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 fluorescent 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).

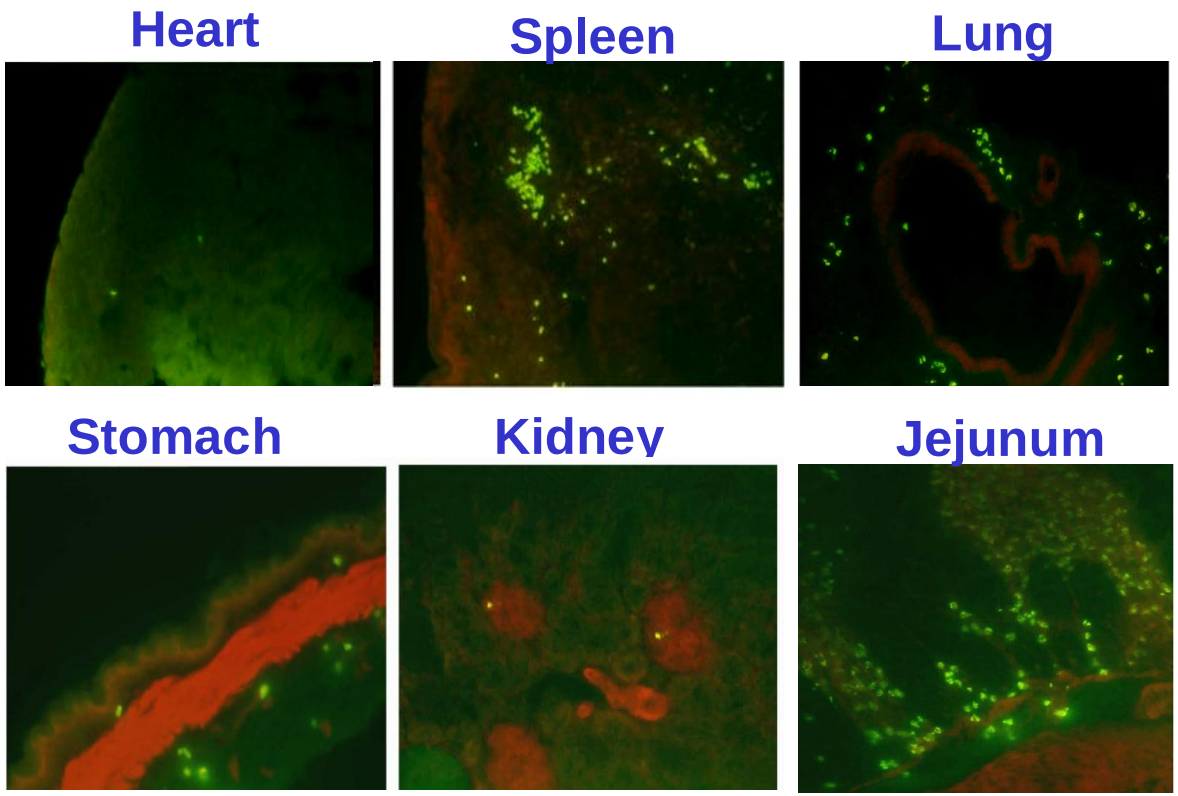
Conclusions: Our results demonstrated that both AT1001 and AT1002 bind to the same receptor with AT1002 showing higher affinity than AT1001. These findings will assist us to develop strategies to properly antagonize the zonulin pathway for the treatment of autoimmune diseases, including CD.

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. ****

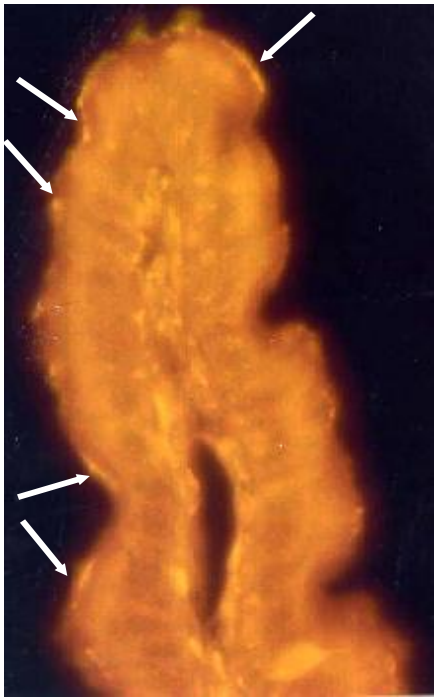
  - *** Fasano et al Lancet 2000 ** Fasano et al Diabetes 2006**

Zonulin Receptor Distribution



As seen on fluorescence microscopy Zonulin receptor is found in various tissues and in a high concentration in the jejunum

