

AQTOX 00590

3-Trifluoromethyl-4-nitrophenol (TFM) toxicity and hepatic microsomal UDP-glucuronyltransferase activity in larval and adult bullfrogs

Andrew S. Kane^a, Wesley W. Day^b, Renate Reimschuessel^a and Michael M. Lipsky^a

^aUniversity of Maryland School of Medicine, Department of Pathology, Aquatic Pathobiology Center, Baltimore, MD, USA and ^bUniversity of Maryland School of Pharmacy, Department of Pharmacology and Toxicology, Baltimore, MD, USA

(Received 28 January 1993; revision received 3 February 1993; accepted 10 March 1993)

Effects of TFM (3-trifluoromethyl-4-nitrophenol) were observed in bullfrog (*Rana catesbeiana*) larvae and adults in vivo by aqueous and intraperitoneal (i.p.) exposures. UDP-glucuronyltransferase (UDPGT) kinetics were examined in vitro from larval and adult hepatic microsomes. The effect of TFM on bullfrog egg development was also examined.

Bullfrog larvae and adults showed similar sensitivities to TFM by i.p. exposure (LD_{50} s = 11.62 and 15.35 mg/kg, respectively), however, larvae were more sensitive than adults in aqueous exposures (LC_{50} s = 0.95 and 12.99 mg/l, respectively). Bullfrog egg development was arrested at the blastula stage at ≥ 1.0 mg/l TFM. No microscopic lesions were observed in tissues from TFM-exposed larvae.

TFM-UDPGT kinetics in bullfrog larvae and adults differed significantly ($P < 0.05$). V_{max} for TFM-UDPGT in bullfrog larvae and adults was calculated to be 1.05 and 2.45 nmol/min · mg, respectively; the apparent K_m for bullfrog larvae and adults was 38 and 64 μ M, respectively. However, the enzyme V_{max}/K_m ratio between the two life stages was not significantly different. Based on data from this and other studies, greater sensitivity to aqueous TFM in gill-respiring larvae (compared with adults) does not primarily appear to be a function of differences in UDPGT-mediated metabolism, but rather may reflect differences in other processes such as uptake.

Key words: Amphibian; Bullfrog; UDP-glucuronyltransferase; Tadpole; 3-Trifluoromethyl-4-nitrophenol

INTRODUCTION

3-Trifluoromethyl-4-nitrophenol (TFM) is a selective pesticide used to control sea lamprey (*Petromyzon marinus*) in tributaries of the Great Lakes. Since its initial appli-

Correspondence to: Andrew S. Kane, University of Maryland School of Medicine, Department of Pathology, Aquatic Pathology Center, 10 South Pine Street, Baltimore, MD 21201, USA.

cation in 1958, many laboratory and field observations have been made on the effects of TFM on a variety of aquatic species. Several reports cite frog larvae (tadpoles) as being sensitive to TFM at lampricidal concentrations (Chandler and Marking, 1975; Gilderhus and Johnson, 1980; GLFC, 1985). Although the toxicity of TFM in terms of median lethal concentrations (LC_{50} s) has been well documented in fish (Marking and Olsen, 1975; Marking et al., 1975; Bills and Marking, 1976) and, to a lesser degree, in frog larvae (Chandler and Marking, 1975; Kane et al., 1985), there are no data on its toxicity in adult frogs.

Frog larvae, particularly large bullfrog tadpoles, are considered pests in warmwater fish culture ponds (Kane et al., 1992). The potential use of TFM as a selective amphibicide to control frog larvae has been explored in preliminary laboratory and field trials (Kane et al., 1985; Kane and Johnson, 1989).

Metabolism of phenol-like compounds in mammals (Mulder, 1982; Caldwell, 1985) and fish (Chambers and Yarbrough, 1976; James, 1986, 1987; Clarke et al., 1991) takes place primarily through glucuronidation. In the case of TFM, the hydroxyl moiety of the substrate is conjugated with α -D-glucuronic acid to form the corresponding β -D-glucuronide (Fig. 1). This reaction is mediated by UDP-glucuronyltransferase (UDPGT, EC 2.4.1.17). UDPGTs are a family of membrane-bound isoenzymes, located mainly within hepatic endoplasmic reticulum, that catalyze the transfer of UDPGA to a variety of xenobiotics and endogenous molecules containing hydroxyl, carboxyl, amino or sulfhydryl groups (Dutton, 1980).

