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Amphiphilic properties of pilocarpine prodrugs

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Abstract

Corneal permeability of pilocarpine has been increased by prodrug derivatization, but ocular irritation is associated with the in vivo use of the prodrugs. To determine possible amphiphilic nature of the prodrugs, we determined the critical micelle concentrations and lipid bilayer disrupting properties of bispilocarpic acid diester prodrugs. Surface activity of prodrugs in water was determined using interfacial tensiometer. Lipid bilayer disruption was tested by calcein release from liposomes and hemolysis of rabbit erythrocytes in vitro. Molar phospholipid and cholesterol contents of the liposomes (EPC:DOPE:DPPG:Ch 8:6:1.5:1.5) were designed on the basis of the lipid content of the corneal epithelium. Surface activities of the pilocarpine prodrugs increased with the increasing lipophilicity of the derivative and increasing pH. The critical micelle concentrations of the prodrugs were 0.1–1 mM at pH 5.0. The concentrations of the prodrugs to induce 50% leakage of calcein were 0.5 mM (V), 1.2 mM (III), 2.2 mM (IV) and 0.25 mM (V), 0.8 mM (III), 3.5 mM (IV) to cause 50% hemolysis. Also, eye irritation of these prodrugs increased with their increasing lipophilicity, pH and concentration. Lipophilic pilocarpine prodrugs show amphiphilic properties which may contribute to the eye irritation. The harmful effects of pilocarpine prodrugs on cell membranes on ocular surface may limit their usefulness. Possible amphiphilicity of lipophilic prodrugs may be a limitation of the prodrug technique in ocular drug delivery.

Keywords: Prodrug; Bispilocarpic acid diester; Irritation; Surface tension; In vitro hemolysis; Bilayer disruption

1. Introduction

Pilocarpine eyedrops are used topically in the treatment of glaucoma. Ocular bioavailability and duration of activity of pilocarpine is low due to its rapid precorneal loss and hydrophilicity (Lee and Robinson, 1979). In an attempt to prolong the duration of action and improve the bioavailability of pilocarpine, various prodrugs have been developed (Druzgala et al., 1992; Bundgaard et al., 1986a; Bundgaard et al., 1986b). Pilocarpic acid diesters showed promising biopharmaceutical properties but their clinical acceptability is limited due to the eye irritation (Mosher, 1986). Järvinen et al. (1991b) synthetized and evaluated numerous dimeric double prodrugs of pilocarpine but they were also irritating in the rabbit eye (Suhonen et al., 1995). Ocular discomfort or irritation was immediate after drug administration and the irritation appeared to increase with increasing lipophilicity of the prodrug.

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When prodrug technology is used to improve corneal or other biomembrane permeability, hydrophilic parent molecule is usually derivatized with a lipophilic substituent. This may result in potentially amphiphilic compounds. For example, pilocarpine prodrugs have hydrophilic (imidazole ring) and hydrophobic (esterified lactone ring) regions in their structures which suggests possible amphiphilicity. Many amphiphilic drugs are known to perturb synthetic and natural lipid bilayer membranes (Lewis et al., 1994; Ellingson et al., 1992; Luxnat and Galla, 1986; Lee, 1976). Since corneal surface is exposed to fairly high concentration of prodrug in eyedrop solution, the amphiphilic properties may cause epithelial damage and/or irritation.

The purpose of this study was to evaluate the amphiphilic nature of bispilocarpic acid diester prodrugs and compare them to some ophthalmic drugs and pharmaceutical adjuvants. In addition to the surface tension measurements, calcein release from liposomes and rabbit erythrocyte hemolysis tests were utilized to test the possible lipid bilayer disrupting properties of the prodrugs. These properties were correlated with eye irritation.

