Screening for Ocular Phototoxicity

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Normally light transmission through the eye is benign and serves to direct vision and circadian rhythm. However, with very intense light exposure, or with ambient light exposure to the aged eye and/or young or adult eye in the presence of light-activated (photosensitizing) drugs or dietary supplements, cosmetics, or diagnostic dyes, light can be hazardous, leading to blinding disorders. Light damage to the human eye is avoided because the eye is protected by a very efficient antioxidant system and the chromophores present absorb light and dissipate its energy. After middle age, there is a decrease in the production of antioxidants and antioxidant enzymes and an accumulation of endogenous chromophores that are phototoxic. The extent to which a particular exogenous photosensitizing substance is capable of producing phototoxic side effects in the eye depends on several parameters, including (1) the chemical structure; (2) the absorption spectra of the drug; (3) binding of the drug to ocular tissue (lens proteins, melanin, DNA); and (4) the ability to cross blood-ocular barriers (amphiphilic or lipophilic). For instance, compounds that have either a tricyclic, heterocyclic, or porphyrin ring structure and are incorporated into ocular tissues are potentially phototoxic agents in the eye. The extent to which these substances might damage the eye (photoefficiency) can be predicted using in vitro and photophysical techniques. With simple, inexpensive testing, compounds can be screened for their potential ocular phototoxicity at the developmental stage. It may be that a portion of the molecule can be modified to reduce phototoxicity while leaving the primary drug effect intact. Preclinical safety testing may prevent ocular side effects that can range from mild, reversible blurred vision to permanent blindness.

Keywords Cataract, Keratitis, Macular Degeneration, Ocular Toxicology, Photosensitization, Phototoxic Drugs

The eye is the organ that is most vulnerable, after the skin, to light damage because it is constantly subjected to ambient radiation. This light serves to direct vision and circadian rhythm (Roberts 2000a) and, therefore, under normal circumstances must be benign. However, chronic exposure to intense light and/or age-related changes can lead to light-induced damage to the eye. Roberts 2001a; Zigman 1993; Dillon 1991; Andley 1987).

There are many drugs, dietary supplements, and diagnostic dyes, both topical and systemic, that absorb in the ultraviolet (UV) or visible range and can be excited by wavelengths of light transmitted to the lens and retina. This absorption can lead to dramatically enhanced ocular damage through the phototoxic side effects of those dyes and drugs (Fraunfelder 1982; Roberts 1996). The extent to which a particular photosensitizing drug will affect the human lens or retina in vivo depends upon (1) its residence time in the lens or retina, which is determined by its structure; (2) the photoefficiency of the particular sensitizer, due to its ability to absorb the appropriate wavelengths of light that have been transmitted to the lenticular or retinal substrates; (3) the mechanism by which it causes that damage, including possible binding of the dye to ocular constituents. Binding can alter the photochemical mechanism and also increase the retention time of the sensitizer in the eye); and (4) the presence of endogenous quenchers or free-radical scavengers that could stop or retard these photochemical reactions. All of these parameters play a role in the photosensitized oxidation of human ocular tissue and its importance as an underlying mechanism in cataractogenesis and retinal damage.

Presented here are several screens that can be used to determine the potential of drugs to cause light damage to the eye. These include a simple screen that takes into account the optical properties of the eye and the structure and absorption spectra of the various drugs that can be used to eliminate various drugs as potential photo-oxidants in the eye. In addition, a second, more detailed screen is presented that can be used to determine (a) the quantitative potential for a drug to cause photo-oxidative damage in the eye, (b) the mechanism by which it occurs, and (c) the in vivo verification of phototoxicity.

Structure of the Eye

The human eye is composed of several layers (Figure 1). The outermost layer contains the sclera, whose function is to

