Video Article

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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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} = 4,200 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.

Video Link

The video component of this article can be found at https://www.jove.com/video/50671/

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 phosphorylation 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 hr, 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, often times 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 min 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 4,200 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.

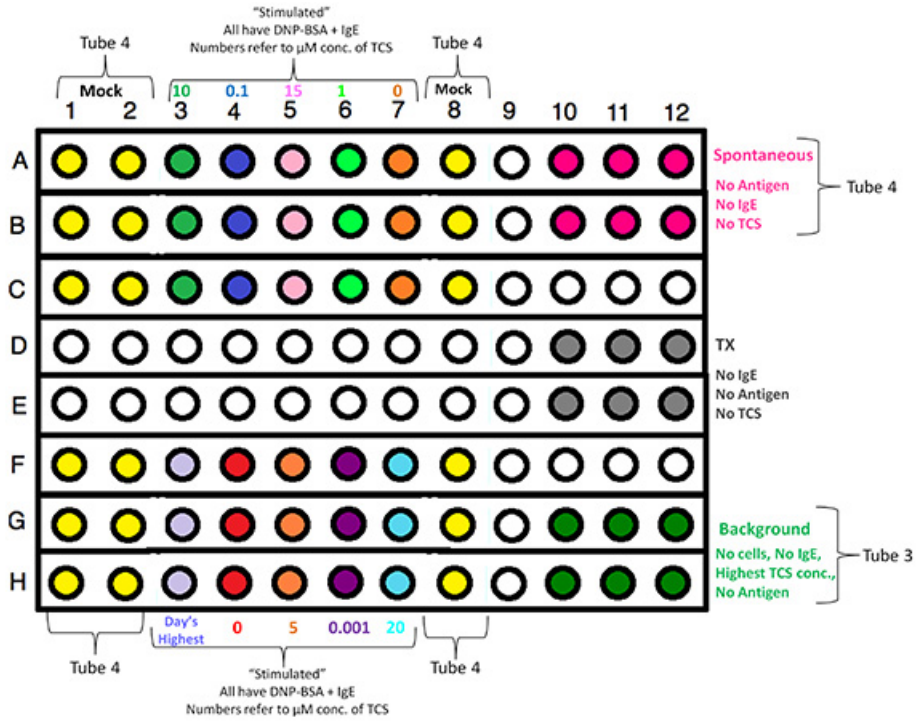
DAY 1:

1. Preparation of Cells

1. 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.
2. Warm RBL media (recipe provided in table) and trypsin in 37 °C water bath.
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



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4. Take the RBL cell flask into the sterile tissue culture (TC) hood. The cells are attached to the bottom of the flask. Working under the TC hood and using standard sterile technique, remove all media from flask with sterile pipette; the cells remain attached to the bottom of the flask. Next, rinse flask with 2 ml trypsin, then discard this wash.
5. Add exactly 2 ml trypsin to cover bottom of the flask. Put into 37 °C incubator for 5 min to allow the cells to detach from the bottom of the flask.
6. After 5 min, 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).
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.
8. Spin the 50 ml tube in centrifuge (with appropriate balance) for 8 min at 500 x g; this force pellets cells effectively.
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 5x 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.
10. Back in the TC hood, remove supernatant from cells that were spun down.
11. Cap the cell tube, and flick pellet at the bottom of the tube to loosen cells.
12. Add media to trypsinized cells to yield a density of 0.5 x 10^6 cells/ml, based on the cell count.
13. Mix well but gently to keep cells suspended during the plating procedure. Using a Pipetman, 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.

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

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. 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.
3. Weigh out 0.0022 g of TCS granules and transfer to "TCS-buffer" flask (which contains 250 ml Tyrodes Buffer). 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.
4. 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.)
    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.
5. Turn on UV/Vis Lamp to warm up the lamp for later use.
6. Heat the "TCS-buffer" solution to 50 °C while stirring constantly. Once up to temperature, time for 90 min. During the 90 min, continue to monitor 50 °C temperature and appropriate stirring speed frequently.
    1. Simultaneously, heat the "control buffer" solution (which is just 250 ml of plain Tyrodes buffer) to 50 °C with continuous stirring. Upon reaching 50 °C, time for 90 min, during which time temperature (keeping at 50 °C) and stirring are both monitored.
7. At end of the 90 min, take both Erlenmeyer flasks off the hot plates and transfer to the benchtop.
8. 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.
9. 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.
    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

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).
2. Warm RBL media and trypsin in 37 °C water bath.
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.
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.
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 min of the 90 min heat/stirring time.
6. Once both solutions have been stirred at 50 °C for 70 min, 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.
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 nonsensitized samples).
8. Dump all media from 96-well plate (that was prepared on Day 1) into sink, and bring the plate under the TC hood.
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.
10. Randomly add 100 µl plain RBL media only to "TX," "spontaneous," and "background" wells.
11. Put plate lid on plate, and then move plate into 5% CO_2 /37 °C incubator for 1 hr.
12. During 1 hr incubation, follow steps 3.13 - 3.24.
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.
14. Once "TCS-buffer" and "control buffer" have been heated and then stirred for 90 min 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.
15. Begin preparing the exposure buffers, with ± Ag, ± TCS. First, from the 249 ml sample of "TCS-buffer" solution (that has already been heated and stirred for 90 min), 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 for a final antigen concentration of 0.0004 µg/ml DNP-BSA. Vortex and invert.
    1. Label this "Tube 1, High TCS/+Ag/+BT." It is used for dilutions and highest TCS concentration exposure.
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 prepared earlier for a final antigen concentration of 0.0004 µg/ml DNP-BSA. Vortex and invert.
    1. Label this "Tube 2, No TCS/+Ag/+BT". Used for TCS dilutions and 0 µM TCS concentration exposure.

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.
    1. Label this "Tube 3, High TCS/No Ag/+BT." This is used for background.

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.)
    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

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 Pipetman.

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

20. After 1-hr 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.)
21. Using a Combitip, randomly wash cells in the 96-well plate with BSA-Tyrodes Buffer (200 µl/well). Release the wash buffer onto the sides of the wells, rather than directly onto the attached cells, in order to avoid disturbing the attached cells. Repeat the process a second time.
22. To prepare treatments for application, vortex and invert dilutions right before addition to the plate.
23. Starting with the top section of the plate: Randomly add triplicates of 200 µl each of the antigen solutions (with correct concentrations of TCS) to the corresponding wells. Continue to bottom of plate. Add "control buffer" solution plus Ag (from "Tube 2" above) to all "mocks" on the plate.
24. Add 200 µl of appropriate solutions to corresponding wells:
    1. Add 200 µl of 0.2% Triton X-100 to "TX"-designated wells.
    2. Next, add 200 µl of Tube 3 to the 3 wells labeled "Background (BkgD)-Highest TCS" on the plate.
    3. Finally, add 200 µl of Tube 4 to 6 wells labeled "Spontaneous."

25. Incubate the plate for 1 hr in 37 °C/5% CO_2.
26. During 1 hr 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 min, keeping in foil because it is light sensitive.
    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.
    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. Get out new box of P200 tips for next step.

27. At the end of 1-hr incubation, put cell plate from incubator onto ice bucket #1, pipette supernatant up and down 4-5x (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.
28. Incubate for 30 min at 37 °C/5% CO_2.
29. After 30 min 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).
30. Run the plate in the fluorescence plate reader (go to section 4).

DAY 2:

4. Fluorescent Plate Reader Instructions and Data Analysis

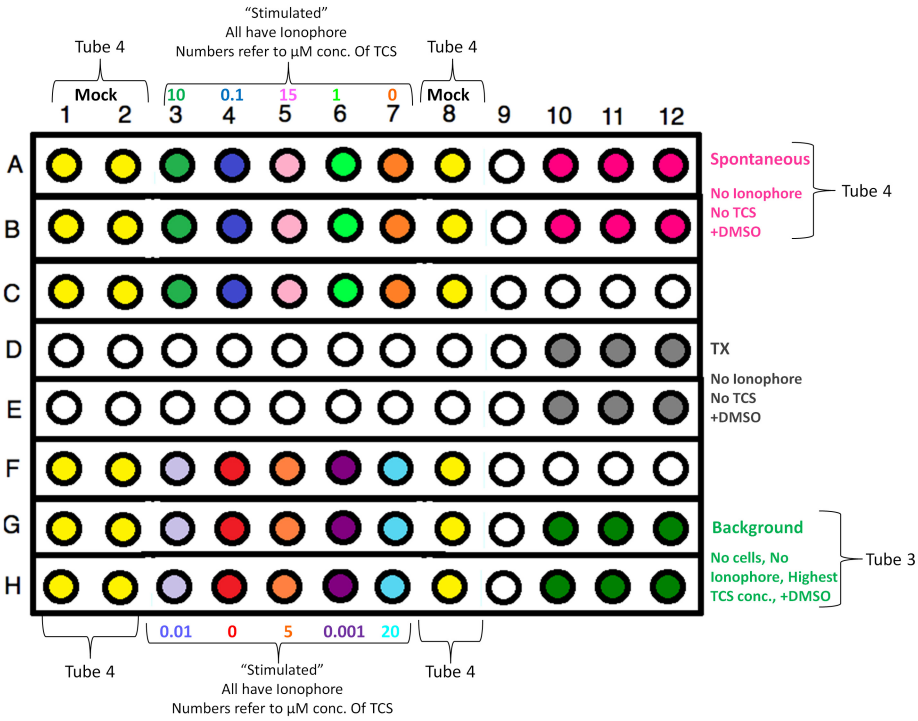
1. Open Gen5 program, and open experiment section.
2. Turn on plate reader and insert plate (upper left corner is A1).
3. Protocol → Procedure → Read to set custom readings. Do not add anything about samples, replicates, *etc.*, in order to collect raw fluorescence data from each well.
4. Under "Read" 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.
5. Choose flat black bottom, 96-well plate (Grenier 96-well, Flat Bottom).
6. Deal with the plate layout: Protocol → plate layout. Set up samples without indicating repeats, dilutions, *etc.*
7. Plate → read.
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.
9. In Excel, subtract the average background reading from every sample, including Triton X-100 wells.
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.
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

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



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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).
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.
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. 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).
    1. Alternatively, if using a frozen stock today, thaw on ice, and check that the 5 mM A23187 ionophore is well mixed and clear. Vortex, flick, and invert this stock before using. Record date of preparation and Lot # of this A23187.

6. Once "TCS-buffer" and "control buffer" have been heated and stirred for 90 min, 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.
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 3x for 8 sec and invert 3x. 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 noncytotoxic to RBL-2H3 cells by cytotoxicity assay (see[2]).
    1. Label this "Tube 1, High TCS/+Ionophore/+BT." Used for dilutions and highest TCS concentration exposure.

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 3x for 8 sec and invert 3x. Final ionophore concentration is 180 nM.
    1. Label this "Tube 2, No TCS/+Ionophore/+BT." This is used for dilutions and 0 µM TCS concentrations.

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 3x for 8 sec and invert 3x; no ionophore is added.
    1. Label this "Tube 3, High TCS/No Ionophore/+BT/+DMSO"; used for background.

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 3x for 8 sec and invert 3x. No Ionophore is added.
    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	✓

11. Calculate and record volumes for dilutions after determining the concentration of the "TCS-buffer" stock. Use calibrated P2 and P1000 Pipetman. 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

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.
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.
14. Add 200 µl of appropriate solutions to corresponding wells:
    1. Add 200 µl of 0.2% Triton X-100 to "TX"-designated wells.
    2. Next, add 200 µl of Tube 3 to the 3 wells labeled "Background (BkgD)-Highest TCS" on the plate.
    3. Finally, add 200 µl of Tube 4 to six wells labeled "Spontaneous."
15. Incubate the plate for 1 hr in 37 °C/5% CO_2.
16. During the 1 hr 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 min, keeping in foil because it is light sensitive.
    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.
    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. Get out new box of P200 tips for next step.
17. At the end of 1-hr incubation, put cell plate from incubator on ice bucket #1, pipette supernatant up and down 4-5x (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.
18. Incubate for 30 min at 37 °C/5% CO_2.
19. After 30 min 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).
20. Run the plate in the fluorescence plate reader (Follow all steps in section 4).

Representative Results

When heated to 50 °C for 90 min, 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 4,200 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 °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 hr 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 (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 hr 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 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.

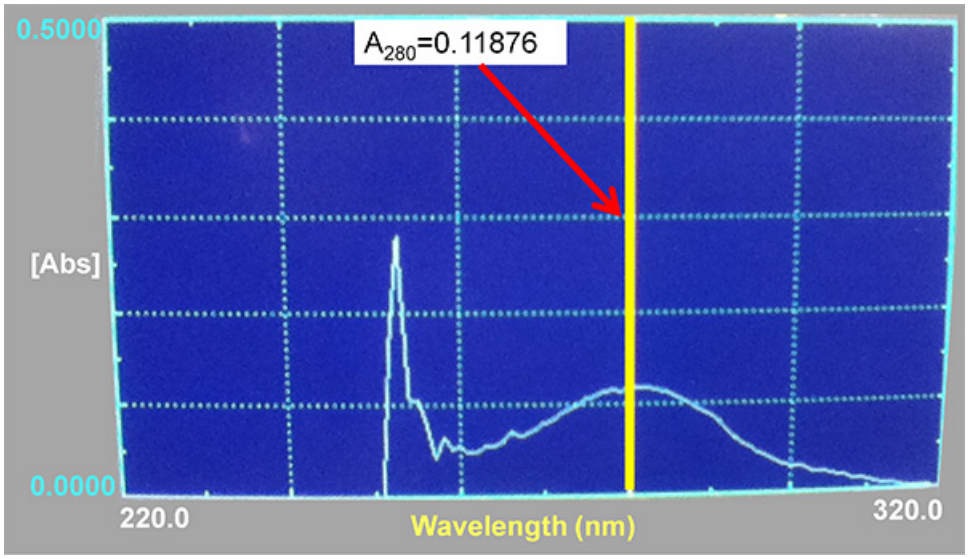

Figure 1: **Representative TCS UV-Vis absorbance spectrum.** TCS has a robust peak at 280 nm, allowing easy determination of A_{280}, as well as affording the ability to use the molar extinction coefficient of 4,200 L/mol/cm[12] to determine the actual concentration of TCS dissolved in tyrodes buffer. 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. Click here to view larger image.

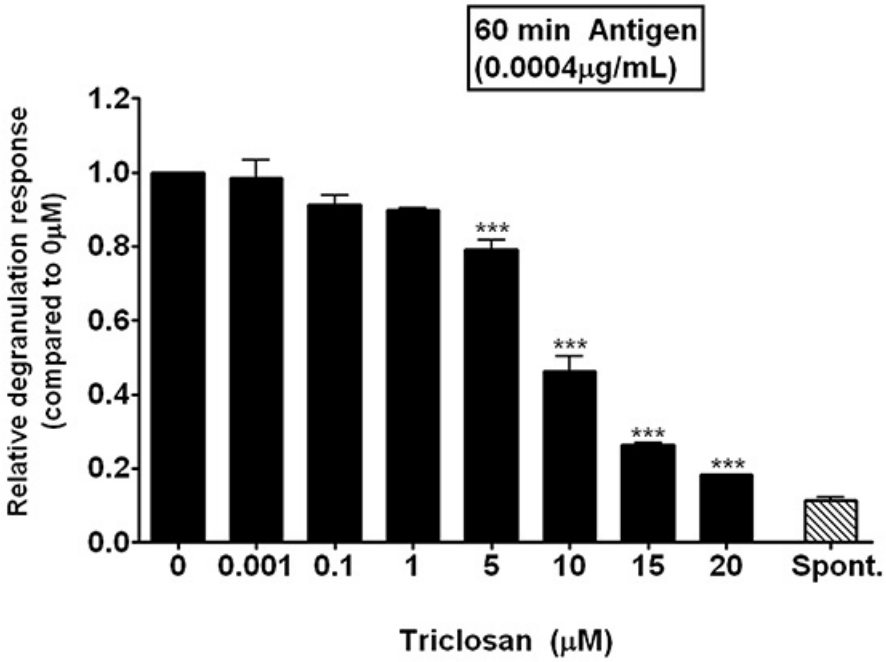

Figure 2: **A representative degranulation response of IgE-sensitized RBL mast cells exposed to 0.0004 µg/ml DNP-BSA antigen and TCS (0-20 µM).** A spontaneous release value (no antigen present) is depicted for reference. Values represent mean ± standard deviation of triplicate samples. As presented, 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. Click here to view larger image.

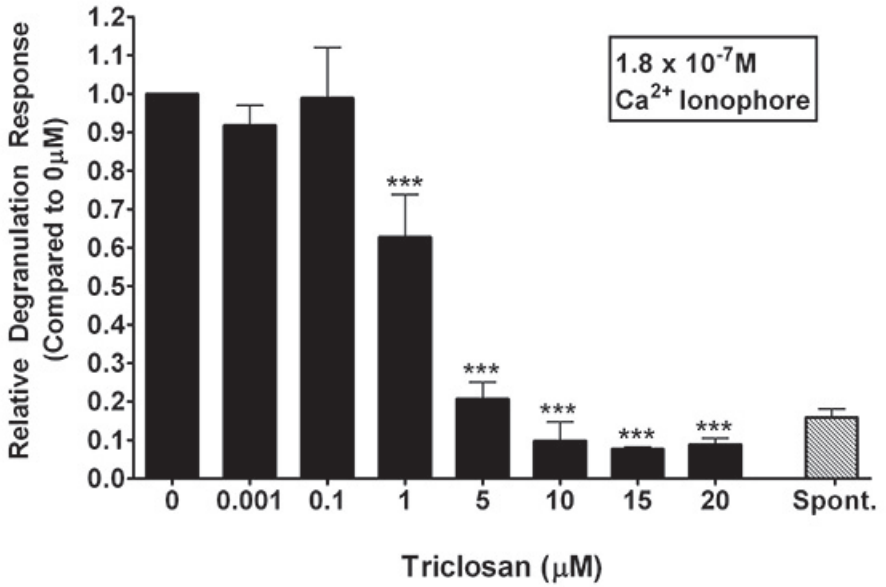

Figure 3: **A representative degranulation response of RBL mast cells stimulated with 180 nM A23187 calcium Ionophore in the presence of TCS (0-20 µM).** A spontaneous release sample (no ionophore present) is depicted for reference. Values represent mean ± standard deviation of triplicate samples. As 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. Click here to view larger image.

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 fluorometers 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 fluorometer. 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 (**Figure 1**), and then the effect of TCS (<30 µM) was examined on mast cell degranulation (**Figures 2** and **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 40-90 min (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 min than 40 min. 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 **Figure 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 remade. 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) 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 min 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	Nonuniform 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 min), 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.

Disclosures

We have nothing to disclose.

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