Arch. Pharm. Res. Vol. 19, No. 5, pp. 435-440, 1996

Effect of Benzalkonium Chloride on Percutaneous Absoption of Antisense Phosphorothioate Oligonucleotides

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(Received March 27, 1996)

The effect of benzalkonium chloride on skin permeability of partially modified antisense phosphorothioate oligonucleotides (PS-ODN), which are designed as scar formation inhibitor, was investigated using Franz Diffusion Cell. When the concentration ratio of PS-ODN-quarternary ammonium salt complex is more than 1:100, the apparent partition coefficient (APC) of each complex was increased in the following order; tetraphenyl phosphonium chloride (TPP) < cetyltrimethyl ammonium bromide(CTAB) < benzalkonium chloride (BZ). The permeability of PS-ODN through the rat skin increased in the presence of BZ. The fluxs of PS-ODN with BZ were increased by addition of Pluronic F 68 or Triton X-100 to phosphate buffered saline (PBS), respectively. When the mole ratio of PS-ODN to BZ is 1:10, the fluxs penetrated of PS-ODN with BZ was greatest. The increase of the permeability in the presence of BZ might be due to the formation of lipophilic ion-pair complex between PS-ODN and BZ. By regulation of mole ratio of PS-ODN to BZ, the development of topical dosage forms using PS-ODN as scar formation inhibitor will be possible with minimal systemic exposure.

Key words : Antisense oligonucleotide, Phosphorothioate, Percutaneous absorption, Apparent partition coefficient (APC), Ion pair, Benzalkonium chloride

INTRODUCTION

The ability of antisense oligonucleotides to bind to complementary sequence in DNA, pre-mRNA, or mRNA and thus block the transfer of information leading to the formation of a specific gene product or protein has aroused the interest of pharmaceutical scientists (Uhlmann *et al.*, 1990). However, the large molecular weights, relative instability in the biological fluid, and the charged nature of oligonucleotide derivatives pose major challenges to the successful delivery of antisense molecules to their intracellular sites of action (Jaroszewski *et al.*, 1991). Different strategies (Dreyer *et al.*, 1985) have been developed to give protection and to increase transport of oligonucleotides to the target cell. Linking intercalating agents (acridine) (Stein *et al.*, 1988), hydrophobic groups (cholesterol) (Letsinger *et al.*, 1989) or polycations (poly-l-lysine) (Leonetii *et al.*, 1990; Leonetii *et al.*, 1988; Martinez-Fong, 1993; Ryser *et al.*, 1978) to oligonucleotides resulted in a higher resistance toward exonucleases and increased their penetration into cell. Besides increasing the ability to across membranes, it might be of major importance to achieve the correct localization of antisense oligonucleotide in the various compartments of the cell. Antisense oligonucleotides, like peptide and protein drugs, are susceptible to enzymatic hydrolysis and are unlikely to be effective by oral administration. Lipophilic drugs have been used for research on and development of transdermal system frequently because of their high permeabilities across the skin. Water soluble drugs, in general, have not been administered through the skin, since the skin permeabilities are often low. However, topical formulation of these water soluble molecules have become possible due to the addition of BZ. The skin transport of macromolecules has become of great interest with the advent of biotechnology methods in drug discovery, development, and production.

Antisense phosphorothioate oligonucleotide (PS-ODN) complementary TGF-β (25 mer) (Sharples *et al.*, 1987; Derynck *et al.*, 1985) was studied as scar formation inhibitor *in vivo* (Simons *et al.*, 1992).

In the present study, the apparent partition coefficient (APC) of PS-ODN-quarternary ammonium salt complex and *in vitro* skin permeability in the presence of quarternary ammonium salt were measured

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in damaged rat skin to circumvent the poor affinity for the skin due to highly water-soluble property of antisense oligonucleotide originated from its polyanionic charge.

MATERIALS AND METHODS

Materials

Antisense agent ® and NAP-10 were purchased from Pharmacia LKB Biotechnology. ^{35}S-ATP γ S and T4 polynucleotide kinase were purchased from Amersham International plc. Microcon-3 (MW 3,000 cut off) was purchased from Amicon, Inc. and Pluronic F 68 was obtained from the BASF. Tetraphenylphosphonium chloride (TPP), cetyltrimethyl ammonium bromide (CTAB), benzalkonium chloride (BZ) were purchased from Sigma chemical company. Other reagents were all of special reagent grade.