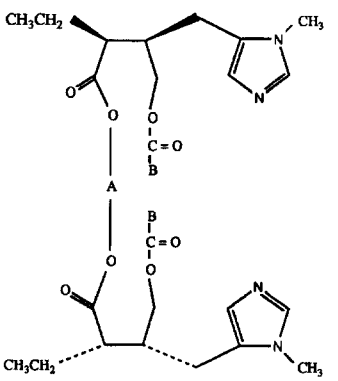
2. Materials and methods

2.1. Materials

Pilocarpine hydrochloride, betaxolol hydrochloride, timolol maleate and dipivefrin hydrochloride were supplied by Leiras Corporation (Tampere, Finland). Benzalkonium chloride was purchased from Fluka Chemie AG (Buchs, Switzerland). Synthesis and analysis of the pilocarpine prodrugs have been described previously (Järvinen et al., 1991a). The chemical structures of pilocarpine prodrugs used in this study are shown in Fig. 1. L-α-phosphatidyl choline (EPC) from egg yolk, L-α-dioleyl phosphatidyl ethanolamine (DOPE), L-α-dipalmitoyl phosphatidyl glycerol (DPPG) and cholesterol (Ch) were from Sigma. All other chemicals were of reagent grade from commercial sources.

2.2. Surface tension measurements

Surface active properties of the studied compounds were determined at 21 ± 1°C at pH 5.0 and 7.4 using the ring method (Wan and Lee, 1974). The concentration ranges were 0.014 μM–19.3 mM and 5 μM–1 mM at pH 5.0 and pH 7.4, respectively. A DuNoüy interfacial tensiometer (K 8600 E/E, Krilss, Hamburg, Germany) was used for the surface tension determinations. An accuracy check on the tensiometer was made by measuring the surface tension of Millipore™ filtered water (72 $mN \cdot m^{-1}$). The tested compounds were dissolved in water, pH was adjusted and the solutions were measured immediately.



Prodrug	A	B
I	$-CH_2-C_6H_4-CH_2-$	$-CH_3$
II	$-CH_2-C_6H_4-CH_2-$	$-CH_2CH_3$
III	$-CH_2-C_6H_4-CH_2-$	$-CH_2CH_2CH_3$
IV	$-CH_2-C_6H_4-CH_2-$	
V	$-CH_2-C_6H_4-CH_2-$	$-C(CH_3)_3$
VI	$-CH_2-CH_2-$	
VII	$-CH_2-CH_2-CH_2-$	
VIII	$-CH_2-CH_2-CH_2-$	

Fig. 1. The chemical structures of the O,O'-(1,4-xylylene) bispilocarpic acid esters (i.e. bispilocarpic acid diesters).

2.3. Liposome studies

Large unilamellar liposomes (LUV) were made by the reverse-phase evaporation method developed by Szoka and Papahadjopoulos (1978). Molar liposome composition was EPC:DOPE:DPPG:Ch, 8:6:1.5:1.5. Briefly, 6.3 mg of EPC, 4.5 mg of DOPE and 0.58 mg of Ch were dissolved in chloroform and 1.1 mg of DPPG in a mixture of chloroform/methanol (2:1). The solvents were removed under reduced pressure by a rotary evaporator. The lipids were redissolved in 1.0 ml of chloroform/diisopropylether (1:1) mixture and 500 μl of isotonic calcein solution (62 mM) in water was added. The mixture was sonicated for 5 min at 45°C (Branson bath sonicator, Model 2200, Danbury, CT, USA) and was then placed on the rotary evaporator to remove the organic solvents. A uniform size distribution of liposomes was formed by extruding the dispersion through polycarbonate membrane filter of 0.2 μm pore size. After extrusion, the unencapsulated calcein was separated twice by column chromatography on Sephadex G-50 with isotonic Hepes buffer of pH 7.4. The size distribution of the vesicles was determined by quasielastic light-scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA). The mean volume size of EPC:DOPE:DPPO:Ch liposomes was 224 nm and 77% vesicles were less than 280 nm in diameter. Phosphorus was determined by the method of Bartlett (1959). The mean concentration of phospholipids in the final liposome suspension was 6 μmol/l.

