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Full Research Paper

Molecular Interactions of Fullerene Derivatives in Human Serum and Inflammatory Cells

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Abstract: Fullerenes are a class of nanomaterials with unique electronic properties that can be harnessed for use in various applications. We have found their biologic function critically depends on the structure of the side chain moieties added to the core carbon cage. A therapeutic candidate termed C_{70}-Tetraglutamate (TGA), previously shown to have potent anti-inflammatory properties, was selected to determine the molecular interactions in human serum and in resting or FcεRI-activated human mast cells (MC). The identity of TGA-binding molecules was analyzed using NanoLC-MS/MS peptide sequencing technology. We found that TGA predominately bound to alpha-2-macroglobulin precursor, Serpin peptidase inhibitor, and serum albumin in human serum. In non-activated MC, TGA interacted predominantly with aminopeptidase N precursor, dipeptidyl peptidase 4, and human fibroblast activation. In MC activated through FcεRI, predominant interactions were observed between TGA and annexin A5, superoxide dismutase, and lysosomal membrane glycoprotein. These studies for the first time identify serum and cellular substrates of a fullerene-based anti-inflammatory compound which serves as a starting point for determining the mechanism of action of this therapeutic candidate.

Keywords: fullerene; human mast cells; protein identification; nano-liquid chromatography; tandem mass spectrometry.

1. Introduction:

The therapeutic usage of fullerenes has been investigated over the past several years as they have a wide range of potential treatment and targeting capabilities. One such property is their unique ability to be functionalized with different side-chain moieties to derive unique compounds for specialized biomedical applications. However, little is known about what molecules they interact with in biological systems such as in the cytoplasm of cells and serum from whole blood. If fullerenes are to be used in biological systems for therapeutics or diagnostics (1-3) it is important to understand what they bind to so that their efficacy and biological compatibility can be optimized.

In previous research, we have demonstrated that a C_{70}-based fullerene derivatized via cyclopropanation with four glutamate molecules attached to its outer shell (C_{70}-Tetraglutamate; TGA) has safe and potent anti-inflammatory activity and is being investigated as a therapeutic for several disease processes (4-6). For example, TGA is a potent mast cell (MC) stabilizer in vitro and in vivo and has not been shown to be toxic to cells using up to 100 μg/ml or in vivo with treated animals having normal organ function (4). While certain insights into the signaling pathways that were affected by TGA have been identified, a clear mechanism underlying its efficacy has not yet been determined. In addition, no information toward the potential of serum binding proteins has been assessed for TGA (as well as most fullerenes being investigated for therapeutic applications). To help understand how TGA exhibits its anti-inflammatory properties, we set out to examine the molecular interactions it exhibits in serum and immune regulatory cells involved in various inflammatory diseases. We found that TGA interacts with a wide range of molecules in serum and human tissue mast cells (MC) which provide clues into its mechanism of action.

