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In situ potentiometric method to evaluate bacterial outer membrane-permeabilizing ability of drugs: Example using antiprotozoal diamidines

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ABSTRACT

We introduced a new assay system, combining tyrocidine A and a K^+-selective electrode, to evaluate the bacterial outer membrane-permeabilizing ability of drugs. Tyrocidine A, in the presence of an outer membrane permeabilizer, increased the permeability to K^+ of the cytoplasmic membrane of *Escherichia coli*, because this antibiotic could markedly increase the permeability of phospholipid layers constituting the cytoplasmic membrane, while it acted weakly on the outer membrane. Hence, the novel function of agents increasing the permeability of the outer membrane could be examined directly by monitoring the tyrocidine A-induced leakage of K^+ from the bacterial cytoplasm using a K^+-selective electrode. We found that antiprotozoal diamidines, such as diminazene, pentamidine, and 4′,6-diamidino-2-phenylindole (DAPI), can increase the permeability of the bacterial outer membrane and appropriate lipophilicity is important for diamidines to permeabilize the outer membrane.

1. Introduction

The outer membrane of Gram-negative bacteria forms an effective barrier to many hydrophobic molecules. Consequently, numerous antibiotics that are active against Gram-positive organisms are much less active against Gram-negative bacteria. This barrier is formed through cross-bridging between lipopolysaccharide molecules via divalent cations (Ca^{2+} and Mg^{2+}). Thus, metal ion chelators, such as EDTA, certain cationic peptides, and polyamines, which can attack the binding sites of divalent cations, are able to disrupt the organization of the outer membrane, increasing its permeability, and therefore sensitize bacteria to hydrophobic antibiotics that otherwise ineffectively traverse the outer membrane (Salmi and Brunel, 2007; Savage et al., 2002; Vaara, 1992; Yasuda et al., 2004). A structural requirement of agents to increase the permeability of the outer membrane at relatively low concentrations is the presence of at least two positively charged groups with an amphiphilic moiety (Katsu, 1991; Katsu et al., 1985).

We are particularly interested in finding an activity to permeabilize the outer membrane among conventional medicines and agents. We chose antiprotozoal and chemopreventive diamidines, along with a general chemical reagent, possessing two amidino groups. The compounds tested included diminazene (Baraldi et al., 2004; Delespaux and De Koning, 2007; Steck et al., 1981), pentamidine (Baraldi et al., 2004; Delespaux and De Koning, 2007; Steck et al., 1981), 4′,6-diamidino-2-phenylindole (DAPI) (Delespaux and De Koning, 2007), *S,S′*-1,4-phenylene-bis(1,2-ethanediyl)bisisothiourea (1,4-PBIT) (Chen et al., 2004), and 1,4-(diamidino)benzene (1,4-DAB). The chemical structure of the agents is shown in Fig. 1. They all possess an amphiphilic structure with two cationic amidino groups. Although aromatic diamidines, such as diminazene, pentamidine, and DAPI, have been known to possess antibacterial activity (Anné et al., 1980), their outer membrane-permeabilizing ability has not yet been investigated. We first examined such ability with a standard microbiological assay (Yasuda et al., 2004; Urakawa et al., 2010), using the synergistic effect of diamidine and an antibiotic that ineffectively traverse the outer membrane of Gram-negative bacteria. We applied novobiocin and tyrocidine A as antibiotics. Although novobiocin is widely used for this purpose (Yasuda et al., 2004; Urakawa et al., 2010), we introduced tyrocidine A to obtain further evidence for outer membrane permeabilization. Because tyrocidine A can increase the permeability of the phospholipid layers constituting the cytoplasmic membrane of living cells (Aranda and De Kruijff, 1988), its use in combination with an outer membrane permeabilizer induced the leakage of K^+ present in the cytoplasm of bacteria. The results obtained through the in situ monitoring of drug actions using a K^+-selective electrode proved directly the outer membrane-permeabilizing ability of the agents. We revealed that appropriate lipophilicity is important for diamidines to permeabilize the outer membrane.

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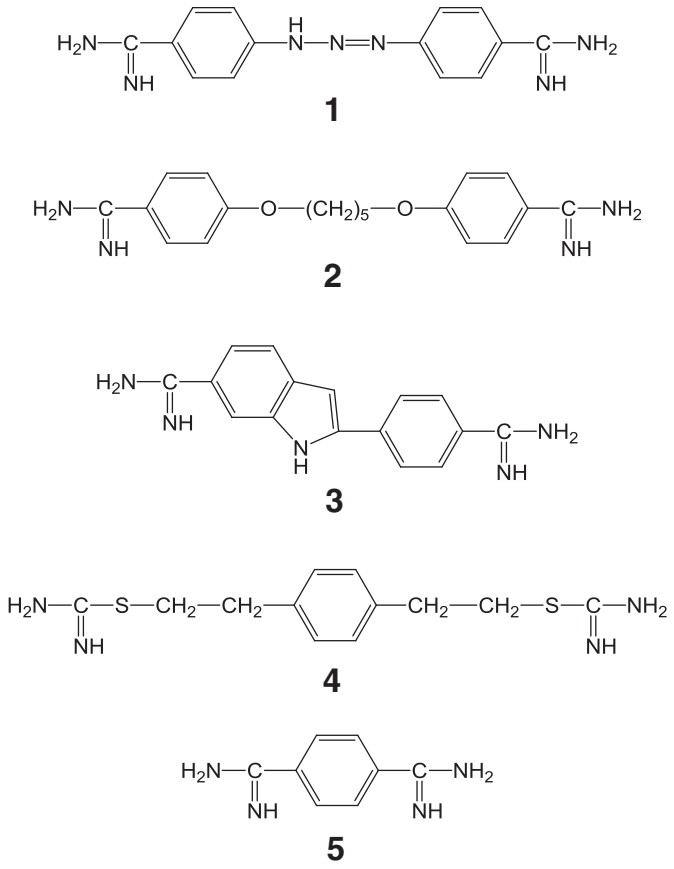

Fig 1. Diamidines tested in this study. Diminazene (1), pentamidine (2), 4′,6-diamidino-2-phenylindole (DAPI) (3), *S,S*′-1,4-phenylene-bis(1,2-ethanediyl)bisisothiourea (1,4-PBIT) (4), and 1,4-(diamidino)benzene (1,4-DAB) (5).

