

Fish Models for Environmental Carcinogenesis: The Rainbow Trout

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Progress over the past 30 years has revealed many strengths of the rainbow trout as an alternative model for environmental carcinogenesis research. These include low rearing costs, an early life-stage ultrasensitive bioassay, sensitivity to many classes of carcinogen, a well-described tumor pathology, responsiveness to tumor promoters and inhibitors, and a mechanistically informative nonmammalian comparative status. Low-cost husbandry, for example, has permitted statistically challenging tumor study designs with up to 10,000 trout to investigate the quantitative interrelationships among carcinogen dose, anticarcinogen dose, DNA adduct formation, and final tumor outcome. The basic elements of the trout carcinogen bioassay include multiple exposure routes, carcinogen response, husbandry requirements, and pathology. The principal known neoplasms occur in liver (mixed hepatocellular/cholangiocellular adenoma and carcinoma, hepatocellular carcinoma), kidney (nephroblastoma), swim bladder (adenopapilloma), and stomach (adenopapilloma). Trout possess a complex but incompletely characterized array of cytochromes P450, transferases, and other enzymic systems for phase I and phase II procarcinogen metabolism. In general, trout exhibit only limited capacity for DNA repair, especially for removal of bulky DNA adducts. This factor, together with a high capacity for P450 bioactivation and negligible glutathione transferase-mediated detoxication of the epoxide, accounts for the exceptional sensitivity of trout to aflatoxin B₁ carcinogenesis. At the gene level, all trout tumors except nephroblastoma exhibit variable and often high incidences of oncogenic *Ki-ras* gene mutations. Mutations in the trout *p53* tumor suppressor gene have yet to be described. There are many aspects of the trout model, especially the lack of complete organ homology, that limit its application as a surrogate for human cancer research. Within these limitations, however, it is apparent that trout and other fish models can serve as highly useful adjuncts to conventional rodent models in the study of environmental carcinogenesis and its modulation. For some problems, fish models can provide wholly unique approaches. — *Environ Health Perspect* 104(Suppl 1):5–21 (1996)

Key words: trout, carcinogenesis, neoplasms, oncogenes, DNA adducts, fish models

Introduction

Fish have gained increasing attention over the past three decades as valuable models for environmental carcinogenesis research. Various fish species have been investigated as nonmammalian vertebrate models for carcinogen testing, as surrogates for understanding mechanisms of human cancer and its prevention, as feral species indicators of ecologic contamination, as indicators of potential human exposure to carcinogens in the water column or aquatic food chain, and for application as *in situ* field monitors of integrated carcinogenic hazard in groundwaters near toxic waste sites. Interest in the use of small aquarium fish species for cancer research arose from the pioneering work of Stanton (1), who in 1965 demonstrated the hepatocarcinogenicity of *N*-nitrosodiethylamine (DEN) to the zebra danio. Table 1 presents a partial list of species used and carcinogens examined since that time. Exposures in these studies have included continuous or acute water bath treatment, dietary intake, or direct injection of embryos or later life stages. Additional species and carcinogens have been explored (23–25), and comprehensive testing of 30 National Toxicology Program carcinogens in one species, the medaka, is in progress at the Duluth laboratory of the U.S. Environmental Protection Agency (R Johnson, personal communication).

Through work with various aquarium fish species, many attributes have been identified: their low cost, portability, and ease of laboratory culture; their potential for *in situ* field monitoring; and their potential for lifetime bioassay, short reproductive cycle, and ease of genetic studies. However, while aquarium fish models have distinct appeal, knowledge of mechanisms of carcinogenesis (e.g., procarcinogen metabolism, DNA adduction and repair, targeted oncogenes) and its modulation (inhibition, promotion–progression) by environmental and dietary factors is at present more advanced in the rainbow trout (*Oncorhynchus mykiss*). Attention was drawn to this species in the early 1960s when epizootics of liver cancer in Pacific Northwest trout hatcheries ultimately led to the identification of aflatoxin B₁ (AFB₁) as a potential human hepatocarcinogen (26–28). This review focuses on the use of the rainbow trout in environmental carcinogenesis research. This model shares some attributes with aquarium species but also has unique features

Manuscript received 9 August 1995; manuscript accepted 26 September 1995.

Technical paper number 10,831 from the Oregon Agricultural Experiment Station. This work was partially supported by U.S. Public Health Service grants ES00210, ES03850, and ES04766 from the National Institute of Environmental Health Sciences.

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Abbreviations used: 2-AAF, 2-acetylaminofluorene; *o*-AAT, *ortho*-aminoazotoluene; AC, adenocarcinoma; ACC, acinar cell carcinoma; AFB₁, aflatoxin B₁; AFG₁, aflatoxin G₁; AFM₁, aflatoxin M₁; AFP₁, aflatoxin P₁; AFQ₁, aflatoxin Q₁; ANF, α -naphthoflavone; Ah, aryl hydrocarbon; B[a]P, benzo[a]pyrene; BeP, benzoyl peroxide; BNF, β -naphthoflavone; CCC, cholangiocellular carcinoma; CYP, cytochrome P450; DBP, dibenzo[a,l]pyrene; DEN, *N*-nitrosodiethylamine; DHEA, dehydroepiandrosterone; DMAB, dimethylaminoazobenzene; DMBA, 7,12-dimethylbenz[a]anthracene; DMN, *N*-nitrosodimethylamine; ER, ethoxyresorufin; GST, glutathione-S-transferase; HA, hepatic adenoma; HCC, hepatocellular carcinoma; HMBA, hydroxymethylbenz[a]anthracene; I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; K, kidney; LA, lauric acid; LV, liver; MAMA, methylazoxymethanol acetate; MMA, 3'-primer mismatch polymerase chain reaction analysis; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methylnitrosourea; NM, *N*-nitrosomorpholine; P, progesterone; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; PCR, polymerase chain reaction; PFOA, perfluorooctanoic acid; RB, rhabdomyosarcoma; RXM, I3C reaction mixture formed *in vitro* upon acid treatment; SB, swim bladder; ST, glandular stomach; T, testosterone; tBuOOH, *t*-butyl hydroperoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholanthrene.

Table 1. Examples of small fish species used in carcinogen bioassays.

Species	Carcinogen	Neoplasms reported	Reference
<i>Danio rerio</i> (zebrafish)	DMN	HCC, CCC, esophageal	(2)
	DEN	HCC, CCC, esophageal ^a	(1,2)
	NM	HCC, CCC, esophageal ^a , intestinal ^a	(2)
<i>Xiphophorus/Platypoecilus</i> sp. (platyfish/swordtail)	MNU	Melanoma, fibrosarcoma, RB	(3,4)
<i>Poecilia reticulata</i> (guppy)	DMN	HCC, CCC	(2,5)
	DEN	HCC, CCC, esophageal ^a	(2)
	NM	HCC, CCC, esophageal ^a	(2)
	<i>o</i> -AAT	HCC, CCC	(6)
	DMAB	HCC, cholangioma	(6)
	MAMA	Pancreatic ACC, AC	(7)
	AFB ₁	Hepatic	(5)
	2-AAF	Hepatic	(5)
	B[a]P	HA, HCC	(8)
DMBA	HA, HCC, RB, renal AC, neurilemmoma, fibrosarcoma	(9)	
<i>Oryzias latipes</i> (medaka)	B[a]P	HA, HCC	(8)
	DEN	Hepatic tumors, HCC	(10-12)
	MAMA	HCC, medulloepithelioma	(12-14)
	<i>o</i> -AAT	HA, HCC	(12)
(medaka)	AFB ₁	HA, HCC	(12)
	AFG ₁	HA, HCC	(12)
	DEN	HCC, CCC, pancreatic AC, Hemangioma, pericytoma	(15-17)
<i>Rivulus marmoratus</i> (rivulus)	DMBA	HCC, lymphosarcoma	(18)
<i>Poeciliopsis</i> sp.	DEN	HCC	(19)
<i>Fundulus grandis</i>	MNNG	Pancreatic ACC	(20)
<i>Gambusia affinis</i>	MAMA	HCC, CCC	(21)
<i>Cyprinodon variegatus</i>	DMBA	HA, HCC	(22)

Abbreviations: DMN, *N*-nitrosodimethylamine; DEN, *N*-nitrosodiethylamine; NM, *N*-nitrosomorpholine; MNU, *N*-methylnitrosourea; *o*-AAT, *ortho*-aminoazotoluene; DMAB, dimethylaminoazobenzene; DMBA, 7,12-dimethylbenz[*a*]anthracene; MAMA, methylazoxymethanol acetate; AFB₁, aflatoxin B₁; 2-AAF, 2-acetylaminofluorene; B[a]P, benzo[*a*]pyrene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; HCC, hepatocellular carcinoma; CCC, cholangiocellular carcinoma; RB, rhabdomyosarcoma; ACC, acinar cell carcinoma; AC, adenocarcinoma; HA, hepatic adenoma. ^aUndefined neoplasms.

such as wide-ranging body size and target organ tissue availability not applicable to small fish models.

Carcinogen Bioassay, Response, and Pathology in Rainbow Trout

Rainbow trout occupy an important niche in the history of carcinogenesis. It was in this species that the carcinogenicity of the aflatoxins was first recognized. Based on this original discovery, two major and several minor research efforts using rainbow trout for cancer research were launched. One of the major programs was centered at the U.S. Fish and Wildlife Service's Western Fish Nutrition Laboratory at Cook, Washington, under the direction of Dr. John Halver. This program started in the late 1950s and was phased out in the early 1970s. The other major program was started in 1963 at Oregon State University under the direction of Russell O. Sinnhuber and continues to date. Carcinogenesis research with rainbow trout in this program

has followed multidisciplinary mechanistic guidelines for many years, with an *in vivo* whole-animal response to carcinogens as its foundation. The remainder of this section will discuss various aspects of this whole-animal research, including routes of exposure, experimental protocols, carcinogens tested, and pathology.