Zonulin Receptor Distribution in Small Intestine: Villous vs Crypt

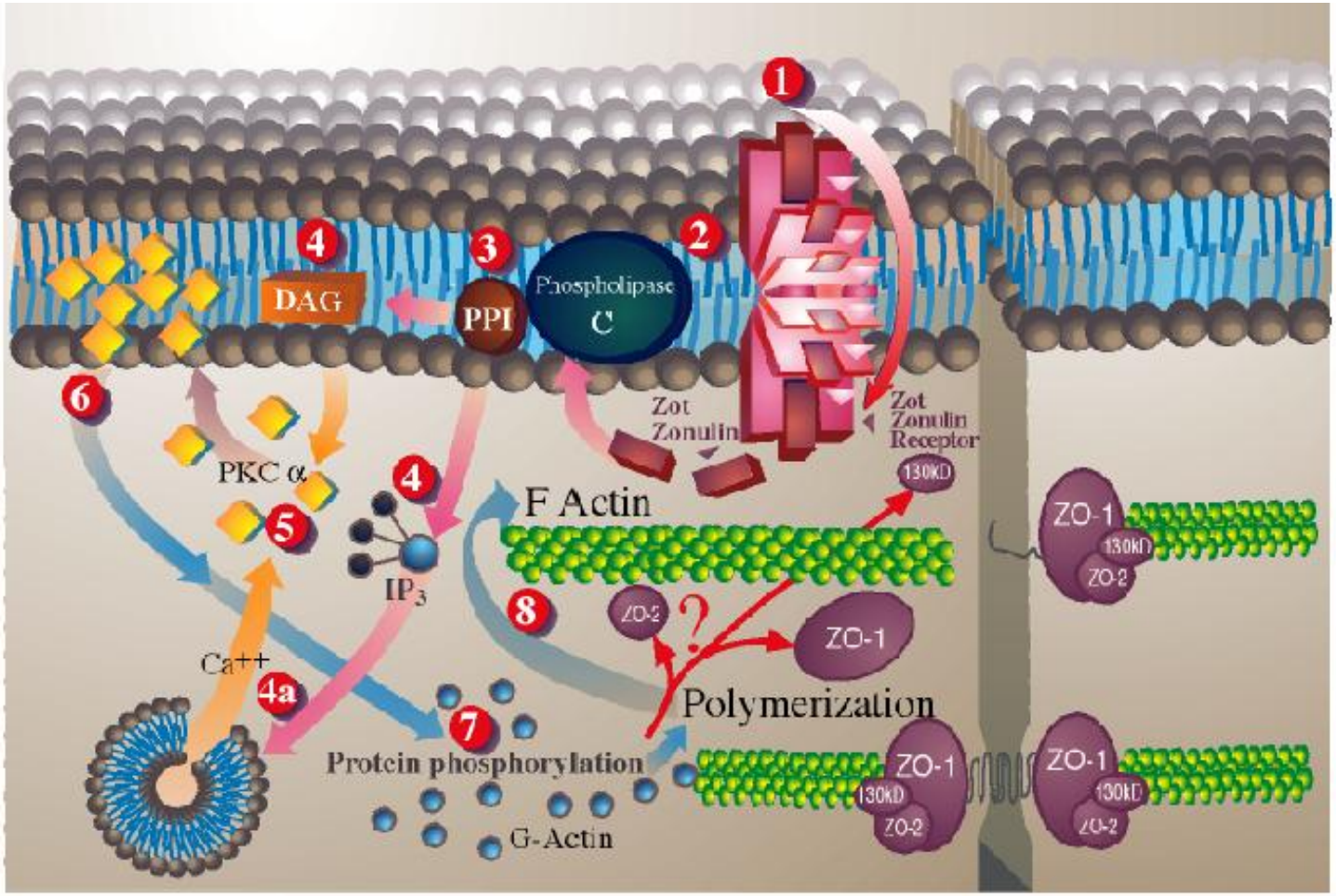


Villous

Crypt

Fluorescence microscopy: Zonulin receptor seen on the villi and absent in the crypts

Proposed Zonulin Mechanism of Action



Background

- **We have demonstrated that the effect of Zonulin on the cell cytoskeleton and tj permeability can be mimicked by its synthetic hexapeptide agonist AT1002 (FCGIRL) and can be inhibited by the synthetic peptide AT-1001 (GGVLVQPG) .[**]**
- **Both peptides seem to bind to the same Zonulin intestinal receptor through a specific binding motif.**

**Clemente et al Gut 2003

Zot, Zonulin, AT-1001

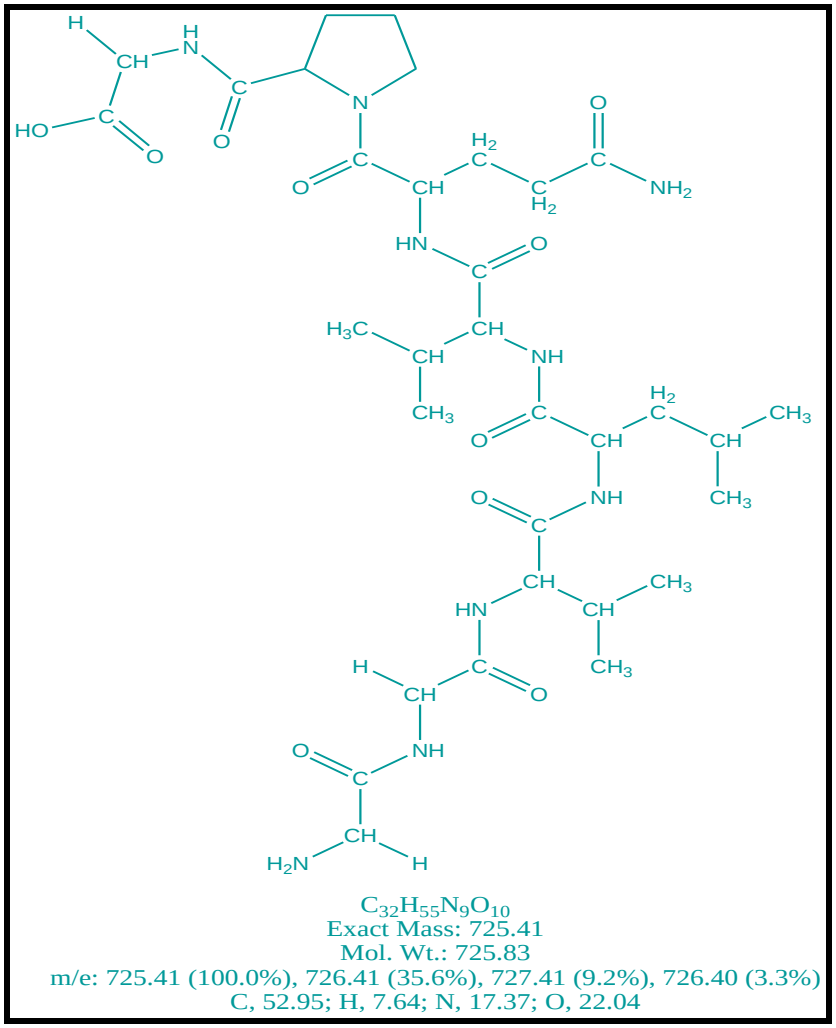
Comparison of the amino-terminal sequences of human intestinal Zonulin and Zot

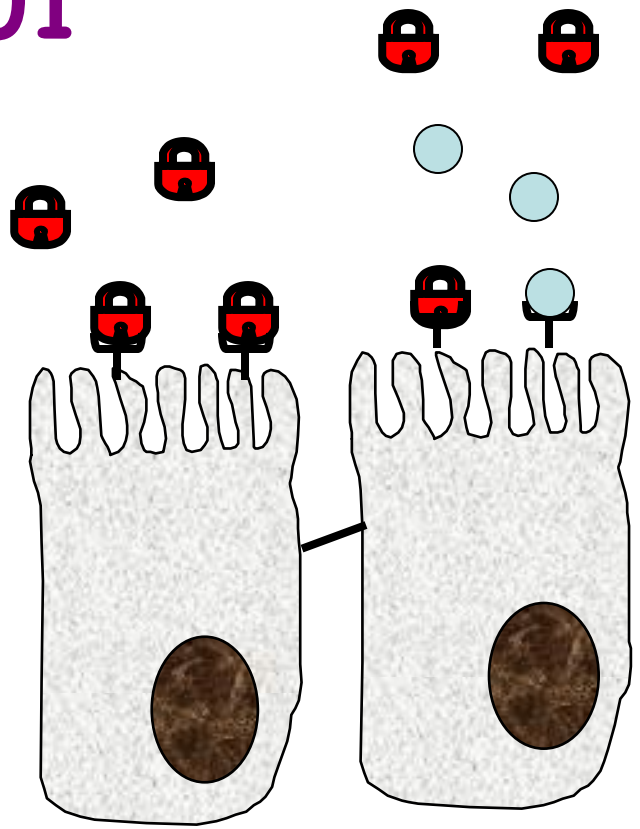
Sample	Source	AA residue* 290	295	300
Zot	*V. cholerae*	F C I	G R L C V Q D G	F V T
		5	10 15	20
zonulin	human intestine	M L Q K A E S	G G V L V Q P G	X S N R L
FZI/0	Synthetic peptide		G G V L V Q P G	

Pierro et al J. Biol. Chem 2001

4 of the 8 amino acid residues are identical (GXXXVQXG)

AT-1001





Zonulin

AT1001

AT1001 shares the same sequence of amino acids as zonulin. It acts on the same zonulin receptor thereby acting as an antagonist

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 lwkdrkilfl fgmvflmfsy

241 sfyglhdnpi f**tggndatieseq sepqska tagnavgska vapasfgfcigrlcvqdgfv**

301 tvgderyrlv dnldipyrgl watghhiykd kltvffetes gsvptelfas syrykvlplp

361 dfnhfvvfdt faaqalwvev krglpikten dkkglnsif

Spanning domain
Mature Zot
Active domain AT1002

AIM OF THE STUDY

To analyze the binding affinity of Zonulin agonist AT1002 and antagonist AT1001 to Zonulin receptor expressing Caco-2 intestinal epithelial cells by fluorescence microscopy

