A new steroid-mimicking nanomaterial that mediates inhibition of human lung mast cells responses

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Abstract:

Water-soluble fullerenes can be engineered to regulate activation of mast cells (MC) and control MC-driven diseases *in vivo*. To further understand their anti-inflammatory mechanisms a C_{70}-based fullerene conjugated to four *myo*-inositol molecules (C_{70}-I) was examined *in vitro* for its effects on the signaling pathways leading to mediator release from human lung MC. The C_{70}-I fullerene stabilizes MC and acts synergistically with long-acting β_2-adrenergic receptor agonists (LABA) to enhance inhibition of MC mediator release through FcεRI-simulation. The inhibition was paralleled by the upregulation of dual-specificity phosphatase one (DUSP1) gene and protein levels. Concomitantly, increases in MAPK were blunted in C_{70}-I treated cells. The increase in DUSP1 expression was due to the ability of C_{70}-I to prevent the ubiquitination and degradation of DUSP1. These findings identify a mechanism of how fullerenes inhibit inflammatory mediator release from MC and suggest they could potentially be an alternative therapy for steroid resistant asthmatics.

From the Clinical Editor: This study investigates the role and mechanism of action of fullerenes in deactivating mast cell-based inflammation, paving the way to the development of a novel, non-steroid therapy in reactive airway disease.

Keywords: Fullerenes | Inhaled corticosteroids | Mast cell | Dual-specificity phosphatase one

Article:

The use of corticosteroids (also known as glucocorticosteroids, glucocorticoids, or steroids) is the hallmark treatment for patients suffering from many chronic inflammatory and immune diseases (such as asthma) yet the treatment response is extremely variable among patients. Specific subsets of patients are non-responsive to such treatments and are termed steroid resistant. These patients often fail to respond to even extremely high dosages of inhaled

corticosteroids (ICS), therefore much research has been focused on understanding the mechanisms of ICS action to better understand non-responsive patients.[1] Several different studies have found that the dual-specificity phosphatase 1 (DUSP1) is a prospective regulator of this ICS response.[2, 3, 4, 5, 6] Dual-specificity phosphatase 1 is one in a family of protein phosphatases which has been shown *in vitro* to inactivate mitogen-activated protein kinases (MAPK).[7] Upregulation of DUSP1 directly results in the de-phosphorylation/inactivation of mitogen-activated protein kinase (MAPK) via dephosphorylation of the threonine and/or tyrosine residues and thus acts as a regulator through a negative feedback mechanism.[8, 9, 10] The induction of the DUSP1 gene and subsequent inhibition of MAPKs result in the reduced expression of pro-inflammatory cytokines[11, 12, 13]

While ICS therapy remains a successful first line treatment for persistent cases of asthma, consistent use of steroids can have many harmful side effects. In addition, polymorphisms in DUSP1 expression are associated with clinical effectiveness of ICS therapy for asthma.[5] For patients nonresponsive to ICS therapy β_2-adrenergic receptor agonists are used. However, the link between DUSP1 expression and patient responses to ICS/β_2-adrenergic receptor agonists is not completely understood. Many cell types respond to steroids by upregulating DUSP1, which dephosphorylates and inactivates both MAPK and JNK resulting in significant reductions in mediator release and the production of pro-inflammatory cytokines.

Lung mast cells (MC) are effector cells in the asthmatic response[14] and release of their asthma-triggering mediators has been shown to be inhibited by steroids[15, 16] and β_2-adrenergic receptor agonists.[17] The role of DUSP1 expression in activated human lung MC following pharmacological interventions has not been studied. Clues for a role for DUSP1 in MC-driven responses came from studies where MC from knockout mice lacking DUSP1 show enhanced degranulation and are highly susceptible to anaphylaxis.[18] Mast cell stabilizing nanomaterials were also shown to increase DUSP1 gene expression which paralleled inhibition of mediator release.[19] Based upon these similarities, we investigated the mechanism underlying C_{70}-Tetrainositol (C_{70}-I) inhibition of lung MC mediator release and compare its effects to those of steroids and β_2-adrenergic receptor agonists. As shown below C_{70}-I inhibited both FcεRI-mediated degranulation and GM-CSF cytokine production which was paralleled with increases in DUSP1 expression. Furthermore, C_{70}-I synergized with long-acting β_2-adrenergic receptor agonists (LABA) to potentiate this inhibition. These findings provide mechanistic insight into how C_{70}-I can mediate MC degranulation and cytokine production and how DUSP1 polymorphisms could influence varying responses in patient ICS treatment[5] through the upregulation of lung MC DUSP1 levels.

Methods

C_{70}-I synthesis and characterization

The C_{70}-I was synthesized by conjugating four *myo*-inositol molecules (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) to each C_{70} carbon cage via Bingel–Hirsch cyclopropanation reaction.[20, 21, 22] C_{70}-I has two inositol moieties at each of the two poles of the oval-shaped C_{70} molecule. These *myo*-inositol moieties solubilize the C_{70} molecule in aqueous media. *Myo*-inositol has been shown to significantly reduce inflammation in two widely used animal models for

inflammation.[23] The final compound was characterized using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), nuclear magnetic resonance (^{1}H and ^{13}C NMR), high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), and dynamic light scatter/zeta potential (DLS/ζ). C_{70}-I was found to be approximately 30 nm in diameter and fully dispersed in buffered saline, it has a molecular formula of $C_{100}H_{44}O_{32}$ with MW of 1756 Da, and a zeta potential of − 16.90 mV (Supplemental Figure 1A). Additionally, cell toxicity was assessed by incubation with concentrations shown effective for inhibition (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) and viability counts taken on days three, six, and nine. No toxicity was observed using trypan blue exclusions compared to control cells (Supplemental Figure 1B). Pre-incubation of MC with a fluorophoretagged-C_{70}-I (Texas Red) revealed uptake of fullerenes by MC with high efficiency at concentrations down to 10^{-10} M after five hours of incubation (Supplemental Figure 1C).

