The effect of hydrophobicity of β-lactam antibiotics on their phospholipid bilayer permeability

Akihito Yamaguchi, Ryoichi Hiruma and Tetsuo Sawai

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba 260, Japan

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Phospholipid bilayer permeability of β-lactam antibiotics was determined using liposomes enclosing β-lactamase. There was good correlation between the permeability and hydrophobicity within the analogous β-lactams. However, the effect of hydrophobic character on the permeability parameter was very different between the groups. Moderately hydrophilic penicillins such as benzylpenicillin and ampicillin showed very high permeability compared with cephalosporins. Penicillins having hindered side chains such as oxacillin and methicillin showed moderate permeability taking into account their hydrophobicity. These observations are suggestive of outer membrane permeation of these β-lactams via routes other than the porin pore, especially in porin-deficient mutants of gram-negative bacteria.

β-Lactam *Lipid-bilayer permeability* *Hydrophobicity* *Liposome* *Outer membrane* *β-Lactamase*

1. INTRODUCTION

Common cephalosporins and penicillins were demonstrated to use porin channels as a permeation route through the outer membrane of gram-negative bacteria in order to reach their targets in the cytoplasmic membrane [1–4]. During the course of our studies on the outer membrane permeation of various β-lactam antibiotics using porin-deficient mutants, we made an interesting observation, namely, that some penicillins such as ampicillin may use the hydrocarbn chain region of the outer membrane as a secondary permeation route in addition to the porin pore [4]. This assumption has been strengthened by the fact that ampicillin easily permeates the lipid bilayer of liposomes prepared from *Escherichia coli* phospholipids, whereas cefazolin and cephaloridine hardly penetrate this lipid bilayer [5], although the hydrophobicity of ampicillin is intermediate between these cephalosporins. This investigation was undertaken as an extension of the previous one [5], to determine the relationship between lipid bilayer permeability and hydrophobicity of β-lactam antibiotics.

2. MATERIALS AND METHODS

2.1. *β-Lactam antibiotics*

β-Lactam antibiotics were a generous gift from the following pharmaceutical companies: benzylpenicillin, ampicillin, cephalosporin-C, dicloxacillin and cloxacillin, Meiji Seika Co. (Tokyo); amoxicillin, ticarcillin, flucloxacillin and clavulanic acid, Beecham Pharmaceutics (Surrey); carbenicillin, cefazolin and ceftezole, Fujisawa Pharmaceutical Co. (Osaka); cephaloridine, Torii Pharmaceutical Co. (Tokyo); cephalexin, Toyama Chemical Co. (Tokyo); oxacillin and methicillin, Banyu Pharmaceutical Co. (Tokyo); sulbenicillin, Takeda Pharmaceutical Co. (Osaka); cefadroxyl, Bristol and Banyu Pharmaceutical Co. (Tokyo); sulbactam, Pfizer Inc. (Groton).

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2.2. *Preparation of liposomes with enclosed β-lactamase*

β-Lactamase-enclosing liposomes were prepared in the presence of RGN823 penicillinase [6], RGN238 penicillinase [7] or *Citrobacter freundii* cephalosporinase [6] as in [5]. A dried mixture of 4 mg *E. coli* phospholipids and 0.37 mg cardiolipin (Sigma) was dispersed in 0.4 ml phosphate-buffered saline or 0.1 M phosphate buffer, pH 7.0 (in the case of RGN238 penicillinase) containing 320 units (ampicillin as substrate) of RGN823 penicillinase, 160 units (ampicillin as substrate) of RGN238 penicillinase or 80 units (cephalothin as substrate) of *C. freundii* cephalosporinase. The enzyme outside the liposome was irreversibly inactivated by an about 500 molar excess of clavulanic acid (for the penicillinase) or sulbactam (for the cephalosporinase). This step is necessary for the enzymatic assay of the permeability, since the enzyme attached on the outside surface of the liposome interfered with the measurement of the rate of hydrolysis by the entrapped enzyme. The degree of inactivation of the entrapped enzyme by these inhibitors was much smaller than that of the enzyme outside the liposome due to the action of the liposomal membrane as a permeability barrier to these inhibitors. Finally, inhibitors and the inactivated enzymes were removed by gel filtration.

