGCACAGAATAATTCC-3′).

Stabilities of prodrugs. Pivaloyl esters are known to be unstable in aqueous media. We therefore tested in detail the stability of PIVA, as well as that of PIMA, under our experimental conditions. Both esters were stable if they were maintained in buffer at 4°C (half-lives, >24 h), so the samples could be handled pre- and postincubation without a significant risk of

TABLE 1. Stability of PIVA and PIMA at 37°C in buffers and in buffers bathing the apical (pH 6.0) and basolateral (pH 7.4) poles of Caco-2 cell monolayer

Ester	Half-life (min)			
	pH 6.0 (apical)		pH 7.4 (basolateral)	
	Buffer alone[a]	Buffer with cells[b]	Buffer alone[a]	Buffer with cells[b]
PIVA	1,505[c]	55	130[c]	115
PIMA	602[c]	273	56[c]	46

[a] Modified Krebs buffer; pH 6, buffered with 25 mM 2-(*N*-morpholino)ethanesulfonic acid; pH 7.4, buffered with 25 mM HEPES.

[b] Same buffers described in footnote *a,* but maintained in contact with Caco-2 cell monolayers.

[c] At 4°C the half-lives were greater than 24 h.

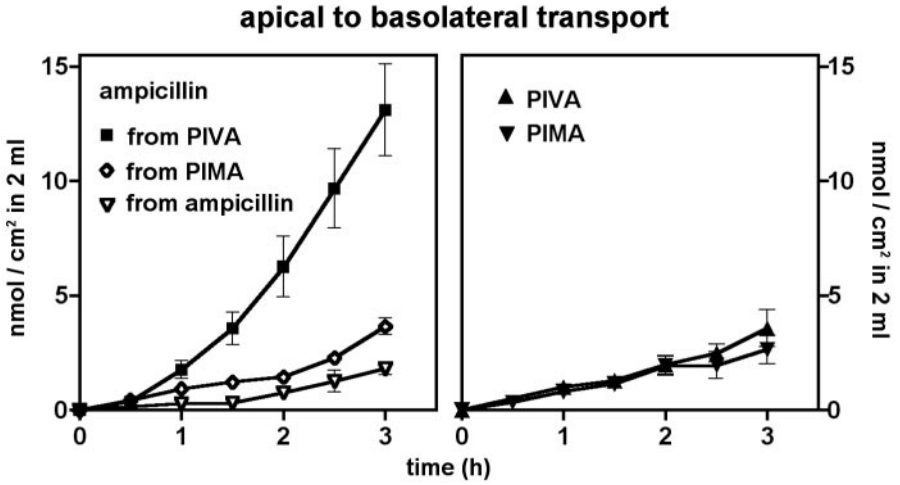

FIG. 2. Apical-to-basolateral transepithelial transport of ampicillin, PIVA, and PIMA through a Caco-2 cell monolayer at 37°C. (Left panel) Cells were incubated with PIVA, PIMA, or ampicillin (all at 0.2 mM) in the apical medium, and the appearance of ampicillin was monitored in the basolateral medium; (right panel) cells were incubated with PIVA or PIMA, as described for the left panel, and the appearance of the corresponding prodrug was monitored in the basolateral medium. At 3 h, the proportions of PIVA, PIMA, and mannitol present in the basolateral side corresponded to 2.1, 1.6, and 2.0% of the total amount present in the apical side, respectively. Each datum point is the mean ± standard deviation of three determinations. This experiment was repeated three times, with similar results each time.

degradation by maintaining them in thawing ice. As shown in Table 1, both prodrugs could also be considered stable at 37°C at pH 6 in buffer within the time frame of our experiments (half-lives, approximately 10 h). In contrast, exposure to buffer in the presence of cells at pH 6 or incubation in buffer with or without cells at pH 7.4 resulted in a considerably faster degradation (half-life range, 46 to 273 min). This led us to restrict our incubation periods to 3 h or less.

Transport of ampicillin from PIVA and PIMA. In a first series of experiments, we examined the net transepithelial transport of ampicillin in cells incubated with free ampicillin or with the prodrugs, together with the transport of the prodrugs themselves. Figure 2 shows the results of these studies with respect to the appearance of ampicillin and the prodrugs at the basolateral pole of cells for which the prodrugs or ampicillin had been added in the medium bathing the apical pole. Concentrating on ampicillin first (Fig. 2, left panel), it immediately appears that its rate of appearance was considerably higher when cells were exposed to PIVA than to ampicillin itself or to PIMA. This appearance proceeded almost linearly with time at a rate of approximately 5 nmol · cm^{-2} · h^{-1} after a first lag period of about 30 min, followed by an acceleration period of about 1.5 h (the concentrations of ampicillin in the basolateral medium were approximately 3.8, 12.4, and 26 μM after 1, 2, and 3 h, respectively). In contrast, the rate of appearance of ampicillin from cells exposed to ampicillin or from cells exposed to PIMA was considerably slower throughout the observation period. This rate was actually quite similar to those of PIVA and PIMA themselves (Fig. 2, right panel) and to that of mannitol (see Materials and Methods). We then measured the basolateral-to-apical fluxes of ampicillin and the prodrugs using the same conditions. In all cases, the flux was very low and essentially similar to that of mannitol (data not shown).

Influences of PEPT1 and OCTN2 inhibitors and energy depletion on the transepithelial transport of ampicillin from PIVA. To determine the influences of PEPT1 and OCTN2 inhibitors and energy depletion on the transepithelial transport of ampicillin from PIVA, we sought to further characterize the process that leads to the release of ampicillin through the basolateral pole of cells when PIVA was added to the medium bathing their apical pole. We therefore examined the influences of selected transporter inhibitors and energy depletion. Table 2 shows that neither the inhibitors of PEPT1 transporter (Gly-Leu, captopril) nor the inhibitor of the OCTN2 transporter (tetraethylammonium) had significant effects on the transport of ampicillin from PIVA. In contrast, ATP depletion effectively reduced the level of ampicillin transport to about one-fourth of the control levels.

