E2012-Induced Cataract and Its Predictive Biomarkers

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E2012, a gamma secretase modulator without affecting Notch processing, aimed at Alzheimer’s disease by reduction of amyloid β-42, induced cataract following repeated doses in the rat. Cataract appeared first at week 10–11 of treatment as a posterior subcapsular area with granular/punctate opaque or shiny dots along the suture line, characterized histologically as lenticular fiber degeneration, which eventually coalesced to form a triangular or circular opacity. It was associated with prolonged and sustained elevation of lenticular desmosterol (24-dehydrocholesterol), the final precursor of cholesterol, and decrease in lenticular cholesterol. In vitro studies to investigate the effect of E2012 on cholesterol metabolism demonstrated that E2012 inhibits 3β-hydroxysterol Δ24-reductase (DHCR24) at the final step in the cholesterol biosynthesis. In vivo lenticular concentration of E2012 after 13-week repeated dose with cataract was well above those where inhibition was observed in vitro. There was no cataract formation at doses where desmosterol did not accumulate in the lens. The elevation of desmosterol and decreased cholesterol levels were also seen in the liver and plasma and preceded those in the lens. These results demonstrate that E2012 induces cataract in the rat by inhibiting DHCR24 at the final step of cholesterol synthesis with associated elevation in desmosterol within the lens, preceded by desmosterol changes that would serve as a predictive safety biomarker for lenticular opacity.

Key Words: cataract; drug-induced; mechanism; DHCR24; desmosterol; biomarker.

Cataract, defined as opacity of the lens associated with lenticular fiber degeneration, is a leading cause of postnatal blindness in men (Moreau and King, 2012). The lens, consisting of highly ordered, hexagonally packed, elongate lenticular fibers in syncytium, continues to grow throughout life by addition of new fibers on previous ones. A new lenticular fiber is provided from the anterior lenticular epithelial cell that acquires nutrients from the aqueous fluid in the anterior chamber with very low proteins and only trace levels of cholesterol. The epithelial cells therefore must obtain all of its cholesterol by de novo synthesis (Cenedella, 1996). The anterior lenticular epithelial cell starts to elongate when it reaches the equator toward both the anterior and the posterior poles; the nucleus and organelles are lost during this process, and eventually, the lenticular fiber depends metabolically on the anterior epithelial cells and the anterior chamber fluid (Bassnett et al., 2011; Samuelson, 2007). A lenticular fiber cell must remain stable for a lifetime to preserve transparency even though there is little or no protein turnover. A variety of protective mechanisms are present in the lens to achieve this, such as high glutathione and ascorbic acid content (Berthoud and Beyer, 2009), and the presence of crystallins with chaperone-like activities (Andley, 2009; Moreau and King, 2012). Cataracts occur in association with a variety of causes including radiation, oxidative stress, and metabolic diseases such as diabetes mellitus (Moreau and King, 2012; Render et al., 2013) and various unrelated classes of compounds including hypolipidemic drugs, steroids, psychotropic compounds, anticancer drugs, and others. Various factors are known to be associated with cataracts such as hypercholesterolemia, cytoskeletal alterations, electrolyte imbalance, alterations in the ion transport, and/or changes in the state of dehydration of fibers (Cheng et al., 2002; Greaves, 2012; Jobling and Augusteyn, 2002; Paterson and Delamere, 2004; Rao et al., 1997). Although the precise causes are mostly unknown, lenticular opacity develops irrespective of the cause when the soluble proteins such as crystallins within the cytoplasm of the fiber aggregate and become insoluble following structural conformational change.

Cholesterol-lowering drugs are well-known as inducers of cataracts in animals and/or humans. These include inhibitors of cholesterol biosynthesis at various steps along the pathway: HMG-CoA reductase inhibitors (Gerson et al., 1990;...
MacDonald et al., 1988), oxidosqualene cyclase (OSC) inhibitors (Cenedella, 1996; Cenedella et al., 2004), and 3β-hydroxysterol Δ24-reductase (DHCR24) inhibitors. It is known that triparanol (MER−29) inhibited DHCR24 and induced a decrease in cholesterol and accumulation of desmosterol (24-dehydrocholesterol) in rats and men with development of cataracts in both species. Cataracts occurred in patients given triparanol for 3 months or more (Kirby, 1967; von Sallmann et al., 1963).