Synthesis of PS-ODN

PS-ODN was synthesized on an automated DNA synthesizer(Plodel, Gene assembler special, Pharmacia LKB Biotechnology, Uppsala, Sweden) at the Korea Biotechnology. The sequence of PS-ODN was as follows; 5'-C(*)AG CCC(*) GGA GG(*)G CGG C(*) AT GGG(*) GGA(*) G-3'. Asterisk represents position of sulfur. PS-ODN was lyophilized and purified by NAP-10 coumn (Pharmacia LKB Biotechnology).

35S-labelling of PS-ODN

PS-ODN was 5'-end labelled with 35S-γ ATP and T 4 polynucleotide kinase (Amersham International plc) in reaction mixture (Shaw *et al.*, 1991). A reaction mixture set up as follows; 2 μl oligonucleotide (20 pmoles), 4 μl 5×T4 polynucleotide kinase buffer containing 300 mM Tris Cl (pH 7.6), 50 mM $MgCl_2$, 1.65 μM ATP, 75 mM 2-mercaptoethanol, 2 μl ^{35}S-γ ATP (specific activity 1,000 Ci/mmole; 10 mCi/ml in aqueous solution)(20 pmoles), 10 μl ddH_2O, 2 μl T4 polynucleotide kinase (10 units/μl). It was mixed well and incubated for 45 minutes at 37°C. The remainder of the reaction was heated for 10 minutes at 68°C to inactivate the T4 polynucleotide kinase. Labelled PS-ODN was purified by Microcon-3 (MW 3,000 cut off, Amicon, Inc.).

Skin preparation

On the day before the experiment, the hair of the dorsal area of rats was removed with an electric clipper and depilatory. On the next day, pieces of full thickness dorsal skin were excised from the rats. The adherent fat and other debris were removed from the under surface. Damaged skin was prepared by removing the stratum corneum by repeated application of cellophane tape (about 15times) until the skin glistened (Bronaugh *et al.*, 1985). Removal of dermis was accomplished by immersion at 60°C water for 1 minute. The dermal side of the skin was soaked in phosphate buffered saline (PBS, pH 7.4) for 4 hrs at 37°C.

In vitro transport studies

In vitro skin transport study was investigated according to the method of Nolen III *et al.* (1994). The receptor compartment of each Franz Diffusion Cell (Crown Gglass Co. USA) was filled with 10 ml of PBS (pH 7.4, 0.05% sodium azide) containing PF68 or Triton X-100. The jacked receptor compartments were kept at 37±0.1°C sufficient to maintain the temperature of the receptor just below the skin at 32±0.5°C. And the available surface area for penetration was 1.77 cm^2. Receptor compartment was mixed through out the experiment with teflon magnetic stirring bar driven by a constant speed (300 rpm) motor. ^{35}S-labelled PS-ODN (10 μg/100 μl) with or without BZ was uniformly applied to the donor side and occluded with a sheet of aluminum foil and parafilm. The effect of mole ratio of PS-ODN to BZ was carried out in 1:1, 1:10, 1:100. Aliquots of 100 μl samples were withdrawn periodically and replaced with an equal volume of fresh normal medium maintained at 37°C. The drug permeation was monitored for 24 hours. The sample of 35S-labelled PS-ODN (100 μl) was mixed with 6 ml of scintillation cocktail (Amersham International plc) and the concentrations were determined using a liquid scintillation system (Beckman LS 7800).

Partition studies between Tris-EDTA (TE) buffer and dichloromethane

Partition studies were investigated according to the modified method of Yong *et al.* (1988). Dichloromethane saturated with TE buffer (pH 7.4) and TE buffer (pH 7.4) saturated with dichloromethane were used. TPP, CTAB and BZ were chosen as quarternary ammonium salts. PS-ODN and quarternary ammonium salt were dissolved in TE buffer (pH7.4) saturated with dichloromethane. The concentration of PS-ODN was kept constant at 1 μM, with only the concentration of quarternary ammonium salt being varied from 10 μM to 500 μM. A 500 μl aliquot of aqueous buffer solution containing PS-ODN and quarternary ammonium salt was mixed vigorously with 500 μl of dichloromethane in an eppendorf tube for about 5 minutes. After standing for 30 minutes at 25°C, two phases were separated by centrifugation at 3,000 rpm for 10 minutes and the aqueous phase was assayed for PS-ODN. The concentration of PS-ODN in the aqueous phase before and after partitioning was

measured using a liquid scintillation system (Beckman LS 7800).

