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Effects of Novel Nanomaterials on Allergic Mediator Release from Human Mast Cells and Basophils through Non-Ige Mediated Pathways

Anthony Dellinger[1], D. Bradford Brooks[2], Beverly Plunkett[3], Becky M. Vonakis[3], Marinella Sandros[1], Zhiguo Zhou[2] and Christopher L. Kepley[1]*

[1]*Joint School of Nanomedicine and Nanoengineering, Greensboro, NC, USA*
[2]*Luna Innovations Incorporated, Nanoworks Division, 521 Bridge St, Danville, VA, USA*
[3]*Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore MD, USA*

Abstract

Mast cells (MC) and peripheral blood basophils (PBB) are well known for their role in the allergic response mediated through high affinity IgE receptors (FcεRI). However, these cells can also be stimulated by other non-allergic secretagogues to release their inflammatory mediators. Certain fullerene derivatives (FD) have already been shown to stabilize FcεRI-mediated MC/PBB responses, but it is not know if they also stabilize these cells through non-IgE-mediated mechanisms. A panel of FD was synthesized and tested for their ability to inhibit non-FcεRI mediated release from human MC and PBB. It was found that specifically engineered FD could significantly inhibit calcium ionophore, compound 48/80, somatostatin, and poly L-lysine induced MC degranulation and cytokine production, as well as blunt degranulation and cytokine production from N-formyl-methionine-leucine-phenylalanine (fMLP), poly L-lysine, and calcium ionophore stimulated PBB. The mechanism of inhibition was due in part to the prevention of secretagogue-induced increases in cellular reactive oxygen species (ROS) and calcium levels as well as the reduced activation of the MAPK signaling intermediates ERK1/ERK2 and LAT. Additionally, preincubation of MC with FD blunted the prostaglandin D_2 (PGD_2) production upon exposure to inflammatory stimuli. In both cell types, the extent of inhibition of mediator release in response to each secretagogue was dependent on the moieties/side chains attached to the carbon cage. These results further extend the utility of fullerene nanomaterials to control mediator release through non-IgE mediated pathways in MC/PBB.

Keywords: Mast cells; Peripheral blood basophils; Fullerenes; Prostaglandin; Reactive oxygen species; Signal transduction; Allergic mediator release; MC degranulation

Introduction

Mast cells (MC) are ubiquitously expressed in almost all tissue and participate as effector cells for immune regulation. Peripheral blood basophils (PBB) are similar to MC in that they have pre-stored, allergy-inducing mediators in their granules (e.g. histamine). Once stimulated, MC/PBB secretes several molecules, including preformed and newly formed inflammatory mediators, via various physiological and non-physiological stimulations [1-3]. While the classical IgE/FcεRI pathway is the most well studied and understood pathway leading to MC/PBB mediator release, these cells can be stimulated by non-IgE secretagogues. Indeed, non-IgE stimulation may be more physiologically relevant in non-allergic conditions such as innate immunity and heart disease [2-4]. Thus, finding new ways to stabilize these cells as a strategy for controlling MC/PBB-diseases is a continuous need.

Fullerenes, which are nanometer-sized tiny spherical carbon cages, are being explored in a wide array of applications including nanomedicine [5,6]. The carbon cage is insoluble without the addition of appropriate side chains that confer water solubility, an important requirement for medical applications. Recent studies suggest that water-soluble fullerene derivatives (FD) can inhibit FcεRI-induced MC responses *in vitro,* which translates to the prevention of MC-driven anaphylaxis and asthma *in vivo* [7-9]. Although it is clear certain FD can stabilize MC activation through FcεRI *in vitro* and *in vivo* it is not known if they can stabilize MC activation through non-FcεRI-mediated stimuli. In these studies, the non-FcεRI/IgE mediated inhibitory effects of a panel of FD were tested for their ability to stabilize MC/PBB.

It is shown that incubation of MC/PBB with fullerene constructs can significantly inhibit mediator release in response to various secretagogues. This inhibition was mediated in part through reductions in the generation of reactive oxygen species (ROS), cellular fluctuations of calcium, and phosphorylation of signaling molecules in activated MC. It is demonstrated that FD inhibit non-IgE mediated pathways and the efficacy of FD relies on several factors: the active side chain moieties added to the fullerene cage, the type of cells evaluated, the secretagogue used to stimulate, and the pathway that is examined.

Materials and Methods:

Reagents

The following reagents: A23187 (calcium ionophore), compound 48/80 (polymer amine synthesized by condensing methyl-p-methoxy phenylethylamine with formaldehyde), somatostatin, poly L-lysine, and N-formyl-methionine-leucine-phenylalanine (fMLP), Fura-2/AM, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (PNP) (all from Sigma-Aldrich Corp., St. Louis, MO.), purified mouse α-human TNF-α (Mouse IgG_1, clone MAb_1), rat α-Human GM-CSF (Rat IgG_{2a}, clone BVD2-23B6), rat α-human IL-13 (Rat IgG_1, clone JES10-5A2), biotin mouse α-human TNF (Mouse IgG_1, clone MAb_1), biotin rat α-human GM-CSF (Rat IgG_{2a}, clone BVD2-23B6), biotin rat α-human