Materials and methods

Intestinal cell cultures

- Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum, 1% nonessential amino acid, 1% L-glutamine, sodium pyruvate and penicillin/streptomycin)
- Cells were first grown in humidified atmosphere (90% relative humidity) with 5% *CO2* at 37°C in 75 cm^2 culture flask, and then were sub-cultured after 4 days using 0.05% trypsin-0.53mM EDTA. The passage number of the cells used were between 46 and 61

Materials and methods

Peptides

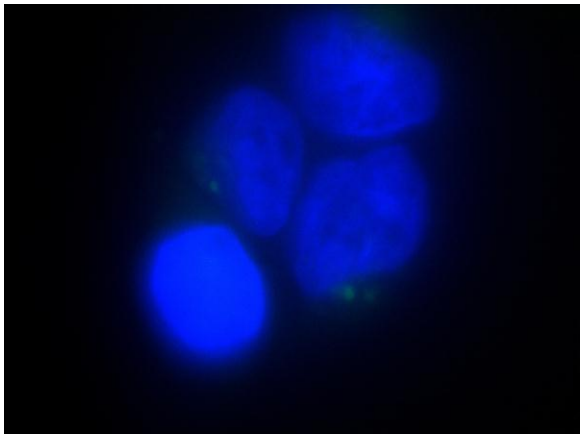
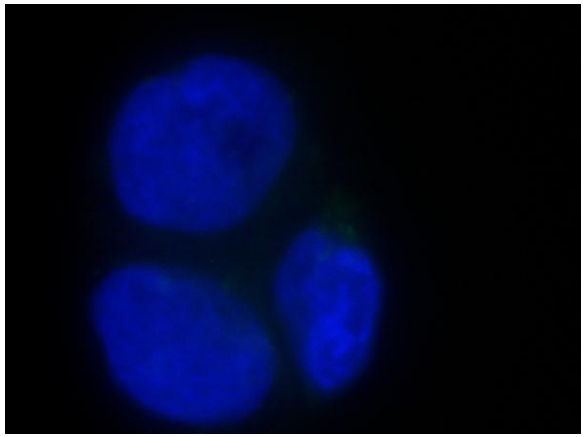
AT1001 untagged and tagged with FITC, AT1002 untagged and tagged with FITC and scrambled peptide (GRLFCI) tagged with FITC were obtained from Biopolymer Laboratories, University of Maryland (Baltimore)

Materials and methods

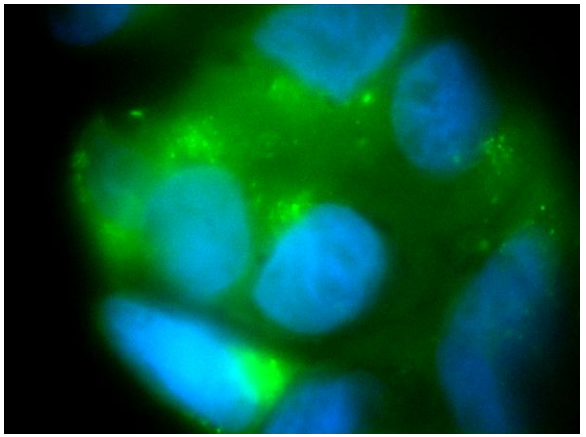
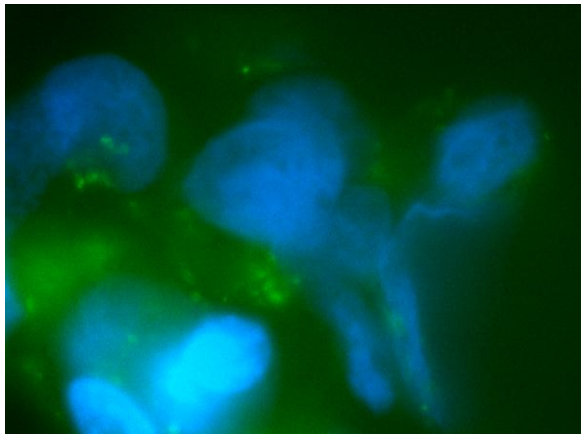
- Cells were cultured on 8 chamber (Lab-Tek II chamber slide system) mounted on glass slide, medium was changed as needed.
- Cells were then fixed in 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature.
- Cells were washed with PBS , and incubated at 37°C for 30 min with FITC labeled peptide (AT1001 or AT1002) either in the presence or absences of unlabelled peptide (in increasing concentrations) at 37°C for 30 min.

Materials and methods

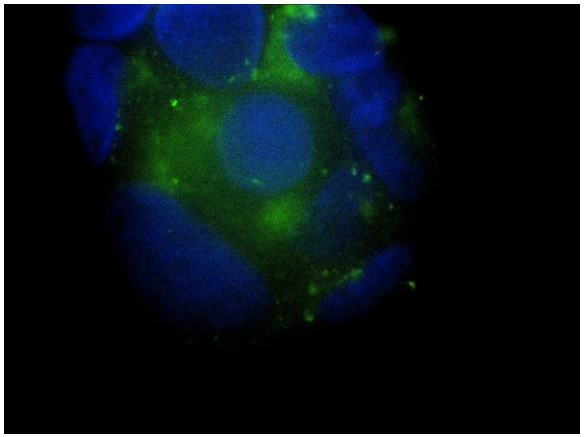
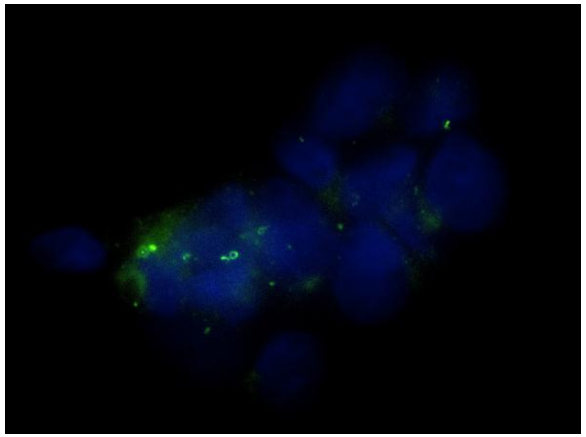
- Dummy peptide was used for control
- Nucleus of the cells were stained with Dapi
- Finally, the cells were washed with PBS. Top of the slide was removed.
- Mounting solution was added, coverslip was placed and slide was sealed with clear nail polish.
- Stored in dark
- Slides were then analyzed in a blind fashion with a fluorescence microscope (Optiphot; Nikon Inc., Melville, NY).