TABLE 2. Influences of selected transporter inhibitors and ATP depletion on rate of appearance of ampicillin at basolateral pole of Caco-2 cells incubated with PIVA (0.2 mM) in apical medium

Condition	Rate of appearance (nmol · cm^{-2} · h^{-1})[a]
Control	3.9 ± 1.1
Inhibitors of PEPT1	
Gly-Leu (10 mM)	4.6 ± 1.4
Captopril (1 mM)	4.3 ± 1.3
Inhibitor of OCTN2 (tetraethylammonium, 10 mM)	4.1 ± 1.1
ATP depletion[b]	0.9 ± 0.1[c]

[a] Calculated from regression analysis of cumulative amount detected in the basolateral medium (2 ml) over 3 h. Values are means ± standard deviations of three determinations.

[b] Depletion for impairment of the ATP binding cassette transporters (which include the MDR and MRP transporters). The cells were preincubated for 1 h with NaN_3 (5 mM) and 2-D-deoxyglucose (60 mM) in both the apical and the basolateral media; these conditions were maintained during the experiment; analysis of the cells at the end of the experiment showed an ATP level of 2.89 ± 0.21 nmol/mg of protein versus a level of 9.19 ± 0.26 nmol/mg of protein for the controls.

[c] Significantly different from the results for the control ($P < 0.01$).

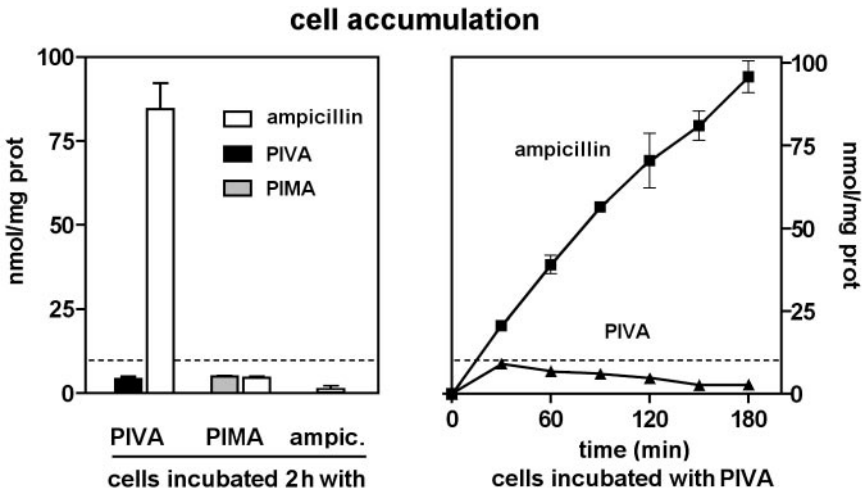

FIG. 3. Accumulation of ampicillin (ampic.), PIVA, and PIMA in cells incubated with PIVA and PIMA and with free ampicillin in Caco-2 cells. (Left panel) Cells were incubated with PIVA, PIMA, or ampicillin (abscissa) in the apical medium for 2 h, as described in the legend to Fig. 2. The ordinate shows the accumulation of ampicillin in each case and of PIVA and PIMA when they were incubated with the corresponding ester. (Right panel) Kinetics of accumulation of ampicillin and PIVA in cells incubated with PIVA, as in the left panel. In both panels, the dotted horizontal line indicates the cell drug content which would correspond to a 10-fold accumulation of PIVA or PIMA (compared to either the actual ampicillin concentration in the same medium [cells incubated with ampicillin] or the concentration of ampicillin that would be created in the same medium if all prodrug was converted to ampicillin [cells incubated with PIVA or PIMA]). Each datum point is the mean ± standard deviation of three determinations. This experiment was repeated three times, with similar results each time. prot, protein.

Accumulation of PIVA, PIMA, and ampicillin by Caco-2 cells. To determine the accumulation of PIVA, PIMA, and ampicillin by Caco-2 cells, we examined directly whether cells would accumulate ampicillin, PIVA, and PIMA when they were present in the apical medium. We also examined whether incubation of cells with PIVA or PIMA would allow the cellular accumulation of ampicillin. We first used a fixed time point of 2 h, and the corresponding results are shown in Fig. 3 (left panel). The accumulation of PIVA and PIMA was relatively modest (corresponding to an apparent cellular concentration-to-extracellular concentration ratio of approximately threefold), and that of ampicillin was negligible when the cells were incubated with free ampicillin. Quite strikingly, however, cells incubated with PIVA showed a considerably higher level of accumulation of ampicillin when PIVA was used at the same molar concentration as free ampicillin itself (corresponding to an apparent cellular concentration 80-fold greater than the maximal extracellular concentration of ampicillin that would have been reached if all extracellular PIVA had been converted without a loss into ampicillin; the actual cellular concentrations of ampicillin were approximately 37, 70, and 95 mmol/mg of protein after incubation with ampicillin, which, on the basis of a volume-to-protein ratio of 3.6 μl/mg of protein [7], would correspond to approximately 10.3, 19.4, and 26.4 mM, respectively). This was not observed for PIMA, from which the amount of ampicillin detected in cells was actually roughly equivalent to that of PIMA itself. The kinetics of accumulation of PIVA and ampicillin in cells incubated with PIVA (in the apical medium) was thereafter studied in more detail. As shown in Fig. 3 (right panel), the cellular accumulation of PIVA was maximal after approximately 30 min of incubation but declined slowly but steadily thereafter, most likely because of the instability of PIVA in the apical medium (Table 1) and the ensuing partial displacement of cell-associated prodrug. In contrast, the accumulation of ampicillin, which was much more stable under these conditions (half-life, approximately 24 h [data not shown]), increased continuously over the entire period of examination (3 h).