The liposomes were incubated at room temperature with the drug solutions as follows: to 2.0 ml of isotonic Hepes buffer, 15 μl of liposomes and 1.0 ml of different concentrations of the tested compounds in Hepes buffer were added. The leakage of calcein from the liposomes was monitored for 5 min. Total fluorescence (100% release) was measured by adding 100 μl of 2.5% Triton X-100. The drug concentrations were 0.0125–20 mM at pH 5.0 and 0.006–3.33 mM at pH 7.4. Fluorescence measurements were carried out using a Perkin Elmer Luminescence Spectrometer Model L5 SOB (UK) (widths of excitation and emission slits = 5 nm, λ_{ex} = 494 nm, λ_{em} = 515 nm).

Leakage of entrapped calcein was calculated according to the following equation:

$$\%\ Calcein\ released = \frac{F - F_0}{Ft - F_0} \cdot 100$$

where F is the fluorescence intensity measured at a specified time, F_0 at time zero and F_t the total fluorescence after disruption with Triton X-100. All reported values are mean values of four determinations.

2.4. Hemolysis studies

The hemolysis assays were carried out according to the method of Reer et al. (1994). The blood was taken from the cannulated ear artery of the rabbits. The heparinized blood was centrifuged for 10 min at 1500 *g* (Eppendorf Centrifuge, Model 5415 C, Eppendorf-Netheler-Hinz GmbH 2000, Hamburg, Germany), the supernatant was discarded and the erythrocytes were resuspended in 0.05 M isotonic phosphate buffer (PBS, pH 5.0). The washing procedure with PBS was repeated twice and the erythrocytes were diluted with PBS (3:14) before assays. One hundred microliters of erythrocyte suspension was added in 1.0 ml of isotonic drug solution in PBS (osmolality was measured with Auto-Osmometer Osmostat OM-6020, Kyoto Daiichi, Kagaku Co. Ltd, Japan) and the mixtures were stirred at room temperature for 30 min. The drug concentrations in incubation media were 1 μM–96 mM. After centrifugation (1000 *g*, 5 min), 100 μl of the resulting supernatant was added in 2.0 ml of an ethanol/HCl mixture (39 parts of 99% v/v ethanol and 1 part of 37% w/v hydrochloric acid) and the hemoglobin concentration was determined spectrophotometrically (λ = 398 nm). All experiments were performed in triplicate. Total hemolysis was obtained by incubating the erythrocytes in water. The absorbance of the samples was corrected for the values obtained with isotonic buffer (PBS, pH 5.0). The percentage hemolysis was expressed as the ratio of the absorbance of the samples to the absorbance of 100% hemolysis.

2.5. Ocular irritation testing

Pilocarpine prodrugs were dissolved in 0.5% sodium chloride solution, the solution was made isotonic with sodium chloride and pH was adjusted to 5.0 with sodium hydroxide. The prodrug solutions were equivalent to 0.5% pilocarpine (12 mM), except III and V, which were equivalent to 0.25% pilocarpine (6 mM). Concentration range of the prodrug solutions was 6.3–12.5 mg/ml. Isotonic pilocarpine hydrochloride solutions (equivalent to 1% pilocarpine base, 48 mM) were prepared in 0.65% sodium chloride solution and pH was adjusted to 5.0.

Adult New Zealand albino rabbits of both sexes, 2.8–4.2 kg, were used in the study. The eye irritation of prodrug I was tested in pigmented rabbits (6 male rabbits weighing 2.7–3.7 kg). The rabbits were housed singly in standard feeding conditions and the animals were treated in accordance with the ARVO resolution on the use of animals in research.

Drops of 25 μl of the test solutions were instilled in one eye of the rabbit, while the contralateral eye was a control. Discomfort was measured on the basis of the eyelid closure time (eyes closed and half-open) after instillation of eyedrops.