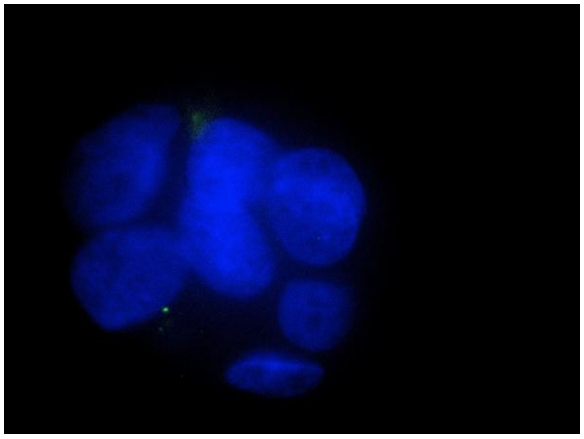
Negative control with PBS

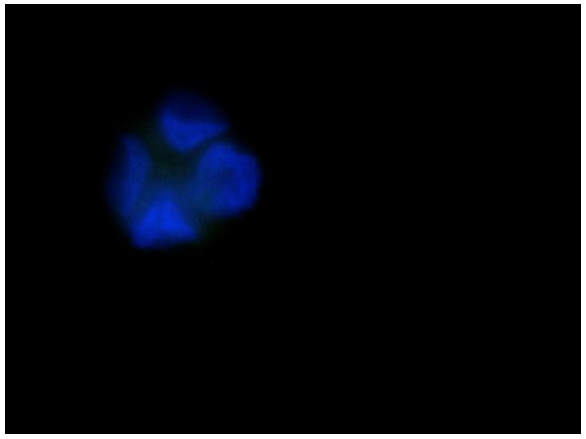



AT1001 tagged with FITC-positive control

FITC AT1001 and cold AT1001X25, 50





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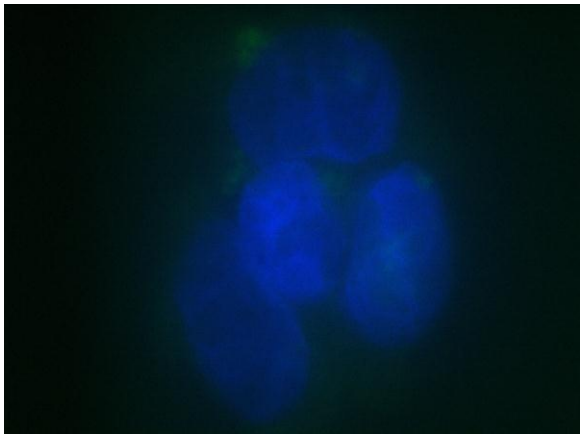
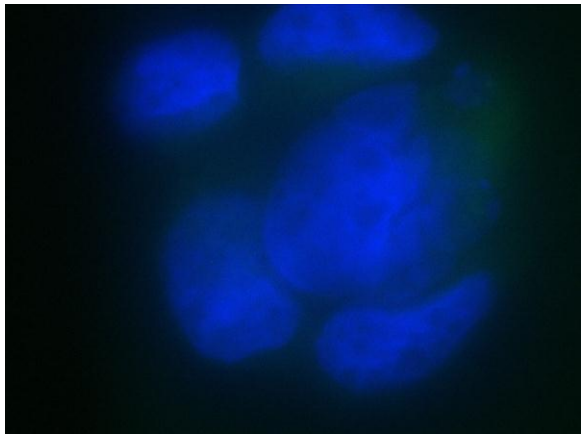
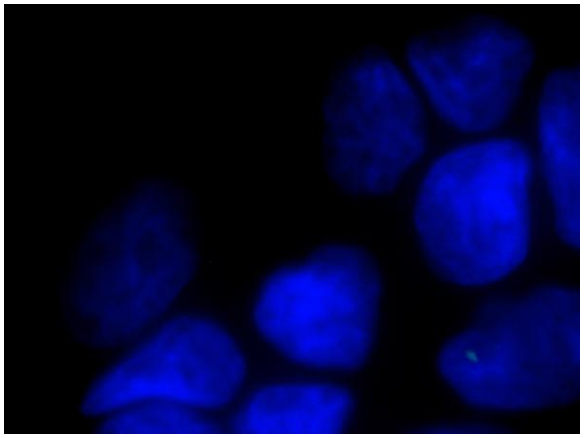
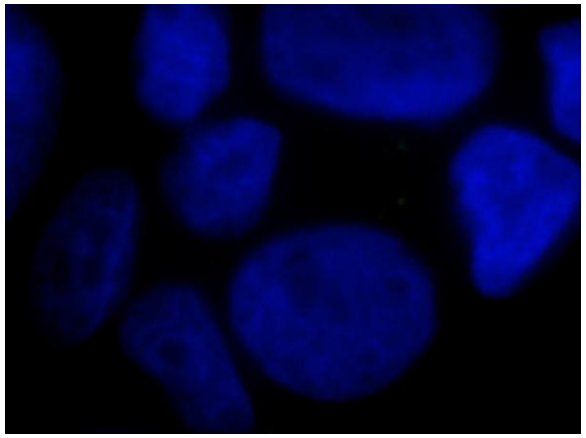




AT1001 FITC and cold AT1001 X100





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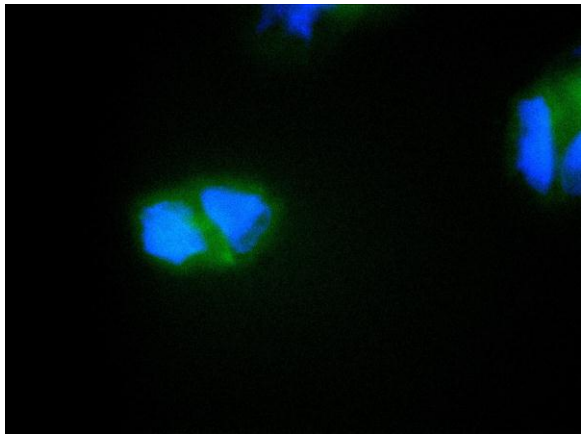
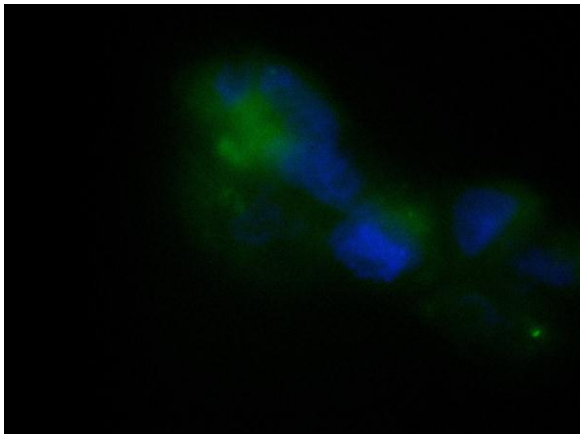
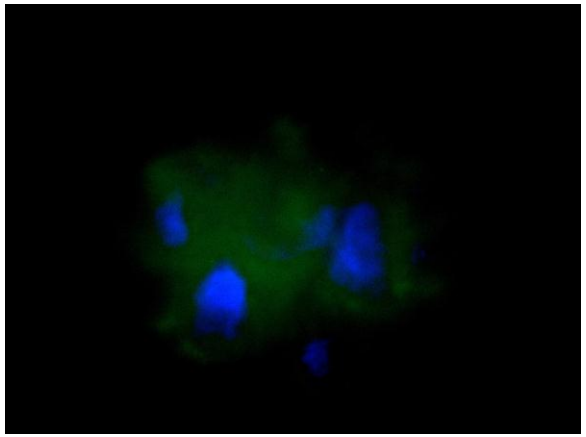
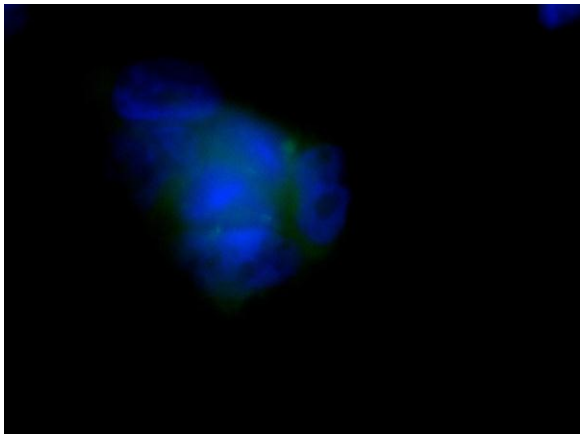
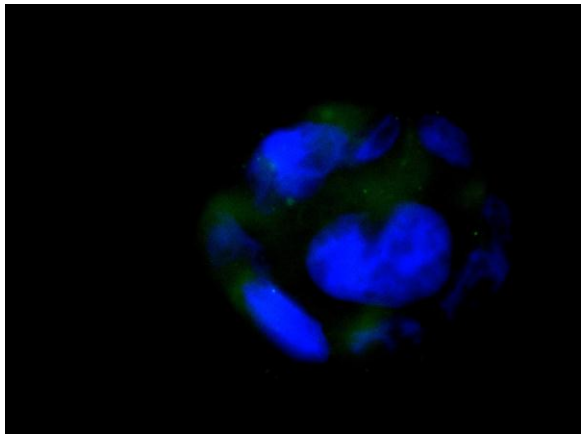
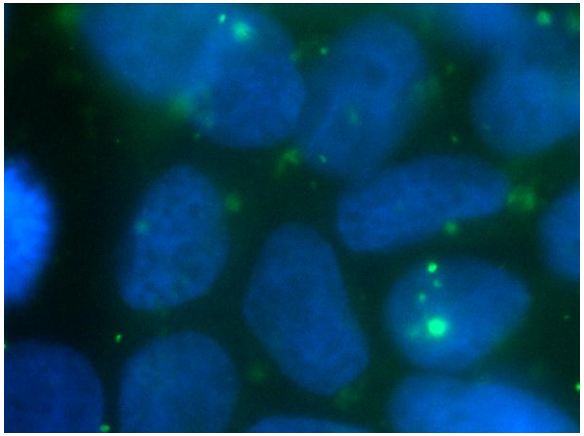