Objectives of this study were to: (i) characterize morphologic alterations caused by aqueous TFM exposure in tadpoles, (ii) observe the effect of TFM on bullfrog egg development, (iii) examine the toxicity of TFM in larval and adult developmental stages of the bullfrog, and (iv) examine hepatic microsomal TFM-UDPGT kinetics in larval and adult bullfrogs.

MATERIALS AND METHODS

Chemicals

Purified (95%) TFM for biochemical studies and intraperitoneal (i.p.) exposures

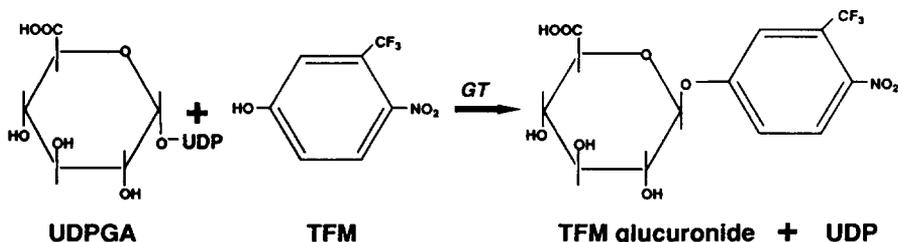


Fig. 1. Conjugation of TFM with UDP-glucuronic acid.

and field-grade (36.1%) TFM for aqueous exposures was supplied by Dr. John Allen, National Fisheries Research Laboratory, La Crosse, WI. All TFM concentrations mentioned in this paper are based on active ingredient. UDPGA (trisodium salt, 98% purity), Triton X-100, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Mixed-sex adult bullfrogs (140–175 g), stage 33–38 (Gosner, 1960) bullfrog tadpoles (20–31 g) and bullfrog egg masses were obtained from a local fish hatchery pond.

Morphology studies

Tadpoles ($n = 10$) were exposed to a lethal concentration of TFM (2.0 mg/l) in a 190-l glass aquarium for up to 4 h. Control animals ($n = 5$) were maintained for 4 h in TFM-free water under similar conditions. Animals were removed from the exposure tank when moribund (no obvious respiration or response to gentle prodding), at which time they were sacrificed and necropsied. Tissues were preserved in 4% phosphate-buffered formaldehyde and embedded in paraffin. Sections (6 μm) were stained with hematoxylin and eosin.

In vivo studies

Egg development

Aqueous exposure of frog eggs in mid-blastula stage (Rugh, 1951; Gosner, 1960) was conducted in 4-l glass beakers. Individual eggs that had been carefully teased out of a single egg mass were exposed in replicate groups of ten at each concentration. Development of eggs in each exposure vessel was compared with that of control animals at the end of 96 h.

LC₅₀ exposures

Adult frogs ($n = 7$) were exposed to TFM, one at a time, in half-filled 15-l polyethylene vessels according to the up-and-down method (Dixon and Massey, 1983). For aqueous exposures, TFM was measured gravimetrically and stock solutions made in distilled, deionized water. Exposure water consisted of filtered, dechlorinated tap water (pH 7.8, hardness 80 mg/l as CaCO₃, 20°C). Exposures began by placing an animal into an exposure vessel. Animals were recorded as dead or alive at the end of 24 h. For i.p. exposures, TFM was dissolved in sterile phosphate-buffered saline (PBS). Control animals received only PBS. After injection, animals ($n = 7$) were maintained in TFM-free water under the same conditions as described above.

Larval frogs were exposed similarly as described above, except that five animals

were exposed at each dose in replicate and exposures were carried out in 38-l glass aquaria. These data were analyzed using probit analysis.

Biochemical studies

Tissue preparation

All animals were euthanized by cervical transection, and livers were carefully removed and dissected free from the gall bladder. Livers were washed in ice-cold PBS, blotted, weighed, frozen in liquid nitrogen and stored at -80°C for no longer than 1 week.

