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UDP-Glucuronyltransferase Kinetics for 3-Trifluoromethyl-4-nitrophenol (TFM) in Fish

ANDREW S. KANE, MYONG W. KAHNG, AND RENATE REIMSCHUESSEL

University of Maryland School of Medicine, Department of Pathology
Aquatic Pathobiology Center, 10 South Pine Street, Baltimore, Maryland 21201-1192, USA

PATSON T. NHAMBURO

University of Maryland School of Medicine, Department of Pharmacology

MICHAEL M. LIPSKY

University of Maryland School of Medicine, Department of Pathology

Abstract.—Studies were conducted to address glucuronidation of 3-trifluoromethyl-4-nitrophenol (TFM) in sea lampreys *Petromyzon marinus*, channel catfish *Ictalurus punctatus*, rainbow trout *Oncorhynchus mykiss*, and bluegills *Lepomis macrochirus*. The ability of these species to biotransform TFM was investigated by determining the kinetics of UDP-glucuronyltransferase (UDPGT; also known as glucuronosyltransferase) in vitro from hepatic microsomal preparations. Maximal velocity (V_{max}, nmol/min·mg) for UDPGT activity toward TFM was significantly greater ($P < 0.05$) in bluegills (1.52), rainbow trout (1.82), and channel catfish (1.46) than in sea lampreys (0.68). Binding affinities (K_m) of UDPGT for TFM varied significantly among species in the following order: bluegill (58 μM) > rainbow trout (97 μM) > channel catfish (172 μM) > sea lamprey (261 μM). Analysis of V_{max}/K_m ratios, a measure of enzyme efficiency (nmol/min·mg·μM TFM), indicated that the efficiency of UDPGT activities in all species examined was influenced more by binding affinity (K_m) than by the V_{max} of the reaction. These calculated ratios were progressively lower for species that were previously reported to be more sensitive to aqueous TFM (i.e., to have lower LC50s, TFM concentrations lethal to half the test fish). Sea lampreys appear to have relatively low UDPGT activity and binding affinity for phenolic substrates. This, in part, may account for the sensitivity of the sea lamprey to aqueous TFM.

In recent years, concern has increased about the effects of organic pollutants, such as phenols, on aquatic biota. Phenols represent a broad group of anthropogenic compounds that enter the aquatic environment from a variety of sources including the coal, petroleum, chemical, and pesticide industries (Buikema et al. 1979; Plumb 1993). Phenolic compounds are also generated in vivo by cytochrome-P_{450}-mediated oxidation of aromatic compounds (Glickman et al. 1977; Stehly and Plakas 1992). Clarke et al. (1991) reviewed the literature on in vivo metabolism of a variety of organic compounds in fish, including phenols, and concluded that glucuronidation is quantitatively the most important pathway for the elimination of many xenobiotics. It is therefore of interest to determine if selective toxicity (tropism) of phenolic xenobiotics is related to metabolic capacity—that is, to glucuronidation.

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is used for selective control of the parasitic sea lamprey *Petromyzon marinus* in the Great Lakes, and it is considered relatively benign at concentrations typically applied in the environment (GLFC 1985). Since the initial application of TFM in 1958, many studies have cited the effects of this phenol on a variety of aquatic plants, invertebrates, and vertebrates. Of more than 20 fish species tested with aqueous TFM (Applegate et al. 1961; Applegate and King 1962; Chandler and Marking 1975; Marking and Olson 1975; Marking et al. 1975; Seelye et al. 1987), sea lampreys appear to be the most sensitive.

The sensitivity of sea lampreys to TFM appears to be caused by a greater accumulation of the toxicant as compared with other fishes. Lech and Statham (1975) reported that uptake of aqueous TFM by adult sea lampreys significantly exceeded that of rainbow trout *Oncorhynchus mykiss*. Furthermore, TFM glucuronide was significantly lower in sea lamprey tissues after aqueous exposure than in tissues obtained from rainbow trout, following similar exposures to TFM. These findings are consistent with the hypothesis that the sensitivity of sea lamprey to TFM resides in a greater uptake and a lower activity of the metabolic inactivation pathway. Additional toxicity studies with inhibitors of glucuronidation pathways indicate that this

inactivation pathway is critical to the elimination of TFM (Lech et al. 1973; Lech 1974; Lech and Statham 1975).

