DPOFA, a Cl⁻/HCO₃⁻ exchanger antagonist, stimulates fluid absorption across basolateral surface of the retinal pigment epithelium

Pavel Iserovich, Qiong Qin and Konstantin Petrukhin*

Abstract

**Background:** Retinal detachment is a disorder of the eye in which sensory retina separates from the retinal pigment epithelium (RPE) due to accumulation of fluid in subretinal space. Pharmacological stimulation of fluid reabsorption from subretinal space to choroid across the RPE has been suggested as a treatment strategy for retinal detachment. DPOFA, (R)-(+)-(5,6-dichloro 2,3,9,9a-tetrahydro 3-oxo-9a-propyl-1H-fluoren-7-yl)oxy)acetic acid, is an abandoned drug capable of inhibiting Cl⁻/HCO₃⁻ exchanger activity. We hypothesized that DPOFA may increase fluid absorption across basolateral surface of the RPE.

**Methods:** Reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA for six different transporters that may act as Cl⁻/HCO₃⁻ exchangers was conducted in bovine and human RPE to confirm that RPE from two species expresses the same repertoire of Cl⁻/HCO₃⁻ exchanger isoforms. The degree of amino acid homology between orthologous human and bovine RPE-specific isoforms was calculated after performing protein alignments. Transport of fluid across bovine RPE-choroid explants mounted in the Ussing chamber was used to assess the ability of DPOFA to modulate fluid absorption across the RPE.

**Results:** Using RT-PCR we showed that three isoforms (SLC4A2, SLC4A3, and SLC26A6) are strongly expressed in human and bovine RPE preparations. Amino acid comparisons conducted for RPE-specific isoforms support the use of bovine RPE-choroid explants as an adequate experimental system for assessing fluid absorption activity for DPOFA. Our data is consistent with the fact that DPOFA stimulates fluid absorption across the RPE in bovine RPE-choroid explants.

**Conclusions:** DPOFA seems to stimulate transport of water across the RPE in bovine RPE-choroid explants. Additional experiments are required to establish dose-dependent effect of DPOFA on fluid absorption in the bovine RPE-choroid experimental system.

Background

Retinal detachment (RD) is the most common cause of blindness in young adults [1-3]. In RD neuro-sensory retina separates from the underlying pigment epithelium due to accumulation of fluid in the subretinal space [4]. The only therapy for RD is surgical reattachment. Surgery is most effective only if performed within 1-3 days after the disease onset. The rate of complications, often in a form of retinal re-detachment, is 10-20%, even if successful surgical re-attachment is performed in time [1]. Even when anatomical recovery in the form of retina reattachment is successfully accomplished, functional recovery after surgery may be poor due to the loss of photoreceptor cells. Identification of the pharmacological treatment for RD that can be used as adjunctive therapy to improve functional outcomes following surgery and reduce the rate of post-operative complications is of utmost importance. Pharmacological up-regulation of fluid reabsorption from subretinal space to choroid across the retinal pigment epithelium (RPE) has been suggested as potential treatment strategy for retinal detachment [5]. In recent years, several drug candidates have been tested in vivo and in vitro for the
ability to stimulate subretinal fluid resorption [6-11]. However, identification of clinically proven pharmacological therapy capable of increasing reabsorption of subretinal fluid in retinal detachment patients remains enigmatic. Removal of fluid from subretinal space across the RPE is mainly driven by transport of K⁺ and Cl⁻ [12,13]. As basolateral Cl⁻/HCO₃⁻ exchanger recycles Cl⁻ back to the RPE thus reducing the rate of fluid absorption from subretinal space, the net movement of water out of the RPE across basolateral surface is determined by activity of the Cl⁻/HCO₃⁻ antiporter [14]. Inhibition of the Cl⁻/HCO₃⁻ exchanger would predictably lead to increase in water transport across the RPE. DPOFA, (R)-(+)-(5,6-dichloro 2,3,9a-tetrahydro 3-oxo-9a-propyl-1H-fluoren-7-yl)oxy]acetic acid, is an abandoned fluorenone drug that has been systemically administered to humans in clinical trials for trauma-induced brain damage [15-17]. While the primary molecular target for DPOFA is thought to be a Cl⁻/HCO₃⁻ exchanger [18-20], a Cl⁻ channel blocker activity has also been suggested for this drug [21]. In the present study we conducted preliminary analysis of the effect of DPOFA on fluid transport using the bovine choroid-RPE ex vivo system.