2. Results and Discussion:

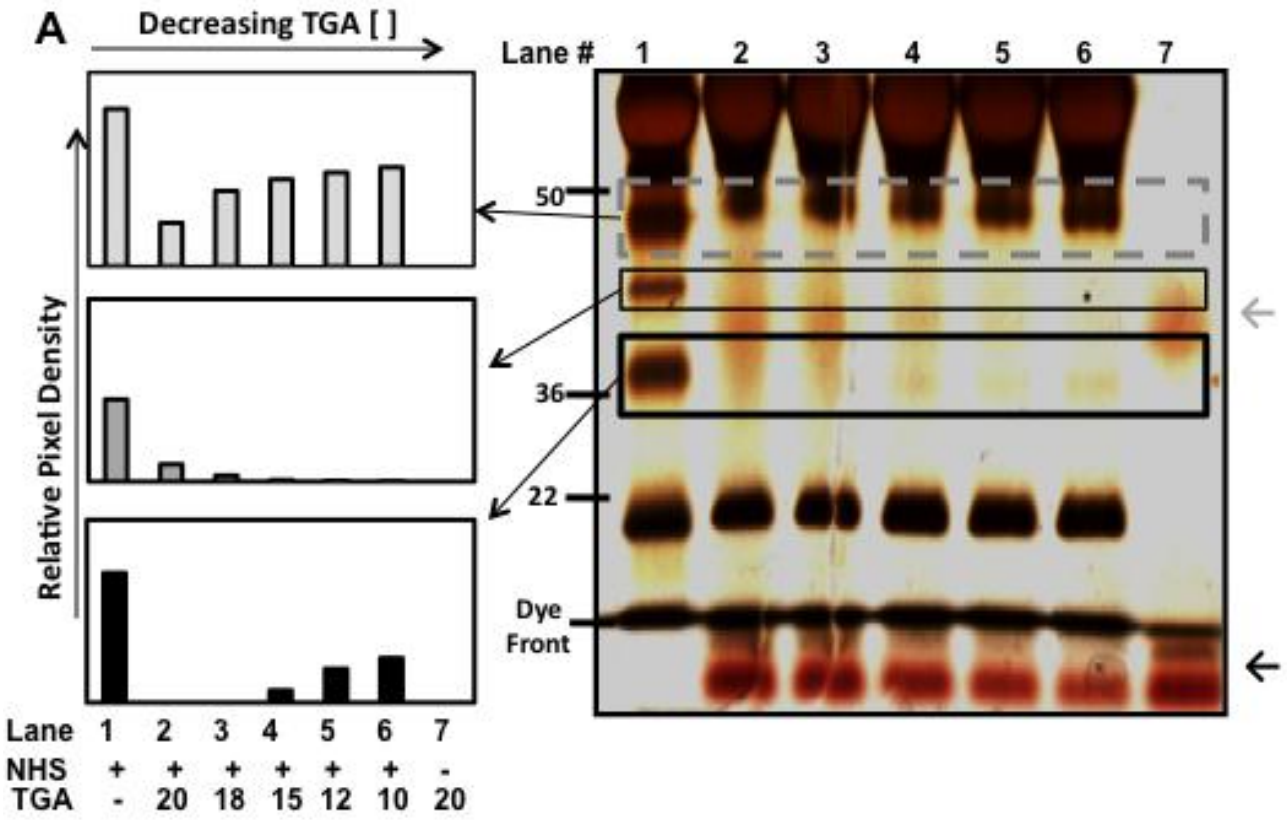
2.1. Identification of Human Serum Binding Proteins Separated by SDS-PAGE and Nano LC/MS.

The C_{70} derivative TGA is being pursued as a lead candidate for anti-inflammatory therapeutics (4;6;9). However, it is still not clear what molecule(s) it binds to in serum or in the major effector cells of inflammation-mast cells. We incubated the TGA with human serum and separated the proteins by SDS-PAGE so that differences in electrophoretic mobility could be assessed. Two separate gels were run simultaneously; one was stained with the silver stain for less time to observe the major constituents (Figure 1) and one for longer times in order to observe low volume proteins (Figure 2).

The gels that were silver stained for a short duration (Figure 1A and 1B) had approximately three proteins that were observed to have significant shifts in mobility. Quantification of each of these three bands is shown in Figure 1A. For example, a band migrating at approximately 45-50 kDa did not appear in the TGA-treated lanes (lanes 2-6) with the TGA becoming clearly visible (compare lanes 2-6 with lane 7). Similarly, bands migrating at approximately 36 kDa and 42 kDa almost completely change migratory distances in the gel with all concentrations of TGA. At 10 μg/ml of TGA the 43 kDa

band is essentially undetectable. In Figure 1B the identity of the proteins in each of these bands was determined. The two sequenced peptides in the 45-50 kDa band were transthyretin precursor and alpha-1-antitrypsin, the four bands in the 42 kDa range were complement component 3, Annexin A1, glyceraldehyde-3-phosphate dehydrogenase, and alpha-1-acid glycoprotein 1 precursor. As for, the 36 kDa band revealed β-actin, alpha-1-acid glycoprotein 1 precursor, apololipoprotein A-IV precursor, and immunoglobin kappa light chain.

Figure 1. Quantification and identification of low abundance normal human serum proteins affected by TGA. Normal human serum (NHS - 0.15 µl) was incubated overnight (37˚C) with or without the indicated concentrations of TGA. Gels were developed with silver stain for 5 minutes to observe minor proteins affected by TGA binding (A). Bands were quantified by calculating relative pixel densities of each band. In B bands were excised and subjected to NanoLC-MS/MS peptide sequencing. All lanes were loaded with equal concentrations of NHS (15 µl) with or without the indicated concentrations of TGA (µg/ml). TGA makes two separate fronts on the gel this is the larger molecular weight (40kDa – grey arrow) and a smaller front (approx 6kDa – black arrow). The molecular weight markers are indicated by the bars on the left or right of the gel.



