

Therapeutic Vision, Inc.

***In Vitro* Lens Culture is a Potent Research Tool for Assessing Chemical Toxicity**

The appearance of lens opacities during the toxicological phase of systemic drug development in long-term rat or dog studies or in preclinical or clinical human studies can quickly result in the death of a promising drug project. The purpose of this presentation is to illustrate that *in vitro* lens culture can be a powerful tool for quickly identifying drug-induced mechanisms through which lens metabolism can be adversely affected. Here, we demonstrate how the specific adverse biochemical mechanism(s) of action of a candidate drug can quickly be identified by *in vitro* lens culture studies so that the project may be saved.

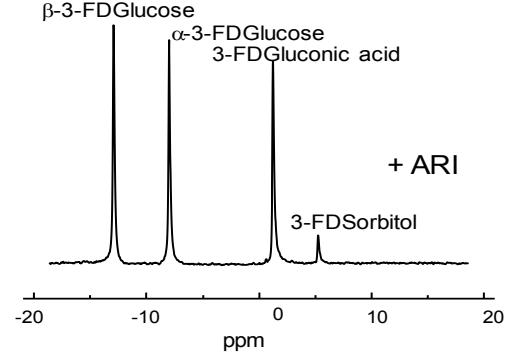
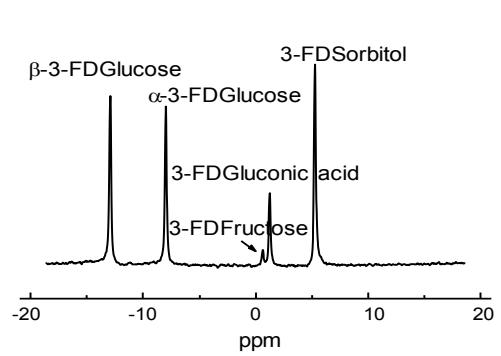
Introduction

The Ocular Lens is Unique

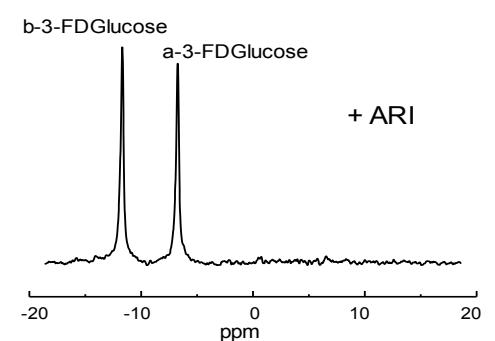
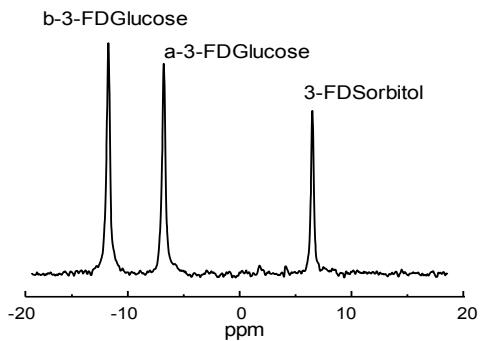
The crystalline lens is a transparent avascular structure composed primarily of structural proteins and water that is surrounded by a collagen capsule. Anteriorly the lens contains a monolayer of epithelial cells which divide, elongate and differentiate to form the regularly arranged lens fibers that are concentrically laid down throughout life. Without a direct blood supply, the lens receives all essential nutrients from the aqueous humor and most of the metabolic, synthetic and transport processes of the lens occur in the epithelial cells. As a result, the lens exists in an environment that is analogous to a single-cell type of tissue culture system. Because of this unique nature, the lens is well-suited for reproducible *in vitro* culture studies aimed at elucidating the complex biochemical pathways of the lens and elucidating the metabolic changes associated with cataract formation. Moreover, lens culture studies can quickly identify species differences in lens metabolism. This is illustrated in the comparison of glucose metabolism by rat, dog and human lenses as monitored by the 24 hr metabolism of 3-fluorodeoxyglucose by ^{19}F -NMR.

Comparative differences in glucose metabolism by aldose reductase in rat, dog and human lenses cultured for 24 hours in medium containing 3-fluoro-deoxy (3-FD) glucose with/without the aldose reductase inhibitor (ARI) AI1576 as measured by ^{19}F -NMR

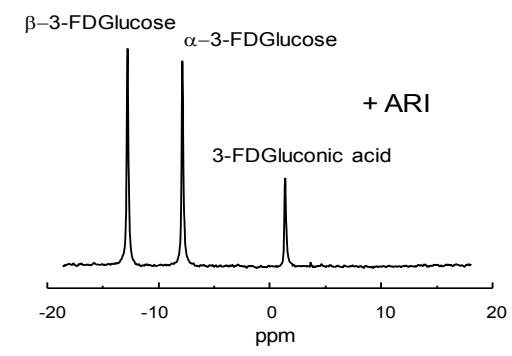
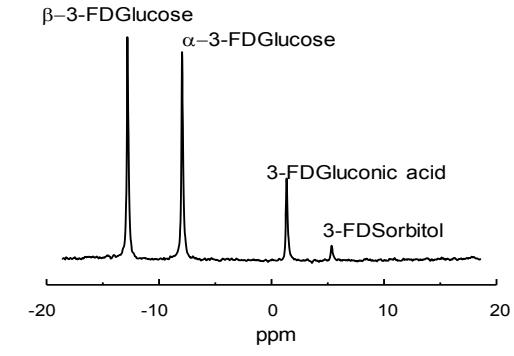
Cultured Dog Lens



