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A STEROID-MIMICKING NANOMATERIAL THAT MEDIATES INHIBITION OF HUMAN LUNG MAST CELL 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 C₇₀-based fullerene conjugated to four *myo*-inositol molecules (C₇₀-I) was examined *in vitro* for its effects on the signaling pathways leading to mediator release from human lung MC. The C₇₀-I fullerene stabilize MC and act synergistically with long-acting β_2 -adrenergic receptor agonists (LABA) to enhance inhibition of MC mediator release through Fc ϵ RI-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 C₇₀-I treated cells. The increase in DUSP1 expression was due to the ability of C₇₀-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.

Keywords

Fullerenes; Inhaled corticosteroids; Mast cell; Dual-specificity phosphatase one

Introduction

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

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the mechanisms of ICS action to better understand non-responsive patients¹. Several different studies have found that the dual-specificity phosphatase 1 (DUSP1) is a prospective regulator of this ICS response^{2–6}. 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 dephosphorylation/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^{8–10}. The induction of the DUSP1 gene and subsequent inhibition of MAPKs results in the reduced expression of pro-inflammatory cytokines^{11–13}.

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 asthma⁵. 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 p38 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 response¹⁴ and release of their asthma-triggering mediators has been shown to be inhibited by steroids^{15, 16} and β_2 -adrenergic receptor agonists¹⁷. 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 anaphylaxis¹⁸. Mast cell stabilizing nanomaterials were also shown to increase DUSP1 gene expression which paralleled inhibition of mediator release¹⁹. Based upon these similarities, we investigated the mechanism underlying C₇₀-Tetrainositol (C₇₀-I) inhibition of lung MC mediator release and compare its effects to those of steroids and β_2 -adrenergic receptor agonists. As shown below C₇₀-I inhibited both Fc ϵ RI-mediated degranulation and GM-CSF cytokine production which was paralleled with increases in DUSP1 expression. Furthermore, C₇₀-I synergized with long-acting β_2 -adrenergic receptor agonists (LABA) to potentiate this inhibition. These findings provide mechanistic insight into how C₇₀-I can mediate MC degranulation and cytokine production and how DUSP1 polymorphisms could influence varying responses in patient ICS treatment⁵ through the upregulation of lung MC DUSP1 levels.

Methods

C₇₀-I synthesis and characterization

The C₇₀-I was synthesized by conjugating four *myo*-inositol molecules (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) to each C₇₀ carbon cage via Bingel-Hirsch cyclopropanation reaction^{20, 21, 22}. C₇₀-I has two inositol moieties at each of the two poles of the oval-shaped C₇₀ molecule. These *myo*-inositol moieties solubilize the C₇₀ molecule in aqueous media. *Myo*-inositol has been shown to significantly reduce inflammation in two widely used animal models for inflammation²³. The final compound was characterized using matrix

assisted laser desorption ionization mass spectrometry (MALDI-MS), nuclear magnetic resonance (^1H and ^{13}C NMR), high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), and dynamic light scatter/zeta potential (DLS/ ζ). $\text{C}_{70}\text{-I}$ was found to be approximately 30 nm in diameter and fully dispersed in buffered saline, it has a molecular formula of $\text{C}_{100}\text{H}_{44}\text{O}_{32}$ with MW of 1,756 Da, and a zeta potential of -16.90mV (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 and compared to control cells (Supplemental Figure 1B). Pre-incubation of MC with a fluorophore tagged- $\text{C}_{70}\text{-I}$ (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^{24–26}. Mast cells ($>95\%$; 1×10^7 cell/condition; each condition performed in triplicate) were incubated with or without $\text{C}_{70}\text{-I}$ (10^{-5} M for 16 hours; 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-Fc ϵ RI Abs ($1 \mu\text{g/ml}$) 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 OneArrayTM gene expression profiling service (Phalanx Biotech Group) report. Microarray analysis was performed and analyzed as described previously¹⁹. The UNCG Human Studies International Review Board approved all studies.