B

Normal Human Serum – Band

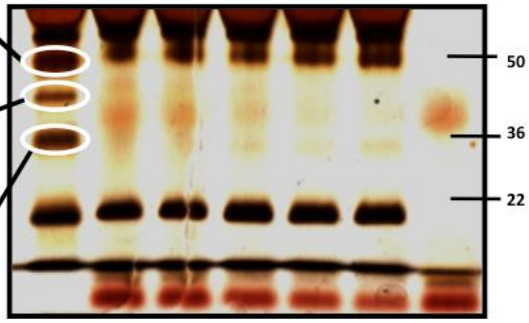
Protein	Mass	Peptides
Transthyretin precursor	15991.08	9
Alpha-1-antitrypsin	46787.05	1

Normal Human Serum – Band

Protein	Mass	Peptides
Complement component C3	188584.75	5
Annexin A1, isoform CRA_c	40284.81	2
Glyceraldehyde-3-phosphate dehydrogenase	36202.43	2
Alpha-1-acid glycoprotein 1 precursor	23752.89	1

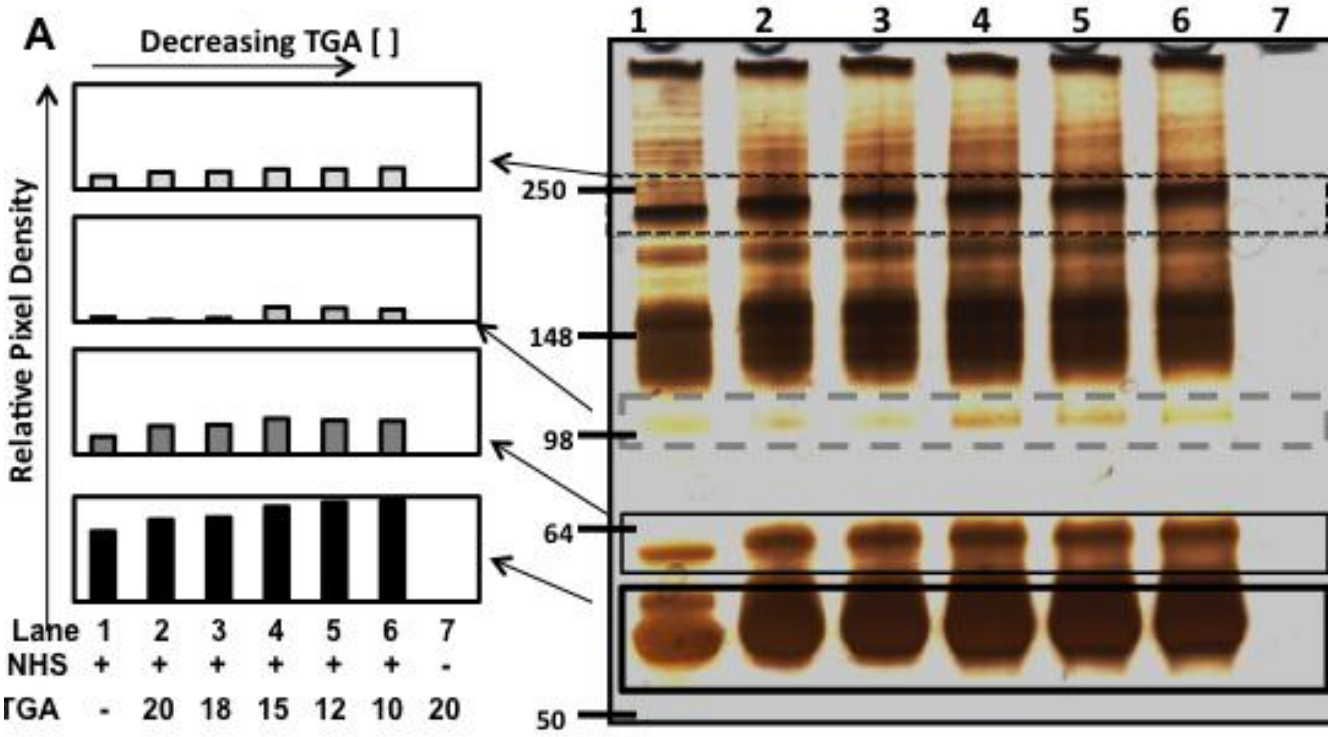
Normal Human Serum – Band

Protein	Mass	Peptides
Beta Actin	41320.57	3
Alpha-1-acid glycoprotein 1 precursor	23579.47	3
Apolipoprotein A-IV precursor	43358.29	2
Immunoglobulin kappa light chain VLJ region	29050.35	2