Routes of Exposure

Dietary. The original discovery of the carcinogenicity of aflatoxins in rainbow trout was the result of aflatoxin contamination of dietary foodstuffs, primarily cottonseed meal. Thus the route of exposure was clearly dietary. All of the early experimental work used the dietary route of exposure, usually by incorporating aflatoxin into a semipurified diet such as the Oregon Test Diet developed at Oregon State University (29,30). Doses in the low (1–20 µg/kg parts per billion [ppb]) range fed continuously for 9 to 18 months (28,31,32) or higher doses (10–80 ppb) fed continuously for shorter periods of time (1–30 days) (33–35) were found to be carcinogenic.

The dietary route of exposure was, and continues to be, a useful procedure for certain desired end points and for specific carcinogens, particularly those with low or negligible water solubility. Its primary weakness is that it is voluntary and inevitably results in unequal exposures within experimental groups of fish housed in the same tank.

Embryo Waterbath. For this reason, alternative and passive routes of exposure have been developed. Wales et al. (36) showed that a brief exposure (0.5–1.0 hr) of trout embryos to a static solution (0.5 ppm or less) of AFB₁ was an effective way to initiate neoplasms. They also demonstrated that embryo sensitivity was a function of age, being very low before liver organogenesis but increasing steadily from that time through and after hatching. This exposure method is especially useful for initiation/promotion protocols in which a lengthy period of time between initiation and subsequent dietary promotion is desirable. Subsequent experiments revealed that this method was effective for a number of carcinogens and that the incidences were dose responsive (Table 2). The subject of embryo initiation of carcinogenesis in rainbow trout has been extensively reviewed (37–39).

Fry Waterbath. Continuous or acute waterbath exposures of free-swimming fish to carcinogens have been used extensively with aquarium fish. This exposure method was not used for many years with rainbow trout, but recently the acute or short-term version of this method has been used with much success (40–43,63–65). Its greatest utility is for inhibition experiments in which the protocol calls for a pre-initiation dietary exposure to a potential inhibitor, followed by a short-term pulse initiation. It works best with small trout, which requires relatively less water and less carcinogen and results in less splashing, an inevitable occurrence with larger fish. This route of exposure is more effective (requiring lower doses to achieve the same response) than embryo waterbath because the protective chorion of the egg is gone. It can also result in different target organ specificity when compared to embryo waterbath exposure. For example, embryo exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) results in the following organ tumor response (liver > stomach > kidney), but fry waterbath exposure produces a different response (stomach > kidney > liver) [JD Hendricks, unpublished results (57)].

Table 2. Carcinogenic response of rainbow trout to chemicals.

Compound	Exposure route	Target organ ^a	Reference
Aflatoxin B ₁	Diet	LV	(28,31,32)
	Water, embryo	LV	(36-39)
	Water, fry	LV	(40-43)
	Microinjection, egg	LV	(44-46)
	Injection (ip)	LV	(47)
Aflatoxin G ₁	Diet	LV	(32)
Aflatoxin M ₁	Water, embryo	LV	(37)
	Diet	LV	(48)
Aflatoxin Q ₁	Microinjection, egg	LV	Unpublished
	Diet	LV	(49)
Aflatoxicol	Diet	LV	(50,51)
	Water, embryo	LV	(51)
	Microinjection, egg	LV	Unpublished
Aflatoxicol M ₁	Diet	LV	Unpublished
	Microinjection, egg	LV	Unpublished
Sterigmatocystin	Water, embryo	LV	(52)
Versicolorin A	Water, embryo	LV	(52)
Diethylnitrosamine	Diet	LV	(53)
	Water, embryo	LV	Unpublished
	Water, fry	LV,ST,SB,K	Unpublished
Dimethylnitrosamine	Diet	LV	(54,55)
	Water, embryo	LV,K	Unpublished
2,6-Dimethylnitrosomorpholine	Diet	LV,ST,SB	(56)
	Water, embryo	LV	Unpublished
2,2'-Dioxo-di- <i>n</i> -propylnitrosamine	Injection (ip)	LV	Unpublished
Nitrosomorpholine	Water, embryo	LV	Unpublished
Nitrosopyrrolidine	Water, embryo	LV	Unpublished
<i>N</i> -Methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine	Diet	ST	(56)
	Water, embryo	LV,ST,K,SB	(57)
	Water, fry	ST,K,SB,LV	Unpublished
	Microinjection, egg	LV,ST,K	Unpublished
	Water, embryo	LV,ST,SB,K	Unpublished
Ethylnitrosourea	Water, embryo	LV,ST,K,SB	Unpublished
Methylazoxy-methanol acetate	Water, fry	SB,LV,ST,K	Unpublished
	Microinjection, egg	LV,ST,K,SB	Unpublished
	Diet	LV	(58)
Benzo[<i>a</i>]pyrene	Injection (ip)	LV	(58)
	Microinjection, egg	LV	Unpublished
	Microinjection, sac-fry	LV	(59)
<i>trans</i> -7,8-Dihydrobenzo[<i>a</i>]pyrene-7,8-diol 7,12-Dimethylbenz[<i>a</i>]anthracene	Diet	ST,LV	Unpublished
	Water, embryo	LV,ST,K	(60)
	Water, fry	ST,LV,SB,K	Unpublished
	Microinjection, egg	LV,ST	Unpublished
	Diet	ST,LV,SB	Unpublished
Dibenzo[<i>a,h</i>]pyrene	Diet	ST, LV	(56)
1,2-Dibromoethane	Diet	LV	(61), Unpublished
Dichlorodiphenyltrichloroethane	Diet	LV	(31,62)
Cyclopropene fatty acids	Diet	LV	Unpublished
Dehydroepiandro-sterone	Diet	LV	(61)
2-Acetylaminofluorene	Diet	LV	(61)
Aminoazotoluene	Diet	LV	(61)
<i>p</i> -Dimethylaminoazobenzene	Diet	LV	(61)

Abbreviations: LV, liver; ST, glandular stomach; K, kidney; SB, swim bladder. ^aOrgans are presented in order of decreasing tumor incidence.

Embryo/Sac-fry Microinjection. Microinjection of rainbow trout embryos was first reported by Metcalfe and Sonstegard (44). The technique was improved by Black et al. (45) and semiautomated at Oregon State University (46). These improvements permit one person to microinject up to 4000 embryos in an 8-hr day. Our current procedure uses a Hamilton Microlab 900 pump (Hamilton

Company; Reno, Nevada) interfaced with a computer. The computer is programmed to automatically fill a microsyringe from a reservoir of injectant and accurately dispense 1- μ l doses when a footswitch is pressed. Fine teflon tubing connects the microsyringe to a 31-gauge stainless steel needle mounted on a micromanipulator. The needle is inserted through the chorion and into the yolk sac where the droplet is

released. We routinely use a carrier of 25% acetone/75% vegetable oil for AFB₁ and experience a low mortality of 5 to 10% from carrier-only injections. The following is an example of results obtained with this technique: AFB₁ doses of 0.5, 1.0, 2.0, and 4.0 ng/ μ l/egg produced hepatic tumor incidences of 26, 34, 45, and 48% nine months later (GS Bailey, unpublished results). The obvious advantages of this procedure include the extremely small doses required to initiate neoplasia and the ability to expose embryos to highly water-insoluble carcinogens.

Sac-fry microinjection was first reported by Metcalfe et al. (66). The procedure is similar to embryo microinjection except the sac-fry are anesthetized in CO₂-saturated water before injection. This allows for more accurate placement of the injection droplet within the yolk sac, less trauma to the immobilized sac-fry, fewer injection-related mortalities, and in general a greater sensitivity to carcinogens because the older organisms may be more metabolically competent than younger embryos.

Intraperitoneal Injection. Intraperitoneal injection (ip) is rarely used as an initiating protocol for trout, with only two such studies known to be in the literature (47,58). However, such injections are used routinely for short-term metabolism experiments or for DNA-binding studies.

Gavage. Gavage or stomach tubing is a problematic route of carcinogen exposure for rainbow trout due to their strong tendency to regurgitate anything that is irritating to the stomach. This reaction was reported by Bauer et al. (67) and has been personally observed repeatedly.

Carcinogens Tested

Table 2 is a compilation of all the carcinogens that have produced neoplasms in rainbow trout. Several interesting features of carcinogenesis in rainbow trout emerge from the data in this table. *a*) The trout liver is the primary organ responding to almost all carcinogens, regardless of the route of exposure. Only the dietary exposure of trout to MNNG, a direct-acting carcinogen, failed to produce liver tumors in all of the experiments where a positive neoplastic response was seen. *b*) A carcinogen that produces only pancreatic neoplasms in Syrian golden hamsters, 2,2'-dioxo-di-*n*-propylnitrosamine (68), produces only hepatocellular neoplasms in rainbow trout. *c*) Only four target organs of the trout have been shown to respond to the carcinogenic stimulus of a wide variety of

chemical carcinogens: the liver, glandular stomach, kidney, and swim bladder (SB); and *d*) different routes of exposure may change the primary target organ but usually not the spectrum of responding organs.

Pathology of Neoplasms in Rainbow Trout

Liver. The subject of the pathology of hepatocellular neoplasms in rainbow trout has been thoroughly described and reviewed (69,70). Here we will only refer to the types of tumors observed and provide corresponding illustrations. The predominant tumor, occurring in response to all the carcinogens tested to date, is a mixed hepatocellular/cholangiocellular carcinoma (59,60,71) (Figure 1). These tumors consist of peripheral hepatic tubules filled with basophilic hepatocytes and centrally located biliary cells together with their connective tissue stroma. The biliary compartment can be either well differentiated into ducts or poorly differentiated and occur as broad sheets of cells. Typically, over 60% of the tumors examined from a termination necropsy will be of this general type. One variant of the mixed carcinoma contains an additional cellular component, pancreatic acinar cells, usually in close association with the biliary portion of the tumor (64) (Figure 2). Another type of mixed carcinoma contains just biliary and pancreatic components.