Immuno-blotting and immuno-precipitation

Mast cells ($\sim 3.0 \times 10^6$ cells/ml) were pretreated with or without $\text{C}_{70}\text{-I}$ and either cross-linked with anti-Fc ϵ RI Abs ($0.5 \mu\text{g/ml}$) or left untreated in a 37°C incubator with 6% CO_2 . Cells were lysed using methods previously described^{24–26}. 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 described²⁴. For highly efficient and quantitative exposure, IR₇₀₀ or IR₈₀₀-conjugated secondary antibodies were measured using Licor's Odyssey infrared imaging system (Licor Inc. Lincoln, NE). The band intensities were quantified as described²⁷ and normalized to the housekeeping 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 hours with or without $\text{C}_{70}\text{-I}$ and ICS alone (at increasing dosages to evaluate dose response), or in combination with β_2 -adrenergic receptor agonists or both overnight (16 hours; 37°C ; 6% CO_2)²⁹. Cells were washed and activated with optimal concentrations ($0.5 \mu\text{g/ml}$) of anti-Fc ϵ RI Abs (3B4) for

two hours (β -Hex) or 16 hours (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 lysosomes²⁹.

Results

Pretreatment of MC with C₇₀-I upregulates DUSP1 gene and protein levels

Microarray analysis was used to obtain a broad overview of those Fc ϵ RI-associated signaling molecules influenced by C₇₀-I pre-incubation following Fc ϵ RI activation¹⁹. Interestingly, C₇₀-I caused a dramatic increase in expression of wide range of genes, including several within the DUSP family. Notable, DUSP1 increased relative gene expression levels approximately 55% compared to cells that were not cross-linked with Fc ϵ RI. Cells pre-incubated with C₇₀-I (10^{-5} M) for 16 hours prior to activation increased gene expression to approximately 155% (Figure 1A). Next, a dose response was performed to find the optimal concentration of C₇₀-I that could affect the protein expression of DUSP1 in MC. Cells incubated with C₇₀-I at 10^{-5} M was found to maximally increase DUSP1 protein expression (Figure 1B). This data was further supported by IB of the DUSP1 protein at two, four, and six hours, where the pre-incubation of MC with C₇₀-I markedly increased protein expression by nearly 99% at four and six hours (Figure 1C). Therefore, the MC inhibition observed with C₇₀-I was paralleled by significant increases in DUSP1 expression.

C₇₀-I upregulation of DUSP1 protein expression modulates activation of MAPK

To further understand the correlation between DUSP1 expression and MC inhibition with C₇₀-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 C₇₀-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 2A); the same time points that showed significant increase in DUSP1 expression (Figure 1C). At six hours phosphorylation of MAPK was nearly 80% lower in cells treated with C₇₀-I as opposed to those that remained untreated (Figure 2B) indicating the C₇₀-I-induced upregulation of DUSP1 directly results in de-phosphorylation of MAPK in MC. These results suggest that increases in DUSP1 from C₇₀-I directly prevents phosphorylation of MAPK which is crucial for MC activation leading to mediator release.

To delineate the possible mechanism of action that C₇₀-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-ubiquitin (Ub), anti-phospho-MAPK, or anti- β -Actin. In Fc RI-crosslinked cells DUSP1 protein was highly ubiquitinated (Figure 2C; compare lane one vs. lane two) which provides for a negative feedback mechanism for inhibition in other cell types³¹. However, when pre-incubated with

C₇₀-I there was a dramatic reduction in DUSP1 ubiquitination (Figure 2C; lane 3). These data indicate that the increased DUSP1 levels (and subsequent decrease in MAPK levels) in cells pre-incubated with C₇₀-I is due to the inhibition of DUSP1 ubiquitination and subsequent degradation through the ubiquitin-proteasome pathway.