However, the gel stained for a longer duration in Figures 2A and 2B revealed approximately four higher molecular weight bands in serum affected by TGA. Quantification of each of these four bands is shown in Figure 2A. Starting with the highest molecular weights, a band migrating at approximately 200 kDa appeared to become more prominent with increasing concentrations of TGA. Bands migrating at approximately 105 kDa, 62 kDa, and 58 kDa had increased intensities with decreasing concentrations of TGA. In Figure 2B, the identity of the proteins in each of these bands was determined. Of the six sequenced peptides in the 200 kDa band the top three included α-2-macroglobin precursor, haptoglobin isoform preprotein, and serum albumin. The four most abundent peptides in the 105 kDa sample included serum albumin, α-1-antitrypsin, and complement components C4a and C3. The four most abundent peptides in the 62 kDa sample included serotransferrin precursor, α-1-antitrypsin, and α-1-antichymotrypsin precursor. Lastly, the 58 kDa band includes peptides serum peptidase inhibitor, serum albumin, inter-α-trypsin inhibitor, and hemopexin precursor.

Figure 2. Quantification and identification of high abundance normal human serum proteins affected by TGA. Normal human serum (NHS - 0.15 µl) was incubated overnight (37˚C) with or without increasing doses of TGA. Gels were developed with silver stain for 0.5 minutes and major proteins affected by TGA binding were quantified (A) or excised and sequenced (B) as in Figure 1.



B

Normal Human Serum – Band		
Protein	Mass	Peptides
Alpha-2-macroglobulin precursor	164599.86	19
Haptoglobin isoform 1 preproprotein	45860.84	8
Serum albumin	71316.41	3
Alpha-1-antitrypsin	46787.05	3
Immunoglobulin alpha-1 heavy chain constant region	38414.91	3
Apolipoprotein B-100 precursor	516662.09	2

Normal Human Serum – Band		
Protein	Mass	Peptides
Serum albumin	71316.41	12
Alpha-1-antitrypsin	46848.16	5
Complement component C4A	194336.06	3
Complement component C3	188584.75	3
Plasma protease C1 inhibitor precursor	55347.55	3
Haptoglobin	39495.84	3
Inter-alpha (globulin) inhibitor H2, isoform CRA_b	106825.88	2
Complement factor B preproprotein (C3/C5 convertase / Properdin factor B / Glycine-rich Beta glyco)	86847.09	2

Normal Human Serum – Band		
Protein	Mass	Peptides
Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin):	46864.12	15
Serum albumin	71316.41	11
Inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP)	103549.17	6
Hemopexin precursor (Beta-1B-glycoprotein)	52384.57	6
Alpha-1-antichymotrypsin precursor (Cell growth-inhibiting gene 24/25 protein):	47791.66	5
Complement C9	61727.89	4
Kininogen 1	48954.02	3
Proapo-A-I protein	30744.91	3

Normal Human Serum – Band		
Protein	Mass	Peptides
Serotransferrin precursor (Transferrin)	79280.5	8
Alpha-1-antitrypsin	46787.05	6
Serum albumin	71316.41	5
Alpha-1-antichymotrypsin precursor	47791.66	5
Alpha-1B-glycoprotein	54808.89	4
Complement cytolysis inhibitor precursor	52900.21	3
Complement component C3	188584.75	2
Histidine-rich glycoprotein precursor	60510.3	2

2.2. Identification of Fullerene-Binding Peptides in Resting and FceRI-Activated Human Mast Cells.

We have shown previously TGA inhibits allergic (4) and dermal delayed type hypersensitivity (9). While an extensive examination of those genes and proteins affected by TGA was assessed, no direct in situ binding study was performed. Here, lysates from TGA treated cells, not activated through FcεRI, were run over a TGA column and the binding proteins analyzed (Figure 3). In non-activated lysates (Figure 3A) TGA bound to aminopeptidase N precursor, dipeptidyl peptidase, human fibroblast activation protein alpha, N-acetylglucosamine-6-sulfatase precursor, manganese superoxide dismutase, and human beta tryptase. In order to verify the interaction of TGA with the molecular intermediates, we used Western blotting with anti-tryptase Abs. As seen in Figure 3A (bottom) tryptase-TGA interactions were detected in lysates from resting cells but not in MC activated through FcεRI verifying the microarray results in Figures 3A and 3B.