E2012 is a gamma secretase modulator without affecting Notch processing aimed at Alzheimer’s disease by reduction of amyloid β-42. E2012-induced cataract in the rat after repeated doses.

The objective of the present investigation was to gain a better understanding of the mechanisms involved in E2012-induced cataract in the rat, particularly in association with cholesterol metabolism. We have shown that perturbations in cholesterol biosynthesis are strongly implicated in the pathogenesis of cataracts by E2012 at the level of DHCR24, the key enzyme in the conversion of desmosterol to cholesterol at the final step of cholesterol biosynthesis, leading to prolonged and sustained accumulation of desmosterol and decrease in cholesterol in the lens. We have managed to discover a new safety predictive biomarker that is altered before the onset of E2012-induced cataract and can be used for monitoring in the plasma.

MATERIALS AND METHODS

All studies have been approved by the Laboratory Animal Care and Use Committee at Eisai Co, Ltd and performed in compliance with Laboratory Animal Policy.

Animals and study design in vivo. Male and female Sprague-Dawley rats (Crl: CD (SD)) aged 8 weeks were given either vehicle, 0.5 wt/vol% methylcellulose solution, or E2012 once a day at various doses up to 1000 mg/kg and various lengths of dosing up to 13 weeks and recovery period up to 26 weeks (Table 1, Fig. 1). Necropsy was undertaken following euthanasia under isoflurane anesthesia or by CO2 asphyxiation. Eyes were immersed in Davidson’s Fixative and processed routinely to prepare paraffin sections for histology with hematoxylin and eosin stain (H&E). Plasma, liver, and the eyes were collected for cholesterol and desmosterol determination.

Ophthalmologic examination. Ophthalmologic exam was periodically undertaken using binocular indirect ophthalmoscope and slit-lamp. The pupils were dilated by topical application of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin P, Santen Pharmaceutical Co, Ltd, Osaka, Japan).

Cholesterol and desmosterol determination. Rats were given E2012 at 0, 3, 10, 30, or 60 mg/kg once or daily for 2, 4, 8, 10, or 13 weeks. The liver and lens were collected on the day following final administration of E2012 at these time points. Separately, rats were given 30 or 60 mg/kg of E2012 daily for 4 weeks followed by a 1-, 4-, 6-, or 14-week recovery period (Fig. 1). All animals were subjected to necropsy at the end of the recovery period to collect the liver and the lens. On days designated for analysis of plasma cholesterol and desmosterol, blood was collected into heparinized syringes prior to dosing and at 6, 12, and 24 hours postdose from the jugular vein of unanesthetized animals.

Blood samples (approximately 0.6 ml) were centrifuged, plasma transferred into amber tubes, and stored frozen below −15°C until analysis. A piece of liver and the entire lens were weighed, transferred to a tube, and stored frozen until analysis. The concentrations of cholesterol and desmosterol in plasma were determined by the liquid chromatography/mass spectroscopy (LC/MS) method using LCMS-2010EV (SHIMADZU) after extraction using alkaline solution, ethanol, and petroleum ether. Following evaporation at 55°C, the lipids were reconstituted in n-hexane-2-propanol (3:2, vol/vol) and applied to LC/MS. For the liver and the lens, KOH solution was added to the liver or lens in tube and the mixture shaken gently at room temperature overnight to allow the tissue dissolve, and used for cholesterol and desmosterol analyses, as described for the plasma.

E2012 determination. Blood samples (approximately 0.25 ml) were collected from jugular vein using heparinized syringe, centrifuged, plasma transferred into amber tubes, and stored frozen below −15°C until analysis. The lens was collected from eyes at necropsy, weighed, transferred to tubes, and stored frozen until analysis. The concentrations of E2012 in plasma were determined using LC-MS-MS. For the lens, E2012 was analyzed in the final supernatant obtained after homogenization with distilled water and addition of acetonitrile including the internal standard followed by centrifugation at 3000 rpm, and the supernatant filtered and centrifuged at 2000 rpm.