2.3. *Determination of permeability parameter*

Uptake of β-lactam antibiotics by liposomes was determined by measuring hydrolysis of β-lactams by intact liposomes using the microiodometric method as in [5]. Enzyme reaction was terminated by addition of 0.15 M sodium tungstate. In this method, hydrolyzed product remaining inside the liposomes could be detectable, since the iodine consumption remained constant before and after sonic disruption of the liposome. The rate of hydrolysis by entrapped enzymes was corrected by subtracting the hydrolysis by the supernatant of the liposome suspension. The permeability parameter was calculated from the ratio of the rate of hydrolysis by intact liposome and that of disrupted liposome in the presence of Triton X-100 as in [5]. The units of the permeability parameter are $min^{-1} \cdot \mu M\ lipid^{-1}$.

2.4. *Reversed-phase thin-layer chromatography*

The hydrophobic character of the β-lactam antibiotics was expressed by the R_f value which was measured by reversed-phase thin-layer chromatography [4].

3. RESULTS

The outer membrane permeability of β-lactam antibiotics is calculated from the rate of their hydrolysis by β-lactamases located in the periplasmic space using Fick's law of diffusion [8]. This method was applied to the permeability measurement of β-lactams through the reconstituted membrane with or without porin using β-lactamase-containing vesicles [5,9,10]. The permeability parameter determined by this method is independent of the kinetic nature of the enzyme used provided the substrate concentration inside the vesicle is well below the K_m value. However, if significant amounts of enzyme were to become attached on the outside surface of the liposome, it would interfere with the calculation of the permeability parameter. In this case, the calculated parameter would decrease with increasing substrate concentration outside the liposome. On the other hand, if the calculated parameter represented the true permeability, it would be independent of the substrate concentration outside the liposome. Fig.1 clearly shows that the former was not the case.

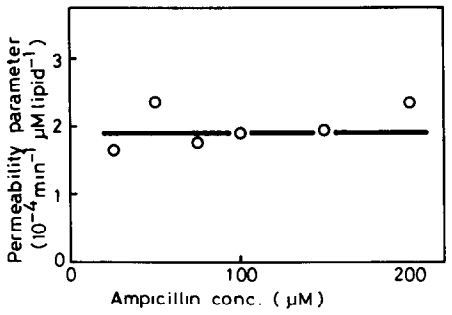

Fig.1. Phospholipid bilayer permeability of ampicillin when the concentration of ampicillin outside the liposomes was varied from 25 μM–200 μM. The permeability parameter was determined as described in section 2.

The β-lactam antibiotics used here are listed in fig.2. These β-lactams can be classified into 3 groups, namely:

(i) cephalosporins;
(ii) broad spectrum penicillins;
(iii) penicillins having hindered 6-β side chains.

The third group are β-lactamase-resistant penicillins which show little antibacterial activity against gram-negative bacteria probably due to their low outer membrane permeability [11,12]. The permeability of these 3 groups to liposomal membrane was mainly measured by *C. freundii* cephalosporinase, RGN823 penicillinase and RGN238 penicillinase, respectively, since these combinations of enzyme and substrate were the best for enzymatic assay. In addition, 3 representative broad spectrum penicillins and 3 cephalosporins were measured by RGN238 penicillinase and RGN823 penicillinase, respectively. Fig.3 shows the plot of the permeability parameter against the R_f value: the greater the hydrophobic character, the smaller the R_f value. These data clearly showed that: (i) the permeability depended on the hydrophobicity of the molecule within each group; while (ii) the effect of hydrophobic character on the permeability parameter was very different between the groups. Broad spectrum penicillins which are moderately hydrophilic showed much larger values for the permeability parameter than cephalosporins having similar hydrophobicity. Penicillins having hindered 6-β side chains showed intermediate permeability between broad spectrum penicillins and cephalosporins.

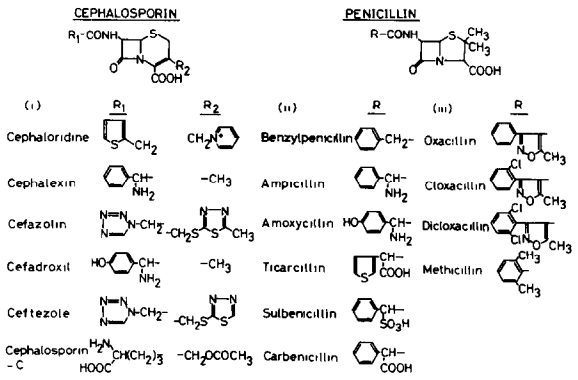

Fig.2. Structures of β-lactams used in this study.