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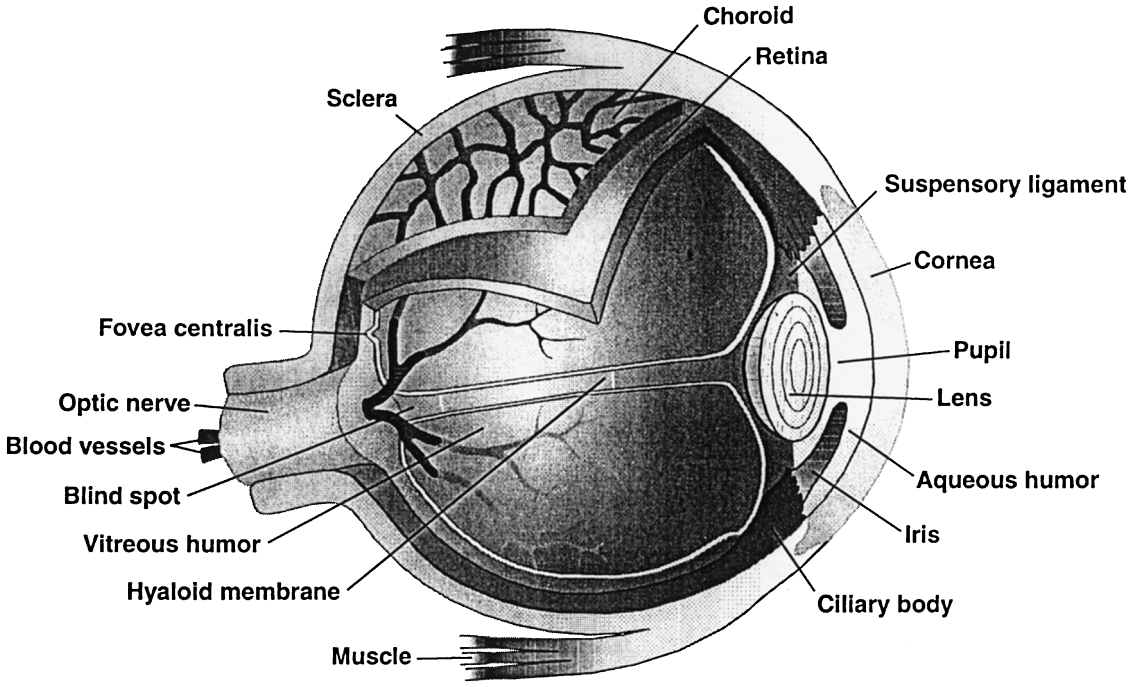


FIGURE 1
The structure of the human eye.

protect the eyeball, and the cornea, which focuses incoming light onto the lens. Beneath this layer is the choroid containing the iris, which is known as the uvea. This region contains melanocytes containing the pigment melanin, whose function is to prevent light scattering. The opening in the iris, the pupil, expands and contracts to control the amount of incoming light. The iris and the lens are bathed in the aqueous humor, a fluid that maintains intraocular pressure; this fluid also contains various antioxidants and supports transport to the lens. The lens is positioned behind the iris; its function is to focus light onto the retina.

Behind the lens is the vitreous humor, a fluid that supports the lens and retina and that also contains antioxidants. The retina is composed of the photoreceptor cells (rods and cones) that receive light and the neural portion (ganglion, amacrine, horizontal and bipolar cells) that transduces light signals through the retina to the optic nerve. Behind the photoreceptor cells are the retinal pigment epithelial cells, Bruch's membrane, and the posterior choroid. The photoreceptor cells are avascular and their nutrient support (ions, fluid, and metabolites) is provided by the retinal pigment epithelial cells. There is transport to the retinal pigment epithelial cells across the Bruch's membrane by the choriocapillaris.

Light Absorption by the Human Eye

For light to damage the eye, it must be absorbed. Each wavelength of light will affect different areas of the eye. Ambient radiation from the sun can contain varying amounts of UV-C (100 to 280 nm), UV-B (280 to 320 nm), UV-A (320 to 400 nm), and visible light (400 to 760 nm). Most UV-C and some UV-B are filtered by the ozone layer. UV light contains shorter wavelengths of light than visible; the shorter the wavelength, the greater the energy and the greater the potential for biological damage.

The primate/human eye has unique filtering characteristics (Bachem 1956). The human cornea cuts off all light below 295 nm, so that all UV-C and some UV-B are filtered from reaching the lens. The adult human lens absorbs the remaining UV-B and all UV-A (295 to 400 nm). Therefore, only visible light reaches the adult human retina. However, the young human lens transmits a small window of UV-B light (320 nm) to the retina and the elderly lens filters out much of the short blue visible light (Barker, Brainard, and Dayhaw-Barker 1991). Aphakia (removal of the lens) and certain forms of blindness may also change the wavelength characteristics of light reaching the retina.

Direct Light Damage in the Eye

Short UV light exposure to the cornea leads to an inflammation reaction (Offord et al. 1999; Pitts, Cullen, and Parr 1976). This is very painful and similar to a sunburn and is known as keratitis. However, these corneal wounds usually heal and do not cause permanent damage. On the other hand, UV damage to the lens and visible light damage to the retina are painless, accumulative, and permanent. It can lead to the formation of

cataracts (clouding of the lens) (Dillon 1991; Balasubramanian 2000), which can only be corrected by surgery, and to macular and retinal degeneration (Ham et al. 1982), leading to permanent blindness for which there is no treatment.

A number of factors suppress or enhance light damage to the eye, including oxygen, antioxidants, repair mechanisms, and biophysical mechanisms.