3. Results and discussion

3.1. Surface activity

The plot of the surface tension of water solutions against concentration of the pilocarpine prodrugs and benzalkonium chloride at pH 5.0 is presented in Fig. 2a. Bispilocarpic acid diesters were clearly surface active, capable of lowering the surface tension below 40 mN/m. Critical micelle concentrations (CMC) were 1.3 μM for BAC (Fig. 2a), 0.1 mM for prodrug V, 0.5 mM for III and 1 mM for IV (Fig. 2b). Surface tension continues to lower above the CMC due to the decreasing monomer concentration above the CMC. The tendency for prodrug molecules to adsorb at the air-water surface and, hence, ability to lower the surface tension increased with increasing lipophilicity of the prodrug (Table 1, Fig. 2b).

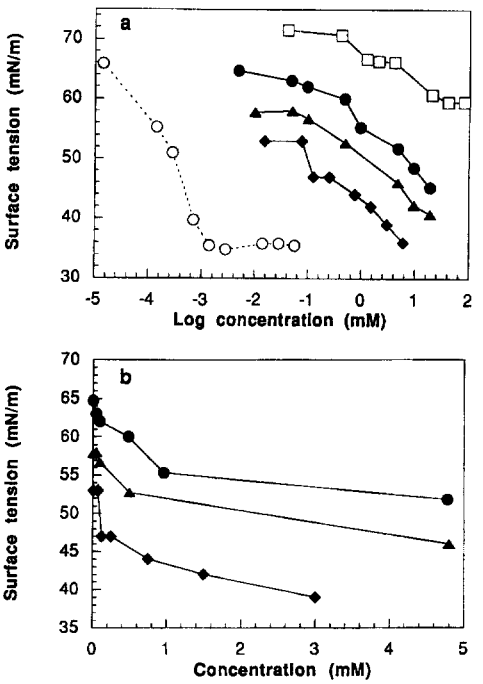


Fig. 2. Surface tension values (mN/m) versus log drug concentration (mM) (a) and drug concentration (mM) (b) at pH 5.0 at 21 ± 1°C. BAC (○); III (▲); IV (●); V (◆); Pilocarpine (□).

The pKa values of the pilocarpine prodrugs in water are about 6.9 (Järvinen et al., 1991a). At pH 5.0, the prodrugs are almost completely ionized and, thus, more hydrophilic having stronger affinity to water than they do at pH 7.4. The effect of pH on the surface tension of prodrug solution is shown in Fig. 3. Equal lowering of surface tension is achieved at 1–3 orders of magnitude higher concentrations at pH 5.0 than at pH 7.4 (Fig. 3). Pilocarpine solution (2%, 96 mM) decreases the surface tension of water from 72 mN/m to 60 mN/m at pH 5.0 and to 55 mN/m at pH 7.4. The equal lowering at pH 5.0 was caused by 15.8 mM (0.5%) timolol, 6.5 mM (0.2%) betaxolol and 0.3 mM (0.01%) dipivefrin solution.

Fig. 4 shows the correlation between the surface tensions of 10-mM solutions and the apparent partition coefficients (log P_{app}) at pH 5.0. The apparent partition coefficients were determined as previously described (Järvinen et al., 1991b) and

Table 1
Apparent partition coefficients (log P_{app}) for the drugs studied

Compound	Log P^a_{app}	
	pH 5.0	pH 7.4
Pilocarpine	−1.74	0.01
I	−0.29	3.04
II	0.66	4.08
III	1.69	5.47
IV	1.04	4.20
V	2.49	—
VI	0.30	3.76
VII	−0.30	3.43
VIII	1.71	—
Dipivefrin	1.14	1.46
Betaxolol	0.14	0.80
Timolol	−0.58	−0.10

[a]Apparent partition coefficient between 1-octanol and phosphate buffer (pH 5.0 or 7.4) at 22°C.

the results are listed in Table 1. The determination coefficient between the surface tension and the log P_{app} is 0.85. The correlation is even better (R^2 = 0.96) when only the results of pilocarpine prodrugs are plotted.