Cultured Rat Lens



Cultured Human Lens



Because the chemical properties of the encapsulated lens allow it to behave both electrically and chemically like a single cell, the lens can serve as a sensitive indicator of chemical toxicity. The use of lens organ culture for toxicology work was first proposed by Edwards (Exp. Eye Res. **10**, 288—292, 1970) who cultured embryonic chick lenses in medium 199 (M-199), containing 0.002% phenol red pH indicator, and supplemented with 10% fetal calf serum. Using this technique, they demonstrated that lenses produced “acid” into the media for at least 2 weeks while similar lenses exposed to 0.5 mM of organic aromatic compounds quickly failed to acidify the media. Subsequently in practice, the Kinoshita Laboratory at the Howe Laboratory at Harvard and later the National Eye Institute found that optimal lens metabolism was maintained with TC-199 - bicarbonate media in the absence of phenol red dye and without fetal calf serum present. Moreover, the TC-199 media had to be supplemented with glutamine prior to culture because glutamine in TC-199 media was not stable. This formulation was adequate for maintaining lens clarity as well as lens biochemical parameters of mouse, rat, dog, monkey and human lenses for culture periods of up to 72 hours. Lens clarity can be maintained for longer periods; however, lens metabolic parameters are quickly reduced at longer culture periods. More importantly, while changes in lens metabolism can result in lens opacification, maintenance of lens clarity does not guarantee that lens metabolism has not been altered or reduced.

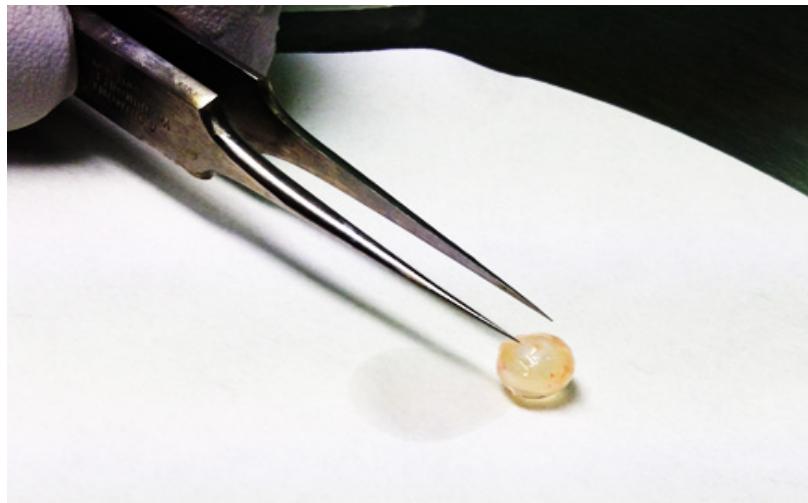
Experimental Procedures

The avascular lens relies on a number of biochemical functions that are critical to maintaining lens redox, transport and osmoregulation systems. Drugs can adversely affect these biochemical systems and these changes can lead to cataract formation. The effect(s) of a drug on lens viability can rapidly be assessed through 24-48 hr *in vitro* lens culture in TC-199 medium with or without the presence of 0.1 mM of the drug in question. A high (0.1 mM) concentration is used to rapidly induce the drug effect. Failure of a drug effect at this concentration suggests that a metabolite may be the toxicological agent.

Preparation of TC-199-Bicarbonate Culture Media The culture medium is composed of bicarbonate buffer based TC-199 media without phenol red, supplemented with glutamine (0.44 mM), glucose (2.4 mM), fructose (30 mM), calcium (1.67 mM) and penicillin (15 units)-streptomycin (15 µg/ml). The culture medium is adjusted to 295-300 mosmol with NaCl and equilibrated with 5% CO₂ for 30-45 min to ensure enough CO₂ has been dissolved to provide proper buffering capability of the buffer. The medium is filtered with 0.2 micron cellulose membrane and pipetted (with a sterile pipetter) into culture plates under sterile condition in the biological laminar flow hood. Rat lenses are cultured in 2 ml of the culture media in 12-well plates.

Lens Dissection Young (100 g) Sprague Dawley rats were asphyxiated with carbon dioxide. After death, the eyes were enucleated and the intact lens from each eye was removed by careful dissection from a posterior approach as illustrated here.

The enucleated eye is placed corneal side down on dry filter paper

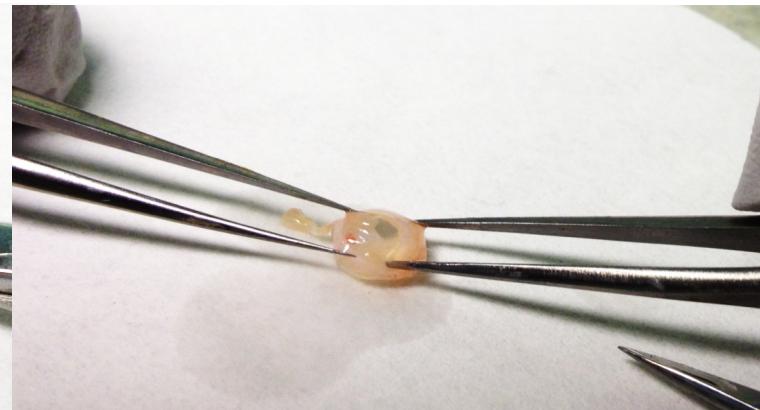
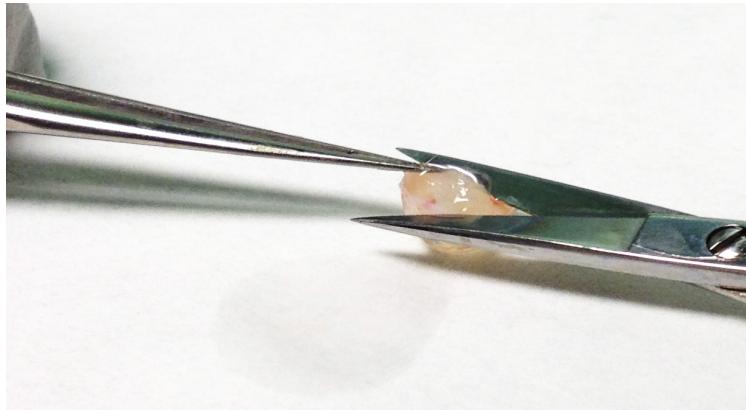