Oxygen Tension in the Eye

The cornea is highly oxygenated. The retina is supplied by the blood so it has varying but high oxygen content in different portions of the retinal tissues. The aqueous humor and the lens have low oxygen content but it is sufficient for photo-oxidation to occur. (McLaren et al. 1998; Roberts et al. 1992a; Kwan, Niinikoske, and Hunt 1971). The higher the oxygen content of the tissue, the easier it is for light damage to occur.

Defense Systems

Because the eye is constantly subjected to ambient radiation, each portion of the eye contains very efficient defense systems. There are antioxidant enzymes (superoxide dismutase [SOD] and catalase) and antioxidants (e.g., vitamins E and C, lutein, zeaxanthin, lycopene, glutathione, and melanin) that serve to protect against oxidative and photo-induced damage (Handelman and Dratz 1986; Giblin 2000; Seth and Kharb 1999; Edge et al. 1998; Khachik, Bernstein, and Garland 1997). Unfortunately, most of these antioxidants and protective enzymes decrease beginning at 40 years of age (Sarna 1992; Khachik, Bernstein, and Garland 1997; Samiec et al. 1998; Sethna, Holleschau, and Rathbun 1983). With the protective systems diminish with age, there is loss of protection against all light-induced damage to the eye and the induction of age related blinding disorders.

Repair

Even if the eye is damaged, the damage does not have to be permanent. The cornea and retina have very efficient repair systems. However, damage to the lens is cumulative and not repairable (Andley 1987).

Biophysical Mechanisms

Ocular damage from light can occur through either an inflammatory response or a photo-oxidation reaction. In an inflammatory response, an initial insult to the tissue provokes a cascade of events that eventually results in wider damage to the tissue (Busch et al. 1999; Wang et al. 1999). In photo-oxidation reactions, a sensitizing compound in the eye absorbs light, is excited to a singlet, then a triplet, state, and from the triplet produces free radicals and reactive oxygen species that in turn damage the ocular tissues (Straight and Spikes 1985).

The photo-oxidation reaction is outlined below:

$$\text{Drug} + \text{light} \rightarrow \text{singlet} \rightarrow \text{triplet} \rightarrow \text{free radicals/}$$
$$\text{reactive oxygen} \rightarrow \text{ocular damage}$$

Drug-Induced Ocular Phototoxicity

Most of the damage to the eye caused by direct irradiation from the sun or artificial sources is from UV radiation. However, in the presence of a light-activated (photosensitized) diagnostic dye or drug, patients are in danger of enhanced ocular injury from both UV and visible light. The extent to which a particular photosensitizer will affect the human cornea, lens, and/or retina in vivo depends upon the following factors: (1) Its residence time in the eye, which is determined by its structure and binding of the dye to ocular constituents. Binding can not only alter the photochemical mechanism but also can increase the retention time of the sensitizer in the eye. (2) The photoefficiency of the particular sensitizer (i.e., its ability to absorb the appropriate wavelengths of light that have been transmitted to the lenticular or retinal substrates). These transmission characteristics change with age. (3) The oxygen content of the ocular tissue. (4) The presence of endogenous quenchers or free-radical scavengers and enzyme detoxification systems that could stop or retard these photochemical reactions.

PREDICTING OCULAR PHOTOTOXICITY

Based on the theoretical considerations stated above, it is relatively easy to predict that a drug cannot cause ocular damage through a photo-induced event. The short screen given in Table 1 will dramatically reduce the number of potential substances needed to be considered for ocular phototoxicity.

Short Screen for Potential Ocular Phototoxicity

Measure the Absorbance Spectrum

In order for a chemical compound (diagnostic dye, drug, endogenous sensitizer) to induce a phototoxic response in any biological tissue, it must first absorb light. This absorption is limited by the filtering characteristics of the biological tissues involved. A comparison of the transmission characteristics of the eye with the absorbance spectrum of the drug may be used as a quick screen for phototoxicity. To have the potential to damage the aqueous or the lens, a drug needs a UV spectrum that consists of absorption wavelengths longer than 295 nm (Bachem 1956). This includes drugs such as chlorpromazine (maximum: 310 nm), tetracycline (maximum: 365 nm), and the porphyrins (maxima: 392 nm, 500–650 nm) (Roberts 1984).

