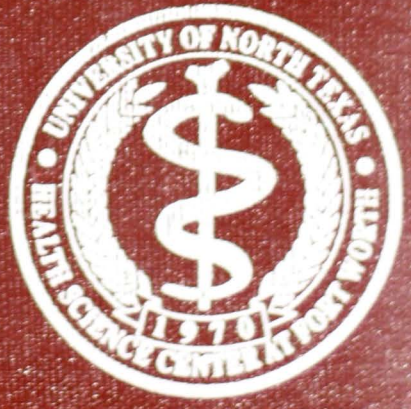
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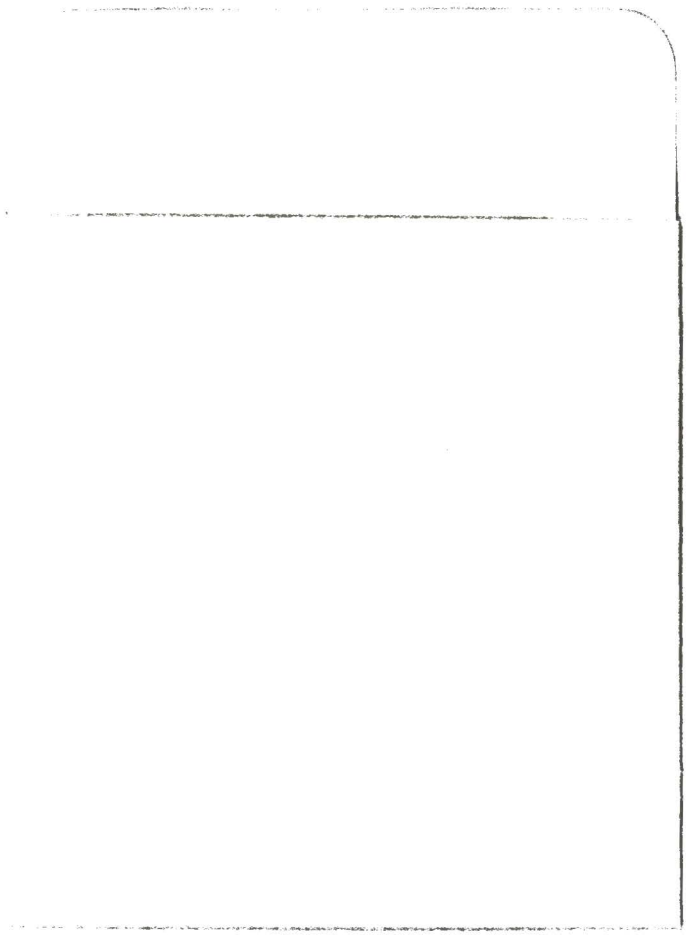

Xu, Guo-Tong, Rat Naphthalene Cataract Studies: Mechanism and Prevention. Doctor of Philosophy (Biomedical Sciences/Pharmacology), June, 1994, 134 pp., 16 tables, 34 figures, references, 153 titles.

The mechanism of naphthalene-induced cataract in rats and the preventive action of AL01576 (an aldose reductase inhibitor, ARI) were studied in both *in vivo* and *in vitro* systems. In the *in vivo* studies, cataracts were induced in five strains of rats (2 pigmented, 3 albino) by naphthalene feeding (1 g/kg/day). The cataractous changes occured in 1 week as watercleft and spoke-like opacities which merged to form a shell-like opacity in the deep cortex by 3 weeks. Semi-quantitation of the opacities with an arbitary six-score grading system showed little difference in the cataract development between the pigmented and albino strains. Major biochemical changes observed were a decrease of 20%-30% in GSH by one week of feeding, the appearance of disulfide cross-linking of lens proteins by 3 weeks, and a more than ten fold increase in the content of protein-GSH mixed disulfide. Neither damage to lens membrane functions as measured by ^{3}H-choline or ^{86}Rb uptake or loss of Na^{+}/K^{+}-ATPase activity was detected. AL01576 (10 mg/kg/day) completely prevented the naphthalene-induced lens changes in both pigmented and albino rats. These results indicate that pigmentation is not required for induction of naphthalene cataract in rats and suggest that tyrosinase action on naphthalene metabolites (such as 1- or 2-naphthol) is not involved in this cataract formation. The *in vitro* "naphthalene cataract" was established by exposing rat lens to each of 5 potential naphthalene metabolites in organ culture system (in modified TC-199 medium) for 48 hrs. When naphthalene dihydrodiol was used, both the morphological and biochemical changes in the lens were very similar to those observed in lenses of naphthalene-fed rats, and AL01576 completely blocked these *in vitro* changes as it did *in vivo*. Other naphthalene metabolites (1,2-dihydroxynaphthalene, 1-naphthol, 2-naphthol and

1,2-naphthoquinone) caused changes which were different from those induced by naphthalene *in vivo* and none of them was prevented by AL01576. Therefore, naphthalene dihydrodiol is the key naphthalene metabolite which reaches the lens via blood and aqueous humor and causes cataract when it is metabolized to 1,2-naphthoquinone. This mechanism is further supported by the detection of naphthalene dihydrodiol in the lens and aqueous humor of naphthalene-fed rats.

Examples of various classes of ARI (AL01576, AL04114, Sorbinil and Tolrestat) were compared for their effects on the formation of naphthalene cataract and a dual cataract induced with simultaneous feeding of galactose and naph-thalene. Both AL01576 and AL04114 (spirohydantoin derivatives) completely prevented the changes in the lenses of naphthalene-fed rats. However, Sorbinil (another spirohydantoin ARI) demonstrated a much weaker efficacy in this model and the carboxylic acid ARI, Tolrestat, showed no efficacy at all. In the dual cataract, Tolrestat prevented galactose cataract formation and reduced the lens dulcitol accumulation, but showed no protection against the shell-like opacity caused by naphthalene. On the other hand, AL01576 protected the lens from the cataractogenic action of both compounds. These results rule out the involvement of aldose reductase in naphthalene cataract formation. Furthermore, AL04114 (not a cytochrome P- 450 inhibitor) showed a similar efficacy as AL01576 (a inhibitor of cytochrome) in naphthalene cataract prevention. Therefore, the inhibition of cytochrome P-450 may not be involved in the prevention of this cataract.

Based on these findings and the fact that AL01576 prevents the changes induced by naphthalene dihydrodiol (ND) but not 1,2-naphthoquinone (NQ), a new mechanism for rat naphthalene cataract formation is proposed: naphthalene is converted by cytochrome P-450 to ND, which reaches the eye via the blood and penetrates into the lens. By the action of dihydrodiol dehydrogenase (DDD), it is further metabolized to 1,2-didydroxynaphthalene which autoxidizes to form NQ and H_2O_2, and thus causes cataract. AL01576 and AL04114 inhibit DDD activity, block NQ and H_2O_2 production and thus prevent the cataract formation.

RAT NAPTHALENE CATARACT STUDIES:

MECHANISM AND PREVENTION

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RAT NAPHTHALENE CATARACT STUDIES: MECHANISM AND PREVENTION

DISSERTATION

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For the Degree of

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LIST OF ABBREVIATIONS

AR	Aldose Reductase
ARI	Aldose Reductase Inhibitor
ARVO	The Association for Research in Vision & Ophthalmology
BND	Bendalina
CSO_3H	Cysteic Acid
D.C.	Deep Cortical Opacity
DDD	Dihydrodiol Dehydrogenase
DN	Dihyddroxynaphthalene
DTNB	Dithio-bis (nitrobenzoic acid)
EC	Enzyme Committee
ECCE	Extracapsular Cataract Extraction
EDTA	Ethylenediaminetetraacetic Acid
Flavo-O	Oxidized Flavoprotein
Flavo-R	Reduced Flavoprotein
GPx	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Glutathione (reduced form)
GSO_3H	Glutathione Sulfonic Acid
GSSG	Glutathione (oxidized form) disulfide
HMW	High Molecular Weight
HPLC	High Performance Liquid Chromatography
IOL	Intraocular Lens
$NADP^+$	Nicotinamide-adenine Dinucleotide Phosphate (oxidized)
NADPH	Nicotinamide-adenine Dinucleotide Phosphate (reduced)

N (or Naph)	Naphthalene
ND (or Diol)	Naphthalene Dihydrodiol
NQ	Naphthoquinone
O.C.	Outer Cortical Opacity
PAD	Pulsed Amperometric Detector
PAGE	Polyacrylamide Gel Electrophoresis
PPP	Pentose Phosphate Pathway
PBS	Phosphate Buffer Saline
PSSC	Protein-cysteine Mixed Disulfide
PSSG	Protein-glutathione Mixed Disulfide
PSSP	Protein-protein Disulfide Aggregate
PTMD	Protein-thiol Mixed Disulfide
Rb	Rubidium
SDS	Sodium Dodecyl Sulfate
TC	Tissue Culture
TCA	Trichloroacetic Acid
UDP	Uridine Diphosphate
UGH Syndrome	Uveitis, Glaucoma and Hyphema
US	Urea Soluble Fraction
UV	Ultraviolet
WHO	World Health Organizition
WI	Water Insoluble Fraction
WS	Water Soluble Fraction
wt	Weight

CHAPTER I

INTRODUCTION

Statement of the Problem

Cataract, an eye disease characterized by progressive lens opacity and visual loss, is a major cause of blindness in humans throughout the world (Goldstein, 1980; Clayton et al., 1982; Leske and Sperduto, 1983). Based on the information provided by the World Health Organization (WHO), there are more than 40 million cases of blindness in the world, about half of which are due to cataracts (WHO Chronicles, 1979; Dawson and Schwab, 1981). The etiology of cataracts is not certain. Studies have demonstrated that cataracts may be related to some risk factors such as oxidative stress (Garner and Spector, 1980; Spector and Garner, 1981; Zigler and Hess, 1983), metabolic disorders like diabetes (Varmar et al., 1979; Ederer et al., 1981), ultraviolet radiation (Zigman et al., 1979; Hu et al., 1989), medicines (Shichi et al., 1978; Lubek et al., 1988; Caballero, 1989; Jaanus, 1991) and malnutrition (Halevi and Landau, 1962; Hollows et al., 1980; Chatterjee et al., 1982; Hiller et al., 1983; Gibson et al., 1986). However, the real causes and initial factors have not yet been identified.

Theoretically, cataract therapy could be achieved by three ways: (1) elimination of risk factors, (2) surgical extraction of opaque lenses and (3) prevention, delay or reversal of cataracts by medicines.

Eliminating the cataractogenesis should be the ideal way to decrease the burden of cataract-induced blindness. However, this can be achieved only after the identification of the real causes of cataracts. A successful example of this approach is the control of miotic-induced cataract. Previously, certain long-acting

cholinesterase inhibitors such as echothiophate, diisopropyl fluorophosphate, paraoxon and demecarium, were used to open the angle of anterior eye chamber to lower intraocular pressure in glaucoma patients by facilitating the outflow of aqueous humor. These agents were effective anti-glaucoma medicines but their use produced a severe side-effect. Their application induced cataracts in tens of thousands of users (Shaffer and Hetherington, 1966; Tarkkanen and Karjalainen, 1966; Dake and Kerlen, 1968; Morton et al., 1969; Levene, 1969; Pietsch et al., 1972; Axelsson, 1973; Pirie, 1973). This source of cataract has been eliminated because the use of these drugs has been significantly curtailed.

Since the introduction of microsurgery, dramatic progress has been made in cataract surgery, including extracapsular cataract extraction (ECCE) by phaco-emulsification, implantation of artificial intraocular lens (IOL), laser treatment of secondary cataract as well as improved management of surgical and postsurgical complications. All these advances make the cataract surgery a very successful therapy against cataract-induced blindness and such a surgery is the number one choice for anti-cataract therapy in many developed countries. However, the IOL implantation is still not a perfect therapy. First, the current IOL cannot completely replace all the functions of a real human lens such as accommodation. Without accommodation, an eye cannot function well. A multifocal IOL may be one of the solutions to the problem. Secondly, the lens, as a living organ, may interact with surrounding tissues and thus involve the regulation of these tissues, and thirdly, the surgery itself can sometimes cause severe complications such as secondary cataract, UGH (uveitis, glaucoma and hyphema) syndrome, decentration (such as sunset syndrome), pupillary capture, and intraocular inflammation (Sheets and Friedbery, 1980; Kratz et al., 1981; Abrahamson and Stein, 1984; Chylack and Cheng, 1985; Pecival, 1985; Vernon and Cheng, 1985; Davies et al., 1986; Cheng, 1987; Watsky et al., 1989). Other limitations include the lack of surgeons and lack of resources in the developing countries (Sommer, 1989), as well as the

expense for such a procedure. Therefore, the reverse, prevention or delay of cataract development by drugs will be a better option, especially for the majority of cataract patients who are living in the developing countries.

The medical treatment for cataracts has made enormous progress in the past two decades. Many drugs have beneficial effects in preventing the development of some types of cataracts in animals (Dvornik et al., 1973; Fukushi et al., 1980; Datiles et al., 1982; Poulsom et al., 1983; Stribling et al., 1985; Kador, 1983), providing a better prospect in medical anti-cataract therapy. Examples include the prevention and delay of sugar cataracts by aldose reductase inhibitors (ARIs) such as Sorbinil, Statil, ICI 105552 and AL01576 (Datiles et al., 1982; Poulsom et al., 1983; Hockwin et al., 1984-5; Stribling et al., 1985); the prevention of cyanate-induced cataract by bendazac and aspirin (Crompton et al., 1985; Lewis et al., 1986); the prevention of corticosteroid-induced cataracts by aspirin and vitamin E (Bucala et al., 1985; Creighton et al., 1983); and the effects of AL01576, which can completely prevent sugar cataracts and/or naphthalene cataract in animals (Hockwin et al., 1984-85; Xu et al., 1989).

In humans, several anti-cataract drugs are being prescribed in many countries. An interesting class of anti-cataract medicines is the aspirin-like analgesics which showed a protective effect against cataract in several case-control studies (Cotlier and Sharma, 1981; van Heyningen and harding, 1986; Chen et al., 1988; Harding and van Heyningen, 1988; Harding et al., 1989; Mohan et al., 1989). Bendalina (BND), the lysine salt of bendazac, has been tested in some European countries and the results showed that bendalina can significantly delay the development of cataract, particularly cortical cataracts (Cvintal et al., 1987; Gandolfo et al., 1987; Courtis, 1987). Sanders et al (1987) evaluated the data from a series of randomized, prospective and double-masked studies of the action of bendalina. They concluded that: "most studies demonstrated a statistically significant improved outcome in the BND group according to the changes in lens opacity" (Sanders et

al., 1987). In Asian countries, Catalin is a very commonly-used anti-cataract drug which was reported to be capable of retarding the occurrence of the lens opacity. Vitamin C and vitamin E also showed beneficial effects in several *in vitro* studies (Libondi et al., 1985; Varma et al., 1986) and *in vivo* animal cataract models (Charalampous and Hegsted, 1950; Nishigori et al., 1987). However, the result from a recent case-control study did not support such findings (Jacques et al., 1988a,b). Zhangyanming, a preparation based on traditional Chinese medical herbs, was reported to be able to delay the progress of age-related cataract and is currently prescribed to thousands of cataract patients who prefers a medical therapy. More than 50 other anti-cataract medicines are now commercially available in the world. However, the reports on their efficacy are either missing or not convincing because they have not been subjected to randomized placebo-controlled double-masked clinical trails (Bron et al., 1987). For preparations like bendalina which was reported to be an effective agent for delaying cataract formation, there still remains controversy on the parameters chosen for comparison and the methods used by the investigators.

A successful medical therapy depends on a thorough understanding of the disease mechanism and powerful tools to evaluate potential drugs. A proper animal model is very useful for both studying the mechanism of cataractogenesis and screening of drugs. During the search for anti-cataract agents, several cataract models have been established in animals (Bachem, 1956; van Heyningen, 1959; Kinoshita, 1965; Koch et al., 1976; Delamere et al., 1981; Matsuda et al., 1981; Garner et al., 1982; Lou et al., 1988; Xu et al., 1989). Some of these models, including diabetic cataract, galactose cataract, X-ray cataract, hypocalcemic cataract and naphthalene cataract, can mimic human cataracts caused by corresponding factors and thus provide useful tools to study the mechanism of human cataract formation.

Among these animal models, the naphthalene cataract model offers more

advantages over other models. First, both the morphological and biochemical changes of this model are very similar to those in human senile cataract (van Heyningen, 1976; Rossa and Pau, 1988). Second, this cataract model can be reproduced in many species of animals (Goldman, 1929; Koch et al., 1976; Iwata and Maesato, 1988; Xu et al., 1989; Wells et al., 1989; Rathbun et al., 1990), and third, it can be completely prevented by some ARIs (Hockwin et al., 1984-84; Xu et al., 1989), so that one can learn why and how some agents prevents cataract formation by studying such a model. Therefore, it has been suggested that naphthalene cataract in animal is a good model to mimic human age-related cataract (Rossa and Pau, 1988), and it is reasonable to believe that this model is a very powerful tool for anti-cataract drug evaluation.

The objectives of the research presented here were to establish the validity and utility of some cataract models including *in vivo* naphthalene cataract in rats and *the in vitro* cataract induced by naphthalene metabolites. Morphological changes were studied semiquantitively so that these parameters can be used for the comparison of the severity of cataract in different strains of rats and in rats with different treatments. Biochemical changes were studied as means to assess the cellular processes altered during cataract formation. Both albino and pigmented rats were compared to examine the role of pigmentation in these models. AL01576 and other ARIs were used as tools throughout the study and the action of AL01576 in naphthalene cataract prevention was investigated. The research provides information on the mechanism of naphthalene cataract formation and the action of the drugs which can prevent such a cataract.

Naphthalene Cataract in Animals

Naphthalene cataracts in animals have been studied for more than a century. The earliest studies were performed in rabbits in the 1880's. Ingestion of naph-

thalene resulted in cataract formation in all the naphthalene-fed rabbits in spite of the variation in the onset and severity of the opacities (Bouchard and Charrin, 1886; Igersheimer and Ruben, 1910; Adams, 1930). Bouchard and Charrin fed rabbits with 1.5-2.0 g/day of naphthalene and bilateral cataracts developed three weeks later (Bouchard and Charrin, 1886). In Dor's experiments, the lens lesions of the rabbits appeared on the 7th day after a daily treatment with 1 g of naphthalene (Dor, 1887). When an extremely large dosage of 10-15 g naphthalene was dosed to rabbits, the lens opacity was observed in 24 hours (Helbron, 1889; Magnus, 1890). A recent study reported that the earliest cataractous changes can be detected on the second day after the application of 3 g naphthalene/kg b.w. in rabbit (Rossa and Guido, 1989). Lubek et al (1989) examined the effect of naphthalene on rabbit lens and reported an interesting relationship between the lens opacities and naphthalene dosage. In their study, both New Zealand white and Chinchilla pigmented rabbits developed cataracts following either a single larger injection of naphthalene (2.0 g/kg, i.p.) or two smaller injections (1.0 g/kg/day, i.p. for 2 days). However, the cataract induced by the single large injection was progressive, leading to permanent cataract in the whole lens, while the opacity generated by the lower multiple dosing was reversible and recovered back to normal in 2 weeks (Lubek et al., 1989).

The morphological changes of naphthalene cataract in rabbit start with lens swelling followed by the appearance of subcapsular vacuoles and radial spokes at the periphery. These spokes fuse to form opaque layers throughout the cortex first and then in the nucleus (Adams, 1930; Pirie, 1968; Rossa and Guido, 1989). When the lens was removed from the eyeball, a yellow-brown color in the lens became apparent (Pirie, 1968).

Naphthalene cataract in rats was first introduced around 1930 (Goldman, 1929; Gifford, 1932). This rat cataract model is more consistent and reproducible than that in rabbit, especially in pigmented rats. Lens opacity could be induced within

1 week by a daily oral administration of 1.0 g/kg/day of naphthalene in mineral oil (Koch et al., 1976; Hockwin et al., 1984-85). However, the morphological characteristics are very different from those observed in rabbits. Instead of a cortical cataract, a naphthalene-fed rat develops a shell-like opacity around the nucleus of the lens (Koch et al., 1976; Hockwin et al., 1984-85; Xu et al., 1989). Koch et al. proposed that the opacities began with the formation of large cortical water clefts in the region under the epithelial layer, and as the lens continued to grow, new fiber layers formed and pushed the opaque layer inwards towards the supranuclear region (Koch et al., 1976). Such an explanation is not satisfactory because the newly formed lens fibers should be also affected and turned opaque when the rats were dosed with naphthalene every day during the test period. Another interesting phenomenon in the rat model is that such a cataract was reported to be inducible only in pigmented rats (Koch et al., 1976), suggesting that pigmented tissues in the eye played an important role in naphthalene cataract formation in rat. This hypothesis has not been challenged yet.

Naphthalene cataract can also be induced in mice (Shichi et al., 1980; Lubek et al., 1986; Wells et al., 1989). Furthermore, the incidence of cataract induced in mice by naphthalene injection (i.p.) is dose-dependent, where a dosage smaller than 500 mg/kg did not cause lens opacity, but a dose of 750 mg/kg or larger induced cataractous changes within 8 hours (Wells et al., 1989). Also, such a cataract can be induced only in some strains of mice such as C57BL/6 mice. Other strains such as DBA/2 mice showed strong resistance to the cataractogenicity of naphthalene and its naphthoquinone metabolites. It was suggested that DBA/2 mice exhibits distal differences in naphthalene biotransformative pathway or in lenticular response to toxic compounds like naphthalene (Wells et al., 1989). The different responses between these two strains may be interesting in revealing the factors which protect the lens from the damages caused by the metabolites of naphthalene.

Naphthalene Cataract in Humans

During the early part of the century, naphthalene was administered internally as an antiseptic and anthelminthic. In some countries it was also used for slimming (Lezenius, 1902). Such naphthalene 'abuse' caused several cases of cataracts in humans (Lezenius, 1902; van der Hoeve, 1906; Ghetti and Mariani 1956). The first case reported was a 36-year-old man who was given 5 g of naphthalene in an emulsion of castor oil in divided doses in the course of 13 hours. He lost his vision the second day. At the examination, a year later, the morphological characteristics of the lens were described as "having countless fine whitish opacities arranged as a zonular about the nucleus with a narrow clear zone at the equator." (Lezenius, 1902), which sounds very similar to the findings in the lenses of naphthalene-fed rats (Koch et al., 1976; Xu et al., 1989).

Besides ingestion of naphthalene, exposure to naphthalene vapor also may be related to cataract development. van der Hoeve reported a 44-year-old man who worked with naphthalene for years and developed cataract in both lenses (van der Hoeve, 1906). In 1956, Ghetti and Mariani examined the workers in a plant producing a dye-intermediate from naphthalene and they reported that among these naphthalene-vapor-exposed workers, 8 out of the 21 employees were found to have pinpoint and diffuse opacities in their lenses, suggesting that the respiratory track can absorb naphthalene vapor and produce toxic naphthalene metabolites (Ghetti and Mariani 1956). Buckpitt and associates provided strong evidence to support such a suggestion (Buckpitt et al., 1984). Their studies showed that some pulmonary microsomal enzymes can convert naphthalene to 1,2-dihydroxynaphthalene which may be the crucial metabolite responsible for naphthalene's cataractogenesis (Buckpitt et al., 1984).

Today, naphthalene itself is no longer used as a medicine and the workers in chemical industries are well protected so that no significant amount of naphthalene will be inhaled. However, naphthalene is still involved in many aspects of

our life. One example of naphthalene-containing products is the mothball which is still used all over the world. Naphthalene accounts for more than 90% of the total weight of mothballs. Drugs are also the source of naphthalene. Many drugs have structures similar to naphthalene or its metabolites and they may share the same metabolic pathway and generate similar toxic products. Furthermore, naphthalene has been proved to be present in a number of fuels and lubricants (Gammage, 1978; Noyes Data Corporation, 1980; Buckpitt et al., 1984) and is used extensively as feedstock in the dye industry (Noyes Data Corporation, 1980).

Another source of naphthalene is cigarette smoke which actually affects the public. Naphthalenes in cigarette smoke arise mainly via pyrolytic formation from leaf constituents and tobacco flavorants. The precursors include: (1) leaf sterols such as stigmasterol and amyrins, (2) terpenoids such as solanesol and neophytadiene, and (3) glycerrhyzin, which is a licorice extract and is used as a tobacco flavorant (Johnston and Quan, 1963; Schlotzhauer and Schmeltz, 1969; Schmeltz et al., 1974; Hoffman et al., 1975; Schmeltz et al., 1976). Schmeltz et al.(1976) reported that a usual 85-mm commercial U.S. nonfilter cigarette can yield more than 0.11 mg naphthalenes (Schmeltz et al., 1976), which are mainly distributed in the sidestream (Table 1). These naphthalenes, after being inhaled, can be converted into dihydroxynaphthalenes by pulmonary microsomal enzymes as mentioned before (Buckpitt et al., 1984). These dihydroxynaphthalenes can then autoxidize, in tissues, to naphthoquinones which have been indicated as crucial factors in cataract formation (van Heyningen, 1970; Ikemoto et al., 1971). These observations are also supported by some clinical and epidemiological studies which reported that the incidence of cataract in smokers is significantly higher than that of the non-smokers (Flaye et al., 1989; West et al., 1989; Leske et al., 1991; Hankinson et al., 1992; Christen et al., 1992). Therefore, naphtha-lene and its metabolites may involve in some types of human cataract formation.

TABLE 1. Amount and Distribution of Naphthalene in Cigarette Smoke.*

Fraction	Naphthalenes (µg/cigarette[a])			
	Naphthalene	2-Methyl-Naph.	1-Methyl-Naph.	Total
Tobacco filler	0.17	0.042	0.018	0.23
Mainstream	2.76	1.210	1.020	4.99
Sidestream	45.50	31.600	30.000	107.10
Total	48.43	32.852	31.038	112.32

* Adopted from Schmeltz et al., 1976 with minor modification.

[a] 85-mm column.

Naph. = naphthalene.

In Vitro Naphthalene Cataract Models

Most of the information about naphthalene cataract are obtained from *in vivo* studies. *In vivo* models can mimic a disease more accurately and usually provide satisfactory tools to evaluate potential drugs. However, such studies are usually very laborious and time consuming. Also, naphthalene is metabolized into several metabolites in the body and it is difficult to identify the metabolite which directly causes cataracts from others. Therefore, an *in vitro* naphthalene cataract model would be very helpful. In an *in vitro* system such as lens culture, one can directly test the effects of each individual metabolite of naphthalene for its cataractogenesis. Furthermore, the cultured lenses are easier to handle than living animals

and the morphological changes in the lenses are easier to observe with our naked eyes or a dissecting microscope. Lastly, the test period in the *in vitro* system can be dramatically shortened by increasing the concentration of the tested chemical in the medium to a higher level which can hardly be reached in a living animals. However, due to the insolubility of naphthalene, *in vitro* studies of naphthalene cataract in organ culture have been difficult to carry out, and consequently the information in this field is very limited.

Several rabbit lens culture studies attempted to examine the effects of naphthalene metabolites on the lens and the results suggested that 1,2-dihydroxynaphthalene and its autoxidative product, 1,2-naphthoquinone might be the toxic agents in these models (Umbreit, Burris and Stauffer, 1949; Ikemoto, Iwata and Narita, 1968; van Heyningen, 1970; Ikemoto et al., 1971). In van Heyningen and Pirie's study, rabbit lenses exposed to 1,2-naphthoquinone in culture medium developed opacities and the lens turned brown. They also tested naphthalene 1,2-dihydrodiol for its cataractogenesis and reported that this compound induced cataractous changes through topical administration (every 30 min) within 5 days and caused lens browning in several hours when rabbit lenses were incubated with 6 mM naphthalene dihydrodiol (van Heyningen and Pirie, 1967). We recently conducted an *in vitro* naphthalene cataract study by exposing rat lens in culture to 1,2-naphthoquinone and naphthalene dihydrodiol. The result showed that both compounds at 2.5 X 10^{-5} M for 48 hours could induce lens opacities but they were different in morphology (Xu et al., 1990). Naphthoquinone caused lens swelling and cortical opacity with significant damage of membrane functions, while naphthalene dihydrodiol induced a yellow-brown opaque shell around the nucleus without membrane disturbance. The morphological change caused in the lens by naphthalene dihydrodiol exposure *in vitro* is very similar to the cataract induced by naphthalene in rat. Biochemical analysis of lens antioxydant system, lens proteins, naphthalene metabolites and related enzymes in these *in vitro* models

will provide very important information.

There are several possible intermediate metabolites (1-naphthol, 2-naphthol, naphthalene 1,2-dihydrodiol and 1,2-dihydroxynaphthalene) produced in the body when naphthalene is metabolized to 1,2-naphthoquinone (van Heyningen, 1979; see Figure 1), and it will be important to test the cataractogenicity of these metabolites in the *in vitro* system. Such studies may identify the metabolites which reach the eye and cause the cataractous changes in naphthalene-fed animals.

The Role of Pigmentation in Naphthalene Cataract

Both rabbits and rats have been commonly used as experimental animals for the production and study of naphthalene cataract. In rabbits, the cataract can be induced in either pigmented or nonpigmented strains; however, in rats only pigmented strains were considered suitable for naphthalene cataract induction (Koch et al., 1976; van Heyningen, 1979).

The relationship between the presence of the iris and the formation of cortical lens spokes was first observed by Lindberg in 1922. He found that in rabbits fed with naphthalene, the spokes first appeared in the portion of the lens covered by the iris. When the pupil of the rabbit was kept dilated, the spokes were distributed more peripherally and when a part of the iris was removed, the colobomatous area remained free of spoke for some time (Lindberg, 1922). One may speculate that the dilation of pupil or excision of the iris relocated the pigmented tissues, changing the polyphenol oxidase (it is now renamed as tyrosinase) distribution and therefore the toxic compound 1,2-dihydroxynaphthalene formed by the action of tyrosinase is released at different places around the lens. Such an explanation is not completely satisfactory because the relocation of iris may also alter the blood supply which delivers the toxic compound to different portions of the lens.