Influence of transporter inhibitors, ATP and Na^+ depletion, and an esterase inhibitor on the accumulation of PIVA and its release of ampicillin. We systematically examined the influences of selected conditions on the accumulation of ampicillin and PIVA described in the previous section. As shown in Table 3, the addition of inhibitors of the PEPT1 and OCTN2 transporters had either no inhibitory effect or only modest inhibitory effects. ATP depletion also had no significant effect. In contrast, and quite interestingly, the esterase inhibitor bis(4-nitrophenyl)-phosphate (BNPP) had divergent effects. Indeed, it reduced the level of accumulation of ampicillin (about 45%), while it markedly increased (about fivefold) the level of accumulation of PIVA.

In vitro conversion of PIVA into free ampicillin by Caco-2 cell lysates. The experiments reported so far suggested that the cellular accumulation of ampicillin could result from the conversion of cell-associated PIVA into free ampicillin because of an esterase-dependent activity. The disappearance of PIVA, in comparison with that of PIMA, was therefore monitored during incubation in buffer (pH 7.4) or in the presence of lysates of Caco-2 cells at the same pH. Figure 4 (left panel) shows that the concentration of PIVA decreased rather slowly in buffer (half-life, >120 min) but much faster in the presence of cell lysates (half-life, ~28 min). In contrast, the concentration of PIMA decreased faster than that of PIVA in buffer (half-life, ~57 min), but this process was not accelerated by the addition of cell lysate. In parallel, we measured the liberation of free ampicillin from both PIVA and PIMA in the presence of cell lysate. Figure 4 (right panel) shows that about three times more ampicillin was released from PIVA than from PIMA within 2 h, both in the presence of Caco-2 cell lysates. It must be noted that the yield of ampicillin from both PIVA and

TABLE 3. Influences of selected transporter inhibitors, ATP and Na^+ depletion, and an esterase inhibitor on cellular accumulation of ampicillin and PIVA in Caco-2 cells incubated for 3 h with PIVA (0.2 mM) in apical medium[a]

Condition	Reference	% of control accumulation[b]	
		Ampicillin	PIVA
PEPT1 inhibitors			
Gly-Sar (10 mM)	49	117.8 ± 10.8	92.1 ± 11.5
Gly-Leu (10 mM)	31	93.0 ± 10.0	88.2 ± 21.4
Captopril (1 mM)	42	80.7 ± 10.1[c]	61.1 ± 9.2[c]
OCTN2 inhibitors			
Tetraethylammonium (10 mM)	16	90.4 ± 4.9	63.8 ± 7.6[c]
Quinidine (1 mM)[d]	34	91.2 ± 5.7	86.3 ± 9.4
Carnitine (1 mM)	16	93.2 ± 6.7	94.1 ± 12.3
ATP depletion[e]		109.5 ± 18.9	91.4 ± 9.7
Na^+ depletion[f]	16	106.4 ± 4.2	141.2 ± 28.9
BNPP (5 mM)	40	56.6 ± 3.9[g]	502.1 ± 52.7[g]

[a] Inhibitors were present only in the apical medium, but they were present at concentrations that proved effective in previous studies (see the references provided).
[b] Values are means ± standard deviations of three determinations in a single experiment. This experiment was repeated three times, with similar results each time
[c] Significantly different from the results for the control ($P < 0.05$).
[d] Quinidine also inhibits P-glycoprotein (53).
[e] See footnote *b* of Table 2.
[f] Na^+ was replaced by Li^+ in both the apical and the basolateral media.
[g] Significantly different from the results for the control ($P < 0.01$).

PIMA was lower than expected from the extent of disappearance of the prodrugs seen in the left panel of Fig. 4. This behavior is, however, not surprising because the degradation of both prodrugs is known to involve at least two pathways, one of which leads to the liberation of free ampicillin, as demonstrated here, and the second of which leads to β-lactam ring opening and the appearance of degradation products (36), for which assays were not conducted in the present study.

Influences of BNPP and other esterase inhibitors on in vitro release of ampicillin from PIVA and on ampicillin transport across the Caco-2 cell epithelium. Because BNPP increased the level of accumulation of PIVA while it decreased that of ampicillin (Table 3), we reasoned that this esterase inhibitor blocks the intracellular transformation of PIVA (taken from the apical pole) to ampicillin and thereby impairs the subsequent release of ampicillin through the basolateral pole. This was tested directly by examining the influence of BNPP on the hydrolysis of PIVA in Caco-2 cell lysates and on the transport of ampicillin across the epithelium of Caco-2 cells incubated with PIVA. Figure 5 (left panel) shows that BNPP was a potent inhibitor of the release of ampicillin from PIVA exposed to the cell lysate. BNPP had no influence on the spontaneous release of ampicillin at a slow rate in buffer (corresponding to the chemical hydrolysis of PIVA; the degradation of PIVA could not be measured in these experiments because of interference from BNPP in the PIVA assay). Figure 5 (right panel) shows also that the appearance of ampicillin in the medium bathing the basolateral pole of cells incubated with BNPP was markedly impaired compared to that of control cells. A series of fruit extracts have also been described to be inhibitors of Caco-2 cell esterases (44). Two of these extracts (strawberry and banana) were tested and were also found to impair the transport of ampicillin from PIVA across Caco-2 cell monolayers in the present model (data not shown).