Methods

DPOFA synthesis

Chemical structure of DPOFA is shown in Figure 1. DPOFA is not commercially available. A non-GMP batch of DPOFA was synthesized by GVK Biosciences, Hyderabad, India. HPLC purity of the synthesized compound was confirmed and estimated to be 98%. Data from the 1H NMR (400 MHz, CDCl₃) and mass spectrometry analyses were in agreement with the compound structure.

Preparation of solutions

DPOFA’s stock solution (40 mM) was prepared in 4.2% NaHCO₃, pH 6.3 as described previously [22,23]. The medium used in fluid transport experiments was HEPES-HCO₃⁻ Ringer solution containing (in mM) 94.1 NaCl, 37 NaHCO₃, 3.8 KCl, 1 KH₂PO₄, 0.8 MgSO₄, 1.7 CaCl₂, 6.9 glucose, and 20 HEPES. The pH was 7.4, and the osmolarity was 290 mosmol/kgH₂O. The solution was pre-incubated at 37°C in 95%/5% air/CO₂ for at least 16 hours before experiments.

Preparation of RPE-choroid explants

Bovine eyes were obtained from Smithfield Beef Group, Souderton, PA and kept in Ringer’s solution until their use within 2.5-5 hours after enucleation. The eye was dissected posteriorly to the ora serrata; lens and vitreous were removed. Neurosensory retina was peeled away; a circular area containing the RPE and choroid was cut out using a cork bore. The RPE-choroid tissue preparation was placed on a metal mesh disk choroid (basal) side down; nylon mesh was positioned on the RPE (apical) side. The tissue explant was inserted between two halves of the Ussing chamber which was pre-equilibrated at 37°C.

Transport of fluid

The Ussing chamber was filled with pre-warmed Ringer’s solution, and electric resistance was tested to assure tissue integrity. If electrical resistance was less than 100Ω, the preparation was discarded. Two cylinders containing measuring capillaries were inserted into apical and basal reservoirs of the Ussing chamber. Levels of fluid in capillaries were adjusted using the specially designed long barrel syringe. DPOFA was added to both apical and basal reservoirs of the Ussing chamber. We
used horizontal observation microscope with automatic position reading in order to measure changes in the position of a meniscus in the measuring capillaries in response to fluid transport from apical to basal reservoir. The pumping rate was recorded as an increase in a meniscus position of the basal capillary expressed in micrometers. SigmaStat 3.0.1 package for Windows from SPSS Inc. was used for data analysis.