TABLE 1

Short screen for potential phototoxicity in the lens/retina

1. Chemical structure
   a. Heterocyclic, tricyclic, porphyrin
   b. Amphiphilic/lipophilic
2. Absorbance spectra
   a. Longer than 295 nm (lens); 400 nm (retina)
   b. Binds to DNA, lens protein, melanin
3. Skin phototoxicity

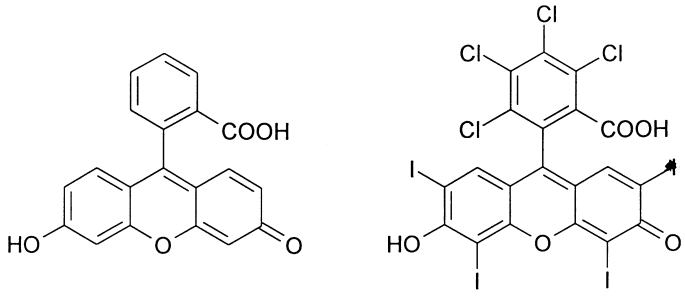
In the older human, drugs/dietary supplements, dyes (i.e., hypericin), and diagnostic dyes (photodynamic therapy porphyrins) that absorb in the 400 to 600 nm region could produce phototoxic damage to both the lens (Schey et al. 2000) and the retina. In very young humans all drugs/dyes absorbing at wavelengths longer than 295 nm are potential photosensitizers in both the retina as well as the lens. Drugs that do not absorb in these regions cannot cause photodamage.

Examine the Chemical Structure

Most drugs must have a tricyclic, heterocyclic, or porphyrin ring to fulfill the energy requirements to produce a stable, long-lived (triplet) reactive molecule that will go on to produce free radicals and reactive oxygen species. The addition of a halide group can enhance the amount of triplet produced (Turro 1978). As seen in Figure 2, fluorescein and rose bengal have similar structures except for the attached iodine groups. Fluorescein is highly fluorescent (singlet state) but is not very efficient at reaching the triplet state (poor quantum yield, 0.03, for the triplet). The singlet state of rose bengal would easily go through intersystem crossing to the triplet (good quantum yield for the triplet (0.60). By simple inspection of the structures of these two diagnostic dyes, we would conclude that rose bengal has a much greater potential to produce phototoxic damage to the eye than fluorescein.

Test the Solubility Properties

Dyes or drugs to be examined should be tested for their partitioning in protic and aprotic solvents. Their hydrophobicity will indicate potential for crossing blood-ocular barriers. More hydrophilic substances are less likely to cross blood-ocular barriers. Compounds that are amphiphilic or lipophilic cross all blood-retinal and/or blood-lenticular barriers. The probable site of damage may also be determined by the hydrophobicity (membranes) or hydrophilicity (cytosol) of the photosensitizing dye or drug.



Fluorescein Rose Bengal

FIGURE 2
The structures of fluorescein and rose bengal. They have heterocyclic ring systems.

Measure Binding of the Drug to Ocular Tissues

Binding of a drug to ocular tissues (DNA—cornea, lens; proteins—lens; melanin—retina) (Roberts and Dillon 1987; Dayhaw-Baker and Barker 1986; Steiner and Buhring 1990; Sarna 1992) would increase its retention time in the eye. Furthermore, binding of a photosensitizing substance to macromolecules increases the lifetime of its triplet state. It is the triplet state of the dye or drug that leads to further oxidative and free-radical reactions. Therefore, drugs/dyes that bind to ocular tissues are very likely to induce phototoxic damage (Roberts et al. 1991a) in that tissue. Binding is determined by measuring the absorption spectrum of the drug in the presence and absence of lens proteins, DNA, and/or melanin (Roberts and Dillon 1987; Roberts, Atherton, and Dillon 1990; Steiner and Buhring 1990). A red shift in the absorbance spectrum of the drug in the presence of any of these biomolecules is an indication of binding of the sensitizer to the biomolecule.

Note Reports of Skin Phototoxicity

Finally, any reports of skin phototoxicity for a particular drug should provide a clear warning of potential ocular phototoxicity. Skin phototoxicity is more readily apparent than ocular phototoxicity, although it is induced by compounds with similar chemical features (Oppenlander 1988).

Detailed Screen for Predicting Ocular Phototoxicity

The short screen can determine a drug's potential for phototoxicity. Once it has been determined that a drug/dye is a possible photosensitizing agent, the more detailed screen given below can determine the potential site of damage (in situ fluorescence techniques), predict the type and efficiency of damage (in vitro assays), and determine the mechanism of damage (photophysical studies of the short lived excited state intermediates). Once the excited state intermediates produced by a particular photosensitizing agent have been determined, the introduction of specific quenching agents may stop the unwanted reactions. (Roberts et al. 1991a).

Determine the Potential Sites for Ocular Damage

The site of damage is determined by the penetration of the drug and the transmission of the appropriate wavelengths of light to that site. At these sites there are numerous substrates for phototoxic damage in the eye.