Microsome preparation

Livers were thawed in ice-cold homogenization buffer (0.01 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ plus 0.15 M KCl, pH 7.4), minced with scissors, and rinsed with buffer. Livers from each adult frog represented an individual sample, while tadpole livers (3–4) were pooled for each sample. Tissue was homogenized in 5 vols. of buffer with a motor-driven (300 rpm) glass-Teflon homogenizer. The homogenate was centrifuged ($10,000 \times g$ for 20 min at 4°C) and the resulting supernatant recentrifuged ($105,000 \times g$ for 60 min at 4°C). Microsomal pellets were suspended in 0.1 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4), frozen in liquid nitrogen, and stored at -80°C for no longer than 2 months.

Enzyme assay

UDPGT activity was determined by using a method modified from Burchell and Weatherill (1981) and Lake (1987). Assays were performed in borosilicate glass tubes (12×85 mm) in a final volume of 0.25 ml. The reaction mixture contained 0.125 ml microsomes (0.3–0.4 mg protein), 0.04% Triton X-100, 2 mM UDPGA, 10 mM MgCl_2 and 25–300 μM TFM, suspended in 0.1 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 7.4). Reactions were initiated by the addition of substrate and incubations were performed in a Dubnoff metabolic incubator (130 oscillations/min) at 24°C . The reaction was terminated after 20 min by adding 0.5 ml ice-cold 40% TCA and vortexing. Samples were then centrifuged at $2,000 \times g$ for 15 min at 4°C . 0.5 ml of the resulting supernatant was made alkaline by the addition of 0.5 ml 5 M NaOH and recentrifuged in 1.5-ml microfuge tubes at $10,000 \times g$ for 15 min at 4°C . This second spin at an alkaline pH was necessary in order to remove remaining turbidity. Absorbance of supernatant was measured at 395 nm to assess reduction in color due to formation of TFM-glucuronide. UDPGA blanks were run concurrently and indicated minimal non-UDPGT degradation. Protein was measured by bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin as the standard. Kinetic data (V_{\max} and $K_m \pm$ standard error) were computer-analyzed by Lineweaver-Burke plots; differences between adults and larvae were compared using Student's *t*-test.

RESULTS

Morphology studies

No TFM-related alterations were observed in any of the tissues examined (gill, liver, kidney, muscle, skin, spleen, heart and gut).

Egg development

Frog eggs in mid-blastula stage that were exposed to 0.1, 0.25 and 0.5 mg/l TFM did not appear affected, and their development continued in a manner similar to that of controls. However, growth was arrested at the mid- to late-blastula stage in eggs exposed to higher concentrations (1.0, 2.0 and 3.0 mg/l). Several animals from each exposure concentration were placed in TFM-free water after the 96 h exposure. Animals from lower concentrations continued development through hatching as did the control animals. However, animals exposed to higher concentrations, where egg development was arrested, did not develop further.

In vivo exposures

Median lethal TFM concentrations for larval and adult bullfrogs are shown in Table 1. Larvae were an order of magnitude more sensitive to aqueous TFM than adults. However, larvae and adults showed similar sensitivities to TFM by i.p. exposure.

Biochemical studies

TFM-UDPGT activity in both larval and adult bullfrogs followed Michaelis-Menton kinetics. The calculated V_{\max} for TFM-UDPGT in hepatic microsomes of adult bullfrogs (2.45 ± 0.30 nmol/min · mg) was significantly greater ($P < 0.01$) than in bullfrog larvae (1.05 ± 0.13 nmol/min · mg), while enzyme binding affinity (K_m) for

TABLE 1

Results from aqueous and i.p. TFM exposures with larval and adult bullfrogs (95% confidence intervals are shown in parentheses).

	Larvae	Adults
LC ₅₀ (mg/l)	0.95	12.99
(aqueous)	(0.72–1.14)	(6.75–19.22)
LD ₅₀ (mg/kg)	11.62	15.35
(i.p.)	(10.71–13.14)	(8.72–21.98)

substrate in larvae ($35 \pm 8 \mu\text{M}$) was significantly greater ($P < 0.05$) than in adults ($64 \pm 7 \mu\text{M}$). However, V_{\max}/K_m ($\text{nmol}/\text{min} \cdot \text{mg} \cdot \mu\text{M}$), an indicator of enzyme efficiency, indicated no significant difference between larvae (0.034 ± 0.008) and adults (0.039 ± 0.004). Hepatic microsomal protein concentrations in larvae ($2.80 \pm 0.27 \text{ mg/g liver}$) were significantly less ($P < 0.01$) than what was observed in adults ($4.75 \pm 0.23 \text{ mg/g liver}$).