While steadyng the eye with forceps a small incision is made into the retina

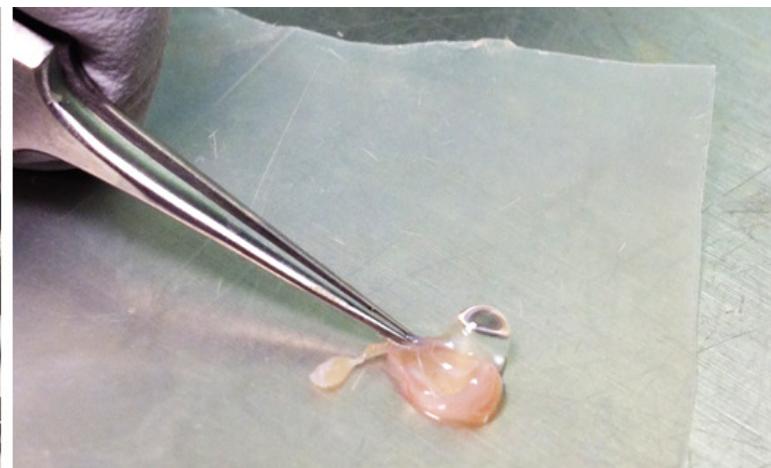
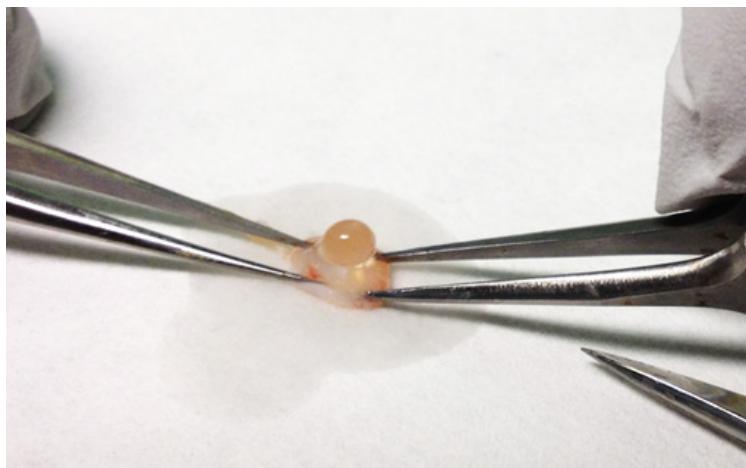


Holding the eye up by the optic nerve, a portion of the posterior segment is removed with scissors

Pressing down at the region of the ora seratta, the lens is expressed – for rabbit, dog or human eyes, the vitreous must be carefully removed and the ciliary fibers cut with scissors prior to expression



After expressing the lens, the eye is placed on parafilm



Using the flat end of a Teflon stirring rod to prevent the expressed lens from moving, the eye is separated



The dissected lens is placed in the incubation media.



Preincubation is generally conducted because lenses are sensitive to physical injury during dissection which generally becomes visible after 4-8 hr in incubation in control media. Lenses free of damage are then transferred to experimental culture media using curved Teflon coated forceps. After culture, lenses are removed from the media, rolled on moist filter paper and immediately weighed. The lenses are then homogenized in ground glass homogenizers.

Biochemical Evaluation

After Incubation, an initial screening of the following select biochemical parameters should be conducted:

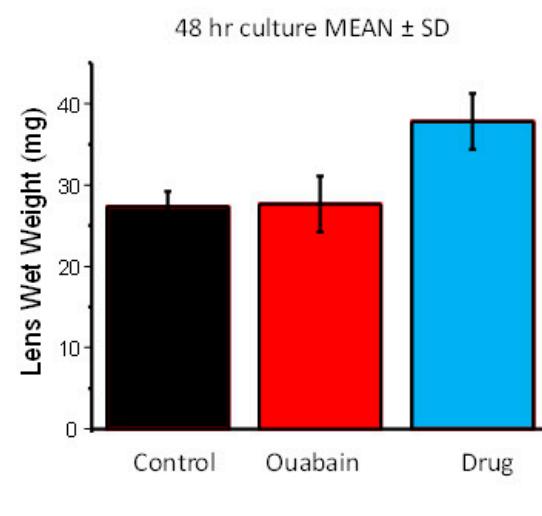
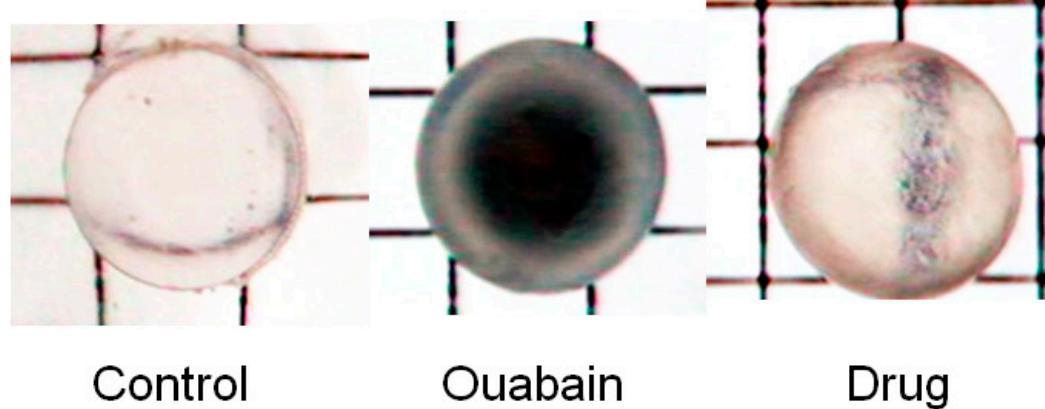
- **Lens Wet Weight** - changes in lens weight reflect osmotic changes that can also induce stress in endoplasmic reticulum (ER) and/or mitochondria in lens epithelial cells with eventual generation of ROS
- **Glutathione (GSH)** levels - a critical component of the lens redox system, GSH levels rapidly decrease as the lens is exposed to oxidative stress
- **Catalase, Glutathione Peroxidase (GPx) and Glutathione Reductase (GR)** activities - these are critical enzymes for reducing reactive oxygen species and oxidation
- **Glucose 6-Phosphate Dehydrogenase (G6PD)** activity - this enzyme in the pentose shunt pathway is essential for the defense of oxidative stress