Efflux of PIVA and ampicillin from Caco-2 cells. Having established that PIVA accumulates in Caco-2 cells and causes the accumulation of large amounts of ampicillin (presumably from its intracellular hydrolysis), we examined how and to what extent both compounds could be cleared from these cells. For this purpose, cells were first incubated with PIVA in the medium bathing the apical side for 1 h. The cells were then washed extensively and transferred to fresh medium for further incubation at either 37 or 4°C to measure the efflux of PIVA and ampicillin from the cells. In the first series of experiments, we monitored the total cell contents of PIVA and ampicillin (with the latter arising from the hydrolysis of either extracel-

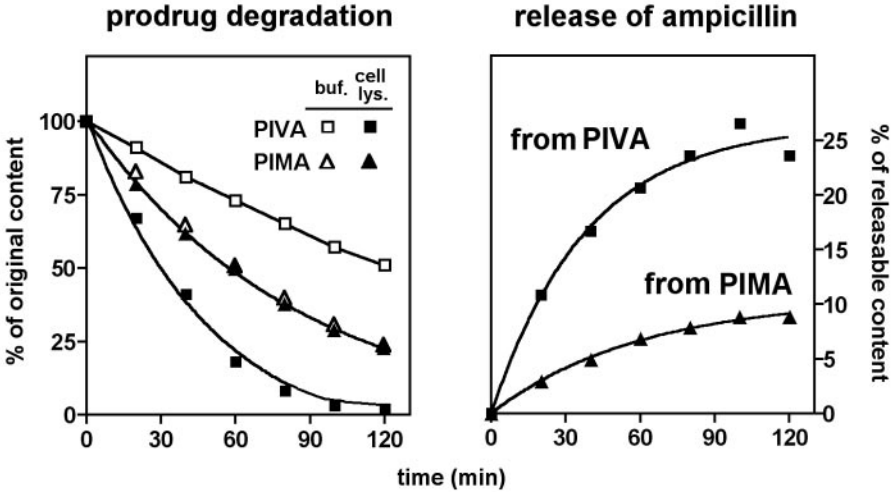

FIG. 4. Degradation of PIVA and PIMA and release of free ampicillin. (Left panel) The prodrugs (0.2 mM) were left either in buffer (pH 7.4) or in the Caco-2 cell lysate at the same pH; (right panel) the prodrugs (0.2 mM) were incubated in the presence of Caco-2 cell lysates. Each datum point represents one determination. This experiment was repeated two times, with similar results each time.

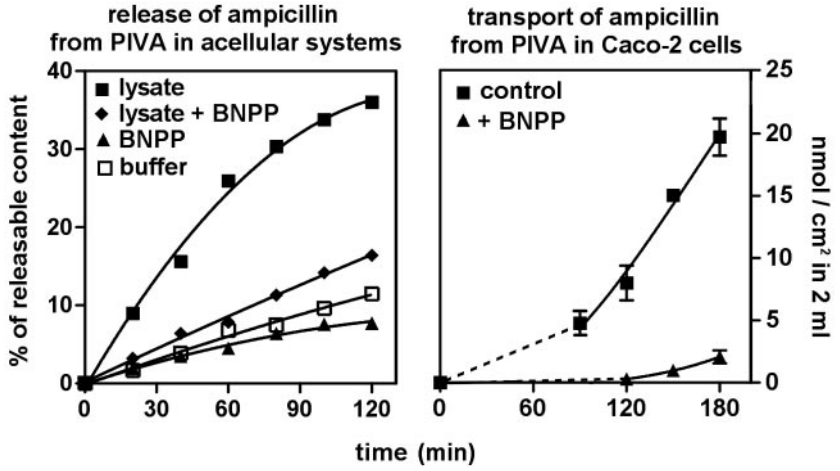

FIG. 5. Influences of BNPP on the release of ampicillin from PIVA and on ampicillin transport through Caco-2 cells incubated with PIVA. (Left panel) PIVA (0.2 mM) was incubated in the presence of Caco-2 cell lysates or buffer, as described in the legend to Fig. 4, with or without BNPP (5 mM). Each datum point represents one determination. This experiment was repeated two times, with similar results each time. (Right panel) Influence of BNPP (5 mM in apical medium) on the appearance of ampicillin through the basolateral side when cells were incubated with PIVA (0.2 mM) in the apical medium. Each datum point is the mean ± standard deviation of three determinations in one experiment. This experiment was repeated two times, with similar results each time.

lular or cell-associated PIVA during the first incubation period or from cell-associated PIVA during efflux). The results are shown in Fig. 6. PIVA was rapidly and extensively cleared not only at 37°C (half-life, ~52 s) but also at 4°C (half-life, ~11 min). In contrast, the clearance of ampicillin was much slower at 37°C (half-life, ~150 min), and no drug clearance could be detected at 4°C. In the second series of experiments, we examined the cell pole through which ampicillin was cleared. Thus, we measured the appearance of ampicillin in both the apical and basolateral media from cells previously incubated for 1 h with PIVA and then reincubated in PIVA-free medium for 2 h. As shown in Fig. 7, about two times more ampicillin was cleared through the basolateral pole than through the apical pole. Figure 7 also shows that depletion of the cells in the presence of ATP or the addition of probenecid or MK-571, two MRP inhibitors, caused a fourfold decrease in the release of ampicillin through the basolateral pole. These treatments also decreased the release of ampicillin through the apical pole, but to a lesser extent. These experiments were repeated with verapamil (1 mM), as inhibitor of P-glycoprotein (MDR1) (53), but no significant effect was seen (data not shown).

DISCUSSION

The present study has revisited the mechanism by which PIVA, a prodrug of ampicillin, may give rise to higher plasma ampicillin concentrations than ampicillin itself after oral administration. PIVA was the first prodrug of β-lactam antibiotics to show a rapid conversion to ampicillin. It has served as a model for other, similar types of enzymatically cleavable esters, such as lactonyl (12) and 1-acetoxyethylesters (22).