Surface activity increases when the lipophilicity of the prodrug is increasing because the more lipophilic prodrug molecules have higher escaping tendency from bulk solution to the air-water surface. The ionization of the prodrugs at acidic pH increases their water-solubility and, thus, decreases the surface activity. CMC values of bispilocarpic acid diesters were high (mM range) and

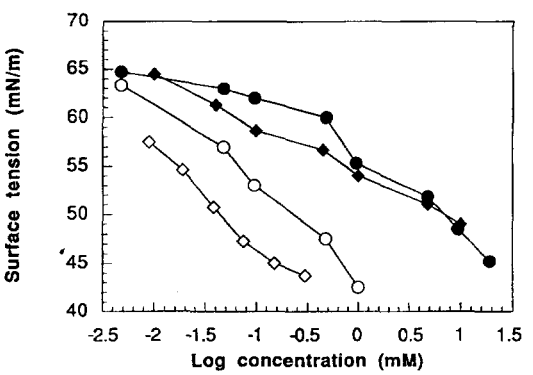


Fig. 3. Effect of pH on the surface tension of prodrug solution II (◆,◇) and IV (●,○) at pH 7.4 (open marks) and pH 5.0 (dark marks).

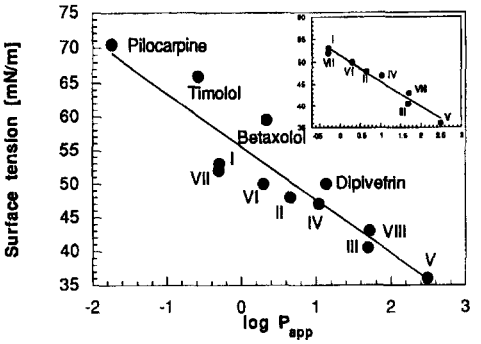


Fig. 4. The relationship between apparent partition coefficients (log P_{app}) at pH 5.0 and surface tension values of 10 mM aqueous drug solutions at pH 5.0.

the inflection points were gradual (Fig. 2a–b). These are typical features in the formation small (n < 10) micelles (Jones and Chapman, 1995). Accordingly, we were not able to detect any micelles with quasi-elastic dynamic light scattering.

3.2. Liposome studies

Bazan and Bazan (1984) have studied the distribution of corneal phospholipids and fatty acids in epithelium, stroma and endothelium of rabbit cornea. Corneal epithelium contains, besides of the common neutral phospholipids phosphatidyl choline and phosphatidyl ethanolamine, also negatively-charged phosphatidyl serine and phosphatidyl inositol. Oleic acid and palmitic acid are the major acyl groups. Corneal epithelium contains also cholesterol, which makes the structure of the cell membrane more rigid.

The lipid composition chosen in the liposomes was based on the data from the literature (Bazan and Bazan, 1984; Broekhuyse, 1968; Feldman, 1967). Negatively-charged liposomes were prepared using dipalmitoyl phosphatidyl glycerol (DPPG) instead of phosphatidyl serine. Phosphatidyl choline from egg yolk was prefered because of the abundance of oleic and palmitic acid chains in EPC (New, 1990).

The percentage release of calcein was monitored for 5 min at room temperature at pH 5.0 (Fig. 5a). The leakage of calcein from the liposomes showed to be dependent on prodrug concentration in solution and the interaction with

lipid bilayer increased with the lipophilicity. From the calcein release-concentration curve, the half-maximal concentrations ($C_{50\%}$) were calculated. The concentrations of the prodrugs to induce 50% leakage of calcein were 0.5 mM (V), 1.2 mM (III) and 2.2 mM (IV). The determination coefficient between the concentration of the prodrug to induce 50% leakage of the marker and log P was 0.98 (data not shown).

At pH 7.4 the maximum leakage of calcein induced by prodrug IV was 15% at the concentration of 0.06 mM. The concentration to produce the equal release of calcein at pH 5.0 was 0.8 mM. Partitioning of the prodrug into lipid bilayer membrane is more favourable at neutral pH where the prodrugs are more hydrophobic. At neutral pH, bispilocarpic acid diesters are poorly soluble in water and, therefore, calcein release as a function of prodrug concentration at pH 7.4 could not be studied.