FITC AT1001 with cold AT1002 x 50

FITC AT1001 with cold AT1002x 75

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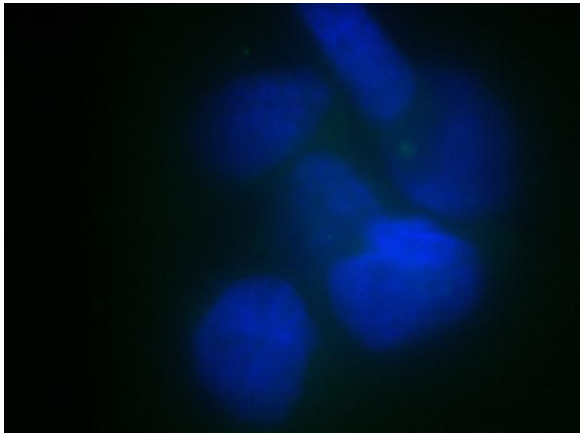
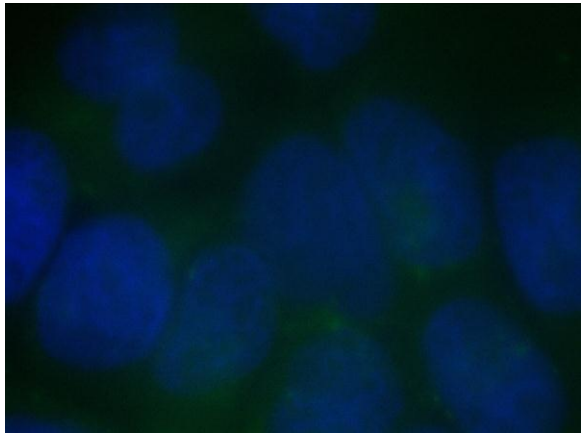
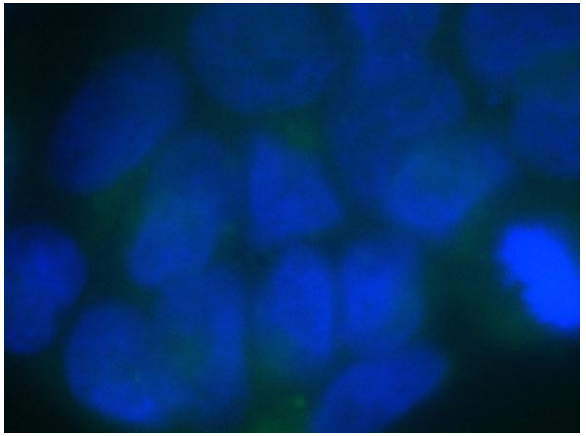
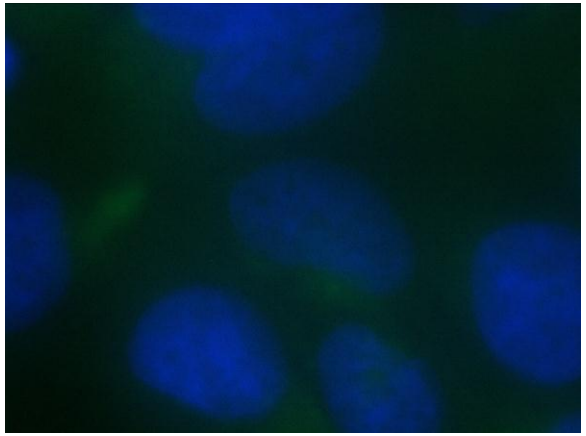


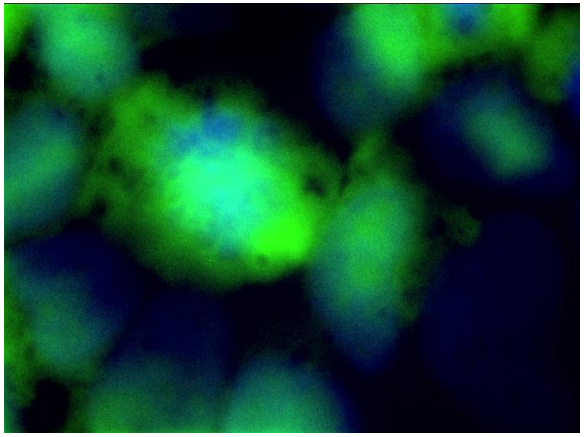


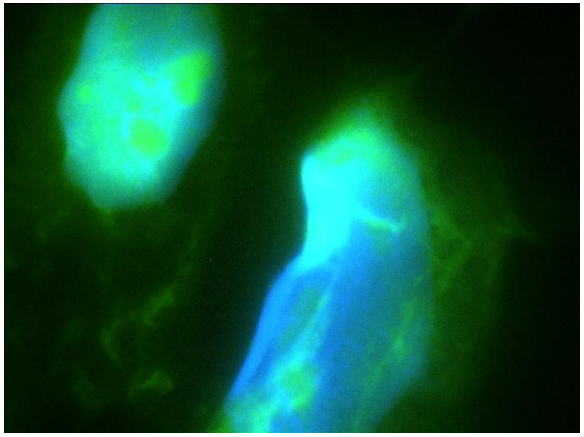
FITC AT1001 and cold AT1002 X 200



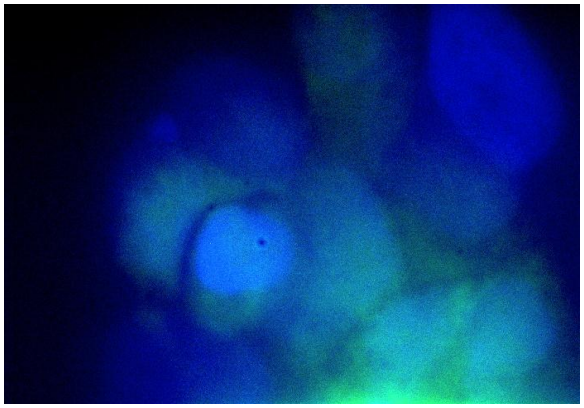


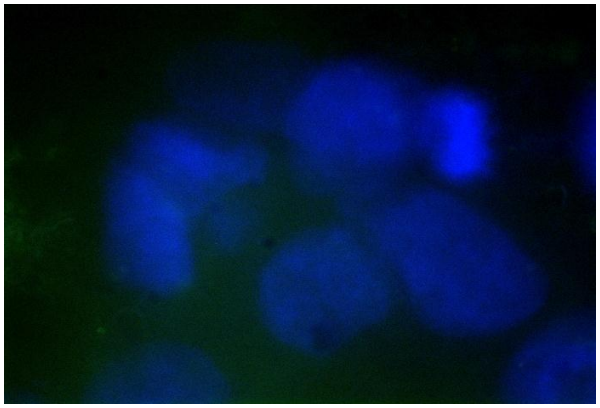
FITC AT1001 and cold AT1002 X 300 and 500