Gene microarray

Mast cells were harvested from tissue received by the Cooperative Human Tissue Network and cultured using methods previously described.[24, 25, 26] Mast cells (> 95%; 1×10^{7} cell/condition; each condition performed in triplicate) were incubated with or without C_{70}-I (10^{-5} M for 16 h; 37 °C; 6% CO_2), washed in fresh warm (37 °C) media and supernatants removed (to remove pre-formed mediators), and fresh warm medium alone (negative) or medium containing anti-FcεRI Abs (1 μg/ml) added for two hours at 37 °C. Cells were centrifuged, the supernatant and the pellet immediately frozen and microarray performed using the Human Whole Genome OneArray™ gene expression profiling service (Phalanx Biotech Group) report. Microarray analysis was performed and analyzed as described previously.[19] The UNCG Human Studies International Review Board approved all studies.

Immuno-blotting and immuno-precipitation

Mast cells (~ 3.0×10^{6} cells/ml) were pretreated with or without C_{70}-I and either cross-linked with anti-FcεRI Abs (0.5 μg/ml) or left untreated in a 37 °C incubator with 6% CO_2. Cells were lysed using methods previously described.[24, 25, 26] For immuno-precipitations (IP), lysates were pre-cleared with Protein A/G agarose beads, followed by overnight incubation with anti-DUSP1 (Abcam, Cambridge, MA) conjugated beads at 4 °C and washed extensively to isolate DUSP1 proteins. Precipitates were subjected to SDS-PAGE (10% SDS gels; Life Technologies, Grand Island, NY) and immuno-blotting (IB) as described.[24] For highly efficient and quantitative exposure, IR_{700} or IR_{800}-conjugated secondary antibodies were measured using Licor's Odyssey infrared imaging system (Licor Inc. Lincoln, NE). The band intensities were quantified as described[27] and normalized to the house-keeping gene, β-actin.

Mast cell mediator release

Lung MC of the MC_T (tryptase-positive, chymase-negative; predominant type seen in the alveoli of the lung > 93%)[28] were incubated for 16 h with or without C_{70}-I and ICS alone (at increasing dosages to evaluate dose response), or in combination with β_2-adrenergic receptor agonists or both overnight (16 h; 37 °C; 6% CO_2).[29] Cells were washed and activated with optimal concentrations (0.5 μg/ml) of anti-FcεRI Abs (3B4) for two hours (β-Hex) or 16 h (cytokines) at

37 °C. Mediator release was measured as described previously.[25, 30] Incubation times and concentrations were previously optimized and it was determined that 70-carbon fullerenes were taken up by MC through an endocytosis-dependent mechanism and persisted in the MC for up to one week, predominantly homing to the ER, but also to a lesser degree found in the mitochondria and lysosomes.[29]

Results

Pretreatment of MC with C_{70}-I upregulates DUSP1 gene and protein levels

Microarray analysis was used to obtain a broad overview of those FcεRI-associated signaling molecules influenced by C_{70}-I pre-incubation following FcεRI activation.[19] Interestingly, C_{70}-I caused a dramatic increase in expression of wide range of genes, including several within the DUSP family. Notably, DUSP1 increased relative gene expression levels approximately 55% compared to cells that were not cross-linked with FcεRI. Cells pre-incubated with C_{70}-I (10^{-5} M) for 16 h prior to activation increased gene expression to approximately 155% (Figure 1, *A*). Next, a dose response was performed to find the optimal concentration of C_{70}-I that could affect the protein expression of DUSP1 in MC. Cells incubated with C_{70}-I at 10^{-5} M were found to maximally increase DUSP1 protein expression (Figure 1, *B*). These data were further supported by IB of the DUSP1 protein at two, four, and six hours, where the pre-incubation of MC with C_{70}-I markedly increased protein expression by nearly 99% at four and six hours (Figure 1, *C*). Therefore, the MC inhibition observed with C_{70}-I was paralleled by significant increases in DUSP1 expression.

C_{70}-I upregulation of DUSP1 protein expression modulates activation of MAPK

To further understand the correlation between DUSP1 expression and MC inhibition with C_{70}-I, experiments were performed examining DUSP1 downstream targets. Given that increases in DUSP1 activity will directly result in reduction of MAPK via de-phosphorylation of threonine and/or tyrosine residues, protein analysis of phosphorylated MAPK (p-MAPK) levels in C_{70}-I MC pretreated with 10^{-5} M (the concentration that maximally activated DUSP1 protein expression; Figure 1) was performed. As expected, phosphorylation of MAPK was dramatically reduced at the four and six hour time points (Figure 2, *A*); the same time points that showed significant increase in DUSP1 expression (Figure 1, *C*). At six hours phosphorylation of MAPK was nearly 80% lower in cells treated with C_{70}-I as opposed to those that remained untreated (Figure 2, *B*) indicating the C_{70}-I-induced upregulation of DUSP1 directly results in de-phosphorylation of MAPK in MC. These results suggest that increases in DUSP1 from C_{70}-I directly prevent phosphorylation of MAPK which is crucial for MC activation leading to mediator release.

To delineate the possible mechanism of action that C_{70}-I inhibits phosphorylation of MAPK and subsequent cell activation, activated cells were subjected to immuno-precipitation (IP) with anti-DUSP1 antibody, followed by immuno-blotting (IB) with anti-ubiquiton (Ub), anti-phospo-MAPK, or anti-β-Actin. In FcεRI-crosslinked cells DUSP1 protein was highly ubiquitinated (Figure 2, *C*; compare lane one vs. lane two) which provides for a negative feedback mechanism for inhibition in other cell types.[31] However, when pre-incubated with C_{70}-I there was a dramatic

reduction in DUSP1 ubiquitination (Figure 2, *C*; lane 3). These data indicate that the increased DUSP1 levels (and subsequent decrease in MAPK levels) in cells pre-incubated with C_{70}-I are due to the inhibition of DUSP1 ubiquitination and subsequent degradation through the ubiquitin-proteasome pathway.