The second most abundant tumor type (25–30%) is the pure hepatocellular carcinoma (Figure 3). These tumors are composed of broad tubules of basophilic hepatocytes, with many cells between adjacent sinusoids and frequent mitotic figures. Cholangiocellular carcinomas are rare, usually less than 1%, but consist of ducts or sheets of cells, have minimal stroma, and are invasive into surrounding liver tissue (Figure 4). All these malignant tumor types are capable of distant metastasis or direct growth into surrounding visceral tissues, but we rarely see this occur within the 9- to 12-months time frame of most of our experiments. Metastases are rather common if the fish are held for 2 years or longer.

Several nonmalignant tumors are also observed. Hepatocellular adenomas tend to be small and noninvasive, with cells that are basophilic but occur within normal-appearing hepatic tubules (Figure 5). This is usually not an end-stage neoplasm but appears to progress to hepatocellular carcinoma. The prevalence varies depending on the time of termination, but in most cases, it is between 5 and 10%. Very rarely

we observe an adenoma that is composed of eosinophilic hepatocytes, but the vast majority are basophilic. Very small foci of basophilic hepatocytes are interpreted to be the beginning stages of either hepatocellular adenomas or carcinomas and not a separate preneoplastic lesion. Cholangioma is an infrequent tumor type consisting of mostly normal-appearing bile ducts and abundant stroma that usually encapsulate the structure (Figure 6). Usually 1 to 2% of the total incidence are cholangiomas. Mixed hepatocellular/cholangiocellular adenomas are tumors having the cellular features of adenomas and cholangiomas together in the same cellular mass (Figure 7). These tumor types are seldom seen.

A final tumor type that has been observed only twice in over 30 years of histopathologic examinations of tens of thousands of liver sections is what we interpret to be a hepatoblastoma. These two tumors consist of deeply basophilic, highly undifferentiated cells with an extremely high rate of mitosis (Figure 8). The cells palisade around vascular channels and outstrip the vascular supply to the rapidly expanding cellular mass. This leads to extensive necrosis that extends into veins and causes serious hemorrhaging (Figure 9).

Kidney. The nephroblastoma is an almost exclusive chemically inducible neoplasm of trout kidneys. It consists of deeply basophilic, highly mitotic blastema cells; abortive, poorly differentiated glomerular structures; incompletely differentiated tubules; and abundant connective tissue stroma (Figure 10). Six carcinogens, listed in Table 2, have produced nephroblastomas, but the treatment of choice for producing a high incidence of these tumors is a static waterbath exposure of rainbow trout fry to a solution of 50 ppm MNNG for 30 min. This will result in about 50% of the fish having one or more large nephroblastomas 6 to 9 months later. These tumors grow rapidly, become very large, and kill the fish through destruction of normal kidney tissue and obstruction of urine flow. To our knowledge, the rainbow trout is the only animal model in which nephroblastoma can be routinely initiated in a high incidence by a chemical carcinogen (72).

Stomach. All the stomach tumors that we have observed in rainbow trout have been benign papillary adenomas of the mucosal lining of the glandular stomach. Typically, they grow upward into the luminal space. Some tumors produced by dietary exposure to MNNG (56) also exhibited

downward growth but never penetrated the basement membrane (Figure 11).

Swim Bladder. As with the stomach tumors described above, the swim bladder adenomas are benign papillary overgrowths of epithelial cells that protrude into the lumen of the swim bladder. The unique feature of the cells of this lesion is the marked increase in size of the tumor cells compared with the normal mucosal cells. The columnar height of these cells is often several times greater than the normal cells (Figure 12).

This brief review of the procedures involved in the initiation and identification of neoplasms in rainbow trout is intended to portray this model as a viable alternative for many aspects of cancer research. The fish are easy and economical to rear, and they respond to classical carcinogens in a predictable, dose-responsive manner.

Pathways of Procarcinogen Metabolism, DNA Adduction, and Repair

Cytochromes P450

As is the case in humans (73), trout cytochromes P450 (CYPs) play a crucial role in the bioactivation of procarcinogens to electrophilic metabolites capable of covalently binding to DNA. The study of properties of the trout CYP-dependent mixed-function oxidase system, response to inducers, and metabolism of xenobiotics was pioneered by a number of laboratories including DR Buhler (Oregon State University), J Lech (Medical College of Wisconsin), and L Forlin and T Andersson (University of Goteborg) [reviewed in (74–79)]. Although relatively few trout CYPs have been sequenced and assigned to a CYP subfamily, a number of trout CYPs have been purified and partially characterized with respect to bioactivation of procarcinogens (Table 3). Much of this work has been performed by the laboratory of D.R. Buhler; the reader is referred to an excellent recent review (97) for more detailed information. Table 3 does not include information on the trout CYPs responsible for steroid synthesis, P450_{scc} (CYP11A1), P450_{C17} (CYP17) or P450_{arom} (CYP19), as these display little or no activity toward procarcinogens.

Role of Trout CYPs in the Bioactivation of Procarcinogens

Aflatoxin B₁. AFB₁ is metabolized by CYP to a number of monohydroxylated metabolites including aflatoxins M₁, Q₁, and P₁ [see (98) for an excellent recent review], all

CARCINOGENESIS IN TROUT

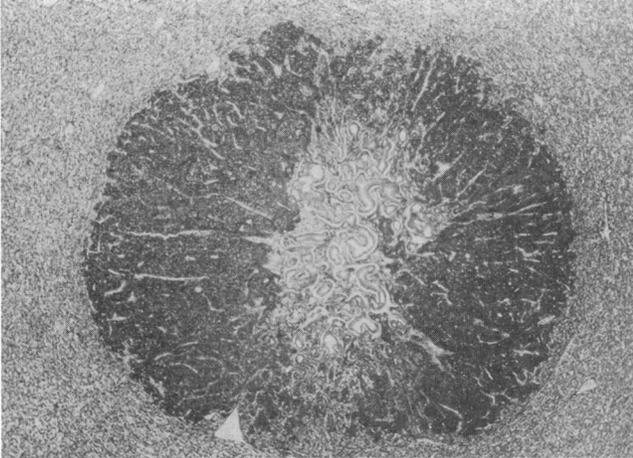


Figure 1. A small mixed carcinoma in a rainbow trout initiated by 20 ppb AFB₁ in the diet for 1 month. Note the deeply basophilic peripheral hepatocellular portion and the central biliary ducts. H & E; × 14.

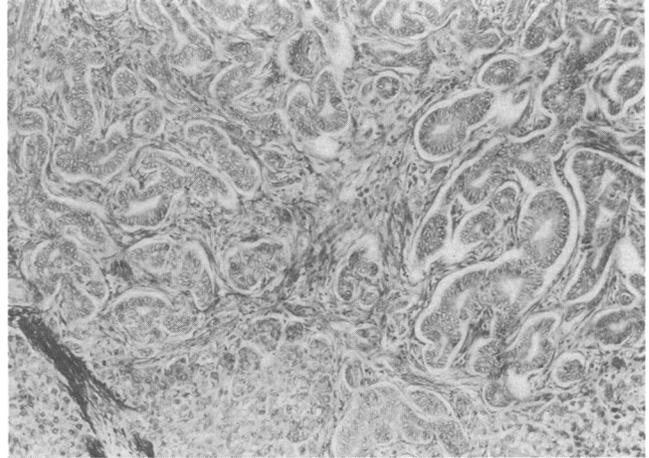


Figure 4. The advancing invasive edge of a cholangiocellular carcinoma initiated by dietary exposure of rainbow trout to 800 ppm *N*-nitrosodimethylamine for 12 months. The tumor is composed of neoplastic bile ducts and minimal connective tissue stroma. H & E; × 56.

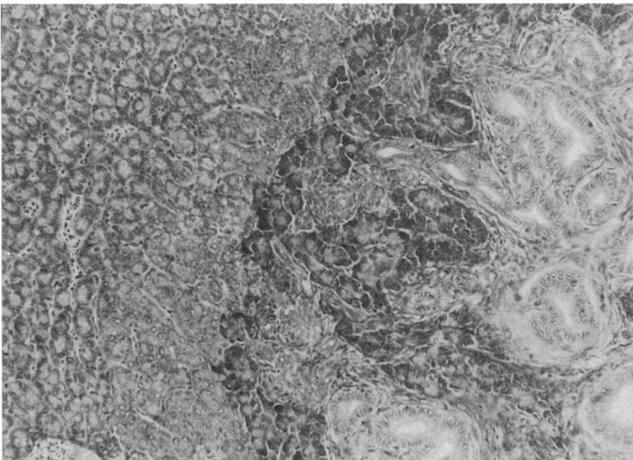


Figure 2. A mixed carcinoma in a rainbow trout initiated by embryo exposure to a 0.05 ppm solution of AFB₁ for 1 hr. The tumor is composed of centrally located biliary ducts (right), adjacent pancreatic acinar units (middle), and peripheral hepatocellular tubules. On the far left is normal liver tissue. H & E; × 56.

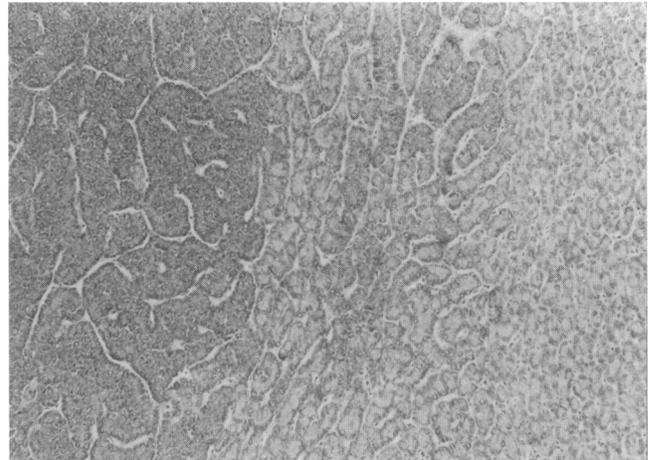


Figure 5. A hepatocellular carcinoma (left) growing within a hepatocellular adenoma (middle), with normal rainbow trout liver tissue on the far right. Contrast the deeply basophilic broad tubules of the carcinoma with the less basophilic two-cell-wide tubules of the adenoma. This occurrence supports the hypothesis that carcinomas develop from adenomas. The tumor was initiated by embryo exposure to aqueous AFB₁ (0.5 ppm) for 1 hr. H & E; × 90.