$$\text{Apparent partition coefficient(APC)} = \frac{[\text{concentration of PS-ODN in dichloromethane}]}{[\text{concentration of PS-ODN in TE buffer}]}$$

Analysis of data

The *in vitro* percutaneous permeation parameters were calculated according to the following equations (Chow *et al.*, 1984),

$$D = l^2/6T$$
$$Js = D\ Km\ Cs/l$$
$$Kp = D\ Km/l$$

where Js is the penetration rate, D denotes the diffusion constant within skin, Km is the skin/vehicle partition coefficient of PS-ODN, T represents the lag time calculated from the intercept of the flux with the time axis. Kp denotes the permeability coefficient through the stratum corneum and, Cs is the PS-ODN concentration.

RESULTS AND DISCUSSION

Partition studies

Table I listed the APC of PS-ODN-quarternary ammonium ion pair complex between TE buffer and dichloromethane. The concentration of PS-ODN was kept constant at 1 mM. The concentration ratios of PS-ODN and quarternary ammonium salt were 1:10, 1:50, 1:100, 1:500. As shown in Table I, the APC of PS-ODN was increased by the addition of quarternary ammonium salt, which indicates the possible formation of the lipophilic ion pair complex between PS-ODN and quarternary ammonium salt. In the case of 1:10, the APC of each complex was 0.235, 2.030, 0.064, TPP, CTAB, BZ, respectively. The APC of PS-ODN-CTAB ion pair complex was greatest. However, in the concentration ratio of 1:100, the APC of each complex was 1.273, 2.390, 5.250, respectively. The APC of PS-ODN-BZ ion pair complex was most large. In the concentration ratio of 1:500, most of each ion pair complex was distributed in dichloromethane. The extent of ion pairing was markedly increased in proportion to the increment of concentration ratio. And the APC of PS-ODN-BZ complex is 0.064 in 1:10 and 7.333 in 1:500. When the concentration ratio of PS-ODN and quarternary ammonium salt is more than 1:100, the APC of complex was increased in the following order; TPP<CTAB<BZ.

Table I. Apparent partition coefficient (APC) of PS-ODN-quarternaryammonium saltion pair complex between TE buffer (pH 7.4) and dichloromethane

Ratio	TPP	CTAB	BZ
1: 10	0.235	2.030	0.064
1: 50	0.538	2.175	1.857
1: 100	1.273	2.390	5.250
1: 500	6.524	6.690	7.333

TPP: Tetraphenyl phosphonium chloride
CTAB: Cetyltrimethyl ammonium bromide
BZ:Benzalkonium chloride

Effect of benzalkonium chloride on permeability of PS-ODN

The transport of ionic and polar solutes across skin is not favored by passive diffusion. *In vitro* skin transport of PS-ODN in the presence of BZ was carried out to test whether BZ can be used for the formulation of PS-ODN designed as scar formation inhibitor. We confirmed that despite of high molecular weight and polyanionic charge, damaged skin made a 3 fold higher amount of PS-ODN penetrated compared with normal skin. BZ was used to improve uptake into target cell and stability. It might be expected that complexation of BZ to PS-ODN resulted in a higher resistance toward nuclease and increase their penetration into cell (Chavany *et al.*, 1992).