Molecular biology-based studies of the transepithelial trans-

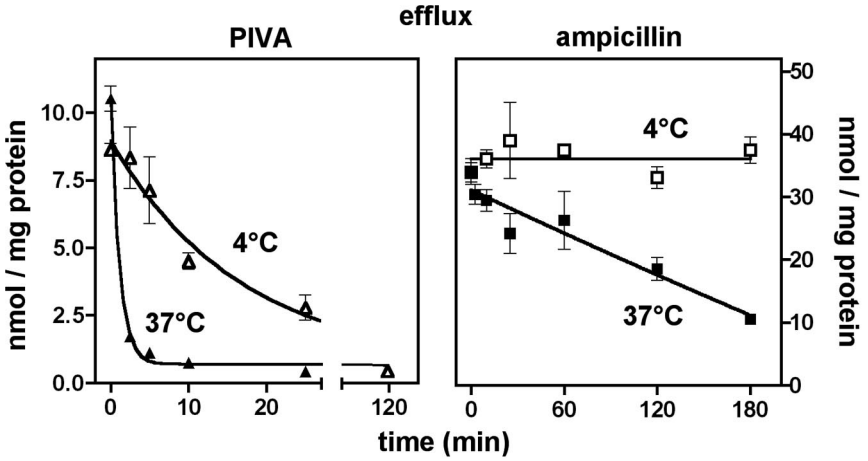

FIG. 6. Clearance of PIVA and ampicillin from cells incubated for 1 h at 37°C with 0.2 mM PIVA in apical medium and thereafter transferred to fresh medium at either 37°C (closed symbols) or 4°C (open symbols). Each datum point is the mean ± standard deviation of three determinations. This experiment was repeated three times, with similar results each time.

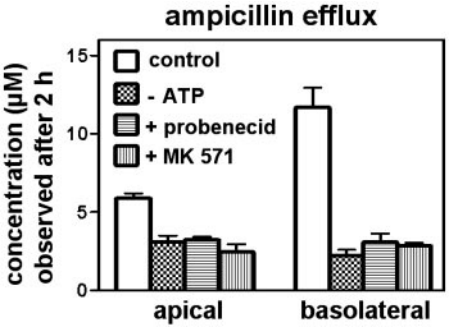

FIG. 7. Appearance of ampicillin in the apical or basolateral medium of Caco-2 cells incubated for 1 h at 37°C with 0.2 mM PIVA in the apical medium (pulse) and then transferred for 2 h in fresh medium (chase). The drug concentrations observed in the corresponding medium at the end of the chase are shown. Control, cells without other treatment; −ATP, cells preincubated for 1 h with 5 mM NaN_3 and 60 mM 2-D-deoxyglucose to obtain ATP depletion (these conditions were maintained during the pulse and chase periods); +probenecid and +MK-571, cells incubated with these MRP inhibitors (5 mM and 100 μM, respectively) in both the apical and basolateral media only during the chase period. Each datum point is the mean ± standard deviation of three determinations. This experiment was repeated three times, with similar results each time.

port of β-lactam antibiotics and their prodrugs have greatly benefited from the development of simple models, such as Caco-2 cell monolayers (28, 30), which nowadays are widely used for absorption screening in drug discovery and development (4, 6). A second critical development has been the recognition of the role played by transporters such as PEPT1, which transports β-lactams possessing an α-amino group (37, 38).

The available data suggest that the mechanism by which pivaloylesters act to enhance the transintestinal resorption of drugs is probably more complex than was originally anticipated for PIVA and similar esters (45, 46). A first hint came from the study of new, original pivaloylesters of anti-human immunodeficiency virus acyclic nucleosides phosphonates developed in the early 1990s (39). These esters showed enhanced transport across Caco-2 cell monolayers, but detailed studies (2, 3, 44) demonstrated (i) that they accumulate in cells and are hydrolyzed therein by an enzyme(s) that is inhibited by PIVA (and other esters) and (ii) that the acyclic nucleoside phosphonates released are exported both apically and basolaterally through an anion transporter inhibitable by indomethacin and previously uncovered in Caco-2 cells (13). In a more recent study (23), PIVA was exposed to Caco-2 cell monolayers for a short period of time to mimic intestinal transit. It was found that the prodrug appeared only transiently and in limited amounts in the basolateral pole, whereas ampicillin was released in large amounts and in a sustained fashion through that pole but was not significantly released through the apical pole. These sets of data clearly suggest the intracellular accumulation and metabolism of PIVA, followed by the oriented transport of ampicillin, rather than the simple diffusion of PIVA through the enterocytes, followed by hydrolysis in the extracellular milieu, as has been proposed for cefuroxime axetil (14).

Our data are consistent with this scheme and provide two additional and important pieces of information. We first show directly that the intracellular accumulation of PIVA remains limited and is not enhanced by the peptide (PEPT1) or cation (OCTN2) transporters (since the corresponding inhibitors have no marked influence on PIVA accumulation). We therefore propose that PIVA remains largely at the cell surface since it is almost immediately released upon transfer in fresh medium, even at 4°C. Both PIVA and PIMA are considerably more lipophilic than ampicillin, and both bind to a large extent to cell membranes and liposomes (11). The apical membrane of Caco-2 cells could therefore act as a reservoir of prodrugs. This, however, is probably not a determining factor per se in the overall ampicillin transport process studied here because PIVA and PIMA accumulate in Caco-2 cells to the same extent. We showed by computer-aided conformational analysis that both PIVA and PIMA are able to move across a lipid bilayer (11). Our present data would suggest that such mobility may give PIVA the opportunity to become exposed to cytosolic esterases and, therefore, to be partly but quickly converted into ampicillin (which is retained intracellularly, since, as will be discussed below, it diffuses much more slowly through membranes).

We have seen that PIVA is also degraded in the extracellular media by both chemical and enzymatic hydrolysis. This relative instability reduces but does not totally prevent the accumulation of PIVA since at any time a proportion of intact prodrug can enter the cells and be hydrolyzed by esterases. This process therefore gives rise to a continuous influx of PIVA into cells (and, through its hydrolysis, of ampicillin), even though the total amount of cell-associated PIVA decreases over time. We believe that this unexpected situation results from the saturation of cytosolic esterases by the high concentrations of cell-associated PIVA (4 mM at 1 h and 1.2 mM at 2.5 h), causing ampicillin to be released at a constant rate.