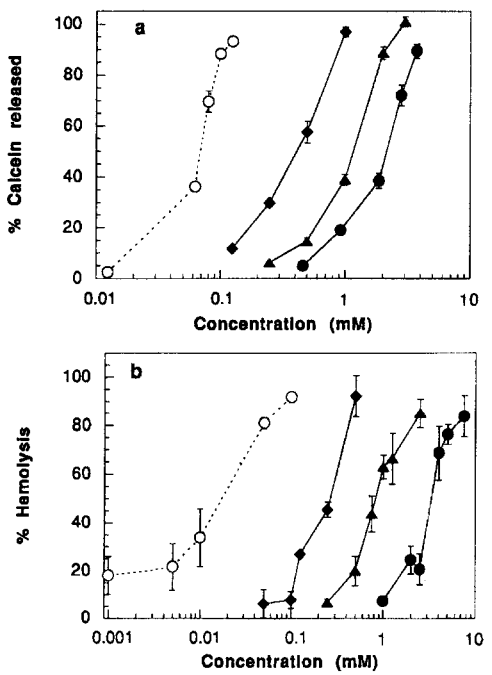


Fig. 5. Effect of concentrations (mM) of BAC (○), prodrug III (▲), IV (●) and V (◆) on the release of calcein from EPC:DOPE:DPPG:Chol (8:6:1.5:1.5) liposomes (a) and on the degree of hemolysis of rabbit erythrocytes in isotonic phosphate buffer (b) (pH 5.0, 21°C). Mean ± S.E., n = 3–4.

The fast calcein release from the liposomes caused by bispilocarpic acid diester solutions at concentrations above the CMC may be a consequence of solubilization of liposomal membrane lipids by prodrug micelles or integration of prodrug molecules to the bilayers, rendering them leaky. The interaction of the prodrugs with lipid bilayers might lead to solubilization or disruption of corneal cell membranes. This is a severe drawback of these prodrugs compared to pilocarpine. Sunamoto et al. (1987) have investigated the use of liposomes for evaluating the eye irritation of surfactants. There was a good correlation between the carboxyfluorescein leakage from polysaccharide-coated EPC liposomes by the surfactants and the in vivo Draize test data. In our experiments, the determination coefficient between the concentration of the prodrug to induce 50% leakage of calcein and irritation in rabbits was 0.91 (data not shown).

3.3. Hemolysis studies

The effects of pilocarpine prodrugs and benzalkonium chloride on rabbit erythrocytes were investigated at pH 5.0 due to their low solubility at physiological pH (Fig. 5b). The low pH of the isotonic buffer causes some hemolysis (3–9%) which has been taken into account in calculations as background. The prodrugs induced the maximum hemolysis within 30 min.

Benzalkonium chloride was the most potent hemolytic agent causing 90% hemolysis at a concentration of 0.1 mM. Drug-induced hemolysis increased with increasing lipophilicity of the prodrug. The concentrations of the prodrugs which caused 50% hemolysis (C_{50}) were 0.25 mM (V), 0.8 mM (III) and 3.5 mM (IV). Pilocarpine (96 mM) did not disrupt neither the liposomal nor the erythrocyte membrane.

Amphiphilic drugs like ellipticine (Lee, 1976) and chlorpromazine (Luxnat and Galla, 1986) induce hemolysis which probably is due to the changes of the arrangement of the phospholipids, leading to an increase in membrane permeability. Chlorpromazine may induce membrane lysis by the formation of mixed micelles containing membrane phospholipids. Pilocarpine prodrugs disrupt

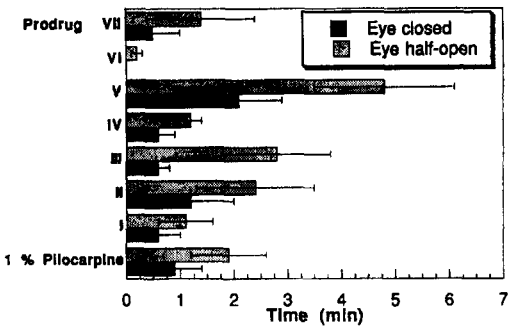


Fig. 6. Duration of eyelid closure after ocular administration of prodrug solutions. Mean ± S.E., $n = 6$.

the model membranes at concentrations above the CMC. The reason for that could be the comicellization of prodrugs and lipids which leads to membrane lysis at higher concentrations. The other reason can be the partition of the prodrug into lipid bilayer membrane and thus disturbing the membrane integrity.

