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A Microplate Assay to Assess Chemical Effects on RBL-2H3 Mast Cell Degranulation: Effects of Triclosan Without Use of an Organic Solvent

--Manuscript Draft--

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Dear Ms. Lewis:

We are pleased to submit for publication our manuscript entitled “A Microplate Assay to Assess Chemical Effects on RBL-2H3 Mast Cell Degranulation: Effects of Triclosan Without Use of an Organic Solvent.”

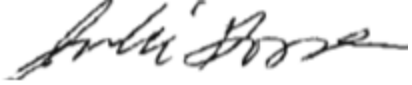
Mast cells are key players in allergy, asthma, and parasite defense. These cells utilize signal transduction cascades to transmit cell surface signals to result in release of pre-formed granules containing histamine and other allergic mediators, in a process called degranulation. Here, we provide detailed instructions for a mast cell degranulation assay, which is applicable to a wide variety of potential test chemicals, both drugs and toxicants, as well as to different types of mast cells. This assay has high-throughput potential, a particularly important feature in light of the need to assess the toxicology of the more than 80,000 untested synthetic chemicals currently in the marketplace. This method may also be useful in the development of new anti-allergy/asthma pharmaceuticals.

In particular, we detail studies in which we have examined the effects of the ubiquitous antibacterial agent triclosan: we have found it to strongly inhibit mast cell degranulation. Found in hundreds of consumer products, triclosan has also been highlighted in the literature recently due to data showing endocrine disruptive effects, and its potential toxic and/or positive effects on mammalian and aquatic biology are only now receiving considerable research interest. Thus, we believe that a method for dissolving triclosan (without use of organic solvents) and for verifying its concentration, which we detail here, is also of interest to the toxicology community.

We the authors declare that there are no conflicts of interest. The three listed authors have all participated deeply in the experimental work, planning, methodology development, and writing of this manuscript. This material has not been submitted to any other journal for publication.

We appreciate your consideration and look forward to your response.

Sincerely yours,



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A Microplate Assay to Assess Chemical Effects on RBL-2H3 Mast Cell Degranulation: Effects of Triclosan Without Use of an Organic Solvent

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Short Abstract

Mast cell degranulation, the release of allergic mediators, is important in allergy, asthma, and parasite defense. Here we demonstrate techniques[1] for assessing effects of drugs and toxicants on degranulation, methodology recently utilized to exhibit the powerful inhibitory effect of antibacterial agent triclosan[2].

Long Abstract

Mast cells play important roles in allergic disease and immune defense against parasites. Once activated (e.g., by an allergen), they degranulate, a process that results in the exocytosis of allergic mediators. Modulation of mast cell degranulation by drugs and toxicants may have positive or adverse effects on human health. Mast cell function has been dissected in detail with the use of rat basophilic leukemia mast cells (RBL-2H3), a widely accepted model of human mucosal mast cells[3-5]. Mast cell granule component and the allergic mediator β-hexosaminidase, which is released linearly in tandem with histamine from mast cells[6], can easily and reliably be measured through reaction with a fluorogenic substrate, yielding measurable fluorescence intensity in a microplate assay that is amenable to high-throughput studies[1]. Originally published by Naal *et al.*[1], we have adapted this degranulation assay for the screening of drugs and toxicants and demonstrate its use here.

Triclosan is a broad-spectrum antibacterial agent that is present in many consumer products and has been found to be a therapeutic aid in human allergic skin disease[7-11], although the mechanism for this effect is unknown. Here we demonstrate an assay for the effect of triclosan on mast cell degranulation. We recently showed that triclosan strongly affects mast

cell function[2]. In an effort to avoid use of an organic solvent, triclosan is dissolved directly into aqueous buffer with heat and stirring, and resultant concentration is confirmed using UV-Vis spectrophotometry (using ε_{280} = 4200 L/M/cm)[12]. This protocol has the potential to be used with a variety of chemicals to determine their effects on mast cell degranulation, and more broadly, their allergic potential.

INTRODUCTION

Mast cells are highly granulated immune effector cells that serve as key mediators in asthma, allergies, parasite defense and carcinogenesis[13-16]. They reside in nearly every vascularized tissue[15], where they safely store allergic and inflammatory mediators in cytoplasmic granules until activated to degranulate. Degranulation is the exocytosis of membrane-bound granules, which results in the release of pharmacologically active mediators such as histamine, tryptase, and leukotrienes[15]. This process results in the initiation of type I hypersensitivity reactions that are critical in mounting defense against parasites as well as initiating allergic, asthmatic, and carcinogenic responses[15].

Mast cells and basophils express FcεRI receptors, the high-affinity receptors for immunoglobulin E (IgE)[17]. Exposure to an allergen or antigen causes aggregation of multiple IgE-bound FcεRI receptors[17], and it is this so-called "crosslinking" of IgE-bound Fc receptors that initiates the degranulation process: a cascade of tyrosine phorphorylation events, the activation of phospholipase C, efflux of calcium from internal stores, and influx of calcium into the cell[18]. This calcium influx is necessary for degranulation, and, further, signals granule fusion with the membrane before causing granule exocytosis[15]. Experimentally, a calcium ionophore can be used to shuttle calcium directly across the cell membrane[19], which essentially bypasses all signal transduction steps prior to the calcium influx step[20], allowing for the identification of a pathway target by a toxicant as being upstream or downstream of calcium signaling[20].

Degranulation can be measured rapidly and effectively by monitoring the release of β-hexosaminidase into cell supernatant, which is released linearly from the granules alongside histamine[6], but is much easier to detect using a simple enzyme-substrate reaction and a microplate reader to assay the fluorescent product. This microplate assay, as detailed in the protocol section, is based upon a robust method originally developed by Naal *et al.*[1], which quantifies the cleavage of the fluorogenic substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide by β-hexosaminidase. We have modified the assay to test effects of drugs and toxicants, with triclosan highlighted here. This method reliably quantifies degranulation, is an inexpensive alternative to, for example, flow cytometric-based detection methods[21], and has the potential to lend itself nicely to high-throughput screening of a wide variety of anti-allergy drugs, as well as immunotoxic or allergenic chemicals. This last point is particularly important in light of the 2007 National Research Council report "Toxicity Testing in the 21st Century: A Vision and a Strategy" (http://www.nap.edu/openbook.php?record_id=11970), which advocates for the development of high-throughput toxicology tests that utilize cell culture to reduce the costly use of traditional lab animals such as mice. The degranulation protocol developed by Naal *et al.*[1] and modified by us[2], utilizes the RBL-2H3 cell line, which is a well-accepted model homologous to human mucosal mast cells or basophils[3-5].(Methods for culturing RBL-2H3 cells

are detailed in Hutchinson *et al.*[22]). This assay could likely be adapted to any attached mast cell type.

Triclosan (TCS) is a broad-spectrum antimicrobial that has been used for more than 30 years in hospitals, personal care products, and consumer goods[23,24]. The mode of action for TCS's antimicrobial characteristic is the inhibition of fatty-acid biosynthesis, likely by inhibiting enoyl-acyl carrier protein reductase[25,26]. It is found worldwide in a wide range of consumer products such as shower gel, hand lotion, toothpaste, mouthwash, and in hand soaps at concentrations up to 0.3% or 10 mM[24]. Widespread use of TCS has resulted in detectable levels in humans[27-29] and in rivers and streams[30]. A study done by Allmyr *et al.*[27] demonstrated that TCS and its metabolites are present in both the plasma and milk from nursing mothers. Importantly, TCS is readily absorbed into the skin[31-37]. Queckenberg *et al.*[37] found ~10% absorption of an ~70 mM TCS cream into human skin within 12 hours, resulting in significant concentration in the skin, where mast cells reside.

TCS has been shown clinically to manage human allergic skin disease[7-11], but the mechanism by which TCS alleviates allergic skin diseases has been unknown[38]. Using the fluorescent microplate assay detailed in this video, we recently demonstrated that TCS, at concentrations as low as 2 μM, significantly dampens mast cell function and degranulation, providing a potential explanation for these clinical data[2]. In addition to providing an explanation for these clinical data, our findings in Palmer *et al.*[2] suggest that TCS targets signaling molecules downstream of calcium influx. Due to the importance of calcium signaling in many immunological and other biological processes, TCS could potentially have adverse effects on a wide variety of necessary biological processes. In fact, Udoji *et al.*[39] showed that TCS suppresses human natural killer cell lytic activity, another important innate immune function.

Beyond its potential as a therapeutic aid in allergic skin disease (or, conversely, as an immunotoxicant), TCS may also be an endocrine disruptor[40-49]. Thus, a clear procedure on how to prepare this chemical in solution is of interest to toxicologists. Because TCS is a small hydrophobic molecule, organic vehicles are often used to make it more soluble in water. In most toxicity studies where TCS has been tested, preparation has involved dissolution in water with the aid of an organic solvent such as ethanol, acetone, or oil[2,50,51]. However, oftentimes these solvents are biologically active themselves, thereby complicating interpretation of the test chemical data[51]. In fact, according to Rufli *et al.*[52] and others[53], it is recommended that test solutions for aquatic toxicity experiments are prepared using physical methods over chemical methods, due to the potential of chemical solvents to create toxicity artifacts. We have previously shown that TCS dissolved in 0.24% ethanol/water (vol/vol) and sonicated for 30 minutes dampens RBL mast cell degranulation[2]. Ethanol at higher concentrations than 0.24% has been shown to dampen mast cell degranulation[54,55]—examples of the potentially confounding effects of organic solvents on toxicity studies.

Not only is it important to consider the effect of solvents on the organism or cells used for study, but also it is important to monitor the effect of a solvent on the test chemical itself. For example, Skaare *et al.*[51] found that dissolving TCS in polyethylene glycol (commonly found

in toothpastes and mouthwash) weakened anti-bacterial and anti-plaque effects in healthy female women while dissolution in oils caused a complete loss of function. Therefore, the ability of different solvents to modulate toxicant and drug, including TCS, effects should be considered in assay design. Use of oils or flavor additives may interfere with the effects of TCS in various products[50,51].

In an effort to eliminate the need to use organic solvents, we improved upon our method for dissolving TCS[2] by eliminating the use of an organic solvent. In the present protocol, we dissolve TCS granules directly into aqueous buffer with heat (≤ 50°C), and then verify the concentration of this TCS stock by UV-Vis spectrophotometry. These improvements are possible because TCS is soluble in water up to 40 µM (http://www.epa.gov/oppsrrd1/REDs/2340red.pdf) and has been shown to resist degradation when heated to 50°C (http://oehha.ca.gov/prop65/public_meetings/052909coms/triclosan/ciba3.pdf)[56,57]. We also have the added benefit of UV-Vis spectrophotometry, as TCS also is known to strongly absorb at 280 nm[58] with a molar extinction coefficient of 4200 L/mol/cm[12].

This protocol provides a simple, yet effective way to dissolve TCS granules into a buffer without the aid of an organic solvent, including low cost and rapid verification of concentration, and describes a powerful fluorescent microplate assay for monitoring chemical effects on mast cell degranulation.

PROTOCOL

Note that all buffer recipes are included in a table at the end of the protocol text.