The fact that cell-associated PIVA is quickly hydrolyzed also explains why it is found only in negligible amounts in the basolateral media, even though its lipophilic character should have allowed the fast and efficient transfer across the monolayer by passive diffusion, as originally hypothesized (46). Our data and the hypothesis that we raise are actually in accordance with recent data also obtained with Caco-2 cells that showed that cell-associated PIVA is almost completely hydrolyzed before it ever reaches the basolateral side of the monolayer (24). Such an intracellular hydrolysis does not take place with PIMA because it is insensitive to esterases. It is highly noteworthy in this context that the esterase inhibitor BNPP, which inhibits the hydrolysis of PIVA into ampicillin in cell lysates, almost entirely blocked the transepithelial transport of ampicillin from PIVA. A similar effect was obtained here with fruit extracts that, according to a previous study (44), act as esterase inhibitors.

We next demonstrated that the ampicillin released through the enzymatic hydrolysis of PIVA is subject to oriented transport through the basolateral pole because of the presence of an ATP-dependent transport system. However, a close analysis of the data presented in Fig. 2 and 3 shows that the movement of ampicillin always goes along a concentration gradient, meaning that the efflux system only facilitates the transport of the drug. Data available from the literature indicate that several efflux transporters of the ATP-binding cassette superfamily are expressed in Caco-2 cells, among which MDR1 (also known as P-glycoprotein) and the group of MRPs (more specifically,

MRP1 through MRP5) appear to be the most important ones. MDR1 can most likely be excluded since verapamil, which affects MDR1 (P-glycoprotein [53]) had no effect. Moreover, localization studies performed with different models of epithelial cells suggest that MDR1 is usually found at the apical surface (41). Moving now to MRPs, we observed marked and similar effects of both the nonspecific inhibitor probenecid and the preferential inhibitor MK-571 (20). We know from data from the literature (17, 27, 51) that MRP2 and MRP4 are usually localized at the apical pole, whereas MRP1, MRP3, and MRP5 are usually found in the basolateral membrane. On the basis of the properties of the basolateral efflux of ampicillin (ATP dependence and impairment by MRP inhibitors) and data on MRPs available from the literature, these transporters appear to be good candidates. The efflux of ampicillin through the apical pole, however, was not negligible, and MRP inhibitors also decreased it to some extent. An MRP transporter(s) could also play a role here, but it may show an affinity lower than those of transporters present on the basolateral membrane. This hypothesis needs to be confirmed in further studies.

In conclusion, the present study stresses the importance of both the intracellular hydrolysis of PIVA and the subsequent basolateral transport of ampicillin in the transepithelial flux of ampicillin in Caco-2 cells incubated with PIVA. If it is assumed that this model correctly describes the situation that prevails in vivo, one may explain, in retrospect, why other pivaloyl esters of β-lactams show quite variable oral bioavailabilities compared to that of ampicillin, since the basolateral transporter may recognize them very differently than it does ampicillin itself. It also suggests that the oral bioavailability of ampicillin will be severely affected by inhibitors of this basolateral transporter if they are present when PIVA is administered.

