UNPRECEDENTED PROTECTION OF THE RETINA FROM GLAUCOMA-INDUCED NERVE DAMAGE BY TOPICAL ADMINISTRATION OF A NOVEL PRODRUG

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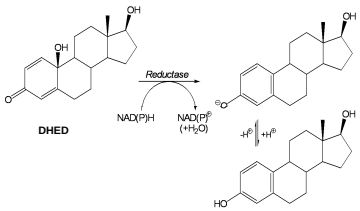
BACKGROUND AND AIMS

- Estrogens are powerful neuroprotectants and have been considered potential drugs to protect the retina against neurodegenerative effects caused by glaucoma [1].
- The "feminizing" effect of estrogens after systemic exposure and their poor ocular bioavailability, however, prevent their development as practical ocular drugs.
- We report here our discovery that a novel type of prodrug [2] for 17β-estradiol (**E2**), 10β,17β-dihydroxyestra-1,4-dien-3-one (**DHED**), overcomes these obstacles due to its improved ocular transport (compared to that of E2) and its selective *in vivo* conversion to E2 inside the eye.



In vivo conversion of **DHED** to **E2** [2].

EXPERIMENTAL METHODS (Medicinal Chemistry & Biopharmaceutics)

- **DHED** was prepared from **E2** by microwave-assisted organic synthesis using lead(IV) acetate oxidation [3].
- Selected physicochemical properties impacting transcorneal permeability (lipophilicity and aqueous solubility) were measured experimentally. Affinities to estrogen-receptor (ER) binding were determined by competitive ligand-binding assays using recombinant ERs. Inhibition of lipid peroxidation was measured using linoleic acid in a cell-free model system by the ferric thiocyanate (FTC) and the thiobarbituric acid reactive substances (TBARS) methods.
- Transcorneal permeabilities were determined *in vitro* using pig cornea.
- *In vitro* conversion of **DHED** to **E2** utilizing NAD(P)H was demonstrated by incubating **DHED** and the cellular reductant in PBS at pH 7.4 and 37°C, followed by liquid chromatography–tandem mass spectrometry (LC/MS/MS) assay [2].
- Metabolic conversion rates in various eye compartments were measured by incubating **DHED** in tissue homogenates (rat and pig) followed by LC/MS/MS assay.
- Evasion of pro-oxidant effect from **DHED** treatment was proven in ovariectomized (OVX) rat retina homogenate by measuring H_2O_2 production as a marker of pro-oxidant (toxic) effects. Estra-1,5(10)-dien-3,4,17-trione (**E1Q**, a profoundly pro-oxidant estrogen metabolite) was used as a positive control.
- Lack of systemic effect after exposure to **DHED** was confirmed by its subcutaneous (s.c.) administration to OVX rats every 48 hours for a period of two weeks for a total of 7 injections (100 μg/kg body weight each) and, then, measuring uterine wet weights. **E2** (administered analogously to that of **DHED**) was used as a positive control.

EXPERIMENTAL METHODS (Pharmacology)

- We used a widely accepted rat model of glaucoma realized through chronic pressure-induced optic nerve damage, which is generated by invasively induced, surgical inhibition of aqueous humor outflow, and characterized by a gradual elevation of IOP [4]. IOP elevation was induced in OVX Brown-Norway rats by injection of hypertonic saline solution in the episcleral vein of the ipsilateral eye using a micro glass needle with an injection pump.
- Using this model of glaucoma, intervention treatments (DHED and E2, respectively, in a vehicle that affords enhancement of transcorneal penetration) were administered as eye-drops in a volume of 10 μl daily for 14-30 days.
- IOP was monitored 2-3 times weekly with a Tono-Pen XL tonometer (Mentor, Norwell, MA) on conscious animals in the presence of a topical anesthetic in both the ipsi- and contralateral eyes for 10-14 days until stabilization of elevated IOP in the ipsilateral eye was achieved.
- Histology and TUNEL assays were used to measure cell death in the retinal ganglion cell layer (GCL) after the rats were sacrificed 19-30 days after IOP elevation.
- Using the OptoMotry system (CerebralMechanics, Lethbride, Alberta, Canada) developed by Prusky *et al.*, we measured functional effects of our small-molecule intervention approach on visual performance. In rats, we took advantage of the optomotor response in which an animal reflexively follows a moving visual stimulus with its eyes.