Metabolism of phenolic compounds in mammals (Mulder 1982; Caldwell 1985) and fish (Chambers and Yarbrough 1976; James 1986, 1987) takes place primarily through glucuronidation, mediated by UDP-glucuronyltransferase (UDPGT, also known as glucuronosyltransferase, number 2.4.1.17: IUBNC 1984). The UDPGTs are a family of membrane-bound enzymes, located mainly within the hepatic endoplasmic reticulum, that catalyze the transfer of UDP-glucuronic acid (UDPGA) to a variety of xenobiotics and endogenous molecules containing hydroxyl, carboxyl, amino, or sulfhydryl groups (Dutton 1980; Burchell and Coughtrie 1989). Glucuronic acid conjugates are generally more polar than their respective parent compounds and thus tend to be more readily excreted.

Previous studies with TFM and other phenolic toxicants have focused on differential uptake and conjugation between species (Lech et al. 1973; Lech 1974; Lech and Statham 1975) or the specific activity of biotransformation enzymes (Dewaide 1971; George et al. 1990; Clarke et al. 1991). The objectives of this study were to examine the biotransformation kinetics of hepatic microsomal TFM-UDPGT in sea lampreys and the relatively more resistant channel catfish *Ictalurus punctatus*, rainbow trout, and bluegill *Lepomis macrochirus*, and to determine whether the measured in vitro enzyme kinetics might underlie differences in the aqueous in vivo sensitivities of these species. We also examined UDPGT kinetics for *p*-nitrophenol (*p*NP), a classic phenolic substrate, in hepatic microsomes isolated from sea lampreys and rainbow trout.

Methods

Materials.—The TFM (95% purity) was supplied by the National Fisheries Research Laboratory, La Crosse, Wisconsin. Concentrations of TFM mentioned in this paper are based on the active ingredient. The UDPGA (trisodium salt, 98% purity), Triton X-100, and buffers were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Fish.—Parasitic-phase sea lampreys (130–168 g) were obtained from Lake Michigan and Lake Champlain. Kamloop strain rainbow trout (294–476 g) were obtained from the Albert Powell Trout Hatchery, Hagerstown, Maryland. Bluegills (72–102 g) and channel catfish (90–101 g) were obtained from Maryland Pride Farms, Aberdeen, Maryland. All fish were procured in late summer, except Lake Champlain sea lampreys were collected in midwinter.

Tissue preparation.—All fish were euthanized by cervical transection. Livers were removed and dissected free from the gall bladder, washed in ice-cold phosphate-buffered saline, blotted, weighed, frozen in liquid nitrogen, and stored at −80°C until used.

Microsome preparation.—Livers were thawed in ice-cold homogenization buffer (0.01 M NaH_2PO_4–Na_2HPO_4 plus 0.15 M KCl, pH 7.4), minced with scissors, and rinsed with buffer. Livers from each rainbow trout, channel catfish, and sea lamprey represented an individual sample, whereas bluegill livers (3–4) were pooled. Tissue was homogenized in five volumes of buffer with a motor-driven (300-revolution/min) glass–Teflon homogenizer. The homogenate was centrifuged (10,000 × gravity for 20 min at 4°C), and the resulting supernatant was recentrifuged (105,000 × gravity for 60 min at 4°C). Microsomal pellets were suspended in 0.1 M NaH_2PO_4–Na_2HPO_4 (pH 7.4), frozen in liquid nitrogen, and stored at −80°C. Microsomes were used within 2 months after preparation.

Enzyme assay.—Activity of UDPGT was determined as previously reported (Kane et al. 1993). The reaction mixture contained 0.125 mL of microsomes (0.3–0.4 mg protein), 0.04% Triton X-100, 2 mM UDPGA, 10 mM $MgCl_2$, and 12.5–300 μM TFM, suspended in 0.1 M NaH_2PO_4–Na_2HPO_4 buffer (pH 7.4). Briefly, reactions were initiated by the addition of substrate and incubations were performed in a metabolic incubator at 24°C. The reaction was terminated after 20 min by adding ice-cold 40% trichloroacetic acid and agitating the mixture with a Vortex machine. Samples were centrifuged and absorbance of the resulting supernatant was measured at 395 nm to assess reduction in color due to formation of TFM glucuronide. Blanks without UDPGA were run concurrently. Protein was measured by the bicinchoninic acid technique (Smith et al. 1985) with bovine serum albumin as the standard. Kinetic data, maximal reaction velocity (V_{max}) and enzyme binding affinity (K_m), were derived for individual microsomal preparations by Lineweaver–Burk analysis. Differences between species were compared with Student's *t*-test.

Ratios of V_{max}/K_m are used as a measure of enzyme efficiency (Tephly et al. 1988; Temellini et al. 1991). Comparison of calculated V_{max}/K_m

TABLE 1.—Maximum velocities (V_{max}) and binding affinities (K_m) calculated for TFM–UDPGT kinetics in four fish species. Data are means ± SEs of independent experiments (N). In each experiment, assays were performed in duplicate.

Species	N	V_{max}[a]	K_m[b]
Sea lamprey	5	0.68 ± 0.01	261 ± 20
Channel catfish	2	1.46 ± 0.07[c]	172 ± 82
Rainbow trout	4	1.82 ± 0.22[c]	97 ± 9[c]
Bluegill	4	1.52 ± 0.10[c]	58 ± 10[c,d]

[a] Units are nmol/min·mg microsomal protein.
[b] Units are μM TFM.
[c] Significantly different from the sea lamprey value ($P < 0.01$).
[d] Significantly different from the rainbow trout value ($P < 0.05$).

ratios for TFM-UDPGT from the present study with previously published in vivo aqueous TFM toxicities was performed by simple linear regression analysis.

Results

TFM-UDPGT Kinetics

Lineweaver–Burk analyses indicated that UDPGT binding affinities (K_m) for TFM varied significantly among species in the following order: bluegill > rainbow trout > channel catfish > sea lamprey. In addition, the maximum velocity (V_{max}) for TFM-UDPGT was highest for rainbow trout and bluegill, less for channel catfish, and lowest for sea lamprey. Kinetic data for TFM-UDPGT are summarized in Table 1. Half the sea lampreys from both Lake Michigan and Lake Champlain lacked TFM-UDPGT concentration-dependent activity, and these fish were not used to calculate the kinetic parameters for this species. There was no significant difference ($P < 0.05$) for V_{max} or K_m between the remaining sea lampreys collected from Lake Michigan or Lake Champlain, and these animals were grouped together for interspecies comparison.

Efficiency of TFM-UDPGT, expressed as V_{max}/K_m ratios, was estimated for the four test species. The order of efficiency of TFM biotransformation was: bluegill > rainbow trout > channel catfish > sea lamprey (Figure 1). The V_{max}/K_m ratio for sea lamprey was significantly less ($P < 0.05$) than those of the other species.

Kinetic Properties of pNP-UDPGT in Rainbow Trout and Sea Lamprey

There was no significant difference ($P < 0.05$) between the calculated V_{max} for rainbow trout *p*NP-UDPGT (1.30 ± 0.18 nmol/min·mg; N=4) and TFM-UDPGT (1.82 ± 0.22 nmol/min·mg). However, the apparent *p*NP-UDPGT binding affinity in rainbow trout (46 ± 3 μM) was signifi-

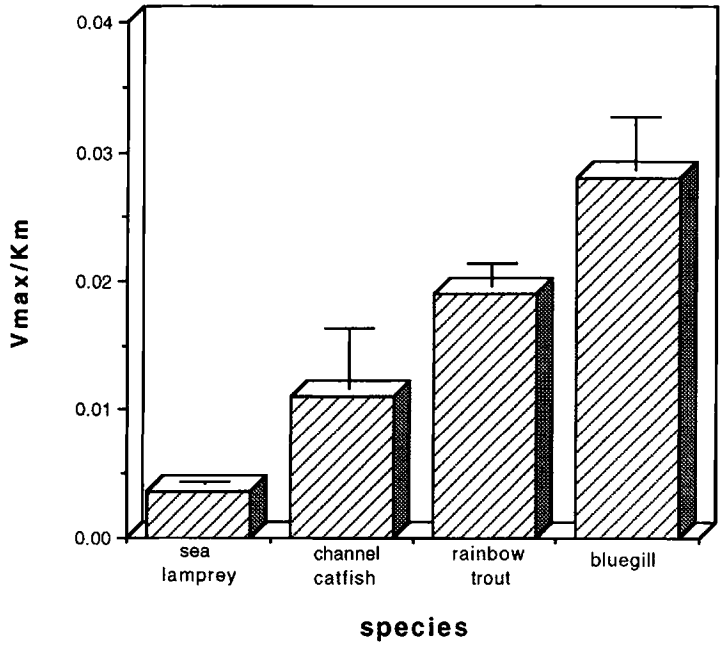

FIGURE 1.—Ratios of V_{max}/K_m for TFM–UDPGT kinetics in fish. Ratios are expressed as nmol/min·mg·μM. Data are means (SE) of independent experiments performed in duplicate.

TABLE 2.—Literature values of aqueous TFM sensitivities of fish.[a]

Species	LC50, mg/L (95% CI)[b]	Source
Sea lamprey	0.57 (0.45–0.72)	Dawson et al. (1975); Lech and Statham (1975)
Channel catfish	1.08 (0.90–1.30)	Marking et al. (1975)
Rainbow trout	1.37 (1.09–1.46)	Marking and Olson (1975)
Bluegill	5.73 (4.81–6.83)	Marking and Olson (1975)

[a] Toxicity tests were conducted in soft water, pH 7.4–7.5, at 17°C; LC50s (concentrations lethal to 50% of test fish) have been corrected for active ingredient.
[b] CI is confidence interval.

cantly less ($P < 0.01$) than the TFM-UDPGT binding affinity (97 ± 9 μM). We did not detect *p*NP-UDPGT concentration-dependent activity in sea lampreys ($N = 4$).

Discussion

In the present study, TFM-UDPGT K_m and, to a lesser extent V_{max}, differed significantly between sea lamprey and the more resistant channel catfish, rainbow trout, and bluegill (Table 1). Kinetic data from these four species indicate that the V_{max}/K_m ratios (Figure 1) follow the same order as previously reported (Table 2) for the in vivo TFM sensitivities of the species (LC50s, the concentrations lethal to 50% of the test fish). Regression analysis of the previously published LC50s with V_{max}/K_m data from the present study shows a positive correlation ($r = 0.85$). The efficiencies of biotransforming TFM (V_{max}/K_m ratios) among relatively sensitive and resistant species appeared to be influenced primarily by the binding affinity (K_m) of the enzyme for the substrate.

The observed lower activity and calculated efficiency of UDPGT in sea lamprey microsomes are consistent with the findings of earlier studies that demonstrated 10 times more glucuronide formation in rainbow trout postnuclear hepatic fractions than in sea lamprey hepatic fractions (Lech and Statham 1975).

Multiple isoforms of UDPGT have been isolated from fish (Clarke et al. 1991, 1992). However, the same UDPGT phenol isoform is probably responsible for mediating the metabolism of both TFM and *p*NP (Burchell and Coughtrie 1989). The results from our studies with rainbow trout microsomes indicated a twofold greater binding affinity (lower K_m) of UDPGT for *p*NP than for TFM. The greater affinity of UDPGT for *p*NP suggests that the enzyme is more efficient at conjugating *p*NP than TFM at low substrate concentrations. This may be due to steric interactions at the active site, because *p*NP lacks the bulky trifluoromethyl side chain present on the TFM molecule. This halogenated side chain on the TFM molecule produces the most favorable lampricidal activity—that is, toxicity plus selectivity (Applegate et al. 1966; Howell et al. 1980). Although close structural similarity between *p*NP and TFM alone cannot necessarily be used to predict toxicity or sensitivity of aquatic organisms to phenols (Murphy 1987), phase II enzyme activities in fish have been observed to abruptly decrease with an increase in chlorine atom number (Kobayashi 1978).

Our studies did not show discernable differences in the TFM-UDPGT kinetic properties between the two sources of sea lampreys collected during different seasons. Our results agree with those of Lindström-Seppä (1985), which showed absence of seasonal variation of *p*NP-UDPGT activity in vendace *Coregonus albula*.

Sea lampreys seem to have relatively poor capacity for glucuronidation, as do cats *Felis catus* (Robinson and Williams 1958; Williams 1974; Kasper and Henton 1980) and Gunn rats *Rattus norvegicus* (Hedrich 1990; Iyanagi 1991). This, in part, may account for the sensitivity of the sea lamprey to aqueous TFM. The trend of decreasing aqueous TFM sensitivity among sea lamprey, channel catfish, rainbow trout and bluegill (Table 2) correlated with the apparent efficiencies (V_{max}/K_m ratios) in the rate of TFM glucuronidation. These values are consistent with previous reports, which indicate that the susceptibility of fish to aqueous TFM follows the order: Petromyzonidae > Percidae, Ictaluridae, Catastomidae > Cyprinidae, Salmonidae > Centrarchidae (Applegate and King 1962; Schnick 1972) when water quality is held constant.

Data from this study indicate that in vivo TFM sensitivity may be related to detoxification enzyme efficiency, based on V_{max}/K_m ratios. Further, this study demonstrates the importance of examining biotransformation kinetics, not just enzyme activity, when the parameters governing enzymatic detoxification processes are elucidated. A more detailed knowledge of the comparative kinetics of biotransformation systems and target toxicities in fish is needed to better understand the mechanisms of selective toxicity in different species.

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