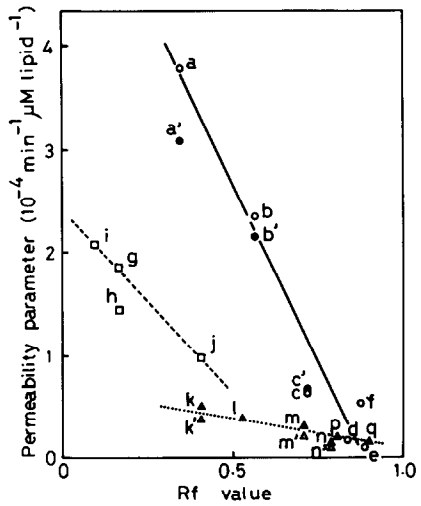

Fig.3. Phospholipid bilayer permeability of various β-lactam antibiotics. Antibiotic concentration added outside the liposomes was 50 μM. The permeability of broad spectrum penicillins was measured by RGN823 penicillinase (○) or RGN238 penicillinase (●) and that of cephalosporins was measured by *C. freundii* cephalosporinase (▲) or RGN823 penicillinase (△). The permeability of penicillins having hindered 6-β side chains was measured by RGN238 penicillinase (□). a,a', benzylpenicillin; b,b', ampicillin; c,c', amoxicillin; d, ticarcillin; e, sulbenicillin; f, carbenicillin; g, oxacillin; h, cloxacillin; i, dicloxacillin; j, methicillin; k,k', cephaloridine; l, cephalexin; m,m', cefazoline; n,n', cefadroxil; p, ceftezole; q, cephalosporin-C. R_f values were obtained as described in section 2.

The difference in the permeability parameters between the groups was not due to the difference in enzymes used, because the parameters of 3 representative penicillins and 3 cephalosporins when they were measured by RGN238 penicillinase and RGN823 penicillinase, respectively, showed essentially the same patterns as those measured by RGN823 penicillinase and *C. freundii* cephalosporinase, respectively (fig.3). In the former case, the calculated parameters tend to be a little smaller than when they were measured by the most appropriate enzymes, but the difference was less significant compared with the difference in the permeability between groups.

The predicted pH drop inside the liposomes during enzymic hydrolysis of β-lactam antibiotics also did not affect the results since the rate of hydrolysis of penicillins and cephalosporins by in-

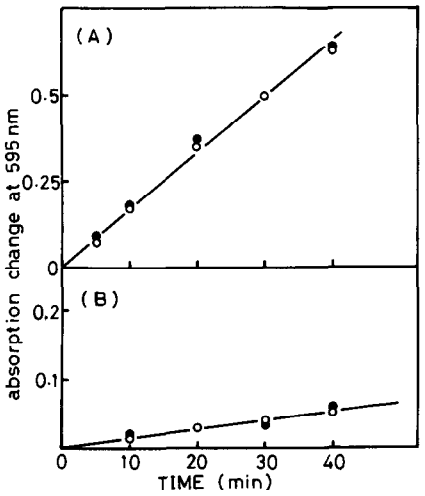

Fig.4. (A) Hydrolysis of ampicillin (50 μM) by RGN823 penicillinase-containing intact liposomes (50 μM lipid) in the absence (●) or presence (○) of 10 μM CCCP was recorded by an absorption change at 595 nm by the microiodometric method. (B) Hydrolysis of cephaloridine (50 μM) was measured in the same way as in (A) except that the lipid concentration was 150 μM.

tact liposomes was not altered by addition of a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) during the course of enzymic hydrolysis (fig.4). In addition, the pH drop inside the liposome could not be detected by continuous-flow dialysis using methylamine (not shown).

These observations supported the view that the difference in the permeability observed in this experiment reflects the true difference in lipid-bilayer permeability of 3 groups of β-lactam antibiotics.

4. DISCUSSION

When molecules pass through a lipid bilayer membrane via a solubility-diffusion mechanism, the permeability coefficient is governed by the product of the partition coefficient between hydrocarbon solvent and water and the diffusion constant in the membrane [13]. Of those two factors, the partition coefficient is predominant [14]. We showed the proportionality between lipid-bilayer permeability and hydrophobicity within the structurally analogous β-lactams. On the other hand, broad spectrum penicillins, cephalosporins and penicillins have hindered side chains showed very different permeabilities compared with their hydrophobicity. The permeability properties of β-lactams are more complex than those of non-electrolytes due to their own electronic properties even though their molecular sizes are similar. The difference between penicillins and cephalosporins might be mainly attributed to the difference in electronic properties of their nuclei such as amide resonance [15].

The unexpectedly high phospholipid bilayer permeability of moderately hydrophilic penicillins gave a clue to understanding their relatively high outer membrane permeability to a porin-deficient mutant [4], although the result here could not be directly extended to the membrane containing lipopolysaccharides and proteins.

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