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REFERENCES

1. **Adibi, S. A.** 1997. The oligopeptide transporter (Pept-1) in human intestine: biology and function. Gastroenterology **113:**332–340.
2. **Annaert, P., R. Kinget, L. Naesens, E. de Clercq, and P. Augustijns.** 1997. Transport, uptake, and metabolism of the bis(pivaloyloxymethyl)-ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine in an in vitro cell culture system of the intestinal mucosa (Caco-2). Pharm. Res. **14:**492–496.
3. **Annaert, P., J. van Gelder, L. Naesens, E. de Clercq, G. van den Mooter, R. Kinget, and P. Augustijns.** 1998. Carrier mechanisms involved in the transepithelial transport of bis(POM)-PMEA and its metabolites across Caco-2 monolayers. Pharm. Res. **15:**1168–1173.
4. **Artursson, P., K. Palm, and K. Luthman.** 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv. Drug Deliv. Rev. **46:**27–43.
5. **Berger, V., A. F. Gabriel, T. Sergent, A. Trouet, Y. Larondelle, and Y. J. Schneider.** 2003. Interaction of ochratoxin A with human intestinal Caco-2 cells: possible implication of a multidrug resistance-associated protein (MRP2). Toxicol. Lett. **140–141:**465–476.
6. **Bohets, H., P. Annaert, G. Mannens, L. Van Beijsterveldt, K. Anciaux, P. Verboven, W. Meuldermans, and K. Lavrijsen.** 2001. Strategies for absorption screening in drug discovery and development. Curr. Top. Med. Chem. **1:** 367–383.
7. **Bretschneider, B., M. Brandsch, and R. Neubert.** 1999. Intestinal transport of beta-lactam antibiotics: analysis of the affinity at the H^+/peptide symporter (PEPT1), the uptake into Caco-2 cell monolayers and the transepithelial flux. Pharm. Res. **16:**55–61.
8. **Carlier, M. B., I. Garcia-Luque, J. P. Montenez, P. M. Tulkens, and J. Piret.** 1994. Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. Int. J. Tissue React. **16:** 211–220.
9. **Carlier, M. B., A. Zenebergh, and P. M. Tulkens.** 1987. Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. J. Antimicrob. Chemother. **20**(Suppl. B)**:**47–56.
10. **Chanteux, H., M. P. Mingeot-Leclercq, E. Sonveaux, F. Van Bambeke, and P. M. Tulkens.** 2003. Intracellular accumulation and activity of ampicillin used as free drug and as its phthalimidomethyl or pivaloyloxymethyl ester (pivampicillin) against Listeria monocytogenes in J774 macrophages. J. Antimicrob. Chemother. **52:**610–615.
11. **Chanteux, H., I. Paternotte, M. P. Mingeot-Leclercq, R. Brasseur, E. Sonveaux, and P. M. Tulkens.** 2003. 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. Pharm. Res. **20:**624–631.
12. **Clayton, J. P., M. Cole, S. W. Elson, H. Ferres, J. C. Hanson, L. W. Mizen, and R. Sutherland.** 1976. Preparation, hydrolysis, and oral absorption of lactonyl esters of penicillins. J. Med. Chem. **19:**1385–1391.
13. **Collington, G. K., J. Hunter, C. N. Allen, N. L. Simmons, and B. H. Hirst.** 1992. Polarized efflux of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein from cultured epithelial cell monolayers. Biochem. Pharmacol. **44:**417–424.
14. **Dantzig, A. H., D. C. Duckworth, and L. B. Tabas.** 1994. Transport mechanisms responsible for the absorption of loracarbef, cefixime, and cefuroxime axetil into human intestinal Caco-2 cells. Biochim. Biophys. Acta **1191:**7–13.
15. **de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. Van Hoof.** 1974. Commentary. Lysosomotropic agents. Biochem. Pharmacol. **23:**2495–2531.
16. **Elimrani, I., K. Lahjouji, E. Seidman, M. J. Roy, G. A. Mitchell, and I. Qureshi.** 2003. Expression and localization of organic cation/carnitine transporter OCTN2 in Caco-2 cells. Am. J. Physiol. Gastrointest. Liver Physiol. **284:**G863–G871.
17. **Evers, R., G. J. Zaman, L. van Deemter, H. Jansen, J. Calafat, L. C. Oomen, R. P. Oude Elferink, P. Borst, and A. H. Schinkel.** 1996. Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. J. Clin. Investig. **97:**1211–1218.
18. **Fan, H. J., I. Paternotte, M. Vermander, K. Li, M. Beaujean, B. Scorneaux, and E. Sonveaux.** 1997. Ester prodrugs of ampicillin tailored for intracellular accumulation. Bioorg. Med. Chem. Lett. **7:**3107–3112.
19. **Ganapathy, M. E., M. Brandsch, P. D. Prasad, V. Ganapathy, and F. H. Leibach.** 1995. Differential recognition of beta-lactam antibiotics by intestinal and renal peptide transporters, PEPT 1 and PEPT 2. J. Biol. Chem. **270:** 25672–25677.
20. **Gekeler, V., W. Ise, K. H. Sanders, W. R. Ulrich, and J. Beck.** 1995. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem. Biophys. Res. Commun. **208:**345–352.
21. **Grasset, E., M. Pinto, E. Dussaulx, A. Zweibaum, and J. F. Desjeux.** 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. Am. J. Physiol. **247:**C260–C267.
22. **Harding, S. M., P. E. Williams, and J. Ayrton.** 1984. Pharmacology of cefuroxime as the 1-acetoxyethyl ester in volunteers. Antimicrob. Agents Chemother. **25:**78–82.
23. **He, X., M. Sugawara, M. Kobayashi, Y. Takekuma, and K. Miyazaki.** 2003. An in vitro system for prediction of oral absorption of relatively water-soluble drugs and ester prodrugs. Int. J. Pharm. **263:**35–44.
24. **He, X., M. Sugawara, Y. Takekuma, and K. Miyazaki.** 2004. Absorption of ester prodrugs in Caco-2 and rat intestine models. Antimicrob. Agents Chemother. **48:**2604–2609.
25. **Hilgers, A. R., R. A. Conradi, and P. S. Burton.** 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. Pharm. Res. **7:** 902–910.
26. **Hu, M., J. Chen, Y. Zhu, A. H. Dantzig, R. E. Stratford, Jr., and M. T. Kuhfeld.** 1994. Mechanism and kinetics of transcellular transport of a new beta-lactam antibiotic loracarbef across an intestinal epithelial membrane model system (Caco-2). Pharm. Res. **11:**1405–1413.
27. **Konig, J., D. Rost, Y. Cui, and D. Keppler.** 1999. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology **29:**1156–1163.
28. **Li, Y. H., K. Ito, Y. Tsuda, R. Kohda, H. Yamada, and T. Itoh.** 1999. Mechanism of intestinal absorption of an orally active beta-lactam prodrug: uptake and transport of carindacillin in Caco-2 cells. J. Pharmacol. Exp. Ther. **290:**958–964.
29. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.