***Corresponding author:** Christopher Kepley, The Joint School of Nanoscience and Nanoengineering, 2901 E. Lee Street, Suite 2200, Greensboro, NC 27401, USA, Tel: 540-808-8993; Fax: 336-217-5179; E-mail: clkepley@uncg.edu

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IL-13 (Rat IgG_1, clone JES10-5A2), avidin-horseradish peroxidase (all from BD Biosciences, San Jose, CA), X-Vivo 15 with gentamicin, L-Glu, phenol red (Lonza, Walkersville, MD), human stem cell factor (SCF; Peprotech, Rocky Hill, NJ), and DCF-DA (Cayman Chemical Company, Ann Arbor, MI.) were obtained from the indicated commercial sources and used as described.

Fullerene derivatives

Luna Innovations Incorporated synthesized all FD. Each FD was characterized using matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF), nuclear magnetic resonance (NMR), and high performance liquid chromatography (HPLC). All FD used were between 1 and 50 nm in aqueous solution as determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-S90 and a NanoSight LM10 with NTA Software allowing individual particles to be tracked and characterized. To evaluate fullerene-specific toxicity, MC viability was determined by incubation with concentrations up to 100 µg/ml over nine days (a 10x higher concentration and longer time period than was needed to control cellular responses to stimuli). No significant ($p<0.05$) toxicity was observed with any of the selected compounds when compared to controls as determined using trypan blue staining and MTT Assay (data not shown).

Human MC and PBB cultures and activation

Human tissue (i.e. skin) was received from the Cooperative Human Tissue Network. All MC studies were approved by their Human Studies Institutional Review Board. Mast cells were purified and cultured as described [10,11]. Peripheral blood basophils were obtained from normal donors (no medications) after informed consent as approved by the Institutional Review Board, Johns Hopkins University and purified to >90% as described previously [12].

A panel of FD was screened at several concentrations to determine their ability to inhibit secretion. Briefly, MC/PBB were first incubated with increasing concentrations of FD (1-40 µg/ml) overnight (18 hours) at 37°C and 6% CO_2, a time point and concentration range found to be optimal for cellular uptake of FD [8,13]. The next day, cells were stimulated with the indicated secretagogues at varied concentrations for 30 minutes (degranulation and PGD_2) or overnight (cytokine production) at 37°C and mediator release measured as described previously or according to the manufactures instructions (Cayman Chemical Company, Ann Arbor, MI, Prostaglandin D_2 Kit-512031). The percent inhibition was calculated as a percent release compared to non-FD treated cells (positive control). *An i*nitial *dose response* was performed to determine the concentration of secretagogues that induced maximal degranulation/cytokine production (not shown). In short, MC responded optimally to 10 µM with A23187 and compound 48/80, 20 µM for somatostatin, and 40 µM for poly L-lysine. Other secretagogues (C5a, morphine, substance P, and LPS) were examined, however preincubation with FD did not produce statistically significant reductions in MC mediator release or stimuli did not induce significant activation (data not shown). For PBB, 10 µM was optimal activation with fMLP, poly L-lysine, and A23187 (data not shown). All studies were performed in triplicate on at least three separate MC cultures or PBB donors.

Reactive oxygen species and calcium measurements

Human skin MC ($2x10^6$/ml) were incubated overnight with FD (those found to significantly inhibit degranulation and/or cytokine release), washed, and loaded with DCF-DA (final concentration of 5 µM) for 30 minutes. Following fluorophore loading, cells were washed and resuspended in fresh media, placed in a cuvette, and activated with various secretagogues (as above) for ~60 seconds. ROS fluorescence intensity was measured at 523 nm wavelengths over a 12-minute time interval using Perkin Elmer LS55 Luminescent Spectrometer (Perkin-Elmer Laboratories). For calcium flux, MC (incubated as above) was loaded with Fura-2 AM at a final concentration of 20 ng/ml for 30 minutes at 37°C in HBSS buffer. The cells were washed twice in the same buffer and incubated for 15 minutes. The cells were then stimulated with appropriate secretagogues and a real time ratio-metric intensity evaluation of fluorescence between wavelengths 340 nm/380 nm was determined over a period of 400 seconds using Perkin Elmer LS55 Luminescent Spectrometer. All samples were measured in duplicate and performed at least three times.

Immunoblotting analysis of signal transduction intermediates

Mast cells ($1x10^7$ cell/condition; each condition performed in triplicate) were prepared for Western blotting as described previously [8]. Briefly, cells were incubated with or without optimal concentrations of the indicated FD overnight, washed, and activated as described above. Cell pellets were lysed and nuclear extracts isolated as described [14]. The cell suspension was heated, passed through a 20-gauge needle, and centrifuged to remove cell debris. Proteins were separated on 12% NuPage tris-glycine gels using tris-glycine SDS running buffer. Signaling molecules were measured using phosphorylated MAPK (ERK1 and ERK2) and LAT primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA or Cell Signaling, Danvers, MA, respectively) and Licor IR-800 anti-mouse F $(ab)_2$ secondary antibodies (1:20,000). The housekeeping protein β-actin was used as a loading control on the same blot and co-stained with Licor IR-600 anti-rabbit F $(ab)_2$ secondary antibodies. Band intensities were quantified using an Odyssey Imaging System as previous described [8].