3.4. Ocular irritation testing

Pilocarpine (1%) caused slight ocular irritation, the eyes were closed for 0.9 ± 0.5 min and half-closed for 1.9 ± 0.7 min. The most irritating prodrug was V, which was also the most lipophilic compound studied. The eyes were closed for 2.1 ± 0.8 min and half-open for 4.8 ± 1.3 min. Prodrugs VI, IV and I caused irritation that was in the same range with 1% pilocarpine at pH 5.0 (Fig. 6). The molar concentrations of the prodrugs in animal experiments (6 mM V, 6 mM III and 12 mM IV) were higher than those which induced 100% leakage of calcein and total hemolysis in vitro and, therefore, these bilayer effects may take place also in vivo in the cornea.

The lipophilicity of the prodrugs correlates fairly well with the ocular irritation ($R^2 = 0.74$, data not shown). Administration of prodrug with high partition coefficient is expected to result in higher concentration in corneal epithelium and, therefore, cause irritation. Upon neutralization of the eyedrop after instillation, the pilocarpine prodrugs are rapidly converted to neutral free base form, which has higher membrane perturbing capacity. Accordingly, by increasing the buffering capacity of the eyedrop, neutralization on ocular surface is slowed down, and absorption and ocular irritation are decreased (Suhonen et al., 1995). The other reason for eye irritation could be the precipitation of the drug in pre-corneal area if the pH of the eyedrop raises from 5.0 to 7.4 in the eye before the drug concentration drops below the water-solubility at neutral pH. On the basis of the rabbit eye irritation data, it is not possible to predict the irritation in human eye due to the limited availability of comparative information. Rabbit eye is, however, generally considered to be more sensitive for eye irritation than human eye (Rubin, 1995).

Despite the good correlations between the surface activity, lipid bilayer disturbance and ocular irritation of pilocarpine prodrugs, the amphiphilic properties do not alone explain the ocular irritation potential. For example, benzalkonium chloride is more surface active than the prodrugs but still less irritating, even when administered with 4% pilocarpine (data not shown). Therefore, the lipid bilayer disruptive effects alone do not explain the pilocarpine prodrug-induced irritation, other mechanisms are also involved.

4. Conclusion

The pilocarpine prodrugs are amphiphilic and their surface activity increases with increasing lipophilicity and solution pH. Amphiphilicity may limit the usefulness of bispilocarpic acid diesters as anti-glaucoma drugs. Possible amphiphilicity and associated bilayer disruptive effects are important considerations in the design of ocular prodrugs in general.

Acknowledgements

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References

Bartlett, G.R., Phosphorus assay in column chromatography. *J. Biol. Chem.*, 234 (1959) 466–468.

Bazan, H.E.P. and Bazan, N.G., Composition of phospholipids and free fatty acids and incorporation of labeled arachidonic acid in rabbit cornea. Comparison of epithelium, stroma and endothelium. *Curr. Eye Res.*, 3 (1984) 1313–1319.

Broekhuyse, R.M., Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids. *Biochim. Biophys. Acta*, 152 (1968) 307–315.

Bundgaard, H., Falch, E., Larsen, C. and Mikkelson T.J., Pilocarpine prodrugs I. Synthesis, physicochemical properties and kinetics of lactonization of pilocarpic acid esters. *J. Pharm. Sci.*, 75 (1986a) 36–43.

Bundgaard, H., Falch, E., Larsen, C., Mosher, G.L. and Mikkelson T.J., Pilocarpine prodrugs II. Synthesis, stability, bioconversion, and physicochemical properties of sequentially labile pilocarpine acid diesters. *J. Pharm. Sci.*, 75 (1986b) 775–783.