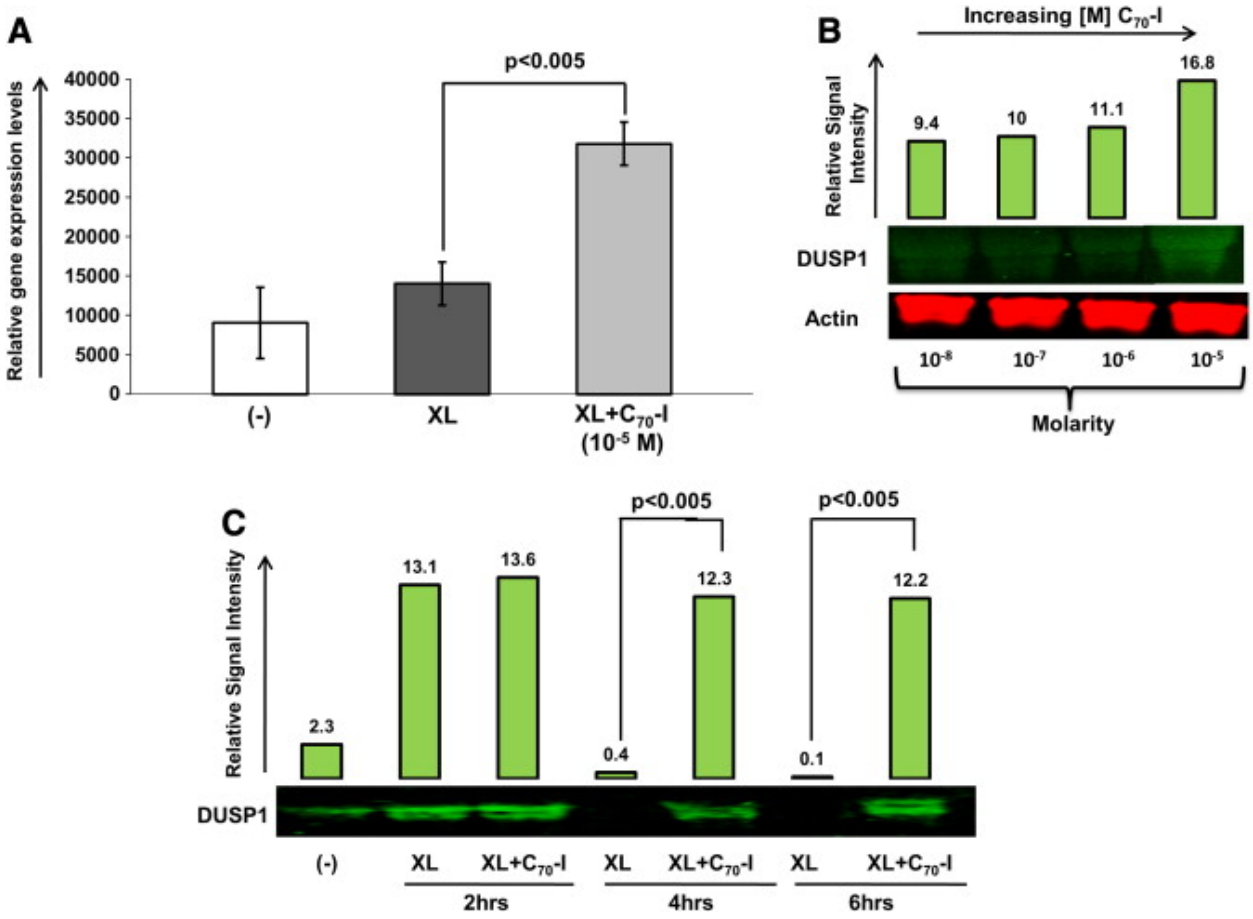


Figure 1. Mast cell pretreatment with C_{70}-I increases DUSP1 gene and DUSP1 protein expression. Fullerene (C_{70}-I [10^{-5} M) treated and untreated MC were challenged with (XL) or without (−) anti-FcεRI (1 μg/ml) for two hours. Cell pellets from each condition in triplicates were used for RNA isolation and gene microarray as described. Gene microarray data conduction by Phalynx Biotechnology **(A)**demonstrates the increases in mean relative intensities of DUSP1 gene expression increases in C_{70}-I pretreated samples ($P < 0.005$; ± SD of six observed values). To evaluate dose responses of C_{70}-I **(B)**, MC were pretreated with C_{70}-I (10^{-8}-10^{-5} M) and lysed as described. The lysates were immuno-blotted with polyclonal rabbit-anti-DUSP1 antibodies (1 μg/ml) and probed on the Odyssey infrared imaging system with anti-rabbit IR_{800} secondary antibodies (results typical in three separate immuno-blots). Relative pixel intensity (green bar graph) of the IR_{800} protein band expression of DUSP1 was quantified and normalized (Licor Odyssey imaging software) to the house-keeping gene, β-Actin to ensure equal protein levels were loaded into each lane (red band). To evaluate time course effects of C_{70}-I **(C)**, MC were cross-linked with (XL) or without (−) anti-FcεRI (1 μg/ml) for two, four, or six hours and immuno-blotted as described in **(B)**.