In vitro: Cholesterol synthesis inhibition in HepG2 cells, rat primary hepatocytes and rat primary lens cultures. HepG2 cells (ATCC, Manassas VA) were plated onto collagen type I-coated 24-well plates at a density of 1 × 10^4 cells/440 μl medium/well and incubated in Dulbecco’s Modified Eagle Medium (Invitrogen Co) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen Co) at 37°C in a humidified atmosphere containing 5% CO2 for 1 day before the assay of cholesterol synthesis.

For rat primary cultures, male Sprague-Dawley rats (Crl: CD [SD]) were used at the age of 6 weeks.

For rat primary hepatocytes, the animal was anesthetized by intraperitoneal Nembutal and given sodium heparin at 500 unit/kg intravenously. The liver was perfused with perfusion medium (Invitrogen Co) for 10 minutes, followed by digestion medium (Invitrogen Co) for 15 minutes. Liver cells were dispersed in culture medium, cells filtered through a mesh, and hepatocytes isolated by centrifugation 4 times at 500 rpm for 1 minute. The cells were plated onto collagen type I-coated 24-well plates at a density of 2 × 10^4 cells/500 μl medium/well and incubated in Williams’ Medium E (Invitrogen Co) containing 10% fetal bovine serum (HyClone), 1% penicillin-streptomycin (Invitrogen Co), L-Glutamine (Invitrogen Co), Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA) (Invitrogen Co), and insulin-transferrin-selenium-G supplement (Invitrogen Co) and 0.1 mmol/l dexamethasone (Sigma) at 37°C in a humidified atmosphere containing 5% CO2. After 3-hour culture, nonadherent cells were removed by washing. 440 μl of the culture medium added, and allowed to settle for 1 day before the assay of cholesterol synthesis.

For rat primary lens, the animal was euthanized by cervical dislocation, eyes excised and lenses collected after making an incision. Lenses were placed in 24-well plates filled with 1 ml of Medium 199 (Invitrogen Co) containing 0.5% penicillin-streptomycin, 1.3 mM L-Glutamine (Sigma), with osmolality adjusted with NaCl to 295–300 mOsm/kg, and incubated at 37°C in a humidified atmosphere containing 5% CO2. After 3-hour culture, nonadherent cells were removed by washing. 440 μl of the culture medium added and allowed to settle for 1 day before the assay of cholesterol synthesis.

Cholesterol synthesis was determined in rat primary hepatocyte culture, HepG2, and rat lens by measuring biosynthesis of cholesterol from [14C]acetate acid sodium salt, with Simonvatiin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) as a positive control (for inhibition of cholesterol synthesis). The rat primary hepatocyte and HepG2 were incubated with E2012 or simvastatin and [14C]acetate acid sodium salt for 2 hours. At the end of this 2-hour incubation, lipids were extracted from the cells by incubation with hexane-2-propanol (3:2, vol/vol), evaporated under a nitrogen stream, and resuspended in petroleum ether. Resuspension was applied onto thin layer chromatography glass plates coated with silver nitrate and developed with chlorform-acetone (85:15, vol/vol). The radioactivity in cholesterol and total radioactivity were analyzed using a BAS 2500 imaging plate system. For the rat lens, the tissues were incubated with E2012 or simvastatin and [14C]acetate acid sodium salt for 8 hours, solubilized with 4 mol/l KOH, and processed similarly.

In vitro: DHCR24 inhibition in rat liver microsome. DHCR24 activity in rat microsome suspension was determined by conversion of desmosterol to 24-reductase (DHCR24) inhibitors. It is known that triparanol and can be used for monitoring in the plasma.
cholesterol (Waterham et al., 2001). Rat liver microsomes were prepared as described elsewhere (Omura and Sato, 1964) with slight modifications; briefly, the liver was perfused with cold 1.15% KCl solution, homogenized, centrifuged at 9,000 × g, 4°C, for 20 minutes, supernatant further centrifuged at 105,000 × g, 4°C, for 60 minutes, and the pellet suspended with 3 ml of cold 0.05 mol/l phosphate buffer (pH 7.4). The effect of E2012 was determined by incubation at 37°C for 8 hours of the reaction mixture containing E2012 at various concentrations up to 10,000 nmol/l, rat liver microsome (1 mg/ml) and desmosterol solution (168 μmol/l).