RPE cell culturing, isolation of RNA, and RT-PCR
Primary fetal human RPE cell culture [24] was obtained from Dr. Sheldon Miller, National Eye Institute. As stated in reference [24], the research on preparation of this fetal human RPE culture followed the tenets of the Declaration of Helsinki and was approved by the NIH institutional review board. Primary fetal human RPE cells were cultured according the published procedure [24]. Bovine RPE was gently brushed off of the eyecup after discarding the lens, vitreous, and neurosensory retina. Total RNA was isolated from human RPE cells and from bovine RPE tissue using the RNeasy kit (Qiagen) and a manufacturer’s protocol. After oligo(dT)-primed cDNA synthesis, which was performed with the ProtoScript First Strand cDNA Synthesis kit (New England Biolabs), we confirmed expression of the established RPE marker, bestrophin 1, using RT-PCR with the following bestrophin 1 primers: 5′-GGCAAGACCAAAGCAGTTTAC-3′ and 5′-CTTCCAAGGGGTTCATGGT-3′ for human RPE; 5′-CAACCCATTTTGGAGGAGTG-3′ and 5′-AAGGTTCTTGGGC TGTTCT-3′ for bovine RPE. To assess expression of anion transporter isoforms we conducted RT-PCR analysis using the following combinations of oligonucleotide primers corresponding to different human and bovine transporters: human SLC4A1, 5′-TCTTCCAGGACCAACACACTA-3′ and 5′-CATGCTGACTCCACAGCAGAC-3′; human SLC4A2, 5′-CAAGTGGATTCTC GGTTGAC-3′ and 5′-ATCCTCGTTAAGGGAGGTTGC-3′; human SLC4A3, 5′-CACCACAGCAGAAGCCTGA-3′ and 5′-GGAAGATCCCAAAGAGCA-3′; human SLC26A3, 5′-CCATCATCGTGCTGATTGCA-3′ and 5′-GGGACTGAA-3′; bovine SLC26A4, 5′-CCATCATCGTGCTGATTGCA-3′ and 5′-GGGACTGAA-3′; bovine SLC26A6, 5′-CCATCATCGTGCTGATTGCA-3′ and 5′-GGGACTGAA-3′; human SLC4A2 [GenBank: NP_001186621], human SLC4A3 [GenBank:NP_005061], human SLC26A6 [GenBank:NP_001035544], bovine SLC4A2 [GenBank:NP_001192593], bovine SLC4A3 [GenBank:NP_001186621], bovine SLC26A6 [GenBank:NP_001186621], guinea pig SLC4A2 [GenBank:NP_001166488], rabbit SLC4A2 [GenBank:NP_001075499].

Results
It has been reported that DPOFA shows significant species specificity in regard of inhibiting its main target, Cl-/HCO3− exchanger. Human, cat, guinea pig, and rabbit constitute responsive species with similar high levels of DPOFA inhibitory activities [18,19,26], while rodents (rat and mouse) represent non-responsive species [20,27]. Before conducting experiments on fluid absorption in bovine RPE-choroid system we wanted to confirm that isoform specificity of Cl-/HCO3− exchanger in bovine RPE is similar to that of the human RPE cells, to assure that data obtained in bovine RPE system would be predictive of outcomes in the human retina. It has been reported that six different transporters from two protein families may act as Cl-/HCO3− exchangers: SLC4A1, SLC4A2, SLC4A3, SLC26A3, SLC26A4, and SLC26A6 [28,29]. We purified total RNA from cultured primary fetal human RPE cells and from RPE tissue...
isolated from bovine eyes. Following cDNA synthesis from human and bovine RNA, we confirmed expression of the established RPE-specific marker, bestrophin 1, using RT-PCR (data not shown). We synthesized oligonucleotide primers from identical isogenic regions of six human and bovine Cl⁻/HCO₃⁻ transporters and conducted RT-PCR analysis in order to compare isoform specificity of expressed anion transporters in human and bovine RPE. As shown in Figure 2, the repertoire of Cl⁻/HCO₃⁻ exchanger isoforms expressed in the RPE is identical in human and Bos taurus with three isoforms (SLC4A2, SLC4A3, and SLC26A6) pronouncedly expressed in both RPE preparations. Control RT-PCR reactions conducted with water in place of cDNA confirmed specificity of PCR amplification (Figure 2, panels B, D). The degree of amino acid homology between orthologous human and bovine Cl⁻/HCO₃⁻ isoforms expressed in the RPE was calculated after performing protein alignments using ClustalW2 algorithm. The bovine-human homology for SLC4A2 and SLC4A3 isoforms is above 95% which is in the range of amino acid similarity between RPE-specific human transporters and Cl⁻/HCO₃⁻ exchangers from three other “responsive” for DPOFA species, cat, guinea pig, and rabbit (data not shown). Lack of sequence information for the SLC26A6 transporter from cat, guinea pig, and rabbit precluded us from performing similar analysis for this isoform. Overall, amino acid comparisons conducted for SLC4A2 and SLC4A3 isoforms are consistent with the idea that Bos taurus, along with human, cat, guinea pig and rabbit may constitute a responsive for DPOFA species further supporting the use of bovine RPE-choroid explants as an adequate experimental system for assessing fluid absorption activity for DPOFA. Attempting to define a dose-dependent effect of the test compound on fluid absorption, we conducted a series of experiments comparing DPOFA concentrations in the 1-20 μM range with the effect of a compound vehicle over the period of 30 minutes. While no statistically significant difference between compound doses could be established, we were able to detect statistically significant increase in fluid absorption after drug treatment versus vehicle control during the first 20 minutes after drug or vehicle addition when data for all DPOFA concentrations were combined. When added in 1-20 μM concentrations, DPOFA significantly (p = 0.013) increased water absorption within first 20 minutes by 3.22 ± 1.4 μl/cm².h while vehicle control decreased absorption by 2.1 ± 1.39 μl/cm².h (Figure 3). Statistical significance could not be reached when 10, 15, and 20 minute timepoints were analyzed individually. The decrease in fluid absorption by the RPE in response to vehicle addition is reminiscent of the previously reported volume flow decline induced by “sham” treatment of choroid-RPE explants mounted in Ussing-type chambers [30]. Table 1 shows numeric data on changes in pumping rate over the period of 30 minutes in response to addition of 1-20 μM DPOFA or vehicle with the number of experiments indicated for each timepoint.