Druzgala, P., Winwood, D., Drewniak-Deyrup, M., Smith, S., Bodor, N. and Kaminski, J.J., New water-soluble pilocarpine derivatives with enhanced and sustained muscarinic activity. *Pharm. Res.*, 9 (1992) 372–377.

Ellingson, C.M., Schoenwald, R.D., Barfknecht, C.F., Rao, C.S. and Laban, S.L., Rapid toxicological model for use in assessing ocular drugs. *Biopharm. Drug Pisposit.*, 14 (1992) 417–436.

Feldman, G.L., Human ocular lipids: Their analysis and distribution. *Surv. Ophthalmol.*, 12 (1967) 207–243.

Jones, M.T. and Chapman, D., The Micellar State. In *Micelles, monolayers and biomembranes*, Wiley-Liss, John Wiley and Sons, Inc., New York, 1995, pp. 64–101.

Järvinen, T., Suhonen, P., Auriola, S., Vepsäläinen, J., Urtti, A. and Peura P., O,O'-(1,4-xylylene) bispilocarpic acid esters as new potential double prodrugs of pilocarpine for improved ocular delivery. I. Synthesis and analysis. *Int. J. Pharm.*, 75 (1991a) 249–258.

Järvinen, T., Suhonen, P., Urtti, A. and Peura, P., O,O'-(1,4-xylylene)bispilocarpic acid esters as a new potential double prodrugs of pilocarpine for improved ocular delivery. II. Physicochemical properties, stability, solubility and enzymatic hydrolysis. *Int. J. Pharm.*, 75 (1991b) 259–269.

Lee, I.P., A possible mechanism of ellipticine-induced hemolysis. *J. Pharmac. Exp. Ther.*, 196 (1976) 525–535.

Lee, V.H.L. and Robinson J.R., Mechanistic and quantitative evaluation of precorneal pilocarpine disposition in albino rabbits. *J. Pharm. Sci.*, 66 (1979) 673–684.

Lewis, R.W., McCall, J.C., Bothman, P.A. and Trebilcock, R., A comparison of two cytotoxicity tests for predicting the ocular irritancy of surfactants. *Toxic. in Vitro*, 8 (1994) 867–869.

Luxnat, M. and Galla, H.-J., Partition of chlorpromazine into lipid bilayer membranes: The effect of membrane structure and composition. *Biochim. Biophys. Acta*, 856 (1986) 274–282.

Mosher, G.L., Theoretical and experimental evaluation of pilocarpine prodrugs for ocular delivery. Ph.D. Thesis, University of Kansas, USA, 1986.

New, R.R.C., Structure of liposomes. In: New, R.R.C. (Ed.), *Liposomes a practical approach*, IRL Press, Oxford, 1990, pp. 1–27.

Reer, O., Bock, T.K. and Müller, B.W., In vitro corneal permeability of diclofenac sodium in formulations containing cyclodextrins compared to the commercial product Voltaren ophtha. *J. Pharm. Sci.*, 83 (1994) 1345–1349.

Rubin, L.F., Development and application of the Draize eye test. Special interest group session at the annual meeting of the association for research in vision and ophthalmology (ARVO) 16.5.1995, Fort Lauderdale, Florida, USA, (Abstract).

Suhonen, P., Järvinen, T., Lehmussaari, K., Reunamäki T. and Urtti A., Ocular absorption and irritation of pilocarpine prodrug is modified with buffer, polymer, and cyclodextrin in the eyedrop. *Pharm. Res.*, 12 (1995) 529–533.

Sunamoto, J., Iwamoto, K., Imokawa, G. and Tsuchiya, S., Possible eye-irritant test using polysaccharide-coated liposomes as a corneal epithelium model. *Chem. Pharm. Bull.*, 35 (1987) 2958–2965.

Szoka, F. and Papahadjopoulos, D., Procedure for preparation, of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA*, 75 (1978) 4194–4198.

Wan, L.S.C. and Lee, P.F.S., CMC of polysorbates. *J. Pharm. Sci.*, 63 (1974) 136–137.