NIH Public Access

Author Manuscript

Cornea. Author manuscript; available in PMC 2014 February 01.

Published in final edited form as:

Cornea. 2013 February ; 32(2): 179–184. doi:10.1097/ICO.0b013e31825646de.

Aliphatic β-nitroalcohols for therapeutic corneoscleral cross-linking: corneal permeability considerations

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Abstract

Introduction—Our recent tissue cross-linking studies have raised the possibility of using aliphatic β-nitro alcohols (BNAs) for pharmacologic, therapeutic corneal cross-linking. The present study was performed in order to determine the permeability of BNAs and to explore the use of permeability enhancing agents.

Methods—Ex vivo rabbit corneas were mounted in a typical Franz diffusion chamber. BNA permeability was determined by assaying the recipient chamber over time using a modification of the Griess nitrite colorimetric assay. The apparent permeability coefficient (Ptot) was determined for 2 mono-nitroalcohols, 2-nitroethanol (2NE) and 2-nitro-1-propanol (2NProp); a nitro-diol (2-methyl-2-nitro-1,3-propanediol=MNPD); and a nitro-triol (2-hydroxymethyl-2-nitro-1,3-propanediol=HNPD). Permeability enhancing effects using benzalkonium chloride (BAC) [0.01 and 0.02%], ethylenediaminetetraacetic acid (EDTA) [0.05%], and a combination of BAC 0.01% + tetracaine (TC) [0.5%] were also studied.

Results—The Ptot (+/−S.E.) values (cm/sec) were as follows: Ptot=4.33×10^{-5} (+/−9.82×10^{-6}) for 2NE (MW=91), Ptot=9.34×10^{-6} (+/− 2.16×10^{-7}) for 2NProp (MW=105), Ptot=4.37×10^{-6} (+/− 1.86×10^{-7}) for MNPD (MW=135), and Ptot=8.95×10^{-7} (+/−1.93×10^{-8}) for HNPD (MW=151). Using the nitrodiol, permeability increased approximately two-fold using BAC 0.01%, five-fold using BAC 0.02% and five-fold using the combination of BAC 0.01% + TC 0.5%. No effect was observed using EDTA 0.05%.

Conclusions—The results indicate that the corneal epithelium is permeable to BNAs with the apparent permeability corresponding to molecular weight. The findings are consistent with previous literature indicating that the small size of these compounds (<10Å) favors their passage through the corneal epithelium via the paracellular route. This information will help to guide dosing regimens for in vivo topical cross-linking studies.

Keywords

nitroalcohols; cornea; permeability; protein cross-linking; keratoconus

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Introduction

Recent studies suggest that aliphatic β-nitro alcohols (BNAs) may represent a useful class of compounds for use as in vivo therapeutic collagenous tissue cross-linking agents. Initial studies have shown that BNAs can induce tissue cross-linking effects at physiologic pH (7.4) and body temperature (37°C).[1-2] In addition, our cell toxicity studies[3] and published literature[4] suggests a reasonable safety profile for these agents. In particular, therapeutic tissue cross-linking with these agents could be applicable to the treatment of destabilizing corneal diseases (i.e. keratoconus and post-LASIK keratectasias) where tissue cross-linking using riboflavin photochemistry has already proven to be efficacious and beneficial.[5-6] In addition, progressive myopia, a disease of widespread prevalence marked by progressive scleral elongation, is potentially treatable through tissue cross-linking of the sclera. Wollensak and Imodina[7] have recently described the use of a subtenon's injection using glyceraldehyde (a chemical cross-linking agent similar in concept to nitroalcohols) to stiffen the rabbit sclera.