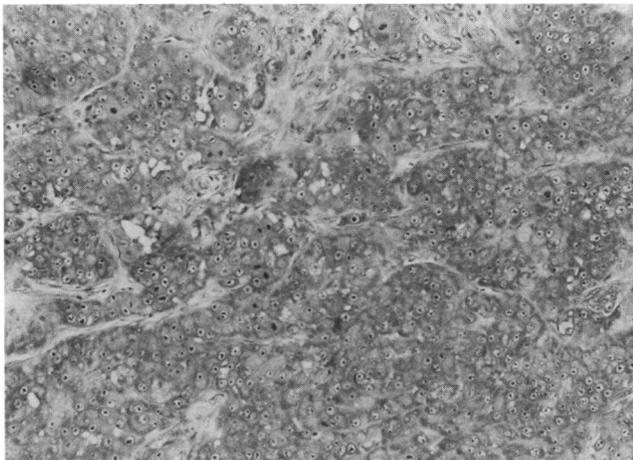


Figure 3. A portion of a large hepatocellular carcinoma in a rainbow trout initiated by dietary exposure to 20 ppb AFB₁ for 1 month. Note the broad tubules of basophilic hepatocytes between adjacent sinusoids and the numerous mitotic figures. H & E; × 140.

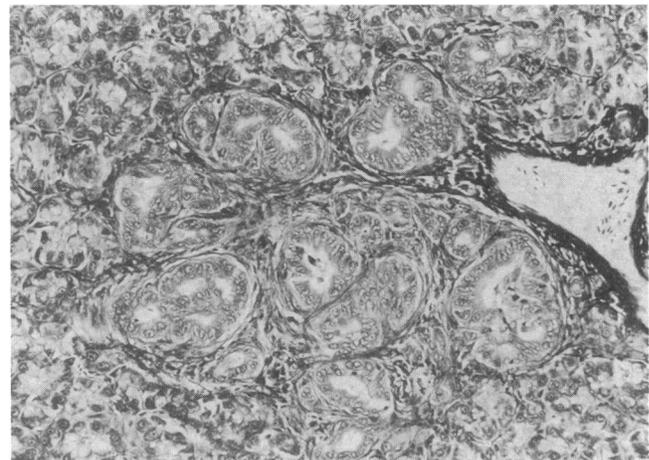


Figure 6. A small cholangioma in a rainbow trout initiated by 20 ppb dietary AFB₁ for 1 month. Note the mostly normal appearance of the ducts and encapsulation by connective tissue stroma. H & E; × 140.

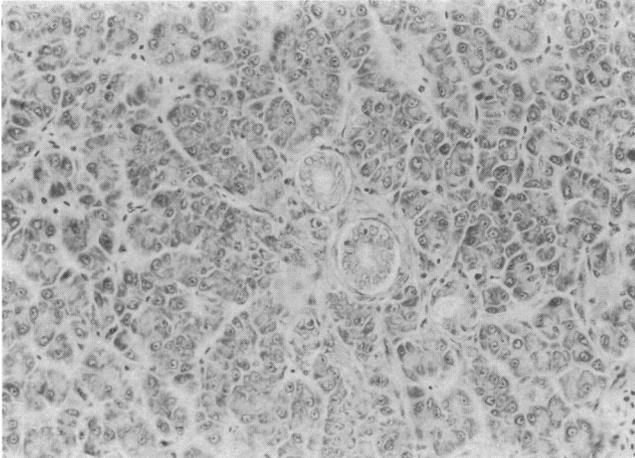


Figure 7. A small mixed adenoma initiated by a 2-week feeding of 800 ppb aflatoxinol-M₁ to rainbow trout. The neoplasm consists of basophilic hepatocytes similar to those observed in hepatocellular adenomas and a beginning proliferation of bile ducts in the central region. H & E; × 224.

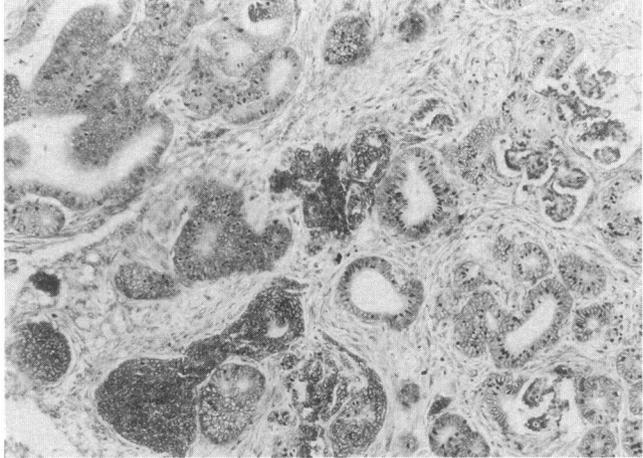


Figure 10. A large nephroblastoma initiated by exposure of rainbow trout embryos to aqueous 10 ppm methylazoxymethanol acetate (MAMA) for 24 hr. Note the deeply basophilic undifferentiated mass of blastema cells (upper right); incompletely differentiated tubules with numerous mitotic figures; abortive, poorly formed glomerularlike structures (lower left); and abundant connective tissue stroma. H & E; × 90.

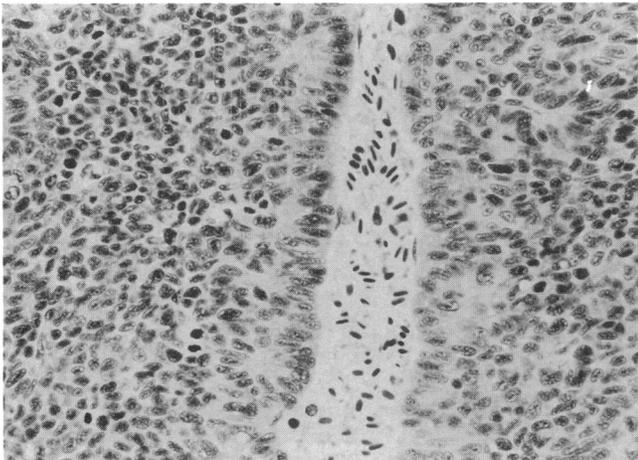


Figure 8. A portion of a presumptive hepatoblastoma initiated by continuous dietary exposure of rainbow trout to 20 ppb AFB₁. Note the poorly differentiated nature of the cells, scanty cytoplasm, numerous mitotic figures, and palisading of cells around a large vein. H & E; × 224.

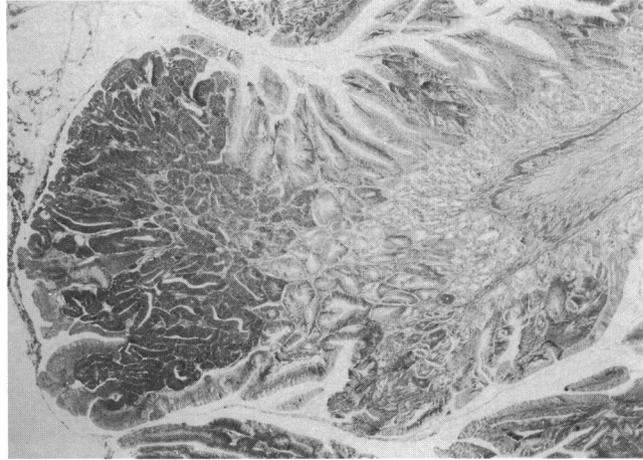


Figure 11. A papillary adenoma of the glandular stomach of a rainbow trout initiated by 500 ppm dietary MNNG for 18 months. Primary proliferation of mucosa cells is upward into the lumen of the stomach, although some neoplastic cells occur in the gastric pits as well. H & E; × 56.

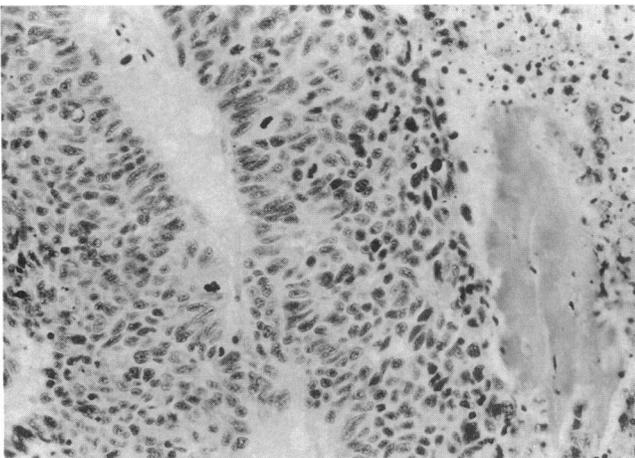


Figure 9. A portion of a presumptive hepatoblastoma initiated by continuous dietary exposure of rainbow trout to 20 ppb AFB₁ (the same tumor shown in Figure 8). Extensive necrosis in the broad bands of cells (upper right) has progressed into a large vein causing hemorrhaging. H & E; × 358.



Figure 12. A papillary adenoma in the swim bladder of a rainbow trout initiated by embryo exposure to aqueous, methylazoxymethanol acetate (MAMA) 10 ppm for 24 hr. Note the tremendous proliferation of cells and their increased size compared to normal swim bladder mucosal epithelium at the right. H & E; × 56.

Table 3. Cytochromes P450 in trout.

Trivial name	Gene nomenclature	Substrates	Expression	References
LM4a ^a	—	BP	—	(80)
LM4b	<i>CYP1A1</i>	ER, BP, AFB ₁	Induced by DMBA, 3-MC, BNF, PCBs, TCDD	(80–84)
—	<i>CYP1A2</i>	—	—	(84)
LMC1	<i>CYP2M1</i>	LA (ω-6)	High in juvenile female kidney	(85–88)
LMC2, LM2	<i>CYP2K1</i>	AFB ₁ , LA (ω-1)	High in mature male kidney	(80)
LMC3	—	DMBA	—	(85,86)
LMC4	—	DMBA	—	(85,86)
LMC5, P450 _{con} ^b	—	T,P (6β)	High in mature male liver; induced by steroids	(85,94,95)
P450KM1 ^c	—	—	—	(96)
P450KM2	—	—	High in mature male kidney; induced by androgens	(96)

Abbreviations: BaP, benzo[*a*]pyrene; ER, ethoxyresorufin; AFB₁, aflatoxin B₁; 3-MC, 3-methylcholanthrene; BNF, β-naphthoflavone; PCBs, polychlorinated biphenyls; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; LA, lauric acid; DMBA, 7,12-dimethylbenz[*a*]anthracene; T, testosterone; P, progesterone. ^aLM4a was purified from liver microsomes of BNF-treated trout (80). The properties of LM4a were similar to the major isoform purified, LM4b (*CYP1A1*). We do not yet know if LM4a corresponds to the protein encoded by the recently cloned trout *CYP1A2* (84). ^bLMC5 and P450_{con} were purified independently by two different laboratories, both of which have preliminary evidence that this trout P450 has many properties in common with mammalian *CYP3A* (85,94,95). ^cTrout P450s KM1 and KM2 were purified from kidney microsomes. Trout P450 KM1 is probably identical to *CYP2K1*.

of which are less mutagenic and carcinogenic than AFB₁ and therefore represent detoxication reactions. These monohydroxylated metabolites are substrates for conjugation by uridine diphosphate-(UDP) glucuronosyltransferase and can be eliminated in the bile. CYP epoxygenation at the 8,9-position results in production of the electrophilic ultimate carcinogen AFB₁-8,9-epoxide. The major human CYPs involved in the bioactivation of AFB₁ to AFB₁-8,9-epoxide are 1A2 and 3A4 (98–101). The relative contribution of these two isoenzymes toward bioactivation of AFB₁ probably varies markedly between individuals due to large interindividual variations caused by genetic and environmental factors (102–104), which along with interindividual differences in DNA repair rates may account for some of the variation between humans with respect to susceptibility to some cancers (105). In trout, the majority of AFB₁ 8,9-epoxygenation is catalyzed by CYP2K1 (89). The covalent adduct produced is the same as in mammals, 8,9-dihydro-8-(*N*⁷-guanyl)-9-hydroxyaflatoxin B₁ (AFB₁-*N*⁷-GUA) (106,107).

As is the case in mammals, trout CYP1A is active in the hydroxylation at the 10 position to produce AFM₁ (108). The induction of CYP1A by compounds such as β-naphthoflavone (BNF), indole-3-carbinol (I3C), and polychlorinated biphenyls (PCBs) was thought to be the mechanism by which these modulators acted as chemopreventors of AFB₁-initiated hepatocarcinogenesis in trout (109,110).

However, recently our laboratory has determined that inhibition of CYP activity may be a more important mechanism of action than CYP1A induction, especially with I3C (111–114). In studies examining the time course and dose response of dietary I3C, trout responded only weakly and transiently to this compound as an CYP1A inducer, and the degree of reduction in covalent binding of AFB₁ to DNA *in vivo* did not correlate to CYP1A induction (111,112). Similarly, BNF was found to significantly reduce AFB₁ covalent binding to DNA at dietary doses too low for CYP1A induction (114). In the course of these studies, we found that both BNF and various acid condensation products of I3C were potent inhibitors of a number of trout and mammalian CYPs, with inhibition contents well below the levels known to be attained in liver after administration of anticarcinogenic doses (112–115).

In addition to possessing a CYP (2K1) with high activity toward bioactivation of AFB₁, the remarkable sensitivity of trout toward AFB₁-initiated carcinogenesis can be explained by their lack of a constitutive or inducible glutathione *S*-transferase (GST) with appreciable activity toward AFB₁-8,9-epoxide (116). The high activity displayed by constitutive mouse Yc GST, compared to rat, is thought to be the major factor for the remarkable resistance of mice (98,117–121). The importance of this enhanced phase II reaction is confirmed by the observation that mice are actually more prolific at production of AFB₁-8,9-epoxide

than rats. Administration of I3C to rats induces a form of GST (Yc2) with high activity toward conjugation of the exo-AFB₁-8,9-epoxide and presumably contributes to chemoprevention in this animal model (122). Trout, however, appear refractory toward induction of this type of GST (116).

Polycyclic Aromatic Hydrocarbons.

Environmental exposures to polycyclic aromatic hydrocarbons (PAHs) (possibly in conjunction with PCBs, dioxins, and dibenzofurans) are thought to be related to epizootic outbreaks of liver neoplasia in feral fish from various regions of the country (123–127). Benzo[*a*]pyrene (B[*a*]P) is hepatocarcinogenic in rainbow trout, but long-term exposures through the diet or intraperitoneally are required (58). The racemic (±)-*trans*-B[*a*]P-7,8-dihydrodiol is a much more potent carcinogen in trout (59). As is the case in mammalian models, the (-) enantiomer is roughly an order of magnitude more potent than the (+) enantiomer (59). Reconstitution studies with purified enzyme and liver microsomes from BNF-treated trout indicate that CYP1A is the predominant subfamily involved in B[*a*]P and B[*a*]P-7,8-dihydrodiol bioactivation to the ultimate carcinogen 7*S-trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-diol-*anti*-9,10-epoxide (59,80,128).

7,12-Dimethylbenz[*a*]anthracene (DMBA) is a much more potent hepatocarcinogen in trout than B[*a*]P and produces tumors in kidney, swim bladder, and stomach as well (Table 2) (60). Trout embryos metabolize DMBA to 12-hydroxymethyl-7-methylbenz[*a*]anthracene (12-HMBA) and 3,4-dihydroxy-3,4-dihydro-DMBA (DMBA-3,4-diol) (60). In addition to these metabolites, juvenile and adult trout metabolize DMBA to the 8,9-dihydrodiol, 7-HMBA, and 2- and 3-hydroxy-DMBA (DR Buhler et al., unpublished data). DMBA is metabolized by both constitutive and induced CYPs (Table 3). Pretreatment of trout with inducers of CYP1A markedly enhances the metabolism, covalent binding, and carcinogenic potency of DMBA, suggesting that this subfamily is very efficient at bioactivation of this PAH (Hendricks et al., unpublished observations). The phenolic and dihydrodiol metabolites of DMBA are substrates for phase II conjugation reactions by UDP-glucuronosyltransferases and perhaps sulfotransferases (129). The ultimate carcinogenic metabolite of DMBA has previously been identified as the bay region DMBA-3,4-diol-1,2-epoxide (130), although recent evidence

suggests the possible contribution of other metabolites (131–133). The DMBA–DNA adducts produced and the resulting mutational spectrum may be different in trout than in mammals. A high percentage of liver tumors in trout treated with DMBA carried activated *Ki-ras*, mostly G→A transitions and G→T transversions in the first and second G, respectively, of the 12th codon, as opposed to the major mutation seen in mouse hepatic tumors (G→C transversion in the first G of codon 13) (60).

We are currently investigating the potential for dibenzo(*a,l*)pyrene (DBP) as a model PAH carcinogen in trout. The advantage of DBP replacing DMBA in studies on PAH carcinogenesis lies mainly in the fact that DBA is a potent environmental contaminant whereas DMBA is not (134). Preliminary evidence indicates that DBP resembles DMBA with respect to trout target tissues but is a more potent carcinogen, especially for liver and swim bladder (GS Bailey et al., unpublished observations).

***N*-Nitrosodiethylamine (DEN).** DEN is a potent hepatocarcinogen in trout (53,63,135,136). The major adducts produced are 7-ethylguanine and *O*⁶-ethylguanine (63,136), indicating that, as in mammals, metabolic activation occurs through *N*-deethylation. In rats, mice and humans, the major CYP catalyzing *O*-deethylation of DEN is CYP2E1, with some contribution from CYP2A6 (137–139). Little information (140) is available on which trout CYPs may be responsible for *N*-dealkylation of DEN or other dialkylnitrosamines. To our knowledge, no ortholog of CYP2E1 has been identified in any fish. Pretreatment of trout with BNF enhances the hepatocarcinogenesis of DEN, suggesting a role for CYP1A (63).

DNA Adduction and Repair

As discussed above, the major initial covalent AFB₁–DNA adduct produced is the same in trout and mammals, *trans*-8,9-dihydro-8-(*N*⁷-guanyl)-9-hydroxyafatoxin B₁, which is spontaneously converted to the more persistent ring-opened form-aminopyrimidine. A dose-dependent linear increase in liver AFB₁-covalent adduction is observed upon feeding trout dietary carcinogenic doses of AFB₁ over a period of 2 to 4 weeks (141). A similar dose-dependent, steady-state linear increase in AFB₁–DNA adduction has been observed with chronic dosing in the rat model (142). The doses used in the trout study (141) were relevant to human AFB₁ consumption and, importantly, provided no

evidence of a threshold dose below which AFB₁ was not genotoxic. The rate of repair of AFB₁–DNA adducts in trout is much slower than in mammals. The pseudo half-life for loss of the initial adduct is 7.5 hr in rats. In contrast, the pseudo half-life for AFB₁–DNA adducts in trout is on the order of 21 days (107). (Note that neither of these values is a true half-life since loss is due to chemical conversion, depurination, and enzymatic removal and is not a first-order process.) The remarkable sensitivity of rainbow trout to AFB₁ hepatocarcinogenesis may be due in large part to this reduced ability to repair bulky DNA adducts. Linear regression analysis of the relative tumor risk versus steady state AFB₁–DNA adducts yields the identical line for both rat and trout (143), indicating that those adducts that form and persist lead to tumors with equivalent efficiency in the two species (51,144–146). These results strengthen the reliance on the molecular dosimetry concept for risk assessment for AFB₁ exposure to humans (147,148).

Relative to AFB₁, little information is available on the identity, kinetics of formation and repair, and relationship to final tumor response for PAHs in trout. Induction of trout CYP1A enhances the covalent adduction of either B[*a*]P or B[*a*]P-diol to liver DNA (128,149). Consistent with tumor data, trout are relatively resistant to the formation of appreciable levels of DNA adduction following exposure to B[*a*]P (150). ³²P-Postlabeling analysis indicates that the major adduct in trout liver DNA following administration of (+) 7*S*-*trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-diol is (+)-*syn*-7*S*-*trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-diol-9,10 epoxide-dG (128). Bath exposure of trout embryos or embryo-derived cells with DMBA produces a concentration-dependent increase in DNA adduction, but the adducts have yet to be identified. Interestingly, a high percentage of liver tumors from trout treated with DMBA as embryos carry activated *Ki-ras* with alleles distinctly different from DMBA-induced liver tumors in mice (60,151). Thus in species as different as trout and mice, biochemical differences in procarcinogen metabolism and adduction can exist, which nonetheless lead to similar oncogenic pathways involving prevalent *ras* activation.

Treatment of trout with DEN produces dose-dependent increases in DNA adduction, primarily 7-ethylguanine and *O*⁶-ethylguanine (63,136). The formation of the latter adduct correlates with tumor incidence and is consistent with the dominance

of a G→A transition mutation in *Ki-ras* isolated from these tumors (53,63). This mutagenic specificity probably derives from ineffective alkyltransferase removal of *O*⁶-ethylguanine in these animals (53,63). Based on total tumorigenic dosage required, however, Shasta trout and F344 rats show comparable sensitivity to hepatocarcinogenesis by DEN (53).

Protooncogene Activation in Trout Tumors

An understanding of the molecular basis for cancer initiation, promotion, and progression is necessary to more readily relate cancer studies in fish to those in mammals including humans. Mutational inactivation of the p53 tumor suppressor gene (152) and activation of the *ras* protooncogene (153) represent two of the most frequently observed and thoroughly studied molecular events in human cancer. Initial studies in our laboratory have not detected p53 codon 248 or 249 mutations as common events in AFB₁-initiated hepatic tumors in trout (GS Bailey, unpublished results); however, we have yet to determine if mutations may occur at other p53 sites or with other carcinogens or protocols, including chronic treatment that may more closely resemble human AFB₁ exposure. In this regard, mutations in p53 have been observed relatively infrequently in rats and mice. By comparison, we have provided partial sequences for several *ras* genes in rainbow trout (154) and have shown that mutational activation of an expressed *Ki-ras* gene is a frequent occurrence in hepatic tumors initiated by the prototypical mycotoxin AFB₁ (155), the polycyclic aromatic hydrocarbon DMBA (60), and the *N*-nitroso compound DEN (53). Table 4 summarizes current knowledge regarding *Ki-ras* mutational activation among the various tumor types elicited by these and additional carcinogens in trout. Overall, the data demonstrate that *Ki-ras* mutagenic activation can be a frequent event in the initiation of liver, stomach, and swim bladder neoplasms in the trout model. We have yet to establish full trout sequences for *N*- and *Ha-ras* homologues and to determine if mutations may also occur in these genes.

The *Ki-ras* mutational data have been accumulated by allele-specific hybridization, 3'-primer mismatch polymerase chain reaction analysis (MMA) and direct sequencing of polymerase chain reaction (PCR) products. Though sequencing provides a more direct identification of any mutant that may exist within the PCR

Table 4. Ki-*ras* codon 12 (GGA), 13 (GGT), and 61 (CAG) oncogenic mutations in tumors elicited by various carcinogens in the rainbow trout.

Carcinogen	Tumor	Mutation [frequency]	Overall incidence	Reference
DMBA ^a	Liver ^e	12(1)G→A[2/9], 12(1)G→C[1/9], 13(1)G→C[1/9]	44%	Unpublished
	Stomach ^{e,n}	12(1)G→C[1/9], 13(1)G→C[4/9], 61(2)A→T[4/9]	100%	Unpublished
	Swim bladder ^e	12(1)G→C[1/9], 13(1)G→C[3/9], 61(2)A→T[2/9]	67%	Unpublished
DMBA ^b	Liver ^e	12(1)G→A[5/27], 12(2)G→T[11/27]	59%	Unpublished
	Stomach ^e	12(1)G→A[1/9]	11%	Unpublished
DMBA ^c	Liver ^f	12(1)G→A[4/11], 12(2)G→T[4/11], 61(2)A→T[1/11]	82%	(60)
DMBA ^b	Liver ^g	12(1)G→A[6/16], 12(2)G→T[1/16]	43%	Unpublished
	Stomach ^g	No mutation [0/16]	0%	Unpublished
DBP ^b	Liver ^{n,o}	12(1)G→A[10/24], 12(2)G→T[9/24]	88%	Unpublished
		12(1)G→T and 12(2)G→T[2/24]		
		12(1)G→A and 12(2)G→T[1/24]		
		12(1)G→A[1/16], 13(1)G→C[2/16]		
AFB ₁ ^d	Liver ^f	12(1)G→A[1/14], 12(2)G→T[7/14], 13(2)G→T[2/14]	71%	Unpublished
		12(1)G→A[2/32], 12(2)G→T[22/32], 13(2)G→T[3/32]	84%	Unpublished
AFB ₁ ^e	Liver ^k	12(1)G→A[1/29], 12(2)G→T[17/29], 13(2)G→T[7/29]	86%	Unpublished
DHEA ^a	Liver ^f	12(1)G→A[8/25]	32%	Unpublished
DEN ^a	Liver ^m	12(1)G→A[6/7]	86%	(53)
MNNG ^a	Liver	12(1)G→A[3/30], 12(2)G→A[25/30]	93%	Unpublished
Control ^a	Spontaneous	No mutation [0/11]	0%	Unpublished

^aDetected by mismatch PCR and some confirmed by direct sequencing. ^bDetected by direct sequencing only. ^cDetected by mismatch PCR and confirmed by cloning and sequencing. ^dDetected by allele hybridization and confirmed by cloning and sequencing. ^eDMBA fry bath exposure/Aroclor 1254 promoter. ^fDMBA embryo bath exposure. ^gDMBA dietary. ^hDBP dietary. ⁱAFB₁ dietary. ^jAFB₁ fry bath exposure. ^kAFB₁ fry bath exposure/DHEA promoter. ^lDHEA promoter only. ^mDEN bath exposure. ⁿOne double mutation 12(1)G→C and 13(1)G→C. ^oTwo double mutations at 12(1)G→T and 12(2)G→T.

product, it has one disadvantage: it fails to detect mutants carried by less than 10 to 20% of the cells in the tumor isolate. Thus, data generated by this method provide only a minimal estimate of the percent of trout tumors bearing Ki-*ras* mutations. For example, MMA detected mutant Ki-*ras* alleles in 100% of stomach tumors elicited by DMBA fry bath exposure, whereas direct sequencing detected only 11% incidence (Table 4). As seen in the table, each carcinogen appears to generate a specific spectrum of Ki-*ras* mutant alleles. AFB₁-initiated liver tumors contain primarily Ki-*ras* codon 12 GGA→GTA and codon 13 GGT→GTT transversions, whereas MNNG elicits entirely GGA→GAA and GGA→AGA transitions. These are compatible with the well-known mutagenic properties of the major DNA adducts elicited by these carcinogens in bacterial systems. Spontaneous liver tumors occur only rarely in trout and few have been available for analysis; of the limited number examined to date (Table 4), we have been unable to detect mutant Ki-*ras* alleles by MMA. These data taken together provide evidence that the mutant alleles that we have observed in carcinogen-treated fish occur as a direct result of carcinogen-DNA adduction in the *ras* gene *in vivo* rather than from amplification of background mutational events in this model.

Most of the data we have generated to date involve liver tumors. The hepatocarcinogens listed here all induce primarily

mixed cholangiocellular/hepatocellular carcinomas, with relatively few pure hepatocellular carcinomas induced. AFB₁, MNNG, DEN, and DBP induce hepatic tumors with a high incidence (71–100%) of activated Ki-*ras* alleles. An interesting exception is dehydroepiandrosterone (DHEA), an endogenous steroid that is also hepatocarcinogenic in the rat but not previously known to be genotoxic. The mutant *ras* incidence of 32% (8/25) is low, but the 12(1)G→A mutation observed is not compatible with indirect damage such as 8-hydroxydeoxyguanosine. The precise origin of the observed *ras* mutations remains to be established. For DMBA, the incidence of Ki-*ras* mutations in liver tumors varied from 44% (7/16) to 100% (9/9) among the various experiments. The number of mutant alleles observed is too small at present to know if the DMBA mutational spectrum is protocol dependent. Among the liver tumors examined, codon 61 A→T transversions have been rarely detected, with codon 12 and 13 guanine-based mutations (G→A, G→T, G→C) more frequently observed. Of 24 liver tumors elicited by the environmental PAH DBP, comparable numbers of codon 12 G→A and G→T mutations were observed and three tumors showed evidence of double Ki-*ras* mutations. Experiments are in progress to establish if these mutations reside on the same or separate *ras* sequences and to establish the specific DBP-DNA adducts that give rise to *ras* mutations in the trout model.

Hepatic tumors from AFB₁-treated trout (Table 4) and rats (156) show frequent *ras* mutation, yet this has not been reported to occur in hepatocellular carcinoma from AFB₁-exposed humans. Mutant Ha-*ras* alleles have, however, been reported in human cholangiocellular carcinoma (157). We are attempting to establish if *ras* mutation in the trout may also be restricted to neoplasms having cholangiocellular involvement. An alternative hypothesis is that the trout and rat laboratory models do not completely mimic AFB₁-related human hepatocarcinogenesis, which may frequently involve tumor progression under the combined influence of chronic carcinogen intake and hepatitis infection.