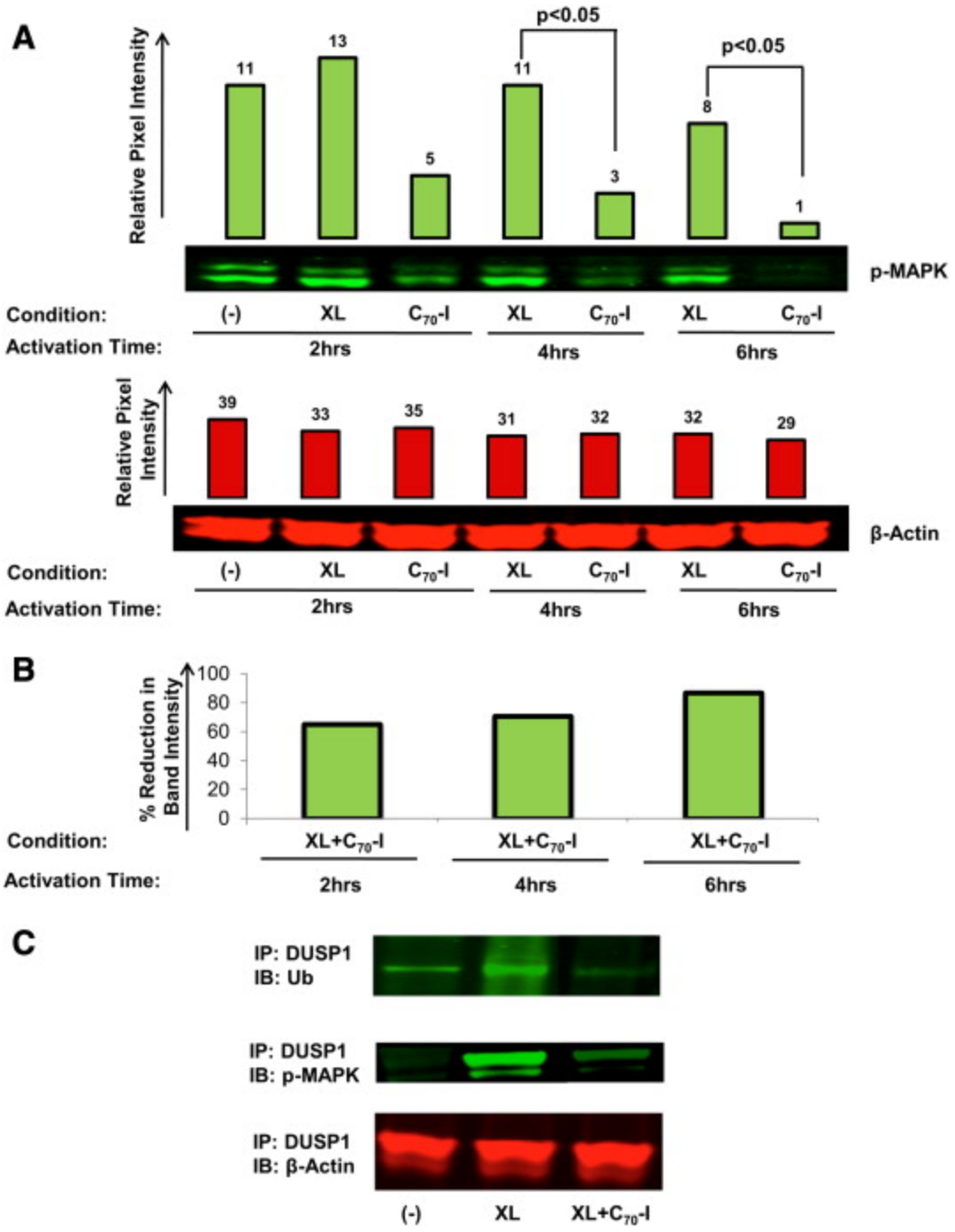


Figure 2. Mast cell pretreatment with C_{70}-I decreases phosphorylation of MAPK1. Mast cells were pretreated with or without C_{70}-I (10^{-5} M), washed, and challenged with (XL) or without (−) anti-FcεRI (1 μg/ml) for two, four, or six hours. Lysates were immuno-blotted with polyclonal rabbit-anti-phospho-MAPK1 antibodies (1 μg/ml) and probed on the Odyssey infrared imaging system with anti-rabbit IR_{800}secondary antibodies **(A)**. Relative pixel intensity of the IR_{800} phospho-MAPK1 protein band (green band and bar graph) and IR_{700} house-keeping gene, β-Actin (red band and bar graph) was quantified. Percent reduction of MAPK1 in untreated MC and C_{70}-I pretreated MC was normalized to the house-keeping gene and pixel density of was calculated **(B)**. Results are typical of three separate immuno-blots. In **(C)** cells were immuno-precipitated with anti-DUSP1 antibodies, run on SDS-PAGE, and blotted with the indicated antibodies.

C_{70}-I and corticosteroids inhibit FcεRI-mediated-MC degranulation

Inhaled corticosteroids can also inhibit MC mediator release, but certain asthmatic patients are less responsive to ICS, due potentially to DUSP1 polymorphisms. Thus, based on the results suggesting a DUSP1-dependent inhibition of lung MC using C_{70}-I, several commonly used ICS were compared in their ability to inhibit FcεRI-mediator release. A battery of ICS was initially evaluated (data not shown) and revealed that fluticasone and budesonide had the most significant impact on blunting mediator release from MC, therefore comparative studies were conducted on these two specific ICS. C_{70}-I (10^{-7}-10^{-5} M) and fluticasone or budesonide ICS (10^{-6} and 10^{-5} M) were capable of significantly reducing MC degranulation without affecting cell viability (> 95% using trypan blue exclusion) compared to their untreated counterparts (Table 1). Optimal inhibition was observed with 10^{-5} M pretreatments of fluticasone, budesonide, and C_{70}-I resulting in inhibitions of 55%, 48%, and 68% respectively. Higher doses did not increase inhibition and resulted in some loss in cellular viability. These data demonstrate that C_{70}-I is capable and comparable to ICS mono-therapy in preventing human lung MC FcεRI-mediated degranulation.

Table 1. Mean % FcεRI-mediated degranulation in C_{70}-I or ICS (fluticasone or budesonide) treated and untreated lung MC.

Treatment/[Conc; M]	Release β-Hex (%) ± S.E.	(%) Inhibition β-Hex ± S.E.
Spontaneous	4.7 ± 0.4	
Positive Control	56.9 ± 4.7	
Fluticasone [8]	52.7 ± 3.7	7% ± 6.8%
Fluticasone [7]	42.3 ± 2.2	26% ± 5.1%
Fluticasone [6]*	34.9 ± 2.3	39% ± 4.0%
Fluticasone [5] *	25.6 ± 0.5	55% ± 1.2%
Budesonide [8]	56.6 ± 3.8	1% ± 8.0%
Budesonide [7]	41.8 ± 3.5	27% ± 7.6%
Budesonide [6] *	35.6 ± 1.9	37% ± 3.4%
Budesonide [5] *	29.6 ± 2.9	48% ± 4.2%
C_{70}-I [8]	53.7 ± 2.1	6% ± 3.9%
C_{70}-I [7] *	34.4 ± 1.4	40% ± 4.1%
C_{70}-I [6] *	24.5 ± 3.6	57% ± 7.2%
C_{70}-I [5] **	18.2 ± 1.5	68% ± 4.1%

Data shown are mean ± standard error (S.E.) of β-Hex release and percent inhibition of β-Hex release compared to untreated, activated MC. Results are representative of three different experiments in triplicates.
ICS: Inhaled corticosteroid; MC: Mast Cell; M: Molar.
* $P < 0.05$.
** $P < 0.005$.