Cornea. Corneal epithelial and endothelial cells may be easily damaged leading to keratitis (Pitts, Cullen, and Parr 1976; Hull, Csukas, and Green 1983). However, these cells have a very efficient repair mechanism and the damage is rarely permanent.

Uvea. Uveal cells are highly melanized and are ordinarily protected against light induced damage. However, melanogenesis may be modified with phototoxic reactions leading to a greater risk from UV radiation (Hu et al. 2000).

Lens. Epithelial cells of the lens have direct contact with the aqueous humor. Their function is to control transport to the lens. They are most vulnerable to phototoxic damage.

Damage to these cells would readily compromise the viability of the lens (Roberts et al. 1994). The lens fiber membrane can be photochemically damaged through damage to the lipids and/or the main intrinsic membrane protein (Roberts, Roy, and Dillon 1985). This will result in a change in the refractive index, causing an opacification. Phototoxic reactions can lead to a modification of certain amino acids (histadine, tryptophan, cysteine) (Roberts 1984; McDermott et al. 1991) and/or a covalent attachment of sensitizer to cytosol lens proteins. In either case, this changes the physical properties of the protein, leading to aggregation and finally opacification (cataractogenesis). The covalently bound chromophore may now act as an endogenous sensitizer, producing prolonged sensitivity to light. Because there is little turnover of lens proteins, this damage is cumulative.

Retina. Phototoxic damage can occur in retinal pigment epithelial tissues, the choroid, and the rod outer segments, which contain the photoreceptors. If the damage is not extensive, there are repair mechanisms to allow for recovery of retinal tissues. However, extensive phototoxic damage to the retina can lead to permanent blindness (Dayhaw-Barker and Barker 1986; Ham et al. 1982).

Determine the Location/Uptake of the Dye/Drug

Radiolabeling. The traditional method of determining uptake into ocular tissues is in vivo radiolabeling. This method is time consuming and expensive, although it is effective in determining which ocular tissues have accumulated the drug in question.

Fluorescence Spectroscopy. An alternative method to determine uptake of a drug into ocular tissues is ocular fluorometry. After a dye or drug has absorbed light and is excited to the singlet state, it can decay to the ground state and is accompanied by the emission of light. The is known as fluorescence. Because most photosensitizers are fluorescent, transmitted or reflective fluorescence provides an accurate means of measuring uptake of a sensitizer into ocular tissue that is simpler, less expensive, and less arduous than using radiolabeled materials. This technique may also be used noninvasively, in vivo, for instance using a slit lamp, to detect uptake of sensitizers into the human, or scanning or reflective fluorometry to determine the presence of endogenous and exogenous fluorescent materials in the retina (Docchio 1989; Docchio et al. 1991; Cubeddu et al. 1999; Sgarbossa et al. 2001).

Determine the Phototoxic Efficiency

The targets of photo-oxidative reactions may be proteins, lipids, DNA, RNA, and/or cell membranes (Straight and Spikes l985). In vitro tests can be designed to determine the specific site(s) of damage to the various ocular compartments (i.e., lens and retinal epithelial cells and photoreceptor cells) and the products of those reactions.

Table 2 presents a summary of additional biochemical and photophysical techniques that can be performed to more accurately predict the potential for and extent of in vivo phototoxicity.

TABLE 2
Detailed screen for ocular technique

1. In vitro studies	
a. Cell culture	DNA, RNA, protein
Enzyme assays	Antioxidant enzymes
Histology	Endothelial, epithelial, photoreceptor cell damage
b. Gel electrophoresis	Protein changes
Amino acid analysis	Protein changes
c. Mass spectrometry	Lipid changes
	Peptide maps
d. TLC	Lipid oxidation
e. HPLC	Lipid peroxides
	DNA adducts
	Protein modification
f. Normalization for photons absorbed	
2. Biophysical techniques	
a. Fluorescence	Uptake
	Binding
	Quantum yields
b. Laser flash photolysis	Triplet detection
	Binding/lifetime
	Quantum yields
c. Luminescence	Singlet oxygen
	Oxygen tension
d. Pulse radiolysis	Radical and oxyradical intermediates
e. ESR	Radical and oxyradical intermediates

The in vitro assays used in determining the phototoxic efficiency include the following:

1. **Cell Culture/Whole Tissues.** The first reported assay for phototoxicity in human ocular cells (Roberts l981) measured changes in macromolecular synthesis in the presence and absence of a light-activated drug. Other studies have assessed damage to corneal, lenticular, and retinal cells by measuring pump function and enzyme activities both in vitro and in situ (Andley et al. 1994; Roberts et al. l994; Organisiak and Winkler 1994; Rao and Zigler 1992; Dayhaw-Barker 1987).
2. **Gel Electrophoresis, Amino Acid Analysis.** Gel electrophoresis has been used to monitor polymerization of ocular proteins (Kristensen et al. 1995; Roberts et al. 1992a; Roberts l984; Zigler et al. 1982). Photopolymerization is one of the most apparent changes in ocular protein induced by photosensitizing dyes and drugs. Quantitative changes can be measured by scanning the gel and determining relative reaction rates. Specific amino acid modifications can be determined using amino acid analysis (Roberts 1984, 1996). Zhu and Crouch (1992) have illustrated the wide variety of classical protein analysis techniques (gel electrophoresis, amino

acid analysis, sequencing, isoelectric point determination, Western blot, enzyme-linked immunosorbent assay [ELISA]) that can be used to investigate phototoxic damage induced by dyes and drugs.

3. **Mass Spectrometry.** Recent innovations in the field of mass spectrometry (liquid secondary ion mass spectrometry [LSIMS] and electrospray ionization [ESI]) have allowed for the identification of specific amino acid modifications within large proteins through molecular weight mapping. These techniques have been applied to determine the specific sites of photooxidative damage in corneal and lenticular proteins (Schey et al. 2000; Roberts et al. 2001). These studies can serve as a model for defining damage from any potential phototoxic agent in the eye.
4. **Thin-Layer Chromatography.** This technique is particularly effective at separating triacyclycerol, free fatty acid, and phospholipids from lens (Fleschner l995) and retinal (Organisiak et al. 1992) membranes. Thin-layer chromotography/gas chromotography/mass spectrometry (TLC/GC/MS) may be used to measure lenticular or retinal lipid modifications (Handelman and Dratz l986). Specific lipids may be modified in the presence of photosensitizing agents and separated on TLC plates. The plates can then be scanned for quantitative analysis of these specific changes.
5. **High-Performance Liquid Chromatography (HPLC).** HPLC is particularly effective at separating and identifying lipid peroxides from the retina (Akasaka, Ohrui, and Meguro 1993). It has also been used to identify adducts formed between DNA nucleotides and phototoxic agents (Oroskar et al. 1994). HPLC has also been used to assess the rates of photo-oxidation of lens proteins in the presence of a sensitizer. Using this technique, it is possible to determine the induced amino acid modification within the protein and their location and to detect possible binding of sensitizing drugs to specific lens crystallins (McDermott et al. 1991).
6. **Normalization for Photons Absorbed.** Whatever the target tissue or extent of damage, the toxic effects of these dyes and drugs are the result of photochemical reactions. As such, their rate of efficiency is dependant on the number of photons absorbed by the sensitizer in the biological tissue. Therefore, in order to get an accurate comparison of the photosensitizing potency of various dyes and drugs with different structures and absorptive characteristics, it is essential to normalize for the number of photons absorbed by each drug in a particular system.

This can be done with a simple computer-generated mathematical formula (Roberts 1996; Kristensen et al. 1995), which takes into account the absorption spectrum of the drug, the output of the lamp source used in the experiments, and the optical properties of the eye. The total relative number of photons absorbed by a drug under particular experimental conditions is the area under the product curve:

$$\text{Photons absorbed} = I \times \text{AB} \times \lambda$$

where I = the intensity of the lamp at various wavelengths adjusted for the transmission characteristics of the cornea or lens, AB is the absorbance of the dye/drug, and λ is the number of photons at those wavelengths. The rates of each photo-oxidative event are then adjusted accordingly for each sensitizer. This in turn can be corrected for the actual transmission characteristics of the cornea and/or lens and the output of the sun to predict in vivo effects.

In summary, in vitro techniques determine the potential damage done to an ocular substrate, which gives information about the photoefficiency of a drug should it be taken up into the various compartments of the eye. Additional information about the site of potential damage can be predicted based on which ocular substrate (DNA, RNA, protein, lipid) is affected.