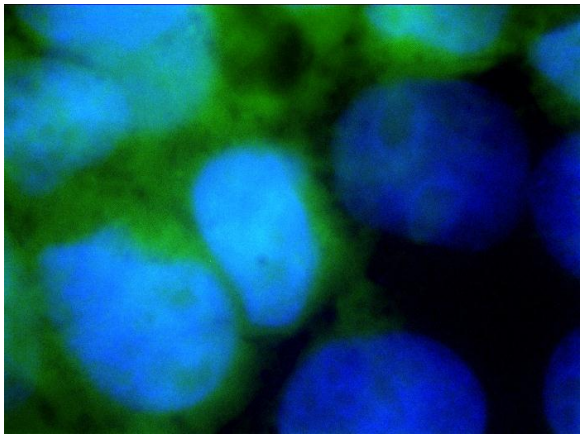
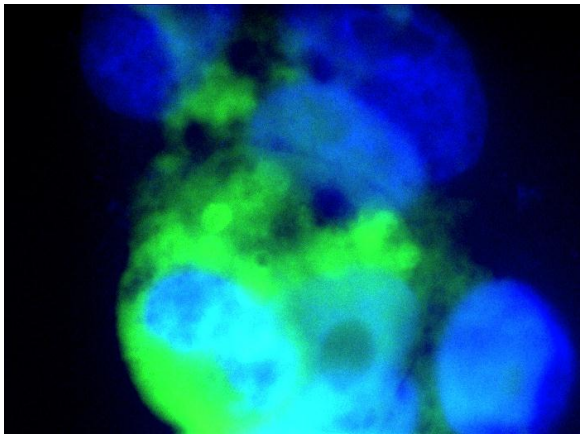


Positive control FITC AT1002 (5mcg)

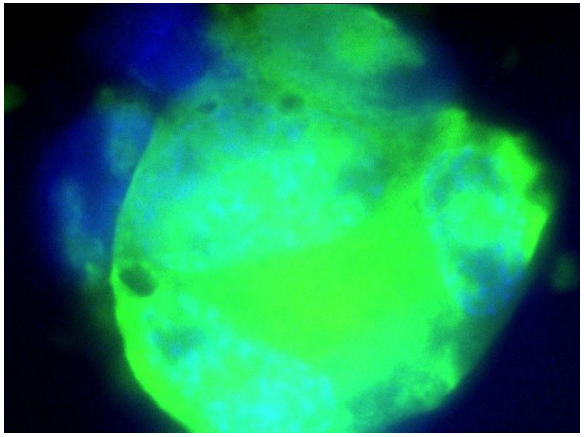
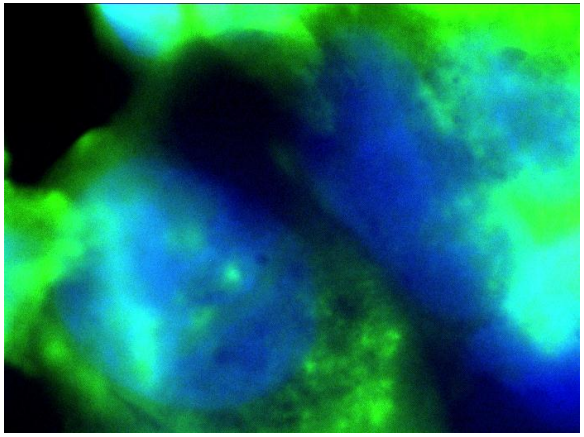




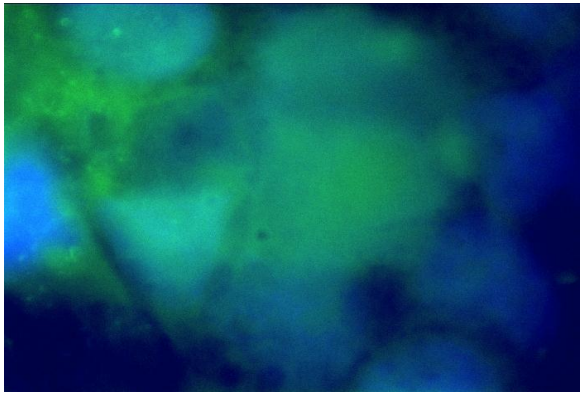
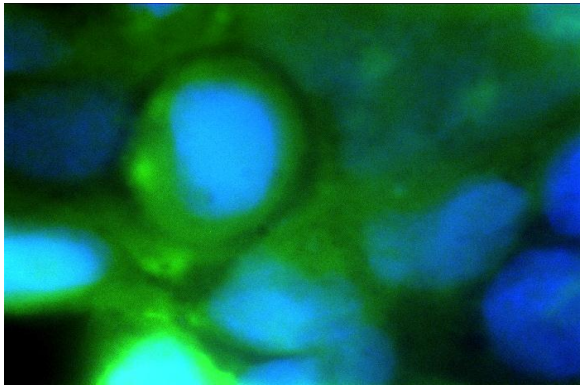
Dummy peptide (GRLFCI) tagged with FITC 5mcg