Co-incubation of C_{70}-I or corticosteroids with long-acting β_2-adrenergic receptor agonists inhibits FcεRI-mediated-MC degranulation

Combination techniques of ICS and LABA are commonly used by physicians to treat patients suffering from persistent asthma. Given the ability of C_{70}-I to inhibit MC inflammatory mediator release through a DUSP1 mechanism and that patient responses to ICS are mediated by DUSP1 the next aim was to investigate the ability of C_{70}-I to synergize with the LABA. Several LABA were investigated at different concentrations, the most efficient inhibitor LABA inhibitor, salmeterol, was selected for synergistic studies. As anticipated, MC treated with C_{70}-I or ICS combined with LABA (dual-agent approach) revealed increased inhibition of degranulation

compared to the single-agent approach. A side-by-side comparison of C_{70}-I and LABA or ICS and LABA combinatory incubation with MC is illustrated in Table 2. The dual treatment response resulted in optimal inhibition of degranulation at concentrations of 10^{-5} M fluticasone, budesonide, or C_{70}-I co-incubated with 10^{-5} M salmeterol with statistically significant inhibitions of 59%, 57%, and 75% respectively, on average an increased inhibition of cellular degranulation by 7% compared to the single-agent approach. Figure 3 represents a comparison of the single-agent (either ICS or C_{70}-I alone) and dual-agent (ICS and LABA or C_{70}-I and LABA) approach validating C_{70}-I as a potential alternative to traditional ICS approaches for asthma therapy aimed at reducing lung MC hyper-reactivity. In addition, these data illustrate both compounds' synergistic abilities with the LABA salmeterol to prevent FcεRI-mediated lung MC degranulation. Although, statistical evaluation of the single-agent versus dual-agent approach did not approach significance, it was observed that inhibition was improved in most cases when MC were treated synergistically with LABAs and/or fullerenes/ICS. However, the evaluation fullerenes and LABA against ICS (fluticasone or budesonide) and LABA at a concentration of 10^{-7} M produced statistically significant results (¥; in Figure 3), indicating that the fullerene molecule may be useful in blunting degranulation with similar efficiency to ICS.

Table 2. Synergistic effects on % FcεRI-mediated degranulation in fullerene (C_{70}-I) and LABA (salmeterol) or ICS (fluticasone or budesonide) and LABA (salmeterol) treated and untreated MC.

Treatment/[Conc; M]	Release β-Hex (%) ± S.E.	(%) Inhibition β-Hex ± S.E.
Spontaneous	6.5 ± 0.5	
Positive Control	35.9 ± 3.1	
Fluticasone [8]/Salmeterol [5]	35.7 ± 0.6	1% ± 1.4%
Fluticasone [7]/Salmeterol [5]	26.8 ± 1.5	26% ± 4.2%
Fluticasone [6]/Salmeterol [5] *	18.1 ± 1.2	50% ± 3.1%
Fluticasone [5]/Salmeterol [5] *	14.8 ± 2.9	59% ± 5.1%
Budesonide [8]/Salmeterol [5]	37.8 ± 1.8	No Effect
Budesonide [7]/Salmeterol [5]	30.3 ± 3.5	16% ± 5.5%
Budesonide [6]/Salmeterol [5] *	19.4 ± 1.5	46% ± 2.9%
Budesonide [5]/Salmeterol [5] *	15.4 ± 2.7	57% ± 4.2%
C_{70}-I [8]/Salmeterol [5]	34.0 ± 0.8	6% ± 1.2%
C_{70}-I [7]/Salmeterol [5] *	15.9 ± 2.0	56% ± 4.6%
C_{70}-I [6]/Salmeterol [5] **	13.8 ± 1.4	62% ± 2.7%
C_{70}-I [5]/Salmeterol [5] **	9.0 ± 1.3	75% ± 4.0%

Data shown are mean ± standard error (S.E.) of β-Hex release and percent inhibition of β-Hex release compared to untreated, activated MC. Results are representative of three different experiments in triplicates.
ICS: Inhaled corticosteroid; MC: Mast Cell; M: Molar.
* $P < 0.05$.
** $P < 0.005$.

C_{70}-I and corticosteroids inhibit FcεRI-mediated-MC GM-CSF cytokine production

Mast cells produce GM-CSF in response to inflammatory stimuli; it is observed that ICS therapy can suppress the release of inflammatory mediators.[1] Therefore, the ability of C_{70}-I and ICS to reduce lung MC FcεRI-mediated-GM-CSF release was investigated. C_{70}-I/ICS were evaluated separately and in conjunction with a LABA. As expected, ICS pre-incubation resulted in a dose dependent, statistically significant reduction in mediator release when compared to untreated controls (Figure 4, *A*). Pre-incubation with C_{70}-I at increasing doses resulted in greater inhibition of mediator release than observed in ICS treatments at similar concentrations (Figure 4, *A*). The

synergistic effect of ICS/C_{70}-I co-incubation with LABA resulted in greater inhibition of GM-CSF production (Figure 4, *B*), just as observed in the degranulation assays. A comparison of the single-agent and dual-agent approach is depicted in Figure 4, *C* demonstrating that C_{70}-I has similar GM-CSF suppressive abilities as traditional ICS, as well as an ability to synergize with LABA to further inhibit cytokine production. Albeit, as was observed in the degranulation studies statistical significance was not revealed when comparing the single-agent and dual-agent therapies, it was found that lower concentrations of C_{70}-I (10^{-8} and 10^{-7} M) with or without LABAs produced statistically significant inhibition when compared to the equivalent ICS (with or without LABA) treatment (¥, Figure 4, *C*).