Results

FD differentially effect MC non-IgE-induced mediator release

A panel of water soluble FD was evaluated to determine efficacy based on various side chain moieties as well as differential responses to numerous secretagogues. Table 1 presents results from a subset (~30%) of FD that was capable of significantly ($p<0.05$) inhibiting MC-

Fullerene Derivative	A23187 + FD*	48/80 + FD*	somatostatin +FD*	poly-L-lysine + FD*
Biotin	no Inh	no Inh	44.6 (±1.8)¥¥¥	no Inh
C70-OH	19.0 (±1.1)¥¥¥	42.0 (±4.6)¥¥¥	13.4 (±5.2)¥	33.4 (±5.5)¥¥¥
CCC	no Inh	no Inh	23.8 (±0.8)¥¥¥	no Inh
DMAE	18.6 (±7.9)¥	16.9 (±10.)¥¥¥	24.3 (±9.7)¥	no Inh
Ethanolamine	36.0 (±3.0)¥¥¥	no Inh	no Inh	no Inh
Niacin	no Inh	59.5 (±1.3)¥¥¥	21.9 (±13.8)¥	20.9 (±12.6)¥
NSAID	21.1 (±11.4)¥	no Inh	no Inh	no Inh
PC4	no Inh	no Inh	21.6 (±2.9)¥¥¥	no Inh
Tetraglutamate	22.3 (±3.9)¥¥¥	no Inh	20.3 (±4.5)¥¥	17.9 (±13.1)¥
Tetrainositol	no Inh	no Inh	32.8 (±1.9)¥¥¥	no Inh
Tetraphosphate	18.9 (±0.4)¥¥¥	no Inh	no Inh	no Inh
Tetrapyridine	14.0 (±1.9)¥¥	34.8 (±8.1)¥¥	no Inh	13.1 (±4.0)¥
Tetrasulfonate	21.1 (±6.3)¥	44.8 (±11.2)¥¥	22.0 (±5.50)¥¥	26.7 (±2.3)¥¥
TGA	49.8 (±8.9)¥¥¥	no Inh	16.7 (±3.8)¥¥¥	43.8 (±10.6)¥¥
TTA	no Inh	no Inh	34.6 (±2.6)¥¥¥	52.4 (±4.3)¥¥¥

* FD Treatment at 10 µg/mL. ¥¥¥ *p* value < 0.01 / ¥¥ *p* value < 0.02 / ¥ *p* value < 0.05 / no Inh = no significant inhibition observed

Table 1: Mean % inhibition of degranulation (±SD) in FD treated and untreated MC.

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degranulation when challenged with 48/80, somatostatin, A23187, and poly-L-lysine. Other secretagogues evaluated included C5a, morphine, substance P, and LPS which did not produce significant mediator release from MC or the pathway was unaffected by FD pretreatment, data not shown. These data demonstrate that inhibition of non-IgE-driven MC degranulation can be generalized across varies stimuli, as in the case of C_{70}-OH and Tetrasulfonate, or selective to a specific stimuli and/or signaling pathway, as observed in the case of Biotin and CCC.

Table 2 represents those FD that were effective at significantly inhibiting TNF-α cytokine production from MC. Out of five separate MC cultures, no activation of cytokine production was observed with compound 48/80 or somatostatin (varying dose and incubation times; positive controls using FcεRI antibodies) as others have reported [15].

Fullerene Derivative	A23187 + FD*	poly-L-lysine + FD*
Biotin	83.9 (±8.0)¥¥¥	no Inh
C70-OH	60.6 (±28.2)¥¥	16.41 (±10.5)¥
CCC	no Inh	no Inh
DMAE	41.1 (±12.9)¥	no Inh
Ethanolamine	52.3 (±3.9)¥¥¥	no Inh
Niacin	73.2 (±9.6)¥¥¥	no Inh
NSAID	no Inh	12.0 (±6.2)¥
PC4	94.1 (±14.2)¥¥	no Inh
Tetraglutamate	59.7 (±3.0)¥¥¥	48.3 (±1.86)¥¥¥
Tetrainositol	95.7 (±2.3)¥¥¥	72.4 (±6.7)¥¥¥
Tetraphosphate	39.3 (±14.3)¥	no Inh
Tetrapyridine	51.7 (±17.5)¥¥	no Inh
Tetrasulfonate	39.9 (±2.5)¥¥¥	no Inh
TGA	93.7 (±4.6)¥¥¥	70.2 (±1.0)¥¥¥
TTA	98.7 (±3.6)¥¥¥	66.8 (±3.1)¥¥¥

* FD Treatment at 10 µg/mL.
¥¥¥ *p* value < 0.01 / ¥¥ *p* value < 0.02 / ¥ *p* value < 0.05
no Inh = no significant inhibition observed

Table 2: Mean % inhibition of TNF-α (±SD) in FD treated and untreated MC.