Nitroalcohols have been used extensively in a wide variety of industrial and commercial applications that include their use in cosmetics, rocket fuels, explosives, toilet deodorizers, and plasticizers.[8] They have also served as convenient starting compounds as well as chemical intermediates for the synthesis of various classes of organic derivatives.[9] The number and scope of industrial applications is vast and includes polymerization chemistry, where nitroalcohols can act as formaldehyde donors to cross-link compounds such as urea, melamine, phenols, resorcinol, etc. in the production of resins, plastics, polyesters, and polyurethane products.[10] Because of their widespread industrial use, the health and safety effects regarding nitroalcohols have been studied extensively, including acute toxicity, teratogenicity, and mutagenicity/carcinogenicity where their profile is quite favorable,[8] and in stark contrast to formaldehyde, whose unfavorable toxicity and carcinogenicity profile has been widely publicized.[11]

These compounds can undergo a base catalyzed thermally driven retronitroaldol condensation (reverse Henry reaction = rHr) to give formaldehyde, a well known cross-linking agent. The BNAs are a family of compounds and thus, more than one member of this class could be used for in vivo cross-linking. We have been focusing on 2 mono-nitroalcohols [2-nitroethanol (2ne) and 2-nitro-1-propanol (2nprop)], a nitrodiol [2-methyl-2-nitro-1,3-propanediol (MNPD)], and a nitro-triol [2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD)], and have confirmed that the triol has greater cross-linking efficacy than the diol, which has greater efficacy than the monols.[12] Such a hierarchy of cross-linking efficacy reflects differences in the number of formaldehyde donating groups per molecule. In this regard, the triol can give three formaldehydes per molecule, the diol gives two, and the monols give one.

Because our intent is to use these compounds topically on the cornea, we felt that it would be useful to study the transcorneal permeability. The present study was undertaken to determine the transcorneal permeability of the various BNAs using ex vivo rabbit corneas mounted in a Franz diffusion chamber specifically designed for studying corneal permeability.

Materials and Methods

2-nitroethanol (2ne), 2-nitro-1-propanol (2nprop), NaH_2PO_4/Na_2HPO_4, benzalkonium chloride (BAC), ethylenediaminetetraacetic acid (EDTA), and tetracaine were all obtained from the Sigma-Aldrich Chemical Co (St. Louis, MO). 2-methyl-2-nitro-1,3-propanediol (MNPD) and 2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD) were obtained from TCI

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America (Portland, OR). Millipore filtered water (<18.2MΩ.cm) was used in all the experiments.

Fresh rabbit corneas were obtained within 60 minutes of sacrifice post-mortem from a local abattoir. A total of 40 rabbit globes were used for this study. The eyes were enucleated and the entire cornea with 4mm associated scleral ring dissected carefully. The corneo-scleral complex was then mounted with the epithelial side facing upward in a 9mm internal diameter (i.d.) Franz corneal diffusion chamber (Permegear, Inc., Hellertown, PA). Intact epithelium was confirmed by visual inspection. The chamber consists of a 3ml volume lower recipient chamber with accompanying sampling port and stirring bar, an outer water jacket with inlet and outlet ports for connection to a water bath with pump for temperature control, a spherical joint tissue sample mounting surface used for corneal work with a 0.64 cm^2 area chamber opening, and an upper donor chamber open to the atmosphere (Figure 1). The outer water jacket was maintained at 37°C throughout the experiments.

Using this setup, trans-corneal permeability was studied. A standard physiologic balanced salt solution (BSS) was used for all experiments both for mixing of BNA solutions as well as in the lower recipient chamber. The sample was carefully mounted with the epithelial side facing upward on the spherical joint surface and the upper donor chamber compartment was attached using a supplied metal clamp. The water bath with pump was kept running throughout the experiments in order to maintain constant temperature at 37°C. The upper donor chamber was then filled with BNA solution at a concentration of 100mM and the recipient chamber was sampled (20ul) via the side port at specific time intervals of 30, 60, 90, and 120 min. The sampled aliquots were then stored and batch assayed for BNA level.

From the data are then generated curves for dc/dt, the change in concentration in the recipient chamber per time. A computer software linear fit equation is then generated using Microcal Origin 6.1 software. The slope of the fitted line is used to calculate the apparent permeability coefficient, using the equation: Ptot=V(dc/dt)/ACo, where V is the lower recipient chamber volume ($1.2cm^3$), dc/dt is the flux across the cornea, A is the surface area of the exposed cornea [1.6cm2 -calculation based on the equation of a sphere (A=4πr2)], and Co is the upper donor chamber initial concentration (100mM).