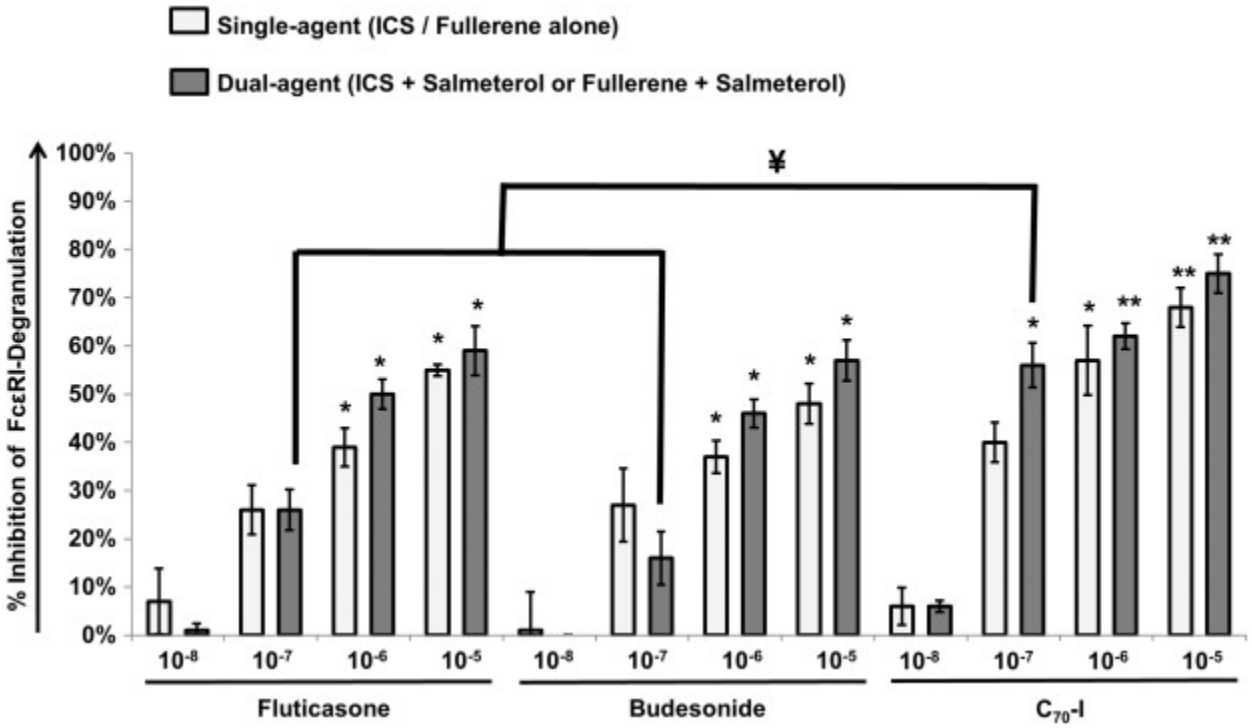


Figure 3. The effects of MC pretreatment with C_{70}-I/ICS individually or in combination with LABA on degranulation. MC were cultured with C_{70}-I, fluticasone, or budesonide (10^{-8}-10^{-5} M) or C_{70}-I, fluticasone, or budesonide at (10^{-8}-10^{-5} M;) in combination with salmeterol (10^{-5} M); each pretreatment condition as well as untreated MC controls (Spontaneous and Positive) was challenged with (XL) or without (−) anti-FcεRI (1 μg/ml) for 30 min at 37 °C and supernatants were collected to measure degranulation as described in previously.[24] All studies were performed using at least three separate MC donor cultures in triplicates. Statistical significance indicated with * ($P < 0.05$) and ** ($P < 0.01$) using the Student's *t* test to compare respective treatment to untreated positive (FcεRI) controls. Statistical significance indicated with ¥ ($P < 0.05$) using the Student's *t* test to compare efficacy ICS versus fullerene treatment at respective concentrations.