Determine the Excited State Intermediates That Cause the Phototoxic Damage

Some photochemical reactions in tissues are summarized below:

EXCITED STATES → INTERMEDIATES

singlet	singlet oxygen
triplet	superoxide
	OH·, ROO·
→ TARGET and DAMAGE	
	proteins = polymers
	lipid = peroxides
	DNA, RNA = cross-links

The biophysical techniques used in determining the phototoxic efficiency include the following:

1. **Quantum Yields.** In predicting the phototoxicity of a dye or drug, it is important to determine what proportion of photons lead to a benign (fluorescence, singlet state) event and what proportion lead to a potentially destructive event (phosphorescence, triplet state). The efficiency of a photoinduced process may be expressed as its quantum yield (Q).

$$Q = \frac{\text{the number of photons used to produce an event}}{\text{the number of photons originally absorbed}}$$

It gives a measure of how likely a photochemical event is to occur. The quantum yield is often expressed as a percentage. For instance, the quantum yield for fluorescence of fluorescein is 0.92 and that of rose bengal is 0.08 (Figure 2). This means that most of the light absorbed by fluorescein is given off in the form of fluorescence energy, making it a relative safe diagnostic dye for the eye. On the other hand, the triplet quantum yield for fluorescein is 0.03 and for rose bengal it is 0.60. This indicates that very little of the singlet fluorescein energy is transformed into a triplet, whereas most of the energy of rose bengal will be available for intersystem crossing and will reach the triplet state from whence it can

produce ocular damage. Therefore, flourescein is appropriate but rose bengal would be an inappropriate diagnostic dye for the eye.

2. **Laser Flash Photolysis.** This method uses a pulse of monochromatic light to promote a specific dye or drug to an excited state (Rodgers 1985).

   i. **Triplet Detection.** Time resolved techniques (either absorption spectroscopy or diffuse reflectance) allow for the detection of the triplet state of the excited chromophore even in intact tissues. This technique has been used to determine the absence (Dillon and Atherton l990) of triplet formation by the endogenous 3-hydroxykynurenine, as well as the presence of triplets from sensitizing drugs in intact lenses (Roberts, Atherton, and Dillon l991b).

   ii. **Lifetime/Binding.** All ocular damage from photooxidation reactions occurs through the triplet state of the drug. The longer the lifetime, the greater the potential for damage. The lifetimes of the triplets of sensitizing drugs were found to be greater when bound to macromolecules or in an intact organ than when free in solution (Roberts, Atherton, and Dillion 1991b). The presence of a triplet and an increase in lifetime when bound to intact ocular tissue is predictive of a drug's causing photo-oxidative damage to the eye in vivo (Roberts et al. 1991a).

3. **Luminescence—Singlet Oxygen.** The presence and lifetime of singlet oxygen can be determined using time resolved infrared luminescence measurements at 1270 nm (Rodgers and Snowden l982). Using this technique, it can be determined whether and how efficiently a dye or drug can produce singlet oxygen (Roberts et al. 2000; Motten et al. 1999). Because singlet oxygen is the most powerful oxidant in a photooxidation reaction, a dye or drug that is an efficient producer of singlet oxygen could be predicted to induce phototoxic damage if present in the eye.

4. **Pulse Radiolysis—Hydroxyl, Peroxy Radicals and Superoxide.** Pulse radiolysis consists of the delivery of a very short intense pulse of ionizing radiation to a sample, the resultant changes in light absorption of the sample being followed by a very fast spectrophotometer (Land 1985). The technique may be used to detect the formation of short-lived radical species of a dye or drug (Roberts, Hu, and Wishart 1998; Roberts et al. 2000). In addition, the interaction of a dye or drug with excited oxygen intermediates (hydroxyl radical, superoxide, peroxy radicals) (Land et al. 1983), which are clearly generated in this system, allows for an understanding of a possible mechanism of in vivo photo-oxidative ocular damage.

5. **Electron Spin Resonance—Hydroxyl, Peroxy, and Carbon-Centered Radicals and Superoxide.** Electron spin resonance (ESR or EPR) spectroscopy detects and characterizes species containing an odd number of electrons, namely free radicals and paramagnetic metal ions. The photooxidation reactions responsible for the phototoxic responses in the eye involve free radicals that are formed via electron transfer (electron exchange) between the sensitizing drug in an excited state and a substrate from the ocular tissues. Although these radicals are very short lived, they can be observed with ESR in situ during their photogeneration. For instance, illumination of rose bengal (RB), an ophthalmic diagnostic dye, in the presence of an electron donor such as NADH, affords a radical anion of the dye $RB^{\cdot -}$ to be directly measured using ESR (Sarna et al. 1991).

   Radicals that are too reactive to accumulate in detectable quantities can frequently be detected by ESR using spin trapping techniques. In this approach, an agent called a spin trap reacts with a short-lived radical R· to give a spin adduct R-T·, which has a much longer lifetime. The original radical R· is identified by the characteristic ESR spectrum of the R-T·. Carbon-, nitrogen-, and sulfur-centered radicals, as well as all of the important oxygen-centered radicals, (hydroxyl, superoxide, alkoxyl and peroxyl) can be identified using ESR either directly or in combination with the spin trapping technique.