30. **Menon, R. M., and W. H. Barr.** 2002. Transporters involved in apical and basolateral uptake of ceftibuten into Caco-2 cells. Biopharm. Drug Dispos. **23:**317–326.
31. **Merlin, D., M. Si-Tahar, S. V. Sitaraman, K. Eastburn, I. Williams, X. Liu, M. A. Hediger, and J. L. Madara.** 2001. Colonic epithelial hPepT1 expression occurs in inflammatory bowel disease: transport of bacterial peptides influences expression of MHC class 1 molecules. Gastroenterology **120:** 1666–1679.
32. **Nielsen, C. U., J. Amstrup, B. Steffansen, S. Frokjaer, and B. Brodin.** 2001. Epidermal growth factor inhibits glycylsarcosine transport and hPepT1 expression in a human intestinal cell line. Am. J. Physiol Gastrointest. Liver Physiol. **281:**G191–G199.
33. **Ohashi, R., I. Tamai, A. Inano, M. Katsura, Y. Sai, J. Nezu, and A. Tsuji.** 2002. Studies on functional sites of organic cation/carnitine transporter OCTN2 (SLC22A5) using a Ser467Cys mutant protein. J. Pharmacol. Exp. Ther. **302:**1286–1294.
34. **Ohashi, R., I. Tamai, H. Yabuuchi, J. I. Nezu, A. Oku, Y. Sai, M. Shimane, and A. Tsuji.** 1999. Na^+-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. J. Pharmacol. Exp. Ther. **291:**778–784.
35. **Okamura, A., A. Emoto, N. Koyabu, H. Ohtani, and Y. Sawada.** 2002. Transport and uptake of nateglinide in Caco-2 cells and its inhibitory effect on human monocarboxylate transporter MCT1. Br. J. Pharmacol. **137:**391–399.
36. **Paternotte, I., H. J. Fan, P. Screve, M. Claesen, P. M. Tulkens, and E. Sonveaux.** 2001. Syntheses and hydrolysis of basic and dibasic ampicillin esters tailored for intracellular accumulation. Bioorg. Med. Chem. **9:**493–502.
37. **Raeissi, S. D., J. Li, and I. J. Hidalgo.** 1999. The role of an alpha-amino group on H^+-dependent transepithelial transport of cephalosporins in Caco-2 cells. J. Pharm. Pharmacol. **51:**35–40.
38. **Snyder, N. J., L. B. Tabas, D. M. Berry, D. C. Duckworth, D. O. Spry, and A. H. Dantzig.** 1997. Structure-activity relationship of carbacephalosporins and cephalosporins: antibacterial activity and interaction with the intestinal proton-dependent dipeptide transport carrier of Caco-2 cells. Antimicrob. Agents Chemother. **41:**1649–1657.
39. **Srinivas, R. V., B. L. Robbins, M. C. Connelly, Y. F. Gong, N. Bischofberger, and A. Fridland.** 1993. Metabolism and in vitro antiretroviral activities of bis(pivaloyloxymethyl) prodrugs of acyclic nucleoside phosphonates. Antimicrob. Agents Chemother. **37:**2247–2250.
40. **Sterri, S. H., B. A. Johnsen, and F. Fonnum.** 1985. A radiochemical assay method for carboxylesterase, and comparison of enzyme activity towards the substrates methyl [1-^{14}C] butyrate and 4-nitrophenyl butyrate. Biochem. Pharmacol. **34:**2779–2785.
41. **Thiebaut, F., T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan, and M. C. Willingham.** 1987. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc. Natl. Acad. Sci. USA **84:**7735–7738.
42. **Thwaites, D. T., M. Cavet, B. H. Hirst, and N. L. Simmons.** 1995. Angiotensin-converting enzyme (ACE) inhibitor transport in human intestinal epithelial (Caco-2) cells. Br. J. Pharmacol. **114:**981–986.
43. **Tyteca, D., P. Van Der Smissen, F. Van Bambeke, K. Leys, P. M. Tulkens, P. J. Courtoy, and M. P. Mingeot-Leclercq.** 2001. Azithromycin, a lysosomotropic antibiotic, impairs fluid-phase pinocytosis in cultured fibroblasts. Eur. J. Cell Biol. **80:**466–478.
44. **van Gelder, J., S. Deferme, L. Naesens, E. de Clercq, G. van den Mooter, R. Kinget, and P. Augustijns.** 2002. Intestinal absorption enhancement of the ester prodrug tenofovir disoproxil fumarate through modulation of the biochemical barrier by defined ester mixtures. Drug Metab. Dispos. **30:**924–930.
45. **von Daehne, E., E. Frederiksen, E. Gudersen, F. Lund, P. Moerch, H. Petersen, K. Roholt, L. Tybring, and W. O. Godfredsen.** 1970. Acyloxymethyl esters of ampicillin. J. Med. Chem. **13:**607–612.
46. **von Daehne, W., W. O. Godtfredsen, K. Roholt, and L. Tybring.** 1970. Pivampicillin, a new orally active ampicillin ester. Antimicrob. Agents Chemother. **10:**431–437.
47. **Watanabe, K., T. Sawano, T. Endo, M. Sakata, and J. Sato.** 2002. Studies on intestinal absorption of sulpiride. 2. Transepithelial transport of sulpiride across the human intestinal cell line Caco-2. Biol. Pharm. Bull. **25:**1345–1350.
48. **Wenzel, U., I. Gebert, H. Weintraut, W. M. Weber, W. Clauss, and H. Daniel.** 1996. Transport characteristics of differently charged cephalosporin antibiotics in oocytes expressing the cloned intestinal peptide transporter PepT1 and in human intestinal Caco-2 cells. J. Pharmacol. Exp. Ther. **277:**831–839.
49. **Wenzel, U., D. T. Thwaites, and H. Daniel.** 1995. Stereoselective uptake of beta-lactam antibiotics by the intestinal peptide transporter. Br. J. Pharmacol. **116:**3021–3027.
50. **Wibo, M., and B. Poole.** 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B1. J. Cell Biol. **63:**430–440.
51. **Wijnholds, J., C. A. Mol, L. van Deemter, M. de Haas, G. L. Scheffer, F. Baas, J. H. Beijnen, R. J. Scheper, S. Hatse, E. de Clercq, J. Balzarini, and P. Borst.** 2000. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleotide analogs. Proc. Natl. Acad. Sci. USA **97:**7476–7481.
52. **Yamaguchi, H., I. Yano, H. Saito, and K. Inui.** 2001. Transport characteristics of grepafloxacin and levofloxacin in the human intestinal cell line Caco-2. Eur. J. Pharmacol. **431:**297–303.
53. **Yumoto, R., T. Murakami, Y. Nakamoto, R. Hasegawa, J. Nagai, and M. Takano.** 1999. Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. J. Pharmacol. Exp. Ther. **289:**149–155.
54. **Zweibaum, A., H. P. Hauri, E. Sterchi, I. Chantret, K. Haffen, J. Bamat, and B. Sordat.** 1984. Immunohistological evidence, obtained with monoclonal antibodies, of small intestinal brush border hydrolases in human colon cancers and foetal colons. Int. J. Cancer **34:**591–598.