

# Mechanistic Considerations in Small Fish Carcinogenicity Testing

*J. McHugh Law*

## Abstract

Historically, small fish species have proven useful both as environmental sentinels and as versatile test animals in toxicity and carcinogenicity bioassays. They can be bred in large numbers, have low maintenance and bioassay costs, and have a low background incidence of tumors. However, more mechanistic information is needed to help validate the information garnered from these models and to keep pace with other more fully developed animal models. This paper focuses on mechanistic considerations when using small fish models for carcinogenicity testing. Several small aquarium fish species have proven useful. The Japanese medaka is perhaps the best characterized small fish model for carcinogenicity testing; however, the zebrafish is emerging as an important model because it is well characterized genetically. Both route and methodology of exposure may affect the outcome of the study. Most studies have been conducted by introducing the test compound into the ambient water, but dietary exposures and embryo microinjection have also been used. Other considerations in study design include use of an initiating carcinogen, such as diethylnitrosamine, and differences in xenobiotic metabolism, such as the fact that fish CYP2B is refractory to phenobarbital induction. The small size of these models has perhaps limited some types of mechanistic studies, such as formation and repair of DNA adducts in response to carcinogen exposure. However, improved analytical methods are allowing greater resolution and should be applied to small fish species. Slide-based methods such as immunohistochemistry are an important adjunct to routine histopathology and should be included in study design. However, there is a need for development of more species-specific antibodies for fish research. There is also a need for more fish-specific data on cytokines, serum biochemistry, and oncogenes to strengthen the use of these important test models.

**Key Words:** carcinogenicity; cytochrome P450; diethylnitrosamine; DNA adduct; hepatocarcinogenesis; medaka; small fish models; zebrafish

## Use of Small Fish Models in Carcinogenicity Testing

Environmental factors play a role in the occurrence of cancer, although the level of this role is the subject of much debate (Povey 2000; Swenberg et al. 1991). Based on epidemiological evidence, Doll and Peto (1981) estimated that environmental factors, including exposure to chemical carcinogens, account for nearly 80% of all human cancers in the United States. Many thousands of synthetic chemicals are currently in use, and numerous new chemicals are put on the market and introduced into the air, water, and soil each year. It is widely recognized that detection and appropriate regulation of these compounds are of great importance for the prevention of neoplasia in man (Harris 1991; Ito et al. 1989).

Historically, the link between exposure to certain chemicals and cancer has been established using whole animal chronic bioassays. In the 1960s and -70s, US government programs such as the National Toxicology Program were established in response to concern over chemical safety. Since the advent of the Clean Air Act Amendments of 1990, federal agencies have been directed to evaluate a large group of priority chemicals in a limited amount of time and for less cost. The standard 2-yr rodent carcinogenesis bioassay has become too costly with regard to both expense and time. Thus, there is a need for alternative, less expensive animal models for detecting environmental carcinogens that might pose hazards for humans.

On the one hand, we are urged to use reduced, more environmentally relevant dose levels in carcinogenicity testing so that we may set more realistic margins of safety for regulatory purposes. On the other hand, statisticians advise that the lower the tumor response, the greater the number of animals (greater "n") needed. Short-term *in vitro* assays such as the Ames test have been useful in mass screening of potentially carcinogenic compounds. Although many of these assays are rapid and economical, their validity has been limited somewhat by false positives and false negatives, and by an inherent inability to determine target organ-specific chemical carcinogenicity or to detect tumor-promoting activity (Ito et al. 1989).

Accordingly, the use of "alternative" animal models in toxicity and carcinogenicity testing has received considerable attention recently (Salem and Katz 1998). In 1993, the US Congress instructed the National Institutes of Health to investigate the use of alternative animal models. Specifi-

J. McHugh Law, D.V.M., Ph.D., Dipl. American College of Veterinary Pathologists, is Associate Professor in the Department of Microbiology, Pathology, and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.

cally, this instruction called for reducing the number of animals used; replacing animals with in vitro tests, chemical reactions, and computer models; and refining current methods to emphasize relief of pain, maximize information obtained from each animal, and utilize animals lower on the phylogenetic tree (Salem and Katz 1998). In 2000, the National Toxicology Program Interagency Coordinating Committee on the Validation of Alternative Methods was established to assist in these efforts. Use of transgenic mouse models, such as the Tg.AC (zeta-globin promoted v-Ha-ras) transgenic mouse (Tennant et al. 1998), can answer some specific questions that require using whole animals; however, these specialized models are costly and often difficult to obtain in the large numbers needed for bioassays.

One possible solution, particularly for presumptive (stage one or tier one) testing, is the use of small fish models (Bailey et al. 1996; Bunton 1996; Couch and Harshbarger 1985; Hawkins et al. 1988a,b; Hendricks 1982; Hoover 1984; Masahito et al. 1988; Metcalfe 1989; Mix 1986; Powers 1989). Unlike in vitro methods, tests using small fish have the advantages of whole animal assays. Many species of fish are available that are easily and economically bred and housed in the laboratory. Scientists have shown that they are sensitive to a variety of known carcinogens and exhibit a short time to tumorigenesis, yet they have an exceedingly low spontaneous tumor rate in potential target organs. Additionally, most appear not to be susceptible to nonspecific cultural conditions or "white noise," which may affect bioassay results when prevailing methods are used (Hawkins et al. 1988a; Hoover 1984).

Although tumors in wild fish have been reported for decades, Dr. Mearl Stanton of the National Cancer Institute appears to have pioneered the use of small aquarium fish for carcinogenicity testing in the controlled laboratory setting in the mid-1960s (Dawe 1984). As a physician, Stanton had an interest in environmental causation of cancer. Using "zebra dannies" (*Brachydanio rerio*), he reported hepatic neoplasia in fish exposed to diethylnitrosamine (DEN<sup>1</sup>) (Stanton 1965) and then cycasin (Stanton 1966).

Also in the mid-1960s, researchers discovered that hatchery-reared rainbow trout (*Onchorhynchus mykiss*) in the Pacific Northwest had liver tumors caused by aflatoxins present in moldy feed (Halver 1967; Sinnhuber et al. 1977). This realization was pivotal, and aflatoxins and their analogs have since been shown to be potent liver carcinogens in many species, including humans. Trout have been used in numerous other carcinogenesis studies since that time (e.g., Hendricks 1982; Hendricks et al. 1984, 1985). For a more exhaustive treatment of this work with rainbow trout, the reader is

referred to an excellent review by Bailey and colleagues (1996).

A number of laboratory studies have focused on small fish hepatocarcinogenesis, particularly involving the Japanese medaka (*Oryzias latipes*) (Bunton 1990; Hawkins et al. 1985; Hinton et al. 1984; Ishikawa and Takayama 1979), platyfish/swordtail hybrids (*Xiphophorus* spp.) (Anders et al. 1984), top minnow (*Poeciliopsis* spp.) (Schultz and Schultz 1982), sheephead minnow (*Cyprinodon variegatus*) (Couch and Courtney 1987), guppy (*Poecilia reticulata*) (Fournie et al. 1987), and zebrafish (*Danio rerio*) (Spitsbergen et al. 2000a,b). Western mosquitofish (*Gambusia affinis*) exhibit sensitivity to chemical induction of liver neoplasia and can be cultured easily in the laboratory (Law et al. 1994). This abundant native small freshwater fish perhaps deserves more attention because it may be used to directly correlate laboratory findings with field studies in warm waters.