The data generated curves for two mono-nitroalcohols, [2-nitroethanol (2NE), 2-nitro-1-propanol (2NProp)], a nitro-diol [2-methyl-2-nitro-1,3-propanediol (MNPD)], and a nitro-triol [2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD)]. We used the nitrodiol as a model compound for studying the permeability enhancing effects of BAC, TC, and EDTA, either alone or in combination. The same apparatus was used for the permeability enhancer experiments, the only difference being that the enhancer was applied first to the upper donor chamber and allowed to dwell for 10 min. The enhancer was aspirated from the upper donor chamber. The corneal surface was rinsed with PBS and the donor solution with BNA then applied. The lower recipient chamber was sampled and assayed in an identical fashion to the previously described experiments. In each case, the enhancing condition effects were determined on a sample cornea and a paired control (from the opposite eye of the same animal) using a second identical Franz chamber setup. The control, in this case, had only the BNA applied in the absence of any enhancer. Restated, each sample enhancer run was paired with a "control" from the opposite eye of the same animal (using no enhancer but testing BNA permeability).

Using a modification of the Griess colorimetric nitrite assay for quantitation of BNAs

We have recently developed a simple colorimetric method for assaying BNAs in solution based upon the detection of nitrite, which is liberated during alkaline heating of BNAs, and utilized this assay in the present study. The full assay description will be reported elsewhere

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(manuscript in press). In that report, we determined the conditions necessary for maximal denitration, which include heating at 100°C for a minimum of 60 minutes, at a pH above 6. The sample was assayed colorimetrically using the Griess colorimetric nitrite assay as described below. A standard curve was generated using commercially available BNAs. Here, we include 4 standard curves which are shown in Figure 2 corresponding to each of the compounds studied. They are 2-nitroethanol (2ne), 2-nitro-1-propanol (2nprop), 2-methyl-2-nitro-1,3-propanediol (MNPD), and 2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD). In each case, the compounds in solution were heated at pH 7.4 at 100°C for 120 minutes prior to colorimetric assay. The linear fit equations from such standard curves were used to determine the transcorneal flux in the Franz chamber.

Liberation of free nitrite was monitored using a modification of the colorimetric Griess assay. Briefly, 20 μl of supernatant were applied to a 400 μl well (96-well microtiter plate). Each sample was assayed in triplicate. Fifty μl of 2N HCl and 50 μl of sulfanilic acid (1mg/ml) were added to the sample and incubated at room temperature for 10 min, followed by 50 μl of N-ethylenediamine hydrochloride (2 mg/ml) and additional incubation for 25 min. The plate was read at 546nm (purple color) on a kinetic microplate spectrophotometer (Benchmark Microplate Reader, Bio-Rad Laboratories, Hercules, CA). A 1 mM stock solution of sodium nitrite (or BNA) was used to create a standard curve by serial dilution and was developed fresh on each day of sampling.

Results and Discussion

Corneal permeability is an important consideration in the development of any topical eye medication and there have been several excellent reviews published over the past several decades regarding the subject.[13-18] A number of techniques for studying corneal permeability have been used, including ex vivo corneas mounted in various types of permeability chambers,[19-22] live animal studies,[23-25] and more recently, cell culture based systems.[26-29] Each has its merits, and information gathered from any of the above mentioned techniques can contribute to our understanding of corneal permeability. A recent review catalogues the permeability characteristics of a number of compounds including many used in ophthalmic practice.[17] Many compounds have been studied for their ability to permeate the cornea and corneal layers. However, this is the first reporting of corneal permeability of BNAs.

Determination of dc/dt for 4 BNAs using ex vivo rabbit cornea

The slope of the fitted line for dc/dt (Figure 3) was used to calculate the apparent permeability coefficient, or Ptot, using the equation: Ptot=V(dc/dt)/ACo, where V is the recipient chamber volume ($1.2cm^3$), dc/dt is the flux across the cornea, A is the surface area of the exposed cornea [$1.6cm^2$ -calculation based on the equation of a sphere ($A=4\pi r^2$)], and Co is the donor chamber initial concentration (100mM).