Tumor Promotion and Inhibition in Trout

Use of the trout tumor model to study modulation of carcinogenesis has been reviewed elsewhere (158–160). The historically low spontaneous liver tumor incidence (0.1%) is a significant advantage in the statistical design of multistage tumor inhibition and promotion experiments. In this overview, we will summarize some past results and also present some recent unpublished findings.

The majority of studies on anticarcinogenesis has focused on inhibition of AFB₁-initiation of hepatocarcinogenesis (Table 5), but we are currently investigating multiple target tissues and combinations of

Table 5. Inhibition of cancer in the trout model.

Carcinogen/exposure	Inhibitor/exposure	Incidence	Mechanism	Reference
AFB ₁ /20 ppb diet (days 57–66)	BNF/500 ppm diet (days 1–114)	↓85%	↑CYP1A	(109)
	I3C/1,000 ppm diet (days 1–114)	↓90%	CYPZK1 inhibition CYPZK1 inhibition	
AFB ₁ /10 ppb diet (weeks 4–8)	BNF/500 ppm diet (weeks 0–8)	↓93%	CYPZK1 inhibition	(108)
AFB ₁ /1–8 ppb diet (12 months)	Aroclor 1254/50 ppm	↓10–45%	↑CYP1A	(161)
AFB ₁ /10–320 ppb diet (weeks 4–6)	I3C/0–4,000 ppm diet (weeks 0–6)	↓ ^a	↑CYP1A	(35)
AFB ₁ /1 ng embryo (microinjection)	I3C/5 µg (microinjection)	↓17%	↑CYP1A	(46)
	I33'/8.6 µg	↓72%		
	RXM/13.5 µg	↓43		
AFB ₁ /10–160 ppb diet (weeks 0–2)	Chlorophyllin/0–4,000 ppm diet (weeks 0–2 with AFB ₁)	↓ ^b	Tight complex	(162)
DEN/250 ppm bath (24 hr-fry)	I3C/2,000 ppm diet (6 weeks prior to DEN)	↓66%	Inhibition of CYP-dependent N-demethylation?	(63)
DMBA/0.5–4 ppm bath (6 hr-fry)	I3C/0–2,000 ppm diet (1 week prior to DMBA)	↓ ^c	CYP inhibition?	Unpublished

Abbreviations: AFB₁, aflatoxin B1; BNF, β-naphthoflavone; CYP, cytochrome P450; I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; RXM, I3C reaction mixture formed *in vitro* upon acid treatment; DEN, N-nitrosodiethylamine; DMBA, 7,12-dimethylbenz[a]anthracene. ^aThis 9,600-animal experiment used five different doses of I3C, each of which also had four different AFB₁ doses. Linear increases in DNA adduct formation with AFB₁ dosage were observed at each level of I3C. Plots of tumor response (as % logit) versus the log AFB₁ dose resulted in a series of parallel lines with increasing I3C resulting in displacement to the right (greater AFB₁ dose required to reach TD₅₀ (dose at 50% incidence). The IC₅₀ (median-inhibiting concentration) was estimated at 1,400 ppm I3C. ^bA 9500-animal experiment similar to the one above was performed with four different dietary levels of chlorophyllin at six different doses of AFB₁. Again, plots of % logit tumor response versus log AFB₁ dose gave parallel lines increasingly displaced to the right with increasing doses of chlorophyllin. A 70 to 80% protection was achieved at dietary levels of 1,500 ppm chlorophyllin. ^cThis was a recently completed 9,600-animal experiment with multiple doses of both I3C and DMBA. I3C was able to completely block tumors in the swim bladder and inhibited tumor formation in the liver and stomach by 60 to 75%.

inhibitors. For example, we recently conducted a preliminary experiment examining the chemoprevention potential of two anticarcinogenic chemicals that have different mechanisms of action. Dietary I3C at 1,500 ppm reduced AFB₁-initiated hepatocarcinogenesis from 31% to 16%, 1,500 ppm chlorophyllin gave 24% incidence (not significant), and the combination of I3C plus chlorophyllin was synergistic (4.5% incidence).

In another recent preliminary collaborative study with Gary Stoner of Ohio State University, postinitiation feeding with ellagic acid suppressed DMBA-dependent stomach carcinogenesis, which represents the first example of postinitiation suppression by any agent in the trout model. By comparison, postinitiation chlorophyllin was without effect. Compounds that have proven negative to date as anticarcinogens in the trout model include BHA, BHT, D-limonene, Oltipraz, menthol, green tea extract, vitamin E, freeze-dried onion or garlic, and mint oil.

A number of environmental agents have been demonstrated to function as postinitiation tumor promoters or enhancers in the trout model (Table 6). Compounds proven ineffective at promotion to date include transition metals (with or without H₂O₂ as prooxidant) and the peroxisome proliferators clofibrate and WY14,643. In addition, some promoters appear to have initiator or tissue specificity. For example, postinitiation dietary Aroclor 1254 promotes hepatocarcinogenesis with DMBA as initiator but not with AFB₁ (160). Postinitiation I3C and BNF enhance carcinogenesis initiated by AFB₁, DMBA, or MNNG in liver but not in other target organs such as kidney and, in fact, they usually reduce the incidence in stomach. We have also tested a number of compounds including vitamin E, green tea extract, nicotinic acid, D-limonene, chlorophyllin, and menthol as potential antipromoters with no success except for one compound, ellagic acid, which was discussed earlier.

Very little is known about the mechanism of action of tumor promoters. In trout, prooxidants such as peroxides, CCl₄, and choline deficiency are effective promoters (Table 6), yet to date, co-treatment with antioxidants has not provided any protection. Our laboratory is currently studying the mechanism of I3C dietary modulation of cancer using trout and murine models. If given before and during initiator exposure, I3C functions in trout as an anticarcinogen, but chronic postinitiation exposure enhances tumorigenesis. These opposing actions have similar potencies (EC₅₀ [median effective concentration] = 1,000–1,500 ppm) in trout (42). The mechanism of I3C inhibition in trout appears to be largely due to inhibition of CYP bioactivation by I3C acid condensation products rather than aryl hydrocarbon (Ah) receptor-dependent induction of CYP1A (111–113).

We are currently investigating the role of the Ah receptor in tumor promotion. Previous studies have documented that a number of I3C acid condensation products have high affinity for the mammalian Ah receptor (167). Initial attempts to block trout Ah receptor-dependent promotion with α-naphthoflavone (ANF) were unsuccessful. In fact, ANF alone in trout was a promoter of hepatocarcinogenesis, and the combination of ANF and BNF was additive (Table 6). Further work has documented that ANF is an Ah receptor agonist in trout. We had hoped to use congenic mice to directly address the role of the Ah receptor in I3C promotion; however, preliminary studies indicated that I3C fails to promote hepatocarcinogenesis in mice (DEN) as it does in rats (AFB₁) (DE Williams, unpublished observations).

Our laboratory has recently found DHEA to be a potent promoter in the trout model (Table 6), and in fact it is a complete carcinogen (165). DHEA has received much attention recently with respect to its chemopreventive properties in humans and animal models with respect to a number of diseases including atherosclerosis, diabetes, obesity, lupus, trauma injury, AIDS, and in aging (168). Currently, a number of clinical trials are being conducted with DHEA, an agent that is also being marketed in health food stores. DHEA is hepatocarcinogenic in rats under protocols requiring high doses (≥4,500 ppm) over long exposure periods (≥1 year). The carcinogenicity of DHEA in rodents is thought to be caused by its actions as a peroxisome proliferator (169).

Humans are relatively insensitive to peroxisome proliferators; therefore, the rodent findings are thought not applicable to human risk assessment (170). Our findings with the trout model are the first to document the carcinogenicity of DHEA in the absence of peroxisome proliferation. Significant promotion in trout is observed in as little as 8 weeks of feeding (5 days per week) at levels that approximate doses previously used in some human clinical trials. The mechanism of DHEA promotion in trout may be hormonal. DHEA is a precursor for both estrogens (already known to promote hepatocarcinogenesis in trout) and androgens, and the plasma levels of both steroids increase markedly in trout fed DHEA. The estrogenic potency of DHEA in trout can be observed by following vitellogenin levels in plasma. The DHEA analog (171) developed by Arthur Schwartz (Temple University), 16 α -fluoro-5-androsten-17-one (8354), was much weaker as a precursor for androgens and estrogens in trout, was not a complete carcinogen, and did not significantly promote AFB₁ hepatocarcinogenesis at a dietary level (444 ppm) for which DHEA was very effective (165). The 8354 analogue is currently undergoing human clinical trials. Based on the findings in rodents and trout, it may be prudent to proceed cautiously with high DHEA supplementation over prolonged periods and to continue developing safer analogues.

Strengths and Limitations of Environmental Carcinogenesis Research in the Trout Model

There should be no expectation that trout will supplant traditional rodent models in carcinogen bioassays or as surrogates for human cancer research. A most evident limitation common to all lower vertebrates is the lack of complete organ homology needed to study cancers of the lung, colon, breast, and prostate, the leading cancers in the United States. While carcinogens that initiate these tumors in rodents can be carcinogenic in trout, organospecificity is lost and liver is the most common target organ. An additional limitation is that trout have late sexual maturity (2–3 years) and a long life span during which the animal continues somatic growth. These limit the potential for genetic studies and preclude lifetime bioassay protocols in carcinogen testing. Under the latter limitation, a negative carcinogen bioassay result would not be considered definitive. (This species

Table 6. Tumor enhancers and promoters in trout.