Calcium ionophore A23187 is a widely used secretagogue which can directly increase intracellular calcium concentration (calcium) levels to induce mediator release [16]. Nine FD significantly (p< 0.05) inhibited degranulation (>10%) (Table 1). Furthermore, 13 FD were capable of inhibiting TNF-α cytokine production (>40%) induced by A23187 (Table 2). Poly-L-lysine is a small polypeptide of the essential amino acid L-lysine that can induce MC activation [17,18]. Seven of the FD inhibited degranulation (>10%) and six were capable of inhibiting TNF-α production (Table 1 and 2). Typical dose response curves of inhibition on degranulation and cytokine production are indicated in Figure 1A-D.

Compound 48/80 is a polybasic secretagogue, which has previously been shown to activate human MC [19,20] presumably through direct interactions with guanosine triphosphate (GTP)-binding proteins and MAPK [21]. As seen in Table 1, five FD inhibited degranulation in MC challenged with compound 48/80 (>20%). Somatostatin is a peptide hormone that has previously been shown to activate human MC [20,22] through the G-protein-coupled somatostatin receptor [20,23]. Table 1 shows a subset of 11 different FD that inhibited degranulation. A representative dose response for inhibition is seen in figure 2A and B.

The effect of FD on non-IgE induced MC PGD_2 release

The panel of FD (Tables 1 and 2) evaluated for mediator release were investigated to determine if they could reduce PGD_2 production in MC. TGA, Niacin, Biotin, and Tetrainositol were selected based on to their ability to inhibit cytokine production and degranulation in activated MC in response to a number of stimuli. Neither Biotin nor Tetrainositol resulted in any inhibition of PGD_2 production. Mast cells preincubated with TGA or Niacin and challenged with A23187, 48/80, or poly-L-lysine decreased PGD_2 production by >60% in all conditions, (Table 3). No detectable levels of PGD_2 where observed in MC-activated with somatostatin. Additionally, PGD_2 production in IgE challenged (FcεRI) MC was reduced nearly 60%.

The effect of FD on non-IgE induced PBB mediator release

The secretagogues that induce mediator release from PBB are

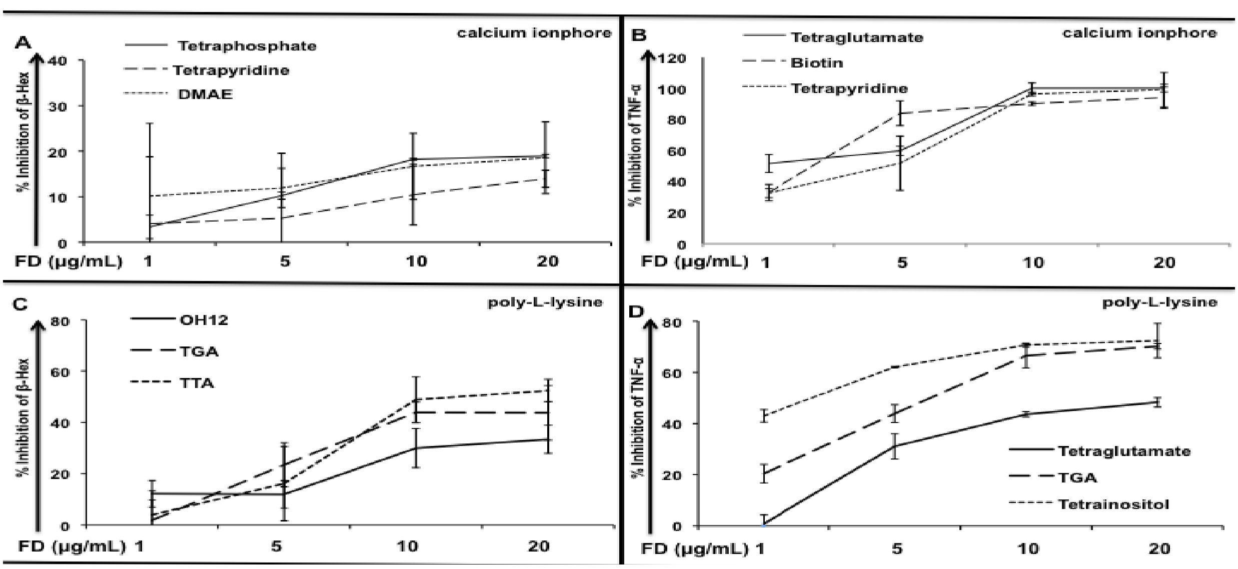

Figure 1: Specific FD inhibit human MC calcium-ionophore and poly-L-lysine-induced mediator release in a dose dependent manner. Mast cells were incubated overnight with FD, washed and activated with increasing concentrations of A23187 (A and B) or poly-L-lysine (C and D) for 30 minutes (degranulation, A and C) or 18 hours (cytokine, B and D). Mediator release was assessed as described in materials and methods. Figures are representative of three separate MC cultures with each condition performed in triplicate.

different from those that induce mediator release from MC [24]. The effects of FD on N-formyl-methionine-leucine-phenylalanine (fMLP) and poly-L-lysine-induced mediator release from PBB are shown in table 4. Only Ethanolamine inhibited fMLP-induced degranulation (14%). Tetrainositol, TGA, and Niacin inhibited IL-13 release when activated with fMLP, but none significantly affected poly-L-lysine induced mediator release as seen in table 5. None of the FD evaluated inhibited A23187-induced PBB degranulation (data not shown).