DAY 1:

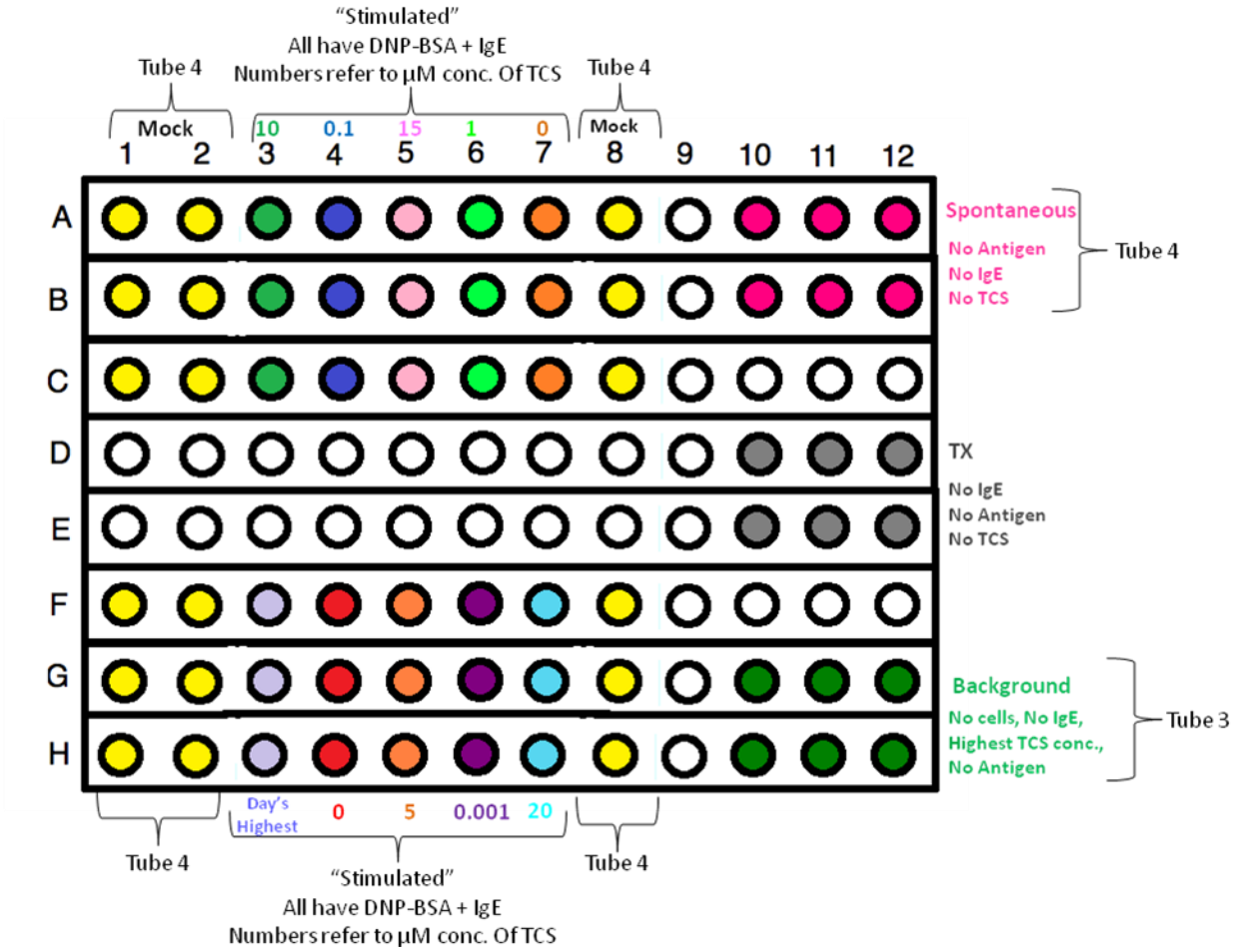
1) Preparation of cells

1.1) Plan out 96-well plate setup scheme, centering test samples on the layout in order to avoid edge effects. Allocate three replicates for each TCS concentration tested (± degranulation stimulant of antigen or ionophore), as well as triplicates for spontaneous release (no degranulation stimulant), maximum release (0.2% Triton X-100 [TX] detergent lysis), as well as wells reserved for background samples (which will contain no cells). For each replicate experimental day, choose a new randomized layout of the TCS sample concentrations.

1.2) Warm RBL media (recipe provided in table) and trypsin in 37°C water bath.

1.3) Check RBL cells in T-25 flask (2-4 days since last passage and less than 3-4 months since they were thawed) for general signs of good health: proper pH indicated by color of media, and lack of cloudiness. Place the flask under a light microscope to confirm that the flask is free of contamination and that the cells appear healthy, properly confluent, and mostly attached. Note that cells should be checked for mycoplasma contamination approximately every six weeks[22].

Treatment	Triplicates
Stimulated, 0 µM TCS	A7, B7, C7, F4, G4, H4
Stimulated, 0.001 µM TCS	F6, G6, H6
Stimulated, 0.1 µM TCS	A4, B4, C4
Stimulated, 1 µM TCS	A6, B6, C6
Stimulated, 5 µM TCS	F5, G5, H5
Stimulated, 10 µM TCS	A3, B3, C3
Stimulated, 15 µM TCS	A5, B5, C5
Stimulated, 20 µM TCS	F7, G7, H7
Stimulated, plus highest [TCS]	F3, G3, H3
Spontaneous, No TCS (includes mocks)	A10, A11, A12, B10, B11, B12 A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8
TX-100, No TCS	D10, D11, D12, E10, E11, E12
No Cells, Background, plus highest [TCS]	G10, G11, G12, H10, H11, H12



1.4) Working under a sterile tissue culture (TC) hood and using standard sterile technique, remove all media from flask with sterile pipette; rinse flask with 2 mL trypsin, and discard this wash.

1.5) Add exactly 2 mL trypsin to cover bottom of the flask. Put into 37°C incubator for 5 minutes to allow the cells to detach from the bottom of the flask.

1.6) After 5 minutes, hit the side of the flask with an open palm to loosen cells. Immediately, add 18 mL RBL media to wash cells off the flask and to quench the trypsin. Immediately take the cell-media-trypsin mixture out of the flask and transfer to a new, sterile 50 mL tube (the total volume in this tube is now 20 mL).

1.7) After mixing gently but thoroughly, remove 50 µL of cell suspension from this tube, and transfer to a 1.5 mL sterile microcentrifuge tube, which is a sample to be counted. Take this sample, as well as the 50 mL tube containing the cell-media-trypsin mixture out of the TC hood to the benchtop.

1.8) Spin the 50 mL tube in centrifuge (with appropriate balance) for 8 minutes at 500 X g; this force pellets cells effectively.

1.9) During the spin time, count the cells in the sample that were isolated before spinning. To do this, first add 50 µL of trypan blue dye to 50 µL of cells in 1.5 mL tube, and gently but thoroughly pipette up and down five times to mix. Immediately, transfer 10 µL of this mixture to the glass hematocytometer, and count cells in the grid area following manufacturer instructions. Count at least 100 cells for reasonable statistical results.

1.10) Back in the TC hood, remove supernatant from cells that were spun down.

1.11) Cap the cell tube, and flick pellet at the bottom of the tube to loosen cells.

1.12) Add media to bring the cells to a density of 0.5 x 10^6 cells/mL, based on the cell count.

1.13) Mix well but gently to keep cells suspended during the plating procedure. Using a pipetteman, put 100 µL cells/well in a 96-well plate (flat, black bottom), following the plate template sheet. Randomize how cells are added to wells to avoid systematic error, and mix after each set of three wells is added. Be sure not to put cells into the wells labeled for the background samples.

1.14) Once all cells have been transferred, place the plate lid onto the plate, and transfer to the incubator (37°C/5% CO_2) overnight. Clean up following standard sterile technique.

DAY 2:

2. Preparation of Triclosan

2.1) Using a graduated cylinder, measure 250 mL Tyrodes Buffer (recipe provided in table) into a 500 mL Erlenmeyer flask labeled "TCS-buffer." Add stir bar. Use glassware, stir bar, thermometer designated for use with TCS only.

2.1.1) Also at this time, measure 250 mL Tyrodes into a separate 500 mL Erlenmeyer flask, labeled "control buffer." Use glassware, stir bar, thermometer that are NOT designated for TCS. Add stir bar.

2.2) Weigh out 0.0022 g of TCS granules and transfer to "TCS-buffer" flask. In order to efficiently transfer granules to Erlenmeyer flask, use 10 mL from the measured 250 mL Tyrodes to wash off weigh boat, making sure all TCS has been transferred.

2.3) Place "TCS-buffer" flask onto a combination hotplate/magnetic stir plate, and set it to stir at a manageably high speed. Once well mixed, this TCS stock will nominally be 30 µM (actual concentration will be calculated after heating). (Do all mixing in a chemical fume hood.)

2.3.1) Also at this time, place the "control buffer" flask (containing no TCS) onto a second combination hotplate/magnetic stir plate, and set it to stir at a similar speed.

2.4) Turn on UV/Vis Lamp to warm up the lamp for later use.

2.5) Heat the "TCS-buffer" solution to 50°C while stirring constantly. Once up to temperature, time for 90 minutes. During the 90 minutes, continue to monitor 50°C temperature and appropriate stirring speed frequently.

2.5.1) Simultaneously, heat the "control buffer" solution to 50°C with continuous stirring. Upon reaching 50°C, time for 90 minutes, during which time temperature (keeping at 50°C) and stirring are both monitored.

2.6) At end of the 90 minutes, take both Erlenmeyer flasks off the hot plates and transfer to the benchtop.

2.7) Using the wavelength scan function on a UV/Vis spectrophotometer, blank the machine on 1 mL of the heated "control buffer" solution before scanning 1 mL of heated "TCS-buffer" solution. Check the shape of the spectrum, and record Absorbance value at 280 nm. To determine the concentration, use the Beer-Lambert equation ($A_{280}=\varepsilon_{280}\ell c$) using an ε_{280} of 4200 L/mol/cm[12] and ℓ of 1 cm.

2.8) After determining the TCS concentration, add 0.249 g bovine serum albumin (BSA) to the remaining 249 mL of the "TCS-buffer" solution, and mix well.

2.8.1) Simultaneously, add 0.249 g BSA to the remaining 249 mL of "control buffer" solution, and mix well.

DAY 2:

3. Antigen-stimulated degranulation assay using RBL-2H3 cells

3.1) Before starting, check the pH of all buffers being used, and ensure that they are clear and not cloudy: this includes Tyrodes buffer, sodium acetate buffer, and glycine carbonate buffer (recipes provided in table).

3.2) Warm RBL media and trypsin in 37°C water bath.

3.3) Make BT (1 mg/mL BSA in Tyrodes buffer): 0.05 g BSA + 50 mL Tyrodes Buffer (X2). Put into 37°C water bath.

3.4) Make 0.2% Triton X-100: 3.136 mL of BT + 64 µL of 10% Triton X-100 (final concentration of Triton X-100 is 0.2%). Mix well by inversion, but do not vortex. Put into 37°C water bath.

3.5) Start preparation of the TCS and heated Tyrodes buffer (steps "2," above). **Note**: Do not start the next step (IgE exposure) until the "TCS-buffer" and "control buffer" solutions reach 50°C and stir for the first 70 minutes of the 90 minute heat/stirring time.

3.6) Once both solutions have been stirred at 50°C for 70 minutes, make up 0.1 µg/mL anti-DNP mouse IgE (Sigma) in RBL media for sample wells to be sensitized (100 µL/well). IgE stock should not be older than 30 days when stored at 4°C; record how old the stock is. Flick to mix, but do not vortex IgE.

3.7) Under a TC hood, add 0.6 µL IgE stock (stock is 1 mg/mL) to 6 mL RBL media in a 50 mL tube. In a second 50 mL tube, add 6 mL of plain RBL media only (which is intended for non-sensitized samples).

3.8) Dump all media from 96-well plate (that was prepared on Day 1) into sink, and bring the plate under the TC hood.

3.9) Randomly add 100 µL media/IgE mixture to wells that should be stimulated (48 wells total). This mixture is not intended for "spontaneous", "TX" and "background" samples.

3.10) Randomly add 100 µL plain RBL media only to "TX," "spontaneous," and "background" wells.

3.11) Put plate lid on plate, and then move plate into 5% CO_2 /37°C incubator for 1 hour.

3.12) During 1 hour incubation, follow steps 3.13-3.24.

3.13) On the benchtop, prepare the antigen dilutions. Add 0.53 µL of 1.6 mg/mL stock DNP-BSA + 850 µL BT to get an antigen concentration of 1 µg/mL. Vortex and invert this stock to mix.

3.14) Once "TCS-buffer" and "control buffer" have been heated and then stirred for 90 minutes at 50°C, continue on with the rest of the preparation for the TCS protocol (go to steps 2.6-2.8.1). After the BSA is dissolved into both solutions, continue below.

3.15) From the 249 mL sample of "TCS-buffer" solution, transfer 50 mL to a new 50 mL conical tube. Remove 20 µL of this 50 mL aliquot and replace it with 20 µL of the 1 µg/mL antigen prepared earlier. Vortex and invert. Final antigen concentration is 0.0004 µg/mL DNP-BSA.

3.15.1) Label this "Tube 1, High TCS/+Ag/+BT." It is used for dilutions and highest TCS concentration exposure.

3.16) From the 249 mL sample of "control buffer" solution, transfer 50 mL to a new 50 mL tube. Remove 20 µL of this new 50-mL aliquot and replace with 20 µL of the 1 µg/mL antigen. Vortex and invert. (Final antigen concentration is 0.0004 µg/mL DNP-BSA).

3.16.1) Label this “Tube 2, No TCS/+Ag/+BT”. Used for TCS dilutions and 0 µM TCS concentration exposure.

3.17) Now take out 50 mL of “TCS-buffer” solution and put into another 50-mL tube. Remove 20 µL from this new 50-mL aliquot and replace it with 20 µL of plain BT. Vortex and invert. No antigen is added.

3.17.1) Label this “Tube 3, High TCS/No Ag/+BT.” This is used for background.