### RESULTS

**E2012 Is Cataractogenic in the Rat**

A single dose of 1000 mg/kg/day, or 4-week treatment at up to 60 mg/kg/day did not induce lenticular lesions even up to, respectively, 26 or 14 weeks of postdose observation (Table 1).

In the repeated dose toxicity studies in rats, there were no lenticular changes in ophthalmological examination up to week 10 at the maximum dose tested (60 mg/kg/day). Lenticular lesions appeared first at approximately 11 weeks of treatment at 30 mg/kg/day and above in a 13-week study. Doses of 3 and 10 mg/kg/day for up to 13 weeks did not lead to cataract (Table 2).

The lenticular lesions were characterized by ophthalmological examination as a posterior subcapsular area with granular/punctate opaque or shiny dots along the suture line that eventually coalesced to form a triangular or circular opacity appearing as an area of altered refraction (Fig. 2c). These were generally dose dependent. Histologically lenticular lesions were characterized by dose-dependent focal lenticular fiber degeneration in the posterior subcapsular region often at or around the Y-suture, with fragmented fibers and homogeneous eosinophilic material.
accumulating in the affected area (Fig. 2d). The incidence was similar between 30 and 60 mg/kg/day groups, but the severity of cataract was dose dependent. When reversibility was assessed following 13-week treatment with up to 26 weeks of recovery (Tables 3 and 4), the incidence of lenticular lesions similar to those at the end of dosing were increased occasionally with increased severity up to 6 weeks of recovery; after 13–26 weeks of recovery, residual lesions (Fig. 3, arrow) were observed without progression with gradual “internalization.” New, normal lenticular fiber (Fig. 3, asterisk) displaced the stable residual lesion deeper into the adjacent posterior cortex. In addition, there were isolated whorl-like structures at the periphery of the lesion, and occasionally in other areas of the cortex.

There was generally a good correlation between ophthalmologic and histopathologic findings. The slightly higher incidence detected by histopathology at 13 weeks was attributed to a greater sensitivity to detection of minute lesions by microscopy, provided that consistent lenticular sectioning was performed close to the Y-suture, where the lesion often started. When lesions became larger or were present at expanded locations within the lens, as was seen at the end of recovery, the reverse was true, with ophthalmology being more sensitive than histopathology. These opacities occurred with plasma E2012 areas under the curve (AUCs) of > 100,000 ng•hr/mL; on the other hand, E2012 AUCs of approximately 35,000 ng•hr/mL were not associated with lenticular opacity (Table 3).

Development of Cataract by E2012 Depends on the Sustained and Cumulative Increase in Desmosterol and Decrease in Cholesterol

| Lens | Repeated doses of E2012 at doses of ≥ 30 mg/kg/day resulted in accumulation of desmosterol, followed by decreased |
cholesterol in the lens (Figs. 5b and 5d) and development of cataract. At lower doses with no cataract development, there was no lenticular desmosterol accumulation or decrease in cholesterol after repeated dosing (Figs. 5b and 5d). Elevations of desmosterol levels occurred prior to the decrease in cholesterol levels in the lens, as in the plasma and the liver (Figs. 5a, 5c, and 5d). The kinetics of desmosterol and cholesterol in the lens was similar but lagged behind temporally to those in the plasma and liver. At doses where cataracts developed, lenticular desmosterol started increasing at week 2, and continued to increase until week 13 (Fig. 5b). When drug treatment was terminated, the return of cholesterol and desmosterol concentrations to control levels in the lens was more gradual than in the liver and plasma (Figs. 5a–d, dotted lines). Decreases in drug concentration in the lens were also gradual when drug treatment was discontinued (Fig. 5e).