The effect of FD on non-IgE mediated induced ROS activity and intracellular calcium flux in MC

We next began to delineate the mechanisms of inhibition focusing on MC given the difficulty in obtaining adequate numbers of PBB for such studies. The activation of MC and subsequent degranulation is calcium-dependent and results in elevated ROS levels [25]. It was hypothesized that FD reduced MC degranulation by blocking ROS production and calcium responses. Therefore, representative FD was selected based on their ability to inhibit A23187, compound 48/80, somatostatin, and poly L-lysine-induced degranulation. A23187-induced increases in ROS and calcium fluctuations were significantly reduced by TGA (~50% for ROS, ~70% for calcium) and Ethanolamine (~50% for ROS, ~90% for calcium) (Figures 3A and 4A). Niacin and Tetrasulfonate significantly reduced compound 48/80-induced ROS activity (>30%; Figure 3B), but selectively effected calcium levels (Niacin > 80% inhibition and Tetrasulfonate was ineffective; Figure 4B). Somatostatin stimulated MC pretreated with Biotin and TTA inhibited ROS and calcium activity by approximately 30% (ROS) and 75% (calcium) (Figures 3C & 4C). Lastly, figures 3D and 4D demonstrate TGA and TTA blunted ROS generation >50% in response to poly-L-lysine as well as inhibited calcium approximately 30% compared to the positive control. Thus, FD appears to inhibit mediator release through the blunting of secretagogue-induced cellular increases in ROS and intracellular calcium stores.

Activator	TGA	Niacin	Biotin	Tetrainositol
A23187	79.8 (±8.4)¥¥¥	no inh	no Inh	no Inh
48/80	no inh	65.2 (±9.8)¥¥¥	no inh	no Inh
somatostatin	ND	ND	ND	ND
poly-L-lysine	62.1 (±12.2)¥¥¥	no inh	no Inh	no Inh
FcERI	59.8 (±7.8)¥¥¥	no inh	no inh	no Inh

* FD Treatment at 10 μg/mL.
¥¥¥ p value < 0.01 / ¥¥ p value < 0.02 / ¥ p value < 0.05 / no Inh = no significant inhibition observed / ND = none detected

Table 3: Mean % inhibition of PGD_2 production (±SD) in FD treated and untreated MC.

Activator	TGA	Niacin	Biotin	Tetrainositol
A23187	79.8 (±8.4)¥¥¥	no inh	no Inh	no Inh
48/80	no inh	65.2 (±9.8)¥¥¥	no inh	no Inh
somatostatin	ND	ND	ND	ND
poly-L-lysine	62.1 (±12.2)¥¥¥	no inh	no Inh	no Inh
FcERI	59.8 (±7.8)¥¥¥	no inh	no inh	no Inh

* FD Treatment at 10 μg/mL.
¥¥¥ p value < 0.01 / ¥¥ p value < 0.02 / ¥ p value < 0.05 / no Inh = no significant inhibition observed / ND = none detected

Table 4: Mean % inhibition of PGD_2 production (±SD) in FD treated and untreated MC.

Fullerene Derivative	FMLP + FD*	poly-L-lysine + FD*
Ethanolamine	no inh	no Inh
Niacin	86.6 (±16.6)¥¥¥	no Inh
Tetrainositol	54.8 (±19.3)¥	no Inh
TGA	83.7 (±19.0)¥¥	no Inh

* FD Treatment at 10 μg/mL.
¥¥¥ p value < 0.01 / ¥¥ p value < 0.02 / ¥ p value < 0.05
no Inh = no significant inhibition observed

Table 5: Mean % inhibition of IL-13 (±SD) in FD treated and untreated PBB.

The effect of FD on non-IgE mediated signaling pathway intermediates

To further delineate the mechanism of action of the FD on non-IgE-mediated degranulation we examined Western blotting using antibodies to the phosphorylation-dependent activated forms of LAT and ERK1/ERK2 [26,27]. As seen in figure 5A neither Biotin or TTA significantly affected A23187-induced changes in MAPK or LAT. Niacin and Tetrasulfatonate both reduced compound 48/80-induced phospho-activation of MAPK and LAT (Figure 5B) while Biotin and TTA both reduced somatostatin-induced changes (Figure 5C). Both TGA and TTA reduced poly-L-lysine induced activation of MAPK with minimal effects on LAT. No changes in the phosphorylation state of other signaling pathways examined including the Src family of kinases and phosphoinositol 3-kinase (PI3K) pathway were observed under these conditions (data not shown).