Which result in changes of several parameters such as partition coefficient (skin/vehicle), diffusivity and activity coefficient of PS-ODN in the skin. The phenomenon of the percutaneous absorption is essentially one of adsorption on to the stratum corneum, diffusion through it and through the viable epidermis, and finally through the papillary dermis and into the microcirculation. Among the aforementioned steps, diffusion through the stratum corneum itself is the ra-

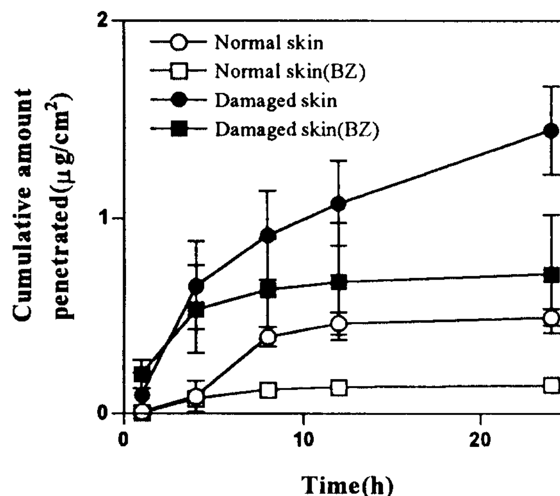

Fig. 1. *In vitro* permeation profiles of PS-ODN with or without BZ across rat skin. Each point represents the mean ±S.E. (n=3).

te-limiting step for the majority of substances. In this paper we focused only on damaged skin because of using in wound healing.

Fig. 1 represents *in vitro* permeation profiles of PS-ODN with or without BZ across rat skin in PBS. In this experiment, mole ratio of PS-ODN to BZ is 1:10. The cumulative amount of PS-ODN penetrating with BZ was unexpectedly smaller than that of PS-ODN alone. The cumulative amount of PS-ODN permeating across normal skin at 24 hrs was 0.498±0. 044 $\mu g/cm^2$ in PS-ODN alone, whereas 0.146±0.008 $\mu g/cm^2$ in PS-ODN with BZ. And the amount of PS-ODN permeating across damaged skin at 24 hrs was 1.446±0.224 mg/cm^2 in PS-ODN alone 0.719±0.302 mg/cm^2 in the presence of BZ. The cumulative amount of PS-ODN over 24 hrs was unexpectedly decreased 3.41 fold in normal skin, 1.59 fold in damaged skin by addition of BZ. It seemed that PS-ODN-BZ complex have an high affinity for more the skin than the receptor medium.

Effect of receptor medium on permeability of PS-ODN

Three different receptor mediums were used in order to increase the solubility of PS-ODN-BZ complex which permeated damaged rat skin. This receptor medium ingredients can be found in the report of Bronaugh *et al.* (1985). The results are shown in Fig. 2 and Table II. The permeation parameters were calculated from the penetration data of PS-ODN with or without BZ. In the case of PBS, the cumulative amount of PS-ODN permeating through damaged rat skin over 8hrs was decreased 1.43 fold in the presence of BZ. However, the presence of detergent such as PF68 and triton X-100 in the receptor resulted in slight enhancement of PS-ODN permeation. In the case of PBS containing 6% PF68, the cumulative amount of PS-ODN was increased 1.22 fold from 1. 077±0.301 $\mu g/cm^2$ to 1.317±0.319 $\mu g/cm^2$ by the addition of BZ.

The presence of Triton X-100 yielded a higher cumulative amount of PS-ODN permeated through damaged rat skin. In the absence of BZ, there is no remarkable difference for the flux by changing of the receptor medium. while, the flux of PS-ODN with BZ permeated through rat damaged skin was increased. The permeability coefficient of PS-ODN with BZ were gradually increased by the addition of PF68 or Triton X-100. These results suggest that the solubility of PS-ODN-BZ complex with skin might be increased by the addition of surfactant to receptor medium.

Effect of mole ratio on permeability of PS-ODN

The effect of mole ratio (PS-ODN :BZ) on skin permeability of PS-ODN across damaged rat skin in PBS

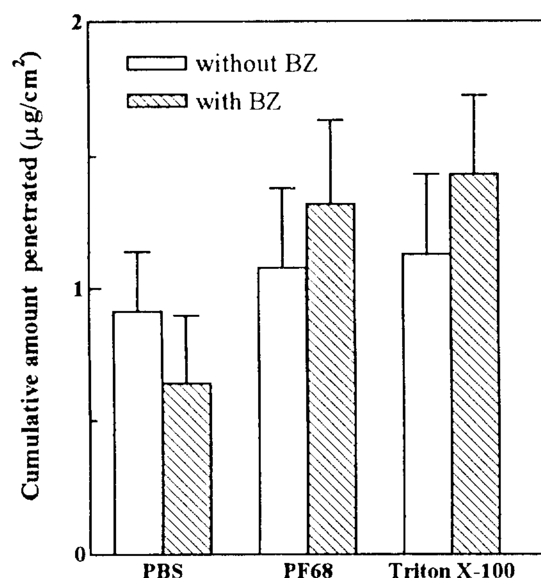

Fig. 2. Effect of receptor medium on the cumulative amount of PS-ODN permeated across damaged rat skin after 8 hrs permeation study. Each bar represent the mean±S.E.(n=3).