C₇₀-I and corticosteroids inhibit Fc RI-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 C₇₀-I, several commonly used ICS were compared in their ability to inhibit Fc RI-mediator release. A battery of ICS were 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. C₇₀-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 C₇₀-I resulting in inhibitions of 55%, 48%, and 68% respectively. Higher doses did not increase inhibition and resulted in some loss in cellular viability. This data demonstrates that C₇₀-I is capable and comparable to ICS monotherapy in preventing human lung MC Fc RI-mediated degranulation.

Co-incubation of C₇₀-I or corticosteroids with long-acting β_2 -adrenergic receptor agonists inhibits Fc RI-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 C₇₀-I to inhibit MC inflammatory mediator release through a DUSP1 mechanism and patient responses to ICS is mediated by DUSP1 the next aim was to investigate the ability of C₇₀-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 C₇₀-I or ICS combined with LABA (dual-agent approach) revealed increased inhibition of degranulation than the single-agent approach. A side-by side comparison of C₇₀-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 C₇₀-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 C₇₀-I alone) and dual-agent (ICS and LABA or C₇₀-I and LABA) approach validating C₇₀-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 Fc RI-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.

C₇₀-I and corticosteroids inhibit Fc RI-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 mediators¹. Therefore, the ability of C₇₀-I and ICS to reduce lung MC Fc RI-mediated-GM-CSF release was investigated. C₇₀-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 4A). Pre-incubation with C₇₀-I at increasing doses resulted in greater inhibition of mediator release than observed in ICS treatments at similar concentrations (Figure 4A). The synergistic effect of ICS/C₇₀-I co-incubation with LABA resulted in greater inhibition of GM-CSF production (Figure 4B), just as observed in the degranulation assays. A comparison of the single-agent and dual-agent approach is depicted in Figure 4C demonstrating C₇₀-I have 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 C₇₀-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 4C).

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 yet been established. However, one protein, DUSP1, has been given significant attention^{1, 6, 11}. Here it is shown that C₇₀-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 C₇₀-I, instead of ICS.

Part of the impetus for these studies was the often unpredictable and varied side effects of ICS in asthma patients³² as well as the donor variability in the benefit of ICS in some patients⁵. This has led to efforts to separate the desired anti-inflammatory effects of ICS³³ 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, 34–39} but instead have an overall anti-inflammatory effect. Additionally, fullerene uptake is not receptor mediated, however is endocytosed by the cell²⁹. Given that this anti-inflammatory effect is now demonstrated in human lung MC, both alone and in synergy with ICS and LABA, they may provide an alternative to ICS for patients that are steroid resistant without the unwanted side effects. 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 GRs¹¹. 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 patients¹¹. 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 C₇₀-I and ICS, although still in need of further investigation, may be advantageous for a C₇₀-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 al⁴⁰. 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 progression⁵. The C₇₀-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 C₇₀-I resulted in greater than a 50% upregulation of DUSP1 gene expression when compared to those untreated, which were analogous to high levels of DUSP1 protein expression in challenged MC at early (4 and 6 hours) time-points. As expected, the upregulation of DUSP1 caused significant (greater than 65%) reductions in MAPK activation at these same time-points, suggesting that C₇₀-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 C₇₀-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 C₇₀-I could be a successful candidate for modulating MC-degranulation and cytokine production. Specifically, optimal doses of all three interventions, fluticasone, budesonide, and C₇₀-I results 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 C₇₀-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 similar doses 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 C₇₀-I to synergize with LABA. Consequently, these strategies mirrored those commonly observed in combinatory therapies of ICS/LABA. Such that C₇₀-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. C₇₀-I combinatory therapies with salmeterol resulted in nearly a 10% increase in inhibition of degranulation compared to C₇₀-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 C₇₀-I functions to increase DUSP1 levels after pretreatment; however it is proposed that C₇₀-I offer 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 C₇₀-I treated MC as displayed in Figure 1C. It is proposed that C₇₀-I 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 C₇₀-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 C₇₀-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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This study focuses on the utility of strategically functionalized fullerene nanomaterials as a potential alternative therapy for inhaled corticosteroids, specifically in regards to mast cell mediated allergic inflammation. Myo-Inositol modified 70-carbon fullerenes (C₇₀-I) are taken up by mast cells and result in down-regulation of MAPK via DUSP1 induction, similar to inhaled corticosteroids, resulting in significant inhibitions of inflammatory mediator release (degranulation and GM-CSF cytokine release).