Discussion

Mast cells and PBB contribute to several disease processes through the release of inflammatory mediators through both IgE and non-IgE mechanisms. Thus, therapies aimed at stabilizing them and preventing the release of their mediators has been the subject of research for many years. For example, omalizumab, the humanized IgE specific IgG_1 monoclonal antibody, which limits the amount of free IgE available to bind FcεRI on the surface of MC/PBB, can control severe allergic asthma [28,29]. Another MC stabilizer, Cromolyn, has been effectively used

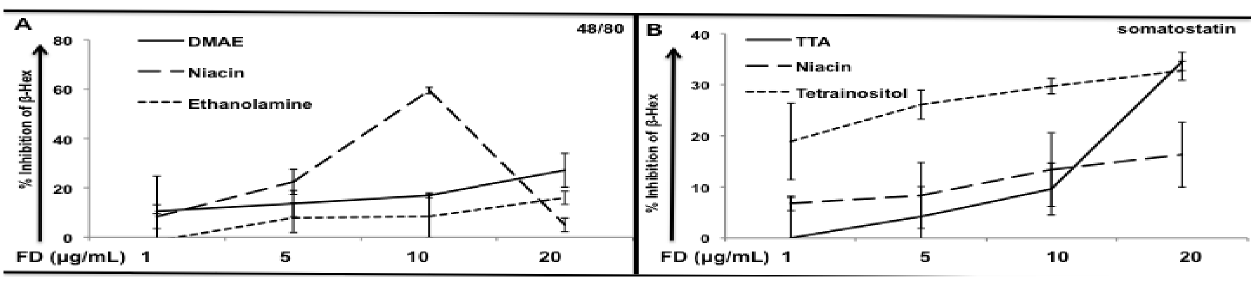

Figure 2: Specific FD inhibit human MC compound 48/80- and somatostatin-induced degranulation in a dose dependent manner. Mast cells were incubated overnight with FD, washed and activated with increasing concentrations of compound 48/80 (A) or somatostatin (B). Mediator release was assessed as described in materials and methods. Figures are representative of three separate MC cultures with each condition performed in triplicate.

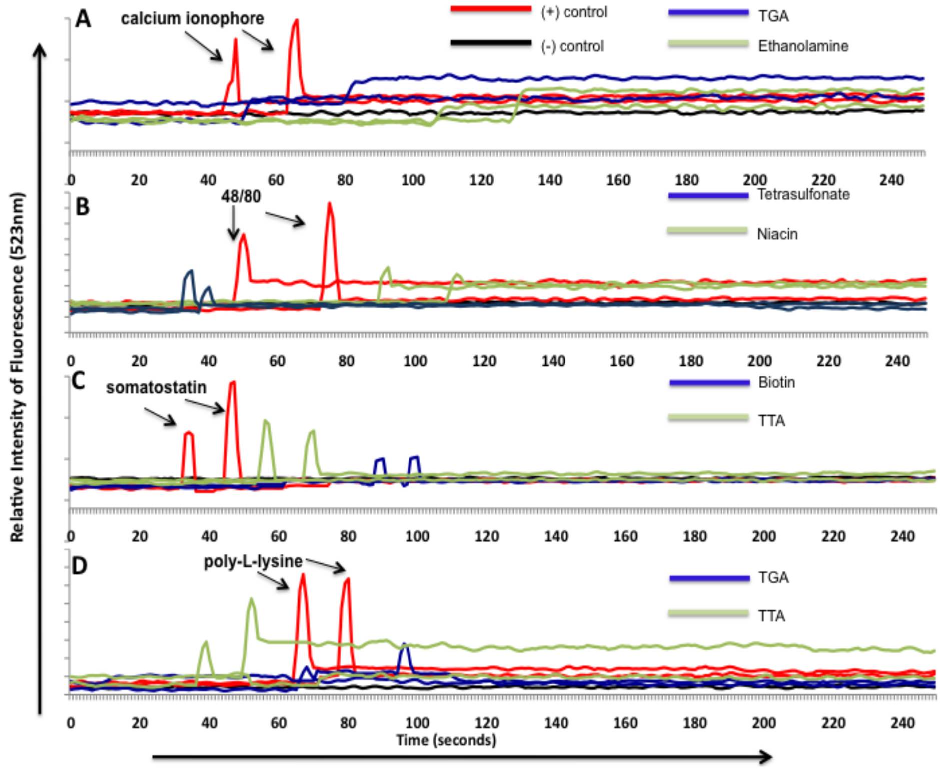

Figure 3: Specific FD inhibit human MC poly-L-lysine-induced mediator release. Mast cells were incubated overnight with FD, washed and activated with optimal concentrations of poly-L-lysine for 30 minutes (A, B) or 4 hours (C, D). Mediator release was assessed as described in materials and methods. Figures are representative of three separate MC cultures with each condition performed in triplicate. **Fullerene derivatives inhibit secretagogue-induced elevations in intracellular ROS levels:** Mast cells were incubated overnight with FD, washed and DCF-DA added to cells for 30 minutes at 37°C. After washing cells were activated with optimal concentrations of the indicated secretagogue and the fluorescence intensity measured at 525nm after establishing baseline. Figures show duplicate samples for each condition and are representative of three separate MC cultures. All positive controls (activated but not pre-incubated with FD) were represented by red traces and all negative controls (non-activated and non-preincubated with FD) are denoted by black traces.