The possible role of the pigmented iris in cataractogenesis in naphthalene-fed rats arose from the inability of some investigators to reproduce the *in vivo* study

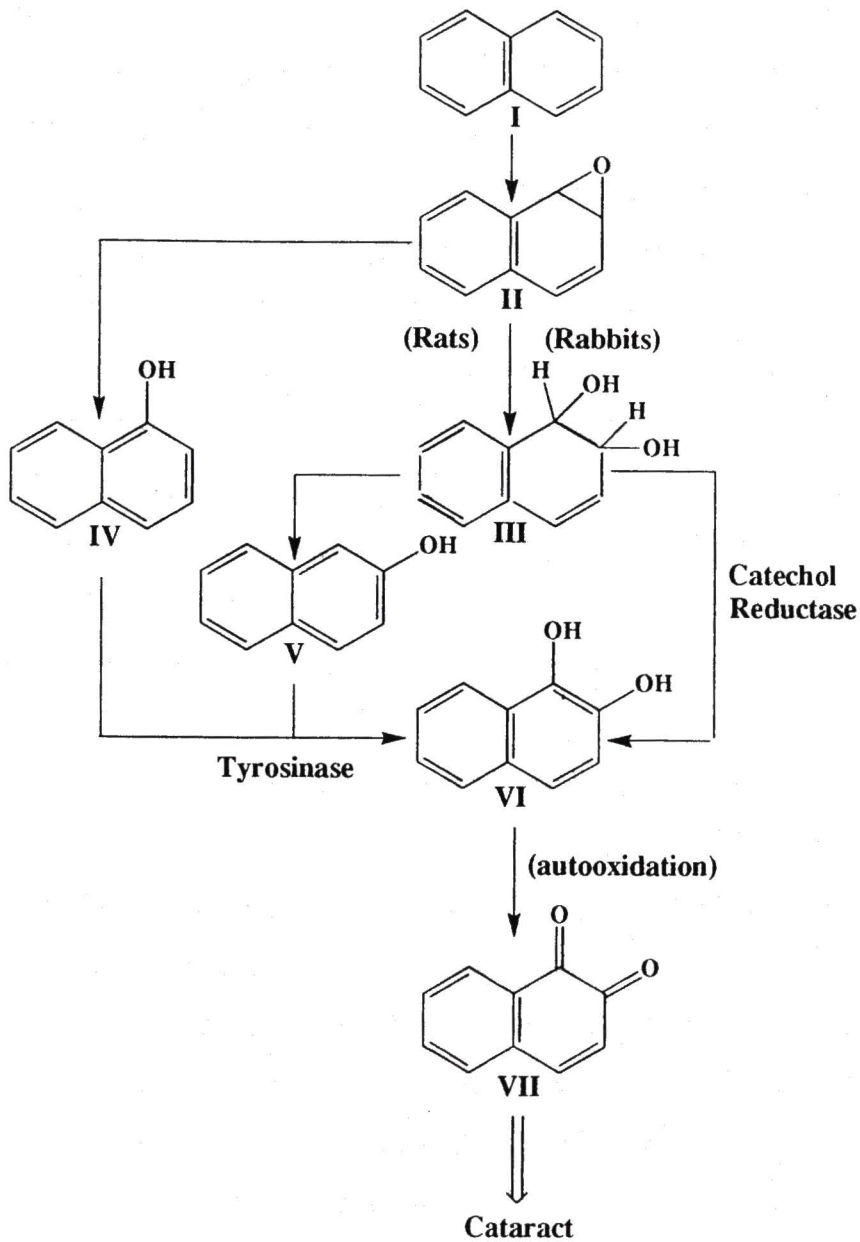


Figure 1. Naphthalene Metabolic Pathway in Animals. (van Heyningen, 1979). In rats: 1,2-dihydroxynaphthalene was formed from 1-naphthol and 2-naphthol by the action of tyrosinase. In rabbits: 1,2-dihydroxynaphthalene was produced through the pathway catalyzed by catechol reductase.

reported by Goldman (Goldman, 1929). Since albino rats became commonly-used strains after the Goldman era, it was suspected that pigmentation might explain this discrepancy (Koch et al., 1976). In order to test the theory that eye pigmentation could be a crucial factor in naphthalene cataract formation, Koch, et al (1976) compared the susceptibility of pigmented rats with albino rats to naphthalene cataract development. They found that all the pigmented rats developed cataract sooner, more consistently and more severely (Koch et al., 1976). Thus, the role of pigmentation in naphthalene cataract formation was established and has never been challenged. Considering the special metabolism in pigmented tissue, Koch et al (1976) suggested that the enzyme tyrosinase, which was found mainly in pigmented tissue (such as the iris in pigmented rats), catalyzed the formation of 1,2-dihydroxynaphthalene from 1-naphthol or 2-naphthol. The 1,2-dihydroxynaphthalene then autoxidized to form the toxic compound, 1,2-naphthoquinone.

In one of our recent experiments on rat naphthalene cataract, we fed naphthalene to several albino rats as controls. To our surprise, all the albino rats developed cataract at the same pace as that of the pigmented rat (Xu et al., 1989), unlike the earlier report that pigmented tissue was an important factor for the formation of naphthalene cataract in rat. The role of pigmentation in naphthalene cataract model is challenged for the first time since the activity of tyrosinase in an albino rat is not significant. If the pigmentation plays no role in this cataract, then the current hypothesis for the metabolic pathway of this model needs to be re-examined.

Naphthalene Metabolic Pathway

Since Bouchard (1886) demonstrated that the feeding of naphthalene to rabbits caused cataract, many attempts have been made to identify what metabolites are produced in the animal and which one reaches the eye and induces cataract.

Studies have shown that the liver is the main organ to metabolize naphthalene and several metabolites have been reported to exist in the blood circulation after naphthalene feeding (Corner and Young, 1954; Boyland et al., 1961; Jerina et al., 1970; Oesch and Daly, 1972; Bock et al., 1976).

The first step in naphthalene metabolism is the oxidation of naphthalene to naphthalene epoxide by a cytochrome P-450 dependent monooxygenase (Jerina et al., 1970; Oesch and Daly, 1972; Bock et al., 1976). The reactive epoxide is then metabolized by at least three pathways. In the first pathway, naphthalene is detoxified by conjugation with glutathione under the action of UDP-glucuronyl-transferase and nonenzymatic conditions (Jerina et al., 1970; Bock et al., 1976; Iwata and Maesato, 1988). This is the major reaction which converts lipid-soluble compounds into polar products which can then be effectively eliminated from the body via the kidney. The second pathway is the non-enzymatic rearrangement of the epoxide to corresponding phenols. The products are mainly 1-naphthol (95%) and some 2-naphthol (5%) (Jerina et al., 1970). Both 1-naphthol and 2-naphthols can be further metabolized to dihydroxynaphthalene and then naphthoquinone (Koch et al., 1976). The third pathway is the hydration of the epoxide by the enzyme epoxide hydratase to yield naphthalene dihydrodiol (Jerina et al.,1970a,b; Bock et al., 1976). This dihydrodiol is still a potential hazard because it can be converted to 1,2-dihydroxynaphthalene and 1,2-naphthoquinone which can cause toxic effects like cataract formation (van Heyningen and Pirie, 1967; Rees and Pirie, 1967).

Based on these reports and the findings mentioned earlier, van Heyningen proposed the metabolic pathways for naphthalene in rabbits and rats, and the relationship between naphthalene metabolism and cataracts formation (van Heyningen, 1979). As shown in Figure 1, the ingested naphthalene (I) is oxidized in the liver first to an epoxide (II) and then is converted into naphthalene dihydrodiol (III). In rabbits, this stable compound is released from liver and reaches the eye

through the blood circulation. It is then converted to 1,2-dihydroxynaphthalene (VI) in the lens by the enzyme catechol reductase (EC 1.3.1.5.). Being unstable at physiological pH, 1,2-dihydroxynaphthalene spontaneously autoxidizes to 1,2-naphthoquinone (VII) and H_2O_2. These oxidants react with lens constituents and cause cataracts (Rees and Pirie, 1967; Pirie, 1968).

In rats, the situation is different. van Heyningen proposed that 1-naphthol (IV) and 2-naphthol (V) produced in liver through epoxide rearrangement is transported into the eye, where tyrosinase in the pigmented tissues catalyses these naphthols to form 1,2-dihydroxynaphthalene (VI) and then 1,2-naphthoquinone (VII) and H_2O_2. These oxidants can damage the rat lens just like they can do to the rabbit lens. Another difference between rat lens and rabbit lens is that the rat lens has only about 3% of the catechol reductase found in the rabbit lens (van Heyningen, 1970). Therefore, naphthalene dihydrodiol (III) obtained from the blood circulation could not be converted by the lens to 1,2-naphthoquinone (VII) in significant amounts. These differences were thought to be the reason of why naphthalene cataract can only be induced in pigmented rats (Koch et al., 1976; van Heyningen, 1979).

Oxidation as A Mechanism for Naphthalene Cataract Formation

As summarized by van Heyningen, the mechanism of naphthalene cataract may be oxidative damage to the lens (Rees and Pirie, 1967; van Heyningen, 1979). In the rabbit model, naphthalene dihydrodiol from the blood circulation is a precursor of the toxic 1,2-naphthoquinone in the eye. This precursor is further converted to 1,2-dihydroxynaphthalene by the action of catechol reductase in the eye. The toxicity of 1,2-dihydroxynaphthalene is due to its ready autoxidation to 1,2-naphthoquinone and H_2O_2 (Figure 2). Rees and Pirie examined the reaction of 1,2-naphthoquinone with the lens in an *in vitro* system and demonstrated that 1,2-naphthoquinone alkylates many constituents of the lens, including proteins,

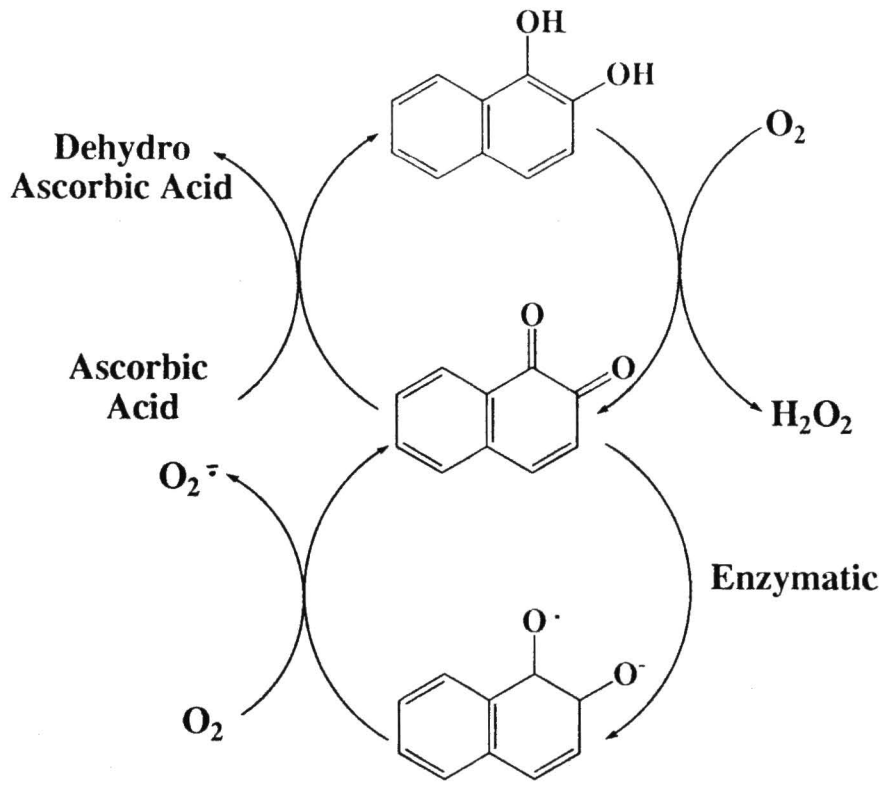


Figure 2. Oxidative molecules produced through naphthoquinone reactions.

amino acids and glutathione (Rees and Pirie, 1967). 1,2-naphthoquinone can also bind to thiol groups of glutathione (GSH) and protein molecules, and thus decreasing the lens defensive abilities and increasing the insolubility of the crystallins, especially the γ-crystallin which is rich in thiol groups. The conjugation of 1,2-naphthoquinone with lens protein causes naphthalene cataract to be yellow or brown in color (Van Heyningen and Pirie, 1967). The amino group in cysteine and proteins is another target for naphthoquinone binding. Under aerobic conditions, the cysteine molecules are linked to 1,2-naphthoquinone (4:1) through their amino groups (Mason and Peterson, 1965; Rees and Pirie, 1967). Pirie also reported that naphthoquinone could cause degenerative changes of the lens epithelium which were probably one of the earliest lesions in naphthalene cataract (Pirie, 1968).

During the autoxidation of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone in the eye, H_2O_2 is produced, and when 1,2-naphthoquinone is reduced back to 1,2-dihydroxynaphthalene, ascorbic acid is oxidized to dehydro ascorbic acid (Figure 2). Therefore, the cycle decreases the reducing power and increases the oxidant levels in the eye. A more important aspect is that only a small amount of 1,2-dihydroxynaphthalene is required to catalyze the formation of H_2O_2, which in turn oxidizes much ascorbic acid (van heyningen, 1976).

H_2O_2 is a stronger oxidizing agent than O_2, being able to oxidize thiol groups in methionine and cysteine. In an *in vitro* study using whole lens culture system, H_2O_2 was found to be a good agent to induce cataract (Cui and Lou, 1993). When H_2O_2 is reduced in the lens to H_2O by glutathione (GSH) under the action of glutathione peroxidase, GSH is oxidized to GSSG which conjugates with the thiol group in protein molecules to form mixed disulfides. Such protein modifications will also lead to protein disulfide cross-linking (PSSP) aggregates, as shown in Figure 3 (Lou et al., 1990). The formation of High Molecular Weight (HMW) disulphide-linked aggregate is found exclusively in cataracts and it may contribute

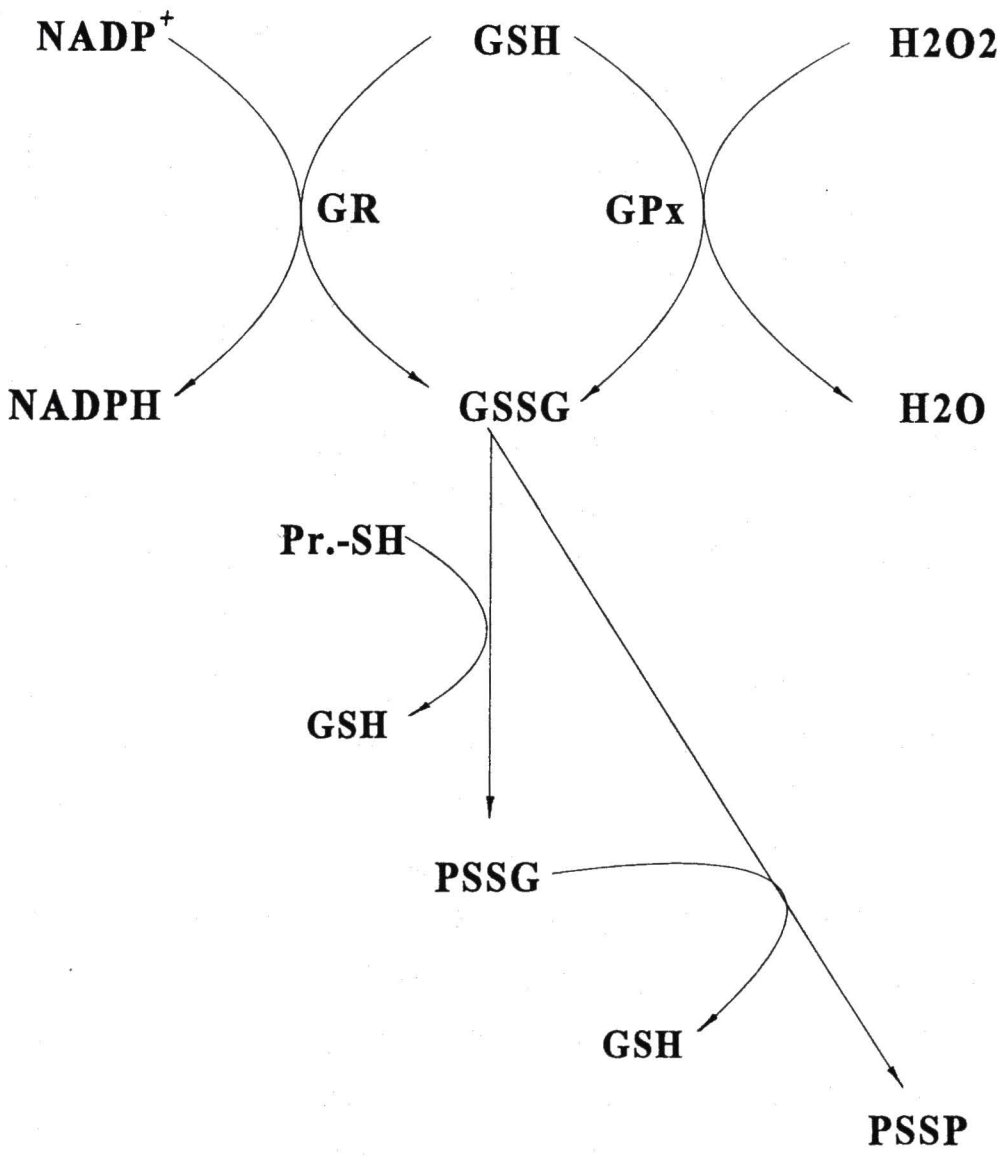


Figure 3. Oxidation of thiol groups and formation of cross-linked HMW aggregates through protein-thiol mixed disulfide. GSH: reduced glutathione; GSSG: oxidized glutathione; PSSG: protein-GSH mixed disulfide; PSSP: protein-protein disulfide cross-linked aggregates.

to the light scattering of the lens and result in the formation of opaque regions (Spector, 1984). Giblin et al observed a stimulated hexose monophosphate shunt activity in H_2O_2-exposed rabbit lens (Giblin et al., 1982.), probably reflecting an increased demand for reducing power via nicotinamide-adenine dinucleotide phosphate (NADPH). NADPH can reduce GSSG, catalyzed by the enzyme glutathione reductase (GR), to GSH which is then used to detoxify H_2O_2. When the amount of H_2O_2 entered into or produced in the lens exceeds the ability of the defence systems of the lens, H_2O_2 reacts with the thiol groups of methionine, cysteine or proteins and leads to the formation of disulfide cross-linked HMW aggregates and the lens protein insolubilization mediated by the protein-thiol mixed disulfide products (Cui and Lou, 1993). Since the lens epithelium and capsule is permeable to H_2O_2 (Pirie, 1965; Fukui, 1976), the H_2O_2 produced outside the lens (such as that yield in the aqueous humor through the autoxidation of ascorbic acid) can insult the lens equally. In naphthalene cataract, GSH concentrations were reported to decrease, therefore we need to examine whether the mixed disulfides are involved in such a cataract model.

In vitro studies also indicated that H_2O_2 in culture medium can cause the uncoupling of the lens Na^+/K^+-ATPase (Garner et al., 1983). The hydrolysis of ATP by Na^+/K^+-ATPase provides energy for the lens so that the lens can maintain normal metabolism and perform its functions such as ions transport. Hydrogen peroxide is one of the oxidants produced in the lens of naphthalene-fed rat and the lens Na^+/K^+-ATPase activity should be examined for its involvement in the formation of naphthalene cataract.

Besides 1,2-naphthoquinone and H_2O_2, free radicals can also be generated through redox cycling of 1,2-naphthoquinone with its semiquinone form or via other enzymatic and non-enzymatic reactions (Brunmark and Cadenas, 1989). Figure 2 shows one of these pathways in which the semiquinone autoxidizes to 1,2-naphthoquinone and yields primarily $O_2^{\cdot-}$, the quinone is then reduced back by

enzymatic reactions. Many enzymes such as cytochrome P-450 reductase and cytochrome b_5 reductase can all catalyze such a typical one-electron transfer reaction. These radicals then decay in several ways to form oxidative products.

Oxidation is considered by many scientists to be one of the most important mechanisms involved in human cataract formation. Examination of the aqueous humor of some patients with cataract has shown remarkably high level of H_2O_2 in a significant number of patients (Spector and Garnar, 1981). The oxidation is believed to be initiated at the membrane, possibly by some external components disrupting pump systems and membrane permeability, so that ionic balance is disturbed and metabolism is impaired. With the defensive systems weakened, the disulfide-linked HMW aggregates formed, the membrane disrupted and subsequently an opacity occurred (Spector, 1984). In order to examine if such a oxidative mechanism is involved in rat naphthalene cataract, the lens membrane transport systems and Na^+/K^+-ATPase activity were studied in this project.

Prevention of Naphthalene Cataract with ARIs

Based on the above information, both naphthalene cataract and human age-related cataract may share similar mechanisms such as oxidation. Therefore, the information from the study on naphthalene cataract will be very helpful for understanding human cataract formation. Since it is important to find an effective anti-cataract drug therapy, the assay of naphthalene cataract models as a tool for potential anti-cataract drug screening can be very beneficial.

Hockwin et al. first demonstrated that some aldose reductase inhibitors from Alcon Laboratories (AL01567, AL01576) could prevent naphthalene-induced cataract in rats (Hockwin et al., 1984-1985; Wegener et al., 1985; Xu et al., 1989). In Wegener's study (1985), AL01567 was tested for its ability to prevent strepto-zotocin-induced diabetic cataract under the additional cataractogenic influences of X-ray radiation or naphthalene. The results clearly showed that AL01567 could

suppress diabetes-related changes and also those from naphthalene (Wegener et al., 1985).

To confirm this effect of ARIs, AL01576 which is more efficient in inhibiting aldose reductase (AR) activity than AL01567 (Muller et al., 1985) was tested for its preventive action on naphthalene cataract. The results demonstrated that this compound can completely prevent naphthalene-induced cataract in rats (Hockwin et al., 1984-1985; Xu et al., 1989). As stated previously, we recently established an *in vitro* cataract model by exposing rat lens to naphthalene dihydrodiol and this model can mimic the cataract in naphthalene-fed rats. We tested the ability of AL01576 in this *in vitro* model and the result proved that AL01576 can prevent this cataract equally well as it did in the *in vivo* model (Xu et al., 1990).

These reports provide a new and powerful tool to study the mechanism of cataract and to search for more effective anti-cataract drugs. Also they raise many new questions. For example, AL01576 is designed for the treatment of diabetes-related changes by inhibiting aldose reductase. Why does it prevent a cataract caused by a toxic compound such as naphthalene? Is aldose reductase involved in naphthalene cataract formation or does AL01576 have an ability to affect other enzymes in the naphthalene metabolic pathway? Does inhibition of cytochrome P-450 by AL01576, which was proved to be true, play any role in AL01576's efficacy? To answer these questions we need to compare the efficacies of different classes of ARIs in naphthalene cataract models. Also, ARIs with and without the ability to inhibit cytochrome P-450 should be compared to clarify the role of cytochrome P-450 in naphthalene cataract formation. These results may also have important ramifications with respect to diabetic cataract and their treatment.

Experimental Rationale

The successful prevention or delay of human cataract is not impossible. When

an anti-cataract agent is simple, safe and effective enough, medical therapy for cataract will be a better and more practical option than surgery. To approach this goal, we need to establish effective models which can mimic human cataract so that we can study the mechanism underlying the disease and search for potential drugs. The main objectives of this proposal were to establish and study naphthalene cataract in animal models and in lens organ culture systems. Through the study on these models, the mechanism of cataract formation and its treatment may be elucidated.

In order to pursue these objectives, the following researches were planned: (1) to establish an *in vivo* rat cataract model by feeding rats naphthalene. Both pigmented rats and albino ones were compared under the identical conditions. In addition, several dosages of AL01576 and other ARIs were given to naphthalene-fed rats to test their effects on this model. AL01576 was also tested for its ability in the intervention of this cataract; (2) to establish *in vitro* models by exposing rat lenses to different metabolites of naphthalene in organ culture systems. Again, we examined the responses of the lenses from both pigmented and albino rats to these metabolites in this system and tested the ability of AL01576 to prevent the changes induced by naphthalene metabolites; (3) to reveal the mechanism underlying naphthalene metabolism and cataract formation. We examined two aspects of naphthalene regarding its metabolism and cataractogenesis in rats: the role of pigmentation and the role of oxidation. Both morphological and biochemical techniques were applied to monitor naphthalene-related changes against normal lens. Different ARIs were compared in this system for their ability to prevent or reverse this cataract. The presence of naphthalene metabolites in the eye was detected by a reversed-phase HPLC system; and (4) to determine the action of AL01576 in the prevention of this cataract by testing the direct effects of this drug on these models, comparing the effect of AL01576 with other classes of ARIs, comparing the efficacy of ARIs with or without inhibition of cytochrome P-450, and

determinating the effect of AL01576 on some of the enzymes in naphthalene metabolic pathway such as dihydrodiol dehydrogenase.

All these experiments were performed in animals or in animal lens cultures. Because of the similarity between rat naphthalene-induced cataract and human age-related cataract, we hope the information from these models can help us to understand human cataract formation and to provide powerful tools to screen and evaluate anti-cataract drugs.

CHAPTER II

METHODS

Chemicals and Radiochemicals

Naphthalene, 1-naphthol, 2-naphthol, 1,2-dihydroxynaphthalene and 1,2-naphthoquinone were ordered from Aldrich Chemical Company, Inc, Milwaukee, WI. Aldose reductase inhibitors (ARI), AL01576 and AL04114, and the vehicle (5 mM sodium acetate-acetic acid buffer, pH 5.5, plus 0.2% Tween 80 and 1% polyvinylpyrrolidone 40), and 1,2-dihydro-1,2-dihydroxynaphthalene (naphthalene dihydrodiol) were synthesized by Alcon Laboratories, Inc., Fort Worth, TX. Other ARIs, Sorbinil and Tolrestat, were gifts from Pfizer Co. and Wyeth-Ayerst Co., respectively. TC-199 medium, penicillin and streptomycin were supplied by Gibco, Inc., Grand Island, NY. Acetonitrile and methanol were obtained from J. T. Baker Inc., Phillipsburg, NJ. All the other chemicals were reagent grade from Sigma Chemical Company, St. Louis, MO.

Galactose (30%) containing chow was purchased from Purina Mills, Inc., Richmond, IN.

The radiochemicals, [methyl-^{3}H]-choline choline and rubidium-86 (^{86}Rb, in 0.5 M Hydrogen chloride), were ordered from New England Nuclear, Boston, MA. The special activities were 80 Ci/mmol for ^{3}H-choline and 8 mCi/mg for ^{86}Rb.

Production of Naphthalene Cataract and Dual Cataract in Rats

Experimental Animals

Five strains of rats were used in the present study. The three strains of albino rats, Sprague-Dawley, Wistar and Lewis, were all supplied by Harlan Sprague

Dawley, Indianapolis, IN. The two strains of pigmented rats were Long-Evans (Charles River Laboratories, Portage, MI) and Brown Norway (Harlan Sprague Dawley, Madison, WI). Only male rats were used in this research and the body weights of the rats were 125-150 g when naphthalene treatment started.

Induction of Naphthalene Cataract in Rats

Naphthalene solution (10%) was made in warm mineral oil by heating at 60°C for 30 min. The rats were dosed with this solution daily with an 18 gauge gavage needle (Popper and Sons, Inc., New Hyde Park, NY) at 0.5 g/kg/day for 3-4 days and 1.0 g/kg/day thereafter up to 6 weeks. Rats used as control were fed with equivalent volume of mineral oil by gavage needle.

AL01576 and other ARIs (10 mg/ml) were suspended in ARI vehicle and be given at desired dosages (typically, 10 mg/kg/day). The suspensions of these compounds were made by sonicating the powder of each drug into the vehicle and were administered by 20-gauge gavage at desired dosages (10 mg/kg/day for most of the ARIs and 50 mg/kg/day for Tolrestat) to rats one hour before the naphthalene administration. AL01576 was also tested for its ability to intervene or reverse the cataract, and in such studies, rats were fed with naphthalene for one or two weeks before starting AL01576 treatment. Typically, each set of experiments included 4 groups of 6-10 rats each: normal control, naphthalene-fed rats, naphthalene-feeding plus an ARI treatment and ARI treatment only. Each experiment lasted up to six weeks.

Induction of Dual Cataracts in Rats

Only Long-Evans rats were used in the dual cataract study. The rats were simultaneously treated with two strong cataract inducing agents, galactose and naphthalene. Galactose was premixed into the rat chow at 30% (w/w). A rat consumes 10-15 g chow/100 g body wt./day, that is 3-5 g galactose/100 g/day.

Naphthalene was prepared in mineral oil as mentioned above and given by a gavage needle at 1.0 g/kg/day. For better observation of both cataracts, naphthalene treatment should start earlier than galactose feeding. When the lens opacity induced by naphthalene was just detectable under a slit-lamp, galactose feeding began. The rats were maintained under these conditions for two weeks. Besides normal control, rats treated with either naphthalene or galactose was also used as control. When ARIs were tested in this system, 10 mg/kg/day of AL01576 or 50 mg/kg/day of Tolrestat was administrated to the rats following the procedure described in the previous section.

Rat Lens Culture and *in vitro* Cataract Models

Preparation of Media

Four types of media were used in the studies: medium-A, a modified TC-199 medium (Zigler and Hess, 1985) with an osmolarity of 298 mOsm, was used for the preincubation of the lenses; medium-B, similar to medium-A, but its osmolarity was adjusted first to 290 mOsm by NaCl and then to 295-300 mOsm upon the addition of the stock naphthalene metabolite solutions (made in 4% ethanol) as described below; medium-C contained 30 mM galactose which replaced the fructose in medium-A, otherwise it was identical to medium-A; medium-D was made the same way as medium-C but it was adjusted to 290 mOsm (Table 2). The addition of stock solution of naphthalene dihydrodiol in 4% ethanol increased the osmolarity to about 297 mOsm.

Preparation of Stock Solutions of Naphthalene Metabolites

Five potential metabolites of naphthalene were investigated: naphthalene 1,2-dihydrodiol, 1-naphthol, 2-naphthol, 1,2-dihydroxynaphthalene and 1,2-naphthoquinone. All these compounds were prepared individually in 4% ethanol at either

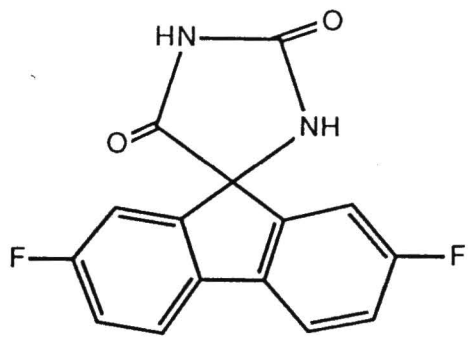
TABLE 2. Media Used in the in vitro Naphthalene-related Cataract Models.

Medium Type	Sugar (30 mM)	Osmolarity	Usage
Medium-A	Fructose	298 mOsm	lens pre-incubation
Medium-B	Fructose	290 mOsm	naphthalene cataract
Medium-C	Galactose	298 mOsm	galactose cataract
Medium-D	Galactose	290 mOsm	dual cataract

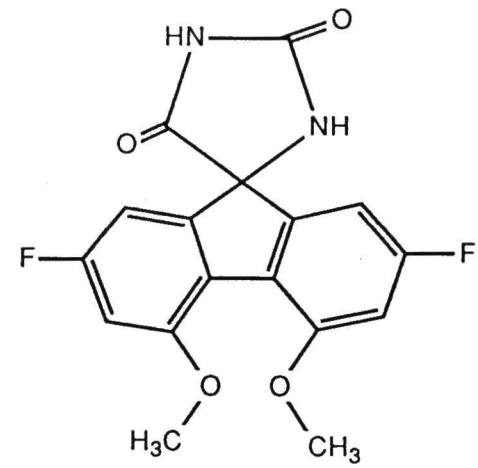
2.5 X 10^{-3} M or 5 X 10^{-3} M concentrations. The stock solution was then added into media-B and medium-D at 1:100 ratio, which brought the final osmolarity to 295-300 mOsm. The osmolarity was measured with an Osmette A osmometer (Preci-sion Instruments). Medium for the control group contained equal volume of ethanol.

Preparation of ARIs' Stock Solutions

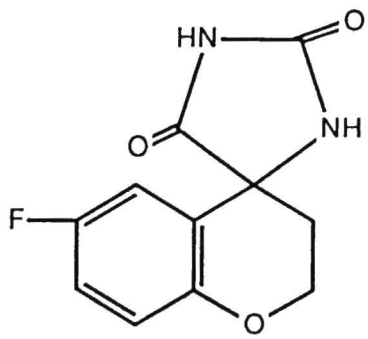
Four different ARIs (see Fig. 4 for their structures) were tested in the present study, including AL01576, AL04114, Sorbinil and Tolrestat. All the ARIs was dissolved in 25 mM NaOH to make a stock solution of 2.5 X 10^{-3} M, or ten-fold more concentrated for Tolrestat. The stock solutions were added to the culture medium at 1 part per 100. The same volume of 25 mM NaOH was used in the groups without ARIs as control. Under this condition, the pH and the osmolarity



AL01576



AL04114



Sorbinil

Tolrestat

Figure 4. Structures of AL01576, AL04114, Sorbinil and Tolrestat.

of the medium remained unchanged.

Lens Culture

At different stages of the studies, rats were sacrificed by CO_2 asphyxiation and the eyeballs were carefully dissected by a posterior approach. The lenses were removed and preincubated in medium-A in 24-well culture plate. The lenses were individually placed in each well holding 1.5 mL medium. The lens culture was maintained at 37°C with 5% CO_2 atmosphere and the media were renewed daily.

Induction of Naphthalene-related Cataract in Cultured Lenses

After preincubation in medium-A for one hour, the lenses were transferred into medium-B containing either alcohol as control or a naphthalene metabolite to test its cataractogenicity. The stock solutions of naphthalene metabolites were diluted 1:100 or 1:50 into medium-B, giving the desired final concentration, usually 2.5 X 10^{-5} M. The lenses were incubated in these media for durations of up to 96 hours with one change of fresh media every day. The contralateral lens was used as normal control, which was incubated in medium containing the same amount of ethanol (0.04%) but no naphthalene metabolite.