## Sentinels of Environmental Degradation

Researchers have shown that fish are useful not only as test organisms but also as sensitive indicators of environmental contamination. Because exposure to toxic chemicals in the environment is difficult to assess because of the great variety of potential exposure routes, differences in bioavailability of toxicants, and differences in pharmacodynamic disposition of xenobiotics, many researchers have turned to the use of biological markers (**biomarkers**). Biomarkers are measurements of body fluids, cells, or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (McCarthy and Shugart 1990). Data from biological systems provide vital information not readily available from chemical analyses of air, water, or soil. Small fish species have the potential of serving as (1) **sentinels** that detect the presence of contaminants and the extent of exposure, (2) **surrogates** that indicate potential human exposure and effects, and (3) **predictors** of long-term effects on populations or ecosystems (McCarthy and Shugart 1990).

Harshbarger and Clark (1990) documented 41 geographic regions in North America in which clusters, or epizootics, of cancer in wild fishes have occurred. The occurrence of neoplasms involving epithelial tissues such as the liver, pancreas, gastrointestinal tract, and some epidermal neoplasms appears strongly correlated with environmental contamination, that is, exposure to chemical carcinogens. Several excellent reviews provide more on these epizootics (Black and Baumann 1991; Couch and Harshbarger 1985; Dawe et al. 1981; Harshbarger and Clark 1990; Harshbarger et al. 1993; Mix 1986). However, several reports of tumors in wild fish have been pivotal and deserve special mention.

English sole from contaminated areas of the Puget Sound, Washington, have high prevalences of liver lesions that range from megalocytosis to neoplasms (Myers et al. 1991). Several detailed studies (e.g., Malins et al. 1984, 1985a,b, 1987, 1988) established statistically significant associations

<sup>1</sup>Abbreviations used in this article: BrdU, bromodeoxyuridine; CYP, cytochrome P450; GC/MS, gas chromatography/mass spectrometry; DEN, diethylnitrosamine; HPLC, high-performance liquid chromatography; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PAH, polycyclic aromatic hydrocarbons; PCNA, proliferating cell nuclear antigen; TCE, trichloroethylene.

between the presence of polycyclic aromatic hydrocarbons (PAHs<sup>1</sup>) in the sediments and the prevalence of liver neoplasia. Malins and colleagues (1990) identified a novel DNA adduct, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, in neoplastic livers of English sole from carcinogen-impacted areas of the Puget Sound.

In relation to the East Coast, Murchelano and Wolke (1985, 1991) have reported epizootic hepatic neoplasia in winter flounder from Boston Harbor, Massachusetts. As in the case of the Puget Sound sole but not as firmly established, the hepatic lesions in the winter flounder were highly correlated with anthropogenic chemical contamination.

Although many incidences of cancer epizootics have occurred in fresh water fishes (Black and Baumann 1991), none have been as well studied as the epizootics in the marine species the English sole and winter flounder. Epizootics of neoplasia in fish populations of brown bullhead catfish (*Ictalurus nebulosus*) and Atlantic tomcod (*Microgadus tomcod*) also should be noted. Sediments rich in PAH have generally been considered the principal causes of skin and liver neoplasia in brown bullheads in the contaminated Black River (Ohio), a tributary of Lake Erie (Baumann 1989; Baumann et al. 1987, 1990). In laboratory tests, medaka exposed to extracts and fractions of PAH-contaminated sediments from tributaries of the Great Lakes, including the Black River, developed liver neoplasia (Fabacher et al. 1991). Similarly, scientists have reported epizootics of hepatic neoplasia from Atlantic tomcod from the Hudson River (Cormier et al. 1989; Smith et al. 1979). Klauda and colleagues (1981) have documented that those liver neoplasms are associated with elevated tissue levels of polychlorinated biphenyls.

White suckers from industrially polluted areas of Lake Ontario exhibited increased prevalences of hepatic and skin neoplasia (Hayes et al. 1990; Sonstegard 1977). As in other epizootics, the neoplasms have been associated with PAH contamination. Stalker and colleagues (1991) showed that the progression of hepatocellular and bile duct neoplasms in the white sucker is accompanied by a loss of immunoreactive glutathione S-transferases, which usually catalyze a major detoxification pathway.

Only a small number of reported cancer epizootics have dealt with small fish species. Vogelbein and colleagues (1990) reported high prevalences of liver neoplasms in mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elizabeth River, Virginia. Later reports documented exocrine pancreatic neoplasms in these fish that were also apparently induced by contaminant exposure (Fournie and Vogelbein 1994; Vogelbein and Fournie 1994).

## Mechanistic Considerations

It should be apparent that studies involving fish models in toxicology may be viewed from different sides of the same coin: on the one side, as surrogates for human health problems; on the other side, as indicators of environmental health. Human health and environmental health are, of course,

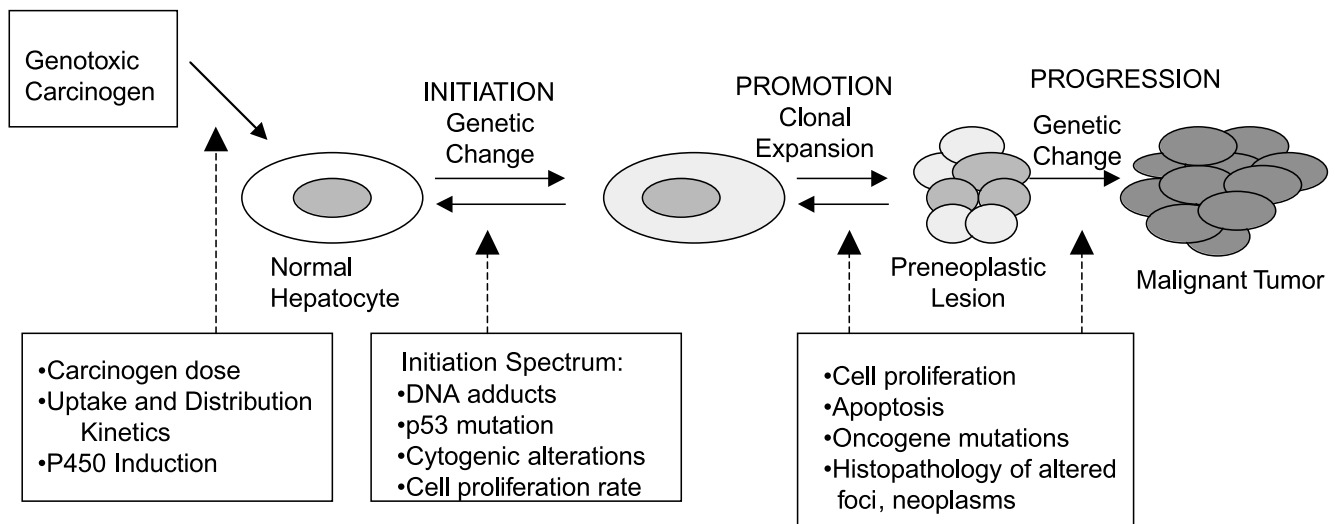
inexorably linked, so the two concepts should not be separated. Instead, it is critical that we provide as much mechanistic information as possible to validate these alternative test methods further and to take advantage of their utility for cancer bioassays and toxicological studies. Mechanistic information garnered across phyletic levels may be more accurately applied to help substantiate findings from field work. Additionally, it may unlock untold mysteries of the basic mechanisms of cellular pathology and neoplasia (recall the wealth of information garnered on apoptosis from the lowly nematode, *Caenorhabditis elegans*) (Fraser 1999).

In Figure 1, the multiple steps thought to be involved in hepatocarcinogenesis are outlined, along with several examples of mechanisms involved at each transition point. A complete look at a potential genotoxic liver carcinogen, for example, should consider factors involved in initiation, promotion, and progression, in addition to the characteristics of the tumors themselves and whether metastasis occurs. Because the liver has been the major target organ in most small fish carcinogenicity studies to date, the focus of this discussion is mainly on hepatocarcinogenesis.