Discussion

Despite significant unmet medical need, there is no pharmacological therapy that was approved by regulatory agencies for treatment of retinal detachment. Pharmacological up-regulation of fluid reabsorption from subretinal space to choroid across the retinal pigment epithelium has been suggested as potential treatment strategy for retinal detachment [5]. Basolateral Cl⁻/HCO₃⁻ exchanger in the retinal pigment epithelium is a reasonable drug target for up-regulation of fluid absorption activity for DPOFA.
reabsorption as it recycles Cl⁻ back to the RPE thus reducing the rate of fluid absorption from subretinal space. DPOFA (shown in Figure 1), also known in the literature as B-3(++) [20] and L-644,711 [18], is a non-diuretic small molecule Cl⁻/HCO₃⁻ exchanger antagonist that has been systemically administered to humans in clinical trials for trauma-induced brain damage [15-17]. It has been shown that DPOFA exhibits remarkable species specificity in regard of inhibiting its main target, Cl⁻/HCO₃⁻ exchanger. While the IC₅₀ of 2 × 10⁻¹¹ M in the K⁺-induced swelling assay is reported in one of the responsive species, cats, the IC₅₀ in a similar rat assay is only 2 × 10⁻⁷ M [16]. It has been shown that DPOFA exhibits remarkable species specificity in regard of inhibiting its main target, Cl⁻/HCO₃⁻ exchanger. While the IC₅₀ of 2 × 10⁻¹¹ M in the K⁺-induced swelling assay is reported in one of the responsive species, cats, the IC₅₀ in a similar rat assay is only 2 × 10⁻⁷ M [16]. It has been shown that human, cat, guinea pig, and rabbit constitute responsive species with similar levels of DPOFA inhibitory activities [18,19,26], while rodents (rat and mouse) represent non-responsive species [20,27]. Bovine RPE-choroid system is a widely used experimental tool for assessing the effect of drug treatment on reabsorption of fluid across the RPE [31-33]. However, in light of significant species-specific difference in response to DPOFA in tissue slice-based CNS experimental systems we wanted to develop additional support for using bovine RPE-choroid system as a tool capable of predicting DPOFA response in human RPE. DPOFA’s selectivity for different Cl⁻/HCO₃⁻ exchanger isoforms is not known. Assuming that species-specific difference in response to DPOFA may be caused by species-specific variations in repertoire of expressed Cl⁻/HCO₃⁻ transporters, we compared isoform specificity of Cl⁻/HCO₃⁻ transporters expressed in human and bovine RPE. Using RT-PCR analysis conducted with isoform-specific oligonucleotide primers that were designed from the isogenic regions of human and bovine genes we showed that three isoforms