FITC AT1002 and cold AT1001 X50

FITC AT1002 and cold AT1001 X 75

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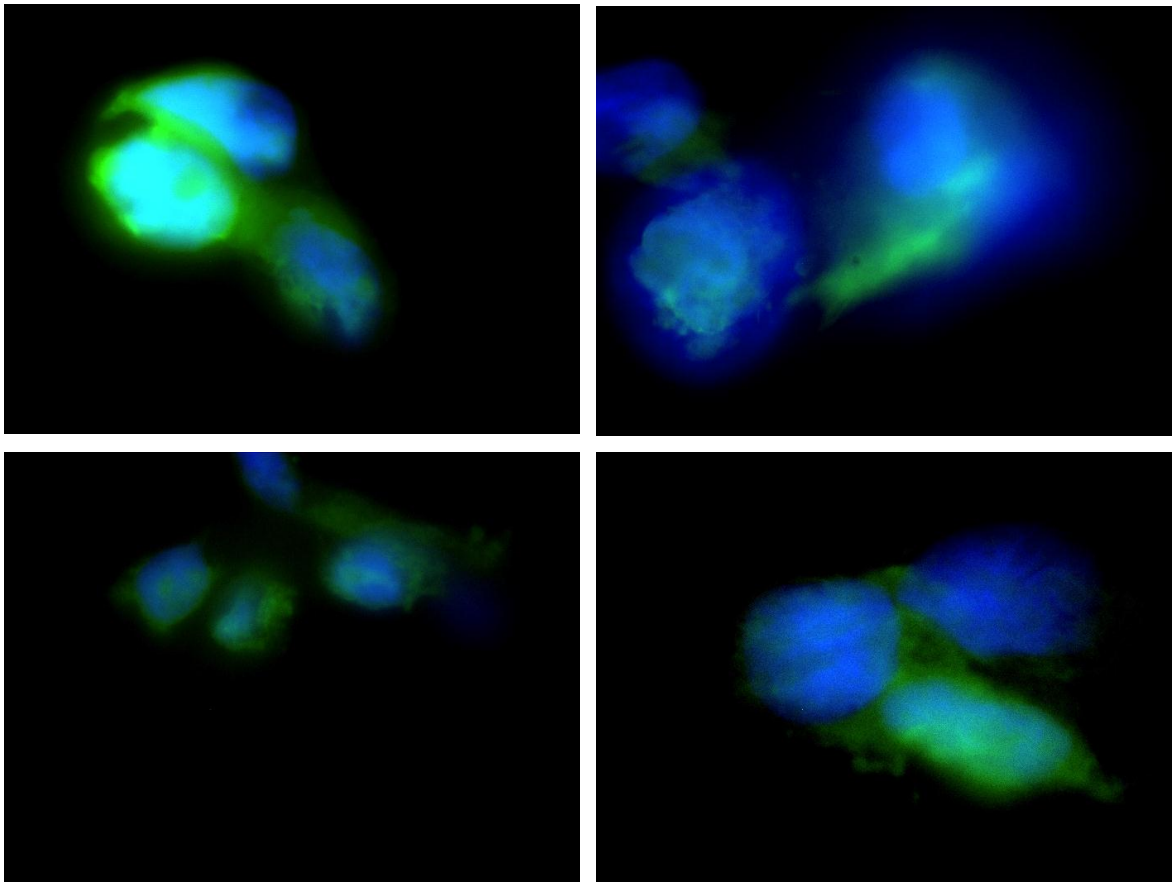
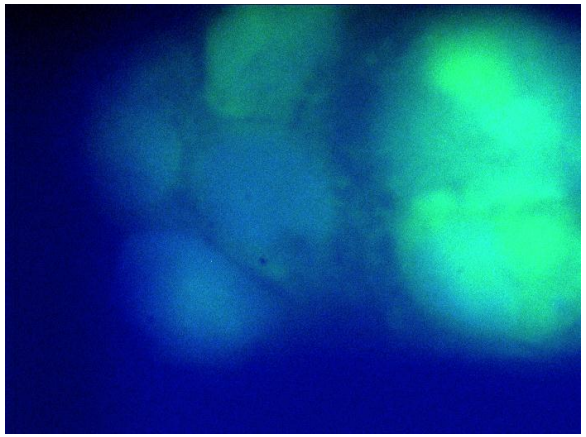
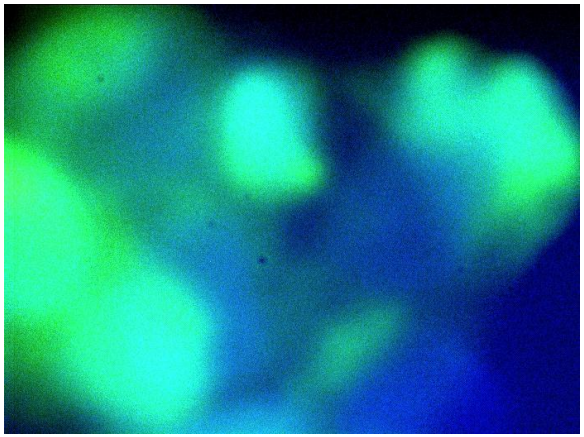
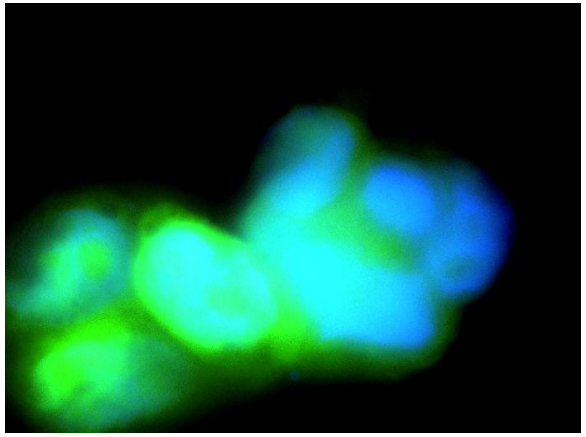
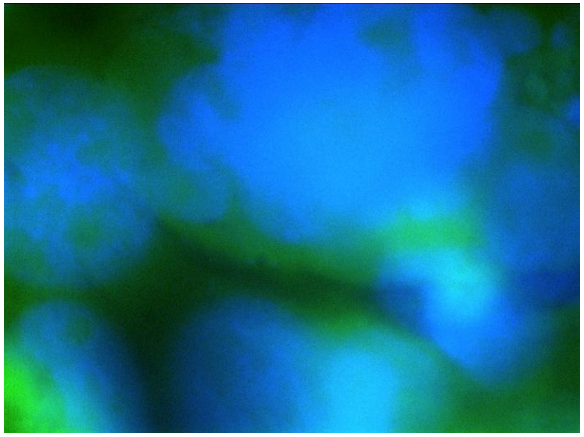


FITC AT1002 and cold AT1001 X150

FITC AT1002 and cold AT1001 X 200

FITC AT1002 and cold AT1002 X 25

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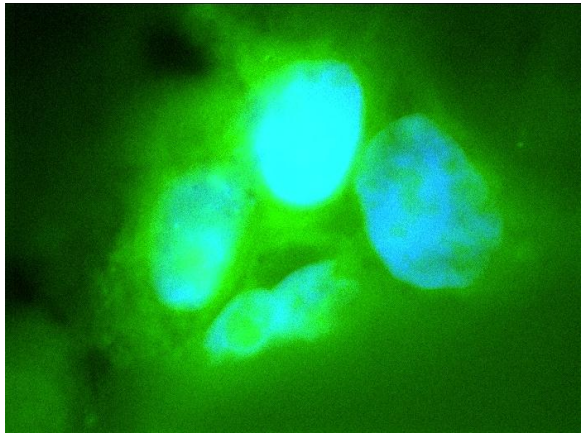
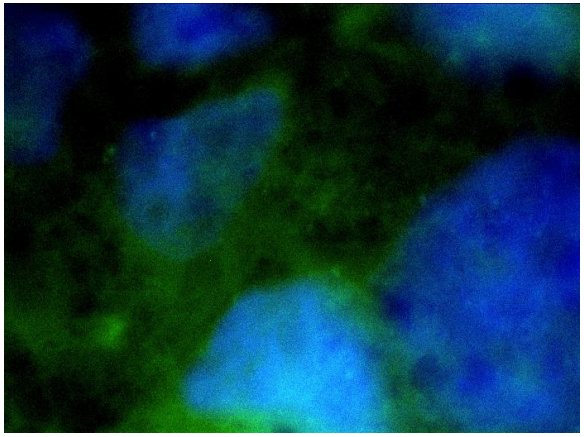