Plasma and liver. The kinetics of desmosterol and cholesterol in the liver was similar and paralleled that of plasma. Desmosterol was decreased and cholesterol increased in both plasma and the liver at 2 weeks at 60 mg/kg/day, and at 30 mg/kg/day more clearly seen at 4 weeks (Figs. 5a, 5c, and 4). When E2012 was given to rats at different doses, there was a dose above which desmosterol, which is undetectable in the control plasma (Below Quantifiable Limit [BQL]: 2 µg/mL), is elevated and accumulated with repeated dosing (at ≥ 30 mg/kg/day, Fig. 4a), eventually reaching a level that was at least 100× normal with development of cataract. The onset of elevation was rapid, as early as 24 hours (Fig. 4a, day 1). At lower doses (at up to 10 mg/kg/day), desmosterol elevation in the plasma was transient within the day without accumulation by repeated dosing (Fig. 4a); E2012 did not induce cataracts at these doses. Similarly, plasma cholesterol levels were decreased at 30 and 60 mg/kg/day but unchanged at 3 and 10 mg/kg/day (Fig. 4b, Table 2). Elevations of desmosterol levels in the plasma and liver were observed prior to decreases in cholesterol; desmosterol was elevated with a single dose of E2012, particularly at 60 mg/kg/day (Figs. 5a, 5c, and 4). Cessation of drug after 4-week treatment, even at doses up to 60 mg/kg/day, resulted

### TABLE 3

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>1–10 Week</th>
<th>11–13 Week</th>
<th>6-Week Recovery</th>
<th>13- to 26-Week Recovery</th>
<th>AUC (ng•hr/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3, 10</td>
<td>No lenticular opacity</td>
<td>No lenticular opacity</td>
<td>Lenticular opacity with higher incidence and degree</td>
<td>Presence of “internalized” residual lesion without progression</td>
<td>10 mg/kg/day: 34,894&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30, 60</td>
<td>No lenticular opacity</td>
<td>First appearance of lenticular opacity</td>
<td></td>
<td></td>
<td>30 mg/kg/day: 110,523&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>AUC of day 87, 10 mg/kg/day from Study V-2.
<sup>b</sup>AUC of day 86, 30 mg/kg from Study V-2.
in rapid return of desmosterol and cholesterol levels to normal in the liver with no development of lenticular opacity (Figs. 5a and 5c, dotted lines). The length of period of elevated desmosterol was also considered to be important for cataractogenesis.

**E2012 Interferes With Cholesterol Synthesis in the Rat by DHCR24 Inhibition**

E2012 was shown to have concentration-dependent inhibitory effects on cholesterol biosynthesis in primary culture of rat hepatocytes (Fig. 6a) and HepG2 cells (Fig. 6b) with IC$_{50}$ values of 11.0, and 15.1 nmol/l, respectively. Cholesterol synthesis in the rat lens was also reduced by E2012 at 100 nmol/l and higher (Fig. 6c). The lenticular concentrations of E2012 after 13-week repeated dose in the rat reached approximately 2.3 µg/g (5.5 µmol/l) at 30 mg/kg/day; this was a concentration that would have inhibited lenticular cholesterol synthesis.

In an enzyme inhibition assay of DHCR24 using rat liver microsome suspension, cholesterol production from desmosterol was decreased (Fig. 7); E2012 was shown to inhibit DHCR24 with a Ki value of 53.0 nmol/l and IC$_{50}$ of 268 nmol/l.

**DISCUSSION**

E2012 is a gamma secretase modulator without affecting Notch processing aimed at Alzheimer’s disease by reduction of amyloid β-42. It induced cataract after repeated doses in the rat in a 13-week repeated dose toxicity study. We investigated the mode of ocular toxicity and discovered a potentially relevant, toxicity mechanism-based biomarker for the cataract. E2012 has been shown to induce cataract in the rat by inhibiting DHCR24, an enzyme involved in a final step of cholesterol biosynthesis where desmosterol is converted to cholesterol. Repeated doses of E2012 in the rat resulted in increased desmosterol and decreased cholesterol in the lens and led to development of cataract.