3.18) Transfer 50 mL of “control buffer” solution to another 50-mL tube. Take out 20 µL from this new 50-mL aliquot and replace it with 20 µL of plain BT. Vortex and invert. (No Ag is added.)

3.18.1) Label this “Tube 4, No TCS/No Ag/+BT.” This is used for background and spontaneous samples.

	BSA	TCS	Antigen
Tube 1	✓	High [ ]	✓
Tube 2	✓	NO	✓
Tube 3	✓	High [ ]	NO
Tube 4	✓	NO	NO

3.19) Calculate and record volumes for dilutions after determining the concentration of the “TCS-buffer” stock. Total volume for each dilution concentration should be 1 mL and should be prepared in a sterile microcentrifuge tube. Use calibrated P2 and P1000 pipettemen.

Concentration	**High Triclosan+Tyrodes+BSA+0.0004 µg/mL Ag (Tube 1 from above)**	**Heated BT+0.0004 µg/mL Ag (Tube 2 from above)**
20 µM		
15 µM		
10 µM		
5 µM		
1 µM		
0.1 µM		
0.001 µM		
0 µM (top of plate)	----------	500 µL plus another 500 µL
0 µM (bottom of plate)	----------	500 µL plus another 500 µL

3.20) After 1-hour IgE incubation, take plate out of incubator and toss all media into sink. (Note: if test chemicals are known to be more toxic than the consumer product TCS, hazardous waste disposal may be necessary.)

3.21) Using a combitip, randomly wash cells in the 96-well plate with BT (200 µL/well). Repeat the process a second time.

3.22) To prepare treatments for application, vortex and invert dilutions right before addition to the plate.

3.23) Starting with the top section of the plate: Randomly add triplicates of 200 µL each of the correct concentration of TCS to the corresponding well. Continue to bottom of plate. Add "control buffer" solution plus Ag (from "Tube 2" above) to all "mocks" on the plate.

3.24) Add 200 µL of appropriate solutions to corresponding wells:

3.24.1) Add 200 µL of 0.2% Triton X-100 to "TX"-designated wells.

3.24.2) Next, add 200 µL of Tube 3 to the 3 wells labeled "Background (BkgD)-Highest TCS" on the plate.

3.24.3) Finally, add 200 µL of Tube 4 to 6 wells labeled "Spontaneous."

3.25) Incubate the plate for 1 hour in 37°C/5% CO_2.

3.26) During 1 hour incubation: Get two buckets of ice (one for "old" plate in incubator and one for new plate). Thaw 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide (4-MU) at room temperature for up to 40 minutes, keeping in foil because it is light sensitive.

3.26.1) Once 4-MU stock is thawed, make up 4-MU working solution: 150 µL stock + 14.85 mL of cold acetate buffer (recipe given in table); vortex and invert. Keep in 50 mL centrifuge tube, wrapped in foil, and on ice until use.

3.26.2) Using combitip, randomly add 100 µL cold 4-MU working solution into the very bottom of each well of a NEW Grenier black 96-well plate (on ice bucket #2). Start first by adding the working solution randomly within the top of the plate, randomly within the bottom of the plate, randomly within Triton X-100 wells, and finally randomly within background wells.

3.26.3 Get out new box of P200 tips for next step.

3.27) At the end of 1-hour incubation, put cell plate from incubator onto ice bucket #1, pipette supernatant up and down 4-5 times (gently, not introducing bubbles), going around the well for good mixing but not touching the cells while mixing. Systematically, take out 25 µL sample from each well and place into the new plate with substrate (same ordering of samples, as

originally planned out). Pipette up and down to mix sample thoroughly when in new well, without introducing bubbles.

3.28) Incubate for 30 minutes at 37°C/5% CO_2.

3.29) After 30 minute incubation, randomly add 200 µL of cold glycine-carbonate buffer per well (using combitip) to fill wells up to 325 µL total. (Make this addition to the Triton X-100 samples last, to avoid Triton X-100 spillover). Check for bubbles before reading plate (poke with clean P10 pipette tip to pop any bubbles).

3.30) Run the plate in the fluorescence plate reader (go to section 4).

DAY 2:

4. Fluorescent plate reader instructions and data analysis

4.1) Open Gen5 program, and open experiment section.

4.2) Turn on plate reader and insert plate (upper left corner is A1).

4.3) Protocol → Procedure → Read to set custom readings. Do not add anything about samples, replicates, etc., in order to collect raw fluorescence data from each well.

4.4) Under "Read" choose "fluorescence," "Endpoint," "Normal Speed," "Gain 40," "Excitation 360/40," "Emission 460/40," Optics position: Top 50%. Top optical offset: 7mm. No shake, no delay, no kinetics, no monitor well, temperature: incubator off.

4.5) Choose flat black bottom, 96-well plate (Grenier 96-well , Flat Bottom).

4.6) Deal with the plate layout: Protocol → plate layout. Set up samples without indicating repeats, dilutions, etc.

4.7) Plate → read.

4.8) Save file: Click the "Excel" button, which will export data file to Excel. Do this for plate layout and for matrix. Save the file on the computer and on a USB drive.

4.9) In Excel, subtract the average background reading from every sample, including Triton X-100 wells.

4.10) Calculate relative % degranulation by dividing each value (already having had background subtracted) by the average Triton X-100 value, and then multiply by 100 to make it a percentage.

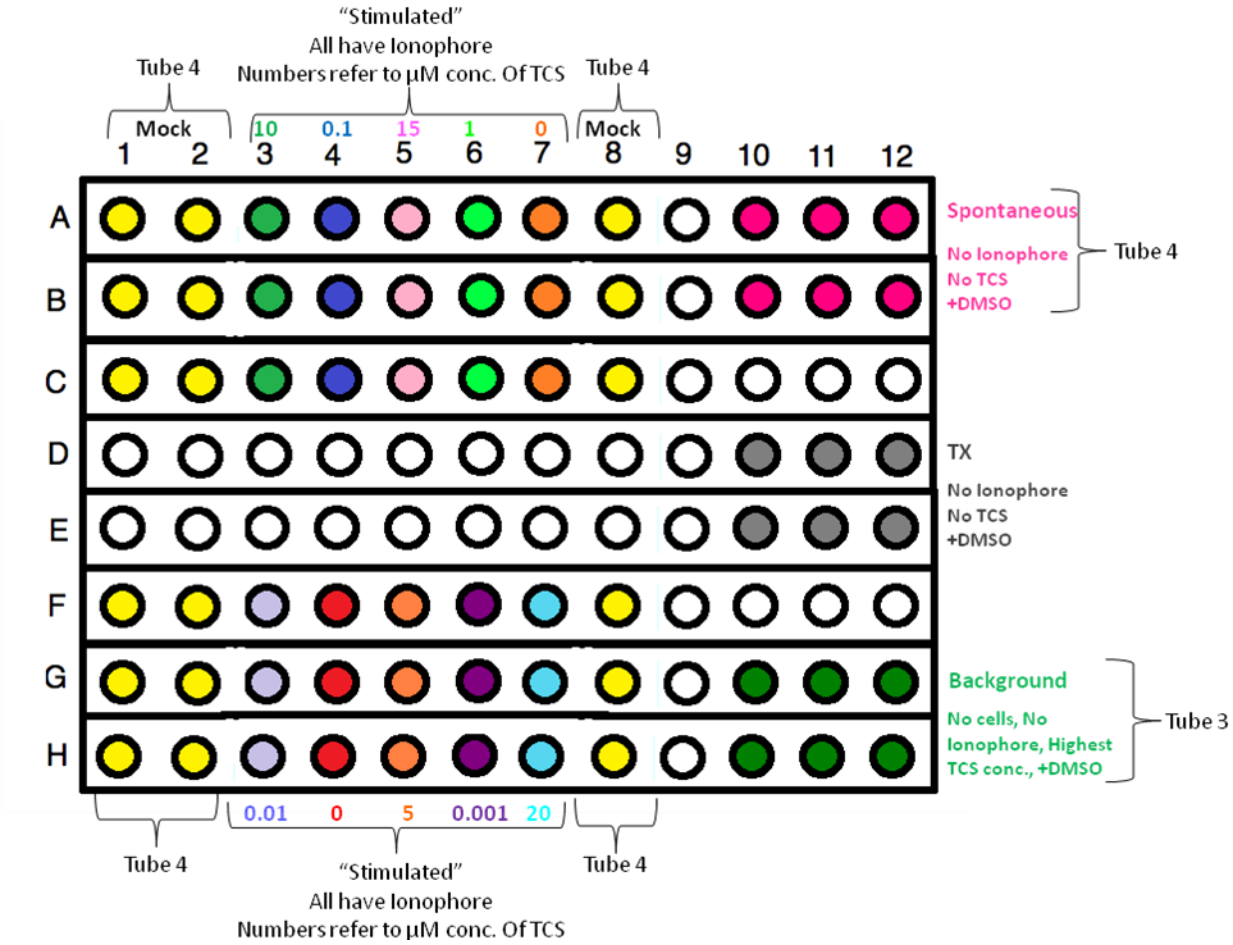
4.11) Average all triplicates, and calculate standard deviation. Graph data in excel as mean values ± standard deviation. For statistical testing, move now to Prism software by Graphpad.

DAY 2:

5. Ionophore stimulated degranulation assay using RBL-2H3 cells

5.1) Follow protocol for "Preparation of cells" (Section 1, Day 1) and "Preparation of triclosan" (Section 2, Day 2), as instructed above. The plate layout example for ionophore stimulation is shown below.

Treatment	Triplicates
Stimulated, 0 µM TCS	A7, B7, C7, F4, G4, H4
Stimulated, 0.001 µM TCS	F6, G6, H6
Stimulated, 0.01 µM TCS	F3, G3, H3
Stimulated, 0.1 µM TCS	A4, B4, C4
Stimulated, 1 µM TCS	A6, B6, C6
Stimulated, 5 µM [TCS	F5, G5, H5
Stimulated, 10 µM TCS	A3, B3, C3
Stimulated, 15 µM TCS	A5, B5, C5
Stimulated, 20 µM TCS	F7, G7, H7
Spontaneous, with DMSO, no TCS (includes mocks)	A10, A11, A12, B10, B11, B12 A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8
TX-100, with DMSO, no TCS	D10, D11, D12, E10, E11, E12
No cells background, with DMSO, plus highest [TCS]	G10, G11, G12, H10, H11, H12



5.2) Before starting, check the pH of all buffers being used, and ensure that they are clear and not cloudy. Tyrodes, sodium acetate buffer, and glycine carbonate buffer (recipes provided in table).

5.3) Prepare one 50 mL conical tube of BT by adding 0.05 g BSA to 50 mL tyrodes buffer and by vortexing to mix well. Incubate in 37°C water bath.

5.4) Make 0.2% Triton X-100 with 0.0032% DMSO (the final calcium ionophore vehicle concentration) by adding 96 µL of 10% Triton X-100 to 4.704 mL BT. Mix well. Next, take out 0.155 µL of this solution and discard. Now add back in 0.155 µL of 100% DMSO.

5.5) Prepare a 5 mM stock (2.5 mg/mL) of A23187 ionophore from powder by adding 400 µL of fresh 100% DMSO into the ionophore vial and vortexing to mix. Once in solution, transfer to a 1.5-mL conical tube, record contents and today's date and expiry (3 months from preparation when stored properly at -20 °C).

5.5.1) Alternatively, if using a frozen stock today, thaw on ice, and check that the A23187 ionophore is well mixed and clear. Vortex, flick, and invert this stock before using. Record date of preparation and Lot # of this A23187.

5.6) Once "TCS-buffer" and "control buffer" have been heated and stirred for 90 minutes, continue the rest of the preparation for TCS protocol (go to steps 2.6-2.8.1). After the BSA is well mixed into both solutions, continue with the remaining protocol steps.

5.7) From the 249 mL sample of "TCS-buffer" solution, transfer 50 mL to a new 50 mL conical tube. Remove 1.8 µL of the 50 mL aliquot and add 1.8 µL of 5 mM ionophore stock. Vortex three times for 8 seconds and invert three times. Final ionophore concentration is 180 nM. Note that this concentration of A23187 will vary depending on stock potency, and an A23187 ionophore dose response is recommended to identify a concentration of A23187 that elicits a degranulation level of roughly 20% maximal release, which has been identified as a non-cytotoxic to RBL-2H3 cells by cytotoxicity assay (see[2]).

5.7.1) Label this "Tube 1, High TCS/+Ionophore/+BT." Used for dilutions and highest TCS concentration exposure.

5.8) From the 249 mL sample of "control buffer" solution, transfer 50 mL to a new 50 mL conical tube. Take out 1.8 µL of the 50 mL aliquot and add back in 1.8 µL of 5 mM ionophore stock. Vortex three times for 8 seconds and invert three times. Final ionophore concentration is 180 nM.