Table II. The penetration parameters calculated from the penetration data of PS-ODN with or without BZ

	Flux ($ng/cm^2.h$)	Lag time (h)	Permeability coefficient ($Kp, cm/h \times 10^3$)	Diffusion constant ($D, cm^2/h \times 10^7$)	Partition coefficient (Km)
PS-ODN without BZ					
PBS	114.25±28.36	0.69±0.01	114.25±28.36	24.89±0.36	1.473±0.37
PF68	134.63±37.67	0.70±0.02	134.63±37.68	24.53±0.70	1.762±0.46
Triton X-100	141.25±35.20	0.67 0.02	141.25±30.34	25.63±0.83	1.769±0.45
PS-ODN with BZ					
PBS	79.75±32.28	0.60±0.05	79.75±32.28	28.62±2.40	0.894±0.29
PF68	164.63±39.88	0.58±0.05	164.63±39.88	29.61±2.36	1.785±0.34
Triton X-100	178.63±29.42	0.54±0.04	178.63±35.32	31.80±3.02	1.803±0.32

PBS: Phosphate buffered saline
PF68: Pluronic F 68
Km is the skin/vehicle partition coefficient of PS-ODN
Each value represent the mean S.D(n=3).

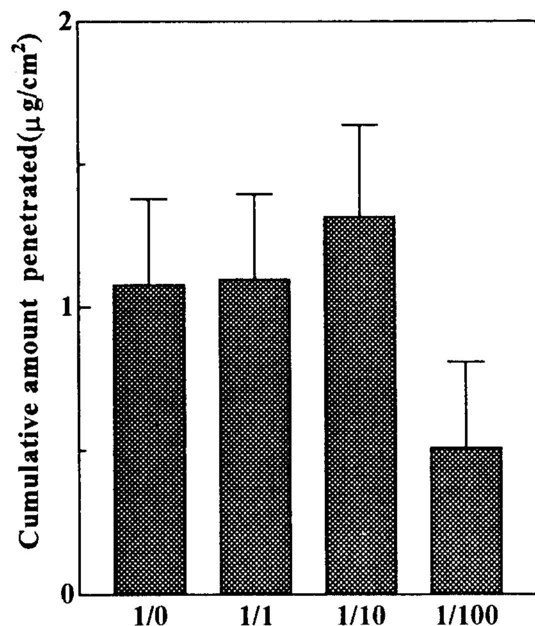

Fig. 3. Effect of mole ratio of PS-ODN to BZ on the cumulative amount of PS-ODN permeated across damaged rat skin after 8 hrs permeation study. Each bar represent the mean±S.E. (n=3).

containing 6% PF68 was shown in Fig. 3. The cumulative amount delivered over 8 hrs was 1.077±0. 301 $\mu g/cm^2$ in control, 1.096 $\mu g/cm^2$ in 1:1, 1.317±0. 319 $\mu g/cm^2$ in 1:10, and 0.508 $\mu g/cm^2$ in 1:100, respectively. 1:1 and 1:10 was increased 1.02 fold, 1. 22 fold, whereas 1:100 was preferably decreased the flux compaired with control. The flux of PS-ODN penetrated into PBS containing PF68 was in the order of 1:100<1:0<1:1<1:10. In a system in which split-thickness rat skin is used as the membrane, the use of a surfactant in the receptor medium was advantageous for absorption measurements of PS-ODN-BZ complex. By variation of mole ratio and receptor medium, the development of topical dosage forms using PS-ODN for treatment of wound healing will be possible with minimal systemic exposure. It might be expected that complexation of BZ to PS-ODN resulted in increase their penetration into cell.

ACKNOWLEGEMENT

This research was done in part by support of WonKwang University (1996). The authors thamk for the help of ILYANG PHARM. CO.

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