DISCUSSION

Most vertebrates form glucuronic acid conjugates of xenobiotics. Some animals, however, such as lamprey (Lech and Statham, 1975), cats (Robinson and Williams, 1958; Finco, 1975; Kasper and Henton, 1980), and Gunn rats (Arias, 1959; Hedrich, 1990; Iyanagi, 1991), are incapable of sufficient glucuronide conjugation for detoxification purposes.

Although adult ranid frogs and toads (Maickel et al., 1958; Smith, 1977; Gorge et al., 1987) form glucuronide conjugates of phenolic compounds, some early reports (Maickel et al., 1958; Dutton, 1964; Lester and Schmid, 1961) suggested that frog larvae do not form glucuronide conjugates. More recent studies, however, showed that the bilirubin glucuronide conjugate was detected in both larval (Cole and Little, 1983a) and adult (Cole and Little, 1983b) bullfrogs. The present study demonstrated UDPGT activity toward at least one phenolic substrate (TFM) in larval and adult bullfrogs. Premetamorphic TFM-UDPGT activity in larvae is consistent with neonatal development of phenol-UDPGT in homeotherms (Dutton, 1980; Coughtrie et al., 1988; Burchell and Coughtrie, 1989).

Data from the i.p.-dosed animals indicates that body burdens of approximately 12–15 mg/kg are lethal to both larval and adult life stages. In aqueous exposure however, bullfrog larvae were significantly more sensitive than adults (Table 1). This difference in aqueous TFM toxicity between gill-breathing larvae and (primarily) air-breathing adults is most likely due to differences in route of uptake. This is consistent with previous observations of aquatic animals (Hunn and Allen, 1974; Hayton and Barron, 1990) indicating that gills are the primary site of uptake for lipophilic organic compounds.

The toxicity of aqueous TFM in bullfrog larvae in our study is supported by findings from earlier studies. Chandler and Marking (1975) determined the LC_{50} in flow-through tests to be 1.40 (95% CI = 1.03–1.90) mg/l, and Kane et al. (1985) determined the LC_{50} to be 1.39 (95% CI = 1.08–1.78) in static tests.

V_{\max}/K_m for TFM-UDPGT in both bullfrog larvae and adults indicated similar enzyme efficiency rates. However, observed differences in V_{\max} and K_m between the two life stages may suggest (i) the involvement of more than one isozyme mediating TFM-UDPGT catalyzed reactions (Tephly and Burchell 1990), (ii) that the same isozyme(s) may have ontogenetically altered kinetic properties (Dutton, 1980), and/or (iii) other factors such as diet (adult bullfrogs are carnivorous while tadpoles are

predominantly vegetarians), may alter the kinetic properties of an enzyme (Castuma and Brenner, 1986a,b; Zakim and Dannenberg, 1992). In addition, greater substrate binding affinity (K_m) of enzymes in certain animals may be of ontogenetic significance when the amount of enzyme in liver cells is low (Castrén and Oikari, 1983). This could hold true for tadpoles in the present study in which levels of hepatic microsomal protein was 1.79-times lower than in adults.

Cessation of egg development in 1.0, 2.0 and 3.0 mg/l TFM is consistent with the effects of pentachlorophenol as described by Crawford and Guarino (1985). Both of these xenobiotics, which are presumed uncouplers of oxidative phosphorylation in aquatic organisms, inhibited post-blastular egg development. This is the expected result when aerobic ATP synthesis is inhibited (Crawford and Wilde, 1966). In consideration of TFM for use as a selective amphibicide, it is an added benefit that relatively low concentrations appear to be effective in controlling tadpoles and frogs as early as the egg stage. This also suggests that, in general, phenolic environmental contaminants may have adverse effects on early amphibian life stages.

ACKNOWLEDGMENTS

The authors are grateful to M.W. Kahng, P.T. Nhamburo and M. Weiner for their insight into the biochemical studies, to J. Allen for supplying the TFM, and to Hunting Creek Fisheries for their support and donation of test animals. We also thank D. Shuman for review of this manuscript.

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