5.8.1) Label this "Tube 2, No TCS/+Ionophore/+BT." This is used for dilutions and 0 µM TCS concentrations.

5.9) From the 249 mL sample of "TCS-buffer" solution, transfer 50 mL to a new 50 mL conical tube. Take out 1.8 µL of the new 50 mL aliquot and add 1.8 µL of 100% DMSO. Vortex three times for 8 seconds and invert three times; no ionophore is added.

5.9.1) Label this "Tube 3, High TCS/No Ionophore/+BT/+DMSO"; used for background.

5.10) From the 249 mL sample of "control buffer" solution, transfer 50 mL to a new 50 mL conical tube. Take out 1.8 µL from the new 50 mL aliquot and add 1.8 µL of 100% DMSO. Vortex three times for 8 seconds and invert three times. No Ionophore is added.

5.10.1) Label this "Tube 4, No TCS/No Ionophore/+ BT/+DMSO"; used for spontaneous release samples.

	BSA	TCS	Ionophore	Added 100% DMSO
Tube 1	✓	High [ ]	✓	NO
Tube 2	✓	NO	✓	NO

Tube 3	✓	High [ ]	NO	✓
Tube 4	✓	NO	NO	✓

5.11) Calculate and record volumes for dilutions after determining the concentration of the "TCS-buffer" stock. Use calibrated P2 and P1000 pipettemen. Total volume for each dilution concentration should be 1 mL, and should be prepared in a sterile microcentrifuge tube:

Concentration	**High Triclosan+Tyrodes+BSA+180 nM A23187 (Tube 1 from above)**	**Heated BT+180 nM A23187 (Tube 2 from above)**
20 µM		
15 µM		
10 µM		
5 µM		
1 µM		
0.1 µM		
0.001 µM		
0 µM (top of plate)	----------	500 µL plus another 500 µL
0 µM (bottom of plate)	----------	500 µL plus another 500 µL

5.12) Take the cells plated yesterday out of the incubator, and empty the media into the sink. Using a combitip, randomly wash cells in the 96-well plate with BT (200 µL/well). Repeat the wash a second time.

5.13) To prepare treatments for application, vortex and invert dilutions right before addition to the plate. Starting with the top section of the plate: Randomly add triplicates of 200 µL each of the correct concentration of TCS to the corresponding well. Continue to bottom of plate. Add "control buffer" solution plus A23187 (from "Tube 2" above) to all "mocks" on the plate.

5.16) Add 200 µL of appropriate solutions to corresponding wells:

5.16.1) Add 200 µL of 0.2% Triton X-100 to "TX"-designated wells.

5.16.2) Next, add 200 µL of Tube 3 to the 3 wells labeled "Background (BkgD)-Highest TCS" on the plate.

5.16.3) Finally, add 200 µL of Tube 4 to six wells labeled "Spontaneous."

5.17) Incubate the plate for 1 hour in 37°C/5% CO_2.

5.18) During the 1 hour incubation: Get two buckets of ice (one for "old" plate in incubator and one for new plate). Thaw 4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide (4-MU) at room temperature for up to 40 minutes, keeping in foil because it is light sensitive.

5.18.1) Once 4-MU stock is thawed, make up 4-MU working solution: 150 µL stock + 14.85 mL of cold acetate buffer (recipe given in table); vortex and invert. Keep in 50 mL centrifuge tube, wrapped in foil, and on ice until use.

5.18.2) Using combitip, randomly add 100 µL cold 4-MU working solution into the very bottom of each well of a NEW Grenier black 96-well plate (on ice bucket #2): start first by adding the working solution randomly within the top of the plate, randomly within the bottom of the plate, randomly within Triton X-100 wells, and finally randomly within background wells.

5.18.3 Get out new box of P200 tips for next step.

5.19) At the end of 1-hour incubation, put cell plate from incubator on ice bucket #1, pipette supernatant up and down 4-5 times (gently, not introducing bubbles), going around the well for good mixing but not touching the cells while mixing. Systematically, take out 25 µL sample from each well and place into the new plate with substrate (same ordering of samples, as originally planned out). Pipette up and down to mix sample thoroughly when in new well, without introducing bubbles.

5.20) Incubate for 30 minutes at 37°C/5% CO_2.

5.21) After 30 minute incubation, randomly add 200 µL cold glycine-carbonate buffer per well (using combitip) to fill wells up to 325 µL total (make this addition to the Triton X-100 samples last, to avoid Triton X-100 spillover). Check for bubbles before reading plate (poke with clean P10 pipette tip to pop any bubbles).

5.22) Run the plate in the fluorescence plate reader (Follow all steps in section 4).

Representative Results:

When heated to 50 °C for 90 minutes, the UV-Vis absorbance spectrum for TCS produces a strong, smooth curve between ~260 and 300 nm, with a peak at 280 nm, as shown in **Figure 1**. UV-Vis spectrophotometry is, therefore, an important tool that can be utilized to calculate concentration, since the published molar absorption coefficient at 280 nm is 4200 L/mol/cm[12]. We have found that TCS does not fall out of solution during the time frame of the entire degranulation experiment, following the 50^{o}C heating (data not shown).

After using this heating method to dissolve TCS directly into aqueous buffer, we examined the effect of TCS on mast cell degranulation using a fluorescence-based assay that was optimized from Naal *et al.*[1] . This assay records the level of β-hexosaminidase released from mast cells after one-hour incubation by detecting a fluorogenic substrate product. Whether stimulated to degranulate by DNP-BSA antigen (**Figure 2**) or calcium ionophore A23187 (**Figure 3**), one can clearly see that TCS causes a significant dose-responsive inhibition of the release of β-hexosaminidase (i.e., degranulation).

Figure 2 is representative of results obtained for IgE-sensitized RBL cells, which were incubated for 1 hour in "TCS-buffer" or "control buffer," and exposed to a DNP-BSA antigen dose of 0.0004 μg/mL. This concentration of DNP-BSA elicited an average absolute degranulation response of 22.5% ± 0.1% (mean ± standard deviation) in the absence of TCS. Statistically significant inhibition of degranulation began at 5 μM, where degranulation levels were 0.79-fold ± 0.05-fold (mean ± SD) of the 0 μM TCS control levels. As the TCS concentration increases, there is a greater dampening effect of TCS, showing a strong dose response relationship. TCS, at 20 μM, almost completely abrogates the degranulation response, to levels roughly equal to spontaneous degranulation (where no antigen is present). Overall, this figure shows strong inhibition of multivalent antigen-stimulated mast cell degranulation due to concentration-verified TCS, without the use of organic solvents.

In **Figure 3,** calcium ionophore A23187 was used as a way to investigate the mechanism of TCS-induced dampening of degranulation in RBL mast cells. A23187 is used as an alternative stimulant because it bypasses the FcεRI crosslinking and other signaling events upstream of calcium influx, but still causes degranulation. RBL mast cells were incubated for 1 hour in "TCS-buffer" or "control buffer," containing a calcium ionophore dose of 180 nM. In the absence of TCS, this concentration of A23187 elicited an average absolute degranulation response of 25.1% ± 4.7% (mean ± standard deviation). Inhibition of degranulation was found with as little as 1 μM TCS (0.63 ± 0.11 [mean ± SD]). As TCS concentration increases, so does the severity of the inhibition: at 5 μM, 0.21-fold ± 0.04-fold of the 0 μM TCS control levels; at 10 μM, 0.09 ± 0.05; at 15 μM, 0.077 ± 0.006; and at 20 μM, 0.09 ± 0.02 (means ± SD). In fact, from 5 μM and higher concentrations of TCS, levels of A23187-induced degranulation were found to be near the level of spontaneous control (where no A23187 is present at all). Overall, Figure 3, in combination with Figure 2, indicates that the molecular events targeted by TCS are likely downstream of calcium influx.

Figure Legends

Figure 1: Representative TCS UV-Vis absorbance spectrum. TCS has a robust peak at 280 nm, allowing easy determination of A_{280}, as well as affording the ability to use the molar extinction coefficient of 4200 L/mol/cm[12] to determine the actual concentration of TCS dissolved in tyrodes buffer. The yellow line indicates the peak at 280 nm. In this example, the absorbance value at 280 nm is 0.11876, which indicates a TCS concentration of 28.28 μM.

Figure 2: A representative degranulation response of IgE-sensitized RBL mast cells exposed to 0.0004 μg/mL DNP-BSA antigen and TCS (0-20 μM). A spontaneous release value (no-antigen present) is depicted for reference. Values represent mean ± standard deviation of triplicate samples. As presented, data were normalized to control (0 μM TCS), and significant differences were determined in Prism software with a one-way ANOVA followed by a Tukey's *post hoc* test (comparisons made to 0.001 μM TCS average response). Significance is represented by ***p<0.001.

Figure 3: A representative degranulation response of RBL mast cells stimulated with 180 nM A23187 calcium Ionophore in the presence of TCS (0-20 μM). A spontaneous release sample (no

ionophore present) is depicted for reference. Values represent mean ± standard deviation of triplicate samples. As presented, data are normalized to control (0 μM TCS), and significant differences were determined in Prism software with a one-way ANOVA followed by a Tukey's *post hoc* test (comparisons made to 0.001 μM TCS average response). Significance is represented by ***p<0.001; **p<0.01.

Discussion:

In 2004, Naal *et al.*[1] developed a mast cell biosensor for high-throughput testing of degranulation. It is a robust assay that we have adapted for our TCS studies and detailed in this video. Prior to the Naal *et al.*[1] assay, mast cell degranulation had been routinely assessed via β-hexosaminidase[59-61], but these early methods utilized fluorimeters in which one sample was read at a time. Importantly, Naal *et al*. established direct concordance between their more high-throughput method utilizing a microplate reader and the earlier method in which samples were read one-at-a-time in a fluorimeter. In sum, Naal *et al.*[1] greatly improved the speed, power, simplicity, and reliability of the assay by adapting it to a high-throughput microplate platform, as well as by incorporating several changes to the workflow. Here, we have further adapted this assay for a study of various test chemicals, in particular, here, the ubiquitous drug TCS. The video details the steps of this very useful assay. Additionally, we have also developed an organic-solvent-free method of applying TCS in aqueous buffer, and we show a simple, low-cost procedure for verifying TCS concentration. These methods should be helpful to the apparently growing field of triclosan toxicology. In this discussion, we detail several considerations for using this degranulation assay to test other chemicals as well.

TCS was prepared directly into aqueous buffer without the aid of organic solvents, concentration was verified by UV-Vis spectrophotometry (**Fig.1**), and then the effect of TCS (< 30 μM) was examined on mast cell degranulation (**Figs. 2,3**), using a fluorescence microplate assay to detect the presence of β-hexosaminidase, a surrogate marker for degranulation. We have found that TCS is able to significantly dampen the release of β-hexosaminidase from RBL mast cells when dissolved in a low concentration of ethanol (0.24% vol/vol)[2] or, as depicted here, directly into aqueous buffer. By foregoing organic solvent, we actually see more pronounced dampening in antigen-induced degranulation as compared our studies in which TCS was dissolved in 0.24% ethanol (vol/vol). For example, here we have demonstrated a >50% reduction in antigen-induced degranulation (0.46-fold ± 0.07), which is much greater than the ~25% reduction we reported for 10 μM TCS dissolved in 0.24% ethanol (0.76-fold ± 0.02)[2]. In the same vein, we determined for A23187-stimulated cells that, by 5 μM, TCS inhibits degranulation to spontaneous release levels; this effect was not demonstrated until 10 μM TCS in our earlier, ethanol-utilizing, study[2]. There are two possible reasons for this discrepancy: either a 0.24% ethanol vehicle[2] attenuates TCS's ability to inhibit active mast cell degranulation, or the TCS we were using was less concentrated than anticipated (since concentrations were not verified by UV-Vis spectrophotometry in the previous study[2]). Regarding the molecular target for TCS's inhibition of mast cell degranulation, it is likely occurring somewhere in the signal transduction cascade downstream of calcium influx[2]. We used calcium Ionophore A23187 as a degranulation stimulant to bypass early signaling events, and TCS's inhibitory effect persisted, indicating that the target for TCS inhibition in the degranulation pathway is not likely

located upstream of calcium influx. We have previously shown that membrane ruffling of these cells is also suppressed due to TCS treatment, suggesting the possibility of a common pathway target[2].

Previous studies have found the absorbance spectrum of TCS having a maximum peak at 280 nm and a molar absorption coefficient was evaluated to be 4200 L/mol/cm at this wavelength (at pH values below the pK_a)[12]. It has been shown that heating the TCS does not lead to thermal degradation[57], and another study has shown success in dissolving TCS in water while being heated to 50°C without aid of an organic solvent[56]. When any new test chemical is used, its solubility in the aqueous buffer, of course, must be carefully considered. We have also found that, when heating the TCS, the shape of the spectral readout is unaffected whether it is heated for 40 minutes or up to 90 minutes (data not shown): this suggests a lack of degradation of the TCS when heated for a longer period of time. Note, however, that TCS dissolution is greater at 90 minutes than 40 minutes. We have also confirmed that TCS does not fall out of solution for the duration of the degranulation experiment (data not shown).

The DNP-BSA antigen and calcium Ionophore concentrations used in this study were chosen on the basis of antigen- and ionophore-dose response assays, and were selected to elicit moderate degranulation levels for the representative **Figures 2 and 3**. An example of an antigen dose response assay can be seen in Figure 1A of our previous work[2]. When determining the antigen or ionophore concentration to be used in your experiment, it is important to be aware that stimulant dose response experiments need to be done periodically, typically at least every two months, since RBL-2H3 cells sometimes function variably. The concentration that yields the desired degranulation percentage can vary depending on the age of the cells and on the antigen/ionophore preparation. Also, as we have seen with inorganic arsenite[22], absolute degranulation percentages (levels of antigen used) can affect levels of toxicant effects on RBL degranulation, so toxicant dose-responses should be done at several different antigen/ionophore concentrations. It is also important to consider the final concentration of DMSO vehicle when stimulating degranulation with ionophore, since degranulation is affected by DMSO[62]. We have found the DMSO concentrations used in this protocol do not affect degranulation, background readings, or 0.2% Triton X-100 values[2].

In addition to the multivalent antigen DNP-BSA and the calcium ionophore A23187, there exist several other methods of RBL-2H3 stimulation. One of these methods is stimulation via exposure to compound 48/80 along with quercetin[63]. Another is crosslinking of IgE-bound receptors with an anti-IgE IgG, as we previously tested along with TCS exposure[2]. Many other stimulation methods exist, and each of these methods addresses a different mechanistic aspect of mast cell degranulation. This plate reader assay can be adapted for use with many of these alternative stimulators, further expanding its utility.

This degranulation protocol has the potential to be used with a wide variety of chemicals. In a study of any test chemical using this assay, controls must be run for the following: (1) effect of the test chemical on background (no cells) readings; (2) effect of the chemical on spontaneous degranulation (cells with no IgE, no antigen, no ionophore); (3) effect

of the chemical on Triton-X-100 values of lysed cells (no antigen, no ionophore). These tests can be easily worked into the plate layout. Previously, we found the TCS affects none of these three parameters[2]. Additionally, tests should be run to determine that the test chemical does not interfere with the β-hexosaminidase enzyme/substrate reaction itself in a cell-free preparation, as described in Fig. S1 of the Appendix A supplementary data section of Palmer *et al*[2]. We found that TCS does not interfere with the ability of β-hexosaminidase to cleave the fluorogenic substrate 4-MU[2]. Effects of any solvents used must also be considered in all these control experiments. For example, we confirmed that DMSO, the solvent for the ionophore, has no effect on Triton-X-100 sample fluorescence levels (data not shown). We also note that we selected all plastics used in this study for not containing the endocrine disruptor bisphenol A; unfortunately, though, all plastics currently on the market probably do contain some endocrine disrupting activity, which could potentially confound data[64].

In the event that troubleshooting is required, several potential aspects of this protocol should be reviewed. For example, it may be that (1) spontaneous release levels are too high (greater than ~7% of lysis values); (2) a dose-response with either stimulant and/or test chemical is not observed; or (3) the TCS concentration in solution is too low (lower than 20 µM). In the first case, a high spontaneous level could be an indication of the cells being in culture too long or being contaminated with mycoplasma; therefore, try these experiments with RBL-2H3 cells that have been in culture between 2-20 weeks, and regularly test for mycoplasma. If a stimulant dose response is not observed, the dissolved stimulant concentration may be too low, and stocks should be re-made. As an example, calcium ionophore typically is provided as a thin film, to be reconstituted with DMSO, requiring careful attention and much vortexing. Additionally, a new ionophore stock with a different lot number could have a different potency simply due to lot-to-lot variation; therefore, a degranulation dose response is recommended with each newly purchased ionophore stock. It is also worth noting that an apparent lack of effect with a given test chemical could be an indication that this chemical may require a longer incubation period in order to cause an effect. If you are not achieving a high TCS yield in solution, check that the temperature has remained constant (50°C ± 5°C) while the granules are dissolving into buffer. The thermometer should never touch the bottom of the flask, a position that would result in an overestimate of the temperature of the solution. Also, make sure there is constant vigorous stirring and that the 90 minute countdown is not started until the temperature has first reached 50°C.

Table for Troubleshooting.

Problem	**Potential Reason**	**Solution**
TCS stock is determined to be < 20 µM	Non-uniform heating of the solution	Ensure that the thermometer is positioned so that it is suspended in the solution and is not touching the bottom of the flask.

	Stirring is not sufficiently vigorous	Increase magnetic stirring on stir-plate to achieve a level of stirring that is vigorous without causing the solution to jump out of the flask. Ensure that an appropriately sized magnetic stir bar is used.
	Problems with spectrophotometer	Allow for proper warmup of UV lamp (typically 10 minutes), or replace bulb if necessary.
Spontaneous degranulation levels are too high (>~7%)	Cells have acquired abnormal genetic mutations due to too much time in culture	Perform experiments with a new cell thaw.
	Cells are dying because of mechanical shearing	When adding buffer or treatments adherent cells, be careful not to disturb the cells, by adding these volumes carefully to the sides of the microwells. Practice using the combitip.
IgE/DNP-BSA does not cause release of beta-hexosaminidase over spontaneous release levels	IgE is older than 30 days or has been subjected to freeze thaw	Use a new, properly stored aliquot of IgE.
	DNP-BSA has not been properly mixed	Be sure to carefully add the small volume of DNP-BSA to the conical tube and to vortex thoroughly.
A23187 ionophore does not cause release of beta-hexosaminidase over spontaneous release levels	A23187 stock has not been properly reconstituted	Product arrives as a "thin film," and must be reconstituted with care and much vortexing. Transfer reconstituted stock to a new 1.5-mL tube for storage.
	A23187 stock has not been properly stored	Stocks are light sensitive. Once reconstituted, parafilm the top, and store wrapped in

		foil at -20°C. If there is a question about the storage of a stock, discard and begin tests with a new stock.
180 nM of A23187 ionophore does not elicit the same level of relative degranulation response, as that found in an earlier assay	Lot-to-lot variation of A23187 ionophore	Perform a dose response experiment for each new lot of ionophore. It is also recommended that stocks from the same lot be tested, due to potential variability in the reconstitution process.

As in any toxicology/pharmacology experiment, the test chemical must not be overtly toxic at the tested concentrations. We recommend using methods that test for both apoptosis and necrosis, either individually or combined (such as with clonogenic assays), as well as tests for general damage to the plasma membrane (such as lactate dehydrogenase leakage). TCS, at concentrations shown in this study, is not cytotoxic to RBL-2H3 cells[2]. A particular note of concern with the ionophore studies is that ionophore plus ionophore vehicle (likely DMSO), plus test chemical, plus any organic solvents used, could be a potentially cytotoxic brew, which must be carefully controlled, as done in Palmer *et al.*[2].

Our protocol for preparing TCS solutions without the use of an organic solvent will be useful for further toxicological testing of this ubiquitous chemical, without the interference of solvent artifacts, a particularly important consideration in aquatic toxicology. These methods also allow verification of the concentration of TCS in solution and quantification of the effects that chemicals, such as TCS, have on mast cell degranulation. This protocol can be used to assess the effects of a wide variety of chemicals on mast cell degranulation, such as suspected endocrine disrupting chemicals[55], and can potentially be scaled up for high throughput screening. Additionally, other mast cell types may be used in this assay in future work.

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Disclosures:
We have nothing to disclose.

Reagents:

Name of Reagent	Company	Catalogue Number	Comments (optional)
RBL-2H3 Cells	ATCC	CRL-2256	The cells we used were a gift, but they are also available from ATCC
Triclosan/Irgasan	Sigma	72779 CAS# 3380-34-5	Should be stored in a low humidity environment
trypsin	Gibco	25300-054 CAS# 3380-34-5	
EMEM	Lonza	12-611F	
Fetal Bovine Serum	Atlanta Biologicals	S11150	
Gentamycin Sulfate	Lonza Biological Sciences	17-518	
Albumin, Bovine Serum	Calbiochem	12659 CAS# 9048-46-8	
Surfact-Amps X-100 (Triton X-100; 10% solution)	Pierce	28314 CAS# 9002-93-1	
HEPES	J.T Baker	4153-01 CAS# 75277-39-3	
Magnesium Chloride	VWR	BDH0244-500G CAS# 7791-18-6	
D-(+)-Glucose	Biomedicals	152527 CAS# 50-99-7	
Potassium Chloride Crystal	J.T Baker	3046-01 CAS# 7447-40-7	
Calcium chloride dihyrdate	Acros Organics	207780010 CAS# 10035-04-8	
Glycine	Sigma	G8898 CAS# 56-40-6	
4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MU)	EMD Biosciences	474502-250MG CAS # 37067-30-4	Wrap in foil – is light-sensitive
Anti-DNP mouse IgE	Sigma	D8406	Reagent has concentration of 1 mg/mL. Aliquot 25 µL of reagent into

			separate microcentrifuge tubes and parafilm. Store aliquots at -20°C that are not being used and store aliquot that is being used at 2-8°C for no longer than 1 month.
DNP-BSA	Gift from Dr. David Holowka and Dr. Barbara Baird, Cornell University		Suggest: life technologies DNP-BSA catalog# A23018
Calcium ionophore A23187	Sigma	C75-22-1mg	Ionophore was made from a powder by adding 400 µL of fresh 100% DMSO into the ionophore vial and is kept at -20°C Note: we have used the ionophore past its 3 month expiration date successfuly
DMSO	Sigma	D2650 CAS# 67-68-5	
Acetic Acid	VWR	BDH3094-2 CAS# 64-19-7	
Anhydrous Sodium Carbonate	Sigma	222321 CAS# 497-19-8	
Sodium Chloride	Sigma	71376 CAS# 7647-14-5	
Hydrochloric Acid	VWR	BDH3026 CAS# 7647-01-0	
Reference Buffer, pH 7	VWR	BDH5046	
Reference Buffer, pH 10	VWR	BDH5072	
Reference Buffer, pH 4	VWR	BDH5018	
pH electrode storage solution	VWR	14002-828	

Equipment:

Material Name	Company	Catalogue Number	Comments (optional)
DU 7500 Spectrophotometer	Beckmann	No longer sold	
Synergy 2 plate reader Uses Gen5 Microplate Data collection and analysis software	BioTek	Module S	
Hematocytometer	Hausser Scientific	3110	
7X7 CER HOT/STIR 120V Combination hot plate/magnetic stir plate	VWR	97042-634	
Centrifuge	Eppendorf	5430	
Tissue culture water bath	VWR	Model# 89032-206	
Tissue Culture biological safety cabinet SafeGARD (TC hood)	The Baker Company	Model# SG403A-HE	
Tissue culture incubator	ThermoScientific	Model# 3598	
Pipettemen	VWR		Range: p2-p1000
Balance	Mettler Toledo	Model# AG204	
pH meter	Symphony/VWR	Model# SB70P	
Pipet-aid	Drummond Scientific	4-000-100	
Combitip dispenser	Eppendorf	4981 000.019	

Recipes:

Name	Recipe	Notes
Acetate Buffer, pH 4.4	• Make 0.12 M acetic acid and titrate to pH 4.4 with 10 N NaOH. • This is 5.3 mL glacial acetic acid into 1 L of MilliQ water: (1L)*(0.12 mol/L)*(60 g/mol)*(mL/1.37 g) = 5.3 mL because density of glacial is 1.37 g/mL	Sterile Filter into autoclaved glass bottle
Substrate (4-MU)	• Sigma M-2133, 250 mg, $C_{18}H_{21}NO_8$, FW 379.4 CAS (37067-30-4) • Store in -20°C • Stock: 0.12 M in DMSO (46 mg in 1 mL DMSO), warm to 37°C, vortex, sonicate 10 min. in water-bath sonicator with warm water, vortex	For each experiment, make fresh solution of substrate in acetate buffer

	again	(100x dilution), for final concentration of 1.2 mM in acetate buffer
Glycine Carbonate Buffer, pH 10	• 26.7 g glycine • 47.1 g anhydrous sodium carbonate • Add deionized water for 1 L, and adjust pH to 10	Sterile filter into autoclaved glass bottle
Tyrodes (2 L), pH 7.4	• 135 mM NaCl: 15.78 g (or 270 mL of 1 M) • 5 mM KCl: 10 mL of 1 M stock • 1.8 mM $CaCl_2$: 7.20 mL of 0.5 M stock • 1 mM $MgCl_2$: 4.00 mL of 0.5 M stock • 5.6 mM glucose: 2.02 g (11.2 mL of 1 M) • 20 mM HEPES: 40 mL of 1 M stock • Using concentrated HCl pH from ~9.7 to 7.4	Sterile filter into autoclaved glass bottle
RBL Cell Media	• Thaw fetal bovine serum (FBS, stored at -20°C) for about 4 hours in 37°C water bath • Follow standard sterile technique • Get out 1 L minimum essential medium (MEM) with L-glutamine (with Earle's salts) • Pour off some MEM to have 800 mL MEM, add 200 mL warm FBS • Add 1 mL gentamicin sulfate antibiotic to 1 L of media with sterile pipette • Only use media bottles that have been autoclaved and marked for cell culture use only.	Sterile filter (0.2 μm) into autoclaved glass bottle

Plastic material used:

Material Name	Company	Catalogue Number	Type of Plastic
200 µL Disposable sterile pipet tips with graduations in 96 rack	VWR	53509-009	polypropylene
1000 µL Sterile aerosol pipet tips with HighRecovery	VWR	89003-420	polyethylene
10 µL micro tip low binding sterile	VWR	14217-704	polypropylene
Disposable/conical Micro-centrifuge tubes for high G-force	VWR	20170-038	polypropylene
Disposable/graduated/conical/sterile 50 mL	VWR	21008-178	polypropylene

centrifuge tubes with screw caps			
Disposable/graduated/conical/sterile 15 mL centrifuge tubes with screw caps	VWR	21008-103	polypropylene
CELLSTAR Tissue Culture Treated T-25 Flask w/ Filter Cap	Greiner bio-one	690175	polystyrene
CELLSTAR Tissue Culture Treated T-75 Flask w/ Filter Cap	Greiner bio-one	658175	polystyrene
CELLSTAR 10 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	607180	polystyrene
CELLSTAR 2 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	710180	polystyrene
CELLSTAR 5 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	606180	polystyrene
CELLSTAR 25 mL Paper /Plastic Wrapped Serological Pipette	Greiner bio-one	760180	polystyrene
1 cm cuvettes	N/A	N/A	polystyrene
CELLSTAR, 96W Microplate, Tissue-Culture Treated, Black, with Lid 96-well Plate	Greiner Bio One	655086	polystyrene
Combitips	Eppendorf	022266501	Polypropylene/ polyethylene

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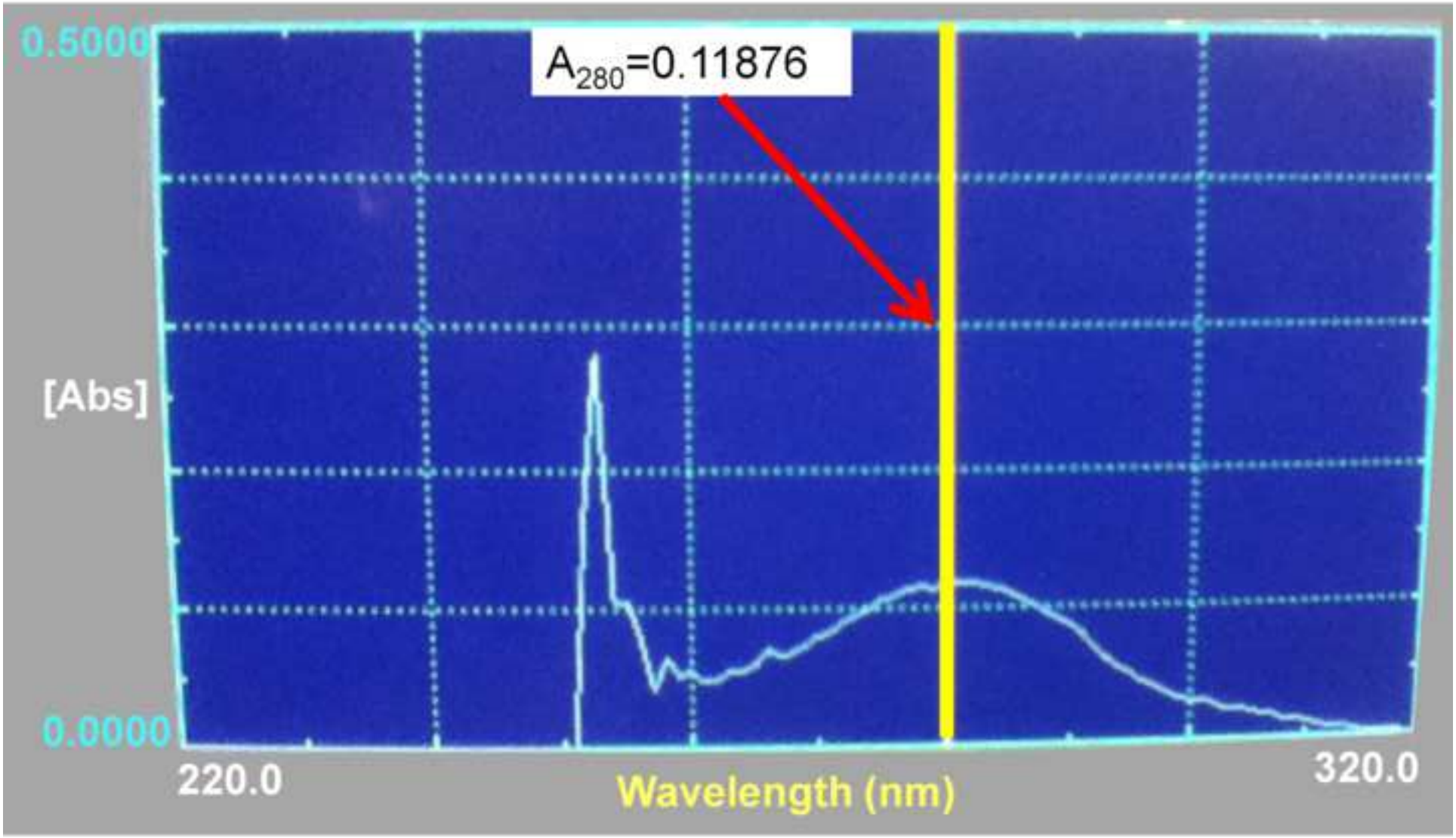

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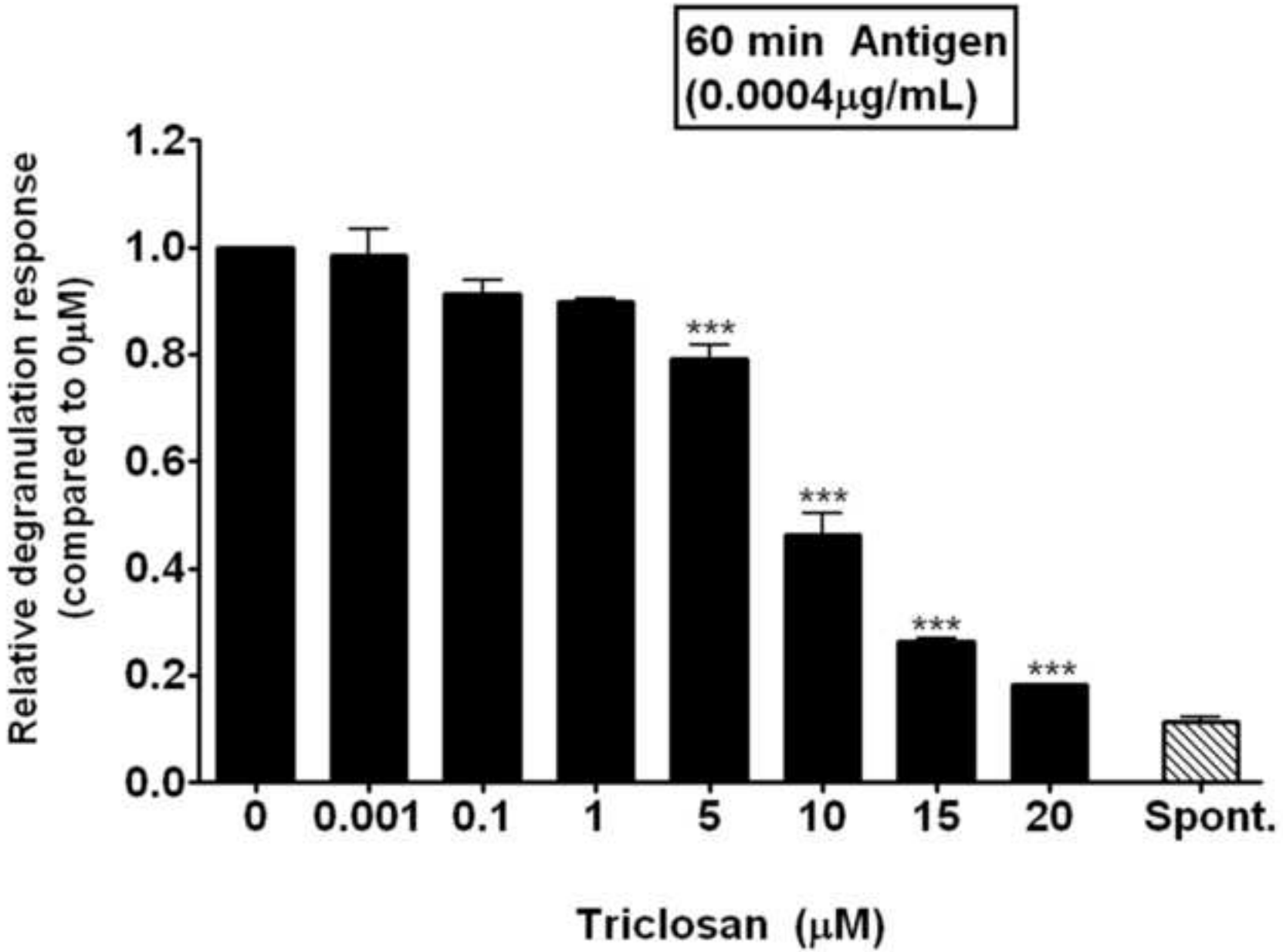

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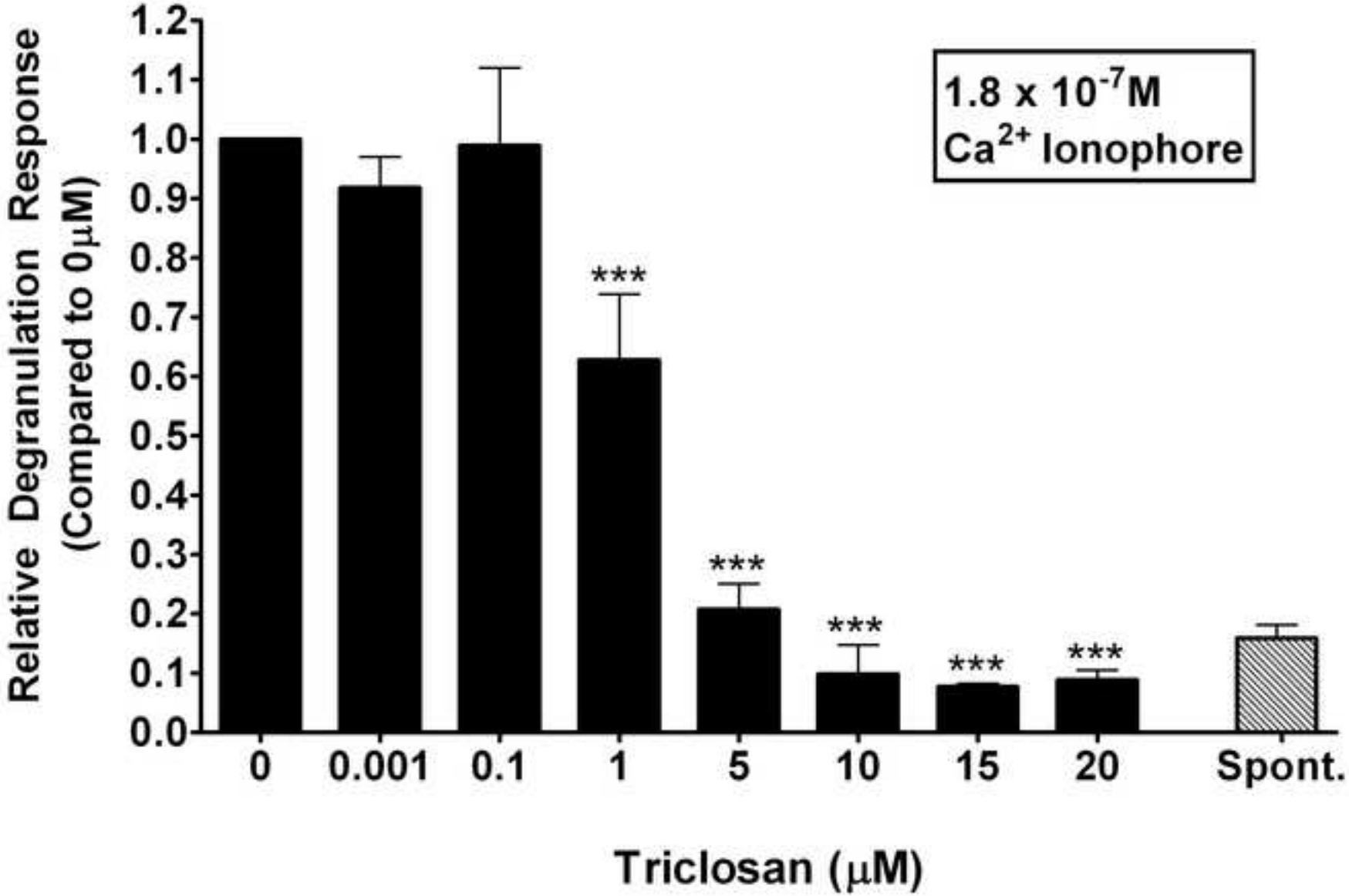

Animated/Video Figure

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*Table of Reagents/ Materials Used

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Reagents:

Name of Reagent	Company	Catalogue Number
RBL-2H3 Cells	ATCC	CRL-2256
Triclosan/Irgasan	Sigma	72779 CAS# 3380-34-5
trypsin	Gibco	25300-054 CAS# 3380-34-5
EMEM	Lonza	12-611F
Fetal Bovine Serum	Atlanta Biologicals	S11150
Gentamycin Sulfate	Lonza Biological Sciences	17-518
Albumin, Bovine Serum	Calbiochem	12659 CAS# 9048-46-8
Surfact-Amps X-100 (Triton X-100; 10% solution)	Pierce	28314 CAS# 9002-93-1
HEPES	J.T Baker	4153-01 CAS# 75277-39-3
Magnesium Chloride	VWR	BDH0244-500G CAS# 7791-18-6
D-(+)-Glucose	Biomedicals	152527 CAS# 50-99-7
Potassium Chloride Crystal	J.T Baker	3046-01 CAS# 7447-40-7
Calcium chloride dihyrdate	Acros Organics	207780010 CAS# 10035-04-8
Glycine	Sigma	G8898 CAS# 56-40-6
4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MU)	EMD Biosciences	474502-250MG CAS # 37067-30-4

Anti-DNP mouse IgE	Sigma	D8406
DNP-BSA	Gift from Dr. David Holowka and Dr. Barbara Baird, Cornell University	
Calcium ionophore A23187	Sigma	C75-22-1mg
DMSO	Sigma	D2650 CAS# 67-68-5
Acetic Acid	VWR	BDH3094-2 CAS# 64-19-7
Anhydrous Sodium Carbonate	Sigma	222321 CAS# 497-19-8
Sodium Chloride	Sigma	71376 CAS# 7647-14-5
Hydrochloric Acid	VWR	BDH3026 CAS# 7647-01-0
Reference Buffer, pH 7	VWR	BDH5046
Reference Buffer, pH 10	VWR	BDH5072
Reference Buffer, pH 4	VWR	BDH5018
pH electrode storage solution	VWR	14002-828

Comments (optional)
The cells we used were a gift, but they are also available from ATCC
Should be stored in a low humidity environment
Wrap in foil – is light-sensitive

Reagent has concentration of 1 mg/mL. Aliquot 25 µL of reagent into separate microcentrifuge tubes and parafilm. Store aliquots at -20°C that are not being used and store aliquot that is being used at 2-8°C for no longer than 1 month.
Suggest: life technologies DNP-BSA catalog# A23018
Ionophore was made from a powder by adding 400 µL of fresh 100% DMSO into the ionophore vial and is kept at -20°C Note: we have used the ionophore past its 3 month expiration date successfuly

Equipment:

Material Name	Company	Catalogue Number
DU 7500 Spectrophotometer	Beckmann	No longer sold
Synergy 2 plate reader Uses Gen5 Microplate Data collection and analysis software	BioTek	Module S
Hematocytometer	Hausser Scientific	3110
7X7 CER HOT/STIR 120V Combination hot plate/magnetic stir plate	VWR	97042-634
Centrifuge	Eppendorf	5430
Tissue culture water bath	VWR	Model# 89032-206
Tissue Culture biological safety cabinet SafeGARD (TC hood)	The Baker Company	Model# SG403A-HE
Tissue culture incubator	ThermoScientific	Model# 3598
Pipettemen	VWR	
Balance	Mettler Toledo	Model# AG204
pH meter	Symphony/VWR	Model# SB70P
Pipet-aid	Drummond	4-000-100
Combitip dispenser	Eppendorf	4981 000.019

Comments (optional)
Range: p2-p1000

Recipes:

Name	Recipe
Acetate Buffer, pH 4.4	• Make 0.12 M acetic acid and titrate to pH 4.4 with 10 N NaOH. • This is 5.3 mL glacial acetic acid into 1 L of MilliQ water: (1L)*(0.12 mol/L)*(60 g/mol)*(mL/1.37 g) = 5.3 mL because density of glacial is 1.37 g/mL
Substrate (4-MU)	• Sigma M-2133, 250 mg, $C_{18}H_{21}NO_8$, FW 379.4 CAS (37067-30-4) • Store in -20°C • Stock: 0.12 M in DMSO (46 mg in 1 mL DMSO), warm to 37°C, vortex, sonicate 10 min. in water-bath sonicator with warm water, vortex again
Glycine Carbonate Buffer, pH 10	• 26.7 g glycine • 47.1 g anhydrous sodium carbonate • Add deionized water for 1 L, and adjust pH to 10
Tyrodes (2 L), pH 7.4	• 135 mM NaCl: 15.78 g (or 270 mL of 1 M) • 5 mM KCl: 10 mL of 1 M stock • 1.8 mM $CaCl_2$: 7.20 mL of 0.5 M stock • 1 mM $MgCl_2$: 4.00 mL of 0.5 M stock • 5.6 mM glucose: 2.02 g (11.2 mL of 1 M) • 20 mM HEPES: 40 mL of 1 M stock • Using concentrated HCl pH from ~9.7 to 7.4
RBL Cell Media	• Thaw fetal bovine serum (FBS, stored at -20°C) for about 4 hours in 37°C water bath • Follow standard sterile technique • Get out 1 L minimum essential medium (MEM) with L-glutamine (with Earle's salts) • Pour off some MEM to have 800 mL MEM, add 200 mL warm FBS

	• Add 1 mL gentamicin sulfate antibiotic to 1 L of media with sterile pipette • Only use media bottles that have been autoclaved and marked for cell culture use only.

Notes
Sterile Filter into autoclaved glass bottle
For each experiment, make fresh solution of substrate in acetate buffer (100x dilution), for final concentration of 1.2 mM in acetate buffer
Sterile filter into autoclaved glass bottle
Sterile filter into autoclaved glass bottle
Sterile filter (0.2 μm) into autoclaved glass bottle

Plastics:

Material Name	Company	Catalogue Number
200 µL Disposable sterile pipet tips with graduations in 96 rack	VWR	53509-009
1000 µL Sterile aerosol pipet tips with HighRecovery	VWR	89003-420
10 µL micro tip low binding sterile	VWR	14217-704
Disposable/conical Micro-centrifuge tubes for high G-force	VWR	20170-038
Disposable/graduated/conical/sterile 50 mL centrifuge tubes with screw caps	VWR	21008-178
Disposable/graduated/conical/sterile 15 mL centrifuge tubes with screw caps	VWR	21008-103
CELLSTAR® Tissue Culture Treated T-25 Flask w/ Filter Cap	Greiner bio-one	690175
CELLSTAR® Tissue Culture Treated T-75 Flask w/ Filter Cap	Greiner bio-one	658175
CELLSTAR® 10 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	607180
CELLSTAR® CELLSTAR® 2 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	710180
CELLSTAR® 5 mL Paper/Plastic Wrapped Serological Pipette	Greiner bio-one	606180
CELLSTAR® 25 mL Paper /Plastic Wrapped Serological Pipette	Greiner bio-one	760180
1 cm cuvettes	N/A	N/A

CELLSTAR®, 96W Microplate, Tissue-Culture Treated, Black, with Lid 96-well Plate	Greiner Bio One	655086
Combitips	Eppendorf	22266501

Type of Plastic
polypropylene
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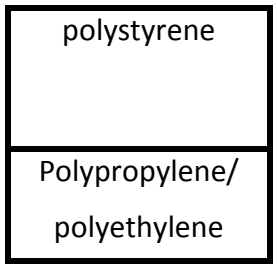
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Title of Article: A Microplate Assay to Assess Chemical Effects on RBL-2H3 Mast Cell Degranulation: Effects of Triclosan Without Use of an Organic Solvent

Author(s): Lisa M. Weatherly, Rachel H. Kennedy, Julie A. Gosse

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Article Title: A Microplate Assay to Assess Chemical Effects on RBL-2H3 Mast Cell Degranulation: Effects of Triclosan without Use of an Organic Solvent

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We thank both reviewers for their thoughtful and constructive criticism. We believe that, after making the reviewers' suggested changes, we are re-submitting an improved manuscript.

Editorial comments:

* Protocol text:
Please see the highlighted portion of your protocol. We would like to clarify the parts of the protocol you would like to include in the video. The highlighted parts should include complete steps and important transitions between actions.
*For example, the very first highlighted text reads: "remove spent media from flask with sterile pipette." What media is being removed? What else is in that flask?
Also, the highlighted portion can only be 3 pages or less. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

In an effort to clarify the parts of the protocol that we would like to include in the video, the highlighted sections have been revised.

Although it has technically not been yellow-highlighted to indicate that we would like it to be part of the video section, we suggest that the video show the colored 96-well plate setup scheme.

Additionally, to avoid possible confusion regarding the word "spent," where the phrase "spent media" once appeared in the manuscript, it has been replaced to "all media," or "media" (the term "spent" has been deleted altogether).

Reviewer #1:
Manuscript Summary:
The authors of this method paper provide a detailed and thorough protocol for a high throughput assay for the measurement of RBL-2H3 mast cell degranulation in the context of assessing chemical inhibition by Triclosan. Their representative results demonstrate that Triclosan dissolved in aqueous buffer is capable of inhibiting antigen-mediated degranulation in a dose dependent manner.

Overall the writing quality was good and there is certainly enough detail to carry out the experiment. The introductory material provides sufficient detail to properly understand the system and scientifically relevant considerations for the experimental design.

Thank you.

Major Concerns:
I have no major concerns over the material covered in this methods paper. The authors provide sufficient information for successful execution of the protocol.

Minor Concerns:
(1) The authors show data indicating that calcium ionophore-triggered degranulation is also inhibited. However, the degranulation values displayed in figure 3 are somewhat concerning. The spontaneous degranulation is quite high (50% of the degranulation in those cells stimulated with ionophore but without Triclosan). This is likely, in part, a result of the fact that the absolute value of degranulation of cells treated with ionophore alone is only 6.9%. So while they see a significant reduction of ionophore-triggered degranulation in the presence of Triclosan, the actual reduction is perhaps not very biologically relevant, because the dynamic range of their data is only 3-4%. The doses used for both antigen and ionophore are quite low, which translate to degranulation percentages are also quite low, so their results are not unreasonable, it is just more difficult to make conclusions based on such small relative differences. That being said, the authors correctly point out in their discussion that balancing antigen and toxicant dose is an important factor.

We thank the reviewer for a close reading of the text and its figures, and for emphasizing that a greater dynamic range of the data would be desirable. In the manuscript we submitted, a concentration of 160 nM A23187 calcium Ionophore was used to elicit a degranulation response from RBL-2H3 cells. Thus, we have addressed the reviewer's comments by repeating the ionophore experiment with a higher concentration of ionophore, 180 nM. This level of ionophore elicited an absolute value of degranulation of ~25% (much higher than the 6.9% in the first manuscript draft), whereas the absolute level of the "spontaneous" degranulation in this same experiment was approximately 4%. Please note that the overall data trends and the statistical significance are very similar to those reported in the first draft of the manuscript.