Prevention of in vitro Naphthalene Cataracts by ARIs

To prevent the naphthalene dihydrodiol-induced cataract, the lenses were preincubated with medium-A containing 2.5 x 10^{-5} M ARIs for one hour before switching to a medium containing both an ARI and naphthalene dihydrodiol. The ARI stock solutions of 5 x 10^{-3} M were made by dissolving the drugs in 25 mM NaOH and later diluted 100-fold with media to be used as the incubation solutions for lenses. A typical experiment consisted of the following four groups with six lenses per group: normal control, naphthalene dihydrodiol, naphthalene dihydrodiol plus ARI and normal control plus ARI.

Induction of in vitro Dual Cataracts

All the media (A-D) listed in Table 2 were used in this study. Healthy Long-Evans rats or Sprague-Dawley rats were sacrificed with CO_2 and the fresh lenses were preincubated in medium-A for one hour before being transferred to different experimental groups. Medium-D containing 30 mM galactose was loaded with naphthalene dihydrodiol to give a final concentration of 5×10^{-5} M. In order to induce dual cataract, the lenses were transferred from medium-A into medium-D with naphthalene dihydrodiol and maintained in this medium for 48 hours with one change of fresh medium at 24 hrs. Medium-B was used either in normal control group when it was added 0.04% ethanol or in naphthalene cataract model when it was loaded with 5×10^{-5} M naphthalene dihydrodiol. Medium-C was used to induce galactose cataract as a control. The effects of various ARIs were tested in this dual cataract system and the testing procedure is the same as that in the previous paragraph.

Morphological Observations

For the *in vivo* studies, the morphological changes in the rat lenses were examined at different stages during the naphthalene feeding, twice a week during the first two weeks and thereafter at weekly intervals. The observations were done with slit-lamp microscopy (Nikon Zoom Photo Slit Lamp Microscope FS-2, both focal and retroillumination) after the pupil was dilated with 1.0% Mydriacyl. All the typical changes were photographed on Kodak Ektachrome color film.

To compare the onset and the degree of the opacities at different stages and in different animals, an arbitrary six-score (0-5) grading system was established for recording and grading the lenticular opacities (Xu et al., 1989, as shown in Table 3):

TABLE 3. The arbitrary 6-score naphthalene cataract grading system.

Score	Morphological Changes
0	clear lens;
1	waterclefts & spoke-like opacities(<1/2 of the shell);
2	waterclefts & spoke-like opacities(>1/2 of the shell);
3	opacities merged to form a shell (thin, gray color);
4	shell became partly thick and white;
5	shell became fully thick and white.

To monitor the cataractous changes in the *in vitro* system, the cultured lenses were kept in the medium and were examined under a dissecting microscope. All the typical changes were photographed through the microscope. Some lenses from naphthalene-fed rats were also put in the same medium and observed under the same condition so that we could compare the morphological characteristics of the *in vitro* cataract models with those in the *in vivo* model.

Sample Collection

At different stages (0-6 weeks) of naphthalene feeding, rats were sacrificed by CO_2 asphyxiation following the guidelines of the ARVO Resolution on the Use of Animals in Research. Aqueous humor was first collected by an anterior chamber puncture technique. Then, the lenses were removed as soon as possible from the enucleated eye for further processing.

To examine the effects of naphthalene on different regions of the lens, some lenses were further dissected into capsule-epithelium, cortex and nucleus by the following procedure: the lens capsule-epithelium was carefully peeled with a pair of forceps, then the decapsulated lens was frozen immediately on dry ice. A core of the lens was removed using a cork borer (2 or 3 mm in diameter depends on the size of lens) and its ends (cortex) were cut off. The central part of the core (about 30% of the lens weight) was taken as lens nucleus and the balance of the decapsulated lens was considered as cortex.

Biochemical Studies

Before the lens opacities are observable, some biochemical alterations must occur at the molecular level. In order to detect these changes, the following biochemical parameters were examined:

Glutathione Assay:

Glutathione (GSH) concentrations were measured either in the whole lens or in separated capsule-epithelium, cortex and nucleus by the (modified) method of Ellman (1959). The capsule-epithelium, cortex (or whole lens) and nucleus were homogenized in glass homogenizers in 0.5, 1.0 and 0.6 mL of 10% TCA, respectively. After centrifugation at 1,650 x g, 4°C, for 30 min, an aliquot of 0.4 mL (for epithelium or nucleus) or 0.2 mL (for cortex or whole lens) supernatant was mixed with 0.59 mL 1.0 M Tris-HCl buffer (pH 8.2, containing 0.2 M EDTA) and 0.01 mL of Ellman's reagent which was made by dissolving 99 mg 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 25 mL methanol. The final volume was brought to 1.0 mL with 0.05 M EDTA, and the absorbance of the solution was measured at 412 nm. The GSH concentration in the lens was expressed as μmole GSH/g wet weight. The pellets of these samples were further processed for protein-thiol mixed disulfides assay as described below.

Lens Protein-thiol Mixed Disulfides Determination:

The pelettes of the samples from GSH assay were washed twice with 1 mL of 10% TCA followed by one wash with 1 mL of methanol-ether (1:1, v:v) mixture. The pellets were then dried overnight on a hot plate at 45°C. Both protein-glutathione mixed disulfide (PSSG) and protein-cysteine mixed disulfide (PSSC) in the dried samples were then oxidized by performic acid to release the non-protein thiol moiety. Into each sample (< 25 mg), 125 µL of 88% formic acid was added, which was followed by 0.5 mL performic acid. The oxidation was stopped by adding 5 mL of chilled water. The non-protein thiol moiety was separated and quantitated by ion exchange chromatography as described previously (Lou et al, 1986). The level of protein-glutathione and protein-cysteine was thus expressed as glutathione sulfonic acid (GSO_3H) and cysteic acid (CSO_3H), respectively.

Lens Protein Electrophoresis:

The lenses were first separated into water-soluble (WS) and urea-soluble (US) fractions by the following procedure: each individual rat lens was homogenized in 1 mL phosphate buffer saline (PBS), pH 7.4, followed by centrifugation at 16,000 X g for 20 min. The supernatant was collected as the WS protein fraction and the pellet, after washing twice with PBS, was suspended in 75-100 µL of 7 M urea, 50 mM Tris, pH 7.4. After standing overnight the urea was diluted with an equal volume of H_2O and the solution was centrifuged again. The supernatant was designated as the US fraction. Both the WS and US proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) on 15% slab gels according to Laemmli (1970). Samples were heated at 100°C for two minutes in 1% SDS with and without 1% 2-mercaptoethanol before being applied to the gels. Equal volume of samples was used for each lane on the SDS gel plate. Cross-linked HMW aggregates and protein profile changes can easily be seen on such a gel.

Choline and Rubidium Transport Systems:

^{3}H-choline and ^{86}Rb were used as tracers to study the lens transport systems. For the in vivo studies, freshly dissected lenses from rats were first incubated in medium-A, the radioisotopes were then added into the media after one hour of equilibration. For the *in vitro* experi-ments, the tracers were added into the culture media directly. After four hours incubation, the lenses were removed, rinsed with PBS and homogenized in 1 mL 10% TCA followed by centrifugation (1,650 x g, 30 min). One-hundred microliters of each supernatant and an equal volume of the corresponding media were counted on a LKB 1219 Rackbeta Scintillation Counter. Using 62% of the lens weight as lens water, the accumulation of choline and rubidium could be expressed as the concentration ratio (L/M) of radiolabeled compound in lens (L) to that in the corresponding medium (M). A decreased L/M value represents a decreased transport function.

Na^+/K^+-ATPase activities:

Rat lens epithelia and decapsulated lenses were homogenized in glass homogenizers in 0.4 mL and 0.8 mL of 0.23M Tris-HCl buffer (pH 7.5 at 20°C), respectively. The supernatant of each sample was added into two reactive systems, with and without the addition of ouabain. Na^+/K^+-ATPase activity was determined by monitoring the free phosphate produced by the action of ouabain-sensitive Na^+/K^+-ATPase according to Bonting et al. (Bonting et al., 1961). The enzyme activity was expressed in μmole Pi/g wet weight/hour.

Dulcitol Analysis:

The lenses were homogenized in 1.0 mL 0.3 N zinc sulfate. The homogenates were then transferred to microfuge tubes and mixed thoroughly with an equal volume of 0.3 N barium hydroxide. The sample was then centrifuged at 15,000 g for 30 minutes in an Eppendorf 5414 microfuge (Brinkman Instruments, Inc.,

Westbury, NY). The supernatant was then transferred to a 5 mL glass vial in which it was frozen and lyophilized. The dried residue was reconstituted in 400 µL H_2O. After further dilution (100-fold) 10 to 40 µL of this sample was typically injected onto the chromatographic system. The chromatographic system is a Dionex BioLC consisting of an HPLC pump, a pulsed amperometric detector (PAD), an eluent degassing module, two HPIC-AS6 anion exchange columns connected in tandem and 2 HPIC-AG6 guard columns (Dionex Corp., Sunnyvale, CA). An SP4270 intergrating chart recorder (Spectra-Physics, Inc., San Jose, CA) was used to collect the data. The mobile phase (45mM NaOH) was delivered by the HPLC pump at 1.0 mL/min, pressure approximately 2,000 psi. The dulcitol peak was identified by coeluting with standard dulcitol. Quantitation of the dulcitol in the sample was accomplished by comparison of sample peak area with that of a known amount of standard.

Naphthalene Metabolites Detection:

The detection of naphthalene metabolites in the lens and aqueous humor may reveal which naphthalene metabolites reache the lens and causes the impairment directly. The most likely metabolites from naphthalene are 1,2-naphthoquinone and naphthalene dihydrodiol. Both compounds can be measured with a reversed-phase (C18, 4.5 X 150mm) HPLC system. Each lens sample was homogenized in 0.45 mL PBS and centrifuged at 1,650 x g for 30 min. The supernatant was adjusted to 2% TCA and centrifuged at 16,000 X g (Eppendorf) for 15 minutes to remove proteins. After filtering through a 0.45 µm filter, this TCA extract was used for HPLC injection. Aqueous humor samples were pooled from six rat eyeballs, diluted (1:3) with water and filtered as above. Two hundred (200) µL of the filtered sample was injected onto the column and eluted with a gradient of 5%-50% acetonitrile at 1.0 mL/min. The eluate was continuously monitored at 250 nm. Our preliminary study (Xu et al., 1989) showed that standard 1,2-naphthoquinone

(at 0.25 nmole) and naphthalene dihydrodiol (at 1 nmole) could be resolved completely and emerged at 13 and 16 minutes, respectively (Figure 5).

Dihydrodiol Dehydrogenase Assay:

The enzyme dihydrodiol dehydrogenase (EC 1.3.1.20.) converts naphthalene dihydrodiol to 1,2-naphthoquinone. If this enzyme is the key step in naphthalene cataract formation, the efficacy of AL01576 on naphthalene cataract formation may be through the inhibition of this enzyme. To study the enzyme activity, two rat lenses were homogenized in 1 mL of 20 mM potassium phosphate buffer (pH 7.5, containing 0.15 M KCl, 0.5 mM EDTA and 5 mM β -mercaptoethanol). The homogenant was first centrifuged at 4°C, 1,650 X g for 10 minutes. Then the supernatant was transferred into an eppendorf tube and centrifuged at 16,000 X g for 30 minutes. The second supernatant contained the enzyme and was used in the assay system. The enzymatic reaction was set up in a cuvette by adding 1.8 mL Glycine buffer (pH 9.0, 0.1 M), 100 µL naphthalene dihydrodiol (20 mM) and 40 µL $NADP^+$ (10 mM). After the baseline was established, the enzyme reaction was started by adding 20 µL lens homogenate (containing the enzyme). The production of NADPH by the reaction was monitored by a fluorescence spectrophotometer at 456 nm (excitation at 340 nm, Perkin-Elmer 650-10S Fluorescence Spectrophotometer) and the enzyme activity could be calculated from the rate of NADPH production (Bolcsak and Nerkand, 1983).

Experimental Protocol

To understand the mechanism of naphthalene cataract formation and the action of AL01576 in the prevention of this cataract, we established the models to mimic such a disease in living animals and in lens culture system as our research

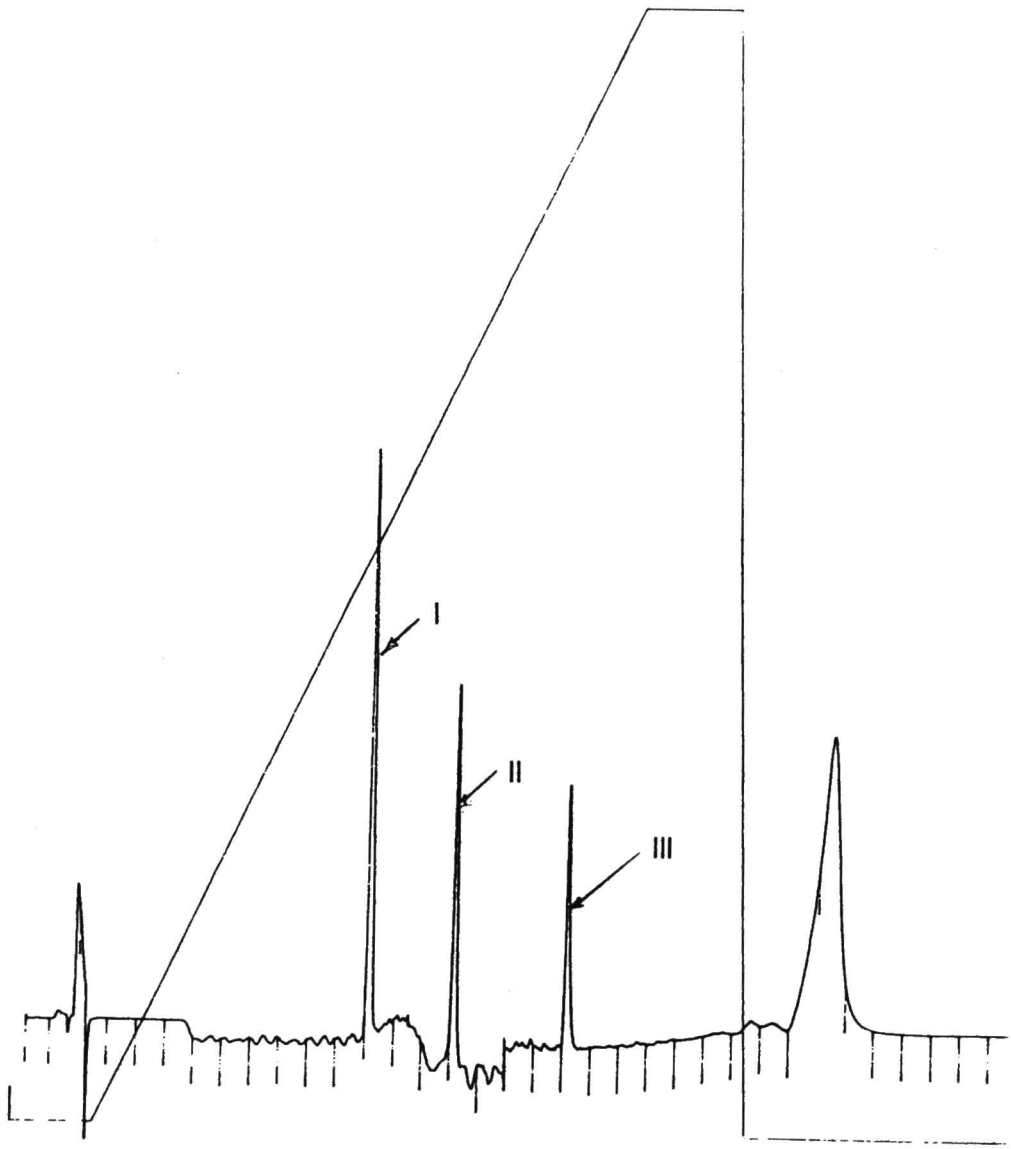


Figure 5. HPLC profiles of naphthalene dihydrodiol, 1,2-naphthoquinone and AL01576. I: Naphthalene dihydrodiol (1.0 nmole); II: 1,2-naphthoquinone (0.25 nmole); III: AL01576 (1.0 nmole). See METHODS section for detail.

tools. The experiment was divided into four distinct studies. **Study I** was planned to establish an *in vivo* naphthalene cataract model in rats and to study the morphological and biochemical characteristics of this cataract. AL01576 was used as a tool to either prevent or intervene with the formation of this cataract. **Study II** was designed to establish cataract models *in vitro* caused by different naphthalene metabolites in an organ lens culture system. Both morphological and biochemical techniques were used to identify which compounds could mimic the lenticular changes observed in the *in vivo* model. The preventive effect of AL01576 was also tested in this system. **Study III** focused on revealing the mechanism underlying naphthalene cataract formation including the function of pigmentation and the biochechemical changes that occurred during cataract development. **Study IV** studied the action of AL01576 in the prevention of naphthalene cataract formation.

Study I: Naphthalene Cataracts in Rats

Induction of Cataract and its Morphological Observations

Morphological changes are usually the most important parameter to evaluate if a cataract model is successful or if an anti-cataract medicine works. Therefore, the morphological changes were observed very carefully in this study. Male rats (125-150 g) were used in the following experiments typically containing 4 groups of rats: normal control, naphthalene, naphthalene + AL01576, and AL01576 only. The dosage of naphthalene and AL01576 were 1.0 g/kg/day and 10 mg/kg/day respectively. The feeding lasted up to 6 weeks and the photographs were taken at different stages for every typical change.

Effects of Various AL01576 Dosages on Naphthalene Cataract Development

Four different dosages of AL01576 were tested for their preventive effects on naphthalene cataract development in Long-Evans rats fed with 1.0 g/kg/day of

naphthalene. The dosages of AL01576 used in this study were 0.4, 1.0, 4.0 and 10.0 mg/kg/day. The 2 control groups were normal control rats which were dosed with mineral oil plus ARI vehicle and naphthalene-fed rats which were given naphthalene plus ARI vehicle. The rats were treated up to four weeks and their lenses were examined with the procedure as described earlier.

Prevention and Intervention of Naphthalene Cataract with AL01576

AL01576 was given to naphthalene-fed (1 g/kg/day) rats at different stages during the course of cataract development. In the prevention study, the rats were treated with AL01576 one hour before naphthalene feeding on the same day. In the intervention studies, the rats were given naphthalene for 1 or 2 weeks before starting ARI dosing. The AL01576 suspension and naphthalene solution were delivered to the rats by gavage needles, and the opacities in the lenses were examined and graded at the intervals of 3, 7,14, 21 and 28 days. Typically each set of experiments consisted of the following four groups with 4-6 rats per group: normal control, naphthalene-fed, naphthalene plus ARI, and normal control plus ARI.

Biochemical Analysis

Biochemical changes were also studied to demonstrate the changes possibly underlying the disease, especially those that occurred before visual damages. The lens GSH levels were studied in two ways. To measure the acute effects of naphthalene on lens GSH, the rats were treated with a single dose (1.0 g/kg) of naphthalene and then sacrificed at intervals of 4, 8, 12 and 24 hours. To study the chronic effects, the rats were dosed daily with naphthalene (1.0 g/kg/day) and the lenses were obtained at intervals of 2, 4 and 6 weeks. For all the other parameters, lens samples were collected at the ends of the 2nd, 4th and 6th weeks of naphthalene feeding.

Study II: Naphthalene-related Cataracts in Lens Culture

Induction of in vitro Cataracts by Naphthalene Metabolites

The cataractogenesis of each naphthalene metabolite can be tested directly in lens culture system. According to van Heyningen (1979), there are five intermediate metabolites which may be involved in naphthalene cataract development. They are: 1-naphthol, 2-naphthol, naphthalene dihydrodiol, 1,2-naphthoquinone and 1,2-dihydroxynaphthalene. In this study, these metabolites were dissolved in 4% ethanol at 2.5 or 5.0 X 10^{-3} M as stock solutions and were added into the medium-B (1:100) to give a final concentration of 2.5 or 5.0 X 10^{-5} M. Rat lenses were preincubated in medium-A for one hour before they were exposed to these metabolites. The media were renewed daily and the lenses were usually cultured for 48 hours.

Comparison of In Vitro Cataracts with the In Vivo Model

To compare the morphological changes, the lenses were examined under a microscope during the course of cataract development. The lenticular opacities were photographed with a Wild Heerbrugg microscope-camera system (Wild Photoautomat MPS 45, Switzerland). In order to compare these changes with the cataract of naphthalene-fed rats, the fresh lenses from *in vivo* studies were collected, transferred into medium-A, and examined or photographed under the same condition. The metabolite which caused a cataract similar to that in the naphthalene-fed rat could be easily identified.

For the biochemical studies, the rat lenses were collected at different stages of naphthalene feeding or harvested from the *in vitro* lens culture. The lenses were processed and assayed under the same conditions. Comparison of the biochemical changes could provide further evidence to support the hypothesis based on the morphological observations.

Effect of Naphthalene Dihydrodiol Concentration on the Cataract Formation

A dose-response study of the effect of naphthalene dihydrodiol on the cultured rat lenses was conducted. The stock solutions were made in 20% ethanol at 5 X 10^{-2} M which was diluted into medium-B to bring the final concentrations of naphthalene dihydrodiol to three levels: 2 X 10^{-5} M, 1 X 10^{-4} M and 5 X 10^{-4} M. Fresh rat lenses were pre-incubated in medium-A for 1 hour and then transferred into medium-B containing different concentration of naphthalene dihydrodiol. The media were renewed after 24 hours and the lenses were cultured for 48 hours. At the end of the study, the lenses were photographed for morphological comparisons and the lens GSH level and lens active transport systems were examined.

Effects of Culture Time on the Cataract Development

The temporal relationship between naphthalene dihydrodiol exposure and cataract development was also studied. The concentration of naphthalene dihydrodiol was fixed at 2.5 X 10^{-5} M throughout the experiment. The rat lenses were pre-incubated as described above and then exposed to naphthalene dihydrodiol for up to 72 hours. Again, the media were refreshed every 24 hours. At 24, 48 and 72 hours, the lenses were photographed and some lenses were harvested for biochemical studies. Some lenses from *in vivo* studies were processed at the same time so that the data were comparable to the *in vitro* experiment.

Effects of AL01576 Dosage on the Cataract Formation

The ability of AL01576 to prevent naphthalene dihydrodiol cataract was tested in two ways. In one experiment, AL01576 at 2 X 10^{-5} M (final concentration) was added to media containing three concentrations of naphthalene dihydrodiol: 2 X 10^{-5} M, 1 X 10^{-4} M and 5 X 10^{-4} M. The lenses were pre-incubated in medium-A containing 2 X 10^{-5} M of AL01576 for 1 hour and then transferred into the above media. The test period is 48 hours with one change of fresh media at 24 hours.

In a separate experiment, the rat lenses were exposed to a constant concentration of naphthalene dihydrodiol (1.0×10^{-4} M) but treated with varied amounts of AL01576. Three concentrations of AL01576 used were 1×10^{-5} M, 2.5×10^{-5} M and 1×10^{-4} M. The lenses were preincubated with AL01576 for one hour and transferred into the media containing both naphthalene dihydrodiol and AL01576. As the other experiment, the lenses were cultured for 48 hours with one renew of media. Both morphological and biochemical studies were performed at 48 hours..

Study III: Mechanism for Naphthalene Cataract Formation

Effects of Pigmentation on the Cataract Formation

The effect of pigmentation on the *in vivo* model was examined by comparing the morphological and biochemical changes in both albino and pigmented rats. Five strains of rats were used in this study, including two pigmented strains and three albino strains. The rats were dosed with naphthalene and AL01576 with the standard procedure for 6 weeks. The morphological changes were observed with a slit-lamp microscope and the biochemical changes were studied on the lenses collected at 0, 2, 4 and 6 weeks.

In a separate study, Long-Evans (pigmented) and Sprague-Dawley (albino) rats at 2-4 per each group were fed with 1-naphthol at 1.0 g/kg/day for 4 weeks. The lenticular changes were studied as above.

The relationship between pigmentation and cataract were also tested in the *in vitro* system. Fresh lenses from both Long-Evans and Sprague-Dawley rats of the same age were cultured and exposed to 2.5×10^{-5} M of naphthalene dihydrodiol under identical conditions. After 48 hours, the lenses from the two strains were compared for lenticular opacities and biochemical changes.

Evidence for the involvement of Oxidation

In the lens, the major targets for oxidants are the thiol groups, either in free glutathione (GSH) or in proteins. Through enzymatic reactions, GSH can reduce oxidants (like H_2O_2) and thus protect the reduced status of proteins. When the oxidative stress exceeds the defensive ability of the lens, oxidized glutathione (GSSG) accumulates and conjugates with protein thiol to form protein-thiol mixed disulfides (PTMD). GSH and PTMD were studied in both the whole lens and the different portions of the lens. In the *in vivo* studies, rat lenses were collected at 2, 4 and 6 weeks of naphthalene feeding, and the samples from the *in vitro* studies were harvested at 24, 48 or 72 hrs. The supernatants of lens TCA homogenants were taken for GSH measurement and the pellets were further processed for ion-exchange chromatographic quantitation of GSO_3H and CSO_3H which represent protein-glutathione and protein-cysteine mixed disulfides respectively.

Oxidants could further react with PTMD and induce the formation of cross-linked HMW aggregates. This potential change was examined using SDS-PAGE electrophoresis on a 15% slab gel. The *in vivo* samples were from control rats, naphthalene-fed and naphthalene plus AL01576 at the 2nd and the 4th weeks of treatment. The *in vitro* samples were from lenses exposed to naphthalene dihydrodiol and to 1,2-naphthoquinone for 48 hours.

Detection of Naphthalene Metabolites in the Eye Tissues

The direct evidence for the involvement of specific naphthalene metabolites in cataract formation could be obtained by detecting those naphthalene metabolites in eye tissues. In this study, Long-Evans rats were treated with naphthalene, or naphthalene plus AL01576 or their vehicles for 6 weeks. The lenses and the aqueous humor were then collected and processed as mentioned above. The naphthalene metabolites could be separated by a reversed-phase HPLC column and quantitated with a UV detector at 250 nm. The detected metabolites and their concentrations were compared with the standard compounds used in the

lens culture system.

Study IV: The Action of AL01576 in Naphthalene Cataract Prevention

In Vivo Dual Cataracts Induced by Galactose and Naphthalene

Naphthalene-induced cataract in rats appeared in the deep cortex while the sugar cataract caused by high galactose (30%) feeding occurred in the outer layer of the cortex. Therefore, it is possible to establish a dual cataract model with 2 distinct zones of opacity when the rats were fed with naphthalene and galactose simultaneously. In this attempt, Long-Evans rats were fed with 30% galactose chow for 2 weeks. During this period, the rats were also dosed with naphthalene by gavage at 1.0 g/kg/day. At the end of the experiment, the lenses were photographed and processed for biochemical studies.

In Vitro Dual Cataracts Induced by Galactose and Naphthalene Dihydrodiol

Production of dual cataracts was also tried in the *in vitro* system. Medium A-D listed in Table 2 were used for this purpose. Fresh rat lenses were pre-incubated in medium-A for 1 hour before being transferred to different experimental groups. Medium-B was used either in control group when it was added 0.04% ethanol or in cataract model when it was loaded with naphthalene dihydrodiol. Medium-C was used to induce galactose cataract and medium-D was used to induce dual cataracts by adding 5×10^{-5} M naphthalene dihydrodiol. The incubation was continued for 48 hours with one change of fresh media at 24 hours. The morphological and bichemical changes were studied and compared with the *in vivo* dual cataract model.

Comparison of Different ARIs on Naphthalene-related Cataracts

The efficacy of different classes of ARIs was compared to determine if aldose

reductase was involved in naphthalene cataract and if AL01576 prevented such a cataract through its inhibition of cytochrome P-450 in the first step of naphthalene metabolic pathway (Figure 1). Four ARIs were tested in this study, including AL01576, AL04114, Sorbinil and Tolrestat. Long-Evans rats were fed with naphthalene as described above and were given one of the ARIs at 10 mg/kg/day. However, when the Ayerst ARI, Tolrestat was used, it was necessary to dose the rats two weeks before naphthalene feeding and at the dosage of 50 mg/kg/day since this ARI's pharmacokinetics is such that it is slow to penetrate into lens tissue and also it is less efficacious than other ARIs in preventing sugar cataract formation. During the four weeks of treatment, opacities were graded and some biochemical changes were studied.

In the *in vitro* study, lenses were pre-incubated with medium-A containing 5 X 10^{-5} M ARI for one hour before switching to medium-B containing both ARI and naphthalene dihydrodiol, both at the concentration of 5 X 10^{-5}M. The experiment consisted of the following six groups with 6 lenses per group: normal control, naphthalene dihydrodiol (ND), ND + AL01576, ND + AL04114, ND + Sorbinil and ND + Tolrestat. The culture period was 48 hours with 1 change of fresh medium at 24 hours.

Comparison of AL01576 and Tolrestat on the Dual Cataract Model

Both AL01576 and Tolrestat have been shown very effective in preventing sugar cataract induced by galactose. However, our preliminary data indicated that only AL01576 completely prevented naphthalene cataract while Tolrestat showed no preventive effect against this cataract. Such a difference between the two ARIs provided a powerful tool to examine the role of aldose reductase in naphthalene cataract formation. It would be very interesting to test these 2 ARIs for their effects on a dual cataract induced by high galactose and naphthalene.

The efficacy of either Tolrestat or AL01576 on the *in vivo* dual cataract model

was compared with groups of rats given either naphthalene alone or galactose alone. Because of the difference of pharmacokinetics and potency of these two ARIs, rats treated with Tolrestat had to be predosed for 2 weeks at 50 mg/kg/day and rats treated with AL01576 were predosed 1 hour at 10 mg/kg/day before starting the feeding and treatment regimens. The efficacious of AL01576 and Tolrestat, both at 5×10^{-5} M, were also tested in the *in vitro* dual cataract model. In either the *in vivo* or the *in vitro* study, a total of 10 groups were used at 3-4 rats or lenses each: control normal, dual cataract, dual cataract + AL01576, dual cataract + Tolrestat, galactose, galactose + AL01576, galactose + Tolrestat, naphthalene, naphthalene + AL01576, naphthalene + Tolrestat (Table 4).

Analysis of Dihydrodiol Dehydrogenase Activity of the Lenses

Dihydrodiol dehydrogenase in the lens catalyzes the formation of 1,2-naphthoquinone from naphthalene dihydrodiol, therefore it may play an important role in such a cataract formation. The study on this enzyme could reveal the possible metabolic pathway of naphthalene in the lens and the possible target of AL01576 action. To prepare the sample, 2 lenses from Long-Evans rats were homogenized in 1.0 mL potassium phosphate buffer. After centrifugation, the supernatant was used to assay the enzyme activity and also the inhibitory effect of AL01576 on its activity.

Data Analysis

All the data are presented as mean $\pm$ S.D. and (n) refers to the number of sample in each group. The data from each individual experiment were subjected to appropriate statistical analysis with $P < 0.05$ considered statistically significant. Statistical analyses were executed by the SAS statistical program (version 6.07)

TABLE 4. Experimental Groups in the Dual Cataract Models.

Group	Galactose	Naphthalene	AL01576	Tolrestat
control	none	none	none	none
galactose 1	*	none	none	none
galactose 2	*	none	#	none
galactose 3	*	none	none	@
naphthalene 1	none	$	none	none
naphthalene 2	none	$	#	none
naphthalene 3	none	$	none	@
dual 1	*	$	none	none
dual 2	*	$	#	none
dual 3	*	$	none	@

* In the *in vivo* study, 30% galactose rat chow was used, and for lens culture 30 mM of galactose was present in the medium..

$ For the *in vivo* study, the dosage of naphthalene was 1 g/kg/day; for the *in vitro* experiments, naphthalene dihydrodiol was used at 5×10^{-5} M.

\# 10 mg/kg/day for the *in vivo* studies and 5×10^{-5} M for the *in vitro* studies.

@ 50 mg/kg/day for the *in vivo* studies and 5×10^{-5} M for the *in vitro* studies.

through an IBM compatible computer. Graphical presentations were plotted with Sigma-Plot for Windows (version 1.0, 1993), a computer software obtained from Jandel Corporation (Sausalito, CA).

CHAPTER III

RESULTS

Study I: Naphthalene Cataract Model in Rats

Morphological Observations

The rats fed with naphthalene developed cataract with special morphological characteristics (Figures 6, 7 and 8). Under the slit-lamp examination, a typical cataract started as waterclefts and spoke-like opacities in the cortex at one week after naphthalene feeding. By the third week these features merged to form an opaque shell in the deep cortex region. With longer naphthalene feeding time (4-6 weeks), the opacification became denser and slightly deeper. The opacities appeared yellow or light brown in color when examining the lenses after they were removed from the eyeballs. AL01576 demonstrated an evidently preventive effect on this cataract. The lenses from all rats treated with naphthalene plus AL01576 or with AL01576 alone remained clear during the six weeks treatment (Figure 8).

Besides cataract, naphthalene feeding caused damages to other eye tissues like retina and vitreous. These eye lesions will not be discussed in the present study. Some systemic disorders were also observed in the naphthalene-fed rats, including diarrhea, decreased growth (Figure 9), loss of hair and occasional death. None of these extraocular insults were prevented or ameliorated by ARI such as AL01576 (Figure 9).

In contrast to naphthalene, 1-naphthol feeding to Long Evans and Sprague-Dawley rats at the same dosage as naphthalene produced no trace of opacity at

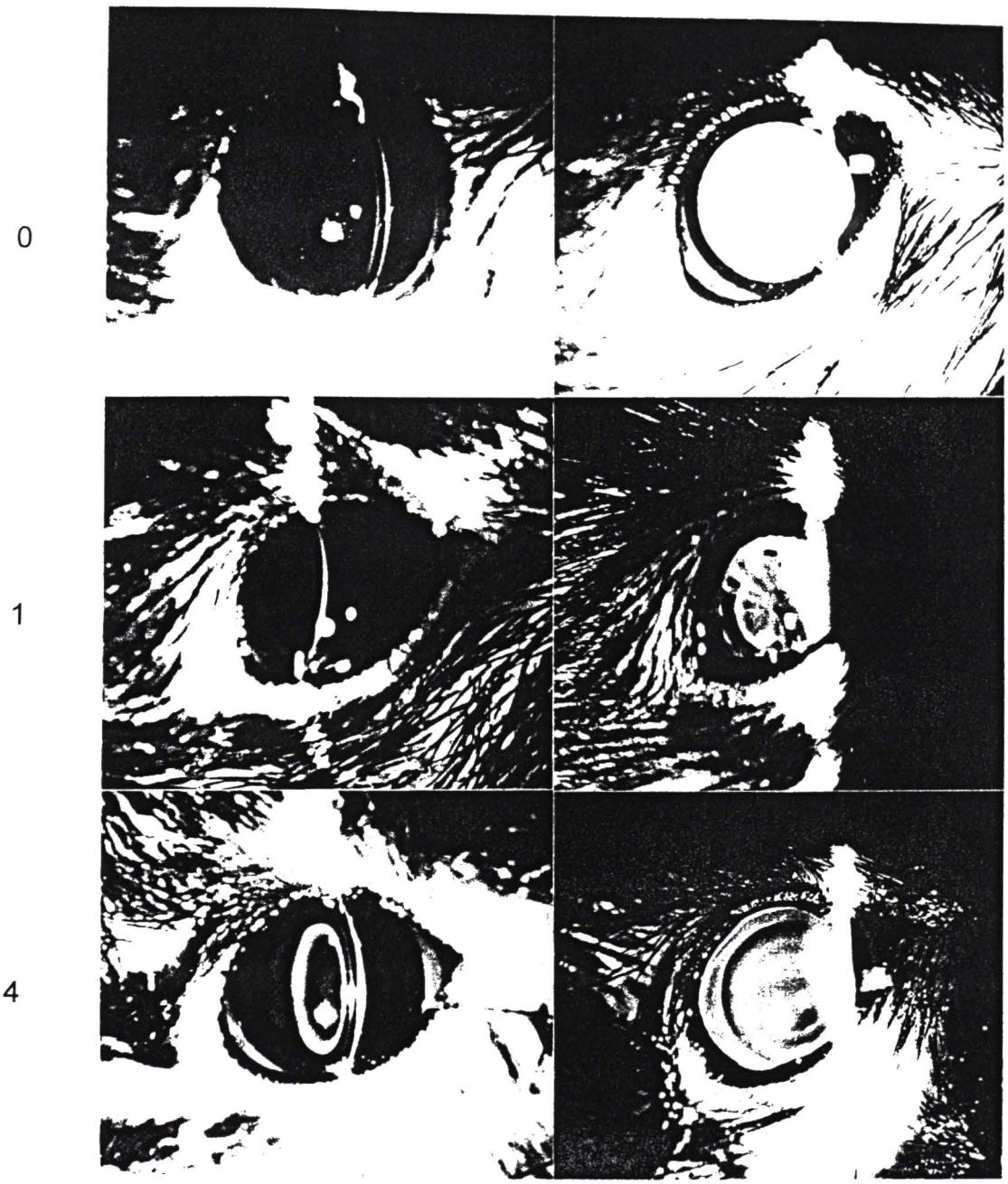


Figure 6. Slit-Lamp Images of Cataract in Naphthalene-fed Rats. Left column: direct illumination view; Right column: retro-illumination view. 0: baseline; 1: one week of naphthalene feeding; 4: four weeks of naphthalene treatment.

Figure 7. Slit-lamp observation of naphthalene-induced cataract in Sprague-Dawley (albino) rat and Long-Evans (pigmented) rat. The rats were fed with naphthalene at 1 g/kg/day for two weeks and the lenses were photographed through a slit-lamp.

Top: Sprague-Dawley rat; Bottom: Long-Evans rat.

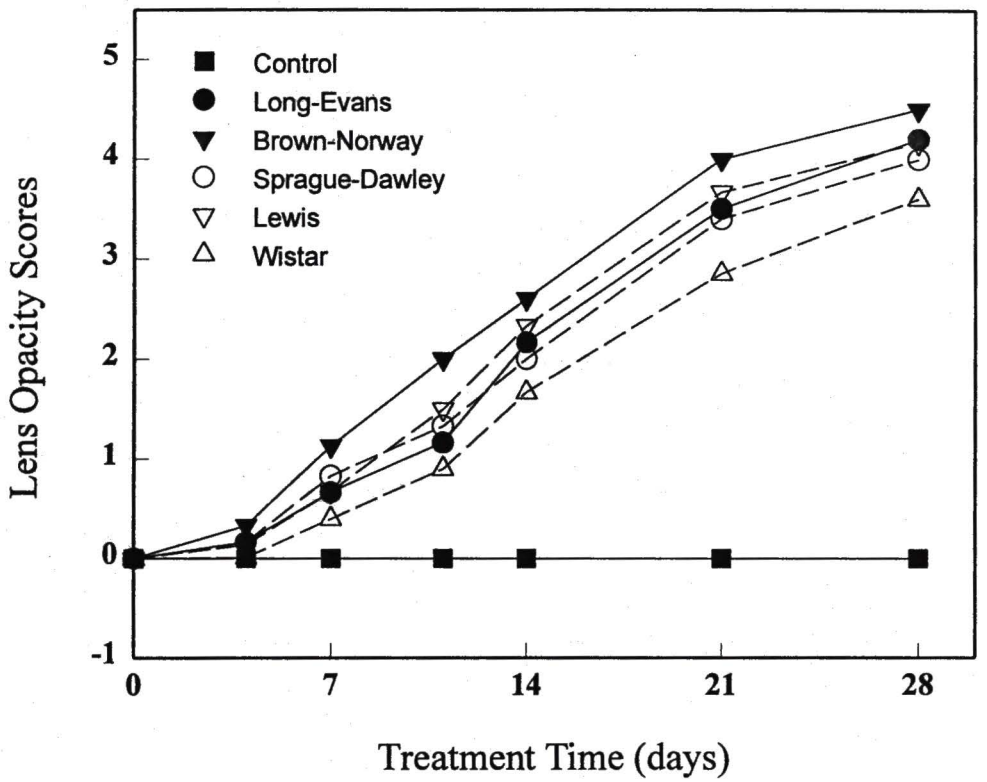


Figure 8. Comparison of the susceptibility of different strains of rats to naphthalene feeding with or without the treatment of AL01576. Naphthalene was given at 1.0 g/kg/day and AL01576 was dosed at 10 mg/kg/day. The lenses were examined by a slit-lamp and the opacities were graded with a six-score system (see METHODS). No significant difference was found among different strains (t-test for slopes , $P > 0.05$). The lens opacity scores for the rats fed with AL01576 or naphthalene plus AL01576 overlaped with those of control rats.

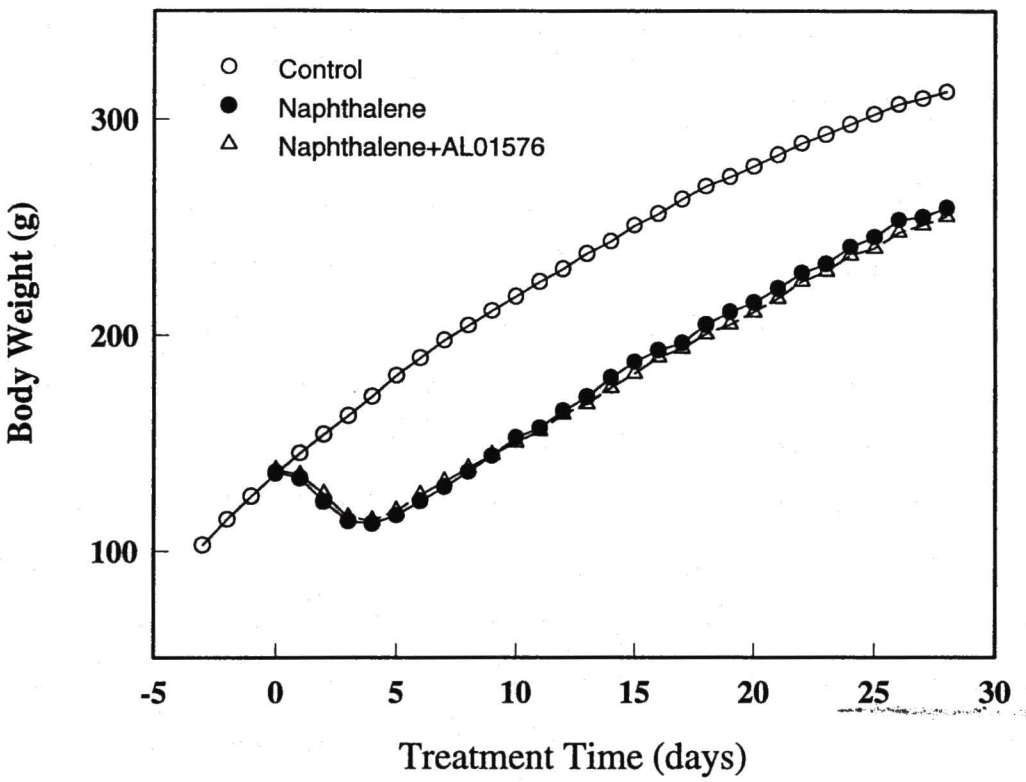


Figure 9. The change in body weight of Long-Evans rats fed with naphthalene (1.0 g/kg/day) or naphthalene plus AL01576 (10 mg/kg/day) during the test period.

the end of 4 weeks (data not shown).

Biochemical Characteristics of the Model

1) Lens Glutathione Level: During the 24 hours following a single dose of naphthalene, only the GSH level of the capsule-epithelium was affected. As shown in Figure 10, GSH significantly decreased to 60% of the normal level at 8 hours and recovered by 12 hours postfeeding.

During long term administration of naphthalene, whole lens GSH concentration was lowered to 70-80% of the control (Table 5). However, when examining the change in different regions of the lens, both the capsule-epithelium (data not shown) and the cortex lost about 30% GSH by one week and remained at this low level throughout the end of the six weeks (Figure 11). GSH depletion in the lens could be prevented by AL01576 treatment (Table 5). In the cortex region, the GSH level recovered from 70% to nearly 90% of the control level at the end of the 10th day (Figure 11) and was maintained at this level throughout the entire six weeks (data not shown).

Glutathione was also determined in 1-naphthol-fed rats. Both the albino rats and pigmented rats were tested. The GSH level remained unchanged after four weeks treatment and showed no difference between the two strains (Table 6).

2) Protein-thiol Mixed Disulfides: The levels of lens protein mixed disulfides were compared in rats treated with naphthalene for four weeks with and without AL01576. Little change was found in the protein-cysteine mixed disulfide (expressed as CSO_3H) content. However, a marked increase was observed in the protein-glutathione mixed disulfide (expressed as GSO_3H) in the naphthalene group. This increase was completely prevented by AL01576 treatment. AL01576 alone did not change either bound cysteine or bound glutathione level in the lens. Table 5 summarizes a typical study of a four week naphthalene experiment. No

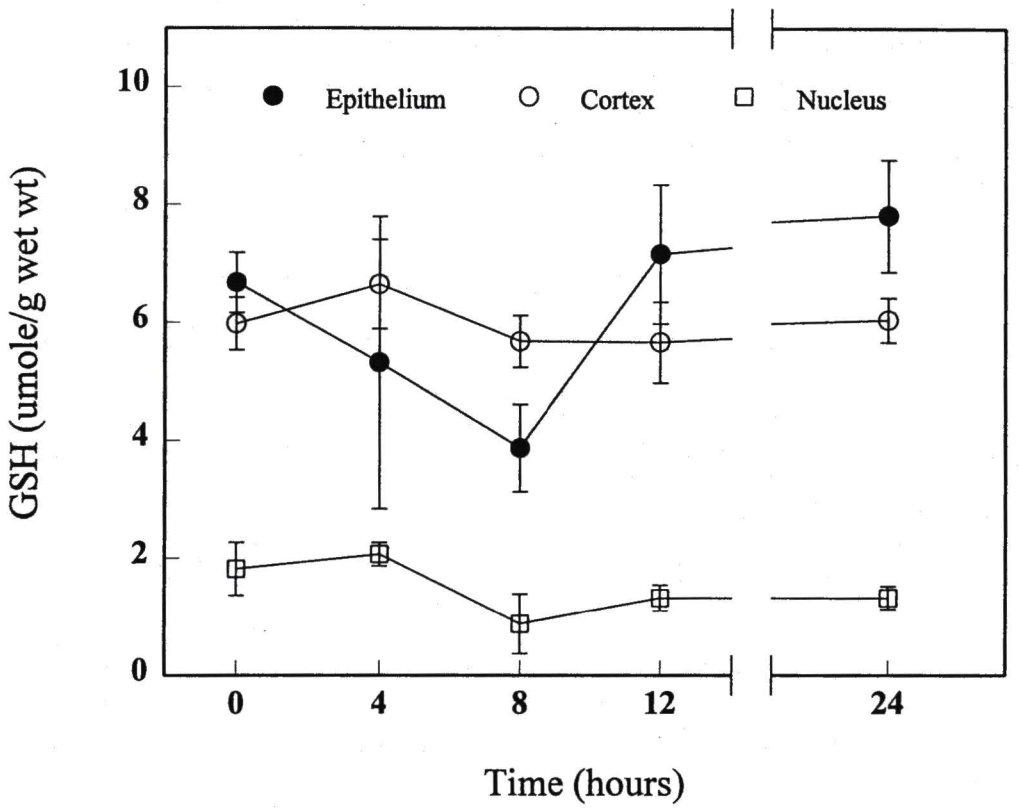


Figure 10. Lens regional GSH levels in Long-Evans rats treated with a single dose of naphthalene (1.0 g/kg). Samples were collected at intervals of 4, 8, 12 and 24 hrs after naphthalene feeding. GSH level was measured in capsule-epithelium, cortex and nucleus. Data are expressed as mean ± S.D. of five samples. Significant difference was only found in the epitheliun between the 8 hours group and the control, the 0 time group (independent t-test, $P < 0.01$).

TABLE 5. Lens GSH and Protein-thiol Mixed Disulfides Levels in Rats Fed With Naphthalene and/or AL01576.

Group (n)	GSH	GSO_3H	CSO_3H
Long-Evans			
Control (6)	5.78 ± 0.45	0.07 ± 0.09	0.69 ± 0.24
Naphthalene (6)	4.74 ± 0.44*	1.40 ± 1.01*	0.98 ± 0.47
Naph.+AL01576 (4)	5.31 ± 0.37	0.05 ± 0.01	0.64 ± 0.08
AL01576 (4)	5.87 ± 0.38	0.04 ± 0.01	0.68 ± 0.02
Sprague-Dawley			
Control (4)	6.76 ± 0.20	0.04 ± 0.01	0.67 ± 0.10
Naphthalene (4)	4.68 ± 0.54*	0.72 ± 0.30*	0.87 ± 0.20
Naph.+AL01576 (3)	6.01 ± 0.61	0.06 ± 0.01	0.58 ± 0.05
AL01576 (4)	7.09 ± 0.04	0.05 ± 0.01	0.64 ± 0.07

The rats were fed with naphthalene at 1 g/kg/day with or without the treatment of AL01576 (10 mg/kg/day) for 4 wks. Data are expressed as µmole/g wet wt and mean ± S.D., and (n) = number of samples.

* $P < 0.01$ (independent t-test, compared with the controls in the same strain).

Mixed Disulfide: GSO_3H = Protein-GSH; CSO_3H = Protein-Cysteine.

Naph. = naphthalene.

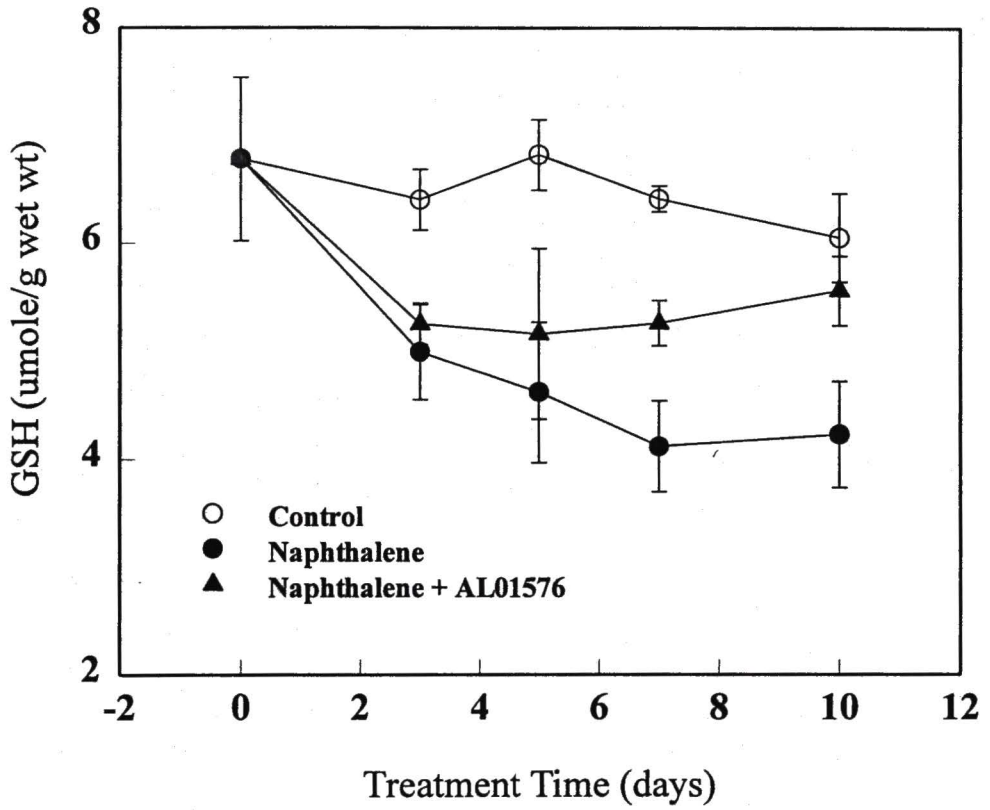


Figure 11. Glutathion levels in the lens cortex of naphthalene-fed rats after multiple dosing. The rats were dosed with naphthalene (1.0 g/kg/day) for 10 days with or without the treatment of AL01576 (10 mg/kg/day). GSH was measured in the cortex region of the rats of normal control, naphthalene-fed and naphthalene plus AL01576. Data are expressed as mean ± S.D. of six samples.

TABLE 6. Lens GSH Levels in Pigmented and Albino Rats Fed With 1-naphthol and/or AL01576*.

Group (n)	GSH (μmole/g wet wt)
Long-Evans	
Control (6)	5.78 ± 0.45
1-naphthol (4)	6.10 ± 0.40
1-naphthol + AL01576 (2)	6.67 ± 0.33
AL01576 (4)	5.87 ± 0.38
Sprague-Dawley	
Control (4)	6.76 ± 0.20
1-naphthol (2)	7.76 ± 0.30
1-naphthol + AL01576 (4)	7.48 ± 0.21
AL01576 (4)	7.09 ± 0.40

* The rats were dosed with 1-naphthol at 1 g/kg/day with or without the treatment of AL01576 at 10 mg/kg/day for four weeks.

Data are expressed as mean ± S.D.; (n) = number of samples. There is no significant difference between any tested group and the control group in the same strain (independent t-test, $P > 0.05$).

significant differences were found in albino versus pigmented strains (t-test, $P < 0.05$).

3) Lens Protein Profiles: Prior to three weeks naphthalene feeding, no change in the electrophoretic profile of the lens proteins was observed. At the end of the third week, protein changes became apparent. As shown in Figure 12, the SDS-PAGE protein profile of the urea soluble fraction was compared before and after β-mercaptoethanol reduction. There is a clear increase in the protein level from the naphthalene-fed rats (compare lane B with lane A). This increase in water insoluble proteins corresponds to a concomitant decrease in water soluble proteins (data not shown). In the reduced sample, the increase in protein content was generalized with no new band evident. In the nonreduced samples, however, there is a large increase in high molecular weight (HMW) material appearing in the stacking gel and at the top of the separating gel. Since these protein bands were not seen following reduction, they must be disulfide cross-linked proteins induced by naphthalene feeding. All these protein changes caused by naphthalene feeding were completely prevented by AL01576 treatment as shown in Figure 12, lane C.

4) Lens Transport Function and Na^+/K^+-ATPase Activity: Throughout the entire study period of 1-6 weeks, lens transport systems were monitored in organ culture by measuring the ability of freshly excised lenses to accumulate ^{3}H-choline or ^{86}Rubidium from the medium. No significant changes were detected in these parameters nor in the activity of the Na^+/K^+-ATPase at any time (t-test, $P < 0.05$). Table 7 summarizes such results after 2 weeks of naphthalene feeding.

Effects of AL01576 Dosage on the Cataract Development

The effects of AL01576 were tested in this model. Only Long-Evans rats were used in this study for the easier observation of the lens opacity on a dark back-

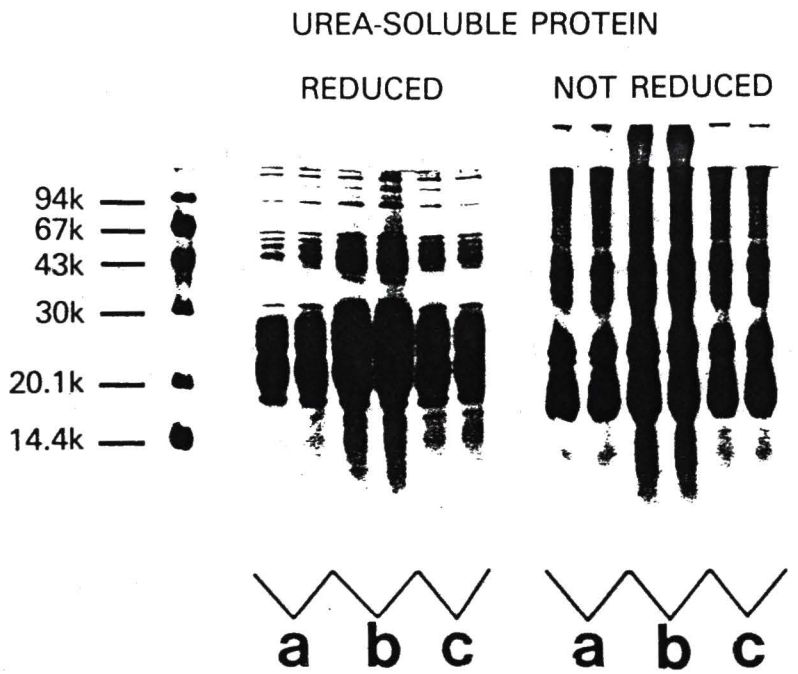


Figure 12. The profiles of the reduced and non-reduced urea-soluble Lens Protein (SDS-PAGE) from Long-Evans rats treated for 3 weeks. The SDS-PAGE patterns of the samples are compared among the following 3 groups: Lane (A): control normal; Lane (B): naphthalene-fed (1.0 g/kg/day); and Lane (C): naphthalene plus AL01576 treatment (10 mg/kg/day).

TABLE 7. Rat Lens Membrane Functions After Two Weeks Treatment of Naphthalene and/or AL01576*

Group	^{3}H-Choline (L/M)	^{86}Rb (L/M)	ATPase Activity (μmole Pi/g wet wt/hr)
Control	8.84 ± 0.79 (5)	7.42 ± 0.43 (5)	17.91 ± 0.88 (3)
Naph.	9.10 ± 2.47 (5)	7.58 ± 1.10 (5)	16.95 ± 1.23 (3)
Naph.+ ARI	8.11 ± 1.86 (5)	7.33 ± 0.67 (5)	17.21 ± 2.01 (3)

* Naphthalene was given at 1.0 g/kg/day and AL01576 was dosed at 10 mg/kg/day. The test period is two weeks.

Data reported as mean ± S.D.; (n) = Number of samples. No significant difference was found among the 3 groups for any parameter (independent t-test, $P > 0.05$).

Naph. = naphthalene; ARI = AL01576.

ground of the pigmented eyeballs. Naphthalene was given with the standard procedure and vried dosages of AL01576 (0.4mg/kg/day to 10mg/kg/day) were given by gavage needle to naphthalene-fed rats one hour before naphthalene feeding. The lens opacity score for each group were compared in Figure 13. The lowest dosage (0.4 mg/kg/day) showed no preventive effect on this *in vivo* cataract formation. Increase of dosage to 1.0 mg/kg/day showed partial prevention of the cataract development. When the dosage of AL01576 is higher than 4.0 mg/kg/day, the naphthalene induced lens opacities were completely prevented.

Although AL01576 showed effective prevention of the cataract formation, the systemic disorders (diarrhea, decreased growth, loss of hair and high mortality) caused by naphthalene feeding could not be prevented or ameliorated by this ARI at all. One of the examples is shown in Figure 9, in which the administration of AL01576 to the rats did not prevent the loss of body weight caused by naphthalene treatment.

Intervention of Naphthalene Cataract with AL01576

Besides being able to prevent naphthalene cataract formation, AL01576 also delayed the cataract development when it was administered at various time intervals after naphthalene feeding began. As shown in Figure 14, when AL01576 was administered (10 mg/kg) to the naphthalene-fed rats at one or two weeks postfeeding and continued to 4 weeks, the opacity scores of these rat lenses were reduced considerably at each treatment point. It is clear that the earlier the intervention started the sooner the cataract was delayed. The 1 week postfeeding intervention allowed the lens to stay at a lower opacification stage and remained so even at the end of the following 3 weeks. The biochemical efficacy of AL01576 was shown in Figure 15. Approximately 2/3 of the GSH loss in naphthalene treated group was prevented in both the 1-week and 2-week intervention groups. Protein-GSH mixed disulfide production which was greatly stimulated by naphthalene feeding (from 0.072 μmole/g wet wt to 2.47 μmole/g wet wt) was decreased

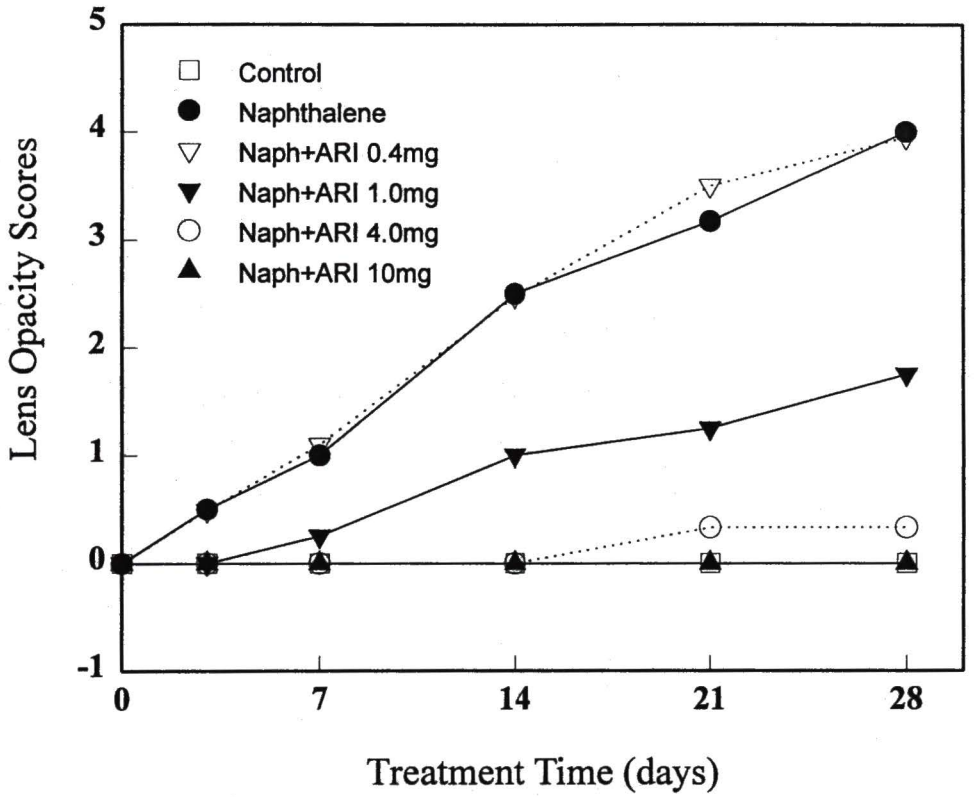


Figure 13. Effect of AL01576 dosage on naphthalene cataract development in Long-Evans rats. The rats were treated for four weeks with naphthalene at 1.0 g/kg/day, with or without AL01576 at desired dosages as shown above. Each data point represents an average of 4-6 rats.

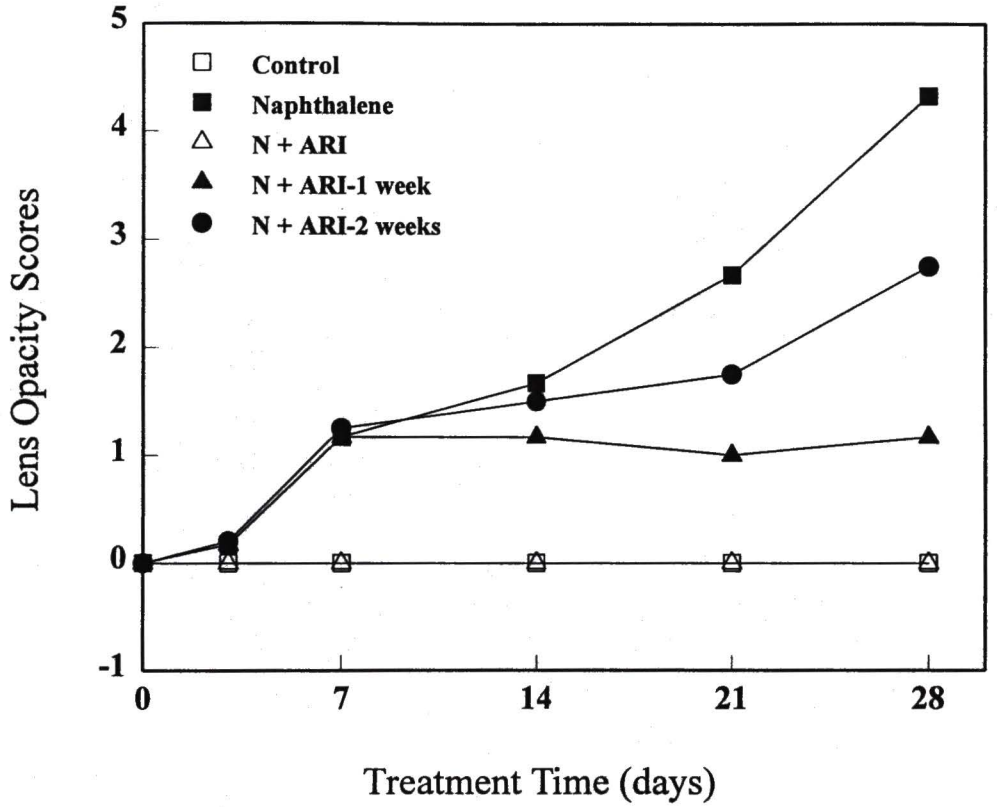


Figure 14. Intervention of rats naphthalene cataract progression by AL01576 adminstration. The rats (Long-Evans) were fed with naphthalene at 1g/kg/day for 4 weeks, and AL01576 was given at 10 mg/kg/day. N+ARI: naphthalene and AL01576 treatments started tigether. N+ARI-1wk: naphthalene feeding started one week earlier than AL01576 treatment. N+ARI-2 wks: naphthalene feeding was two weeks earlier than AL01576 treatment. Each data point represents an average of 4-6 rats.

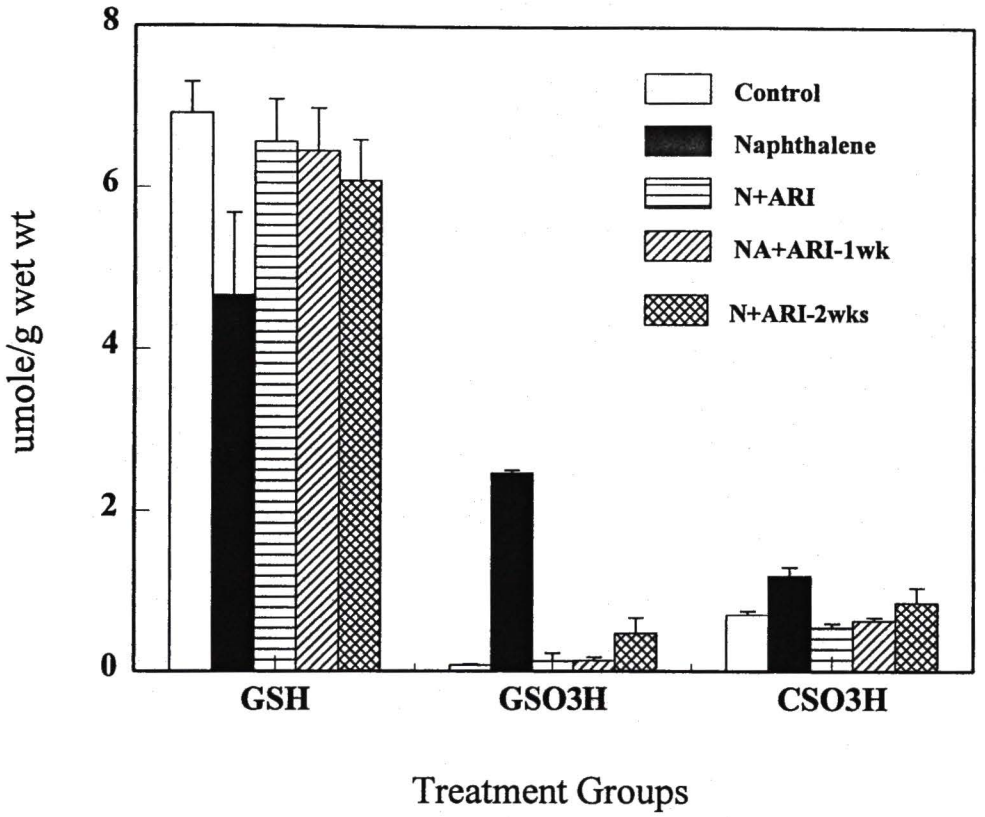


Figure 15. Intervention of naphthalene-induced cataractous changes in lens GSH and mixed disulfides by AL01576. The experimental conditions and group name initials are the same as Fig. 14. Data are expressed as mean ± S.D.; numbers in parentheses indicate the number of samples. Data from the rats treated with naphthalene and AL01576 together overlap with the control group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (independent t-test, compared with the control for the same parameter).

to 0.11 µmole/g wet wt at one week and to 0.47 µmole/g wet wt at two weeks. A similar trend in the protein-cysteine formation was observed in this intervention study.

Study II: Naphthalene-related Cataracts in Lens Culture

Induction of In Vitro Cataracts by Naphthalene Metabolites

Five potential naphthalene metabolites were tested for their ability to induce cataract in the lens culture system. After 48 hours of incubation, the lenses exposed to three compounds, naphthalene dihydrodiol, 1,2-dihydroxynaphthalene and 1,2-naphthoquinone (at the same concentration of 5 X 10^{-5} M), all developed opacities. However, the changes in naphthalene dihydrodiol exposed lenses differed from those caused by the other naphthalene metabolites. Both 1,2-dihydroxynaphthalene and 1,2-naphthoquinone caused brown pigmentation in the lens and generated opacities which were concentrated in the outer layer of the cortex with regions of obvious liquefaction. These lenses were swollen and thus heavier than the normal lens. In contrast, naphthalene dihydrodiol induced no hydration and the cataract was well inside the lens with the opacity forming a shell in the deep cortical region while the nucleus and the outer cortex remained clear (Figure 16). The morphological changes were remarkably similar to those produced *in vivo* by naphthalene feeding. Unlike the above three metabolites, 1-naphthol or 2-naphthol at 5 X 10^{-5}M induced no morphological changes *in vitro* although some biochemical changes were detected (Table 8).

Biochemical Comparison of In Vitro Cataracts with the In Vivo Cataract

Under the same culture conditions (5 X 10^{-5} M and 48 hours incubation), 1,2-naphthoquinone and 1,2-dihydroxynaphthalene showed similar effects of GSH depletion, Na^+/K^+-ATPase activity loss and severe choline and rubidium transport

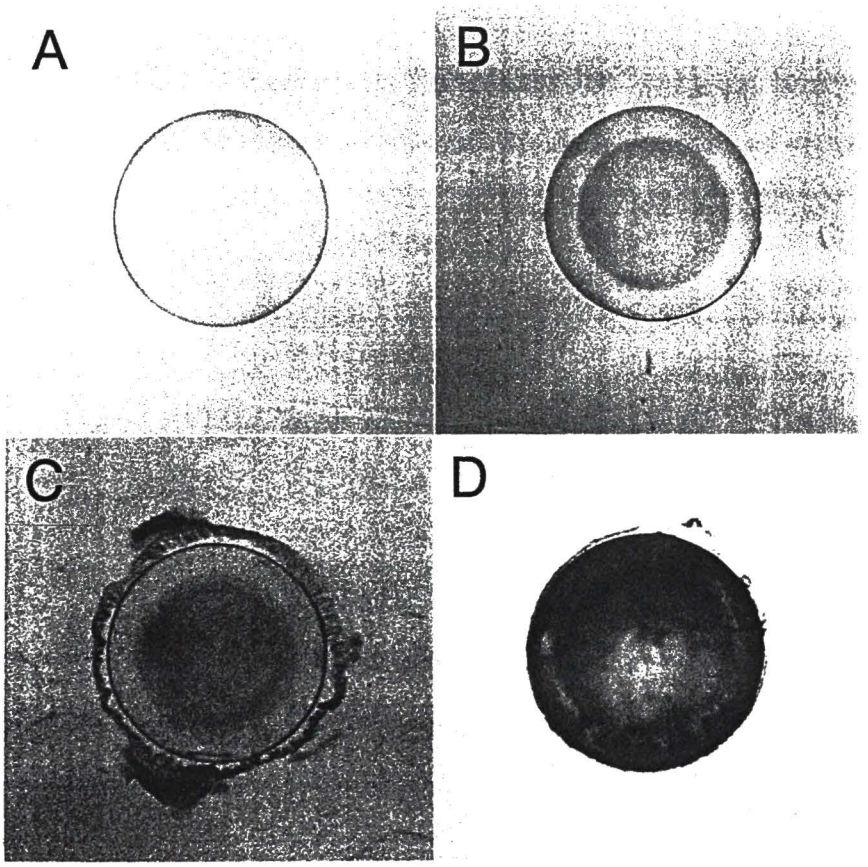


Figure 16. Comparison of the morphology of the cataracts induced by naphthalene in vivo and naphthalene metebolites in vitro. The morphology is compared by photographs taken under a dissecting microscope.

A: a normal control lens;

B: lens from a rat fed with naphthalene for six weeks (1.0 g/kg/day);

C: lens exposed to naphthalene dihydrodiol in vitro (5 X 10^{-5}M, for 48 hrs);

D: lens exposed to naphthoquinone in vitro (5 X 10^{-5} M, for 48 hours).

TABLE 8. Rat Lens Membrane Functions in Cataracts Induced by Naphthalene (*in vivo*) and Its Metabolites (*in vitro*)*

Group	(n)	^{3}H-Choline (L/M)	^{86}Rb (L/M)	ATPase (μmole Pi/g/hr)
in vivo				
Control	(5)	8.84 ± 0.79	7.42 ± 0.43	17.90 ± 0.88
Naph.	(5)	9.10 ± 2.47^{ns}	7.58 ± 1.10^{ns}	16.95 ± 1.23^{ns}
in vitro				
Control	(4)	9.42 ± 0.59	8.06 ± 0.78	10.33 ± 2.87
Dihydrodiol	(5)	9.12 ± 1.81^{ns}	7.87 ± 1.13^{ns}	10.74 ± 2.68^{ns}
Dihydroxy Naph.	(5)	1.45 ± 0.33**	2.43 ± 0.40**	6.82 ± 1.19*
Naphthoquinone	(5)	1.56 ± 0.76**	2.67 ± 1.08**	6.10 ± 1.23*
1-naphthol	(5)	5.93 ± 0.47**	6.98 ± 0.57*	9.87 ± 0.82^{ns}
2-naphthol	(5)	6.55 ± 0.51**	7.58 ± 0.64^{ns}	10.52 ± 1.28^{ns}

* *in vivo*: the rats were dosed with naphthalene at 1.0 g/kg/day for two weeks;
in vitro: the lenses were exposed to each naphthalene metabolite at 5 X 10^{-5}M in the media for 48 hours.

Data reported as mean ± S.D.; (n) = Number of samples.
ns = not significant (P > 0.05); * P < 0.05; ** P < 0.001 (independent t-test).
Naph. = naphthalene; Dihydrodiol = naphthalene dihydrodiol.

reduction. It is likely that the latter compound spontaneously oxidizes to 1,2-naphthoquinone thus explaining the similar effects of the two compounds. The lenticular changes caused by these two compounds are very different from those in naphthalene-induced cataract *in vivo*. Another two compounds, 1-naphthol or 2-naphthol showed little effect on the lens GSH level and Na^+/K^+-ATPase activity. Although they caused impairment of the ability of the lens to accumulate choline, they had essentially no effect to rubidium transport (Table 8). Only naphthalene dihydrodiol demonstrated effects nearly identical to the naphthalene cataract *in vivo* where 30% GSH loss and no membrane transport function or Na^+/K^+-ATPase activity change was observed. These results are summarized in Table 8 and Table 9. Furthermore, the naphthalene dihydrodiol produced effects on the lens protein profile similar to those seen *in vivo*, with formation of disulfide cross-linked insoluble proteins (Figure 17). As seen in the *in vivo* system, a marked increase of protein-GSH mixed disulfide was observed (Table 9) in naphthalene dihydrodiol-induced cataract. In addition, AL01576 which showed efficacy in preventing naphthalene cataract *in vivo* also prevented the cataract induced by naphthalene dihydrodiol but not the cataracts induced by other naphthalene metabolites (Table 10).

Effects of Naphthalene Dihydrodiol Concentration on the In Vitro Model

A dose-response effect of naphthalene dihydrodiol from 10^{-5}M to 5 X 10^{-4}M was observed on the lens transport systems (choline and rubidium) and the GSH concentration (Figure 18). Similarly, the morphological changes in these lenses also showed dose-response relationships. As shown in Figure 19, at a low concentration (2 X 10^{-5}M) a shell-like opacity was formed very deep in the cortex just around the nucleus. This shell moved outwardly as the concentration of naphthalene dihydrodiol in the medium was increased. When its concentration reached 5 X 10^{-4} M, the epithelium and outer cortex region were visibly damaged, similar to the effect of 1,2-naphthoquinone but the opacities still appeared to be

TABLE 9. Comparison of the GSH and Protein-thiol Mixed Disulfides Levels in Cataracts Induced by Naphthalene (*in vivo*) and its Metabolites (*in vitro*)[$].

Group (n)	Lens wt (mg)	GSH	GSO_3H	CSO_3H
in vivo				
Control (6)	28.73 ± 0.76	4.91 ± 0.42	0.07 ± 0.09	0.69 ± 0.24
Naph. (6)	29.21 ± 0.88	4.16 ± 0.55*	1.40 ± 1.01*	0.98 ± 0.47
in vitro				
Control (9)	23.15 ± 0.88	3.68 ± 0.17	0.05 ± 0.03	0.44 ± 0.16
Dihydrodiol(7)	23.55 ± 1.05	3.02 ± 0.17**	0.48 ± 0.08***	0.43 ± 0.13^{ns}
D.N. (5)	24.78 ± 1.06	1.93 ± 0.17***	-	-
N.Q. (5)	25.07 ± 0.55	1.64 ± 0.35***	0.06 ± 0.01^{ns}	0.38 ± 0.06^{ns}
1-naphthol (5)	23.42 ± 0.93	3.34 ± 0.52^{ns}	-	-
2-naphthol (5)	23.67 ± 0.48	3.46 ± 0.28^{ns}	-	-

[$] Experiment conditions are same as Table 8.

Data are expressed as µmole/g wet wt, mean ± S.D.; (n) = Number of Samples.
ns = Not significant ($P > 0.05$); * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ (t-test).

GSO_3H: protein-GSH; CSO_3H: protein-cysteine.
Naph.: Naphthalene; D.N.: Dihydroxynaphthalene; N.Q.: Naphthoquinone.

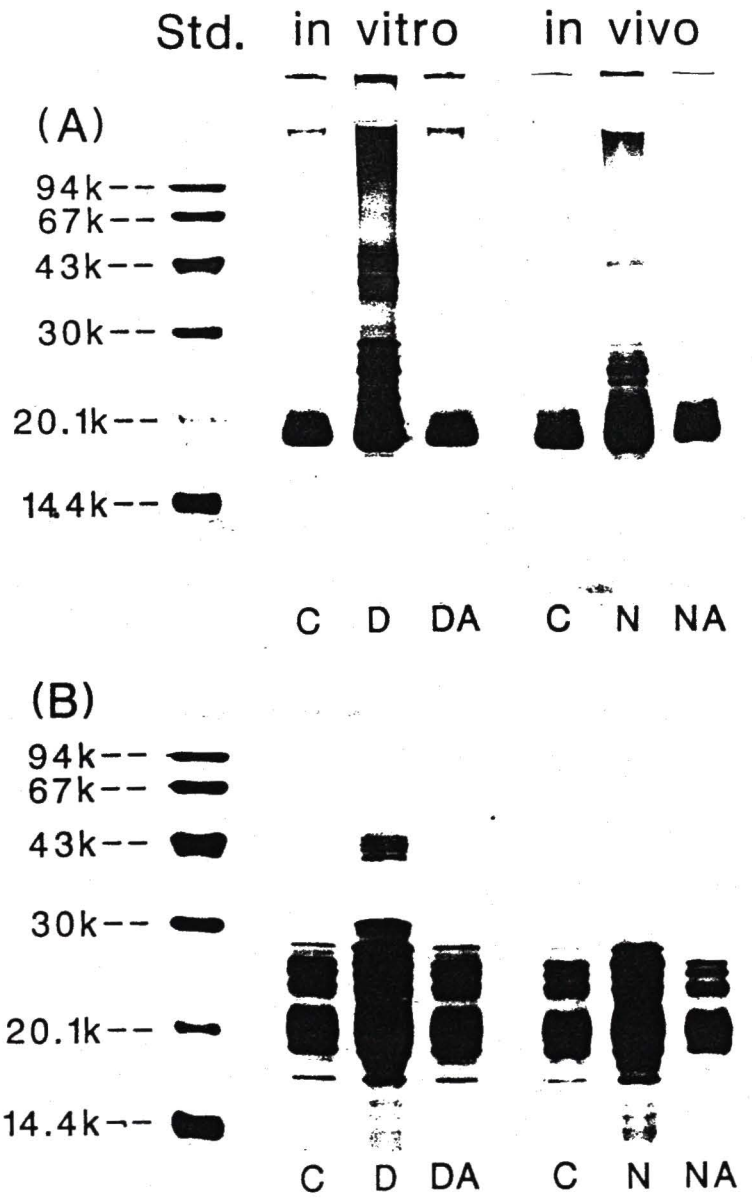


Figure 17. SDS-PAGE profiles of urea-soluble proteins of the lenses from *in vivo* and *in vitro* naphthalene cataract models. *in vivo*: the rat was fed wiht naphthalene (1 g/kg/day) for 3 wks. *in vitro*: the lens was exposed to dihydrodiol at 5 X 10^{-5}M for 72 hrs.

(A), non-reduced samples; (B), reduced samples.

C: control; D: naphthalene dihydrodiol; DA: naphthalene dihydrodiol+AL01576 (10^{-5}M); N: naphthalene-fed; NA: naphthalene+AL01576 (10 mg/kg/day).

TABLE 10. Effects of AL01576 on Lens GSH and Mixed Disulfides of Naphthalene-Related Cataracts *in vivo* and *in vitro*.

Group (n)	GSH	GSO_3H	CSO_3H
in vivo			
Control	4.74 ± 0.30 (5)	0.07 ± 0.09 (6)	0.69 ± 0.24 (6)
Naphthalene	3.64 ± 0.75[a](5)	1.40 ± 1.01[b](6)	0.98 ± 0.47 (6)
Naph.+ AL01576	4.39 ± 0.75 (5)	0.05 ± 0.01 (4)	0.64 ± 0.08 (4)
in vitro			
Control	3.79 ± 0.43 (3)	0.05 ± 0.03 (9)	0.44 ± 0.16 (9)
Dihydrodiol	3.21 ± 0.12[a](4)	0.48 ± 0.08[c](7)	0.43 ± 0.13 (7)
Diol + AL01576	3.73 ± 0.45 (3)	0.08 ± 0.03 (7)	0.44 ± 0.16 (7)

Data are expressed as µmole/g wet wt and mean ± S.D.; (n) = 4 for each group.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ (independent t-test).

Mixed Disulfides: GSO_3H = Protein-GSH; CSO_3H = Protein-Cysteine.

Naph.= naphthalene; Diol = naphthalene dihydrodiol.

in vivo: rats were fed with naphthalene (1 g/kg/day) and AL01576 (10 mg/kg/day) for 4 weeks;

in vitro: lenses were exposed to 2.5×10^{-5} M naphthalene dihydrodiol in the media for 48 hours; AL01576 was 2.5×10^{-5} M in media and preincubated with lens for 1 hour before exposing to the dihydrodiol.

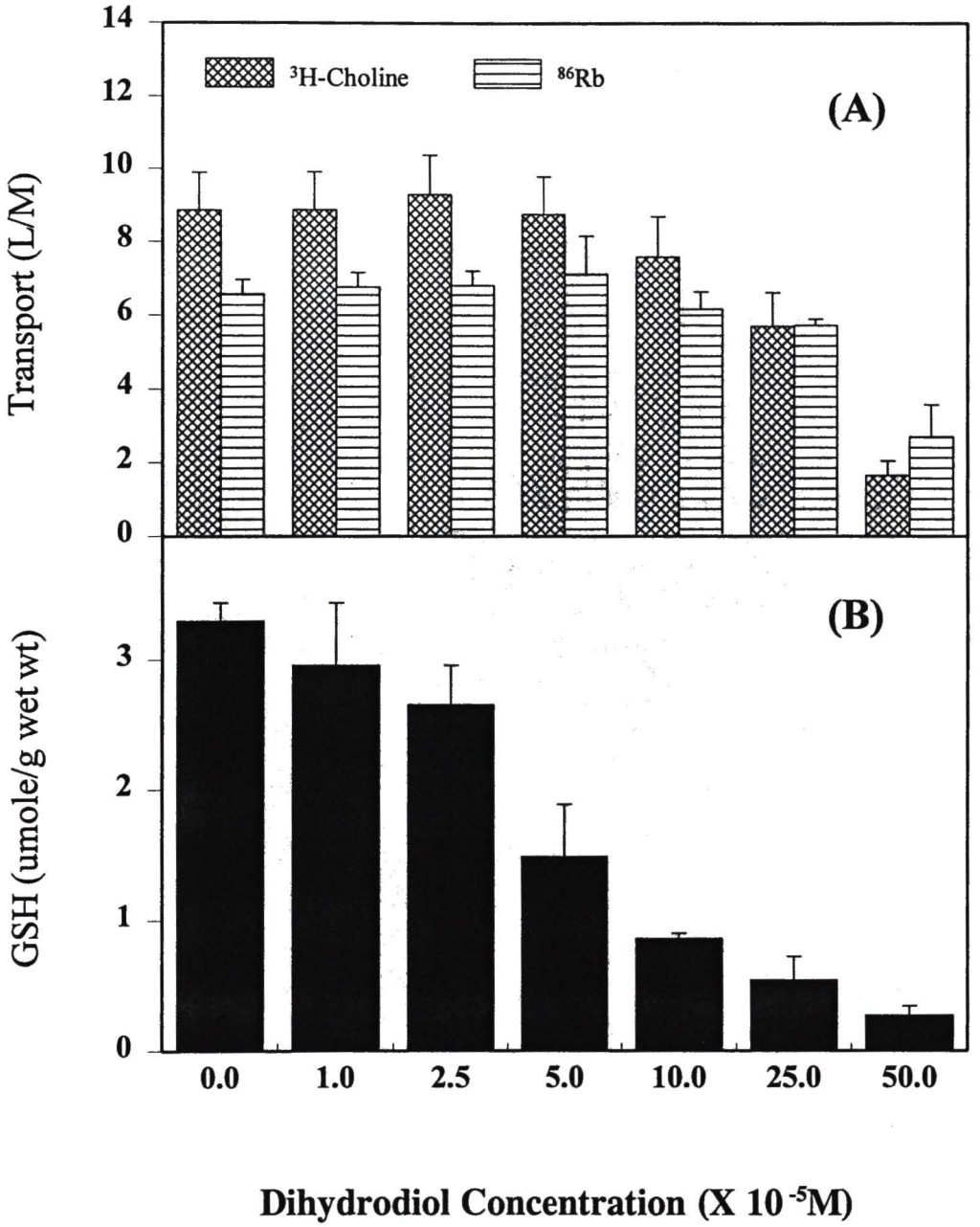


Figure 18. Effect of dihydrodiol concentrations on GSH level and membrane transport in the cultured rat lenses. The lenses were exposed to naphthalene dihydrodiol for 48 hrs at a concentration ranging from 10^{-5}M to 5×10^{-4}M. Data are expressed as mean ± S.D. with n = 4. (A) Active Transport. (B) GSH. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (t-test).

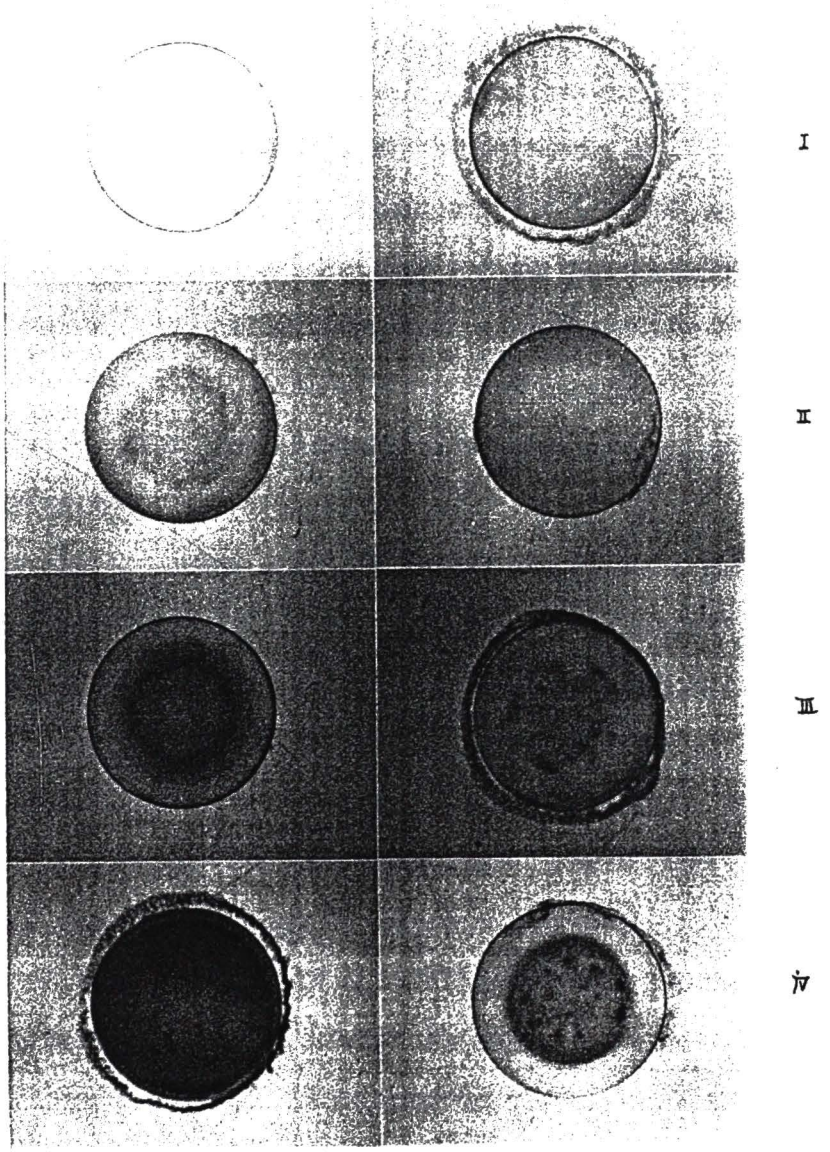


Figure 19. Morphological comparison of the lenses exposed to naphthalene dihydrodiol (ND) or ND + AL01576 (2 x 10^{-5}M) for 48 hours. Lane A: the lenses exposed to ND. A-I: Normal lens; A-II: Lens exposed to 2 x 10^{-5}M ND; A-III: Lens exposed to 10^{-4}M ND; A-IV: Lens exposed to 5 x 10^{-4}M ND. Lane B: the lenses exposed to ND + AL01576, otherwise the condition of B I-IV was identical to A I-IV.

confined to discrete layers of the cortex with the remaining portions staying clear.

Effects of Naphthalene Dihydrodiol Exposure Time on the In Vitro Model

Induction of the dihydrodiol cataract *in vitro* showed a clear correlation with time of exposure. At a constant concentration of 2.5 X 10^{-5} M, lenses were quite clear during the initial 24 hours but gradually formed the shell-like opacity around the nucleus by 48 hrs. The opacification became more peripheral and widespread after 72 hrs. There was a progressive decrease of lens GSH (Figure 20), but the transport systems (rubidium and choline) remained intact throughout the time course (data not shown).

Based on the above studies, the best condition to induce a cataract with naphthalene dihydrodiol in the *in vitro* which mimics the *in vivo* naphthalene cataract is to expose rat lenses to 2.5 X 10^{-5} M naphthalene dihydrodiol in culture medium for 48 hours.

Effects of AL01576 Dosage on the Dihydrodiol Cataract Development

AL01576 was tested in two ways for its preventive effects in the *in vitro* model. In one experiment, a fixed dosage of AL01576 was tested in cataract induced by varied amount of naphthalene dihydrodiol. As shown in Figure 19, AL01576 at 2 X 10^{-5}M completely prevented the cataractous change induced by naphthalene dihydrodiol when a low dose (2 X 10^{-5} M) was used in the culture media. This drug concentration partially prevented the opacity induced by 1 X 10^{-4} M naphthalene dihydrodiol and it also effectively reduced the opaque area and the pigmentation when the highest dose (5 X 10^{-4} M) of naphthalene dihydrodiol was used in the media.

In a separate experiment, when rat lenses were exposed to a constant dihydrodiol concentration (1 X 10^{-4} M) for 48 hours, AL01576 at varied concentrations also showed efficacy against the *in vitro* cataract. As shown in Table 11, AL01576 at 10^{-5} M partially prevented the cataractous changes but was more effective

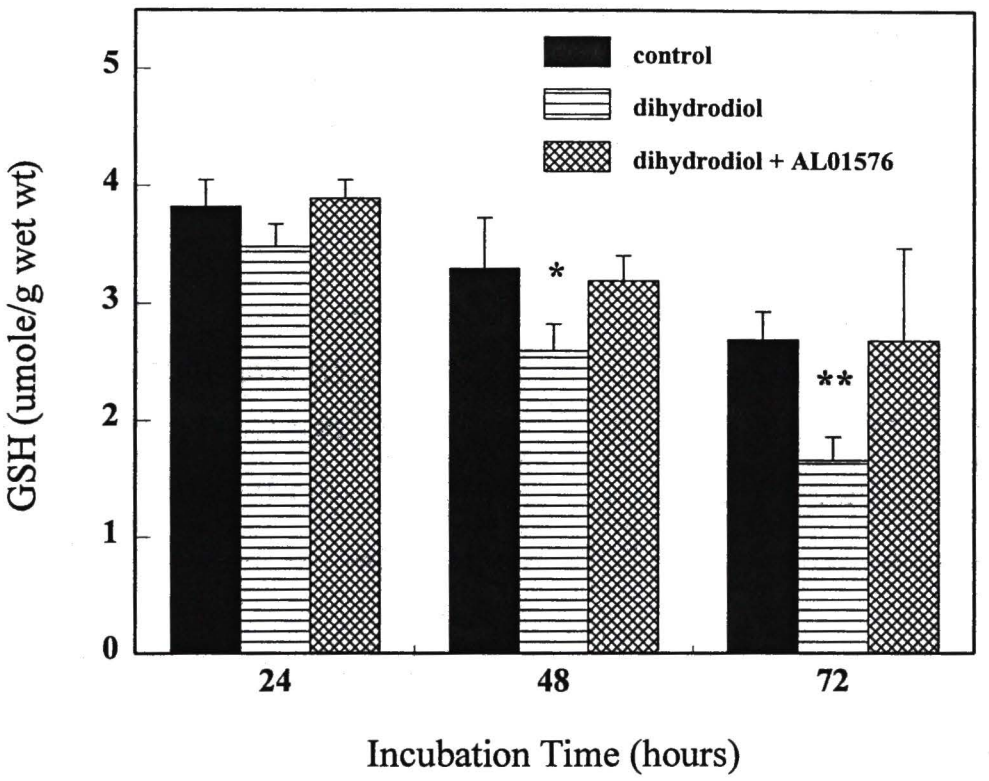


Figure 20. Effects on GSH level of exposure time of rat lenses to naphthalene dihydrodiol in organ culture. The lenses were exposed to 2.5 x 10^{-5}M naphthalene dihydrodiol for 24, 48 and 72 hrs in the presence and absence of AL01576 (2.5 X 10^{-5}M). Data are expressed as mean ± S.D.; (n) = 4; * $P < 0.05$; ** $P < 0.001$ (independent t-test, samples were compared to the control at the same time point).

TABLE 11. Efficacy of AL01576 in Naphthalene Dihydrodiol Induced Cataract (in vitro).[$]

Group (n)	Lens Opacity	GSH (μmole/g wet wt)
Control (3)	None	2.81 ± 0.08
ND (4)	+3	1.00 ± 0.29**
ND + 10^{-5}M ARI (3)	+1	2.03 ± 0.31*
ND + 2.5 x 10^{-5}M (5)	None	2.51 + 0.33^{ns}
ND + 10^{-4}M ARI (3)	None	2.53 ± 0.20^{ns}

[$] Cataract was induced by exposing to 2 X 10^{-4}M Naphthalene dihydrodiol (ND) for 48 hrs. ARI (AL01576) was preincubated with lenses for one hour before addition of ND in the media.

Data are expressed as mean ± S.D. with (n) = number of samples.
** = $P < 0.001$; * = $P < 0.05$; ns = not significant = $P > 0.05$ (t-test).

when it was raised to 2.5 X 10^{-5} M and 10^{-4} M. At the high concentrations, lens GSH was maintained at 90.0% of the control value instead of 35.5% when the lens was untreated. Above the concentration of 10^{-4} M, the solubility of AL01576 became a limitation. The most practical and efficacious concentration of AL01576 in preventing naphthalene dihydrodiol cataract was 2.5 X 10^{-5}M.

Effect of AL01576 on the in vitro Naphthoquinone-induced Cataract

AL01576 was also tested in the *in vitro* system for its ability to prevent cataracts caused by other naphthalene metabolites such as 1,2-dihydroxynaphthalene and 1,2-naphthoquinone. Neither of these two cataracts could be prevented by AL01576. Figure 16, 21 and 22 showed typical changes caused by 1,2-naphthoquinone in the *in vitro* system. This compound induced lens swelling, outer layer cortical opacity and significantly decreases in lens GSH level and active transport system (t-test, $P < 0.001$). AL01576 did not prevent these changes at all (Figures 21 and 22).

Study III: Mechanism for Naphthalene Cataract Formation

Effects of Pigmentation on the Cataract Development

In order to compare the susceptibility of pigmented rats and albino rats to naphthalene cataract formation, 5 strains of rats, 2 pigmented strains and 3 albino strains, were fed with naphthalene for 4 weeks. The opacities were graded under a slit-lamp with an arbitrary score system (see methods). As shown in Figure 8, Long-Evans (pigmented), Lewis and Sprague-Dawley (both albino) rats showed a similar rate of cataract formation. The Brown-Norway rat (pigmented) developed cataract slightly faster than the above groups and the Wistar rat (albino) formed opacity at a slower pace. No significant difference (at 0.05 level) was detected among these strains. The lenses from both the pigmented and the albino rats

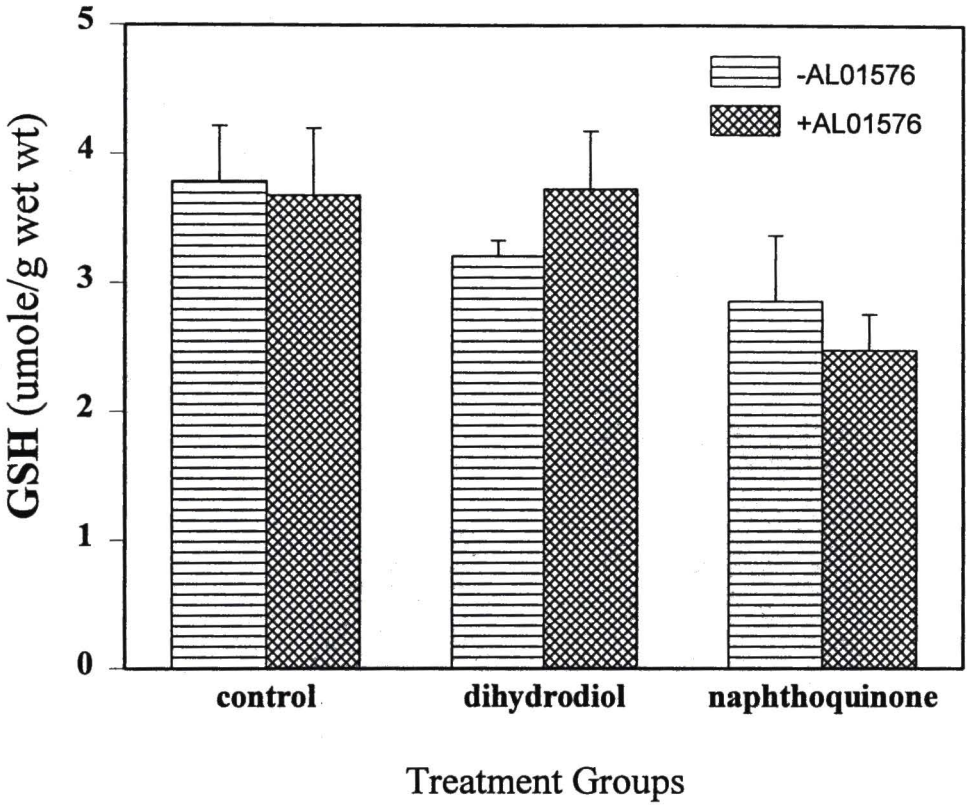


Figure 21. Glutathione concentration in the cultured rat lenses exposed to naphthalene dihydrodiol or 1,2-naphthoquinone, with or without AL01576 treatment. The concentrations of all the 3 compounds are 2.5 x 10^{-5}M, and the test period was 48 hrous. Independent t-test indicates no significant difference between naphthoquinone-exposed lenses and naphthoquinone + AL01576 treated lenses ($P > 0.05$). ^{A} and ^{B} indicate significant differences between those groups and the control (^{A} = $P < 0.05$; ^{B} = $P < 0.001$).

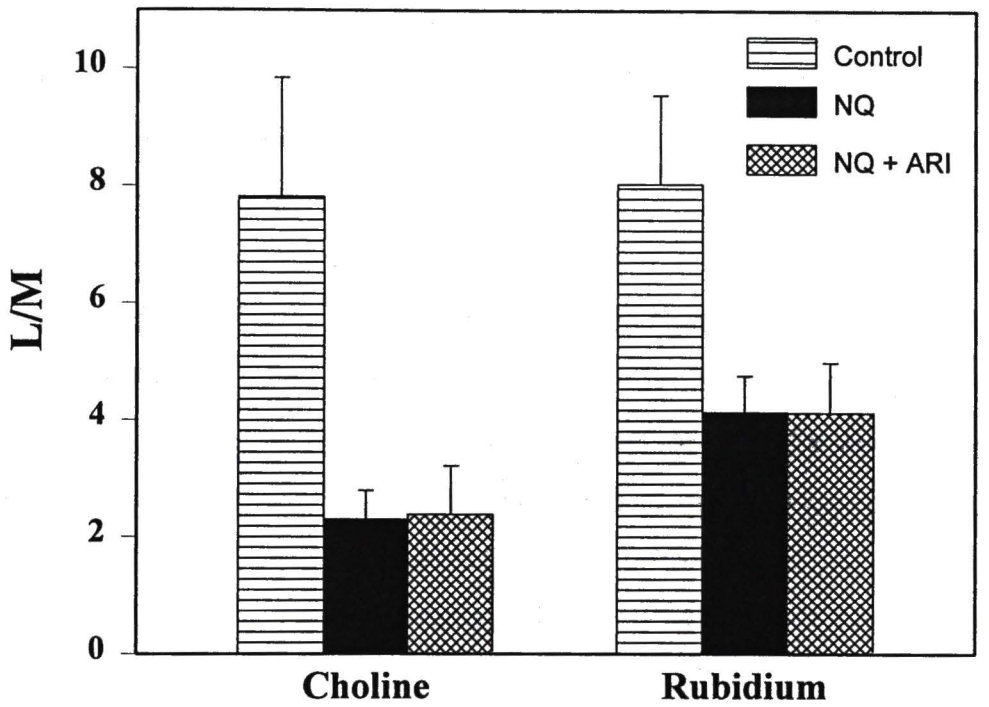


Figure 22. Choline and rubidium transports in the cultured rat lenses exposed to 1,2-naphthoquinone, with or without AL01576 treatment. The concentrations of both 1,2-naphthoquinone and AL01576 are 2.5 x 10^{-5}M, and the test period was 48 hrous. Independent t-test indicates no significant difference between naphthoquinone-exposed lenses and naphthoquinone plus AL01576 treated lenses ($P > 0.05$). ^{A} indicates a significant difference between that group and the control (^{A} $P < 0.001$).

treated with naphthalene plus AL01576 or with AL01576 alone remained clear during the four weeks treatment.

Lenses from both albino and pigmented rats of the same age were tested for their susceptibility to naphthalene dihydrodiol cataract induction *in vitro*. Upon exposure to naphthalene dihydrodiol at 5 X 10^{-5} M for 48 hrs, the opaque shells in both groups of lenses looked similar and the lens GSH level was depressed by about the same extent (30% depletion). These changes were inhibited equally in both strains by AL01576 (Table 12).

Detection of Naphthalene Metabolites in the Eye Tissues

To detect the existence of naphthalene metabolites in the eye tissues, Long-Evans rats were fed with naphthalene for six weeks and the lens and aqueous humor were collected for naphthalene metabolite analysis by a HPLC system. As shown in Figure 23, standard naphthalene dihydrodiol and 1,2-naphthoquinone were resolved completely by reversed-phase HPLC (Figure 23A). Naphthalene dihydrodiol was the only naphthalene metabolite which could be detected in either the aqueous humor (Figure 23B) or the lens (Figure 23C) from naphthalene-fed rats with this method.

A similar approach was also tried in the in vitro system. The attempt to measure 1,2-naphthoquinone in the lens after exposure to either naphthalene dihydrodiol or 1,2-naphthoquinone in the media was not successful. Naphthalene dihydrodiol, however, was successfully identified in the lens and the amount detected was proportional to the concentration of naphthalene dihydrodiol used in the culture media. As shown in Figure 24E, the amount of naphthalene dihydrodiol in the lens after exposure to 5 X 10^{-4} M naphthalene dihydrodiol is five-fold higher than that in the lens exposed to 1 X 10^{-4}M (Figure 24D), and about 25 fold higher than that in the lens exposed to 2 X 10^{-5} M (Figure 24C). These results indicated that naphthalene dihydrodiol penetrated or was transported into the lens where it was futher metabolized. Interestingly, the amount of naphthalene dihydrodiol

TABLE 12. Comparison of Lens GSH and Lens Membrane Transport Systems of the Dihydrodiol-induced Cataract (*in vitro*) in Albino and Pigmented Rats.[a]

Group (n)	GSH (μmole/g wet wt)	^{3}H-Choline (L/M)	^{86}Rb (L/M)
Sprague-Dawley			
Control (4)	3.68 ± 0.17	5.21 ± 0.57	5.83 ± 0.43
Dihydrodiol (5)	3.02 ± 0.17*	6.07 ± 0.97^{ns}	6.30 ± 0.60^{ns}
Diol + AL01576 (5)	3.51 ± 0.34^{ns}	5.40 ± 0.26^{ns}	6.52 ± 1.30^{ns}
Long-Evans			
Control (4)	3.39 ± 0.26	7.63 ± 0.41	6.82 ± 0.30
Dihydrodiol (4)	2.71 ± 0.18*	7.05 ± 0.91^{ns}	7.08 ± 0.53^{ns}
Diol + AL01576 (4)	3.25 ± 0.31^{ns}	7.03 ± 0.45^{ns}	7.19 ± 0.63^{ns}

[a] Lenses were exposed to 5 X 10^{-5}M Diol (naphthalene dihydrodiol) or Diol plus AL01576 (5 X 10^{-5}M) in the media for 48 hours.

Data are expressed as mean ± S.D.; (n) = number of samples.
ns = not significant =P > 0.05; * = P < 0.01 (independent t-test).

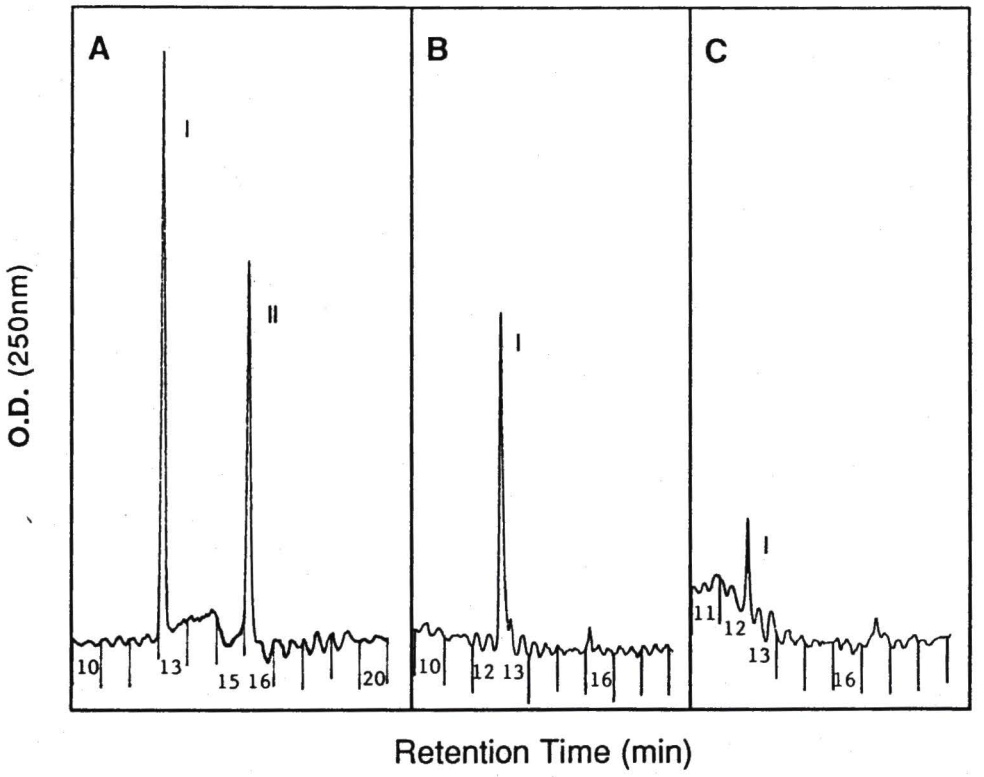


Figure 23. HPLC profiles of naphthalene metabolites in the lenses and aqueous homor of naphthalene-fed rats. Details of the analytical techniques are described in METHODS.

(A) Standards: Peak I: naphthalene dihydrodiol 1 nmole;
Peak II: 1,2-naphthoquinone, 0.25 nmole.

(B) Aqueous humor from naphthalene-treated rats (1.0 g/kg/day, 4wks).

(C) Lens from the same group of rats as (B).

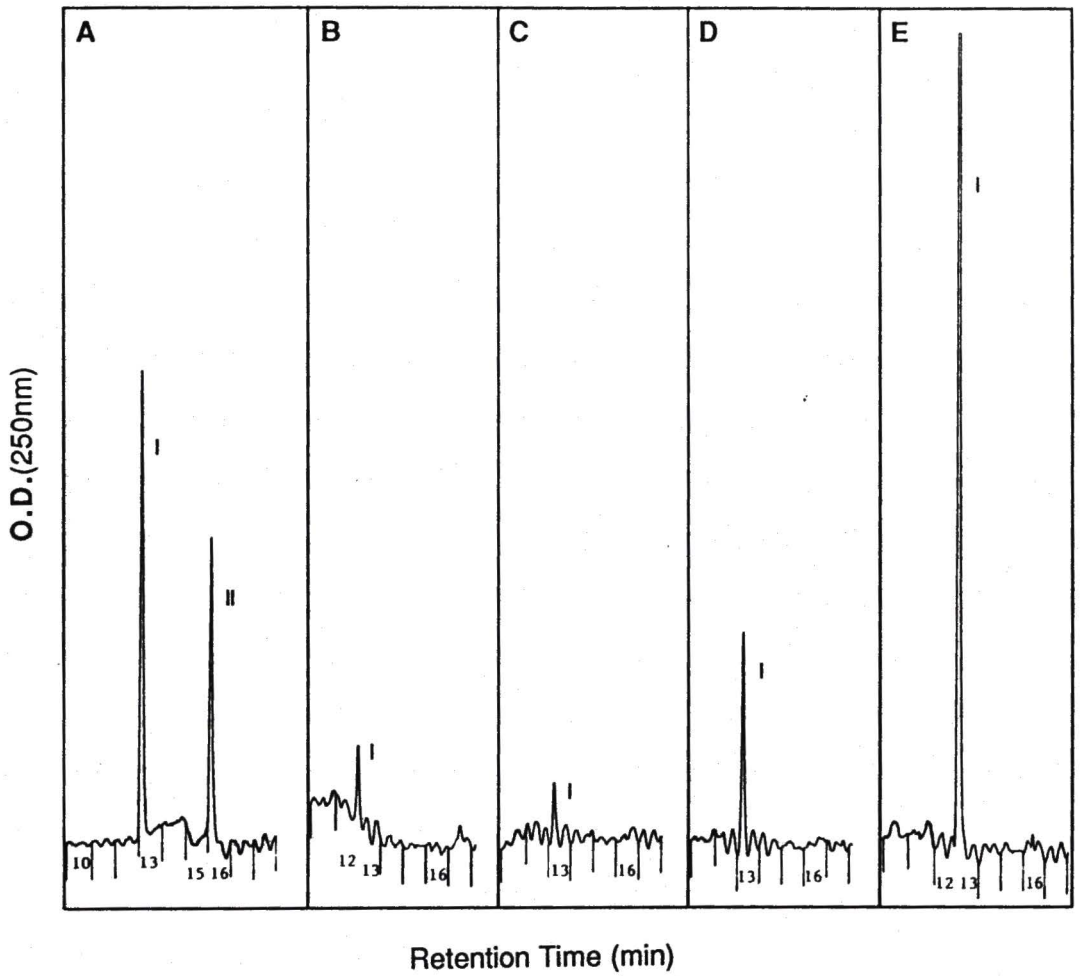


Figure 24. HPLC profiles of naphthalene metabolites in the rat lenses from *in vivo* and *in vitro* naphthalene cataract models. Details of analytical conditions are described in the METHODS.

(A). Standards: same as Figure 23; (B). Lens of a rat fed with naphthalene for 4 weeks. (C, D and E). lenses exposed to 2 x 10^{-5}M, 10^{-4}M and 5 x 10^{-4}M naphthalene dihydrodiol for 48 hours, respectively.

detected in lenses exposed to 2 X 10^{-5} M naphthalene dihydrodiol containing medium, was about the same level as the naphthalene dihydrodiol found in the lens of rats fed 1 g naphthalene/kg/day (Figure 24, B and C).

Study IV: The Action of AL01576 in Naphthalene Cataract Prevention

Comparison of the Efficacies of Several ARIs on Naphthalene Cataract

Four ARIs including AL01576, AL04114, Sorbinil and Tolrestat were tested for their ability to prevent naphthalene cataract by comparing lens opacification with an arbitrary cataract score system. All the ARIs were given at the same dosage of 10 mg/kg/day and delivered daily by a gavage needle 1 hr before naphthalene feeding except Tolrestat which was given to the rats beginning two weeks before naphthalene treatment and continued daily along with naphthalene feeding. As shown in Figure 25, during the course of 4-week study, treatment of naphthalene-fed rats with Tolrestat essentially showed no benefit to their lenses. At the same dosage, Sorbinil provided a very weak protection but both AL04114 and AL01576 ameliorated the morphological changes and kept the lenses clear and indistinguishable from the normal control group. Since naphthalene feeding causes GSH loss and protein-thiol mixed disulfide production (Xu et al., 1992), the efficacy of these four ARIs in preventing GSH loss was compared. As shown in Table 13, Tolrestat and Sorbinil each showed no protection but AL01576 and AL04114 were able to prevent GSH loss almost completely.

These ARIs (except Tolrestat) were also used for dose response studies on naphthalene cataract prevention. The Sorbinil study was conducted for 6 weeks with two dosages at 4 mg/kg and 16 mg/kg. As shown in Figure 26, the opacity scores (by slit lamp observations) of the lower dosage showed a slightly less cataract progression as compared to the untreated group. The higher dosage of 16 mg/kg reduced the opacity score to nearly 50% during the entire course of

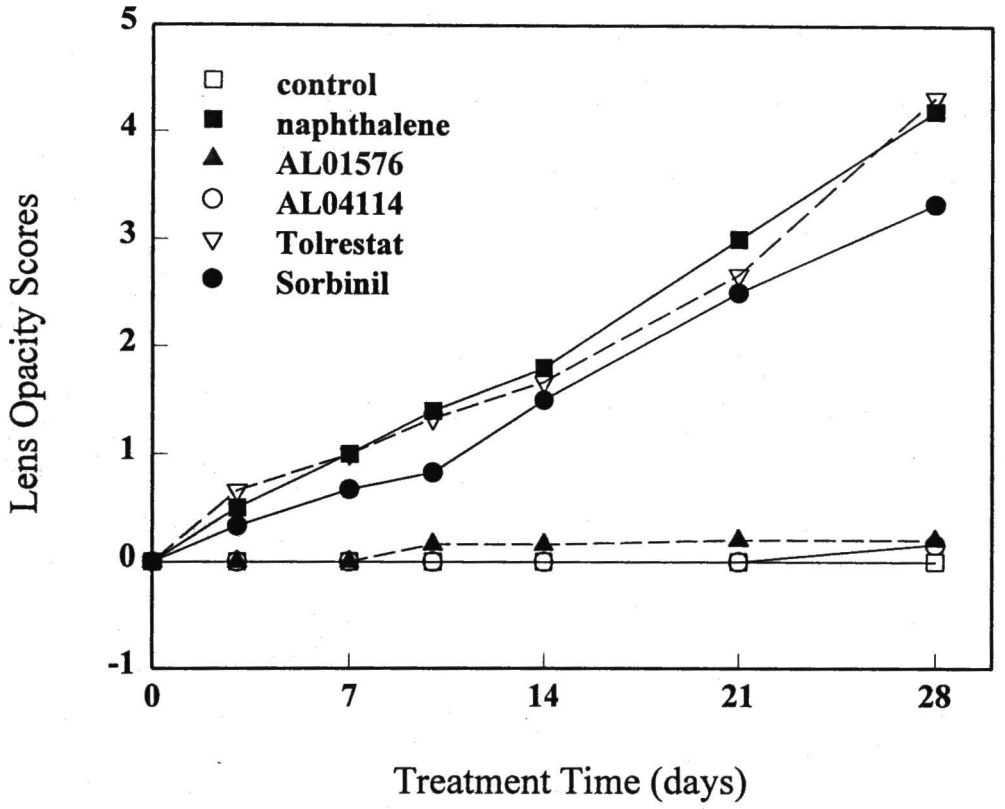


Figure 25. Comparison of the efficacies of 4 ARIs on naphthalene cataract formation in rats. The rats were fed with naphthalene (1.0 g/kg/day) for 4 wks, with or without the treatment of one of these ARIs. All the ARIs were given daily at the same dosage of 10 mg/kg, 1 hour before naphthalene feedings. A1: AL01576; A2: AL04114; T: Tolrestat; S: Sorbinil; Naph: naphthalene. The t-test for slopes indicates no significant difference among the high score groups (Naph, Naph + T and Naph + S), or among the low score groups (control, Naph + A1 and Naph + A2) at 0.05 level. But there is a significant difference between the above two groups ($P < 0.01$).

TABLE 13. Comparison of Naphthalene-Induced Glutathione Changes After Treatment With Several ARIs.[$]

Group (n)	ARI Dosage (mg/kg)	GSH	
		μmole/g wet wt	%
Control (4)	0	5.27 ± 0.20	100
Naph. (5)	0	3.73 ± 0.47*	70.7
Naph. + AL01576 (3)	10	4.76 ± 0.25[ns]	90.3
Naph. + AL04114 (2)	10	5.21 ± 0.09[ns]	98.8
Naph. + Sorbinil (6)	10	3.76 ± 0.34*	71.3
Naph. + Tolrestat (3)	10	3.70 ± 0.86*	70.2

[$] The rats were fed with naphthalene at 1.0 g/kg/day for 4 weeks. ARIs were dosed at the same dosage of 10 mg/kg/day 1 hr. before naphthalene feeding. Naph.= naphthalene.

Data are expressed as Mean ± S.D.; (n) indicates the number of lenses.
* = $P < 0.01$; ns = not significant = $P > 0.05$ (independent t-test, the samples were compared to the control).

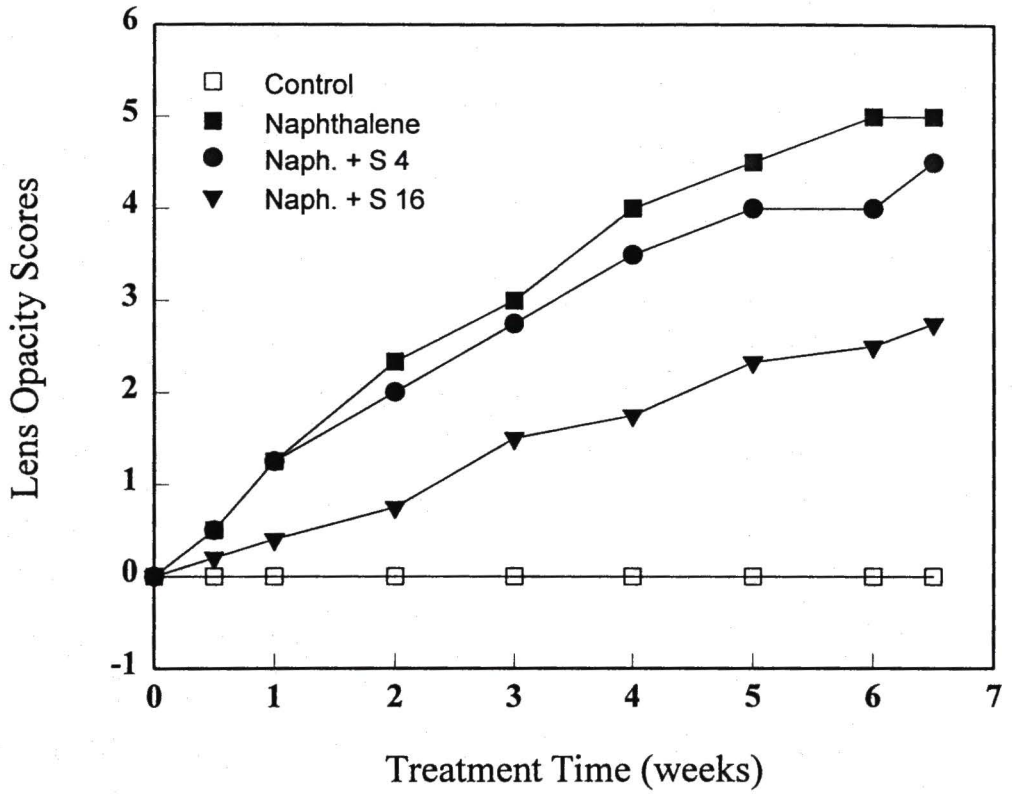


Figure 26. Comparison of naphthalene cataract progression in rats treated with lower dosage (4.0 mg/kg/day) and higher dosage (16 mg/kg/day) of Sorbinil. Condition of experiment was the same as Figure 25 except the study was carried out for 6 weeks. Naph. = naphthalene; S4 = sorbinil 4 mg/kg/day; S16 = sorbinil 16 mg/kg/day. The t-test for slopes ind icates no significant difference between Naph and Naph + S4 ($P > 0.05$), but the lens opacity scores in Naph + S16 group is significantly low than those in Naph rats ($P < 0.05$).

cataract development. The above study also demonstrated a dose-dependent effect on biochemical changes. Sorbinil showed only a trend toward dose-dependent protection of lens GSH whereas its ability to suppress protein-thiol mixed disulfide production was very clearly dose dependent. As shown in Figure 27, at 4 mg/kg/day, Sorbinil suppressed protein-GSH level to nearly 30% of that of the untreated naphthalene-fed rats. At 16 mg/kg/day, protein-GSH production was reduced to only 10% of naphthalene group. Similar but less dramatic effect on protein-cysteine mixed disulfide was observed. AL01576 was a much potent inhibitor of naphthalene cataract formation. As shown in Figure 13, AL01576 at 1.0 mg/kg/day maintained lens clarity more than 50% during the four week study period. At a higher level of 4 mg/kg/day, AL01576 kept the lenses all clear and nearly identical to the normal control group (Figure 13). The effect of ARIs' dosage on lens GSH level of the above study is summarized in Table 14, which shows that AL01576 at a dosage higher than 1.0 mg/kg/day kept the lens GSH level nearly identical to that of the normal control. AL04114, a dimethoxy derivative of AL01576, was as potent as AL01576 in the prevention of naphthalene cataract. Although both AL01576 and AL04114 are equally effective in the inhibition of aldose reductase activity, their effects on cytochrome P-450 are totally different. AL01576 has strong inhibitory effect on cytochrome P-450, but AL04114 showed no inhibition on these enzymes (Kiss et al., 1992). In fact, drug metabolic study has demonstrated that when AL04114 was added to rat liver microsomal preparation, it was metabolized very quickly to its monomethoxy metabolite (Kiss et al., 1992).

The above mentioned four ARIs were also compared for their efficacious in preventing the *in vitro* naphthalene cataract model. At 5×10^{-5} M, AL01576, AL04114 and sorbinil all maintained lens clarity and lens GSH level. Tolrestat, however was not only ineffective in preventing cataract formation but also showed adverse effect on lens GSH level (Table 15). Due to the pharmacological characteristics, Tolrestat builds up its concentration in the tissues very slowly. There-

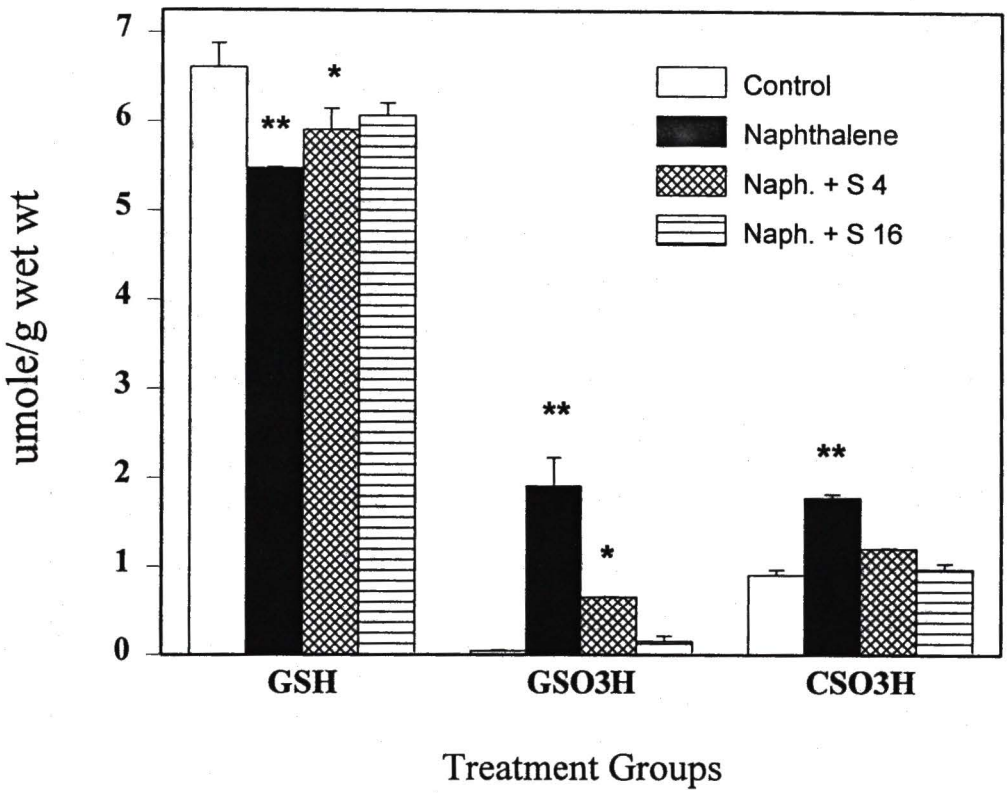


Figure 27. Effects of various dosages of Sorbinil on lens GSH and mixed disulfide levels of naphthalene-fed rats. Condition of experiment and group name initials were the same as Figure 26. Data are expressed as mean ± S.D. with (n) = 4. * = $P < 0.01$; ** = $P < 0.001$ (independent t-test, compared with the control in the same group).

TABLE 14. Dose Response Studies of AL01576 and AL04114 on Rats Naphthalene Cataract *in vivo*.

Group (n)	ARI Dosage (mg/kg)	Lens Morphology	GSH	
			μmole/g wet wt	%
Normal Control (6)	0	Clear	6.59 ± 0.75	100
Naphthalene (6)	0	Shell (4+)	5.38 ± 0.64*	81.6
AL01576 (6)	0.4	Shell (4+)	5.68 ± 0.48*	86.2
AL01576 (6)	1.0	Shell (1+)	6.10 ± 0.75^{ns}	92.6
AL01576 (6)	4.0	Clear	6.11 ± 0.41^{ns}	92.7
AL01576 (5)	10.0	Clear	6.36 ± 0.48^{ns}	96.5
AL04114 (6)	4.0	Clear	6.39 ± 0.48^{ns}	97.0
AL04114 (5)	10.0	Clear	6.34 ± 0.62^{ns}	96.2

The rats were treated with naphthalene (1 g/kg/day) or naphthalene plus one of the ARIs at the dosages indicated above for four weeks.

Shell: shell-like opacity in deep cortex.

Data are expressed as Mean ± S.D., (n) indicates the number of lenses.

* = $P < 0.05$; ns = not significant = "$P > 0.05$" (independent t-test).

TABLE 15. Comparison of ARIs' Efficacies in the Naphthalene Dihydrodiol-Induced Cataract *In Vitro.*

Condition	(n)	Morphology	GSH	
			μmole/g wet wt	%
Control	6	Clear	4.17 ± 0.24	100
ND	6	D.C.	3.30 ± 0.24*	79.1
ND + AL01576	6	Clear	4.04 ± 0.24^{ns}	96.9
ND + Sorbinil	6	Clear	4.03 ± 0.47^{ns}	96.6
ND + Tolrestat	6	D.C.	2.07 ± 0.11**	49.6
ND + AL04114	6	Clear	4.08 ± 0.23^{ns}	97.8

The lenses were exposed to naphthalene dihydrodiol (ND) or ND plus one of the ARIs for 48 hrs. All the compounds were used at the same concentration (5×10^{-5}M). D.C.= deep cortical opacity (induced by naphthalene dihydrodiol).

Data are expressed as Mean ± S.D.; (n) = the number of samples.
* = $P < 0.01$; ** = $P < 0.001$; ns = $P > 0.05$; (independent t-test).

fore, the ineffectiveness of Tolrestat in this cataract model may be due to its slow accumulation in the lens at the given dosage. To avoid such a possibility, several dose levels up to 2.5×10^{-4} M of Tolrestat were examined and similar ineffective results were found (data not shown).

Establishment of Dual Cataract

An *in vivo* dual cataract model was successfully established in Long-Evans rats. The rats were made galactosemic by feeding rat chow containing 30% galactose and were given naphthalene (1 g/kg/day) simultaneously for 2 weeks. The lenses not only developed a deep cortical shell opacity (induced by naphthalene) but also produced an outer cortical opacity (induced by galactose feeding). The galactose-induced cataract started as numerous small vacuoles around the equator and then merged to form opacities in the cortex. Biochemical study on this dual cataract showed a dramatic accumulation of dulcitol and an extensive depletion of lens GSH (Figures 28 and 29, Table 16).

Similar to the *in vivo* studies, dual cataracts were successfully induced in cultured rat lenses when they were exposed for 48 hours to 30 mM galactose and 5×10^{-5} M naphthalene dihydrodiol simultaneously. After 48 hours in the culture medium, a characteristic cortical opacity was induced in lenses exposed to high galactose but a deep cortical shell opacity was only seen in the lenses exposed to either naphthalene dihydrodiol alone or jointly with galactose (Figure 30). The biochemical changes correlated well with the above morphological findings. As shown in Table 16, lenses exposed to high galactose or high galactose plus naphthalene dihydrodiol hydrated extensively, as evident by the morphological observation and their weight gain. Both lens GSH level and transport systems of ^{86}Rb and ^{3}H-Choline were drastically decreased under high galactose stress. The double stress of naphthalene and high galactose lowered lens GSH to 21% of the normal value and impaired the ability of the lens to accumulate choline and rubidium to the same degree as the galactose stress alone.

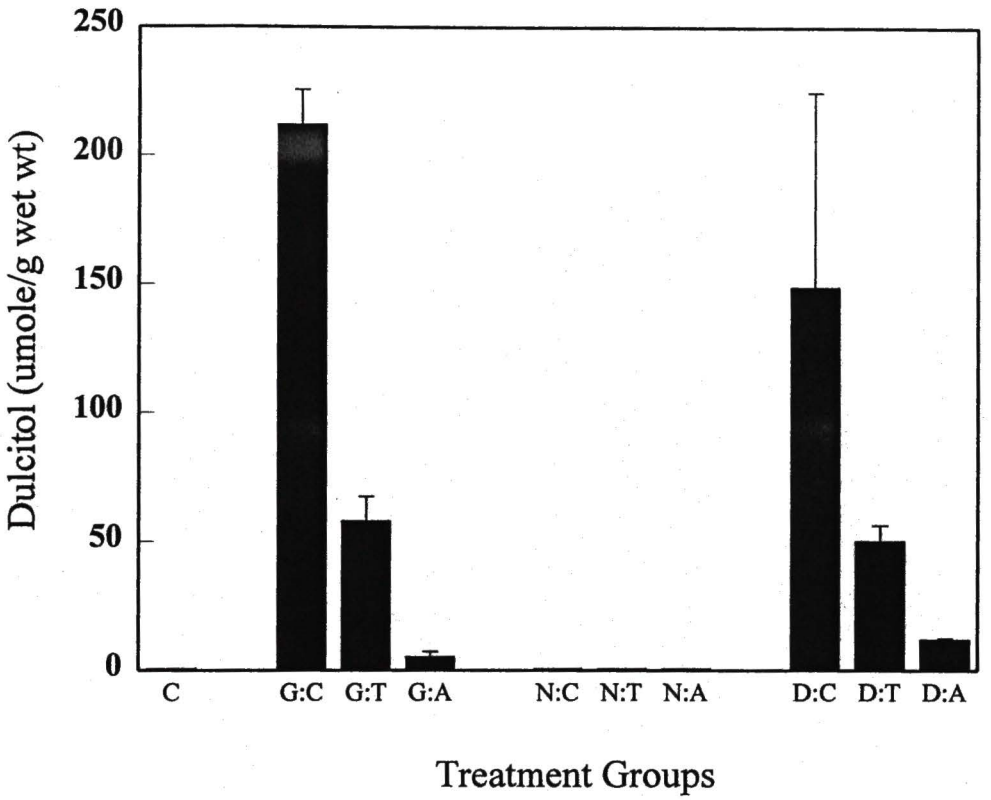


Figure 28. Comparison of the effects of AL01576 and Tolrestat on lens dulcitol level of rat cataracts induced by galactose and/or naphthalene. The rats were fed for 2 weeks with 30% galactose chow or naphthalene (1.0 g/kg/day) or both together. Each bar represents an average and S.D. of 4-6 samples.

C: control; T: Tolrestat treated; A: AL01576 treated;

G: galactose-fed; N: naphthalene-fed; D: dual cataract model.

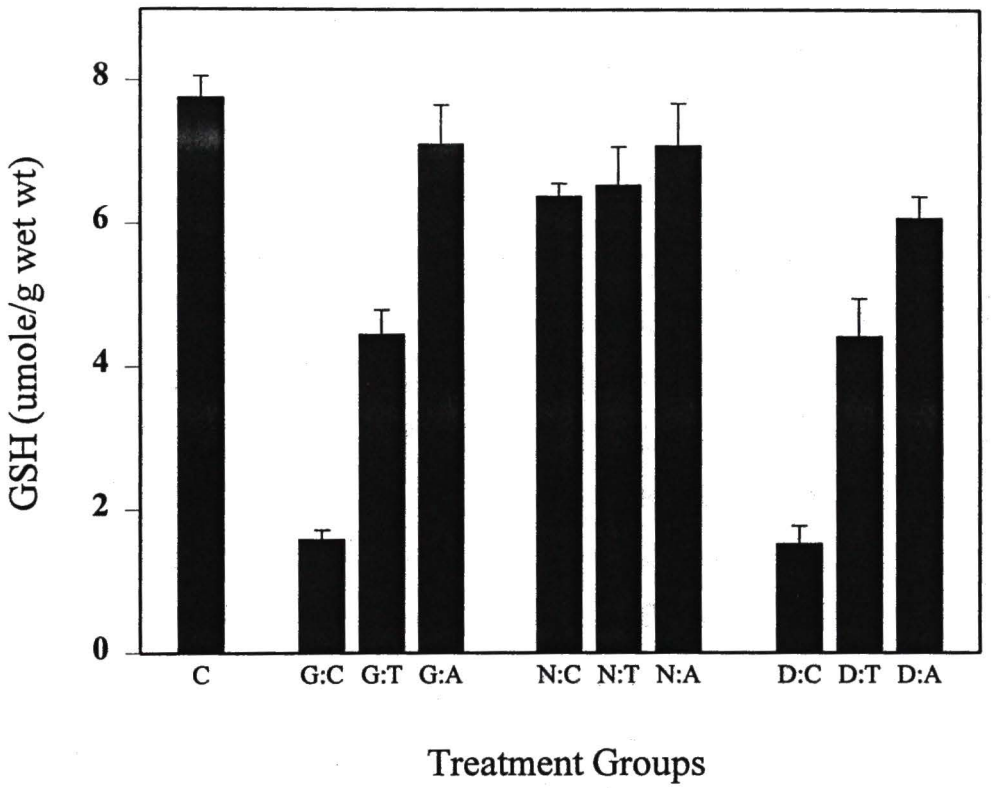


Figure 29. Comparison of the effects of AL01576 and Tolrestat on lens GSH levels of rat cataracts induced by galactose and/or naphthalene. The experimental conditions and the symbols for each group are the same as those in the Fig. 28. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ (independent t-test, all the data are compared to the same control, C).

TABLE 16. Comparison of the ARI Treatment on an In Vitro Induced Dual Cataract by High Galactose and Naphthalene Dihydrodiol.[a]

Group (n)	Lens wt. (mg)	Morphology	GSH μmole/gwt	^{86}Rb (L/M)	^{3}H-Choline (L/M)
C	22.89 ± 1.44	Clear	4.02 ± 0.60	7.74 ± 0.84	7.61 ± 1.02
G	28.24 ± 1.76*	O.C.	1.09 ± 0.18	3.38 ± 0.93*	2.70 ± 0.84*
G + T	24.11 ± 0.78	Clear	2.37 ± 0.44	4.88 ± 0.64*	4.43 ± 0.75*
G + A	23.47 ± 1.05	Clear	3.47 ± 0.62	6.21 ± 0.93	5.86 ± 1.04
ND	23.41 ± 0.83	D.C.	3.09 ± 0.28	6.41 ± 1.11	6.19 ± 1.31
ND + T	23.71 ± 1.19	D.C.	2.21 ± 0.72	5.87 ± 0.59*	5.54 ± 0.46*
ND + A	24.10 ± 1.09	Clear	3.67 ± 0.46	6.24 ± 0.93	5.76 ± 1.18
G + ND	27.43 ± 1.56*	Dual	0.84 ± 0.28	3.30 ± 0.74*	2.54 ± 0.68*
G + ND + T	24.53 ± 0.60	D.C.	1.51 ± 0.44	4.35 ± 0.72*	3.85 ± 0.47*
G + ND + A	23.25 ± 0.37	Clear	3.37 ± 0.81	6.05 ± 1.24	5.50 ± 1.48

Opacity: O.C.= outer cortex; D.C.= deep cortex; Dual = outer and deep cortex.

C: Control; G: Galactose (30 mM); ND: Naphthalene Dihydrodiol (5 X 10^{-5}M);

T: Tolrestat (5 X 10^{-5}M); A: AL01576 (5 X 10^{-5}M).

[a] Data are presented as Mean ± S.D., n = 4 for each group.

* indicated a significant difference between that group and the control ($P < 0.05$). The others are not significantly different from the control at the 0.05 level (independent t-test).

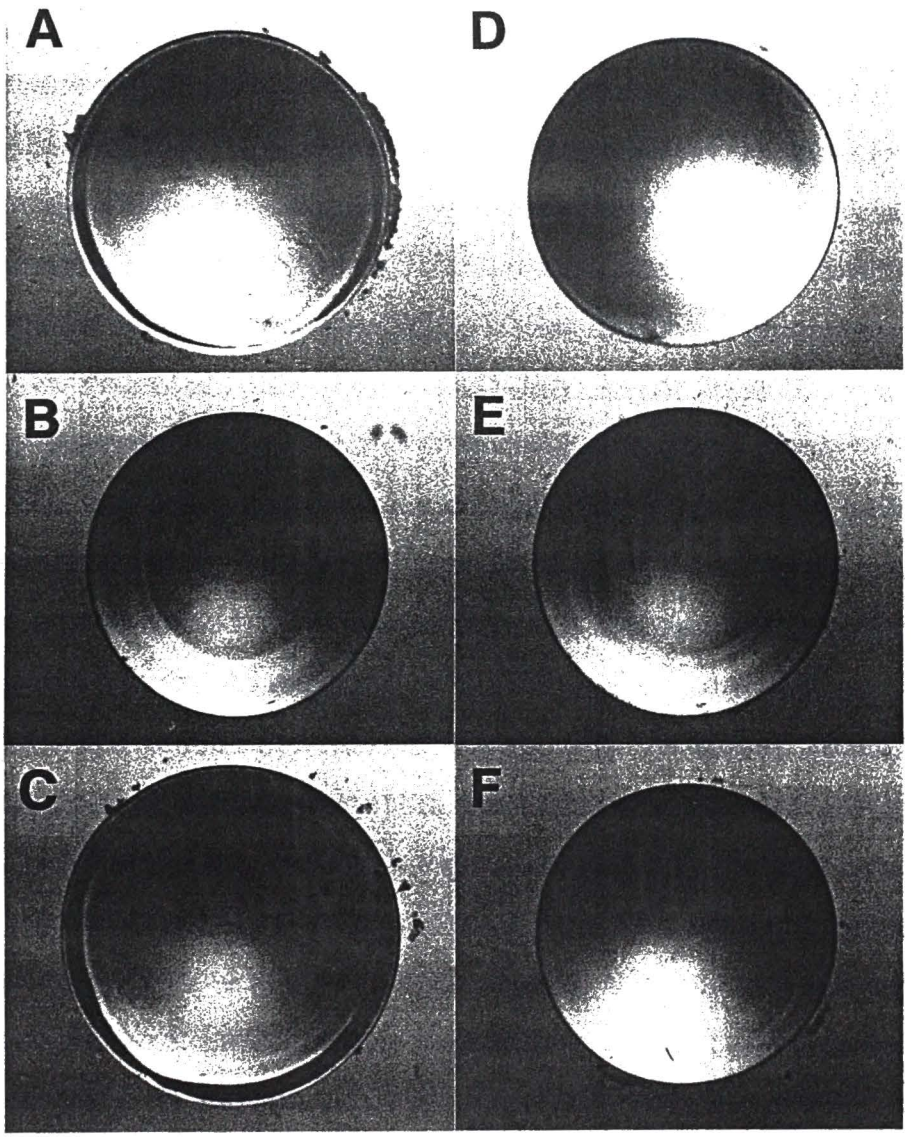


Figure 30. Comparison of the morphological changes of *in vitro* cataracts induced by galactose and/or naphthalene dihydrodiol and the effects of Tolrestat and AL01576 (both at 5 x 10^{-5}M). Test period: 48 hours.

A: Galactose, 30 mM;

B: Naphthalene Dihydrodiol, 5 x 10^{-5}M;

C: Dual cataract (induced by A + B);

D: Control;

E: Dual cataract + Tolrestat;

F: Dual cataract + AL01576.

Comparison of AL01576 and Tolrestat on the Dual Cataract Model

In order to clarify whether the ARI's preventive effect in naphthalene cataract is strictly a result of aldose reductase inhibition or involves some other mechanism, a potent ARI (AL01576) and a weak ARI (Tolrestat) were compared in the dual cataract model made by feeding 30% galactose chow and naphthalene (1 g/kg/ day) simultaneously. AL01576 and Tolrestat were each administered to these doubly stressed rats at a dosage which is known to prevent galactose cataract formation (Dvornik et al., 1986; Lou et al., 1988). AL01576 at 10 mg/kg/day completely prevented both outer and deep cortical cataracts whereas Tolrestat at 50 mg/kg/day only prevented the outer cortical opacity induced by galactose but not the deep cortical ring induced by naphthalene. At the same time, control experiments were carried out on lenses from animals exposed to only one of the cataractogenic agents. As expected, both ARIs prevented galactose cataract but only AL01576 prevented naphthalene cataract. Figures 28 and 29 summarize the biochemical findings of this experiment. When the rats were fed with galactose alone, the lens dulcitol accumulation (Figure 28) was only partially reduced by Tolrestat but was almost totally eliminated by AL01576. Since naphthalene feeding did not contribute to dulcitol accumulation, the dual cataract group showed a similar lens dulcitol pattern to galactose cataract and responded to the two ARIs similarly. In addition, AL01576 largely preserved lens GSH in all three cataract groups (galactose, naphthalene and dual cataracts) whereas Tolrestat had a much weaker effect in preventing GSH depletion in all 3 cataract groups (Figure 29).

In the *in vitro* studies, the effects of Tolrestat and AL01576 on the dual cataract caused by galactose and naphthalene dihydrodiol were also examined. As shown in Figure 30, the cortical opacity induced by high galactose was ameliorated after treatment either by AL01576 or Tolrestat. However, the opacities in deep cortex induced by naphthalene dihydrodiol was only eliminated by AL01576, but not by

Tolrestat (Figure 30). In the dual cataract group, Tolrestat treatment effectively abolished the outer cortical opacity induced by galactose but left the deep cortical shell intact. Biochemical changes correlated well with the above morphological findings. As shown in Table 16, the hydration in the lenses exposed to high galactose-containing media was effectively prevented by both AL01576 and Tolrestat. In addition, the high galactose induced lens GSH loss and ^{86}Rb and ^{3}H-Choline transport reduction were prevented more effectively by AL01576 than by Tolrestat. The double stress lowered lens GSH to 21% of the normal value. However, the Tolrestat treatment maintained lens GSH at 40% and the AL01576 treated group maintained the lens GSH at 84% of the control level.

Dihydrodiol Dehydrogenase Activity of Rat Lens

Using a fluorescence spectrophotometer, the activity of dihydrodiol dehydrogenase (DDD) of rat lens was studied in test tube. Figure 31 demonstrated the production of NADPH through this enzyme reaction. It is clear that the rat lens extract contains the enzyme DDD or another enzyme which used $NADP^+$ and naphthalene dihydrodiol as substrates and yielded 1,2-dihydroxynaphthalene and NADPH. As shown in the plot, the enzyme reaction was clearly inhibited by the addition of AL01576.

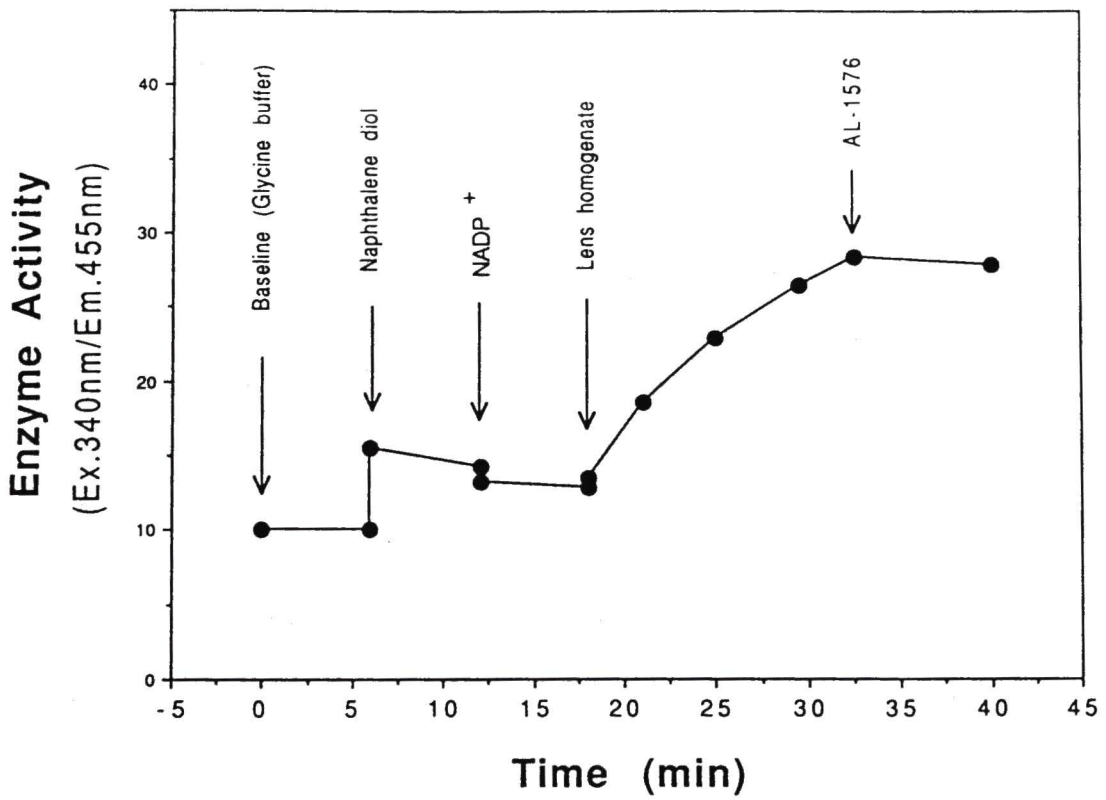


Figure 31. Dihydrodiol dehydrogenase activity in rat lens extract and the inhibition by AL01576. For detail, see METHODS.

CHAPTER IV

DISCUSSION

Study I: Naphthalene Cataract Model in Rats

The existence of lens opacities is the most important parameter to evaluate if a cataract model is successful or if a medicine can prevent the cataract formation. Therefore, the morphological changes in our rat naphthalene cataract model were observed and recorded very carefully during the entire period of naphthalene treatment. Based on slit-lamp examination, all the rats fed with naphthalene alone developed typical cataracts which were similar to the descriptions by Hockwin et al.(1984-85). Waterclefts and spoke-like opacities were seen in the cortex within one week of naphthalene feeding. The spokes merged to form a shell-like opaque layer around the nucleus. The most interesting observation was that the nucleus inside the opaque shell and the outer layer cortex surrounding the shell remained clear. One hypothesis for this phenomenon is that the opaque layer first formed in the outer layers of cortex and being pushed inwards when the new fiber layers next to the epithelium were continuously made and laid down during the experimental period (Koch et al., 1976). Such an explanation is not very convincing because naphthalene was given continuously for the entire test period and the new fibers were under the same stress caused by naphthalene and should also develop cataract. Therefore, if naphthalene targeted at the lens bifers near the epithelium, the entire outer cortex should become opaque without a cleat zone between epithelium and the opacity. In our observation, during the first week of naphthalene feeding the earliest visible opacity was separated from the capsule-epithelium by layers of clear cortex (Figure 6). The lenses could not grow so fast

to form a rather deep clear zone around the opaque layers in several days. Thus, it is reasonable to assume that the toxic metabolites of naphthalene penetrated into the lens and induce opacities in certain layers of cortex. Such speculation is supported by the studies on the *in vitro* naphthalene cataract models which will be discussed below.

Results of our biochemical findings in this model clearly indicate that naphthalene-induced lens damage was oxidative in nature. The fact that decrease in GSH was the earliest biochemical change observed suggests that oxidative stress may be the primary mechanism of damage in this model. A transient decrease in GSH was observed only in the lens epithelium after a single naphthalene dosing, while daily feeding for 1 week produced a 30% permanent loss of GSH in both epithelium and cortex (Figure 10 and 11). Only at later stages did we observe oxidative effects on lens proteins, such as protein-protein disulfide cross-linking and protein insolubilization. The most pronounced oxidative change was the high elevation of protein-GSH mixed disulfide, a conjugate usually observed in the lens cortex. Protein-cysteine mixed disulfide which is normally associated with water insoluble proteins and proteins from the lens nucleus region (Lou et al., 1990) only slightly increased. Interestingly, the naphthalene cataract lens had an intact membrane transport function as well as normal Na^+/K^+-ATPase activity (Table 7). In view of these findings, we believe the damaging agent must arise within the lens since toxic species such as H_2O_2 entering the lens from the surrounding fluids invariably produce damages at the lens periphery, generally evident by impaired membrane transport functions (Garner et al., 1983). It would seem likely that the toxic metabolites caused by naphthalene feeding were generated in the metabolically active epithelial and outer cortical regions where they conjugate 20%-30% of the free GSH pool. However, the GSH biosynthesis machinery was not damaged and more than 60% of the defense systems were found intact even after the rats were fed naphthalene for 4-6 weeks (Rathbun et al., 1990). Therefore, the cells of this

region should be reasonably competent to defend themselves from toxic insults. In contrast, the deeper cortical layers are much less metabolically active and have weaker antioxidative defense ability. Any toxic insult present in this region is likely to produce permanent damage. Indeed, the morphological changes in the lenses of naphthalene treated rats were most pronounced in the deep cortical area where we also observed that opacity was enhanced with each additional week of naphthalene feeding.

Some recent reports demonstrated that certain ARIs such as AL01567 and AL01576 could effectively prevent the cataract induced by naphthalene or naphthalene along with diabetes (Hockwin et al., 1984; Wegener et al., 1985; Hockwin et al., 1984-85; Xu et al., 1989; Rathbun et al., 1990). The present study confirmed the preventive effect of AL01576 on naphthalene cataract development in rats. Morphologically, the lenses remained perfectly clear throughout the six weeks test period if the rats were treated with naphthalene plus a small dosage of AL01576 (Figure 8). Under slit-lamp, we could not distinguish these lenses from those of control rats. Biochemical studies also showed the ability of AL01576 to suppress naphthalene-induced changes. In the rats treated with naphthalene and AL01576, the lens GSH level was maintained at 90% of normal concentration (Table 5, Figure 11), protein-GSH mixed disulfide was kept at the same low level as the control (Table 5) and no protein-protein disulfide crosslinking was detected on SDS-PAGE gel (Figure 12, lane C).

The action of AL01576 was previously assumed to be through its inhibition of tyrosinase. The hypothesis is that the enzyme tyrosinase in the iris and ciliary body (especially in pigmented rats) catalyzes the productions of 1,2-dihydroxynaphthalene from 1-naphthol and 2-naphthol. 1,2-dihydroxynaphthalene penetrates into the lens and induces cataract through its further metabolism. AL01576 blocks the production of 1,2-dihydroxynaphthalene through its inhibition of tyrosinase and thus prevents the cataract formation. However, an *in vitro* study showed that the activity of tyrosinase, derived either from fungi or from bovine iris

and ciliary body, was not affected by AL01576 (Hockwin et al., 1984-85). Considering that AL01576 prevented only the changes in the lens but not any of the systemic effects, it is reasonable to assume that AL01576 penetrated into the lens and selectively inhibited certain lens enzymes in the naphthalene metabolic pathway, such as dihydrodiol dehydrogenase. Another possibility is that other tissues lack of the pretective barrier which the eye has, and thus expossed to other naphthalene metabolites in the blood directly. These assumptions were supported by the *in vitro* studies and the enzyme assay.

Study II: Naphthalene-related Cataracts in Lens Culture

The *in vivo* naphthalene cataract model provided a powerful tool to study the mechanism underlying the disease. But such studies are usually very laborious and time consuming, and also it is difficult to identify the real cause of the cataract in the *in vivo* system because many factors and reactions are involved. In contrast, *in vitro* models can be used to examine the direct effects of each naphthalene metabolite so that one can determine which metabolites are most likely involved in the cataractogenesis of naphthalene.

Naphthalene was reported to produce 5 metabolites in animals (see introduction). All of them were tested for their abilities to induce cataracts in the *in vitro* system. At 5 X 10^{-5} M concentration and 48 hours of incubation, 1-naphthol and 2-naphthol induced no cataract although they decreased choline transport considerably (Table 8). Both 1-naphthol and 2-naphthol were proposed to be important precursors for the formation of 1,2-dihydroxynaphthalene by Koch et al. (1979). They suggested that the enzyme tyrosinase in the pigmented eye tissues can convert 1-naphthol and 2-naphthol to 1,2-dihydroxynaphthalene and thus cause damage to the lens. Our current studies do not support this hypothesis. First of all, the feeding of 1-naphthol to rats at 1 g/kg/day for a month induced no cataract in the lens. The dosage of 1 g/kg/day is a larger load of 1-naphthol to the rats

than naphthalene feeding at the same dosage because only part of naphthalene is converted to 1-naphthol in the rats. If naphthalene induced cataract through 1-naphthol, the treatment of larger dosage of 1-naphthol would result in cataract too. Secondly, the exposure of rat lens to 1-naphthol or 2-naphthol at 5 X 10^{-5} M in lens culture induced no detectable lens opacity. Considering that the conversion of naphthols to 1,2-dihydroxynaphthalene needs the catalysis by the enzyme tyrosinase, the enzyme was added into the above culture system to test the effects of this combination. After 48 hours of culture, the lens remained clear in the medium containing both 1-naphthol and tyrosinase (unpublished data). Again, the in vitro study did not support the hypothesis that naphthols and tyrosinase are involved in naphthalene cataract formation. Thirdly, biochemical analysis revealed no change in the tested parameters except the mild decrease in choline transport (Table 8 and 9). It seems that the reaction between naphthols and choline transport is selective, either through the binding of naphthols to choline transporter or through the competition of naphthols with choline molecules for the transport sites. The significance of this decrease has not been determined yet and also this change is not a characteristic of naphthalene cataract. Based on these data, we proposed that 1-naphthol and 2-naphthol are not involved in naphthalene cataract formation. Naphthalene is converted to the culpable metabolites such as 1,2-dihydroxynaphthalene and 1,2-naphthoquinone through another pathway which bypasses the intermediates naphthols.

At the same concentration and exposure time, the other three metabolites all induced cataracts *in vitro*, but only naphthalene dihydrodiol induced a cataract which was similar both morphologically and biochemically to the naphthalene cataract induced *in vivo* (Figure 16 and 17, Table 8, 9 and 10). 1,2-dihydroxynaphthalene or 1,2-naphthoquinone each produced very severe damage to the lens. However, the site affected by these two compounds was at, or very near, the outer surface of the lens, as evident by the opacities in the outer cortex (Figure 16D) and the decreases in Na^+/K^+-ATPase activity and membrane trans-

port function (Table 8 and 9). In contrast, naphthalene dihydrodiol caused damage that was concentrated in the inner layers of the cortex (Figure 16C). The cellular membrane transport systems and the key transport enzyme, Na^+/K^+-ATPase, were unaffected (Table 8). The only biochemical change observed was the depletion in lens GSH concentration with a concomitant increase in protein-GSH mixed disulfide and in protein-protein disulfide crosslinking (Table 9, Figure 17). All the above morphological and biochemical findings induced by naphthalene dihydrodiol were similar to the observation in the *in vivo* naphthalene-induced cataract. Therefore, it is reasonable to assume that naphthalene was metabolized to naphthalene dihydrodiol which reached the lens and caused the opacity.

These findings in the *in vitro* model therefore supported the conclusion mentioned above that *in vivo* naphthalene-induced cataract changes involve oxidative processes. Furthermore, they also support the hypothesis that naphthalene dihydrodiol was the metabolite which reached the aqueous humor through the circulation. This metabolite is quite stable and penetrates into the lens, where presumably the enzyme dihydrodiol dehydrogenase (EC 1.3.1.20.), or another enzyme with similar activity, converts it to 1,2-dihydroxynaphthalene which autoxidizes to 1,2-naphthoquinone. This autoxidation must occur inside the lens, otherwise 1,2-naphthoquinone would insult the epithelium and the outer layers of cortex while it penetrated these regions, and the morphological characteristics would be different from the *in vivo* model too. This toxic product can cause damage to the tissue either by conjugating with lens protein (Rees and Pirie, 1967) or by modifying the protein through free radical action (Brunmark and Cadenas, 1989). These events may be more evident in the inner section of the cortex since intrinsic antioxidants such as GSH are higher in the outer cortex where they may be able to successfully counteract the toxic effect of the naphthalene metabolite. The hypothesis that naphthalene dihydrodiol is metabolized *in situ* is also consistent with the findings that higher concentrations of naphtha-

lene dihydrodiol in the media quantatively induces the expansion of the opaque area outward towards the capsule since it is likely that the more naphthalene dihydrodiol in the media the more will penetrate into the lens (Figure 24). The location of the opaque shell in the lens can also be manipulated by the amount of AL01576 in the medium perhaps because AL01576 inhibits the production of 1,2-naphthoquinone from naphthalene dihydrodiol in the lens. Any increase in oxidative stress, either by increasing the naphthalene dihydrodiol concentration or decreasing AL01576 causes the opacification to occur closer to the periphery of the lens. Conversely, decreasing the level of the stress causes the opacity to form deeper in the lens cortex (Figure 19). It is clear, therefore, that the formation of naphthalene cataract is not limited to a specific zone of lens fibers which are particularly susceptible. Rather it appears that the location of the lesion depends upon a balance between the level of oxidative stress and the capacity of the lens cells to combat the stress. Since lens fibers become metabolically less active from the superficial layers toward the center, it is likely that the deeper within the lens that a fiber is located, the less able it is to protect itself from the oxidative stress.

Study III: Mechanism for Naphthalene Cataract Formation

The currently accepted hypothesis for naphthalene cataract formation was proposed by van Heyningen in 1979. She suggested that in both rats and rabbits 1,2-dihydroxynaphthalene is the toxic agent through its autoxidation to 1,2-naphthoquinone. In the rabbit, catechol reductase in the lens catalyzed the production of 1,2-dihydroxynaphthalene from naphthalene dihydrodiol; whereas in the rat the enzyme tyrosinase in the iris or ciliary body converts 1-naphthol and 2-naphthol to 1,2-dihydroxynaphthalene (van Heyningen, 1979). The hypothesis emphasizes the role of pigmented tissues in rat naphthalene cataract, which and the different catechol reductase activities in lens are the two major differences between the

mechanisms of naphthalene cataracts in rat and rabbit.

A possible role of pigmentation in cataractogenesis in naphthalene-fed rats arose from the inability of various investigators to reproduce the first success reported by Goldmann in 1929. Since albino rats came into common usage after Goldmann's era, it was suspected that pigmented tissues might explain this discrepancy. Koch et al. (1976) investigated this possibility by comparing the susceptibilities of pigmented rats with albino rats to naphthalene cataract induction and found that the pigmented rats developed cataract sooner, more severely and more consistently. Thus the dependence of the production of rat naphthalene cataract on the pigmentation was established and has never been challenged. In the present study, the procedure for naphthalene cataract induction followed closely with that of Hockwin et al. (1984-85). We were able to produce naphthalene cataract in pigmented rats as others, but we also had no difficulty in producing naphthalene cataract in albino rats. When 3 albino strains were compared with 2 pigmented strains in this study, the result showed little difference in the cataract morphology, severity, or rate of opacity formation between the rats with and without pigmentation (Figures 7 and 8). Even the biochemical parameters were comparable (Table 5 and 12). While re-evaluating the report of Koch et al. (1976), we are aware that they did observe zonular cataract in 3 out of the 5 Sprague-Dawley rats. In this study, we used retroillumination to capture the entire lens changes in the albino eye, which was hard to catch with direct illumination due to the poor contrast in an albino eye. Besides these, we also observed the presence of yellow or brown opacification in all lenses from naphthalene-fed rats after excising and examining them under a dissecting microscope.

Since 1- or 2-naphthol has been considered to be the key metabolite in this pigmentation hypothesis, we fed 1-naphthol to both pigmented and albino rats for four weeks but failed to observe any lens opacity. AL01576, which was originally reported to prevent naphthalene-induced cataract in the pigmented rats (Hockwin et al., 1984-85), also inhibited naphthalene cataract induction in the albino rats

(Table 5 and 12). Furthermore, we have confirmed the finding of Hockwin et al. (1984-1985) that AL01576 exhibited no ability to inhibit enzyme tyrosinase, the key enzyme implicated in the pigmentation hypothesis (unpublished results). We have thus concluded that the earlier claim on the role of pigmentation in naphthalene cataract formation is unwarranted.

Similar results were obtained from our *in vitro* naphthalene cataract studies which demonstrated that lenses from both albino and pigmented rats formed cataracts similarly after exposure to naphthalene dihydrodiol *in vitro* (Table 12). The similarity of the *in vitro* naphthalene dihydrodiol-induced cataract to the *in vivo* naphthalene cataract strongly supports the hypothesis that naphthalene dihydrodiol is the key metabolite of naphthalene which circulates to the eye and affects the lens. Inhibition of this process by AL01576 possibly results from its inhibition of one or more key enzymes involved in the metabolism of naphthalene dihydrodiol in the lens.

The involvement of oxidation in naphthalene cataract formation was first indicated by the decrease of GSH in early stages of the cataract (Figure 10 and 11). Glutathione, a tripeptide consisting of three amino acids, glutamate, cysteine and glycine, is the major component of the lens defensive system against oxidation. It exists in two forms, reduced (GSH) and oxidized (GSSG) and its concentration is regulated by several factors because it is involved in several metabolic pathways as shown in Figure 32: (1) it is regulated by its anabolism and its catabolism. In the anabolic pathway, peptide bonds are formed between glutamate and cysteine by the action of γ-glutamylcysteine synthetase and between this dipeptide and glycine catalyzed by glutathione synthetase. In the catabolism, it is first degraded by the enzyme γ-glutamyltranspeptidase to cysteinylglycine and γ-glutamate conjugated with an amino acid. Cysteinylglycine is further broken down by dipeptidase to cysteine and glycine, and glutamyl-AA is converted to glutamate by two enzymatic reactions; (2) Reduced glutathione can conjugate with some metabolites such as quinones to facilitate their metabolism and elimination. The

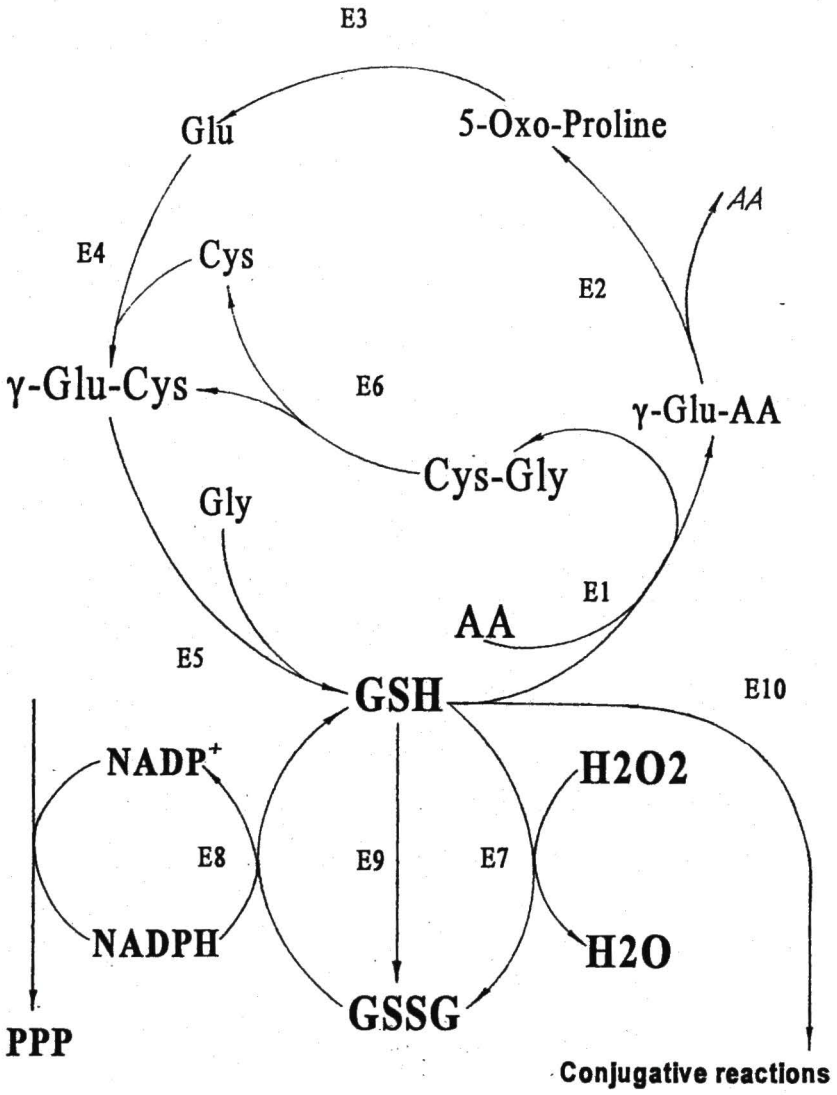


Figure 32. Metabolic pathways involved in the maintenance of GSH level.

E1: γ-Glutamyltranspeptidase;
E2: γ-Glutamylcyclotransferase;
E3: 5-Oxoprolinase;
E4: γ-Glutamylcysteine Synthetase;
E5: Glutathione Synthetase;
E6: Dipeptidase;
E7: Glutathione Peroxidase;
E8: Glutathione Reductase;
E9: Transhydrogenase;
E10: Glutathione S-Transferase.

reaction is catalyzed by the enzyme glutathione S-transferase; (3) An enzyme transhydrogenase converts GSH to GSSG directly, and its significance has not been recognized yet; (4) The dynamic equilibrium between the two forms of glutathione. This cycle is very important to maintain the glutathione molecules in the reduced status. Under the action of glutathione peroxidase (GPx), GSH reduces oxidant H_2O_2 to H_2O. The GSSG yielded in this reaction is reduced back to GSH by glutathione reductase (GR) with NADPH as the H^+ donor. The NADPH molecule is mainly provided by the pentose phosphate pathway (PPP). When oxidative stress exceeds the defense ability, the oxidants such as H_2O_2 and free radicals start to attack protein functional groups such as thiol groups and amino groups (Mason and Peterson, 1965; Rees and Pirie, 1967; van Heyningen and Pirie, 1967). In addition, the accumulated GSSG can conjugate with the thiol group in protein to form mixed disulfides. The dramatic increase in the lens mixed disulfides in naphthalene treated rats (Table 5 and 9) provided the second evidence for the oxidative mechanism. At later stages, disulfide cross-linked protein aggregates were detected by SDS-PAGE (Figure 12 and 17), which again supported the oxidation hypothesis.

A point of emphasis is that the oxidants did not attack the lens from outside but were produced inside the lens. When the lens was exposed to oxidants like H_2O_2 or naphthoquinone in culture medium, the lens membrane was damaged badly as indicated by lens swelling and impaired membrane functions (Figure 16, Table 8). The cataractous lenses from naphthalene-fed rats showed no hydration and the membrane function as well as ATPase activity were perfectly normal (Figure 16, Table 8). It is thus reasonable to assume that certain precursors to the oxidant must have reached the lens and were converted to oxidants *in situ*. This was further supported by the failure to detect any naphthoquinone and other oxidants in the aqueous humor of naphthalene-fed rats as discussed below.

Our attempt to analyze the naphthalene metabolites in the ocular tissues of

naphthalene-fed rats revealed that only naphthalene dihydrodiol existed in detectable amounts in the aqueous humor and lens (Figure 23). Furthermore, the amount of naphthalene dihydrodiol detected in lens after it was exposed to 2.5 X 10^{-5} M naphthalene dihydrodiol in the culture medium was similar to the amount present in lenses of naphthalene fed rats after 4-6 weeks feeding (Figure 24). On the other hand, while we failed to detect any 1,2-naphthoquinone in the aqueous humor or in the lens in this rat model, Iwata and Maesato (1988) reported that they could detect 1,2-naphthoquinone in substantial amounts in rabbit ocular tissues and serum after only a single administration of naphthalene. This discrepancy can be explained by the fact that rat lens was reported to have only 3% of the amount of catechol reductase present in the rabbit lens (Van Heyningen, 1970). Hara et al. (1989) isolated a key enzyme, now called dihydrodiol dehydrogenase, which converts naphthalene dihydrodiol to the toxic 1,2-dihydroxynaphthalene from lenses of several species, including rat. The low concentration of this enzyme in rat and the reactive nature of its end-product 1,2-naphthoquinone with biochemical molecules in the lens could explain our difficulty in detecting the free quinone in ocular tissues.

Based on the above information and findings, we believe that the ingested naphthalene is first metabolized to naphthalene dihydrodiol in the liver by cytochrome P-450 system (Jerina et al., 1970; Bock et al., 1976; D'Arey Doherty and Cohen, 1984). Naphthalene dihydrodiol is a stable compound which is released into the blood circulation from the liver and is eliminated via kidney. When it reaches the aqueous humor via the circulation, it penetrates into the lens, where it is converted by dihydrodiol dehydrogenase and/or other unknown enzymes to the toxic end product of 1,2-naphthoquinone. This reaction scheme is depicted in Figure 33, which is similar to the cataract mechanism in naphthalene-fed rabbit as proposed by van Heyningen (1979). The autoxidation of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone also yields H_2O_2, whereas free radicals could be

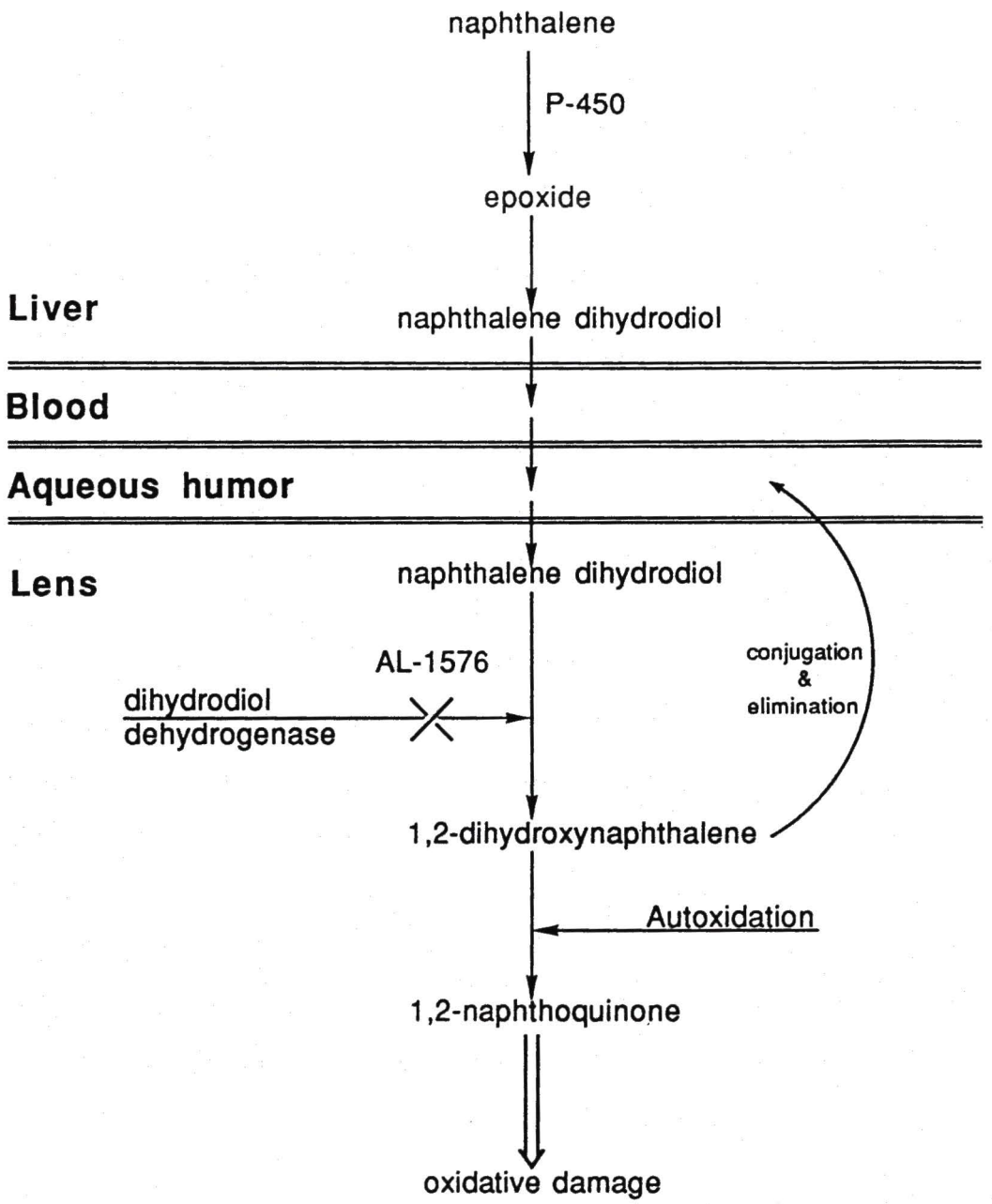


Figure 33. Proposed mechanism for naphthalene cataract formation and the action of AL01576 in the prevention of this cataract. I: naphthalene; II: naphthalene epoxide; III: naphthalene dihydrodiol; IV: 1,2-dihydroxynaphthalene; V: 1,2-naphthoquinone.

produced through the redox cycling of 1,2-naphthoquinone with its semiquinone or other reactions (Brunmark and Cadenas, 1989). All these oxidants may contribute to the cataract formation in naphthalene-fed rats.

Study IV: The Action of AL01576 in Naphthalene Cataract Prevention

The preventive effect of some ARIs in rat naphthalene cataract was first reported by Hockwin et al. (1984-85) and Wegener et al. (1985) as they studied diabetic cataract and naphthalene cataract in rats. They demonstrated that a relative low dose of AL01567, another Alcon's ARI which is less efficient than AL01576, was able to prevent or slow down the development of both diabetic and naphthalene cataracts. Hockwin et al.(1984-85) tested the effect of AL01576 in similar experiments and reported that AL01576 had also effectively prevented cataract in naphthalene-fed rats. This new efficacy of AL01576 in the prevention of naphthalene cataract *in vivo* was confirmed recently (Tao et al., 1989; Xu et al., 1989). The extensive studies on Alcon's ARIs (AL01576 and AL04114) in this thesis have demonstrated the efficacious of these ARIs for preventing naphthalene cataract *in vivo* as well as *in vitro* (Table 9 and 10, Figure 17 and 25). Other naphthalene metabolites such as 1-naphthol, 2-naphthol, 1,2-naphthoquinone and 1,2-dihydroxynaphthalene produced totally different types of damages to the lenses in the *in vitro* system, which could not be inhibited by AL01576 (Xu et al., 1990).

The action of AL01576 in naphthalene cataract was previously assumed to be the inhibition of tyrosinase. However, an *in vitro* study showed that AL01576 did not affect the activity of this enzyme at all (Hockwin et al., 1984-85). In this study, the role of pigmentation in naphthalene catarct formation was totally eliminated because the albino rats, which have little tyrosinase activity in their iris or ciliary body, developed naphthalene cataract equally well as pigmented ones (Figure 7

and 8, Table 5 and 12). Therefore, the inhibition of tyrosinase by AL01576 can not explain the effect of this agent.

The other possibility is that the enzyme aldose reductase (AR) is involved in naphthalene cataract formation. AL01576 is a potent inhibitor for AR, and if AR is involved in the metabolism of naphthalene metabolites, the mechanism would be readily apparent. Recently Sato et al. (1993) reported that both the purified rat lens AR and its recombinant protein displayed activity with naphthalene dihydrodiol as a substrate. This activity can be inhibited equally with AL01576, Tolrestat and Sorbinil. Based on these *in vitro* findings, Sato et al. concluded that aldose reductase may be the same enzyme as dihydrodiol dehydrogenase, therefore the inhibition site of ARI for naphthalene cataract could be simply targeted at AR. To test this possibility, several ARIs with different characteristics were examined in a dual cataract model induced simultaneously by galactose and naphthalene *in vivo* and the dual cataract induced *in vitro* by galactose and naphthalene dihydrodiol exposure together. The logic behind this testing system is that ARIs are known to prevent the sugar cataract, induced by high glucose, galactose or xylose, through the inhibition of polyol accumulation in the lens. If naphthalene cataract is induced via the involvement of aldose reductase similar to the sugar cataract, any ARI that is a potent inhibitor to sugar cataract should also be effective in naphthalene cataract prevention. In this dual cataract study, Tolrestat inhibited galactose cataract formation in the outer cortical layer with concomitant reduction of lens dulcitol accumulation but provided no protection against naphthalene induced damage in the deep cortical zone, whereas AL01576 protected the lens from both galactose and naphthalene induced opacification (Figure 28 and 29, Table 13). The *in vitro* studies also showed similar results (Figure 30, Table 16). Therefore, the action of AL01576 for naphthalene cataract prevention may not be associated with the aldose reductase inhibition.

Another possible action site for AL01576 is cytochrome P-450 system, which

is a lager family of isozymes related to most oxidative reactions in our body. All the members in this family are associated with smooth endoplasmic reticulum membrain. As shown in Figure 34, many compounds can be metabolized by this system to their oxidized form. In the case of naphthalene metabolism, this enzyme system catalyzes the first step of naphthalene metabolism by converting naphthalene to its epoxide before being further metabolized. The oxidative reactions are coupled a reductive reaction which is catalyzed by P-450 reductase and requires NADPH. It has been known for a long time that the cytochrome P-450 system can be induced or inhibited by many drugs. For example, phenobarbital and aromatic hydrocarbons can induce the activity of the system, while cimetidine, SKF 525A and ethanol can inhibite its activity. As indicated by Wells et al., naphthalene cataract induction in mice can either be inhibited or enhanced by agents which either inhibit or induce cytochrome P-450, respectively (Wells et al., 1989). Therefore, it is reasonable to believe that naphthalene feeding induced the oxidative activity of cytochrome P-450 and thus produced more metabolites such as epoxide and 1,2-naphthoquinone. Because GSH is in limited supply in liver and kidney and can be depleted, the metabolites like 1,2-naphthoquinone may reach a sufficient level to react with cell constituents. In this study, however, we clarified that cytochrome P-450 is not involved in either the formation of rat naphthalene cataract or in the action of ARIs in the prevention. We compared the effects of both of Alcon's ARIs, AL01576 and AL04114 on naphthalene cataract formation and found that both AL01576 and AL04114 were equally efficacious *in vivo* and *in vitro* (Table 13, 14 and 15, Figure 25). It is known that AL01576 is a cytochrome P-450 inhibitor whereas AL04114 is not (Kiss et al., 1992). In addition, the efficacy of these ARIs is limited to the prevention of ocular damages and neither AL01576 nor AL04114 showed any effect on systemic toxicity caused by naphthalene feeding. As reported in the lectures, there is no cytochrome P-450 activity in the lens. Therefore, the preventive effect of AL01576 in naphthalene

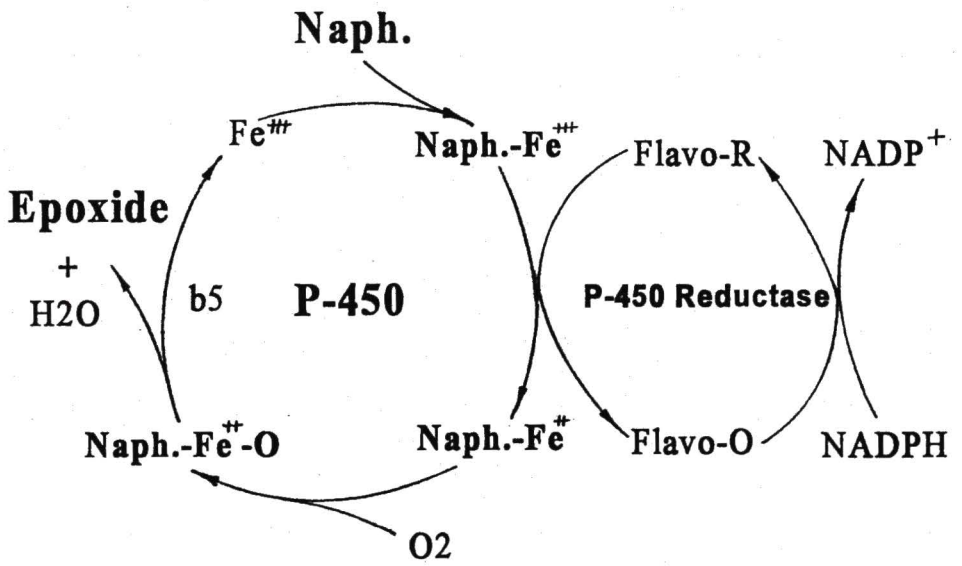


Figure 34. The oxidation of drugs (naphthalene) by cytochrome P-450.

Naph.: naphthalene; Flavo-R: reduced flavoprotein;

P-450: cytochrome P-450; Flavo-O: oxidized flavoprotein.

cataract could not be through its inhibition of cytochrome P-450. The tissue-selected sensitivities to the protection of AL01576 against naphthalene-induced insults suggested that the mechanism of cataract formation in naphthalene feeding is different from that for other insults. Naphthalene was converted to several metabolites which are all released into the circulation and delivered to all the tissues and except the organs with a protective barrier. One of these organs is the eyeball which has a barrier between the blood and the aqueous humor. This barrier blocked the entrance of these metabolites into the aqueous humor and protected the lens from direct insult by those metabolites such as 1,2-naphthoquinone. By an unknown mechanism, naphthalene dihydrodiol penetrated the barrier and entered the lens via aqueous humor. In the lens, naphthalene dihydrodiol was metabolized as mentioned above by the enzyme dihydrodiol dehydrogenase, which was inhibited by AL01576 and AL04114. The situation in other tissues is different. They were not protected by any barrier and the culpable metabolites released from the liver were already exist to attack them directly. Therefore, the inhibition of the enzyme by AL01576 could not protect them.

The fact that AL01576 could inhibit cataract induced by naphthalene dihydrodiol but not by 1,2-naphthoquinone (Xu et al., 1989) suggests the possibility that the AL01576 action site may be between the naphthalene dihydrodiol and 1,2-naphthoquinone in the naphthalene metabolic pathway. As soon as 1,2-naphthoquinone formed, AL01576 could not stop its attack towards the lens. The possible target is the enzyme dihydrodiol dehydrogenase which metabolizes naphthalene dihydrodiol to 1,2-naphthoquinone through 1,2-dihydroxynaphthalene. This inhibition may be a specific property of the spirohydantoin class of ARIs such as AL01576, AL04114 and Sorbinil but not the other ARI classes such as Tolrestat or Statil (data not shown). The direct inhibition of this enzyme by AL01576 in the test tube may explain that the drug action of AL01576 is targeted on inhibiting naphthalene dihydrodiol dehydrogenase (Figure 31).

Based on the above findings, a new mechanism for naphthalene cataract in rats is proposed as the following (Figure 33): naphthalene is first metabolized in the liver by cytochrome P-450 to naphthalene dihydrodiol, which reaches the aqueous humor via the blood and penetrates into the lens. Under the action of dihydrodiol dehydrogenase, it is further metabolized to 1,2-dihydroxynaphthalene and ultimately autoxidizes to 1,2-naphthoquinone. Naphthoquinone reacts with the lens components and thus causes cataract. AL01576 can inhibit the enzyme dihydrodiol dehydrogenase and therefore it can block the production of the toxic species of 1,2-naphthoquinone and H_2O_2, and thus prevent naphthalene cataract formation.

CHAPTER V

SUMMARY

The mechanism of naphthalene-induced cataract in rats and the preventive action of AL01576 (an aldose reductase inhibitor), which succesfully prevented this cataract, were studied in both *in vivo* and *in vitro* systems. In the *in vivo* studies, cataracts were induced in five strains of rats (2 pigmented, 3 albino) by oral administration of naphthalene at 1.0 g/kg/day for up to 6 weeks. Initial lens changes of watercleft and spoke-like opacities were observed after one week by slit-lamp. By three weeks the spokes merged to form a distinct shell-like opacity in the deep cortex. Semiquantitation of the opacities with an arbitary 6-score (0-5) grading system showed little difference in the course of cataract development between the pigmented and albino strains. Major biochemical changes observed were a decrease of 20%-30% in glutathione (GSH) by one week of feeding; the appearance of disulfide cross-linking of lens proteins by 3 weeks; and a more than ten fold increase in the content of protein-GSH mixed disulfide. There was no damage to lens membrane functions as measured by ^{3}H-choline or ^{86}rubidium uptake from the medium in lens culture nor loss of lens Na^{+}/K^{+}-ATPase activity. AL01576 (10 mg/kg/day) completely prevented all the morphological and biochemical changes in the lenses of naphthalene-fed rats in both pigmented and non-pigmented strains. These results indicate that pigmentation is not required for the induction of naphthalene cataract in rats.

In order to study the direct effects of individual naphthalene metabolites on the rat lenses, we established *in vitro* "naphthalene-related cataract" models by exposing rat lenses to 5 individual naphthalene metabolites in modified TC-199

medium for 48 hours. Under these conditions, we analyzed several biochemical parameters as mentioned above. When naphthalene dihydrodiol was used, the results showed that both the morphological and biochemical changes were very similar to those observed in lenses of naphthalene-fed rats (1 g/kg/day). Furthermore, AL01576 completely blocked the *in vitro* changes as it did *in vivo*. Other naphthalene metabolites such as 1-naphthol, 2-naphthol, 1,2-dihydroxynaphthalene and 1,2-naphthoquinone were also studied *in vitro* and the results showed that the effects of these naphthalene metabolites were very different from those observed in naphthalene cataracts *in vivo* and none of them could be prevented by AL01576. These results demonstrated that naphthalene dihydrodiol is the key metabolite which causes the cataract when it reaches the lens where it is metabolized to 1,2-naphthoquinone. This mechanism is further supported by the detection of naphthalene dihydrodiol in the lens and aqueous humor of naphthalene-fed rats.

To further understand the action of AL01576, we compared the effects of four classes of ARI (AL01576, AL04114, Sorbinil and Tolrestat) on naphthalene cataract and a dual cataract induced with simultaneous feeding of galactose (30%) and naphthalene (1.0 g/kg/day). At 10 mg/kg/day, both AL01576 and AL04114 (both spirohydantoin derivatives) completely prevented the morphological and biochemical changes in the lenses of naphthalene-fed rats. However, Sorbinil, another spirohydantoin ARI, demonstrated a much weaker efficacy in this model and the carboxylic acid ARI compound, Tolrestat showed no efficacy at all. In the dual cataract model, Tolrestat provided the classical anti-sugar-cataract efficacy by preventing galactose cataract formation and reduced the lens dulcitol accumulation, but showed no protection against the shell-like opacity caused by naphthalene. On the other hand, AL01576 protected the lens from both stresses. These results ruled out the possible involvement of aldose reductase in naphthalene cataract formation. Furthermore, AL04114, a dimethoxy derivative of

AL01576 and a noninhibitor to cytochrome P-450, demonstrated similar efficacy as AL01576 in the prevention of naphthalene cataract. Therefore, cytochrome P-450 may not be involved in the prevention of this cataract. All these results and the fact that AL01576 can only prevent the changes caused by naphthalene dihydrodiol but not those induced by 1,2-naphthoquinone indicate that the action site of AL01576 or AL04114 in naphthalene cataract prevention may be targeted at enzymes involved in the naphthalene dihydrodiol metabolism, such as the enzyme dihydrodiol dehydrogenase.

Based on the above findings, a new mechanism for naphthalene cataract in rats is proposed that naphthalene was metabolized in the liver by cytochrome P-450 to naphthalene dihydrodiol, which reaches the aqueous humor via the circulation and penetrates into the lens. Under the action of dihydrodiol dehydrogenase, it is further metabolized to dihydroxynaphthalene and ultimately autoxidizes to form naphthoquinone and H_2O_2, which are both involved in cataract formation. AL01576 and AL04114 can inhibit the enzyme dihydrodiol dehydrogenase and therefore block the production of the toxic species of 1,2-naphthaoquinone and H_2O_2, and thus prevent naphthalene cataract formation.

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