The Ptot (+/−S.E.) for each of the 4 BNA compounds studied are included in Table 1 and were as follows: 4.33×10^{-5} (+/−9.82×10^{-6}) cm/sec for 2NE (MW=91), 9.34×10^{-6} (+/−2.16×10^{-7}) cm/sec for 2NProp (MW=105), 4.37×10^{-6} (+/−1.86×10^{-7}) cm/sec for MNPD (nitro-diol, MW=135), and 8.95×10^{-7} (+/−1.93×10^{-8}) cm/sec for HNPD (nitro-triol, MW=151). The trend for the Ptots corresponds to molecular weight (MW). In other words, the smallest molecule, 2ne (MW=91.07) was the fastest, followed in size order by 2nprop (MW=105.09), MNPD (MW=135.12), and HMPD (MW=151.12). Although clearly more effective as cross-linking agents, the higher order nitroalcohols (HONAs)[12] are larger in size and as such, appear to be slightly less permeable through the intact cornea. Such a difference in corneal permeability could influence the way in which these compounds are used in clinical practice. For example, more frequent dosing regimens or higher concentrations for

the HONAs may be needed in order to achieve a given targeted effect, given the differences in permeability noted in this study.

The optimal logP values for lipophilicity associated with good corneal penetration are in the 2-4 range. Such hydrophobic compounds permeate the cornea via the transcellular route, traversing the cell membrane lipid bilayer. Compounds entering the cornea via this route can then encounter a barrier of hydrophilic corneal stroma, where passage is inhibited for hydrophobic compounds. In contrast and as shown in Table 1, the aliphatic BNAs are hydrophilic compounds as evidenced by their relatively low logP (octanol/water partition coefficient) which are all less than 0 [see Table 1]. Thus, their passage via the transcellular route would be unlikely. However, as small hydrophilic compounds, these BNAs are likely to pass via the paracellular pathways, where adjacent corneal epithelial cells are separated by on average 2 nanometers, with tight junctions present only on the most superficial epithelial layer. It is via this paracellular route that many small hydrophilic molecules travel into the stroma. The four compounds that we studied range in size from 91-151Da. This corresponds to a molecular radius in the range of less than 0.5 nanometers (or 5Å), favoring their passage via a paracellular route, which Prausnitz and Noonan estimate to be <10Å. In addition, the values that we determined for these compounds are comparable to other molecules of similar size and hydrophilicity such as glycerol (MW92, Ptot=4.5×10^{-6}cm/sec) and mannitol (MW182, Ptot=2.4×10^{-6}cm/sec).[16]

Studies using permeability enhancers

Using the same apparatus, we screened 4 different permeability enhancing conditions in order to measure their effects on permeability of MNPD. The enhancing conditions were benzalkonium chloride at two concentrations (0.01 and 0.02%), EDTA (0.05%), and the combination of BAC (0.01%) and tetracaine (0.5%). In addition, we used BAC at one concentration (0.01%) in order to measure the effect on HNPD (i.e. nitrotriol) permeability. The results are summarized in Table 2 which shows the amount of permeability increase between paired samples either receiving the enhancing condition or not, prior to studying either MNPD or HNPD permeability. The greatest effect was observed using the higher concentration of BAC (0.02%), which was also possible using the lower concentration of BAC (0.01%) in combination with TC (0.5%). A more modest permeability enhancing effect was observed with BAC (0.01%) alone for the nitrodiol MNPD and with BAC (0.02%) alone for the nitrotriol HNPD. We found no permeability enhancing effect using EDTA alone for the nitrodiol MNPD.

The subject of using agents to enhance corneal permeability has received a significant amount of attention over recent years and several groups have reported increases in corneal permeability following BAC application using both ex vivo[30-33] and live animal[34-37] based techniques. Due to its widespread use as a preservative in ophthalmic solutions, BAC has received a significant amount of attention both from a toxicologic standpoint as well as from a permeability enhancing perspective. It is generally accepted that BAC can cause disruption of tight junctions located on the very surface of the corneal epithelium and thereby enhance permeability. Thus, the significant increases in permeability that we observed using both 0.01% and 0.02% BAC support the assumption that the BNAs travel via the paracellular pathway.