- **Glyceraldehyde-3-Phosphate Dehydrogenase (G3PD)** - this is a glycolytic enzyme that is a major target protein in oxidative stresses that is extremely sensitive to oxidative stress resulting in loss of ATP production
- **Lactate Dehydrogenase (LDH)** - this enzyme rapidly leaks out of a damaged lens; therefore, it serves as a marker for cell injury
- **4-Hydroxynonenal (4-HNE)** - an oxidation product of lipids, it is a measure of lipid peroxidation activity
- **ATP levels** - this is a critical energy supply for the lens, especially for maintaining transport systems
- **C¹⁴- α -aminoisobutyric acid (AIB)** - this is a good marker for the general uptake of neutral amino acids
- **H³-choline** - the facilitated uptake of choline is an established sensitive marker for cellular biochemical changes that are associated with cataract formation
- **Rubidium⁸⁶** - a good substitute for potassium, its uptake indirectly assesses lens ATPase activity which is critical for maintaining appropriate intracellular levels of potassium and sodium

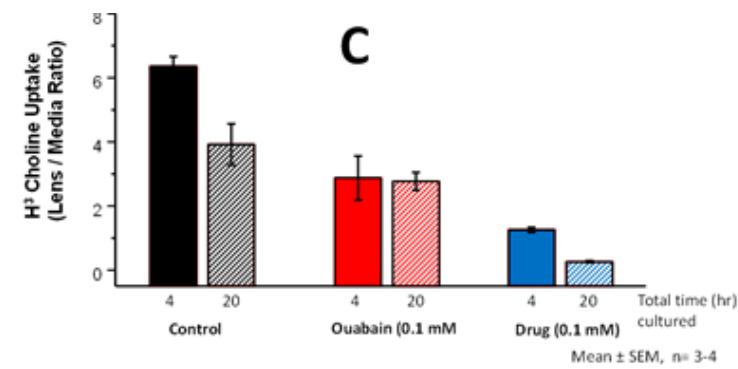
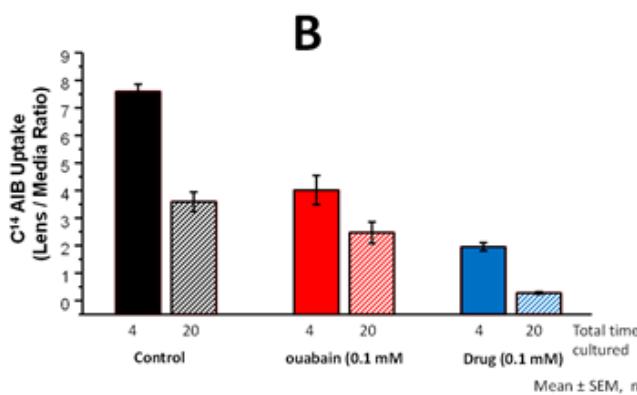
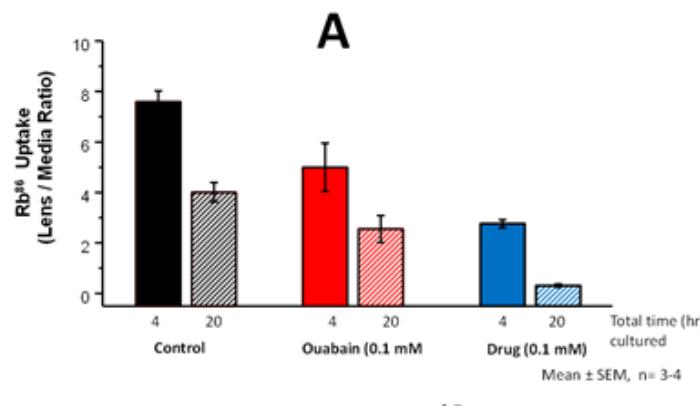
Example of a Drug Evaluation

The utility of this technique for toxicological studies is illustrated where the oral 3-month feeding of a “drug” for thoracic disease resulted in cataract formation in rats.

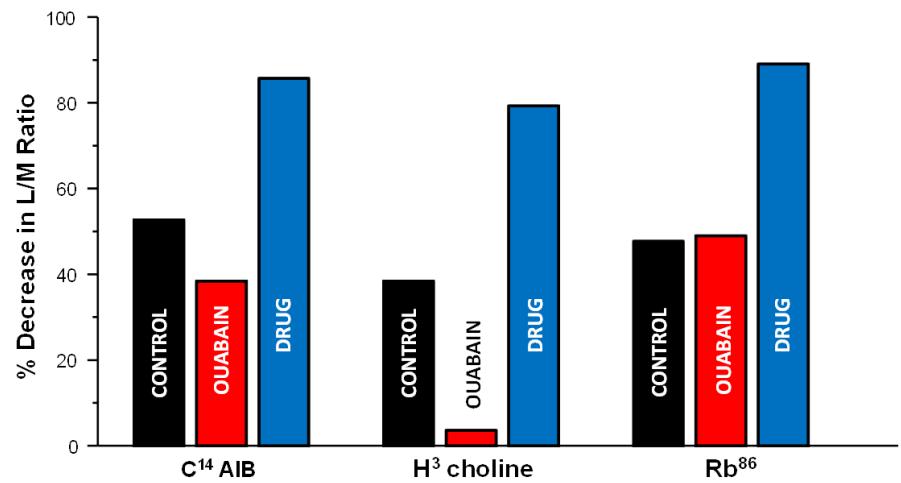
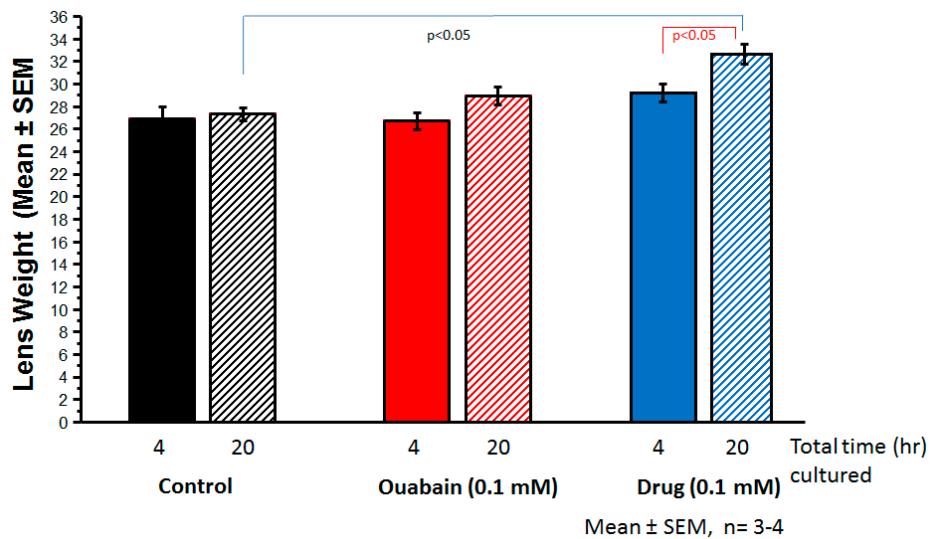
As an initial step, lenses were incubated with/without 0.1 mM of drug (in 0.4% DMSO) for 48 hrs. Lenses were also incubated with 0.1 mM of the Na⁺,K⁺-ATPase inhibitor ouabain as a cataractogenic control. During this time period, the appearance of the ouabain and drug exposed lenses significantly changed as seen below. Weight measurements revealed that the drug-exposed lenses had higher wet weights suggestive of the development of an osmotic cataract. A similar increase in wet weight was observed at 24 hours.