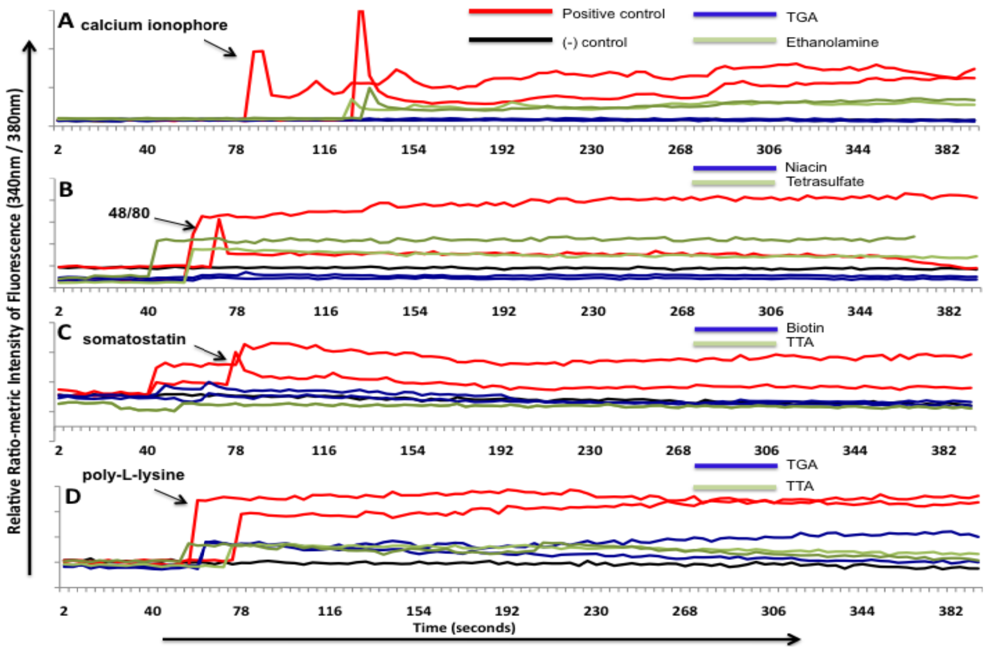

Figure 4: Fullerene derivatives inhibit secretagogue-induced elevations in intracellular calcium levels. Mast cells were incubated overnight with FD, washed and FURA 2 added to MC and incubated for 20 minutes at 37°C in the dark. After washing cells were activated with optimal concentrations of the indicated secretagogue and intensity of fluorescence was read as a ratio 340 nm and 380 nm wavelengths. Figures show duplicate samples for each condition and are representative of three separate MC cultures.

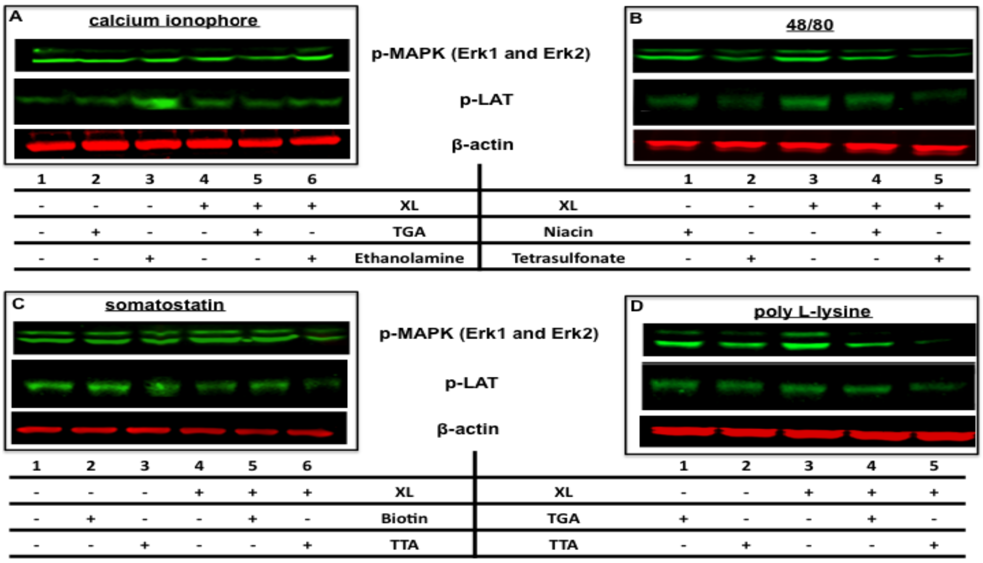

Figure 5: Inhibition of mediator release in compound 48/80, poly-L-lysine, and somatostatin challenged MC involves the down-regulation of MAPK and LAT phosphorylation. MC were incubated with or without the FD overnight, washed and activated with the indicated secretagogue. Western blotting was performed as described previously in Methods with the indicated Abs and Odyssey IR800-anti-rabbit secondary antibodies. In both cases, the total protein content in lysates was measured with the house keeping gene β-Actin and Odyssey IR680-anti-mouse secondary antibody.

to treat a wide range of MC-driven diseases [30-32]. The significance of these studies is that FD has a broad range of inhibitory capabilities toward inflammatory cells and has not demonstrated acute toxicity *in vitro*. Since MC/PBB can be induced to secrete their inflammatory mediators through several non-overlapping pathways these molecules represent a new strategy for therapeutics aimed at those disease mediated by these cells such as allergy, asthma, and arthritis.