Figure 3. Identification of TGA-binding proteins in human MC. Mast cell lysates (25 x 10^6 per condition) from resting (A) or activated with optimal concentrations of FcεRI stimuli (B) were precleared over non-TGA agarose gel columns before circulation overnight over TGA-conjugated agarose. Columns were washed, column-binding proteins eluted, and subjected to NanoLC-MS/MS as described in Methods. Results show the number of sequenced peptides, their molecular weights, and a brief functional analysis. In separate experiments three separate MC preparations were incubated with or without FcεRI-stimulus as above and lysates incubated overnight with TGA-agarose, washed with centrifuged, and subjected to Western blotting with anti-tryptase Abs.

A	Human Mast Cells Non-Activated		
Protein	**Mass**	**Peptides**	**Notes/Function**
Aminopeptidase N Precursor	109841	26	Membrane protease expressed by the tumor neovasculature.
Dipeptidyl Peptidase 4	88906	20	Intrinsic membrane glycoprotein and a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides.
Human Fibroblast Activation Protein Alpha	83775	11	Highly expressed in epithelial cancers and has been implicated in extracellular matrix remodeling, tumor growth, and metastasis.
N-acetylglucosamine-6-Sulfatase Precursor	62840	8	The product of this gene is a lysosomal enzyme found in all cells involved in the catabolism of heparin, heparin sulphate, and keratan sulphate.
Manganese Superoxide Dismutase	22189	8	Manganese superoxide dismutase (MnSOD) protects mitochondria from oxidative damage associated with electron transport (d/e).
* Human Beta Tryptase	27799	7	Human tryptase, a mast-cell-specific serine protease
Triosephosphate Isomerase Isoform 1	26937	6	Catalyzes the isomerization of glyceraldehydes 3-phosphate (G3P) and dihydroxy-acetone phosphate (DHAP) in glycolysis and gluconeogenesis.
Human Neutral Endopeptidase Complexed with Phosphoramidon	80070	5	Neutral endopeptidase is a mammalian type II integral membrane zinc-containing endopeptidase, which degrades and inactivates a number of bioactive peptides.
Acid Alpha-Glucosidase	106126	4	
Lysosomal Membrane Glycoprotein-1	45200	4	Plays key role in proper function of lysosomes.
Lysosomal Membrane Glycoprotein-2	45374	3	Plays key role in proper function of lysosomes
Leukocyte Elastase Inhibitor	42828	3	Proteinase inhibitor that regulates the activity of the neutrophil proteases: elastase, cathepsin G and proteinase-3.
Cathepsin B Complexed with Dipeptidyl Nitrile Inhibitor	29570	3	Member of the papain superfamily of cysteine proteases.
Membrane protein band 7.2b Somatin	34185	3	Integral membrane phosphoprotein.
Syntenin-1 Isoform 1	32594	2	May represent a promising potential therapeutic target for preventing cancer progression and metastatic spread (N).
Ferritin Light Chain	20064	2	Ferritin is the major intracellular iron storage protein in prokaryotes and eukaryotes involved in iron storage.
Dipeptidyl-Peptidase 7	54749	2	Essential for maintaining cells in G(0).
Beta-Hexosaminidase Subunit Alpha	61106	2	Responsible for the degradation of GM2 gangliosides, and a variety of other molecules containing terminal N-acetyl hexosamines, in the brain and other tissues.

Blot: anti-tryptase

FcεRI activation	-	-	-	+	+	+
TGA-beads	+	+	+	+	+	+
MC lysate #	1	2	3	1	2	3

B	Human Mast Cells FcεRI-Activated		
Protein	**Mass**	**Peptides**	**Notes/Function**
Annexin A5	35971.41	5	Belongs to the annexin family of calcium-dependent phospholipid binding proteins
Manganese Superoxide Dismutase	22189.24	5	Protects mitochondria from oxidative damage associated with electron transport.
Lysosomal Membrane Glycoprotein-1	45200.98	4	Plays key role in proper function of lysosomes
Lysosomal Membrane Glycoprotein-2	45374.46	3	Plays key role in proper function of lysosomes.
Aminopeptidase N Precursor	109841.89	3	Membrane protease expressed by the tumor neovasculature / exploited for ligand-directed delivery of various drugs and particles to tumor vessels, in the attempt to increase their antitumor activity (a/b).
N-acetylglucosamine-6-Sulfatase Precursor	62840.41	3	The product of this gene is a lysosomal enzyme found in all cells. It is involved in the catabolism of heparin, heparin sulphate, and keratan sulphate. Deficiency of this enzyme results in the accumulation of undegraded substrate and the lysosomal storage disorder mucopolysaccharidosis type IIID (Sanfilippo D syndrome). Mucopolysaccharidosis type IIID is the least common of the four subtypes of Sanfilippo syndrome.
Cathepsin B Complexed with Dipeptidyl Nitrile Inhibitor	29570.65	3	Member of the papain superfamily of cysteine proteases and has been implicated in the pathology of numerous diseases, including arthritis and cancer (K).
Dipeptidyl Peptidase 4	88906.77	2	The protein encoded by this gene is identical to adenosine deaminase complexing protein-2, and to the T-cell activation antigen CD26. It is an intrinsic membrane glycoprotein and a serineexopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides.
Leukocyte Elastase Inhibitor	42828.79	2	Proteinase inhibitor that regulates the activity of the neutrophil proteases: elastase, cathepsin G and proteinase-3. Gene may be associated with cornary atherosclerosis (I/J).