RESULTS

Compound	$LogP_{calc}$ [a]	S_w [b]	Trans-corneal Flux [c]	Receptor Binding: IC_{50} (nM) [d] ERα	ERβ	LPO: IC_{50} (μM) [e] FTC	TBARS
Estra-1,3,5(10)-triene-3,17β-diol (**E2**)	4.01	4.2 ±0.3	13 ± 3	1.3	0.7	11.8 ± 1.6	3.9 ± 0.4
10β,17β-dihydroxyestra-1,4-diene-3-one (the prodrug **DHED**)	1.67	60.3 ±1.7	126 ± 14	>10,000	>10,000	n.i.	n.i.

[a] P denotes the *n*-octanol/water partitioning coefficient, which is a measure of attraction to lipid phase *versus* an aqueous phase. The logarithm of *P* (logP) is considered the measure of lipophilicity. [b] Intrinsic water-solubility at 25 °C; μg/ml (average ± SEM, n=3). [c] pmol·cm^{-2}·s^{-1} (average ± SEM, n=3) through pig cornea (obtained from a local abattoir); measured *in vitro* using a simple diffusion chamber, 10-μM initial concentration in the donor compartment, 2-h transport period at 35 °C, and LC/MS/MS as an assay method. [d] Reference 2. [e] Concentration to inhibit lipid peroxidation by 50% in the cell-free model system used; n.i. - no inhibition.

Table 1. Selected physicochemical data (lipophilicity and water-solubility), transcorneal permeability, ER-binding and capacity to inhibit lipid peroxidation for **DHED** *versus* **E2**.

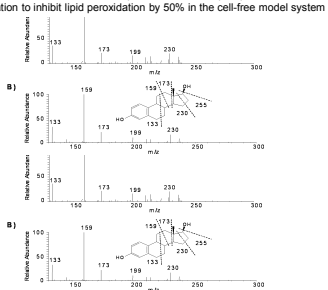


Fig. 1. Conversion of DHED to E2 (LC/APCI-MS/MS analysis, 5 cm x 2.1 mm i.d. Discovery HS C18 column, mobile phase 65/34/1 water/acetonitrile/acetic acid at 0.3 ml/min): **A)** Chromatographic traces are selected reaction monitoring (SRM) of m/z 271 $[M+H-H_2O]^+$ → m/z 161, 175 and 189 for DHD and SRM of m/z 255 $[M+H-H_2O]^+$ → m/z 133, 159, 173 for E2. **B)** The peak at t_R = 3.5 min is unequivocally identified, based on coelution with an authentic reference compound and identical MS/MS (shown in the chart together with the probable origin of the major fragments observed), as E2. Measured initial rate of conversion at 100-nmol/ml substrate concentration was 33 ± 3 nMmin^{-1}.

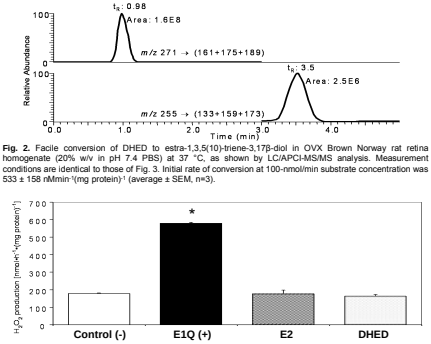


Fig. 2. Facile conversion of DHED to estra-1,3,5(10)-triene-3,17β-diol in OVX Brown Norway rat retina homogenate (20% w/v in pH 7.4 PBS) at 37 °C, as shown by LC/APCI-MS/MS analysis. Measurement conditions are identical to those of Fig. 3. Initial rate of conversion at 100-nmol/min substrate concentration was 533 ± 158 nMmin^{-1}(mg protein)$^{-1}$ (average ± SEM, n=3).

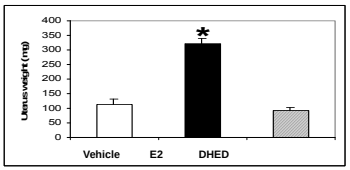


Fig. 4. Uterine wet weights (mg) in OVX rats after chronic administration of corn oil vehicle, E2 and DHED (700μg/kg, s.c., over two weeks). Uterine weights were measured 48 hours after last injection. *Statistically significant differences, ANOVA followed by Tukey test (n = 6, p < 0.05).

