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ORTHO-HYDROXYBENZOATES MAY ACT AT THE PROTEIN FRACTION TO ENHANCE MEMBRANE PERMEABILITY

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Experimental observation suggests that the ability of *ortho*-hydroxybenzoates to alter the permeability of rat rectal epithelial membrane may involve an interaction between the *ortho*-isomer and the membrane protein. Such an interaction may cause a temporary structural modification of the protein rendering the membrane permeable to compounds normally poorly absorbed from the rectal compartment.

The adjuvant effect of sodium salicylate on rectal absorption of drugs has been shown to be substantially higher than the adjuvant effect of *meta*- and *para*-hydroxybenzoate isomers [1]. Our previous studies [2,3] using human red blood cells have indicated that the ability of salicylate to permeate the barrier membrane may be essential for enhanced drug uptake to occur. Evidence in the above studies also suggested that the site of salicylate permeation may be the protein fraction. In the present report, we examined the membrane permeation of *ortho*-hydroxybenzoates using brush-border membrane vesicles and artificial protein-containing lipid membranes in order to understand how the *ortho*-hydroxybenzoates' mechanism of permeation may be related to their adjuvant action.

Permeation of brush-border membrane vesicles from male Sprague-Dawley rat rectal mucosa was carried out by the method of Kessler et al. [4]. A medium was prepared with isotonic buffer containing 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-Tris (pH 7.0), 40 mM NaSCN and brush-border membrane vesicles (20 mg of protein/ml). The osmotic pressure of the medium was adjusted with mannitol. Uptake of compounds into rat rectal brush border membrane vesicles was determined after a 10-min incubation in the medium at 30 °C. Other procedures were performed according to the method of Hopper et al. [5]. The amount of compound taken up into the vesicles was measured by high-pressure liquid chromatography following extraction of the compound from the vesicles with acetonitrile.

Model artificial lipid membranes and artificial protein-containing lipid membranes were prepared using glass-fiber filter circles (2.4 cm diameter, 0.58 mM thickness and 120 g/m^2). Artificial protein-containing lipid membranes were prepared by submerging the filters for 10 min in 50 ml of 0.01 M phosphate buffer (pH 7.0) containing 500 mg of protein extracted from rat rectal brush-border membranes [6]. After drying under a flow of nitrogen gas for 6 h, the filters were immersed for 5 min in 10 ml of a chloroform/methanol (6 : 4,v/v) solution containing 1 g phosphatidylcholine, 200 mg cholesterol and 100 mg diacetyl

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phosphate. The filters were then dried at room temperature. Artificial lipid membranes without protein were prepared by immersing the filters in a phosphate buffer which did not contain protein. Each membrane was positioned in the middle of an apparatus dividing it into two separate chambers, the donor side and the receptor side. A volume of 4 ml of buffer solution containing cefoxitin and/or a benzoate analog was placed on the donor side and 1 ml of buffer solution was placed on the receptor side. Compound permeation of the membrane was determined by measuring the concentration of the compound on the receptor side after a 1-h incubation at 20 °C.

o-Hydroxybenzoate analogs increased the uptake of sodium cefoxitin, a low lipophilic cephamycin antibiotic [7], into brush-border membrane vesicles prepared from rat rectal membrane (Fig. 1). Other benzoates not having the hydroxy group at the *ortho* position only slightly affected cefoxitin uptake.

Before the mechanism of adjuvant action of *o*-hydroxybenzoates can be explained, the mechanism of membrane permeation for the benzoate analogs should be examined. *Ortho* analogs were taken up into brush-border membrane vesicles to a greater degree than the *meta*- and *para*-hydroxybenzoate isomers (Fig. 1). This difference in total uptake after a 10-min incubation could indicate that the mechanism of membrane permeation for *ortho*-isomers is not the same as that for *meta*- and *para*-isomers. The evidence below strongly suggests that unlike the *meta*- and *para*-isomers, the *ortho*-isomers have an affinity for the membrane protein.

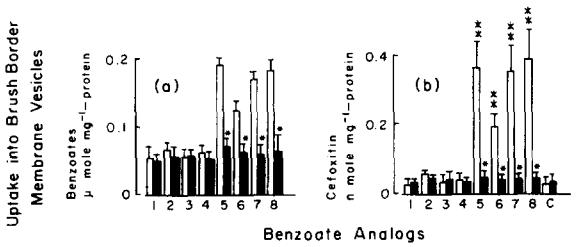

Fig. 1. (a) Uptake of benzoate analogs into rat rectal brush-border membrane vesicles (20 mg of protein/ml) at pH 7.0 in absence of any inhibitor (□) and in the presence of 5 μM DIDS (■) after a 10-min incubation at 30 °C. Initial concentration of benzoate analogs in the medium was 20 μmol/ml. The analog corresponding to each number is as follows: 1, sodium benzoate; 2, sodium *o*-methoxybenzoate; 3, sodium *m*-hydroxybenzoate; 4, sodium *p*-hydroxybenzoate, 5, sodium salicylate; 6, sodium 2,4-dihydroxybenzoate; 7, sodium 5-methoxysalicylate; 8, sodium *p*-aminosalicylate. (b) Uptake of cefoxitin into rat rectal brush-border membrane vesicles (20 mg of protein/ml) in absence of any inhibitor (□) and in the presence of 5 μM DIDS (■) after a 10-min incubation at 30 °C. Initial concentration of cefoxitin in the benzoate medium was 40 nmol/ml. The benzoate analogs corresponding to each number are described above. 'C' is the control which contained 20 μmol of NaSCN/ml instead of a benzoate species. For both (a) and (b), each value represents the mean ± S.D. ($n \geq 4$). For *, $P < 0.001$ versus without DIDS, Student's *t*-test; for **, $P < 0.001$ versus control.

