Analysis of the Binding Affinity of the Zonulin Agonist (AT1002) and Antagonist (AT1001) to the Zonulin Intestinal Receptor

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ABSTRACT

Background: Zonulin is a modulator of intestinal tight junction (tj) permeability whose physiological role within the GI tract is to protect against proximal bowel contamination. We have found that Zonulin upregulation is involved in a series of autoimmune diseases, including type 1 diabetes and celiac disease (CD). We have also demonstrated that the effect of Zonulin on the cell cytoskeleton and tj permeability can be mimicked by its synthetic peptide agonist AT1002 and can be inhibited by the synthetic peptide AT1001. Both peptides seem to bind to the same Zonulin receptor through a specific binding motif. **Aim:** To analyze the binding affinity of Zonulin agonist and antagonist to Zonulin expressing Caco2 intestinal epithelial cells by fluorescence microscopy. **Materials and methods:** CaCo2 cells were used to analyze the affinity of AT1001 and AT1002. AT1001 untagged and tagged with FITC, AT1002 untagged and tagged with FITC and scrambled peptide tagged with FITC was obtained. Cells were cultured and fixed on 8 chamber mounted on glass slide and incubated with FITC labeled peptide (AT1001 or AT1002) either in the presence or absences of unlabelled peptide. Slides were then analyzed in blind fashion with a fluorescence microscope. **Results:** Both AT1001 and AT1002 bind to CaCo2 cells, while no detectable binding was observed with the scrambled peptide. FITC-AT1001 was displaced when untagged AT1001 was used at a concentration >100 times that of the tagged peptide. FITC-AT001 was also displaced by untagged AT1002 but at lower concentrations (75 times). Similarly, FITC-AT002 was displaced by untagged AT1002 and AT1001 but at higher concentrations (>150 times for untagged AT1002 and >200 for untagged AT1001). **Conclusions:** Our results demonstrated that both AT1001 and AT1002 bind to the same receptor with AT1002 showing higher affinity than AT1001. These findings will assist us to develop strategies to properly antagonize the zonulin pathway for the treatment of autoimmune diseases, including CD.

BACKGROUND

- CD is an autoimmune enteropathy triggered by ingestion of gliadin containing grains in genetically susceptible individuals
- Zonula occludens toxin (Zot) is an enterotoxin obtained from the bacterium *V.cholerae* that has been shown to reversibly open the tight junction (tj) and enhance paracellular transport by interacting with a mammalian cell receptor
- Zonulin is the Zot mammalian analogue
- Zonulin is a modulator of intestinal tight junction permeability whose physiological role within the GI tract is to protect against proximal bowel contamination (innate immune function). *
- We have found that Zonulin up regulation is involved in a series of autoimmune diseases, including type 1 diabetes and CD. **

*** El Asmar Gastroentrology 2002 ** Sapone et al Diabetes 2006

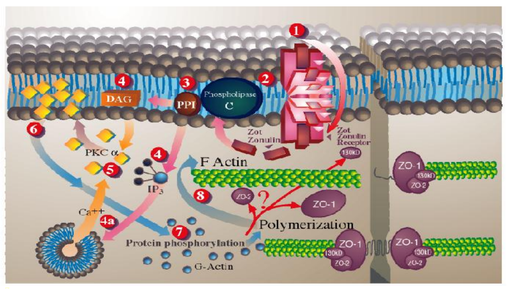
Zot: Structure-Function Analysis

***V. cholerae* cleavage site**

1 msifihhgap gsyktsgalw lrllpaiksg rhiitnvrgl nlermakylk | mdvsdisief
61 idtdhpdgrl tmarfwhwar kdaflfidec griwpprlta tnlkaldtpp | dlvaedrpes
121 fevafdmhrh hgwdiclttp niakvhnmir eaaeigyrhf nratvglgak ftltthdaan
181 sgqmdshalt rqvkkipspi fkmyastttg kardtmagta lwkdrkilf fgmvflmfsy
241 sfyglhdnpi **ftggndatieseq sepqska tagnavgska vapasfgfcigrlcvqdgfv**
301 tvgderyrlv dnldipyrgl watghhiykd kltvffetes gsvptelfas syrykvlplp
361 dfnhfvvfdt faaqalwvev krglpikten dkkglnsif

Spanning domain
Mature Zot
Active domain (AT 1002)

Proposed Zonulin Mechanism of Action



1 Zonulin interacts with a specific surface receptor 2 is then internalized and activates phospholipase C 3 Which then hydrolyses phosphatidyl inositol 4 To release inositol 1,2,5-triphosphate (PPI 3) and diacylglycerol (DAG) 5 Protein kinase Cα (PKCα) is then activated, either directly (via DAG;4) or through the release of intracellular Ca 2+ (via PPI 3; 4a) 7 PKCα catalyses the phosphorylation of target protein(s) with subsequent polymerization of soluble G actin into F actin 8 This polymerization causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO-1) from the junctional Complex. As a result, intestinal tight junctions become looser, allowing the passage of macromolecules through the paracellular pathway

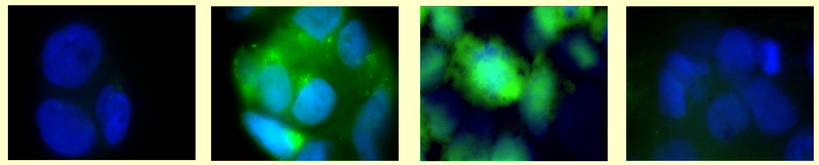
AIM

To establish whether the Zonulin agonist AT1001 and antagonist AT1002 bind to the same receptor