2. Materials and methods

2.1. Reagents

The sources of reagents were as follows: diminazene aceturate, pentamidine isethionate, novobiocin, melittin, and polymyxin B from Sigma (St. Louis, MO, USA); DAPI as a dihydrochloride salt from AnaSpec (San Jose, CA, USA); 1,4-PBIT as a dihydrobromide salt from Cayman (Ann Arbor, MI, USA); 1,4-DAB as a dihydrochloride salt from Aldrich (Milwaukee, WI, USA); polypepton and agar powder from Wako (Osaka, Japan); Mueller–Hinton broth and yeast extract from Becton Dickinson (Sparks, MD, USA); and nutrient broth from Nissui (Tokyo, Japan). Tyrocidine A was synthesized according to procedures described previously (Ösapay et al., 1990). All other reagents were of analytical grade.

2.2. Minimum inhibitory concentrations (MICs)

MICs were determined by the liquid microdilution method, using serially diluted (two-fold) agents. Cells (1×10^4) were cultured at 37 °C for 20 h in 100 μL of Mueller–Hinton broth containing the test agent in 96-well microtiter plates. The MIC was determined as the lowest concentration of agent at which cells were unable to grow (Yasuda et al., 2004; Urakawa et al., 2010). The synergistic inhibitory effect of the antibiotic in the presence of diamidines was measured as follows. Cells (1×10^4) were inoculated in Mueller–Hinton broth containing a serial two-fold dilution (1–128 μg/mL) of novobiocin or tyrocidine A, and then, an appropriate concentration of the diamidine was added. The final volume in each microtiter plate well was 100 μL. After incubation at 37 °C for 20 h, the MIC was determined as described above.

2.3. Preparation of bacteria

Escherichia coli K12 strain W3110 and *Staphylococcus aureus* FDA 209P were used. *E. coli* cells were grown at 37 °C in a minimal salt medium supplemented with 1% polypepton, while *S. aureus* cells were grown at 37 °C in a nutrient broth (Katsu et al., 1989; Nakao et al., 2011). Cells were harvested in the exponential phase of growth, washed twice with buffer (100 mM choline chloride and 50 mM 4-morpholinepropanesulphonic acid (Mops) — Tris, pH 7.2), and suspended in this buffer at a final concentration of 2×10^9 CFU/mL.

2.4. Measurements of cell viability and K^+ leakage

E. coli cells (2×10^9 CFU/mL) were incubated with the test agent in the presence and absence of tyrocidine A at 37 °C for 30 min. The final volume of the cell suspension was 1 mL. The viability of cells was determined by counting colonies. After incubation, 100 μL of the cell suspension was taken, diluted with physiological saline, and dispersed on an agar plate prepared with 1% polypepton, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar (pH was adjusted to 7 by adding NaOH). Colonies were counted after standing for 20 h at 37 °C. The remaining cell suspension was centrifuged (14,000×*g* for 1 min), and the amount of K^+ in the supernatant was measured with a K^+-selective electrode (Katsu et al., 1986, 1989; Komagoe et al., 2011; Nakao et al., 2011). The total amount of K^+ was determined by disrupting the outer and cytoplasmic membranes of cells with polymyxin B (Komagoe et al., 2011).

3. Results

Because aromatic diamidines have been known to possess antibacterial activity (Anné et al., 1980), we first investigated the MICs of the present compounds against *E. coli* cells. As summarized in Table 1, DAPI showed moderate antimicrobial activity, while other compounds were less effective against *E. coli* cells, in accordance with the previous results (Anné et al., 1980). The diamidines, however, were expected to increase the permeability of the outer membrane of *E. coli* cells, because they possessed two positively charged groups with an amphiphilic moiety, which is a minimum structural requirement of agents to increase the permeability of the outer membrane at relatively low concentrations (Katsu, 1991; Katsu et al., 1985). Such ability can be examined based on the synergistic effect of diamidines and antibiotics that ineffectively traverse the outer membrane of Gram-negative bacteria, an experiment widely accepted in the field of microbiological research (Urakawa et al., 2010; Vaara, 1992; Yasuda et al., 2004). In the present study, novobiocin and tyrocidine A were used as the antibiotics. Although novobiocin, which acts on DNA gyrase (Hooper et al., 1982), is widely used for this purpose, we introduced tyrocidine A, which acts on the phospholipid layers constituting the cytoplasmic membrane of the living cells (Aranda and De Kruijff, 1988). As shown in Table 2, when novobiocin or tyrocidine A was added in the presence of diamidines at concentrations below their MICs, both antibiotics became remarkably effective, suggesting that diamidines had the ability to increase the permeability

Table 1
MICs of diamidines against *E. coli*.

Diamidine	MIC (μg/mL)
Diminazene	32
Pentamidine	128
DAPI	8
1,4-PBIT	>128
1,4-DAB	>128