The lens consists of lenticular fibers and is equipped with several remarkable and unique characteristics to keep its transparency for a lifetime with little or no turnover of cells or proteins. These include the absence of fiber nuclei and organelles and highly ordered hexagonal arrangement of fiber cells in close apposition. A new lenticular fiber is provided from the anterior lenticular epithelial cell that starts to elongate when it reaches the equator toward both the anterior and posterior poles until the fiber ends meet those from the opposite side at the Y-suture and undergo membrane remodeling. It continues to grow throughout life by addition of new fibers on previous ones (Bassnett et al., 2011; Rao, 2008). E2012-induced lenticular lesions were characterized by posterior subcapsular opacity along the suture line that later coalesced to form a triangular or circular opacity and correlated histologically with lenticular fiber degeneration. The fact that the lesion is limited to the posterior subcapsular area and took approximately 11 weeks to develop is suggestive that the toxicity results from effects on new fiber elongation, differentiation, or reorganization rather than effects on pre-existing fibers.

**TABLE 4**

**Time-Course of Incidence for Lenticular Changes in SD Rats (Study No. V-6)**

<table>
<thead>
<tr>
<th>Study Week</th>
<th>2–10</th>
<th>12</th>
<th>13</th>
<th>19</th>
<th>27</th>
<th>39</th>
</tr>
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<tbody>
<tr>
<td>Recovery</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>Ophthalmology</td>
<td>No change</td>
<td>Initial lesion</td>
<td>End of treatment</td>
<td>Cataract</td>
<td>Cataract and new normal layer</td>
<td>Residual lesion</td>
</tr>
<tr>
<td>30 mg/kg/day</td>
<td>0/30</td>
<td>1/30</td>
<td>1/30</td>
<td>12/15</td>
<td>12/14</td>
<td>12/14</td>
</tr>
<tr>
<td>60 mg/kg/day</td>
<td>0/30</td>
<td>2/29</td>
<td>5/29</td>
<td>12/15</td>
<td>12/15</td>
<td>12/15</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Degeneration, lenticular fiber, subcapsular posterior</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mg/kg/day</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5/15</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1+</td>
<td>—</td>
<td>—</td>
<td>5/15</td>
<td>—</td>
<td>—</td>
<td>5/14</td>
</tr>
<tr>
<td>2+</td>
<td>—</td>
<td>—</td>
<td>5/15</td>
<td>—</td>
<td>—</td>
<td>6/14</td>
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<td>60 mg/kg/day</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>6/14</td>
<td>—</td>
<td>—</td>
<td>9/15</td>
</tr>
</tbody>
</table>

**FIG. 3.** E2012-induced cataract after recovery period. Arrow: “internalized” residual lesions without progression after 26 weeks of recovery. * New, normal lenticular fiber layer displacing the stable residual lesion deeper into the adjacent posterior cortex.
than direct cellular damage, in which case the lesion would appear earlier and also involve wider areas within the lens, as in the case of compounds that cause oxidative stress or inhibitors of lenticular ion channels and pumps (Jacob, 1999). Another compound with different chemical structure but with the same mode of action, a gamma secretase modulator without affecting Notch processing, did not affect cholesterol synthesis and did not induce cataract in the rat in 13-week toxicity studies (data not shown). Taken all these together, the primary mechanism of lenticular toxicity is considered not a class effect but the inhibition of cholesterol synthesis involving DHCR24, leading to impaired new lenticular fiber formation and development of cataract.

The incidence of lenticular lesions increased up to several weeks of recovery. This apparent “progression” of the lesion during recovery is not a true progression but is likely attributed to slow decreases in lenticular drug concentration after cessation of treatment and slow recovery in sterol levels. Indeed, there was clear recovery as indicated by the formation of new, normal lenticular fiber that “internalize” the stabilized, residual lesions formed in the past. This is explained by the nature of lenticular growth by addition on top of older layers and a lesion once formed remain because of no protein or cellular turnover.