Figure 1. Mast cell pretreatment with C₇₀-I increases DUSP1 gene and DUSP1 protein expression

Fullerene (C₇₀-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 C₇₀-I pretreated samples (*p* < 0.005; ± SD of six observed values). To evaluate dose responses of C₇₀-I (**B**), MC were pretreated with C₇₀-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 IR₈₀₀ secondary antibodies (results typical in three separate immuno-blots). Relative pixel intensity (green bar graph) of the IR₈₀₀ protein band expression of DUSP1 was quantified and normalized (Licor Odyssey imaging software) to the housekeeping gene, β-Actin to ensure equal protein levels were loaded into each lane (red band). To evaluate time course effects of C₇₀-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 C₇₀-I decreases phosphorylation of MAPK1

Mast cells were pretreated with or without C₇₀-I (10⁻⁵ M), washed, and challenged with (XL) or without (–) anti-FcεRI (1 μg/ml) for two, four, or six hours. Lysates were immunoblotted with polyclonal rabbit-anti-phospho-MAPK1 antibodies (1 μg/ml) and probed on the Odyssey infrared imaging system with anti-rabbit IR₈₀₀ secondary antibodies (A). Relative pixel intensity of the IR₈₀₀ phospho-MAPK1 protein band (green band and bar graph) and IR₇₀₀ house-keeping gene, β-Actin (red band and bar graph) was quantified. Percent reduction of MAPK1 in untreated MC and C₇₀-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.

Figure 3. The effects of MC pretreatment with C₇₀-I/ICS individually or in combination with LABA on degranulation

MC were cultured with C₇₀-I, fluticasone, or budesonide (10^{-8} – 10^{-5} M) or C₇₀-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) were 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 students t-test to compare respective treatment to untreated positive (FcεRI) controls. Statistical significance indicated with ¥ ($p < 0.05$) using the students t-test to compare efficacy ICS versus fullerene treatment at respective concentrations.

Figure 4. The effects of MC pretreatment with C₇₀-I/ICS individually or in combination with LABA on GM-CSF cytokine production

MC were cultured with C₇₀-I, fluticasone, or budesonide at (10⁻⁸ – 10⁻⁵ M) (A) or C₇₀-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) were 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 students t-test to compare respective treatment to untreated positive (FcεRI) controls. Statistical significance indicated with ¥

($p < 0.05$) using the students t-test to compare efficacy of ICS versus fullerene treatment at respective concentrations.

Table I

Mean % Fc RI-mediated degranulation in C₇₀-I or ICS (fluticasone or budesonide) treated and untreated lung MC