3. Experimental Section

3.1. Cells and Reagents.

Pooled normal human serum was obtained from Sigma (H422, Saint Louis, MO). Human skin derived mast cells (MC) were cultured and activated for 10 minutes with optimal concentrations of anti-FcεRI-α antibodies (3B4, 1 μg/ml) as previously described (7).

3.2. Synthesis of Agarose-Bound TGA.

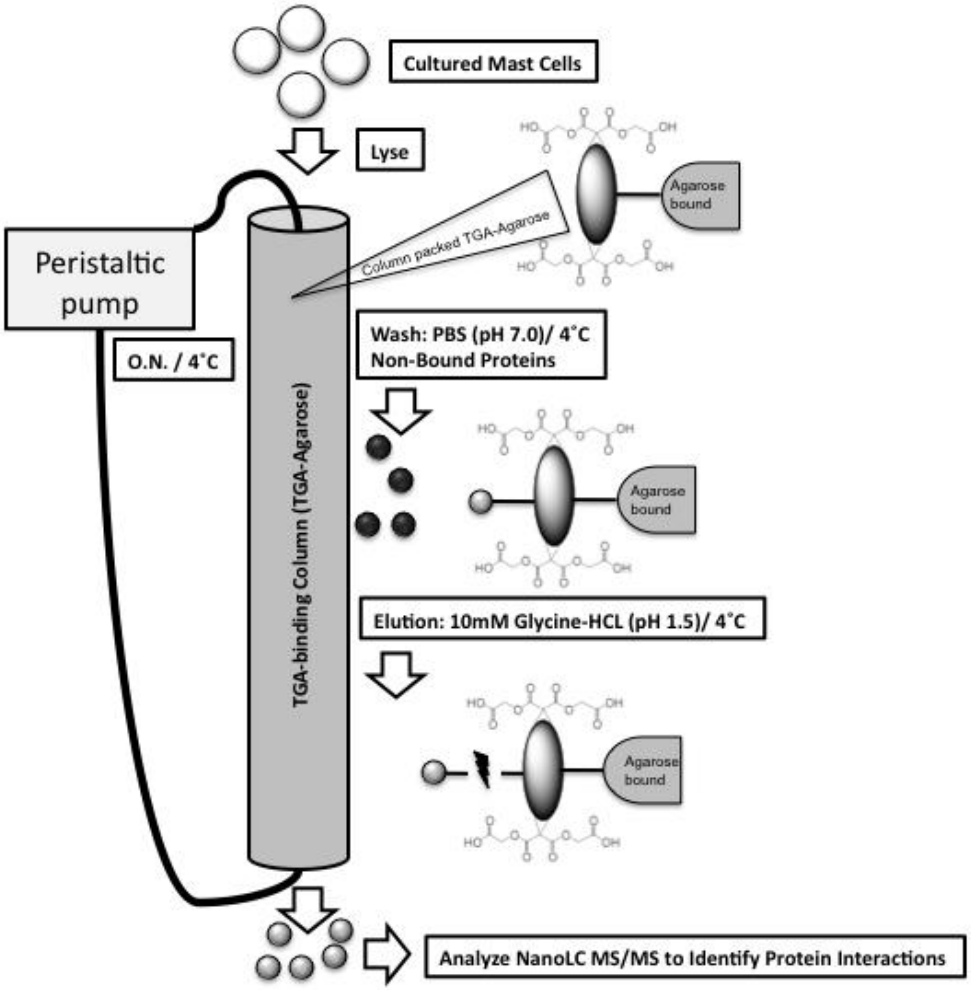
C_{70} (2.94 g), di-t-butylacetoxymalonate (1.25 g), iodine (I_2; 1.0 g) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 1.2 g) in 10 ml xylene were combined under nitrogen, with the DBU added last and dropwise over several minutes. The reaction was stirred until judged complete by thin-layer chromatography (TLC) using alumina on polyester plates and eluted using the solvent toluene. The first fraction was unreacted C_{70}, the second was the C_{70}-mono-adduct (desired product), and C_{70}-bis-adduct (bis-addition product) is removed with Tetrahydrofuran (THF). To the mono-adduct synthesized above, a second Bingle reaction was performed (8). C_{70} (monoadduct; 250 mg), I_2