Carcinogen/Exposure	Enhancer/Exposure	Incidence	Mechanism	Reference
AFB ₁ /20 ppb diet (weeks 1–4)	BNF/500 ppm diet (weeks 5–16)	↑ 3-fold	Ah receptor?	(163)
	I3C/2,000 ppm diet (weeks 5–16)	↑ 6-fold	Ah receptor?	(163)
AFB ₁ /fry bath (multiple doses)	I3C/0, 750, 1,500, and 2,000 ppm diet (various durations)	↑ ^a	Ah receptor?	(42)
AFB ₁ /120 ppb (fry bath)	Temperature, 11°	4%	Not growth related	(43)
	14.5°	35%		
	18°	61%		
AFB ₁ /5–125 ppb (embryo bath)	β-estradiol/5–15 ppm (alternate weeks)	↑ 5–8-fold ^b	↑ Proliferation ODC	(71)
AFB ₁ /10 ppb (fry bath)	H ₂ O ₂ /1,000, 3,000 ppm	↑ 1.6-fold	Oxidative stress?	(164)
	BeP/500, 1,500 ppm	↑ 1.5-fold, 1.6-fold		
	<i>t</i> -BuOOH/500, 1,500 ppm diet (10 months)	↑ 1.6-fold, 1.9-fold		
	Choline-deficient diets	↑ Multiplicity		
	CCl ₄ /500 ppm diet	↑ 1.5-fold		
AFB ₁ /10 ppb (fry bath)	PFOA/1,800 ppm diet	↑ 2-fold	?	(165)
	DHEA/1,800 ppm diet (6 months)	↑ (to 100%)		
		↑ Multiplicity		
AFB ₁ /50 ppb (embryo bath)	H ₂ O ₂			Unpublished
	β-estradiol			
AFB ₁ /10 ppb (fry bath)	β-estradiol + H ₂ O ₂			(165)
	DHEA/0–888 ppm diet (30 weeks)	↑ Dose dependent significant ≥ 222 ppm	^c	
		↑ Multiplicity ≥ 222 ppm		
MNNG/25 ppm (embryo bath)	H ₂ O ₂ /0, 600, and 3,000 ppm diet (8 months)	↑ 1.6-fold, 3-fold (liver)	^e	(166)
MNNG/50 ppm (embryo bath)	H ₂ O ₂ /0, 1,800, and 2,500 ppm diet (8 months)	↑ Liver ↑ Stomach and kidney		Unpublished
MNNG/35 ppm (fry bath)	DHEA/0, 55, 111, ^f 222, 444, and 888 ppm diet (6 months)	↑ Liver and kidney	^c	(165)
		↓ Stomach and swim bladder		
DMBA/0.5–4 ppm (fry bath)	I3C/800 ppm diet	↑ Liver and swim bladder → Stomach		Unpublished
DMBA/1.5 ppm (fry bath)	BNF/200 ppm diet ANF/200 ppm diet ANF+BNF/200 ppm each	↑ Liver 3.4-fold		Unpublished
		↑ Liver 2.3-fold		
		↑ Liver 4.7-fold ↓ Stomach (all groups)		

Abbreviations: AFB₁, aflatoxin B₁; BNF, β-naphthoflavone; I3C, indole-3-carbinol; BeP, benzoyl peroxide; *t*-BuOOH, *t*-butyl hydroperoxide; PFOA, perfluorooctanoic acid; DHEA, dehydroepiandrosterone; MNNG, *N*-methyl-*N'*-nitrosoguanidine; DMBA, 7,12-dimethylbenz[*a*]anthracene. ^aThis study examined the effect of timing, duration and dose on I3C-enhancing potency. A linear dose–response relationship between tumor incidence and length of exposure to 2000 ppm dietary I3C was observed. Promotional potency was still effective if feeding was delayed post-initiation and if fed alternate months, weeks, or days. A tumor study employing three different doses of dietary I3C and three different doses of AFB₁ demonstrated a linear relationship between % logit tumor incidence and log AFB₁ with increasing doses of I3C displacing the line to the left. The calculated P₅₀ (dose producing 50% promotion) for I3C promotion of AFB₁ hepatocarcinogenesis was 1000 ppm. We are currently repeating this study with more AFB₁ and I3C doses to determine if a threshold exists for I3C promotion. ^bβ-Estradiol also enhanced tumor multiplicity and tumor size. ^cDietary DHEA produced marked hypertrophy and cell proliferation in trout. In addition, we have evidence that DHEA may disrupt control of the cell cycle as evidenced by alterations of p53 and PCNA. DHEA also dramatically enhances blood levels of vitellogenin in these sexually immature fish. ^dTrout were fed diets containing 1800 or 2500 ppm H₂O₂ alone, 5 or 10 ppm β-estradiol alone, or combinations of these two doses. Both compounds promoted AFB₁ hepatocarcinogenesis. The combination diets were less than additive. ^eH₂O₂ also enhanced tumor multiplicity. The degree of enhancement of tumor incidence significantly correlated to oxidative damage to DNA, as evidenced by liver DNA levels of 8-hydroxy-2'-deoxyguanosine. ^fIn liver, DHEA produced a dose-dependent enhancement of tumor incidence from 1% at 0 ppm to 99% at 888 ppm. Tumor multiplicity and size were also significantly increased in a dose-dependent manner. Kidney tumors were also significantly enhanced at 111 and 888 ppm DHEA. In contrast, DHEA significantly inhibited MNNG-initiated tumors of the stomach and swim bladder.

instead assesses cancer risk through the embryonic and juvenile lifestages, which are not unimportant. Moreover, any chemical that is positive in a trout carcinogen bioassay should reasonably be considered a potential human carcinogen, barring mechanism information to the contrary.) A final limitation of the trout model is the continuing lack of knowledge in this species of fundamentals in the genetics and cell biology of cancer; owing to the relatively few investigators involved in its development, this is likely to remain a chronic problem.

Even with these limitations, there are many attributes of trout and other fish models that afford unique approaches in the study of cancer. Where mechanistically reasonable; the use of trout and other fish models can substantially reduce our dependence on small mammals for health research. Cost is a considerable advantage, especially for investigating statistically challenging issues that can be addressed only with large numbers of animals. Our molecular dosimetry studies using 8,000 to 10,000 animals to quantify the interrelationships among carcinogen dose, anticarcinogen dose, DNA adduct formation, and final tumor outcome exemplify this (35,162). Similarly, the low husbandry costs for fish permit tumor studies designed to define the shape of cancer dose-response curves down to 0.1% incidence. The design of any such experiment requires at least 31,000 animals to provide adequate statistical power, given the expectation of only 10 tumors among 10,000 animals at the targeted lowest dose. One such experiment is in progress testing DEN in the medaka model. DBP and DEN will be similarly tested in the next 2 years in the trout model, together with biochemical studies of metabolism, DNA adduction and repair, *Ki-ras* activation, target organ toxicity, and proliferation that aim to define mechanisms accompanying any departure from linearity. An advantage of trout inherent in this study is its historically zero background tumor rate in two of the target organs, which assures that all observed

tumors in these organs can be ascribed to carcinogen treatment. In the third organ, liver, the historic background tumor rate of 0.1% will become important at the lowest carcinogen dose only. Even here, given the high incidence of activated *Ki-ras* in carcinogen-initiated tumors only, it may be possible to separate almost all carcinogen-related and spontaneous tumors. The entire tumor study, including in-house pathology as well as the proposed mechanism studies, is to be carried out with trout for a total budget that is only 5 to 10% of the per-diem costs alone for a comparable 40-week single-sex, single species rat or mouse experiment. Given current budgetary restrictions, it seems unlikely that the 24,000-animal megamouse study of 2-acetylaminofluorene dose-response will be extended to additional carcinogens; the more affordable fish models can help in addressing at least some important mechanistic questions in dose-response.

Sensitivity of fish models can be an important attribute. Trout embryos, which are readily available in the thousands at any specified stage of development, provide a highly sensitive early life stage model enabling nanogram to microgram bioassay of scarce materials (e.g., high-performance liquid chromatography [HPLC] fractions). For example, we have been able to bioassay scarce HPLC-purified metabolites of the phytochemical indole-3-carbinol to identify the anticarcinogenic intermediates by co-injection with AFB₁ (46), and we have generated entire tumor dose-response curves with 2,000 individuals, requiring less than 100 µg total of AFM₁ and related aflatoxins (GS Bailey, unpublished data). We have determined that a measured dose of 0.5 ng AFB₁ per trout embryo will induce 26% incidence of hepatic tumors 9 months after embryo injection; this is nine orders of magnitude lower than the dose of AFB₁ required to elicit a similar response in monkeys. No other established cancer model offers this sensitivity.

Finally, the nonmammalian status of trout also has its inherent advantages in

that a comparative approach in cancer research is no less useful than in other fields of biology. Thus the establishment of *ras* activation as a common oncogenic pathway in trout strongly supports a commonality in molecular mechanisms of cancer in lower and higher vertebrates. The fact that trout are more humanlike than rats in their resistance to the phenomenon of hepatic peroxisome proliferation makes them an attractive species in which to establish if peroxisome proliferators might pose carcinogenic risk by mechanisms other than peroxisome proliferation itself—as discussed above, we now know that some of these compounds are complete carcinogens and are possible genotoxins in trout. AFB₁ hepatocarcinogenesis can be blocked in rats by co-treating animals with the antioxidant ethoxyquin (172) or the antischistosomal drug Oltipraz (173), apparently through induction of GST Yc2 with high specificity for AFB₁-8,9-oxide. The importance of this mechanism to planned human intervention trials in China with Oltipraz is underscored by our determination that neither Oltipraz nor antioxidants induce AFB₁-glutathione detoxication in the trout model, and hence both fail to provide protection against AFB₁ hepatocarcinogenesis [GS Bailey, unpublished results; (108)]. There is notable uncertainty that such an enzyme is inducible in human liver *in vivo*. The demonstration, first in trout (34) and later confirmed in rats (174), that the candidate anticarcinogen indole-3-carbinol can behave as a potent tumor promoter under some exposure protocols has raised as yet unresolved safety concerns over its proposed use in human breast cancer chemoprevention.

In summary, it is evident that there are many experimental situations in which trout and other fish models are inadequately developed or inappropriate to address certain issues in cancer research. It is equally apparent that these species can serve as highly useful adjuncts to the traditional rodent models in the study of human environmental cancer risks and in some situations can provide wholly unique approaches.

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