## Carcinogen Dose and Exposure Route

Factors such as carcinogen exposure concentration (“dose”), along with uptake and distribution kinetics of the compound(s) of interest, are important in determining the amount of a compound to which a target tissue is ultimately exposed. The relative importance of these factors depends on the particular experimental protocol, solubility of the test compound, and physiology of the individual fish. These and other factors must be considered when designing a bioassay. Because of the relative ease with which they can be administered in the ambient water, water-soluble compounds have been most commonly used in fish carcinogenicity testing. However, scientists have administered a number of relatively hydrophobic compounds with various exposure systems (Hawkins et al. 1988b; Spitsbergen et al. 2000a; Walker et al. 1985). DEN, an alkylating carcinogen that predominantly targets the liver, is perhaps the best characterized chemical carcinogen in small fish bioassays and is used as a model for consideration of mechanistic endpoints in this discussion. DEN has been used for bioassays in fish at concentrations ranging from <10 mg/L to 1000 mg/L. However, it has been used most often in a continuous exposure at 15 to 100 mg/L of DEN in the ambient water for several weeks followed by an additional 8-wk grow-out period in clean water to allow for tumor development (Boorman et al. 1997). It is important to consider the dynamics of cell injury, cell loss, and regenerative cell proliferation when choosing a particular dose inasmuch as cellular defense mechanisms such as DNA repair may be overwhelmed at higher doses (Williams et al. 2000).

Equally important is the **route** of exposure. Numerous exposure protocols have been used for small fish bioassays (Hawkins et al. 1988b) and are discussed briefly below.



**Figure 1** Stages of hepatocarcinogenesis, including some examples of mechanistic factors that should be considered at each transition point when designing a carcinogenicity bioassay.

1. In **embryo microinjection**, the test material is administered by injection into the perivitelline space of embryonated eggs. With this method, the selectively permeable chorion of the egg is avoided, although pretreatment methods are available that allow penetration of the chorion by the test material via bath exposure (Steve Manning, Gulf Coast Research Laboratory, Ocean Springs, Mississippi, personal communication, 1995). Fish are grown out in clean water for variable periods of time and examined for neoplastic lesions. Embryo microinjection appears to be useful for testing poorly water-soluble compounds, or compounds available only in small quantities. However, one must keep in mind that carcinogen sensitivity may be vastly different at this life stage due to differences in cell physiology and biotransformation enzyme chemistry. In a recent study, zebrafish embryos given microinjections of 96 ng/egg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG<sup>1</sup>) were quite responsive to the compound and developed liver neoplasms and various mesenchymal neoplasms (Spitsbergen et al. 2000b). In contrast, juvenile (2-mo-old) zebrafish fed diets containing up to 2000 ppm of MNNG were refractory to MNNG-induced neoplasia. In addition, as in all early life stage exposures, the background cell growth rate should be considered, especially when examining cell proliferation dynamics.
2. **Early life stage (pulse) exposures** have resulted in important advances (Hendricks 1982). Fish embryos or fry are exposed to several brief pulses of carcinogen separated by clean water rinses, then grown out in clean water for several months. Hawkins and colleagues (1985) used this method to examine the carcinogen sensitivity of seven small fish species to find suitable models for chronic studies. This method has the advantages and

disadvantages of exposures using the ambient water. The exposures are uniform and probably involve carcinogen uptake via all three exposure routes—gills, digestive tract, and skin—simultaneously. Additionally, compounds or extracts that are available in only limited quantities can be tested. Note that even for brief exposures, compound concentrations should be monitored analytically.

3. **Dietary exposures** have been developed, perhaps most extensively, at Oregon State University (Hendricks 1982). Scientists have performed an extensive list of elegant experiments in carcinogenicity and anticarcinogenicity using rainbow trout and a standardized test diet (Bailey et al. 1996). In contrast, relatively few dietary exposure studies have been performed with small fish species. DeKoven and colleagues (1992) developed a purified casein-based diet for the medaka, which compared favorably with a combination of commercial flake food and newly hatched brine shrimp. A standardized test diet has the advantage of consistent, defined nutrition and the ability to premix test compounds for dietary exposures. In addition, it is less likely to contain the extraneous compounds that may be present in whole live food, which could confound test results. Using dietary exposures with a defined test diet affords the ability to test poorly soluble compounds and may more closely model biomagnification of toxicants through the food chain. Drawbacks of dietary exposures include the relatively large amount of carcinogen required, large amounts of carcinogenic wastes produced, and uneven dosing caused by aggressive feeders in the exposure tank (Hawkins et al. 1988b).
4. In **static exposures**, the test compound is simply added to the aquarium water and allowed to remain, with respiking of compound into the test vessel as needed.

This method has probably been the most popular with small fish species such as Japanese medaka, guppy, and zebrafish. It is the least technically challenging exposure methodology, and it allows constant bath exposure over a defined exposure time. However, if the exposures are lengthy, animal waste products may build up in the tanks, and infectious diseases may become difficult to control unless the water is changed frequently. In addition to the deleterious effects on the fish themselves, nitrogenous wastes may affect water quality parameters such as tank pH. This characteristic can alter the chemical state of the test compound and its uptake, distribution, and metabolism, depending on the compound's pKa and the buffering capacity of the test water. In addition, the effects of the parent compound recycling through body tissues (e.g., enterohepatic cycling) as well as exposure to metabolites of the test compound must be considered. In my laboratory, we have used static exposures predominantly for brief treatments with alkylating carcinogens. For example, western mosquitofish (*Gambusia affinis*) exposed to the methylating agent, methylazoxymethanol acetate, at 10 mg/L for 2 hr, then grown out in clean water, exhibited a 33 and 52% incidence of hepatobiliary neoplasms at 25 and 40 wk after exposure, respectively (Law et al. 1994). Colleagues and I have treated Japanese medaka with DEN for a total of 48 hr (24-hr static exposure, then replaced test water with fresh DEN at the same concentration) to study early mechanisms of hepatocarcinogenesis (Law et al. 1998a).

We have also used medaka in longer static exposures. For example, medaka exposed continuously for 2 wk to the drinking water disinfection by-product dichloroacetic acid developed a dramatic accumulation of cytoplasmic glycogen within hepatocytes, a lesion thought to be pre-neoplastic in rodent studies (Law et al. 1998b). However, when we attempted to extend these static exposures for longer periods of time, we experienced increasing problems with water quality and infectious diseases, despite frequent water changes (Lopez-Perez 2000). The lesson is to limit static exposures to relatively brief exposure periods in which adequate water quality and stability of the test compound can be maintained.

- 5. Flow-through exposures** can overcome many of the problems associated with static exposures because the ambient water is continuously replaced in the test vessels. Walker and colleagues (1985) developed an intermittent flow-through system in which even volatile and hydrophobic compounds can be delivered at stable concentrations for long exposure periods. The test compound is delivered into the diluent water by a computer-controlled precision injector. Although more technically demanding, flow-through exposures afford the ability to test constant exposure to relatively low environmentally realistic carcinogen concentrations (Hawkins et al. 1988b). Furthermore, water quality is easier to maintain, which avoids a large source of potentially confounding factors. Proper disposal of the larger amounts of contaminated

effluent water is an additional consideration before undertaking these exposures.

## Study Design: Initiation/Promotion

An additional consideration in studying weakly carcinogenic or nongenotoxic compounds, or mixtures with unknown carcinogenic properties, is whether to include an initiating dose of a genotoxic agent. Such inclusions increase the cost and complexity of the bioassay yet may provide important weight of evidence as to the carcinogenicity of the test compound. Gardner and coworkers (1990) developed mobile biomonitoring laboratories for on-site assessment of the toxic hazards of contaminated groundwater. Groundwater is pumped into an 8 × 24-ft trailer equipped with aquaria and flow-through diluter systems. Japanese medaka are most commonly used for the carcinogenicity bioassays, but other species can be used for specific studies as needed. They use DEN at 10 mg/L for 48 hr as an "initiating" dose. Use of an initiator can help to determine whether one or more components in the unknown mixture act as a tumor promoter. Using this methodology, Gardner and coworkers (1998) discovered that groundwater contaminated with trichloroethylene (TCE<sup>1</sup>) has carcinogenic properties beyond what had been shown with TCE alone in a companion laboratory-based study, which suggested that unidentified compounds in the mixture may have had promoting properties alone or synergistically with TCE. However, the difference in tumor response may not have been detected without prior initiation with DEN. Such an initiation protocol should be standardized across laboratories that perform carcinogenicity tests with small fish models to increase the consistency of results.