   Using these techniques, the photosensitized generation of superoxide in protic (Reszka, Lown, and Chignell l992) and aprotic (Reszka et al. 1993) media can be monitored. These are model systems for the hydrophilic (aqueous) and hydrophobic (rod outer segment membrane) portions of the eye. ESR has recently been used to define the photochemical mechanisms involved in the light activation of endogenous pigments in the lens (Reszka et al. 1996) and the retina (Reszka et al. 1995). With these systems defined, ESR can be used to predict potential phototoxic events induced by exogenous photosensitizing dyes and drugs and natural pigments (Roberts et al. 2000; Motten et al. 1999).

In summary, the molecular mechanism involved in the phototoxic damage induced in the eye is photosensitized oxidation reactions. This mechanism begins with the absorption of light by the sensitizing compound (endogenous pigment, dye, or drug), which promotes the compound to an excited singlet state (short lived) and then, through intersystem crossing, goes to the triplet state (long lived). The excited triplet state of the drug/dye then proceeds either via a type I (free radical) or type II (singlet oxygen) mechanism, causing the eventual biological damage (Straight and Spikes 1985). Therefore, information about the efficiency and excited state intermediates for a phototoxic reaction in the eye obtained by using photophysical techniques (fluorescence, flash photolysis, pulse radiolysis, esr) can be predictive of phototoxicity in vivo. We have confirmed that photophysical studies collate well with in vivo data (Roberts et al. 1991a). For instance, tetrasulphonatophenylporphyrin (TPPS), which binds to lens proteins, shows a long-lived triplet in the intact calf and human lens, and produces singlet oxygen efficiently, causes photo-oxidative damage in vivo in pigmented mouse eyes, whereas uroporphyrin (URO), which produces an efficient triplet but does not bind to ocular tissues, does not cause photooxidative damage in vivo.

In Vivo Testing

The use of either the short or more detailed (Tables 1 and 2) screens for ocular phototoxicity will not totally eliminate the need for accurate in vivo experiments. The function of these studies is to limit the need for in vivo testing for ocular phototoxicity of large numbers of drugs. Those drugs found in screening to be highly likely to produce phototoxic side effects in the eye should be tested further with animal studies to determine the exact site and extent of damage to be expected in humans (Roberts et al. 1991a).

Prolonged use of a phototoxic drug is most probably of greater long-term risk to the eye than short-term dosage because of cumulative damage. Because there is an active repair system in the cornea, there should be little or no long-term side effects of phototoxicity there. However, because there is no turnover in the lens constituents, any modification in that tissue will tend to stay and accumulate with age. Thus cataractogenesis may not develop until much later than the initial insult. In addition, phototoxic damage to the lens may not only cause direct damage to cell viability but may undermine its defense system, so that gross morphological effects may appear much later than the original insult.

The susceptibility of both the lens and retina to light-induced damage increases with the age-related changes in chromophores (Roberts et al. 2000, 2001, 2002), with concurrent decrease in antioxidant status (Giblin 2000; Samiec et al. 1998; Sarna 1992). Therefore the elderly may be particularly sensitive to drugs and other agents that induce phototoxic side effects. Sight may be regained after cataract surgery; however, damage to the retina that is not repaired leads to permanent blindness. The environmental lighting, particularly the constant presence of intense ambient light, must always be taken into account when assessing the potential in vivo ocular toxicity of a drug.

Protection

Even if a drug has the potential to produce phototoxic side effects in the eye, no damage will be done if the specific wavelengths of optical radiation absorbed by the drug are blocked from transmittance to the eye. This can be easily done with wrap-around eyeglasses (Gallas and Eisner 2001; Sliney 1999; Merriam 1996) that incorporate specific filters. Furthermore, nontoxic quenchers and scavengers could be given in conjunction with the phototoxic drug to negate its ocular side effects while allowing for the primary effect of the drug (Roberts 1981; Roberts et al. 1991a; Roberts and Mathews-Roth 1993).

CONCLUSION

With simple, inexpensive in vitro testing, compounds can be monitored at their developmental stage for potential ocular phototoxicity. It may be that a portion of the molecule can be modified to reduce phototoxicity while leaving the primary drug effect intact. This may reduce the necessity of later, more costly, drug recalls. In the future, the more effective use of ocular fluorometry will allow for a more accurate assessment of the uptake and location of exogenous photosensitizing dyes, drugs, and herbal supplements in the eye, which may potentially harm the eye. Also, for those drugs that must be continued, in spite of their phototoxicity (i.e., antimalarial drugs; Motten et al. 1999), appropriate protection against light (sunglasses) and specific supplementary antioxidants may be prescribed to retard or eliminate the most severe blinding side effects (Roberts and Mathews-Roth 1993).

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