4,4′-Diisothiocyano-2-2′-disulfonate stilbene (DIDS, 5 μM), reported to irreversibly interact with the amino group in the protein structure [8], inhibited the uptake of salicylate and 5-methoxysalicylate into brush-border membrane vesicles but did not affect the uptake of *m*- and *p*-hydroxybenzoates. Salicylate-enhanced uptake of cefoxitin into brush border membrane vesicles was also significantly suppressed by DIDS (Fig. 1).

To further study the involvement of the membrane protein in the permeation of *o*-hydroxybenzoates, model artificial lipid membranes and artificial protein-containing lipid membranes were employed. As shown in Fig. 2a, the permeation of *o*-hydroxybenzoates across the artificial protein membrane was more rapid than their permeation of the artificial non-protein membrane. *Meta*- and *para*-isomers were taken up across the two membranes at a similar rate (Fig. 2a). Cefoxitin by itself did not significantly permeate either the protein or non-protein artificial membranes (Fig. 2b, control). Coadministration with *o*-hydroxybenzoates, however, substantially increased cefoxitin permeation of the protein membrane whereas the permeability of the non-protein membrane to cefoxitin was not affected.

To verify membrane integrity after exposure to salicylate, the protein membrane was immersed in a buffer solution containing 20 mM salicylate for 30 min and then rinsed and inserted into the apparatus. When the medium containing only cefoxitin was placed on the donor side, the concentration of cefoxitin on the receptor side 1 h after incubation was minimal (0.31 ± 0.12 μM, $n = 6$). Therefore, at the salicylate concentration used in this study, the change in membrane permeability following salicylate pretreatment ap-

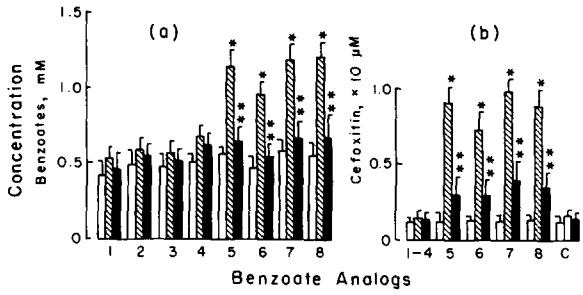

Fig. 2. (a) Benzoate analog permeation of the artificial non-protein membrane (□) and the artificial-protein membrane (□) determined by the concentration of the analog on the receptor side after a 1-h incubation at 20 °C (20 mM initial analog concentration, analog number described in Fig. 1). The effect of DIDS (5 μM), added to the donor side, on benzoate permeation of the artificial protein membrane is also shown (■). Each value represents the mean ± S.D. ($n = 8$). (b) Effect of benzoate analogs on the permeability of the artificial non-protein membrane (□) and artificial-protein membrane (▨) to cefoxitin. (Analog numbers described in Fig. 1). Initial concentration of cefoxitin on the donor side was 40 nmol/ml. 'C' is the control which contained 20 μmol of NaSCN/ml instead of a benzoate species. The effect of DIDS (5 μM), added to the donor side, on enhanced cefoxitin permeation of the artificial protein membrane is also shown (■). Results for the benzoate analogs 1–4 were combined in the figure. In both (a) and (b), each value represents the mean ± S.D. ($n = 8$). For *, $P < 0.001$ versus non-protein membrane, Student's t-test; for **, $P < 0.001$ versus without DIDS.

pears to be reversible and not a result of structural damage [9].

The addition of DIDS to the donor side suppressed the permeation of the *ortho*-isomer across the artificial protein membrane and caused a decrease in the *o*-hydroxybenzoate-enhanced uptake of cefoxitin (Fig. 2b). These artificial-membrane studies imply the existence of an affinity between *o*-hydroxybenzoates and the membrane protein. Furthermore, since DIDS, which blocked the transport of *o*-hydroxybenzoates, also inhibited *ortho*-isomer-enhanced cefoxitin uptake across both vesicular and artificial membranes, *o*-hydroxybenzoates' enhancement of cefoxitin permeation may be dependent on concurrent uptake of the antibiotic and the *ortho*-isomer.

The interaction between orthohydroxybenzoates and the membrane protein may, therefore, produce a transitory change in the physicochemical properties of he protein. We speculate that this *o*-hydroxybenzoate-induced structural change in the protein alters the membrane permeability thus permitting low lipophilic compounds to be taken up across the membrane barrier. Investigations of the mechanism underlying the salicylate/protein interaction are currently ongoing in our laboratories.

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