EDTA did not have a significant effect in our study. The reason for this is not clear since others have observed corneal permeability enhancement using EDTA under comparable experimental conditions.[31,33,38] As well, TC did not show an effect as a single agent in preliminary studies (data not shown), although a significant enhancement was observed when used in combination with BAC. This combination has been previously reported to improve corneal permeability for fluorescein in normal humans.[34]

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Conclusion

In summary, we report a study on the transcorneal permeability of BNAs using ex vivo rabbit cornea in a Franz cell setup. The results indicate that the corneal epithelium is permeable to BNA compounds and differences in permeability between compounds correspond to molecular weight, with the fastest Ptot noted for the smallest compound (2ne) and the slowest Ptot noted for the largest compound (HNPD). The findings are consistent with existing literature which indicates that for small hydrophilic compounds (i.e. <10Å), passage through the corneal epithelium via the paracellular route is favored. The information from this study will help to guide dosing regimens for topical cross-linking studies in living rabbit eyes.

Acknowledgments

Supported in part by Research to Prevent Blindness and by National Institutes of Health/National Eye Institute Grants R21EY018937 and R01EY020495.

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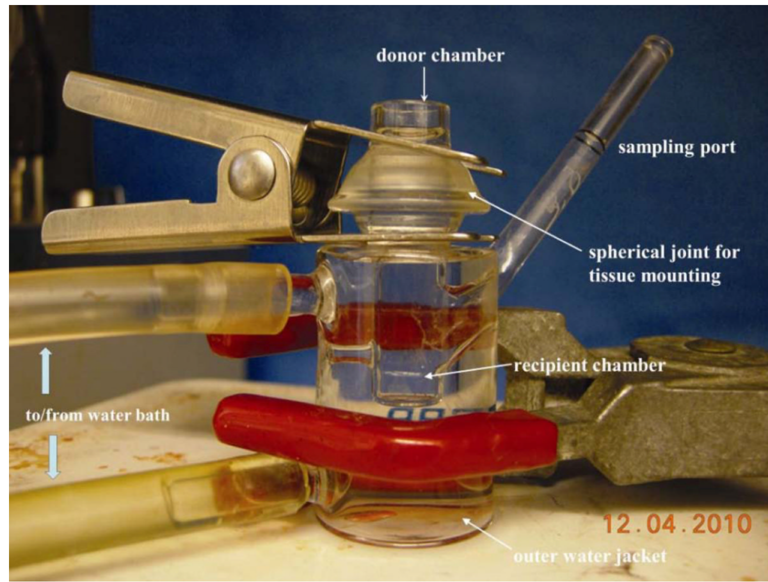

Figure 1.
Apparatus for studying transcorneal permeability of ex vivo rabbit cornea. Franz static diffusion cell with i.d.=0.9cm and a spherical joint option designed for corneal work (PermeGear, Inc., part #4G-03-00-09-03). The chamber outer water jacket is connected to a circulating water bath and placed on a stirring plate as shown.

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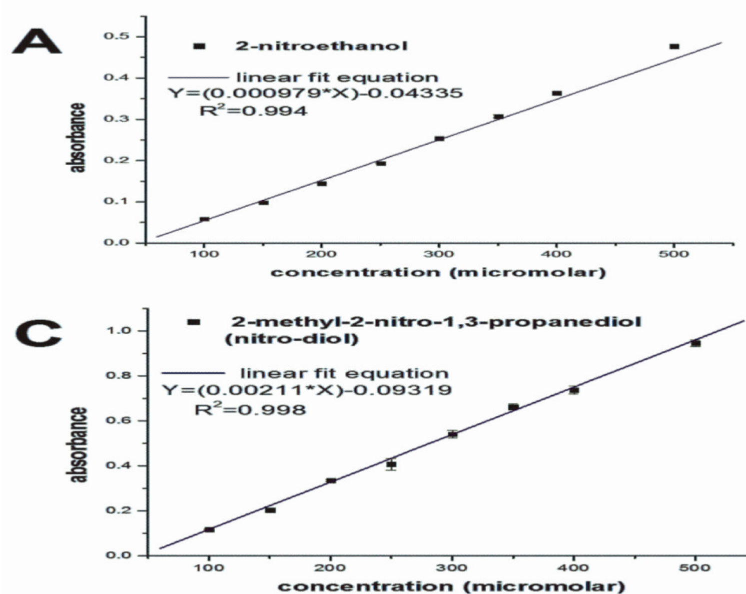

Figure 2.