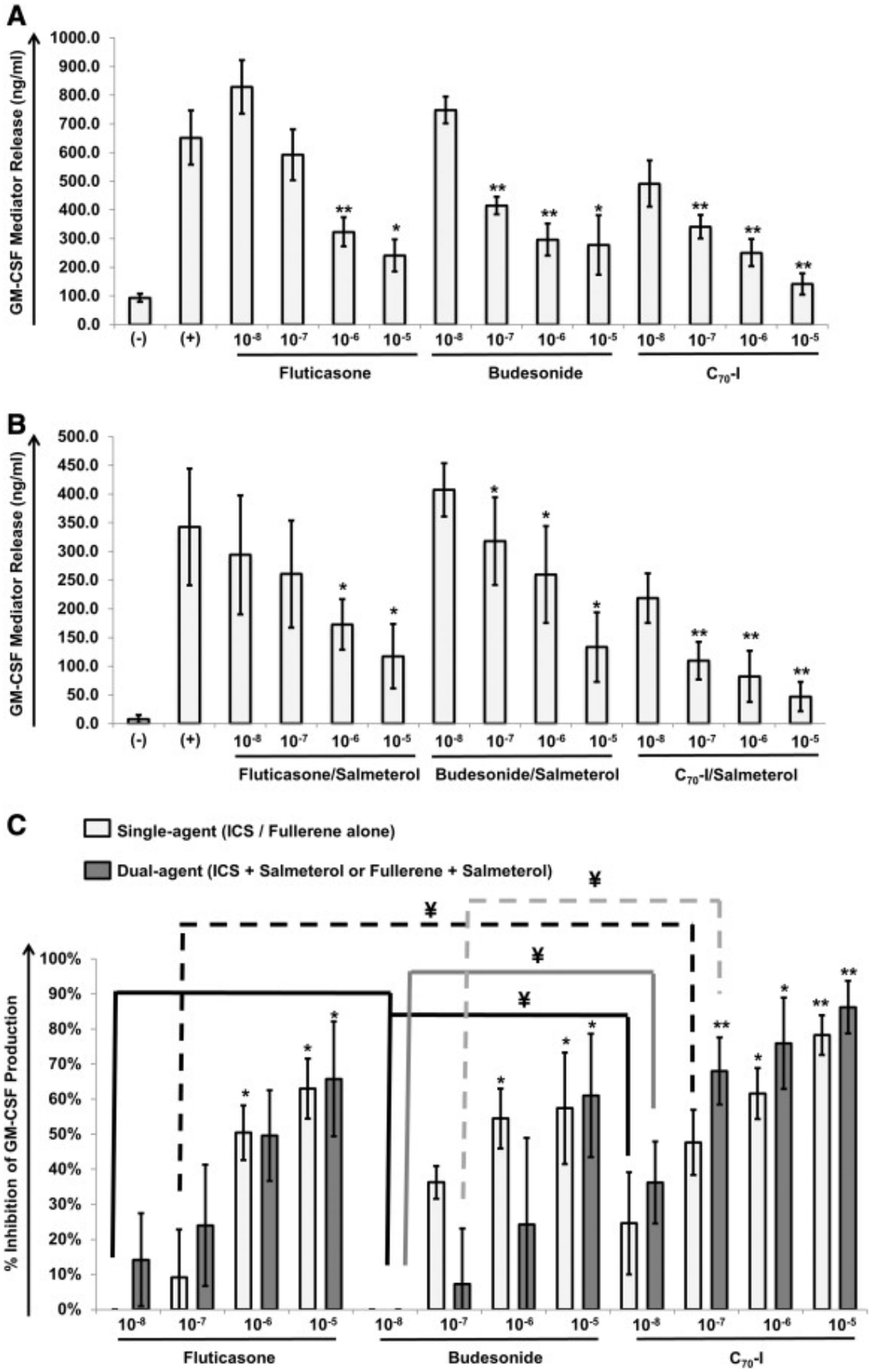


Figure 4. The effects of MC pretreatment with C_{70}-I/ICS individually or in combination with LABA on GM-CSF cytokine production. MC were cultured with C_{70}-I, fluticasone, or budesonide at (10^{-8}-10^{-5} M) **(A)** or C_{70}-I, fluticasone, or budesonide at (10^{-8}-10^{-5} M) in combination with salmeterol (10^{-5} M) **(B)**; each pretreatment condition as well as untreated MC controls (Spontaneous and Positive) was challenged with (XL) or without (−) anti-FcεRI (1 μg/ml) for 30 min at 37 °C and supernatants were collected to measure mediator release as described. Values are presented as total mediator release ng/ml and are representative of three experiments analyzed in triplicate. Percent inhibition of GM-CSF production of fullerene/ICS alone (white bar) and fullerene/ICS in combination with LABA is displayed in **(C)** Statistical significance indicated with * ($P < 0.05$) and ** ($P < 0.01$) using the Student's *t* test to compare respective treatment to untreated positive (FcεRI) controls. Statistical significance indicated with ¥ ($P < 0.05$) using the Student's *t* test to compare efficacy of ICS versus fullerene treatment at respective concentrations.

Discussion

Inhaled corticosteroids are regarded as the hallmark treatment for patients suffering from asthma. Their beneficial effect has been shown to be due in part to the inhibition of pro-inflammatory genes. Mast cells play a key role in IgE-dependent inflammatory diseases such as asthma. In many cases, MC mediate both the sustained and immediate inflammatory responses via degranulation and production of cytokines. Stimulation through the IgE-receptor, FcεRI, results in the activation of MAPK and subsequently induces degranulation of preformed inflammatory mediators. The role that ICS play in modulating MC activation is of much interest, yet a defined understanding of the inhibitory mechanism of ICS at the cellular level has not been established. However, one protein, DUSP1, has been given significant attention.[1, 6, 11] Here it is shown that C_{70}-I upregulates the DUSP1 gene in human lung MC resulting in higher protein levels and lower MAPK activation through an ubiquitination pathway. Furthermore, combinatory treatments of ICS and LABA can further inhibit MC activation and similar inhibition was observed when pre-treating with C_{70}-I, instead of ICS.

Part of the impetus for these studies was the often unpredictable and varied side effects of ICS in asthma patients[32] as well as the donor variability in the benefit of ICS in some patients.[5] This has led to efforts to separate the desired anti-inflammatory effects of ICS[33] and better understand the link between DUSP1 polymorphisms and their link between asthma lung function. Our studies have demonstrated that fullerenes are not toxic *in vitro* or *in vivo*[19, 22, 34, 35, 36, 37, 38, 39] but instead have an overall anti-inflammatory effect. Additionally, fullerene uptake is not receptor mediated, however it is endocytosed by the cell.[29] Given that this anti-inflammatory effect is now demonstrated in human lung MC, both alone and in synergy with LABA, they may provide an alternative to ICS for patients that are steroid resistant without the unwanted side effects observed in high-dosage therapies. It is established the anti-inflammatory actions of ICS are known to modulate gene expression via the glucocorticoid receptor (GR).[11] Side effects of ICS are commonly attributed to gene induction by ligand-activated GRs.[11] While the ICS treatment strategy is deemed an extremely potent anti-inflammatory therapy, their use often comes with some reticence from physicians due to the range and severity of their side effects. Furthermore, it has been shown that mutated GRs have failed to activate the ICS response in a subset of patients.[11] Consequently, the non-receptor mediated mechanism of fullerenes may kinetically favor therapeutic intervention in cases of patients that fail to respond adequately or inversely to traditional ICS treatment regimes. Additionally, the differences in mechanisms between C_{70}-I and ICS, although still in need of further investigation, may be advantageous for a C_{70}-I-inspired therapy through a decrease in ICS-associated side effects.

As a protein tyrosine phosphatase, DUSP1 is known to modulate immune responses. Specifically, DUSP1 is considered an ICS-induced phosphatase that is capable of inhibiting phosphorylation of MAPK in MC as first identified by Kassel et al.[40] Furthermore, clinical effectiveness of ICS in patients with DUSP1 polymorphisms can be variable, suggesting that new therapies should be examined to help better control disease progression.[5] The C_{70}-I was capable of increasing both gene expression and protein levels of the inhibitory regulator DUSP1, just as in ICS therapies. Pre-incubation of FcεRI challenged MC with C_{70}-I resulted in greater than a 50% upregulation of DUSP1 gene expression when compared to those untreated, which was analogous to high levels of DUSP1 protein expression in challenged MC at early (4 and 6 h)

time-points. As expected, the upregulation of DUSP1 caused significant (greater than 65%) reductions in MAPK activation at these same time-points, suggesting that C_{70}-I could function similarly to ICS intervention in modulating MC responses to stimuli.