MATERIALS AND METHODS

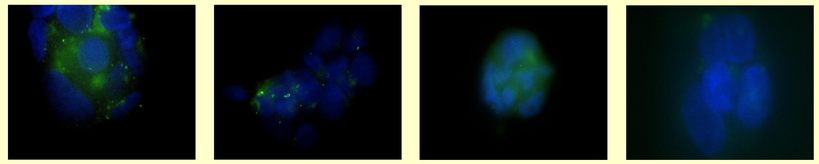
- **Caco-2 cells passage number 46 and 61 were used**
- **AT1001 untagged and tagged with fluorescein (FITC), AT1002 untagged and tagged with FITC and scrambled peptide (dummy peptide-GRLFCI) tagged with FITC were obtained from Biopolymer Laboratories, University of Maryland (Baltimore)**
- **Cells were cultured on 8 chamber (Lab-Tek II chamber slide system) Cells were then fixed and incubated at 37°C for 30 min with FITC labeled peptide (AT1001 or AT1002) either in the presence or absences of unlabelled peptide (in increasing concentrations). Dummy peptide was used for control**
- **Nucleus of the cells were stained with DAPI (**forms fluorescent complexes with DNA**). Slides were then analyzed in a blind fashion with a fluorescence microscope (Optiphot; Nikon Inc., Melville, NY).**

RESULTS

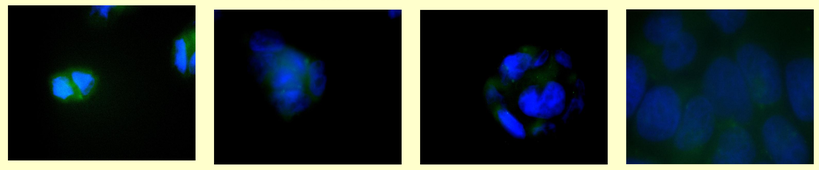


Negative control Only PBS, no peptides | **FITC tagged AT1001 - positive control** | **FITC tagged AT1002 - positive control** | **FITC tagged dummy peptide (GRLFCI) - negative control**

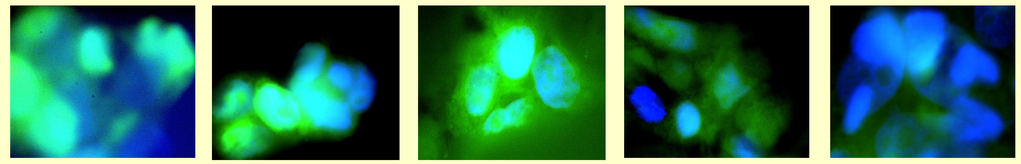
Both AT1001 and AT1002 bind to CaCo2 cells, while no detectable binding was observed with the scrambled peptide



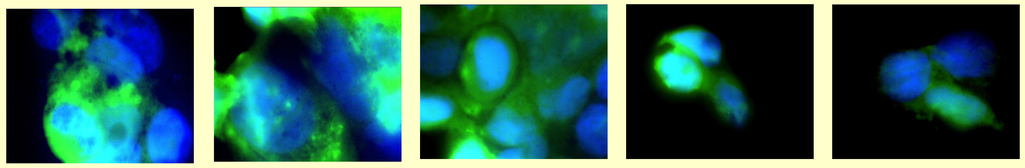
FITC tagged AT1001 + cold AT1001X25 | **FITC tagged AT1001 + cold AT1001X50** | **FITC tagged AT1001 + cold AT1001X75** | **FITC tagged AT1001 + cold AT1001X100**



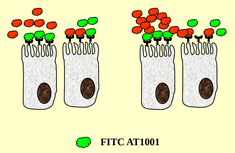
FITC tagged AT1001 + cold AT1002 x 50 | **FITC tagged AT1001 + cold AT1002 x 75** | **FITC tagged AT1001 + cold AT1002 x 100** | **FITC tagged AT1001 + cold AT1002 X 200**



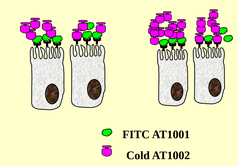
FITC AT1002 and cold AT1002 X 25 | **FITC AT1002 and cold AT1002 X 50** | **FITC AT1002 and cold AT1002 X 75** | **FITC AT1002 and cold AT1002 X100** | **FITC AT1002 and cold AT1002 X150**



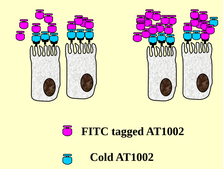
FITC AT1002 and cold AT1001 X50 | **FITC AT1002 and cold AT1001 X 75** | **FITC AT1002 and cold AT1001 X 100** | **FITC AT1002 and cold AT1001 X150** | **FITC AT1002 and cold AT1001 X 200**



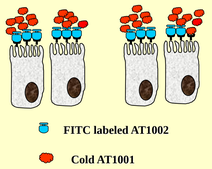
FITC-AT1001 was partially displaced when untagged AT1001 was used at a 50x concentration of the tagged peptide and completely displaced when used at a concentration 75x of the tagged peptide



FITC-AT001 was also displaced by untagged AT1002 but at lower concentrations (75x of the tagged peptide).



FITC-AT002 was only partially displaced by untagged AT1002 at concentrations >150x of the tagged peptide



FITC-AT002 was only partially displaced by untagged AT1001 at concentrations >200x of the tagged peptide

CONCLUSIONS

- **Our results demonstrated that both AT1001 and AT1002 bind to the same receptor with AT1002 showing higher affinity than AT1001.**
- **These findings will assist us to develop strategies to properly antagonize the zonulin pathway for the treatment of autoimmune diseases, including CD.**