Table 1 Change in pumping rate in response to DPOFA or vehicle treatment

| Time after addition, min. | DPOFA, Change in pumping rate ¹ | DPOFA, SEM² | Vehicle, Change in pumping rate ¹ | Vehicle, SEM² |
|---------------------------|---------------------------------|-------------|---------------------------------|-------------|
| 10                        | 3.64 (6)                        | 3.49        | -2.44 (5)                       | 2.03        |
| 15                        | 3.17 (4)                        | 0.72        | -1.71 (4)                       | 3.08        |
| 20                        | 2.83 (6)                        | 1.22        | -1.89 (4)                       | 1.30        |
| 30                        | 0.47 (10)                       | 2.04        | -0.44 (7)                       | 0.68        |

¹ Change in pumping rate in comparison with time 0 when DPOFA or vehicle were added is expressed in (μl/cm².h); ²SEM, Standard Error of Means; Number of experiments is shown in parenthesis.
(SLC4A2, SLC4A3, and SLC26A6) are strongly expressed in both RPE preparations (Figure 2). After defining transporters expressed in the RPE, we conducted their amino acid comparisons in order to confirm that human-bovine protein homology for RPE-specific transporters is in line with the degree of similarity between human and three other responsive species (cat, guinea pig, and rabbit). These protein comparisons confirmed that human-bovine homology for SLC4A2 and SLC4A3 does not differ from homology of RPE specific transporters in responsive for DPOFA species which may further indicate that effect of the drug in bovine RPE-choroid system is likely to adequately reflect the behavior of DPOFA in human RPE. In an attempt to define a dose-dependent effect of DPOFA on reabsorption of fluid in the bovine RPE-choroid system, we conducted multiple experiments assessing compound activity in the 1-20 μM dose range on stimulation of water absorption across the tissue explant (Table 1 and Figure 3). While no statistically significant difference between compound doses could be discerned, we were able to detect statistically significant (p = 0.013) increase in fluid absorption after drug treatment versus vehicle control during the first 20 minutes post drug/vehicle addition when data for all DPOFA concentrations within the 1-20 μM range were pooled for the analysis. We speculate that our inability to detect dose dependence in compound activity is consistent with reported complexity of biological response to DPOFA in another biological system. It has been shown that tiritations of DPOFA in a cerebrocortical slice swelling assay produced the U-shaped dose response curve with a maximum reduction of K+-induced swelling in a ling assay produced the U-shaped dose response curve that titrations of DPOFA in a cerebrocortical slice swelling assay produced the U-shaped dose response curve that titrations of DPOFA in a cerebrocortical slice swelling assay produced the U-shaped dose response curve that titrations of DPOFA in a cerebrocortical slice swelling assay produced the U-shaped dose response curve...
17. Musson DG, Tobias RJ Jr, Maglietto BK, Ramjit HG, Bayne WF: A sensitive gas chromatographic mass spectrometric assay for a novel dopamine agonist (+)-trans-3,4,4A,5,6,10b-hexahydro-4-propyl-2H-naphth(1,2-B)(1,4) oxazin-9-ol in human plasma. Biomed Environ Mass Spectrom 1988, 17:203-8.

18. Thakran P, Nelson RM Jr, Leuschen MP: Loop diuretic derivative L-644,711 inhibits K(+) stimulated cellular injury in neonatal guinea pig cortical astrocytes. Mol Chem Neuropathol 1994, 21:23-39.