## Metabolism

The basic metabolic machinery in small fish species, with regard to Phase I and Phase II metabolism, is similar to that in mammals. The Phase I metabolizing enzyme system, the cytochromes P450 (CYPs<sup>1</sup>), have been perhaps the best characterized in aquatic species (Stegeman and Hahn 1994; Stegeman and Lech 1991). It appears that in fish only members of the CYP1A subfamily are induced by environmental toxicants and thus would have a major impact on the activation or detoxification of carcinogens (Williams et al. 1998). As in mammals, compounds such as PAHs, polychlorinated biphenyls, and polychlorinated dioxins have been shown to induce CYP1A in fish. One important difference to note between fish and mammals, however, is in the response to phenobarbital. Whereas phenobarbital classically induces the mammalian CYP2B subfamily, fish CYP2B appears to be refractory to phenobarbital induction (Kleinow et al. 1987, 1990). Other studies indicate that phenobarbital can instead induce CYP1A in fish, perhaps via enhancement of Ah receptor activation (Elskus and Stegeman 1989; Sadar et al. 1996). In a recent review, Williams and colleagues (1998)

point out that xenoestrogens, an important class of aquatic pollutants, may alter the response to carcinogens in fish through modulation of CYPs.

Although these relationships are best characterized in the rainbow trout model, comparatively little information is available for small fish models. CYP1A was found to be deficient in preneoplastic and neoplastic lesions in mummichog (*Fundulus heteroclitus*), an estuarine small fish model that shows great promise for environmental toxicology research (Van Veld et al. 1992). This same group recently demonstrated tissue-specific expression of CYP1A in mummichog exposed to benzo[a]pyrene in both aqueous and dietary exposures, and they developed a grading system for CYP1A staining intensity (Van Veld et al. 1997). Studying the metabolism of trichloroethylene, a common groundwater contaminant, Lipscomb and coworkers (1998) found that CYP1A was readily detectable in medaka liver by immunohistochemistry, whereas CYP2E1 was present at very low levels.

Other enzyme systems have shown somewhat more variable results in fish studies. Immunostaining for gamma-glutamyl transpeptidase, an important enzyme marker in rodents, detected foci of cellular alteration in medaka exposed to DEN (Hinton et al. 1988). However, gamma-glutamyl transpeptidase staining showed conflicting results in rainbow trout studies (Bunton 1996). Studies with glutathione-S-transferase, an important Phase II biotransformation enzyme, have also had variable results with regard to preneoplastic and neoplastic lesions in rainbow trout (Bunton 1996).

These reports support the utility of tissue-specific induction patterns for biotransformation enzymes in fish carcinogenesis research. However, it is also clear that there is a need for more information on these enzyme systems, particularly in small fish models. Future carcinogenesis bioassays using these models should include a battery of immunohistochemical stains, such as those used by Van Veld and colleagues (1997) or by Lipscomb et al. (1998). Immunostains could provide valuable mechanistic information on carcinogen metabolism and help delineate enzyme-altered foci that might otherwise be missed using routine staining methods. Furthermore, basic research is needed on induction of enzymes by various classes of carcinogens in small fish models such as the CYPs, glutathione S-transferase, DNA repair enzymes, and the caspases involved in apoptosis. This information will be forthcoming as long as adequate funding is maintained and balanced between basic, applied, and clinical cancer research.

### DNA Adducts: Mutagenesis as a Mechanism of Carcinogenesis

An approach that integrates all of the various factors involved in chemical exposure (e.g., uptake and biotransformation) is to compare levels of specific covalent adducts of DNA in a target tissue (La and Swenberg 1996; Swenberg et al. 1990). It is generally accepted that most chemical carcinogens act

by interacting with the genetic material of the cell, in particular the DNA template (Lawley 1984). Chemical modification of DNA is the first in a series of steps that lead to mutation, cell transformation, and tumor development (Wogan 1988). In fact, it has been suggested that any chemical that forms DNA adducts even at low levels is potentially mutagenic and carcinogenic (de Serres 1988).

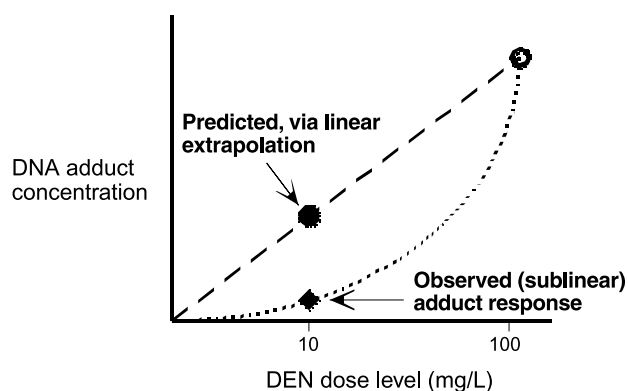
That DNA adducts are critical to tumorigenesis is supported by a number of observations, including the facts that (1) most carcinogens are also mutagens, (2) the mutagenic and carcinogenic properties of most compounds depend on their *in vivo* conversion to electrophilic derivatives that attack nucleophilic sites in DNA to form adducts, (3) the degree of DNA adduct formation in a tissue can often be positively correlated with tumorigenic response, and (4) the activation of protooncogenes has been demonstrated through the interaction of chemical carcinogens with DNA (Beland 1989). DNA adduct determinations can provide crucial information on metabolic pathways as well as chemical effects on DNA structure, transcription, synthesis, and repair. Adduct analyses can also provide a direct test of the somatic mutation theory. Finally, DNA adducts can be considered dosimeters of exposure to chemicals in cancer risk assessments (Beland 1989).

Several recent reviews focus on analysis of the significance of DNA adducts in fish, although few studies with small fish species are reported (Law et al. 1996b; Maccubbin 1994; Stein et al. 1994). Sensitive methods for detection of DNA adducts are essential for mechanistic studies of mutagenesis and carcinogenesis and for biomonitoring populations at risk for environmentally caused cancer. Adducts have been detected with such methods as <sup>32</sup>P-postlabeling, immunoassays, high-performance liquid chromatography (HPLC<sup>1</sup>) with fluorescence or electrochemical detection, and gas chromatography/mass spectrometry (GC/MS<sup>1</sup>) (Cadet and Weinfeld 1993). HPLC coupled to mass spectrometry shows much promise because the derivitization steps needed to make compounds volatile enough for GC/MS can be avoided.

Alkylating agents such as DEN are archetypal carcinogens, in that most other carcinogens are active only after they are metabolized to alkylating or aralkylating agents (Lawley 1984). Alkylation (e.g., methylation and ethylation) at the N-7 position of guanine is a preferential site of attack for most alkylating agents, and attack at the O<sup>6</sup> position of guanine (less common) is most highly correlated with carcinogenesis (Beranek et al. 1980; Lawley 1984; Loveless 1969; Swenson and Lawley 1978). Fong and coworkers (1988) reported formation and persistence of O<sup>6</sup>-ethylguanine in rainbow trout exposed to DEN when using HPLC with fluorescence detection. Few studies, however, have attempted to measure specific adduct levels in small fish species exposed to small alkylating carcinogens. This paucity is perhaps due to the fact that older methods were not sensitive enough to utilize such small amounts of tissue available from these species. Although <sup>32</sup>P-postlabeling is extremely sensitive, it cannot identify chemical structures for specific adducts. Immuno-

chemical methods can be sensitive and very specific; however, they are limited to detection of adducts against which specific monoclonal antibodies are available. Colleagues and I recently reported on guanine and thymidine adducts in medaka exposed to DEN in a study that used an immunoslot-blot technique (Law et al. 1998a). Ethyl-DNA adducts appear to accumulate in medaka liver tissue in a sublinear fashion; that is, a much lower than linear dose response was seen in medaka exposed to 10 mg/L of DEN versus those exposed to 100 mg/L. Thus, critical DNA repair enzymes, which are relatively efficient at lower carcinogen levels, are probably saturated after exposure to 100 mg/L of DEN (Figure 2).

GC/MS with single ion monitoring is a method that can unequivocally identify specific adducts at extremely low detection limits (femtomole levels) (Maccubbin 1994). This method was used by Malins and coworkers to detect hydroxyl radical-induced DNA adducts in liver neoplasms of feral English sole from Puget Sound (Malins 1993; Malins et al. 1990). Highly accurate measurements of adduct levels can be obtained using stable isotope-labeled analogs of analytes as internal standards in a method known as reverse isotope-dilution mass spectrometry (Dizdaroglu 1993). This method has been adapted for use in small fish species in our laboratory. Western mosquitofish were shown to acquire O<sup>6</sup>-methylguanine adducts in liver tissue in a dose-dependent manner after exposure to methylazoxymethanol acetate in the ambient water (Law et al. 1996a). Additional experiments are needed to characterize more accurately the dose response of small fish models to this potent methylating agent. Because DNA repair enzymes specific for O<sup>6</sup>-alkylguanine adducts have been demonstrated in fish, it is likely that the dose-response curve for methylazoxymethanol acetate will be shown to be sublinear, similar to that for DEN.



**Figure 2** Linear versus sublinear dose response. DNA adduct concentration in liver tissue of Japanese medaka exposed to 10 mg/L of diethylnitrosamine (DEN) is less than the predictable response by linear extrapolation from the 100 mg/L dose response, most likely due to saturation of DNA repair at higher carcinogen levels.

## Immunohistochemistry

Perhaps their small size has limited the number of mechanistic studies that have been performed using dissected single organs and tissues of small fish species. However, although a wealth of membrane, cytoplasmic, and nuclear markers are available using immunohistochemistry (see review by Bunton 1996), it is important to note that preparations for immunohistochemistry must be considered in the study design well in advance. In a 30 mm-long medaka, there is little tissue available in a given study for multiple investigators to use. If the pathology protocol calls for sectioning through most or all of a specimen for complete histopathological examination and tissue accountability, then extra fish must be included in each treatment group to allow for immunohistochemical studies. Alternatively, a protocol may be modified to section through only one half the fish, leaving extra sections from the same fish for subsequent studies. Adequate sections of liver can usually be obtained from both halves of the paraffin block.

It is important to minimize the fixation time so as not to destroy important epitopes in the tissue of interest. We have found that routine use of 10% neutral buffered formalin and limiting fixation time to less than 48 hr works well. Fixation is followed by overnight demineralization in 10% formic acid to facilitate sectioning. This concentration does not appear to be too harsh for most immunostaining protocols. If processing is delayed, tissue samples may be rinsed and held in 70% ethanol. Regardless of the protocol used, it is important to process all specimens equally. Unstained sections of consistent thickness should be mounted on coated slides for immunostaining. Thicker tissue sections, which can take up more stain, appear to have a stronger signal in the target tissue and may promulgate a false interpretation.

Important areas for mechanistic developments with small fish species are cell proliferation and cell cycle dynamics. Studies using these markers can help identify proliferative rate as well as the proliferative cell type. They may also help determine the level of cytotoxicity and regenerative cell proliferation versus neoplastic proliferation (Butterworth 1991; Butterworth et al. 1992). The two methods most commonly used include detection of proliferating cell nuclear antigen (PCNA<sup>1</sup>) and bromodeoxyuridine (BrdU<sup>1</sup>):

- PCNA can be used on formalin-fixed paraffin-embedded tissues. Thus, it requires less disruption of protocol and can be used on archived specimens (if they are fixed properly). However, questions exist as to the specificity of PCNA for replicating cells. In addition, PCNA tags only currently replicating cells, so there is a much lower window of detection. Ortego and colleagues (1994) devised the specific application of PCNA to small fish species. Antigen retrieval and use of a super-sensitive detection kit were important factors. Medaka exposed to DEN exhibited a dose-dependent increase in proliferative index.

- BrdU staining is more technically difficult than PCNA because live animals must be allowed time to incorporate BrdU into their cells. BrdU is integrated into the DNA of replicating cells and so becomes a detectable nuclear antigen. Thus, more proliferating cells are identified, and it is not necessary to rely simply on a one-time “snapshot” of cells in the active phases of the cell cycle. BrdU also labels only DNA replication, not DNA repair, and so may give a truer indication of cell proliferation than PCNA. The method was reported in fish as a bath exposure by Moore and coworkers (1994). Colleagues and I have used a modification of this protocol with medaka in which fish were exposed to 30 mg of BrdU per liter of tank water for 3 days with daily replacement of tank water and BrdU (Lopez-Perez 2000).

Besides immunohistochemistry, there is a whole battery of older yet reliable histochemical stains that may also be applied to available tissue sections. For example, medaka exposed for 2 wk to the drinking water disinfection byproduct dichloroacetic acid developed cytomegaly, nuclear atypia, and severe cytoplasmic vacuolation within hepatocytes. Because the hepatocellular vacuoles were clear and the nuclei were centrally placed, the material was suspected to be glycogen. This suspicion was confirmed with the histochemical stain, periodic acid Schiff, followed by periodic acid Schiff with diastase digestion (which specifically removes glycogen staining) (Law et al. 1998b). This work provided important mechanistic information, inasmuch as glycogen accumulation is considered to be a preneoplastic lesion in rodents exposed to dichloroacetic acid.

## Oncogenes/Tumor Suppressor Genes

A virtual explosion of information is occurring in the area of molecular biology with small fish models, much of which is beyond the scope of this paper. Readers are directed to recent reviews for more detailed discussion of these developments (Talbot and Hopkins 2000; Van Beneden and Ostrander 1994). These molecular findings can serve only to bolster the validation of these models. Recent discoveries have supported the use of small fish models for human health risks from the environment. For example, the *ras* oncogene of fish has a high homology to human *K-ras*; in goldfish, this homology is approximately 96% (Van Beneden and Ostrander 1994). Point mutations in *Ki-ras* occurred in a high proportion of rainbow trout liver tumors induced by aflatoxin B1, dimethylbenzanthracene, MNNG, and DEN. Another important example of molecular markers is the p53 tumor suppressor gene, which scientists have recently cloned and sequenced in medaka (Krause et al. 1997), although they have not yet demonstrated specific mutations. However, we have seen overexpression of the p53 protein product in liver neoplasms in several fish bioassays (unpublished work). Stabilization of a nonfunctional form of p53 protein is thought to account for the increased expression of p53 in

tumors and suggests a failure of this important gate keeper of the cell cycle.

## Future Research Needs

Clearly, a gap exists between the mechanistic information available for the more traditional rodent models for carcinogenicity testing and those for small fish models. If work with these valuable alternative animal models is to continue, it is vital that we obtain more mechanistic data in these species. A critical balance must be achieved between applied cancer research in the form of carcinogenicity testing/safety assessment, in which fish models have proven utility, and the kinds of basic research efforts that help to divulge the meanings of bioassay results as well as lesions detected in sentinel species in the aquatic environment. Development of transgenic and knockout fish, such as the transgenic medaka or mummichog, holds great promise for the future of mechanistic research with fish. Besides the molecular arena, another area that needs to be developed in small fish species is clinical pathology. There is increasing interest in blood/serum markers that can be monitored in rodent models, but very little is known concerning the small fish species. This area has tremendous potential. In conclusion, mechanistic work with small fish species can provide information in basic research that is applicable to human health. By the same token, we should also pursue studies on “fish-specific” processes. Although often not immediately apparent, the true benefits will likely come later.

## References

- Anders F, Scharfl M, Barnekow A, Anders A. 1984. *Xiphophorus* as an in vivo model for studies on normal and defective control of oncogenes. *Adv Cancer Res* 42:191-275.
- Bailey GS, Williams DE, Hendricks JD. 1996. Fish models for environmental carcinogenesis: The rainbow trout. *Environ Health Perspect* 104(Suppl 1):5-21.
- Baumann PC. 1989. PAH, metabolites, and neoplasia in feral fish populations. In: Varanasi U, ed. *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*. Boca Raton: CRC Press.
- Baumann PC, Harshbarger JC, Hartman KJ. 1990. Relationship between liver tumors and age in brown bullhead populations from two Lake Erie tributaries. *Sci Total Environ* 94:71-87.
- Baumann PC, Smith WD, Parland WK. 1987. Tumor frequencies and contaminant concentrations in brown bullheads from an industrialized river and a recreational lake. *Trans Am Fish Soc* 116:79-86.
- Beland FaMP. 1989. DNA Adducts and Carcinogenesis. . The Pathobiology of Neoplasia. New York: Plenum Press. p 57-80.
- Beranek DT, Weis CC, Swenson DH. 1980. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. *Carcinogenesis* 1:595-606.
- Black JJ, Baumann PC. 1991. Carcinogens and cancers in freshwater fishes. *Environ Health Perspect* 90:27-33.
- Boorman GA, Botts S, Bunton TE, Fournie JW, Harshbarger JC, Hawkins WE, Hinton DE, Jokinen MP, Okihiro MS, Wolfe MJ. 1997. Diagnostic criteria for degenerative, inflammatory, proliferative nonneoplastic and neoplastic liver lesions in medaka (*Oryzias latipes*): Consensus of a National Toxicology Program working group. *Toxicol Pathol* 25:202-210.

- Bunton TE. 1990. Hepatopathology of diethylnitrosamine in the medaka (*Oryzias latipes*) following short-term exposure. *Toxicol Pathol* 18:313-323.
- Bunton TE. 1996. Experimental chemical carcinogenesis in fish. *Toxicol Pathol* 24:603-618.
- Butterworth BE. 1991. Chemically induced cell proliferation as a predictive assay for potential carcinogenicity. In: Butterworth BE, Slaga TJ, Farland W, McClain M, eds. *Chemically Induced Cell Proliferation: Implications for Risk Assessment*. New York: Wiley-Liss Inc. p 457-468.
- Butterworth BE, Popp JA, Conolly RB, Goldsworthy TL. 1992. Chemically induced cell proliferation in carcinogenesis. In: Vainio PNM, McGregor DB, McMichael AJ, eds. *Mechanisms of Carcinogenesis in Risk Identification*. Lyon: IARC Scientific Publications 116. p 279-305.
- Cadet J, Weinfeld M. 1993. Detecting DNA damage. *Analyt Chem* 65:675A-682A.
- Cormier SM, Racine RN, Smith CE, Dey WP, Peck TH. 1989. Hepatocellular carcinoma and fatty infiltration in the Atlantic tomcod, *Microgadus tomcod* (Walbaum). *J Fish Dis* 12:105-116.
- Couch JA, Courtney LA. 1987. N-nitrosodiethylamine-induced hepatocarcinogenesis in estuarine sheepshead minnow (*Cyprinodon variegatus*) neoplasms and related lesions compared with mammalian lesions. *J Natl Cancer Inst* 79:297-321.
- Couch JA, Harshbarger JC. 1985. Effects of carcinogenic agents on aquatic animals: An environmental and experimental overview. *Environ Carcin Rev* 3:63-105.
- Dawe CJ. 1984. Dedication: An appreciation of Dr. Mearl F. Stanton. In: Hoover KL, ed. *Use of Small Fish in Carcinogenicity Testing*. Bethesda: National Cancer Institute Monograph 65. p 1-2.
- Dawe CJ, Harshbarger JC, Kondo S. 1981. Phyletic Approaches to Cancer. Tokyo: Japan Scientific Societies Press.
- de Serres FJ. 1988. Banbury Center DNA Adducts Workshop, meeting report. *Mutat Res* 203:55.
- DeKoven DL, Nunez JM, Lester SM, Conklin DE, Marty GD, Parker LM, Hinton DE. 1992. A purified diet for medaka (*Oryzias latipes*): Refining a fish model for toxicological research. *Lab Anim Sci* 42:180-189.
- Dizzardoglu M. 1993. Quantitative determination of oxidative base damage in DNA by stable isotope-dilution mass spectrometry. *FEBS Lett* 315:1-6.
- Doll R, Peto R. 1981. The causes of cancer: Quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 66:1191-1308.
- Elskus AA, Stegeman JJ. 1989. Further consideration of phenobarbital effects on cytochrome P-450 activity in killifish, *Fundulus heteroclitus*. *Comp Biochem Physiol* 92C:223-230.
- Fabacher DL, Besser JM, Schmitt CJ, Harshbarger JC, Peterman PH, Lebo JA. 1991. Contaminated sediments from tributaries of the Great Lakes: Chemical characterization and carcinogenic effects in medaka (*Oryzias latipes*). *Arch Environ Contam Toxicol* 20:17-34.
- Fong AT, Hendricks JD, Dashwood RH, Van Winkle S, Bailey GS. 1988. Formation and persistence of ethylguanine in liver DNA of rainbow trout (*Salmo gairdneri*) treated with diethylnitrosamine by water exposure. *Food Chem Toxicol* 26:699-704.
- Fournie JW, Hawkins WE, Overstreet RM, Walker WW. 1987. Exocrine pancreatic neoplasms induced by methylazoxymethanol acetate in the guppy *Poecilia reticulata*. *J Natl Cancer Inst* 78:715-725.
- Fournie JW, Vogelbein WK. 1994. Exocrine pancreatic neoplasms in the mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site. *Toxicol Pathol* 22:237-247.
- Fraser AG. 1999. Programmed cell death in *C. elegans*. *Cancer Metastasis Rev* 18:285-294.
- Gardner H, Brennan L, Toussaint M, Rosencrance A, Boncavage-Hennessey E, Wolfe M. 1998. Environmental complex mixture toxicity assessment. *Environ Health Perspect* 106(Suppl 6):1299-1305.
- Gardner H, Schalie WVD, Wolfe M, Finch R. 1990. New methods for on-site biomonitoring of effluent water quality. In: Sandu S, Lower W, Serres Fd, Suk W, Tice R, eds. *In Situ Evaluations of Biological Hazards of Environmental Pollutants*. New York: Plenum Press. p 61-69.
- Halver JE. 1967. Crystalline aflatoxin and other vectors of trout hepatoma. In: Halver JE, Mitchell IA, eds. *Trout Hepatoma Research Conference Papers*. Washington DC: Bureau of Sports Fisheries and Wildlife. p 78-102.
- Harris CC. 1991. Chemical and physical carcinogenesis: Advances and perspectives for the 1990s. *Cancer Res* 51(Suppl):5023S-5044S.
- Harshbarger JC, Clark JB. 1990. Epizootiology of neoplasms in bony fish of North America. *Sci Total Environ* 94:1-32.
- Harshbarger JC, Spero PM, Wolcott NM. 1993. Neoplasms in wild fish from the marine ecosystem emphasizing environmental interactions. In: Couch JA, Fournie JW, eds. *Pathobiology of Marine and Estuarine Organisms*. Boca Raton: CRC Press. p 157-176.
- Hawkins WE, Overstreet RM, Fournie JW, Walker WW. 1985. Development of aquarium fish models for environmental carcinogenesis: Tumor induction in seven species. *J Appl Toxicol* 5:261-264.
- Hawkins WE, Overstreet RM, Walker WW. 1988a. Carcinogenicity tests with small fish species. *Aquatic Toxicol* 11:113-128.
- Hawkins WE, Overstreet RM, Walker WW. 1988b. Small fish models for identifying carcinogens in the aqueous environment. *Water Resources Bull* 24:941-949.
- Hayes MA, Smith IR, Rushmore TH, Crane TL, Thorm C, Kocal TE, Ferguson HW. 1990. Pathogenesis of skin and liver neoplasms from industrially polluted areas in Lake Ontario. *Sci Total Environ* 94:105-123.
- Hendricks JD. 1982. Chemical carcinogenesis in fish. In: Weber LJ, ed. *Aquatic Toxicology*. New York: Raven Press. p 149-211.
- Hendricks JD, Meyers TR, Shelton DW. 1984. Histological progression of hepatic neoplasia in rainbow trout (*Salmo gairdneri*). *Natl Cancer Inst Monogr* 65:321-336.
- Hendricks JD, Meyers TR, Shelton DW, Casteel JL, Bailey GS. 1985. Hepatocarcinogenicity of benzo[a]pyrene to rainbow trout by dietary exposure and intraperitoneal injection. *J Natl Cancer Inst* 74:839-851.
- Hinton DE, Couch JA, Teh SJ, Courtney LA. 1988. Cytological changes during progression of neoplasia in selected fish species. *Aquatic Toxicol* 11:77-112.
- Hinton DE, Lantz RC, Hampton JA. 1984. Effect of age and exposure to a carcinogen on the structure of the medaka liver: A morphometric study. *Natl Cancer Inst Monogr* 65:239-249.
- Hoover KL, ed. 1984. *Use of Small Fish Species in Carcinogenicity Testing*. Bethesda: National Cancer Institute.
- Ishikawa T, Takayama S. 1979. Importance of hepatic neoplasms in lower vertebrate animals as a tool in cancer research. *J Toxicol Environ Health* 5:537-550.
- Ito N, Tatematsu M, Hasegawa R, Tsuda H. 1989. Medium-term bioassay system for detection of carcinogens and modifiers of hepatocarcinogenesis utilizing the GST-P positive liver cell focus as an endpoint marker. *Toxicol Pathol* 17:630-641.
- Klauda RV, Peck TH, Rice GK. 1981. Accumulation of polychlorinated biphenyls in Atlantic tomcod (*Microgadus tomcod*) collected from the Hudson River estuary, New York. *Bull Environ Contam Toxicol* 27:829-835.
- Kleinow KM, Haasch ML, Williams DE, Lech JJ. 1990. A comparison of hepatic P450 induction in rat and trout (*Onchorhynchus mykiss*): Delineation of the site of resistance of fish to phenobarbital-type inducers. *Comp Biochem Physiol* 96C:259-270.
- Kleinow KM, Melancon MJ, Lech JJ. 1987. Biotransformation and induction: Implications for toxicity, bioaccumulation, and monitoring of environmental xenobiotics in fish. *Environ Health Perspect* 71:105-119.
- Krause M, Rhodes L, Van Beneden R. 1997. Cloning of the p53 tumor suppressor gene from the Japanese medaka (*Oryzias latipes*) and evaluation of mutational hotspots in MNNG-exposed fish. *Gene* 189:101-106.
- La DK, Swenberg JA. 1996. DNA adducts: Biological markers of exposure and potential applications to risk assessment. *Mutat Res* 365:129-146.
- Law JM, Bull M, Nakamura J, Swenberg JA. 1998a. Molecular dosimetry of DNA adducts in the medaka small fish model. *Carcinogenesis* 19:515-518.
- Law JM, Hawkins WE, Overstreet RM, Walker WW. 1994. Hepatocarcinogenesis in Western mosquitofish (*Gambusia affinis*) exposed to methylazoxymethanol acetate. *J Comp Path* 110:117-127.

- Law JM, Lopez L, DeAngelo AB. 1998b. Hepatotoxicity of the drinking water disinfection by-product, dichloroacetic acid, in the medaka small fish model. *Toxicol Lett* 94:19-27.
- Law JM, McMillin DJ, Swenson DH, Means JC. 1996a. DNA adduct analysis in small fish species using GC/mass spectrometry. In: Ostrander GK, ed. *Techniques in Aquatic Toxicology*. Boca Raton: CRC Press. p 185-204.
- Law JM, McMillin DJ, Swenson DH, Means JC. 1996b. Quantification of DNA adducts in small fish exposed to alkylating agents. In: Bengtson DA, ed. *Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (ASTM STP no. 1306)*. Philadelphia: American Society for Testing and Materials. p 117-137.
- Lawley PD. 1984. Carcinogenesis by alkylating agents. In: Searle CE, ed. *Chemical Carcinogens*. Washington DC: American Chemical Society Monographs. p 325-484.
- Lipscomb J, Confer P, Miller M, Stamm S, Snawder J, Bandiera S. 1998. Metabolism of trichloroethylene and chloral hydrate by the Japanese medaka (*Oryzias latipes*) in vitro. *Environ Toxicol Chem* 17:325-332.
- Lopez-Perez L. 2000. The toxic and preneoplastic effects of chronic dichloroacetic acid exposure in the Japanese medaka small fish model [M.S.]. Raleigh: North Carolina State University.
- Loveless A. 1969. Possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* 223:206-207.
- Maccubbin AE. 1994. DNA adduct analysis in fish: Laboratory and field studies. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Boca Raton: Lewis Publishers. p 267-294.
- Malins DC. 1993. Identification of hydroxyl radical-induced lesions in DNA base structure: Biomarkers with a putative link to cancer development. *J Toxicol Environ Health* 40:247-261.
- Malins DC, Krahn MM, Brown DW, Rhodes LD, Myers MS, McCain BB, Chan S-L. 1985a. Toxic chemicals in marine sediment and biota from Mukilteo, Washington: Relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). *J Natl Cancer Inst* 74:487-494.
- Malins DC, Krahn MM, Myers MSea. 1985b. Toxic chemicals in sediments and biota from a creosote-polluted harbor: Relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophrys vetulus*). *Carcinogenesis* 6:1463-1469.
- Malins DC, McCain BB, Brown DW, Chan SL, Myers MS, Landahl JT, Prohaska PG, Frideman AJ, Rhodes LD, Burrows DG. 1984. Chemical pollutants in sediments and diseases of bottom-dwelling fish in Puget Sound, Washington. *Environ Sci Technol* 18:705-713.
- Malins DC, McCain BB, Landahl JT. 1988. Neoplastic and other diseases in fish in relation to toxic chemicals: An overview. *Aquatic Toxicol* 11:43-67.
- Malins DC, McCain BB, Myers MSea. 1987. Field and laboratory studies of the etiology of liver neoplasms in marine fish from Puget Sound. *Environ Health Perspect* 71:5-16.
- Malins DC, Ostrander GK, Haimanot R, Williams P. 1990. A novel DNA lesion in neoplastic livers of feral fish: 2,6-Diamino-4-hydroxy-5-formamidopyrimidine. *Carcinogenesis* 11:1045-1047.
- Masahito P, Ishikawa T, Sugano H. 1988. Fish tumors and their importance in cancer research. *Jpn J Cancer Res* 79:545-555.
- McCarthy JF, Shugart LR, ed. 1990. *Biomarkers of Environmental Contamination*. Boca Raton: Lewis Publishers.
- Metcalfe CD. 1989. Tests for predicting carcinogenicity in fish. *CRC Rev Aquatic Sci* 1:111-129.
- Mix MC. 1986. Cancerous diseases in aquatic animals and their association with environmental pollutants: A critical review of the literature. *Mar Environ Res* 20:1-141.
- Moore MJ, Leavitt DF, Shumate AM, Alatalo P, Stegeman JJ. 1994. A cell proliferation assay for small fish and aquatic invertebrates using bath exposure to bromodeoxyuridine. *Aquatic Toxicol* 30:183-188.
- Murchelano RA, Wolke RE. 1985. Epizootic carcinoma in winter flounder, *Pseudopleuronectes americanus*. *Science* 228:587-589.
- Murchelano RA, Wolke RE. 1991. Neoplasms and nonneoplastic lesions in winter flounder, *Pseudopleuronectes americanus*, from Boston Harbor, Massachusetts. *Environ Health Perspect* 90:17-26.
- Myers MS, Landahl JT, Krahn MM, McCain BB. 1991. Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the US West Coast. *Environ Health Perspect* 90:7-15.
- Ortego LS, Hawkins WE, Walker WW, Krol RM, Benson WH. 1994. Detection of proliferating cell nuclear antigen (PCNA) in tissues of three small fish species. *Biotech Histochem* 69:317-329.
- Povey AC. 2000. DNA adducts: Endogenous and induced. *Toxicol Pathol* 28:405-414.
- Powers DA. 1989. Fish as model systems. *Science* 246:352-358.
- Sadar MD, Ash R, Sundquist J, Olsson PE, Andersson TB. 1996. Phenobarbital induction of CYP1A1 gene expression in a primary culture of rainbow trout hepatocytes. *J Biol Chem* 271:17635-17643.
- Salem H, Katz S, eds. 1998. *Advances in Animal Alternatives for Safety and Efficacy Testing*. Washington DC: Taylor & Francis.
- Schultz ME, Schultz RJ. 1982. Induction of hepatic tumors with 7,12-dimethylbenz[a]anthracene in two species of viviparous fish (genus *Poeciliopsis*). *Environ Res* 27:337-351.
- Sinnhuber RO, Hendricks JD, Wales JH, Putnam GB. 1977. Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. *Ann NY Acad Sci* 298:389-408.
- Smith CE, Peck TH, Klauda RJ, McLaren JB. 1979. Hepatomas in Atlantic tomcod *Microgadus tomcod* (Walbaum) collected in the Hudson River estuary in New York. *J Fish Dis* 2:313-319.
- Sonstegard RA. 1977. Environmental carcinogenesis studies in fishes of the Great Lakes of North America. *Ann NY Acad Sci* 298:261-269.
- Spitsbergen JM, Tsai HW, Reddy A, Miller T, Arbogast D, Hendricks JD, Bailey GS. 2000a. Neoplasia in zebrafish (*Danio rerio*) treated with 7,12-dimethylbenz[a]anthracene by two exposure routes at different developmental stages. *Toxicol Pathol* 28:705-715.
- Spitsbergen JM, Tsai HW, Reddy A, Miller T, Arbogast D, Hendricks JD, Bailey GS. 2000b. Neoplasia in zebrafish (*Danio rerio*) treated with N-methyl-N'-nitro-N-nitrosoguanidine by three exposure routes at different developmental stages. *Toxicol Pathol* 28:716-725.
- Stalker MJ, Kirby GM, Kocal TE, Smith IR, Hayes MA. 1991. Loss of glutathione S-transferases in pollution-associated liver neoplasms in white suckers (*Catostomus commersoni*) from Lake Ontario. *Carcinogenesis* 12:2221-2226.
- Stanton MF. 1965. Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. *J Natl Cancer Inst* 34:117-130.
- Stanton MF. 1966. Hepatic neoplasms of aquarium fish exposed to *Cycas circinalis*. *Fed Proc* 25:661.
- Stegeman JJ, Hahn ME. 1994. Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Boca Raton: Lewis Publishers. p 87-206.
- Stegeman JJ, Lech JJ. 1991. Cytochrome P-450 monooxygenase systems in aquatic species: Carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ Health Perspect* 90:101-109.
- Stein JE, Reichert WL, Varanasi U. 1994. Molecular epizootiology: Assessment of exposure to genotoxic compounds in teleosts. *Environ Health Perspect* 102(Suppl 12):19-23.
- Swenberg JA, Fedtke N, Fennell TR, Walker VE. 1990. Relationship between carcinogen exposure, DNA adducts and carcinogenesis. In: Clayson DB, Munro IC, Shubik P, Swenberg JA, eds. *Progress in Predictive Toxicology*. Amsterdam: Elsevier. p 161-184.
- Swenberg JA, Hoel DG, Magee PN. 1991. Mechanistic and statistical insight into the large carcinogenesis bioassays on N-nitrosodiethylamine and N-nitrosodimethylamine. *Cancer Res* 51(Suppl):6409-6414.
- Swenson DH, Lawley PD. 1978. Alkylation of deoxyribonucleic acid by carcinogens dimethyl sulphate, ethyl methanesulphonate, N-ethyl-N-nitrosourea, and N-methyl-N-nitrosourea. *Biochem J* 171:575-587.
- Talbot W, Hopkins N. 2000. Zebrafish mutations and functional analysis of the vertebrate genome. *Genes Dev* 14:755-762.

- Tennant R, Tice R, Spalding J. 1998. The transgenic Tg.AC mouse model for identification of chemical carcinogens. *Toxicol Lett* 102-103:465-471.
- Van Beneden RJ, Ostrander GK. 1994. Expression of oncogenes and tumor suppressor genes in teleost fishes. In: Malins DC, Ostrander GK, eds. *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*. Boca Raton: Lewis Publishers. p 295-325.
- Van Veld P, Vogelbein W, Cochran M, Goksoyr A, Stegeman J. 1997. Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicol Appl Pharmacol* 142:348-359.
- Van Veld P, Vogelbein W, Smolowitz R, Woodin B, Stegeman J. 1992. Cytochrome P450IA1 in hepatic lesions of a teleost fish (*Fundulus heteroclitus*) collected from a polycyclic aromatic hydrocarbon-contaminated site. *Carcinogenesis* 13:505-507.
- Vogelbein WK, Fournie JW. 1994. Ultrastructure of normal and neoplastic exocrine pancreas in the mummichog, *Fundulus heteroclitus*. *Toxicol Pathol* 22:248-260.
- Vogelbein WK, Fournie JW, Van Veld PA, Huggett RJ. 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. *Cancer Res* 50:5978-5986.
- Walker WW, Manning CS, Overstreet RM, Hawkins WE. 1985. Development of aquarium fish models for environmental carcinogenesis: An intermittent-flow exposure system for volatile, hydrophobic chemicals. *J Appl Toxicol* 5:255-260.
- Williams DE, Lech JJ, Buhler DR. 1998. Xenobiotics and xenoestrogens in fish: Modulation of cytochrome P450 and carcinogenesis. *Mutat Res* 399:179-192.
- Williams GM, Iatropoulos MJ, Jeffrey AM. 2000. Mechanistic basis for nonlinearities and thresholds in rat liver carcinogenesis by the DNA-reactive carcinogens 2-acetylaminofluorene and diethylnitrosamine. *Toxicol Pathol* 28:388-395.
- Wogan GN. 1988. Detection of DNA damage in studies on cancer etiology and prevention. In: Bartsch H, Hemminki K, O'Neill IK, eds. *Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention*. Lyon: IARC Scientific Publication no. 89, International Agency for Research on Cancer. p 32-51.