E2012 was shown to inhibit cholesterol synthesis in the lens. After 13 weeks of treatment *in vivo*, lenticular E2012 concentration was well above the level that inhibited lenticular cholesterol biosynthesis *in vitro*, with desmosterol accumulation and development of cataracts. The lens is an avascular tissue in contact with the aqueous humor that is very low in protein or cholesterol content. The lens needs to obtain its cholesterol by *de novo* synthesis in the lenticular epithelial cells. The lens has the highest content of cholesterol within the body (Cenedella, 1996). The lenticular fiber cell membrane has special cholesterol domains that are essential for functions of membrane proteins that localize to these domains. Cholesterol domains are also required to prevent cataractogenic aggregation of soluble lens proteins (Mason et al., 2003). Lenticular plasma membrane is associated with proteins including crystallins that have chaperone-like activities to prevent aggregation of other proteins (Andley, 2009; Ifeanyi and Takemoto, 1990). Alterations

FIG. 4. Desmosterol and cholesterol levels in the plasma (Study V-5). Bars: standard deviation. Control 2 and 3 mg/kg/day dosed up to 4 weeks only.
in cell membranes and perturbations in cholesterol metabolism would interfere with these critical functions or structures of the lenticular fiber to keep its transparency and lead to aggregation of proteins and development of cataract.

Although cataracts are not seen in the few cases reported in desmosterolosis (Schaaf et al., 2011; Zolotushko et al., 2011), inborn perturbations in cholesterol synthesis are associated with inherited cataracts including in Conradi-Hünermann-Happle syndrome (CDPX2, with elevated 8(9)-cholesterol and 8-dehydrocholesterol; Cañueto et al., 2012) and Smith-Lemli-Opitz syndrome (SLOS, with elevated 7-dehydrocholesterol; DeBarber et al., 2011). Perturbations in cholesterol metabolism are also implicated in the pathogenesis of cataract by hypolipidemic drugs such as HMG-CoA reductase inhibitors, OSC inhibitors, and DHCR24 inhibitor triparanol, by affecting various lenticular functions associated with plasma membranes. Similarly, perturbations in cholesterol biosynthesis are strongly implicated in the pathogenesis of cataracts by E2012.
Of note, elevation of plasma desmosterol in the monkey was mild, lower than those in rats at 30 mg/kg/day that resulted in lenticular changes, and without decreases in plasma cholesterol levels; E2012 did not induce cataracts in the monkey (data not shown) up to the highest tolerated dose for 13 weeks.

The final step of cholesterol synthesis involving DHCR24 is common between the lens and the liver; the levels of desmosterol and cholesterol in the liver paralleled those in the plasma. Indeed, repeated doses of E2012 at ≥ 30 mg/kg/day led to accumulation of desmosterol and decreased cholesterol in the liver/plasma prior to the lens, as early as 24 hours for desmosterol. Accumulation of desmosterol in the lens was first detected at week 2. At doses where there was no cataract, plasma or liver desmosterol or cholesterol changes were transient without accumulation over time; there were no accumulations in the lens either. Cessation of treatment at 4 weeks instead of 13 weeks did not result in cataract. The accumulation of lenticular desmosterol and initiation of cataract formation were therefore preceded and could be speculated to some extent from the elevations in plasma desmosterol and prevented by cessation of treatment at an early time point where desmosterol accumulation was observed.

It is noteworthy that we managed to discover a toxicity mechanism-based biomarker for a sensory organ that rarely has a good biomarker. It is even more remarkable in that the changes in the biomarker occur prior to the toxicity change itself, unlike common markers that often are associated with the results of damage. Also of note is that the final step of cholesterol metabolism is common between species, as indicated by the case of triparanol where cataracts occurred in both animals and humans. Desmosterol is undetectable in the untreated plasma, and its elevation is easily and accurately detected and represents an earlier signal of the same process that would later follow within the lens. Thus, although more work needs to be done to verify this in other animal models and humans, because there is evidence that desmosterol increases as an early consequence of the likely causal mechanism (inhibition of the final step of cholesterol synthesis) and its elevation in plasma precedes the initiation of the lenticular changes, desmosterol would be a prodromal biomarker for E2012-induced cataract.

We have succeeded in demonstrating the mechanism of E2012-induced cataract. E2012 inhibits DHCR24 with resultant increases in desmosterol and decreases in cholesterol in the lens. Sustained and prolonged changes in these sterols in the lens have been shown as the prerequisite for cataractogenesis by E2012 in the rat, which can be monitored by the same preceding parameters in the plasma. Further investigation will allow us to better understand the precise pathways leading to cataractogenesis and, ultimately, physiology of the lens.

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