19. Kimmelberg HK, Rose JW, Barron KD, Wanieowski RA, Cragoe EJ: Astrocytic swelling in traumatic-hypoxic brain injury. Beneficial effects of an inhibitor of anion exchange transport and glutamate uptake in glial cells. Mol Chem Neuropathol 1989, 11:1-31.

20. Song CW, Lyons JC, Griffin RJ, Makepeace CM, Cragoe EJ Jr: Increase in thermosensitivity of tumor cells by lowering intracellular pH. Cancer Res 1993, 53:1599-601.

21. Kimmelberg HK: Cell Volume in the CNS: Regulation and Implications for Nervous System Function and Pathology. The Neuroscientist 2000, 6:1-25.

22. Kohut JJ, Bednar MM, Kimmelberg HK, McAuliffe TL, Gross CE: Reduction in ischemic brain injury in rabbits by the anion transport inhibitor L-644,711. Stroke 1992, 23:93-7.

23. Kimmelberg HK, Cragoe EJ Jr, Nelson LR, Popp AJ, Szarowski D, Rose JW, Woltersdorf OW Jr, Pietruszkiewicz AM: Improved recovery from a traumatic-hypoxic brain injury in cats by intracisternal injection of an anion transport inhibitor. Cent Nerv Syst Trauma 1987, 4:3-14.

24. Mamnishiakas A, Chen S, Jalickeee S, Ranzon T, Shi G, Wang FE, Ehalt T, Hammer JA, Miller SS: Confluent monolayers of cultured human fetal retinal pigment epithelium exhibit morphology and physiology of native tissue. Invest Ophthalmol Vis Sci 2006, 47:3612-24.

25. Petrukhin K, Koisti MJ, Bakall B, Li W, Xie G, Marknell T, Sandgren O, Forman K, Holmgren G, Andreason S, Vujic M, Bergen AA, McGarty-Dugan V, Figueroa D, Austin CP, Metzker ML, Caskey CT, Wadelius C: Identification of the gene responsible for Best macular dystrophy. Nature Genet 1998, 19:241-7.

26. Cragoe EJ: Drugs for the treatment of traumatic brain injury. Med Res Rev 1987, 7:271-305.

27. Rutledge EM, Aschner M, Kimmelberg HK: Pharmacological characterization of swelling-induced D-[3H]aspartate release from primary astrocyte cultures. Am J Physiol 1998, 274:C1511-20.

28. Pushkin A, Kurtz I: SLC4 base (HCO3-,CO3(2-)) transporters: classification, function, structure, genetic diseases, and knockout models. Am J Physiol Renal Physiol 2006, 290:F580-99.

29. Mount DB, Romero MF: The SLC26 gene family of multifunctional anion exchangers. Pflugers Arch 2004, 447:710-21.

30. Tsuboi S: Measurement of the volume flow and hydraulic conductivity across the isolated dog retinal pigment epithelium. Invest Ophthalmol Vis Sci 1987, 28:1776-82.

31. Rymer J, Miller SS, Edelman JL: Epinephrine-induced increases in [Ca2+] (in) and KCl-coupled fluid absorption in bovine RPE. Invest Ophthalmol Vis Sci 2001, 42:1921-9.

32. Edelman JL, Miller SS: Epinephrine stimulates fluid absorption across bovine retinal pigment epithelium. Invest Ophthalmol Vis Sci 1991, 32:3033-40.

33. Zhang N, Kannan R, Okamoto CT, Ryan SJ, Lee VH, Hinton DR: Characterization of brimonidine transport in retinal pigment epithelium. Invest Ophthalmol Vis Sci 2006, 47:287-94.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2415/11/33/prepub

doi:10.1186/1471-2415-11-33
Cite this article as: Iserovich et al.: DPOFA, a Cl-/HCO3- exchanger antagonist, stimulates fluid absorption across basalateral surface of the retinal pigment epithelium. BMC Ophthalmology 2011 11:33.

Submit your next manuscript to BioMed Central and take full advantage of:
• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit