Supporting Information

Effects of triclocarban, triclosan, and methyl triclosan on thyroid hormone action and stress in frog and mammalian culture systems

Ashley Hinther[1], Caleb M. Bromba[2], Jeremy E. Wulff[2], and Caren C. Helbing[1,3]

[1]Department of Biochemistry & Microbiology, University of Victoria, P.O. Box 3055 Stn CSC, Victoria, B.C., Canada, V8W 3P6

[2]Department of Chemistry, University of Victoria, P.O. Box 3065 Stn CSC, Victoria, B.C., Canada, V8W 3V6

[3]Corresponding Author: Caren C. Helbing, Phone: (250) 721-6146, Fax: (250) 721-8855, email: chelbing@uvic.ca

Content

Supporting Materials and Methods

mTCS Synthesis and Characterization. A flame-dried 100 mL round-bottom flask was charged with TCS (184.2 mg, 0.636 mmol) and K_2CO_3 (177.9 mg, 1.29 mmol, 2 equivalents). The contents of the flask were placed under an atmosphere of argon. Tetrahydrofuran (20 mL, distilled over sodium and benzophenone) and iodomethane (0.16 mL, 2.6 mmol, 4 equivalents) were added via syringe, and the mixture was heated to reflux for 16 h. After cooling to room temperature, the reaction mixture was diluted with saturated aqueous ammonium chloride, and extracted three times with dichloromethane. The combined organic layers were washed with 10% aqueous NaOH, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford 187 mg (97%) of mTCS as a colorless oil. An analytical sample was purified by flash-column chromatography over silica gel (4:1 hexanes:ethyl acetate) prior to use. Spectral data: ^{1}H-NMR ($CDCl_3$, 300 MHz, δ) 7.41 (d, 1H, *J* = 2.5 Hz), 7.08 (dd, 1H, *J* = 8.8, 2.5 Hz), 6.96 (d, 1H, *J* = 2.2 Hz), 6.88 (dd, 1H, *J* = 8.5, 2.2 Hz), 6.82 (d, 1H, *J* = 8.5 Hz), 6.65 (d, 1H, *J* = 8.8 Hz), 3.79 (s, 3H); ^{13}C-NMR ($CDCl_3$, 75 MHz, δ) 152.1 (C), 151.6 (C), 143.2 (C), 130.5 (C), 130.4 (CH), 128.4 (C), 127.9 (CH), 125.0 (C), 121.3 (CH), 121.1 (CH), 118.6 (CH), 113.8 (CH), 56.4 (CH_3).

Organ Culture of Tail Fin Biopsies. Preparation of the tail fin biopsy cultures was adapted from conditions described previously *(1, 2)*. Premetamorphic (Taylor and Kollros (TK) stage VI-VIII *(3)*) *R. catesbeiana* tadpoles were euthanized in 0.1% tricaine methanesulfonate (Syndel Laboratories, Vancouver, BC) in 25 mM sodium bicarbonate, and subsequently washed three times in 100 ml per tadpole of sterile magnesium-free (MFM) solution (7.5 mM Tris-HCl pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.88 mM $CaCl_2$). Eight biopsies were obtained per animal, from the dorsal and ventral tail fins using a 6 mm dermal biopsy punch (Miltex Inc., York PA), to allow the

assessment of eight treatments per animal. Biopsies were cultured in individual wells at 25ºC in air for 48 h in 24-well multi-well culture plates (Primaria, BD Biosciences, Mississauga, ON) in 1 ml per biopsy of 70% strength Leibovitz's L15 medium (Gibco-Invitrogen) supplemented with 10 mM HEPES pH 7.5, 50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (Gibco-Invitrogen) and 50 μg/ml neomycin (Sigma-Aldrich) *(1)*.

For the chemical treatments, the media additionally contained the vehicle control, 10 nM 3,3', 5-triiodothyronine (T_3), or 10 nM T_3 in combination with the test chemicals at different concentrations or an equal volume of the test chemical solvent. T_3 was prepared as a 10^{-5} M stock in 400 μM NaOH and was applied at 1 μl/ml of media, giving a final concentration of 10 nM T_3 (400 nM NaOH final). An equal volume of vehicle was applied to those treatments lacking T_3. The vehicle concentration was kept constant throughout.

The biopsies were pretreated with 0.5 ml of the appropriate concentration of the test chemical in culture media for 2 h prior to the addition of T_3. After the 2 h incubation, 0.5 ml of the appropriate concentration of the test chemical plus 20 nM T_3 (in 800 μM NaOH) were added into the wells giving final concentration of 10 nM T_3 (in 400 nM NaOH). For the wells not containing T_3, 0.5 ml of the appropriate concentration of the test chemical plus 800 μM NaOH were added. Each test chemical stock was applied at 1 μl/ml medium giving final nominal concentrations as indicated in the text with a solvent dilution of 1:1000. At the end of the 48 h incubation period for each treatment, the biopsy was stored in 100 μl of RNA*later* (Ambion Inc., Austin TX) for 24 h at 4°C and then transferred to -20ºC until processed for RNA outlined below.

Cell Culture. A rat GH3 cell line originating from the pituitary was purchased from American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained F-12 Kaighn's medium (Gibco-Invitrogen, Burlington, ON) supplemented with 50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (Gibco-Invitrogen), 1 mM sodium pyruvate (Gibco-Invitrogen) and 10% fetal bovine serum (FBS; Gibco-Invitrogen). FBS was inactivated prior to use by heating at 60°C for 20 minutes. Cells were initially grown on a 10 cm diameter sterile culture dish (Sarstedt, Inc., Montreal, PQ) at 37°C in air and 5% CO_2. At confluence, the cells were harvested by the addition of 0.05% (w/v) trypsin (Gibco-Invitrogen) in 0.5 nM ethylenediaminetetraacetic acid (EDTA) to each 10 cm dish and plated on sterile Falcon 6-well plates (VWR International, Mississauga ON) at 2×10^5 cells/well (1×10^5 cells/mL) in modified F-12 Kaighn's media supplemented with penicillin streptomycin, sodium pyruvate and 10% heat-shocked FBS in 37°C in air and 5% CO_2. Following a 24 h incubation, the media was subsequently removed and the GH3 cells were washed three times with 2 mL sterile phosphate buffer saline solution (PBS). Two mL F-12 Kaighn's media supplemented with penicillin streptomycin and sodium pyruvate and also containing 10% heat-inactivated hormone-stripped FBS (Fisher Scientific Canada, Ottawa ON) was then added and the cells were incubated for a further 48 h at 37°C in air and 5% CO_2. After this time, treatment of GH3 cells in the presence and absence of T_3 took place.

For the chemical treatments, the media additionally contained the vehicle control, 10 nM 3,3', 5-triiodothyronine (T_3; Supplementary Figure 1), or 10 nM T_3 in combination with the test chemicals at different concentrations or an equal volume of the test chemical solvent. T_3 was prepared as a 10^{-5} M stock in 400 μM NaOH and was applied at 1 μl/ml of media, giving a final concentration of 10 nM T_3 (400 nM NaOH final). An

equal volume of vehicle was applied to those treatments lacking T_3. The vehicle concentration was kept constant throughout. Each test chemical stock was applied at 1 μl/ml medium giving final nominal concentrations as indicated in the text with a solvent dilution of 1:1000. Two mL of media containing the appropriate test chemical was applied to the cells. Cells were incubated in the test chemicals for 48 h followed by removal of the growth medium and addition of 1 mL TRIzol to each well for isolation of total RNA as described below.

Isolation of RNA and Quantification of Gene Expression. RNA was isolated using TRIzol reagent as described by the manufacturer (Gibco-Invitrogen). Mechanical disruption of the tail biopsies utilized 300 μl TRIzol reagent, a 1 mm diameter tungsten-carbide bead, and safe-lock Eppendorf 0.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific) at 20 Hz two times for 1.5 min with the chambers being rotated in between the cycles. Twenty μg of glycogen (Roche Diagnostics, Laval PQ) were added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 10 μl diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at -70ºC. cDNA was synthesized from 5 μl (~0.5 μg) total RNA as per manufacturer's protocol using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington ON) with minor modifications: RNA was first annealed with 200 ng random hexamer primer in the presence of dNTPs and then cDNA was synthesized by adding the mixture of reaction buffer, ribonuclease inhibitor and RevertAid H Minus M-MuLV reverse transcriptase and incubating at 25ºC for 10 min and then at 42ºC for 1.5 h. The cDNA products were diluted five-fold prior to PCR amplification and stored at -20ºC. Selected transcripts, designed using the computer software Primer Premier (version 5.00) (Supplementary Table 1) were analyzed using a MX3005P real-time

quantitative PCR system (Stratagene, La Jolla CA). Each 15 µl amplification reaction contained 10 mM Tris-HCl (pH 8.3 at 20ºC), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene OR), 200 µM dNTPs, 69.4 nM ROX reference dye (Gibco-Invitrogen), the appropriate concentration of the test primer as listed in Table 1, 2 µl of diluted cDNA, and one unit of Hot Start Taq DNA polymerase (Fermentas). The thermocycle program for both gene targets included an initial enzyme activation step at 95ºC (4 min) followed by 40 cycles of 95ºC denaturation (15 sec), appropriate annealing for each primer as listed in Supplementary Table 1 (30 sec), and 72ºC elongation (45 sec). Controls lacking cDNA template and an inter-plate standard containing a mixture of cDNA were included to determine the specificity of target cDNA amplification as well as the quality of each QPCR run. Quadruplicate reactions were performed for each sample and data were averaged and normalized to the expression of the invariant control gene encoding the ribosomal protein L8 using the comparative C_t method *(4)*. The integrity of amplification reactions was confirmed by the presence of a single DNA product following gel electrophoresis and by amplicon sequencing. Additionally, the efficiency of the target amplifications was validated to be approximately equal allowing the use of the comparative C_t method. The average C_t values obtained for the controls are indicated in the Supplementary Table 1 to give an idea of the relative abundance of the transcripts in the tissue and cells examined.

Statistical Analyses. All of the data sets were non-parametric and statistical analyses were performed using SPSS Ver. 12.0 (Chicago, IL) software. For the GH3 cell data, Kruskal-Wallis (KW) analysis was done on all treatments for each transcript followed by a subgrouping of vehicle control plus chemical treatment or T_3 plus chemical treatment.

Pairwise comparisons relative to the vehicle control or T_3 alone treatment was done using the Mann-Whitney U (MWU) test.

The C-fin data were analysed using the Kendall's W and paired Wilcoxon tests since these data were generated from a repeated-measures type of experimental design. The types of comparisons made were as described previously *(1)*. The test chemical results were examined relative to the vehicle control and it was verified that an expected TH-dependent response was obtained. Then, the test chemicals in combination with T_3 results were compared relative to T_3 alone and the response to a test chemical in the presence of T_3 was expressed as a fold change relative to the each individual's response to T_3 alone to eliminate inter-animal variation thus enabling us to better identify chemical-induced perturbations relative to each individual's ability to respond to T_3. Therefore, the T_3 values in this comparison were given a value of one and the graphs show the fold change relative to the T_3-induced response.

Literature Cited

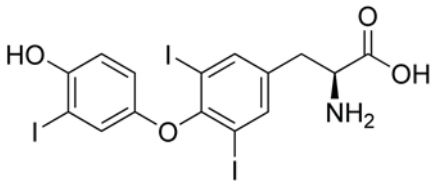
(1) Hinther, A.; Domanski, D.; Vawda, S.; Helbing, C. C. C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals. *Env. Toxicol. Chem.* 2010, 29, 380-388.

(2) Hinther, A.; Vawda, S.; Skirrow, R. C.; Veldhoen, N.; Collins, P.; Cullen, J. T.; van Aggelen, G.; Helbing, C. C. Nanometals induce stress and alter thyroid hormone action in Amphibia at or below North American water quality guidelines. *Environ. Sci. Technol.* 2010, 44, (21), 8314-21.

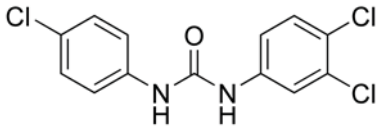
(3) Taylor, A. C.; Kollros, J. J. Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* 1946, 94, 7-24.

(4) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, 25, (4), 402-8.

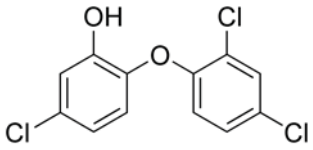
3,3',5-triiodothyronine (T_3)



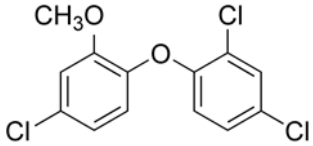
triclocarban (TCC)



triclosan (TCS)



methyl triclosan (mTCS)



Supplementary Table 1. Gene transcripts, primers, and QPCR reaction conditions

Gene transcript	Species	GenBank Accession Number	Primer Name	Primer Sequence (5' → 3')	Amplicon Size (bp)	PCR Temperature (°C)	Picomoles of primers per reaction	Average C_t of controls
Ribosomal protein L8	*Rana catesbeiana*	AY452063	L82up	AGGGGACAGAGAAAAGGTG	270	55	5	17.9±0.4
			UL8dn	TGAGCTTTCTTGCCACAG				
TRβ	*Rana catesbeiana*	M95194	FE022up	AGCAGCATGTCAGGGTAC	538	55	10	27.5±0.4
			FE022dn	TGAAGGCTTCTAAGTCCA				
RLKI	*Rana catesbeiana*	EF156435	DDKerF3	GTTGGCGTTGGTGTTAGCGG	336	55	5	15.4±0.5
			DDKerRQ	GGCACTGCTTCTTGCAACTTG				
Catalase	*Rana catesbeiana*	GQ222411	Q556a	GAATGGTTACGGCTCACA	174	60	5	21.5±0.4
			Q556b	GCAATGGCTTCATACAGA				
Heat shock protein 30	*Rana catesbeiana*	U44894	FN028up	GCCTCCACCAGACT	198	61	5	28.0±0.4
			RCHSP30dn	GTCTCCTTCCTTCCG				
Ribosomal protein L8	*Rattus norvegicus*	NM_001034916	UL8-up	GGTGTGGCTATGAATCCTGT	126	55	5	18.4±0.2
			L82-dn	ACGACGAGCAGCAATAAGAC				
Growth hormone	*Rattus norvegicus*	NM_001034848	NOR1	TTGATCCAGCAGACCTACATT	305	60	5	21.0±0.2
			NOR2	CTCCCTGGCTCCTGACCTT				
Deiodinase-I	*Rattus norvegicus*	NM_021653	NOR3	GGACTCTGCTACAAGGGTAA	304	60	5	30.4±1.1
			NOR4	GCTCGGTATTGCTTTATCT				
Prolactin	*Rattus norvegicus*	NM_012629	NOR7	CCTCCTGCTGATGATGTC	498	60	5	19.8±0.9
			NOR8	TGTGACCAAACCAAGTAGAT				
Heat shock protein 70	*Rattus norvegicus*	NM_031971	NOR9	GGCTTCCACTAGACCACG	117	60	5	32.2±0.2
			NOR12	AACTGGATCGAAGGCGTA				

Supplementary Table 2. Comparison of C_t values obtained for *rpL8* mRNAs in the C-fin assays

Experiment	Treatment	Median[a]	25th percentile	75th percentile
1	Control	18.8	18.4	19.4
	1 nM TCS	19.7	18.9	20.4
	10 nM TCS	19.8	18.1	20.4
	100 nM TCS	18.8	18.3	19.0
	T_3	19.2	18.7	20.1
	T_3 + 1 nM TCS	18.5	17.9	19.4
	T_3 + 10 nM TCS	19.6	18.7	20.5
	T_3 + 100 nM TCS	18.9	18.4	19.8
2	Control	16.8	16.4	17.1
	1 nM mTCS	16.1	15.3	17.0
	10 nM mTCS	15.6	15.4	16.3
	100 nM mTCS	16.0	15.6	17.0
	T_3	16.7	16.2	17.2
	T_3 + 1 nM mTCS	16.7	16.2	17.1
	T_3 + 10 nM mTCS	17.2	16.5	18.2
	T_3 + 100 nM mTCS	17.0	16.6	17.8
3	Control	17.6	16.9	18.4
	10 nM TCC	17.8	16.8	18.6
	100 nM TCC	17.5	16.9	18.3
	1000 nM TCC	19.0	18.1	20.5
	T_3	18.4	17.9	19.7
	T_3 + 10 nM TCC	18.6	17.7	19.5
	T_3 + 100 nM TCC	18.3	17.5	19.7
	T_3 + 1000 nM TCC	19.0	18.3	19.7

[a]The median and percentiles are presented since the data were non-parametric.

Supplementary Table 3. Comparison of C_t values obtained for *rpL8* mRNAs in GH3 cells

Experiment	Treatment	Median[a]	25th percentile	75th percentile
1	Control	18.58	18.41	19.46
	1 nM TCS	18.30	18.15	18.92
	10 nM TCS	18.41	17.99	19.05
	100 nM TCS	18.65	18.52	19.06
	1000 nM TCS	18.91	18.58	19.53
	T_3	18.49	18.20	19.01
	T_3 + 1 nM TCS	18.46	18.14	18.73
	T_3 + 10 nM TCS	18.49	18.07	19.18
	T_3 + 100 nM TCS	18.67	18.32	19.25
	T_3 + 1000 nM TCS	20.00	19.71	20.00
2	Control	18.06	17.77	18.61
	1 nM mTCS	18.32	17.89	18.89
	10 nM mTCS	17.96	17.31	18.58
	100 nM mTCS	16.97	16.96	17.41
	1000 nM mTCS	16.90	16.84	16.98
	T_3	17.11	16.91	17.50
	T_3 + 1 nM mTCS	17.49	17.30	17.86
	T_3 + 10 nM mTCS	18.06	17.51	18.86
	T_3 + 100 nM mTCS	17.08	16.98	17.19
	T_3 + 1000 nM mTCS	17.35	17.06	18.82
3	Control	18.06	17.63	18.44
	10 nM TCC	18.82	18.30	19.43
	100 nM TCC	17.85	17.43	18.33
	1000 nM TCC	17.96	17.43	18.38
	T_3	17.15	16.96	17.56
	T_3 + 10 nM TCC	16.93	16.81	16.97
	T_3 + 100 nM TCC	17.11	17.01	17.27
	T_3 + 1000 nM TCC	17.39	17.16	17.62

[a]The median and percentiles are presented since the data were non-parametric.