The gene and protein expression results led to examination of commonly used *in vitro* functional assays to compare two ICS, fluticasone and budesonide, to C_{70}-I as a possible alternative to traditional asthma-ICS regulated therapy. Being that both compounds were capable of mediating the phosphatase DUSP1, we sought to discover if C_{70}-I could be a successful candidate for modulating MC-degranulation and cytokine production. Specifically, optimal doses of all three interventions, fluticasone, budesonide, and C_{70}-I result in significant inhibitions of degranulation by 55%, 48%, and 68% respectively, with fullerenes being nearly 20% more effective than traditional ICS treatments. These results were paralleled in GM-CSF cytokine production, where optimal concentrations of fluticasone and budesonide inhibit release by 63% and 57% respectively, the same concentration of C_{70}-I revealed nearly 30% greater inhibitions with significant reductions in cytokine production by 78%. In light of the degranulation and cytokine release assays, fullerenes may be an alternative to traditional ICS treatment. Overall, the fullerene intervention was capable of providing statistically significant reductions in MC-mediator release when compared to ICS treatment at the lower concentrations evaluated (10^{-7} M). In situations of persistent asthma, patients are often given a dual-therapy of ICS and LABA. Therefore, it was considered important to evaluate the ability of C_{70}-I to synergize with LABA. Consequently, these strategies mirrored those commonly observed in combinatory therapies of ICS/LABA. Such that C_{70}-I co-incubation with the LABA, salmeterol, resulted in increased inhibitions compared to fullerene mono-therapy. The synergistic effects were similar to those in the ICS/LABA dual-therapeutic strategy, fluticasone/salmeterol, or budesonide/salmeterol, while not statistically significant, repeatedly revealed enhanced inhibition of degranulation between 4% and 10% respectively when compared to the mono-therapeutic approaches. C_{70}-I combinatory therapies with salmeterol resulted in nearly a 10% increase in inhibition of degranulation compared to C_{70}-I alone. These same trends were observed in cytokine production. Taken together, each of these results provides a heretofore undiscovered mechanism for controlling asthma using novel water-soluble fullerenes that are functionally similar to the present first-line treatment method, ICS.

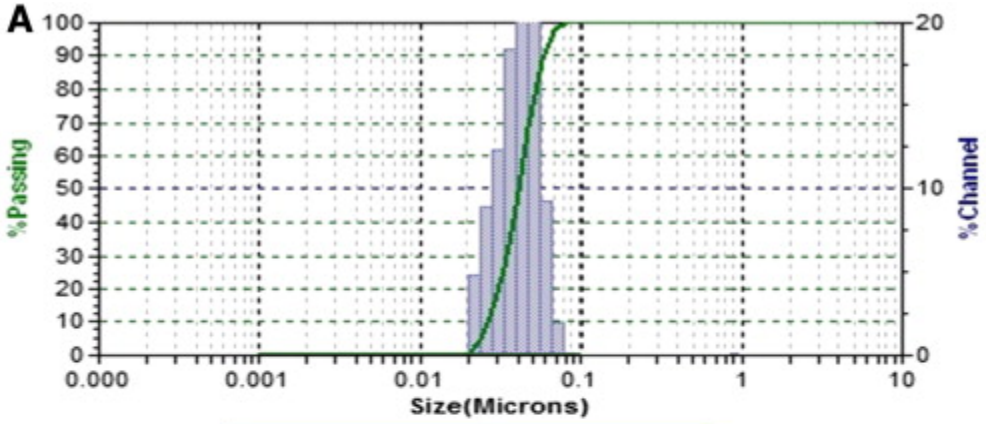
Further studies will be required to understand how C_{70}-I functions to increase DUSP1 levels after pretreatment; however it is proposed that C_{70}-I offers some protective qualities that prevent DUSP1 ubiquitination and subsequent activation-induced degradation. Following MC-activation, untreated cells displayed a rapid increase in DUSP1 production, a production that diminishes nearly 95% after two hours and 100% after four hours. However, the duration of this spike in production persists significantly longer in C_{70}-I treated MC as displayed in Figure 1, *C*. It is proposed that C_{70}-I can prevent proteolysis degradation of the phosphatase, allowing it to act on MAPK well into six hours after stimulation with FcεRI. Our efforts are now focused on understanding what C_{70}-I interacts with directly or if it is indirectly reducing ubiquitination through another mechanism, possibly reduced oxidative stress as has been demonstrated in other cell types.[41]

In conclusion, these studies reveal that strategically functionalized fullerene nanomaterials, like ICS, can control human lung MC mediator release through a DUSP1-mediated mechanism.

However, unlike ICS, their efficacy is not mediated through the glucocorticoid receptor. Since the fullerenes are taken up by MC via endocytosis, this may prove beneficial in patients with receptor mutations, who subsequently fail to respond to ICS treatment. These findings show that using water-soluble fullerenes with side-chain moieties such as C_{70}-I could be pursued as a non-steroidal based therapy for patients that fail to respond clinically or respond adversely to ICS treatment. Additionally, these findings may not only provide a more robust controller therapy for patients with asthma, but a therapy with reduced adverse effects commonly associated with ICS treatment.

Appendix A. Supplementary data

A

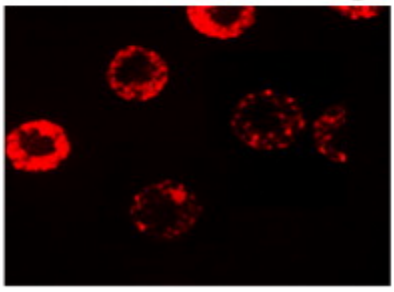


Mean Diameter (µm)	Zeta-Potential (mv)
0.0341	-16.90

B

% Viability	Day 3	Day 6	Day 9
Untreated	97.8%	95.6%	92.9%
C_{70}-I (10^{-8} M)	98.5%	94.1%	91.0%
C_{70}-I (10^{-7} M)	99.4%	92.9%	91.9%
C_{70}-I (10^{-6} M)	98.3%	92.6%	91.2%
C_{70}-I (10^{-5} M)	97.5%	92.5%	92.5%

C



Supplementary Figure 1.

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