Treatment/[Conc; M]	Release β -Hex (%) $\pm$ S.E.	(%) Inhibition β -Hex $\pm$ S.E.
Spontaneous	4.7 $\pm$ 0.4	
Positive Control	56.9 $\pm$ 4.7	
Fluticasone [10 ⁻⁸]	52.7 $\pm$ 3.7	7% $\pm$ 6.8%
Fluticasone [10 ⁻⁷]	42.3 $\pm$ 2.2	26% $\pm$ 5.1%
Fluticasone [10 ⁻⁶] [*]	34.9 $\pm$ 2.3	39% $\pm$ 4.0%
Fluticasone [10 ⁻⁵] [*]	25.6 $\pm$ 0.5	55% $\pm$ 1.2%
Budesonide [10 ⁻⁸]	56.6 $\pm$ 3.8	1% $\pm$ 8.0%
Budesonide [10 ⁻⁷]	41.8 $\pm$ 3.5	27% $\pm$ 7.6%
Budesonide [10 ⁻⁶] [*]	35.6 $\pm$ 1.9	37% $\pm$ 3.4%
Budesonide [10 ⁻⁵] [*]	29.6 $\pm$ 2.9	48% $\pm$ 4.2%
C ₇₀ -I [10 ⁻⁸]	53.7 $\pm$ 2.1	6% $\pm$ 3.9%
C ₇₀ -I [10 ⁻⁷] [*]	34.4 $\pm$ 1.4	40% $\pm$ 4.1%
C ₇₀ -I [10 ⁻⁶] [*]	24.5 $\pm$ 3.6	57% $\pm$ 7.2%
C ₇₀ -I [10 ⁻⁵] ^{**}	18.2 $\pm$ 1.5	68% $\pm$ 4.1%

Data shown are mean $\pm$ 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;

*
p<0.05,

**
p<0.005.

ICS: Inhaled corticosteroid; MC: Mast Cell; M: Molar.

Table II

Synergistic effects on % Fc RI-mediated degranulation in fullerene (C₇₀-I) and LABA (salmeterol) or ICS (fluticasone or budesonide) and LABA (salmeterol) treated and untreated MC

Treatment/[Conc; M]	Release β -Hex (%) $\pm$ S.E.	(%) Inhibition β -Hex $\pm$ S.E.
Spontaneous	6.5 $\pm$ 0.5	
Positive Control	35.9 $\pm$ 3.1	
Fluticasone [10 ⁻⁸]/Salmeterol [10 ⁻⁵]	35.7 $\pm$ 0.6	1% $\pm$ 1.4%
Fluticasone [10 ⁻⁷]/Salmeterol [10 ⁻⁵]	26.8 $\pm$ 1.5	26% $\pm$ 4.2%
Fluticasone [10 ⁻⁶]/Salmeterol [10 ⁻⁵] [*]	18.1 $\pm$ 1.2	50% $\pm$ 3.1%
Fluticasone [10 ⁻⁵]/Salmeterol [10 ⁻⁵] [*]	14.8 $\pm$ 2.9	59% $\pm$ 5.1%
Budesonide [10 ⁻⁸]/Salmeterol [10 ⁻⁵]	37.8 $\pm$ 1.8	No Effect
Budesonide [10 ⁻⁷]/Salmeterol [10 ⁻⁵]	30.3 $\pm$ 3.5	16% $\pm$ 5.5%
Budesonide [10 ⁻⁶]/Salmeterol [10 ⁻⁵] [*]	19.4 $\pm$ 1.5	46% $\pm$ 2.9%
Budesonide [10 ⁻⁵]/Salmeterol [10 ⁻⁵] [*]	15.4 $\pm$ 2.7	57% $\pm$ 4.2%
C ₇₀ -I [10 ⁻⁸]/Salmeterol [10 ⁻⁵]	34.0 $\pm$ 0.8	6% $\pm$ 1.2%
C ₇₀ -I [10 ⁻⁷]/Salmeterol [10 ⁻⁵] [*]	15.9 $\pm$ 2.0	56% $\pm$ 4.6%
C ₇₀ -I [10 ⁻⁶]/Salmeterol [10 ⁻⁵] ^{**}	13.8 $\pm$ 1.4	62% $\pm$ 2.7%
C ₇₀ -I [10 ⁻⁵]/Salmeterol [10 ⁻⁵] ^{**}	9.0 $\pm$ 1.3	75% $\pm$ 4.0%

Data shown are mean $\pm$ 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;

* p<0.05,

** p<0.005.

ICS: Inhaled corticosteroid; MC: Mast Cell; M: Molar.