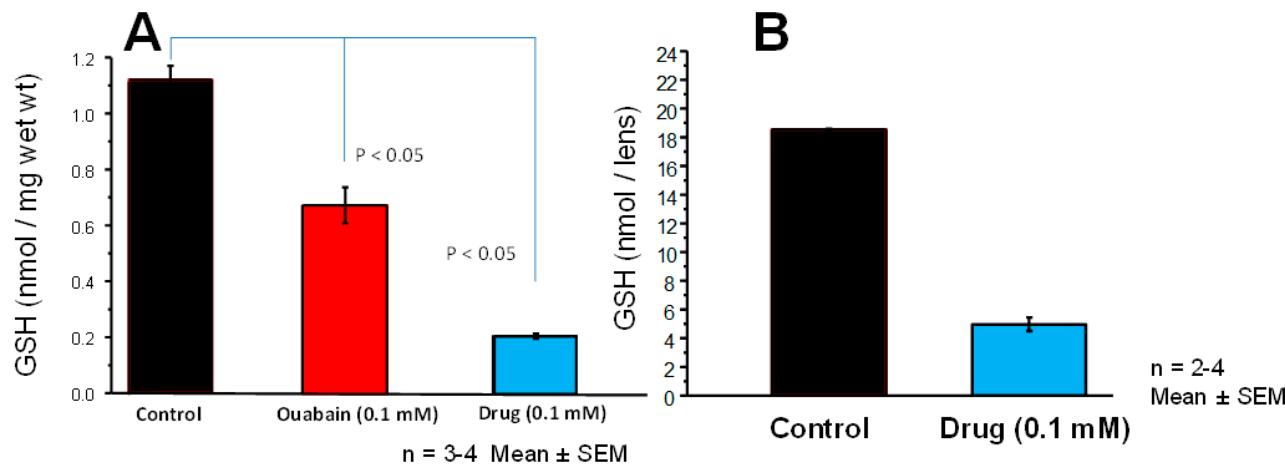
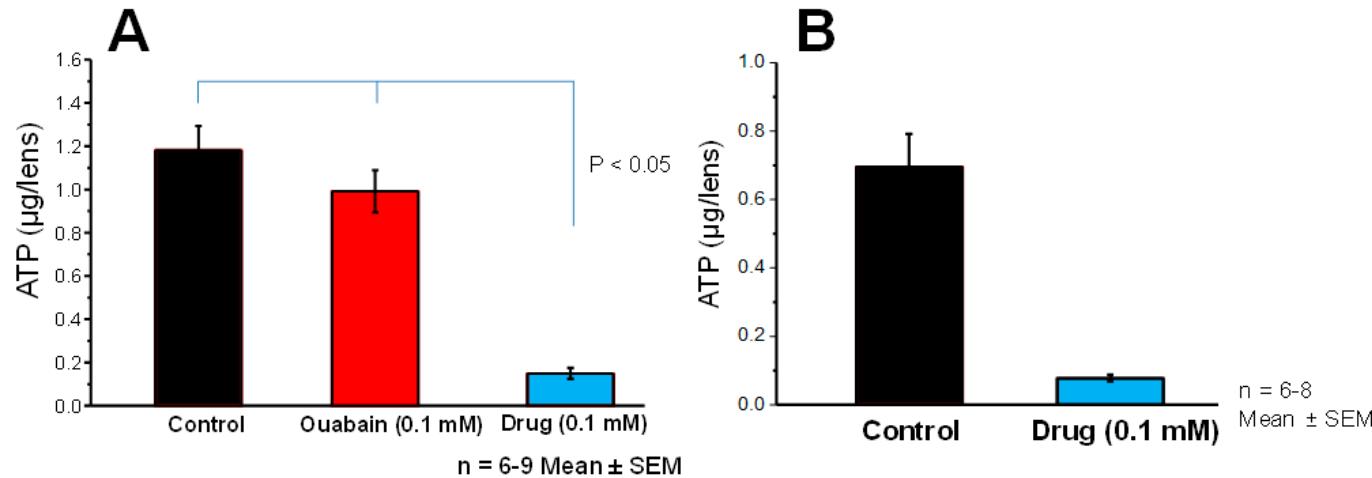
Osmotic cataract development leads to membrane permeability changes that are generally associated with either a malfunction of the Na^+,K^+ -ATPase system of the lens or the accumulation of osmolytes. The accumulation of polyols as osmolytes was ruled out because the incubation media contained primarily 30 mM fructose which cannot be converted to polyols and the amounts of glucose present in the TC-199 media was not adequate to induce osmotic changes. Therefore, uptake-leak-out studies were conducted using a cocktail mixture of Rb^{86} , [methyl-H^3]-choline and [$1-\text{C}^{14}$]-methylaminoisobutyric acid (AIB). The 4 hr uptake and 16 hour efflux of radiolabeled Rb^{86} (A), C^{14} -AIB (B) and H^3 -choline (C) for cultured rat lenses is shown.