(60 mg), mixed malonate (ethoxy ester /amide-[$(CH_2CH_2O)_3CH_2CH_2NH(BOC)$; 154 mg] were combined in toluene under nitrogen (N_2), and DBU (79 mg in 10 ml toluene) added drop-wise over several minutes. The reaction mixture was stirred overnight at RT, and then chromatographed on silica (THF/ether 1:1). The final product was made by de-protecting the above C70 derivative by reaction with trifluoroacetic acid followed by 1M HCl. After solvent removal under vacuum, the fullerene component was used without further purification. Next, 1,1'-Carbonyldiimidazole-agarose (1 ml of 1 g/ml slurry in acetone) was combined with fullerene derivative (14 mg in 1.0 ml dionized-H_2O with K_2CO_3; 105 mg) and the slurry placed in a shaker for 20 hours. The slurry was filtered, with no color in the filtrate, indicating that all fullerenes were bound to agarose. The agarose was then washed with acetone and taken into a tris/EDTA buffer (pH=8.1; 3 ml).

3.3. C_{70}-Tetraglutamate-agarose column fractionation of human mast cell lysates.

To determine what molecules interact with human MC we used lysates from resting and FcεRI activated cells and ran them over the agarose-TGA column. Following activation with optimal concentrations of anti-FcεRI receptor antibodies (1 µg/ml), cells (25 x 10^6/condition) were washed twice in PBS, and lysed in 500 µl of lysis buffer as described previously (7). The samples were lysed on ice for 15 minutes, freeze-thawed three times, and passed through a 26 gauge-syringe 10 times to further lyse cells. Non-lysed cellular debris was removed through centrifugation at 15,000 rpm for 10 minutes at 4˚C and supernatants were collected for column analysis (schematic below). Cellular lysates were first pre-cleared over non-TGA agarose-gel columns for 2 hours at 4˚C to reduce any non-specific binding. The pre-cleared lysates were then circulated over the TGA-conjugated agarose column O.N. at 4˚C using a peristaltic pump. The column was washed with 50 ml of ice cold PBS at physiological pH (7.4) and elution of TGA-binding molecules was performed using 5 ml of 10 mM Glycine-HCL elution buffer at pH 1.5 (Biacore, Piscataway, NJ). The elution and washes were dialyzed against PBS and run on SDS-PAGE for silver stain or Western blotting analysis as described (4). Aliquots of whole eluent (5 ml) analyzed using Nano liquid chromatography (LC), mass spectroscopy (MS) as described below.

Schematic 1: TGA – Agarose Column Schematic.



3.4. C_{70}-Tetraglutamate binding to human serum components.

Normal human serum was incubated overnight in a 37˚C/6% CO_2 incubator with or without TGA (10, 12, 15, 18, or 20 µg/ml). The following day, aliquots were separated by SDS-PAGE and proteins stained using an ultra sensitive silver stain kit (Thermo-Scientific, Rockford, IL). Briefly, gels were washed in dionized-H2O two times for five minutes, and then fixed for 30 minutes in ethanol/acetic acid. Fixed gels were washed with 10% ethanol twice for five minutes, and incubated in silver stain sensitizer for one minute, wash, and incubated with silver stain for 30 minutes. Stained gels were visualized using silver stain developer and stopped with 5% acetic acid once optimal visualization was achieved (0.5-7 minutes). When non-TGA treated serums were visualized next to TGA-treated serums differences in electrophoretic mobility and serum component concentration levels indicated possible binding partners. Relevant bands were excised and analyzed using MS/MS as described below.

3.5. NanoLC-MS/MS identification of Protein Interactions with C_{70}-Tetraglutamate

Nano liquid chromatography (LC), mass spectroscopy (MS)/MS (tandem MS) identification of the unknown TGA-binding proteins was carried out by ProtTech, Inc. (Norristown, PA). The highly

sensitive NanoLC-MS/MS platform for protein identification is able to analyze simple in-gel protein bands or very complex protein mixtures. This is possible because each peptide is independently sequenced and produces a unique fingerprint that can be identified using protein databases, such as GenBank.