Standard curves of 4 BNAs using a simple modification of the Greiss colorimetric nitrite assay. The samples are heated for 120min at pH 7.4 and 100°C in order to release free nitrite which is then colorimetrically assayed. Excellent linearity is noted in the 100-500uM range for 2 mono-nitroalcohols 2ne (**A**) and 2nprop (**B**), a nitrodiol (**C**), and a nitrotriol (**D**).

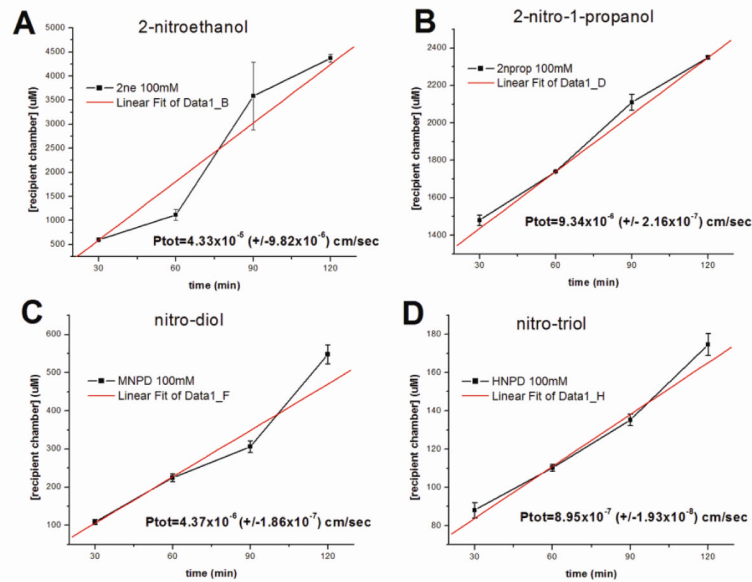

Figure 3.

Shown above are time dependent changes in recipient chamber concentration (i.e. flux) for 4 BNAs studied. The linear fit equations were then used in order to determine the apparent permeability coefficient (Ptot) using the equation Ptot=V(dc/dt)/ACo as described above (see Methods). The Ptot (+/−S.E.) for each compound is included with each graph. Each data point represents the average of a minimum of 3 independent determinations. BNA quantitation was performed using the Greiss colorimetric nitrite assay following a denitration step (see Figure 2.).

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Table 1

Some aliphatic β-nitroalcohols being considered as in vivo therapeutic cross-linking agents.

Compound **[cas#]**	**order**	**MW (Da)**	**pKa**	**logP**	**genotoxicity risk**	**Ptot (cm/sec)** **[corneal permeability]**
2-nitroethanol (2ne) [625-48-9]	monol	91.07	8.42+/−0.29	−0.420+/−0.266	No	2.70×10^{-5}+/−7.5×10^{-6}
2-nitro-1-propanol (2nprop) [2902-96-7]	monol	105.09	7.36+/−0.13	−0.066+/−0.269	No	9.35×10^{-6}+/−2.15×10^{-7}
2-methyl-2-nitro-1,3-propanediol (MNPD) [77-49-6]	diol	135.12	12.86+/−0.10 14.4+/−0.50	−0.018+/−0.564	NA	4.37×10^{-6}+/−1.86×10^{-7}
2-hydroxymethyl-2-nitro-1,3-propanediol (HNPD) [126-11-4]	triol	151.12	12.32+/−0.10 13.26+/−0.10 14.20+/−0.10	0.115+/−0.770	No	8.95×10^{-7}+/−1.90×10^{-8}

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Table 2

Comparison of permeability enhancer effect for nitrodiol (and nitrotriol) across ex vivo rabbit corneas. Each value is the average of 3 independent determination using paired samples.

compound studied	enhancer and concentration	*average change (+/– SE) in Ptot using enhancer
MNPD		
	BAC 0.01%	2.01+/–0.30
	BAC 0.02%	5.26+/–2.20
	EDTA 0.05%	1.10+/–0.08
	BAC 0.01% + tetracaine 0.5%	5.16+/–1.35
HNPD		
	BAC 0.02%	2.01+/–0.40

*The difference in Ptot values between each animal and its paired control (i.e. control side is without the enhancer and the test side is with the enhancer) was determined. This difference was then averaged between a minimum of 3 independent determinations in order the express the average change induce by each enhancing condition.