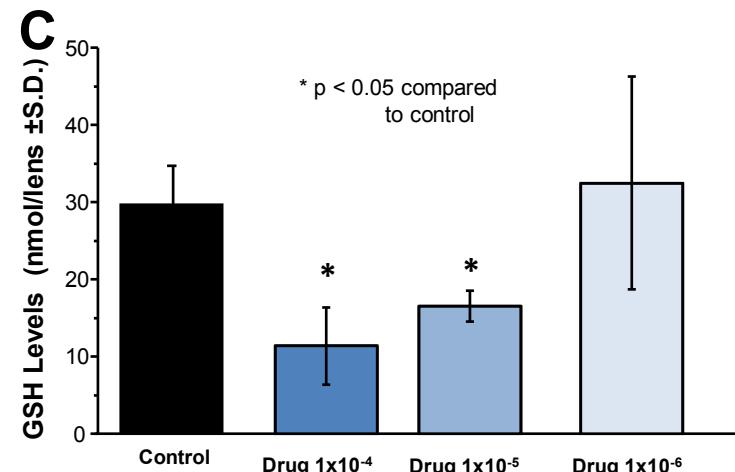
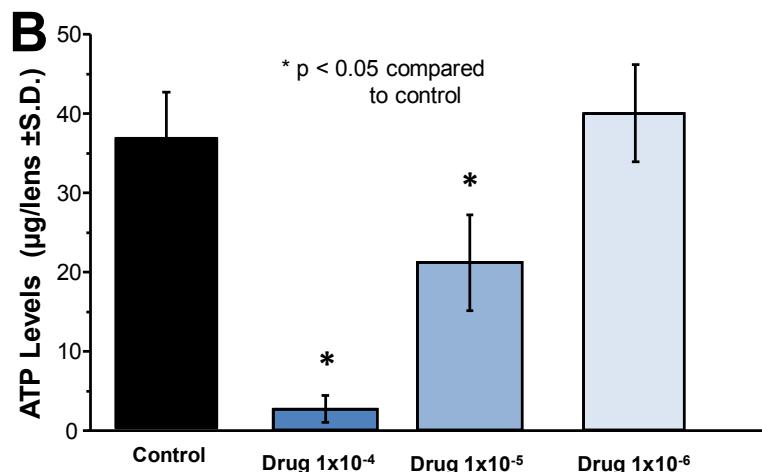
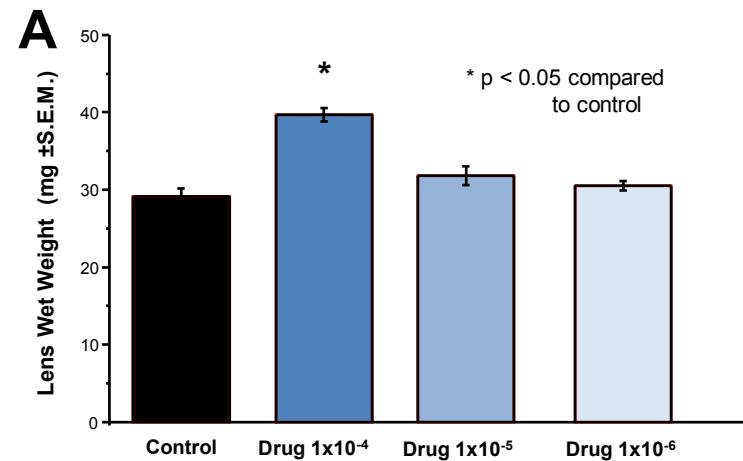
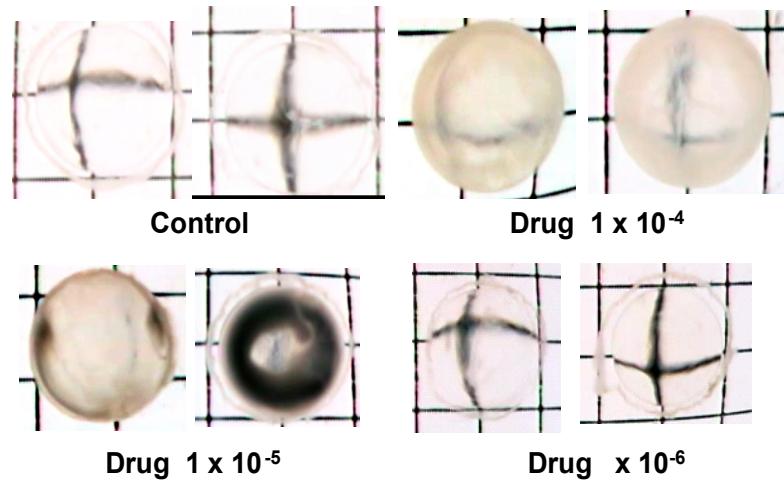
As initially observed in the first experiment, the lens wet weights were significantly increased by 20 hr exposure to the drug (left) and these osmotic changes appear to be associated with increased efflux of radiolabel suggestive of lens membrane permeability changes (right). Similar results were observed with Cl³⁵.



The results of the uptake and efflux studies suggest that the drug may reduce cellular energy required for the transport systems. ATP levels were only significantly reduced by drug both after 48 hr (A top) and 24 hr (B top) exposure. In contrast, GSH levels are significantly reduced with ouabain and drug both after 48 hr (A bottom) and 24 hrs (B bottom).