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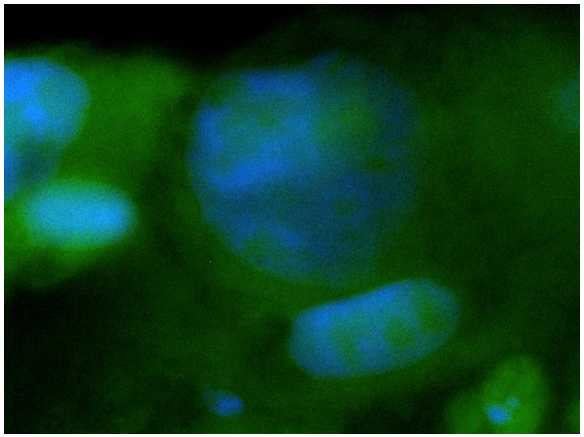
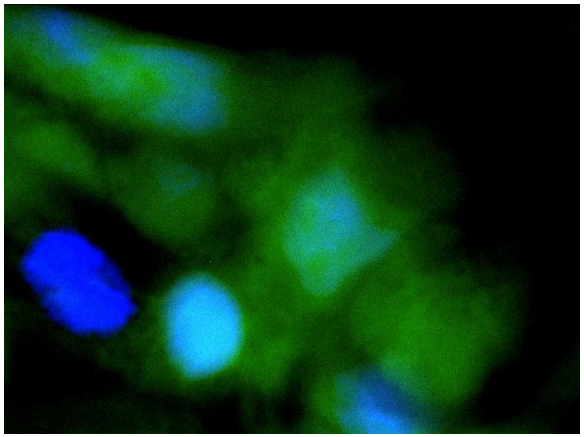
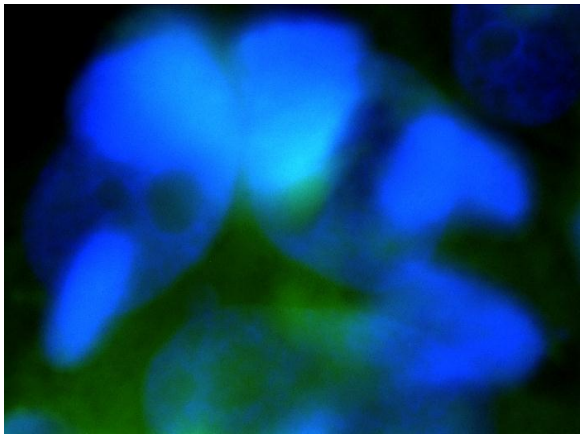
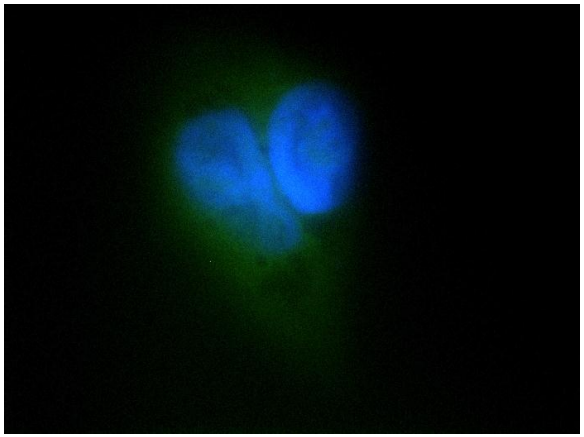




FITC AT1002 and cold AT1002 X100





FITC AT1002 and cold AT1002 X150

RESULTS

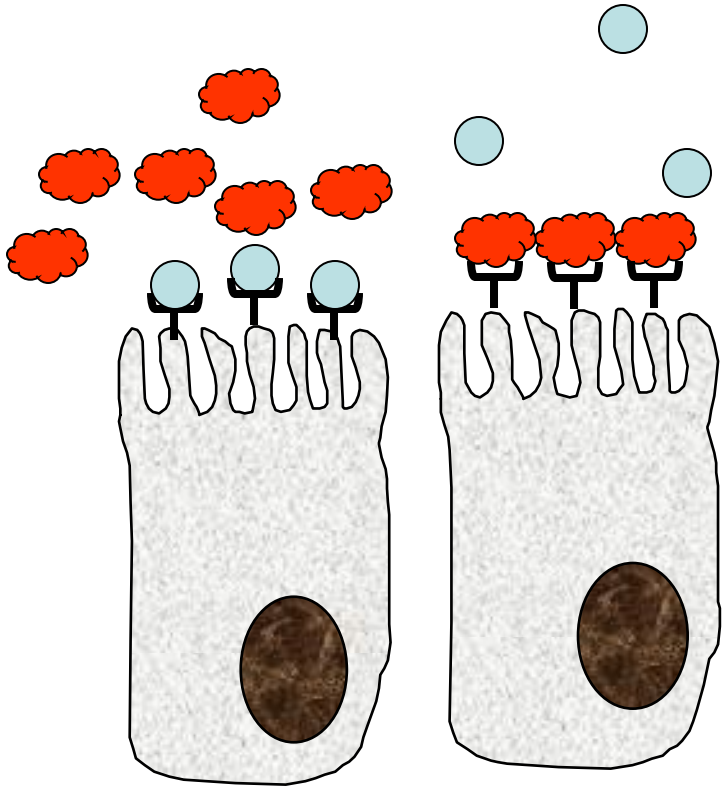
- Both AT1001 and AT1002 bind to CaCo2 cells, while no detectable binding was observed with the scrambled peptide.
- FITC-AT1001 was displaced when untagged AT1001 was used at a concentration >100 times that of the tagged peptide.
- FITC-AT001 was also displaced by untagged AT1002 but at lower concentrations (75 times that of the tagged peptide).
- 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.

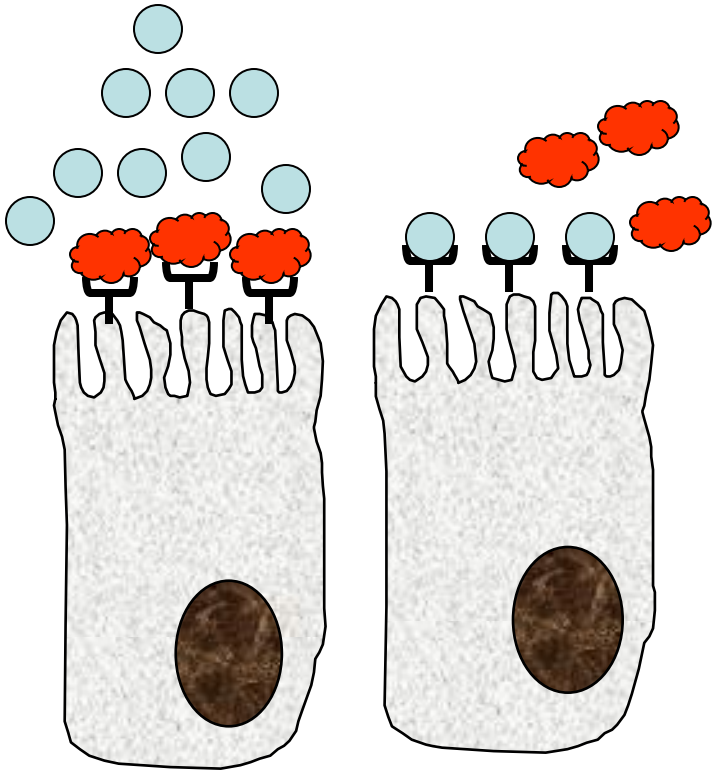
FUTURE DIRECTIONS

- Identify target receptor using colocalization experiment in cells transfected with putative receptor gene.



AT1002

AT1001



AT1002

AT1001