We are therefore submitting a revised Figure 3 that reflects these changes. The representative results and legend for Figure 3 have been changed to represent these revisions (changes are highlighted in green):

change in the "Results" section:
"RBL mast cells were incubated for 1 hour in "TCS-buffer" or "control buffer," containing a calcium ionophore dose of 180 nM. In the absence of TCS, this concentration of A23187 elicited an average absolute degranulation response of 25.1% ± 4.7% (mean ± standard deviation). Inhibition of degranulation was found with as little as 1 µM TCS (0.63 ± 0.11 [mean ± SD]). As TCS concentration increases, so does the severity of the

inhibition: at 5 µM, 0.21-fold ± 0.04-fold of the 0 µM TCS control levels; at 10 µM, 0.09 ± 0.05; at 15 µM, 0.077 ± 0.006; and at 20 µM, 0.09 ± 0.02 (means ± SD). In fact, from 5 µM and higher concentrations of TCS, levels of A23187-induced degranulation were found to be near the level of spontaneous control (where no A23187 is present at all)."

change in the figure legend:
"**Figure 3**: A representative degranulation response of RBL mast cells stimulated with 180 nM A23187 calcium Ionophore in the presence of TCS (0-20 µM)."

(2) I found some sections of the protocol a little hard to follow. For example, while I understood the concept behind the 96 well plate scheme, it was a difficult easily determine what was what on the layout provided. A table of what samples to include might be clearer than the list of given in step 1.1.

For the sake of clarity, we have added tables in addition to the 96-well plate layouts. The following tables were added to section 1.1 and section 5.1, respectively, of the manuscript.

Antigen Table

Treatment	**Triplicates**
Stimulated, 0 µM TCS	A7, B7, C7, F4, G4, H4
Stimulated, 0.001 µM TCS	F6, G6, H6
Stimulated, 0.1 µM TCS	A4, B4, C4
Stimulated, 1 µM TCS	A6, B6, C6
Stimulated, 5 µM TCS	F5, G5, H5
Stimulated, 10 µM TCS	A3, B3, C3
Stimulated, 15 µM TCS	A5, B5, C5
Stimulated, 20 µM TCS	F7, G7, H7
Stimulated, plus highest [TCS]	F3, G3, H3
Spontaneous, no TCS (includes mocks)	A10, A11, A12, B10, B11, B12 A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8
TX-100, no TCS	D10, D11, D12, E10, E11, E12
No cells background, plus highest [TCS]	G10, G11, G12, H10, H11, H12

Ionophore Table

Treatment	**Triplicates**
Stimulated, 0 µM TCS	A7, B7, C7, F4, G4, H4

Stimulated, 0.001 µM TCS	F6, G6, H6
Stimulated, 0.01 µM TCS	F3, G3, H3
Stimulated, 0.1 µM TCS	A4, B4, C4
Stimulated, 1 µM TCS	A6, B6, C6
Stimulated, 5 µM [TCS	F5, G5, H5
Stimulated, 10 µM TCS	A3, B3, C3
Stimulated, 15 µM TCS	A5, B5, C5
Stimulated, 20 µM TCS	F7, G7, H7
Spontaneous, with DMSO, no TCS (includes mocks)	A10, A11, A12, B10, B11, B12 A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8
TX-100, with DMSO, no TCS	D10, D11, D12, E10, E11, E12
No cells background, with DMSO, plus highest [TCS]	G10, G11, G12, H10, H11, H12

(4) The addition of a trouble shooting section (either in the discussion or in an accompanying table), should readers have experimental difficulties, would be a useful.

Although it was not specifically labeled as a troubleshooting section in the text, we had detailed possible confounding effects in paragraphs 5 and 6 of the Discussion section. To make it clearer that we are addressing troubleshooting in these paragraphs, we have added the following paragraph to the "Discussion" section, as well as a table:

In the event that troubleshooting is required, several potential aspects of this protocol should be reviewed. For example, it may be that (1) spontaneous release levels are too high (greater than ~7% of lysis values); (2) a dose-response with either stimulant and/or test chemical is not observed; or (3) the TCS concentration in solution is too low (lower than 20 µM). In the first case, a high spontaneous level could be an indication of the cells' being in culture too long or being contaminated with mycoplasma; therefore, try these experiments with RBL-2H3 cells that have been in culture between 2-20 weeks, and regularly test for mycoplasma. If a stimulant dose response is not observed, the dissolved stimulant concentration may be too low, and stocks should be re-made. As an example, calcium ionophore typically is provided as a thin film, to be reconstituted with DMSO, requiring careful attention and much vortexing. Additionally, a new ionophore stock with a different lot number could have a different potency simply due to lot-to-lot variation; therefore, a degranulation dose response is recommended with each newly purchased ionophore stock. It is also worth noting that an apparent lack of effect with a given test chemical could be an indication that this chemical may require a longer incubation period in order to cause an effect. If you are not achieving a high TCS yield in solution, check that the temperature has remained constant (50°C

± 5°C) while the granules are dissolving into buffer. The thermometer should never touch the bottom of the flask, a position that would result in an overestimate of the temperature of the solution. Also, make sure there is constant vigorous stirring and that the 90 minute countdown is not started until the temperature has first reached 50°C.

Table for Troubleshooting.

Problem	**Potential Reason**	**Solution**
TCS stock is determined to be < 20 µM	Non-uniform heating of the solution	Ensure that the thermometer is positioned so that it is suspended in the solution and is not touching the bottom of the flask.
	Stirring is not vigorous enough	Increase magnetic stirring on stir-plate to achieve a level of stirring that is vigorous without causing the solution to jump out of the flask. Ensure that an appropriately sized magnetic stir-bar is used.
	Problems with spectrophotometer	Allow for proper warm up of UV lamp (typically 10 minutes), or replace bulb if necessary.
Spontaneous degranulation levels are too high (>~7%)	Cells have acquired abnormal genetic mutations due to too much time in culture	Perform experiments with a new cell thaw.
	Cells are dying because of mechanical shearing	When adding buffer or treatment to plates that have adherent cells, be careful not to force cells to detach, by adding these volumes carefully to the sides of the microwells. Practice using the combitip.
IgE/DNP-BSA does not cause release of beta-hexosaminidase over spontaneous release levels	IgE is older than 30 days or has been subjected to freeze/ thaw	Use a new, properly stored aliquot of IgE.

	DNP-BSA has not been properly mixed	Be sure to carefully add the small volume of DNP-BSA to the conical tube and to vortex thoroughly.
A23187 ionophore does not cause release of beta-hexosaminidase over spontaneous release levels	A23187 stock has not been properly reconstituted	Product arrives as a "thin film," and must be reconstituted with care and much vortexing. Transfer reconstituted stock to a new 1.5-mL tube for storage.
	A23187 stock has not been properly stored	Stocks are light sensitive. Once reconstituted, parafilm the top, and store wrapped in foil at -20°C. If there is a question about the storage of a stock, discard and begin tests with a new stock.
180 nM of A23187 ionophore does not elicit the same level of relative degranulation response	Lot-to-lot variation of A23187 ionophore	Perform a dose response experiment for each new lot of ionophore. It is also recommended that stocks from the same lot be tested, due to potential variability in the reconstitution process.

(5) The third paragraph on pg. 4 the following phrase is used: "also very much recent attention being paid", this phrasing is awkward, and I would suggest rewording.

The sentence was reworded: "Beyond its potential as a therapeutic aid in allergic skin disease (or, conversely, as an immunotoxicant), TCS may also be an endocrine disruptor."

Additional Comments to Authors:
Overall I found this manuscript well thought out and executed.

Thank you.

Reviewer #2:
Manuscript Summary:

Weatherly et al. reported to use a microplate assay as a high-throughput to assess the effect of Triclosan on RBL-2H3 mast cell degranulation. In addition, the authors reported a method to resolve triclosan into aqueous, which is different than using the traditional organic solvent. This article provided a detailed description of a method for detecting mast cell degranulation. The rationale and background for the method is adequately explained.

Major Concerns:
Traditional method (beta-hexosaminidase) for examining mast cell degranulation should be used as positive control for evaluating the microplate assay.

In this manuscript we describe using β-hexosaminidase as a measure for mast cell degranulation. We believe the reviewer is referring to β-hexosaminidase assays that, historically, were performed with samples measured one-at-a-time in a fluorimeter, rather than, as we describe here, using a 96-well plate, a microplate reader, and batteries of toxicant tests. As stated in the manuscript, our methods are an extension of Naal et al. (2004), who previously rigorously demonstrated concordance between the "old" β-hexosaminidase assay and this assay[1]. Additionally, prior to new toxicant-test assays, we always perform antigen and/or ionophore-dose responses (in the absence of test chemical) to ensure that the cells are responding normally, compared with the previous literature. To make this clearer in the text, we have added lines to the first paragraph of our "Discussion" section:

> Prior to the Naal *et al.*[1] assay, mast cell degranulation had been routinely assessed via β-hexosaminidase[2-4], but these early methods utilized fluorimeters in which one sample was read at a time. Importantly, Naal *et al.* established direct concordance between this more high-throughput method utilizing a microplate reader and the earlier method in which samples were read one-at-a-time in a fluorimeter. In sum, Naal *et al.*[1] greatly improved the speed, power, simplicity, and reliability of the assay by adapting it to a high-throughput microplate platform, as well as by incorporating several changes to the workflow. Here, we have further adapted this assay for a study of various test chemicals, in particular, here, the ubiquitous drug TCS.

Minor Concerns:
Other factors, such as Stem cell factor should be used as a positive control to evaluate the impact of Triclosan on mast cell degranulation.

The reviewer has made an interesting point–that there are multiple ways to stimulate degranulation of RBL-2H3 mast cells. There exist several methods of RBL-2H3 stimulation in addition to the corsslinking of IgE-bound receptors (multivalent antigen)

and ionophore induced Ca^{2+} influx. One of these methods is stimulation via exposure to compound 48/80 along with quercetin[5]. Another, as suggested by this reviewer, is stimulation via stem cell factor. Yet another is crosslinking IgE-bound receptors with an anti-IgE IgG antibody. In fact, we have previously found that Triclosan's (TCS's) inhibition of IgE-mediated degranulation is not dependent upon the type of crosslinker used (whether antigen or anti-IgE IgG antibody)[6], and this paper is cited in our *JoVE* manuscript. In light of this reviewer's insightful comment, we have added the following statement to our "Discussion" section:

> In addition to the multivalent antigen DNP-BSA and the calcium ionophore A23187, there exist numerous other methods of RBL-2H3 stimulation. One of these methods is stimulation via exposure to compound 48/80 along with quercetin[5]. Another is crosslinking of IgE-bound receptors with an anti-IgE IgG, as we previously tested along with TCS exposure[6]. Many other stimulation methods exist, and each of these methods addresses a different mechanistic aspect of mast cell degranulation. This plate reader assay can be adapted for use with many of these alternative stimulators, further expanding its utility.

Other edits:

-Due to her assistance with producing a revised Figure 3 and her anticipated role in the filming aspects of this manuscript, we are including Juyoung Shim as an additional author of the manuscript.

-At this time, we also have a publication number assigned, and this number now appears in the "Acknowledgements" section: "This is Maine Agricultural & Forest Experiment Station publication number 3311."

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2 Soto, E. O. & Pecht, I. A monoclonal-antibody that inhibits secretion from rat basophilic leukemia-cells and binds to a novel membrane component. *Journal of Immunology* **141**, 4324-4332 (1988).

3 Pierini, L., Harris, N. T., Holowka, D. & Baird, B. Evidence supporting a role for microfilaments in regulating the coupling between poorly dissociable IgE-Fc epsilon RI aggregates and downstream signaling pathways. *Biochemistry* **36**, 7447-7456, doi:10.1021/bi9629642 (1997).

4 Aketani, S., Teshima, R., Umezawa, Y. & Sawada, J. Correlation between cytosolic calcium concentration and degranulation in RBL-2H3 cells in the presence of various concentrations of antigen-specific IgEs. *Immunology Letters* **75**, 185-189, doi:10.1016/s0165-2478(00)00311-4 (2001).

5 Senyshyn, J., Baumgartner, R. A. & Beaven, M. A. Quercetin sensitizes RBL-2H3 cells to polybasic mast cell secretagogues through increased expression of Gi GTP-binding proteins linked to a phospholipase C signaling pathway. *Journal of Immunology* **160**, 5136-5144 (1998).

6 Palmer, R. K. *et al.* Antibacterial agent triclosan suppresses RBL-2H3 mast cell function. *Toxicology and Applied Pharmacology* **258**, 99-108, doi:10.1016/j.taap.2011.10.012 (2012).