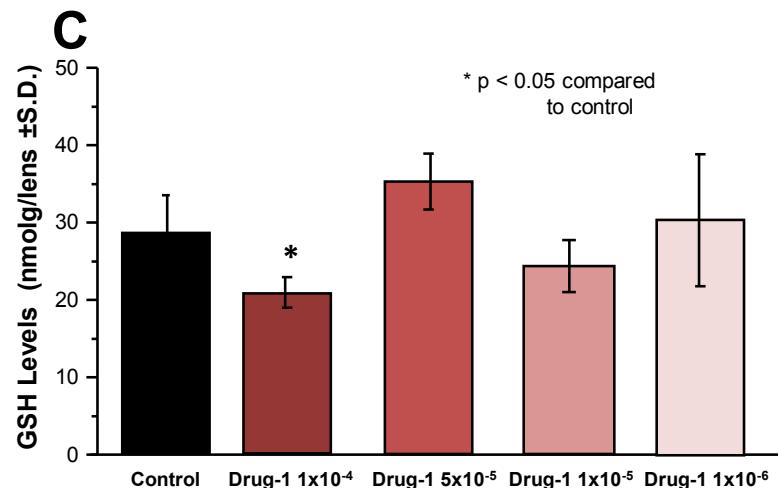
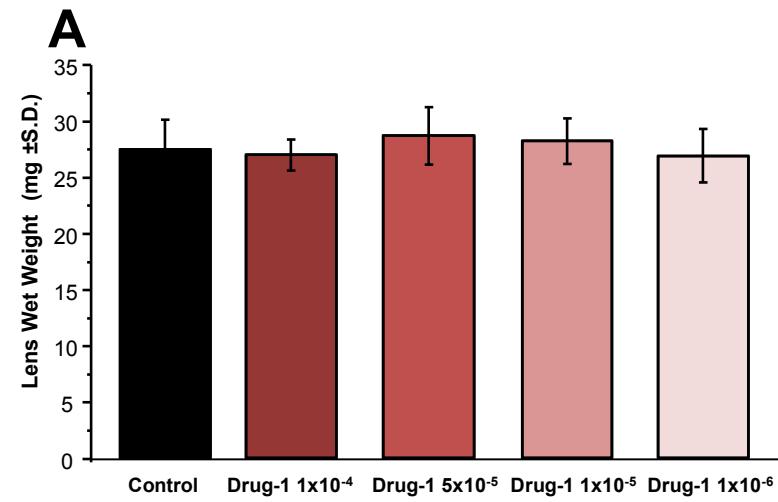
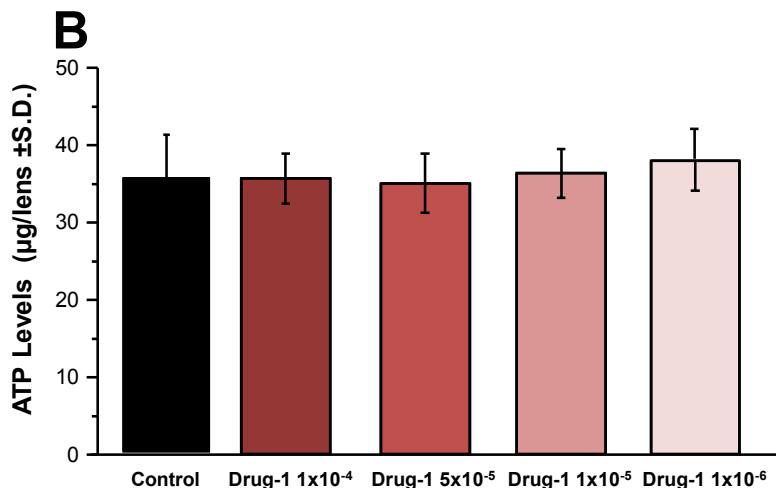
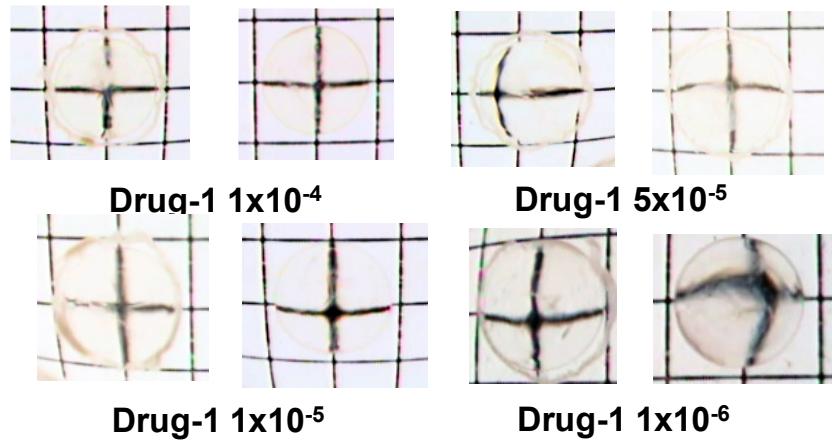


To determine whether the drug-induced increase in lens wet weight and decrease in lens ATP and GSH levels are dose-dependent, 48 hr lens culture studies were conducted. This confirmed that the exposure of drug to lenses resulted in dose-dependent changes in appearance, increased wet weight (A), decreased ATP (B) and GSH levels (C).