As in our previous studies examining IgE-FcεRI-mediated degranulation and cytokine production [7,8], the efficacy in of FD was strongly dependent upon specific side chains additions; approximately 20% of the FD tested exhibited significant inhibition. In addition, certain secretagogues were unaffected by FD preincubation, indicating that the pathway in which the stimuli activates the cell is critical to FD efficacy. That is, the FD does not result in a blanket protection of the cells response to poster all stimuli, but the FD specifically regulates specific signaling pathways. Mast cells and PBB responded differently to fullerene preincubation, suggesting that the signal transduction pathways leading to mediator release are dissimilar as has been previously reported [33]. Further variation in how FD affected cell responses was demonstrated in the type of mediator release (degranulation, cytokine, prostaglandin production, or a combination thereof). Our strategy, including these studies, is to identify those FD that stabilize both IgE and non-IgE pathways and pursue more in depth toxicity, pharmacokinetic, and biodistribution studies. To this end, TGA is currently a top candidate demonstrating no toxicity *in vitro* and *in vivo* [9,34], is a potent human MC/PBB stabilizer to -FcεRI - dependent stimuli [8], prevents and reverses MC-dependent asthma in mouse models [9], and can inhibit non--FcεRI responses as demonstrated herein.

There were no clear structure-activity relationships that were established from these studies. Particular FD was more effective controllers of numerous stimuli, for example Tetrasulfonate showed statistically significant reductions in degranulation regardless of stimulant used. However, compounds such as CCC were only successful at blunting somatostatin-induced degranulation, while it did not have any effect on the mediator release elicited by any of the other compounds. Mechanistically, these compounds significantly prevented activation of MAPK and LAT suggesting they either block the signaling intermediates directly or some other intermediate upstream. The inhibition of mediator release by FD was paralleled by reductions in secretagogue-induced elevations in ROS/calcium levels as well as the signaling intermediates MAPK and LAT. These findings are similar to those previously demonstrated when examining ROS/calcium levels and phosphorylation-of signaling intermediates in FcεRI-challenged MC [7,8]. Current studies are aimed at identifying potential intracellular binding partners of FD similar to experiments performed to identify binding partners in response to FcεRI stimulus [35].

Another example in our attempts to rationally designed FD for specific disease involves the role of MC in atherosclerosis. A link between MC activation and atherosclerosis has now been clearly demonstrated [4,36] as their numbers are greatly increased in the intima at sites of arterial plaque rupture [36], in advanced plaque lesions in the carotid artery [37], and patients who died of acute myocardial infarction have an increased number of degranulated MCs at the actual site of plaque erosion or rupture [38]. Therapies aimed at treating atherosclerosis include Niacin which blocks vascular inflammation, ROS, and inflammatory cytokine production in conjunction with diminishing NF-κB activation [39]. Thus, the Niacin FD was constructed and demonstrated to inhibit mediator release from several non-IgE pathways through reduced cellular ROS and ERK1/2 phospho-activation. Mast cell-stabilizing therapies such as Cromolyn have shown promise in ApoE models of atherosclerosis (the classic animal model for atherosclerosis) where it prevents intra-plaque hemorrhage [40]. Thus, strategies aimed at blocking MC activation before it occurs could represent a new strategy for treating atherosclerosis.

Prostaglandin D_2 is produced by MC and recruits Th2 cells, eosinophils, and PBB and it is critical to development of allergic diseases such as asthma [24]. In mammalian organs, large amounts of PGD_2 are found only in the brain and in MC. Previous studies found

MC-produced PGD_2 is the primary mediator of vasodilatation (the "niacin flush") after ingestion of niacin (nicotinic acid) [41]. Previously reported data reveals that non-IgE induced PGD_2 production was attenuated when ROS production was reduced (REF in comment). This may be in part due to the mechanism that ROS facilitates the formation of a hydrogen bond necessary for PGD synthase activation [42]. In our study, the FD evaluated that was effective at reducing ROS generation in MC was also effective regulators of PGD_2 production, possibly under the proposed mechanism of modulating PGD synthase activity.

In conclusion, these studies further delineate the ability of FD to affect human MC and PBB responses so that diseases mediated by these cell types may be a target for FD-derived therapies. It is demonstrated that certain FD can differentially modulate mediator release from MC and PBB in response to a variety of secretagogues. This inhibition can involve the blunting of activation-induced increases in ROS, the release of intracellular stores of calcium, and the phospho-activation of MAPK and LAT. Furthermore, the inhibition is strictly governed by several factors, the degree and type of functionalization of the fullerene nanomaterials, the type of cells that are analyzed, the mediator being evaluated, and how the cell is being activated to illicit the mediator release. These studies further extend the utility of FD as inhibitors of MC mediator release and cytokine production through non-IgE mediated pathways.

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