Either complex protein mixtures (obtained from MC column elutions) or excised bands (obtained from human serum SDS-PAGE) were digested using modified sequencing grade trypsin. Next, the peptide mixture was separated via HPLC system running a 75 μM inner diameter C18 column. A tandem mass spectrometer on-line coupled with the HPLC system was used and peptides eluted from the HPLC column were fragmented by collision-induced dissociation (CID) and a MS/MS spectra acquired for each fragmented peptide. Each MS/MS spectrum is used to search protein data bases (Protein Information Resources data base and GenBank) for matched peptides. Finally, data base hits were manually evaluated to ensure proper protein identification. All experiments were performed at least twice.

4. Conclusions

This study aimed to identify the binding partners that a C_{70} fullerene derivative has in human serum and immune effector cells. This derivative was chosen for further analysis due to its potential as an anti-inflammatory agent (4;6;9;10). The TGA has also demonstrated a favorable safety profile with no toxicity detected in vitro and in vivo. Given that each unique fullerene derivative will have different biological function, our strategy was to first identify a promising therapeutic candidate before extensive binding experiments were performed.

While others have investigated protein-binding techniques using fullerenes, including human serum (11-15), there is still little information regarding the identification of protein binding interactions. Here, it was demonstrated that TGA interacted with a wide range of proteins in human serum. The top three interactions included alpha-2-macroglobulin precursor, serpin peptidase inhibitor, and serum albumin. Human alpha-2-macroglobulin is a tetrameric protease inhibitor mainly synthesized in the liver and functions as a broad range irreversible proteinase inhibitor. The serpin peptidase inhibitor/alpha-1 antitrypsin is also produced in the liver neutralizes the enzyme neutrophil elastase.

We showed previously that TGA can inhibit IgE-mediated degranulation and cytokine production through the blunted release of intracellular calcium stores, reduced levels of reactive oxygen species (ROS), and differential effects of several signaling molecules (4;16). Here we show that TGA binds to several proteins on and within the MC that may help explain how they mediate this inhibition. For example, in non-FcεRI-challenged cells TGA bound predominately to the cell surface molecules aminopeptidase N precursor (CD13), dipeptidyl peptidase 4 (DPP4; CD26), and human fibroblast activation protein (FAP) alpha. Aminopeptidase N is a membrane protein and an important target of tumor vasculature-targeting drugs (17). Similarly, DPP4 is a cell surface aminopeptidase which acts as a Type-II serine proteinase (18). It is a target for several anti-diabetic treatments and inflammatory disease therapeutics (18). These top three TGA binding molecules all belong to the post-prolyl peptidases class of enzymes that are inducible and active on the cell surface and are uniquely capable of cleaving Pro-XAA bonds and have demonstrated to be potential targets for cancer therapy. Given that TGA's structure contains carboxyl groups the binding to these three molecules may occur at the

lysine, histidine, or arginine residues in their primary structure (17;19). None of these top-binding molecules have previously been shown to affect FcεRI signaling pathways.

The TGA also bound to intracellular proteins including N-acetylglucosomine-6-sulfate, superoxide dismutase mimetic (SOD), and tryptase. Inhibition of MC mediator release has been demonstrated with inhibitors of N-acetylglucosamine (20). In addition, IgE MC activation is closely linked to the function of serglycin proteoglycan core proteins; building blocks for the glycosaminoglycans (21). Thus, it is likely that TGA could prevent MC mediator release through its interactions with N-acetylglucosamine, which could disrupt the signaling pathway induced by FcεRI. Of particular interest is TGA's binding to human beta tryptase, a MC-specific marker (22) which was used to verify binding using Western blotting. In addition, it has been shown that the C_{60} fullerene C_3 acted as a superoxide dismutase mimetic and treatment of SOD2 (-/-) mice, which lack expression of mitochondrial manganese superoxide dismutase (MnSOD), increased their life span by 11-15% (23). We are currently examining the significance of TGA binding to these molecules.

In conclusion, these studies demonstrate binding of TGA, which has demonstrated anti-inflammatory properties, with serum and cellular proteins. These studies were performed to help delineate the mechanisms of action of this molecule in vivo and in vitro. Further studies are needed to understand how the derivation of the fullerene cage can change the binding affinities for serum and cellular proteins.

Acknowledgement

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