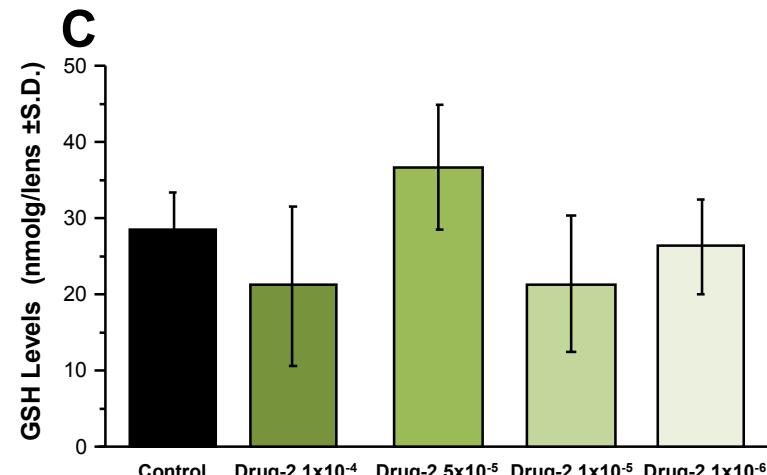
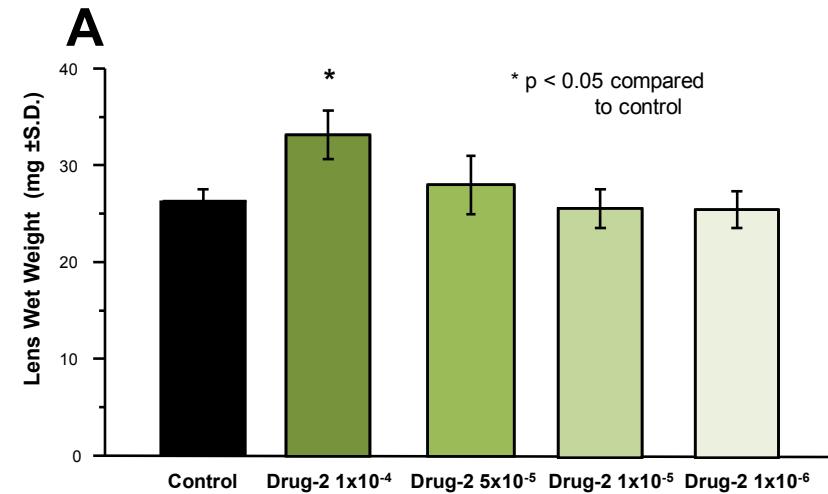
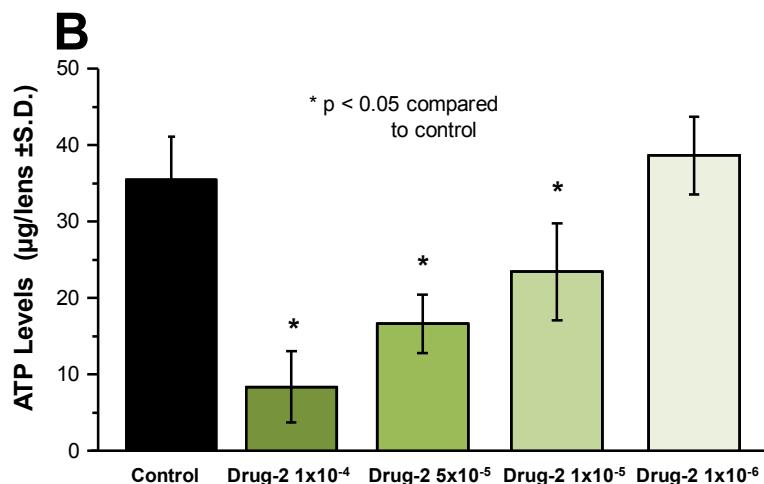
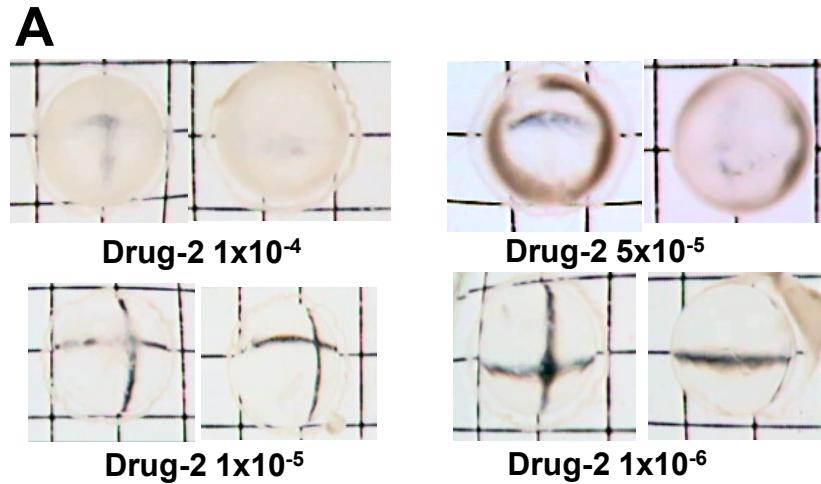


At this point, it was not clear whether these adverse lenticular effects were linked to the desired systemic thoracic activity or to the general chemical structure of the drug. To gain insight into this question, two additional analogs (Drug-1 and Drug-2) were synthesized and evaluated in lens culture studies. Each possessed structural changes from the opposite ends of the structure of the drug molecule. As a result neither retained the desired thoracic activity.

Studies with Drug-1 indicated that 48 hr exposure of rat lenses to this drug did not have any dose-dependent effect on either lens appearance, wet weight (A) or ATP levels (B). Only a slight but significant decrease in GSH levels was observed at a 1×10^{-4} M concentration.



Similar culture studies conducted with Drug-2 indicated that 48 hr exposure of rat lenses to this drug retained the adverse dose-dependent effects on lens appearance, wet weight (A) or ATP levels (B) and GSH levels (C).



The results of these studies suggested that the adverse lenticular effects of the parent drug are due to its structural attributes rather than its intrinsic biological activity because similar lenticular effects were seen to a lesser extent with the two inactive analogs examined.

Conclusion

- Lens culture studies not only can elucidate the experimental mechanism(s) of cataract formation but also identify the toxicological mechanism(s) of action of how a drug can alter lens biochemistry and clarity.
- In the above example, while drug-induced cataract developed in a 3 month *in vivo* time-frame, significant lens effects were detected within 24 hr of *in vitro* lens culture.
- These studies indicate that *in vitro* lens culture is a powerful research tool for drug development that can rapidly identify the specific adverse mechanism(s) of action of a drug candidate as well as determine whether the parent compound or its metabolite initiates the adverse effect(s).