Fig. 3. Rates of H_2O_2 production (mean ± standard deviation, n=3) at 37°C in retina homogenate (0.2%, w/v, pH 7.4) of OVX rats in the absence (control) and presence of estra-1,5(10)-dien-3,4,17-trione (3,4-E1Q, a potent prooxidant estrogen metabolite; positive control), E2 and DHD (100 nmol/ml, respectively). Asterisks indicate statistically significant differences (ANOVA followed by *post hoc* Dunnett's test, P<0.05) from the respective controls.

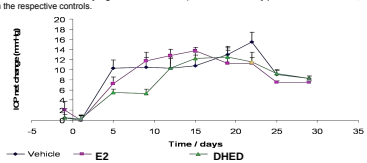


Fig. 5. Summary diagram of changes in IOP with and without intervention treatments for neuroprotection of the retina. IOP is not affected significantly by topical (corneal) administration of neuroprotectants.

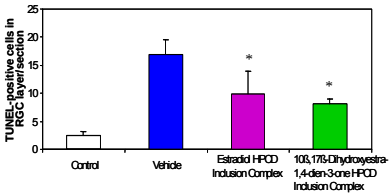


Fig. 6. Summary diagram of analyses of TUNEL-labeling of apoptotic cells in the RGC layer of glaucomatous retinae with and without intervention treatments. RGC death is significantly reduced by topical (corneal) administration of neuroprotectants in both E2– and DHED–treated groups (p<0.05; n=3).

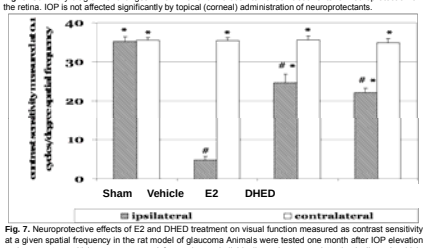


Fig. 7. Neuroprotective effects of E2 and DHED treatment on visual function measured as contrast sensitivity at a given spatial frequency in the rat model of glaucoma Animals were tested one month after IOP elevation and contrast sensitivity was measured for each eye individually and compared to the ipsilateral vehicle control (asterisks, p<0.05) or to the sham surgery vehicle control (#, p<0.05; n=3 per group).

DISCUSSION

While presently a majority of pharmacological approaches target IOP lowering therapies for glaucoma [5], non-intraocular pressure lowering glaucoma therapies are needed to complement these strategies, because [6]:

- Not all forms of glaucoma display elevated IOP and are responsive to IOP lowering therapies,
- Preservation or restoration of visual performance by IOP-lowering intervention is often incomplete due to intrinsic properties of the therapy, dosing regimens or patient compliance,
- Cellular degeneration in the nervous retina including apoptotic signaling cascades develops kinetics and patterns of progression that are or become independent of changes in IOP.

Clinical studies argue for a potential role of estrogen and related compounds in the protection of retinal neurons [7,8]. Retinal ganglion cell (RGC) death either by apoptosis and/or necrosis is a common feature of many ophthalmic disorders including glaucoma [9], and estrogen is a powerful neuroprotectant to prevent RGC death by a variety of genomic and non-genomic mechanisms.

Here, we report the preclinical development of a prodrug strategy that allows for targeting a potent estrogen (**E2**) into the eye, including the retina, after topical administration as eye drops [10]. **DHED** is a prodrug of **E2** (Table 1, Fig. 1) with improved physicochemical properties compared to those of **E2**, which affords enhanced transcorneal transport. The initial conversion rate in the rat retina homogenate (Fig. 2) has been the fastest among all ocular and non-ocular (brain, anterior pituitary, liver and uterus) tissues. This observation has indicated that a rapid intraocular bioactivation in retinal tissue may result in sequestration and preferential release of the neuroprotective agent in this compartment of the eye. Treatment with **DHED** is not accompanied with toxicity (Fig. 3) and, unlike treatment with the parent estrogen, endocrine side-effects (Fig. 4). In a rat model for glaucoma, **DHED** treatment not only reduced the number of apoptotic RGCs (without affecting IOP, see Fig. 5), but a trend was also observed that its topical application reduced apoptosis more than **E2** treatment and was less variable among sample points (Fig. 6). In addition, **DHED** treatment significantly preserved visual function and prevented loss of vision, measured as contrast sensitivity at a given spatial frequency (Figure 7).

In conclusion, our pharmacological approach has great promise for the development of a novel alternative and/or complementary approach to glaucoma therapy.

ACKNOWLEDGEMENTS

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