The active motif of Zot, AT1002, increases ZO-1 and Myosin 1 Beta serine phosphorylation, their interaction, and intestinal permeability

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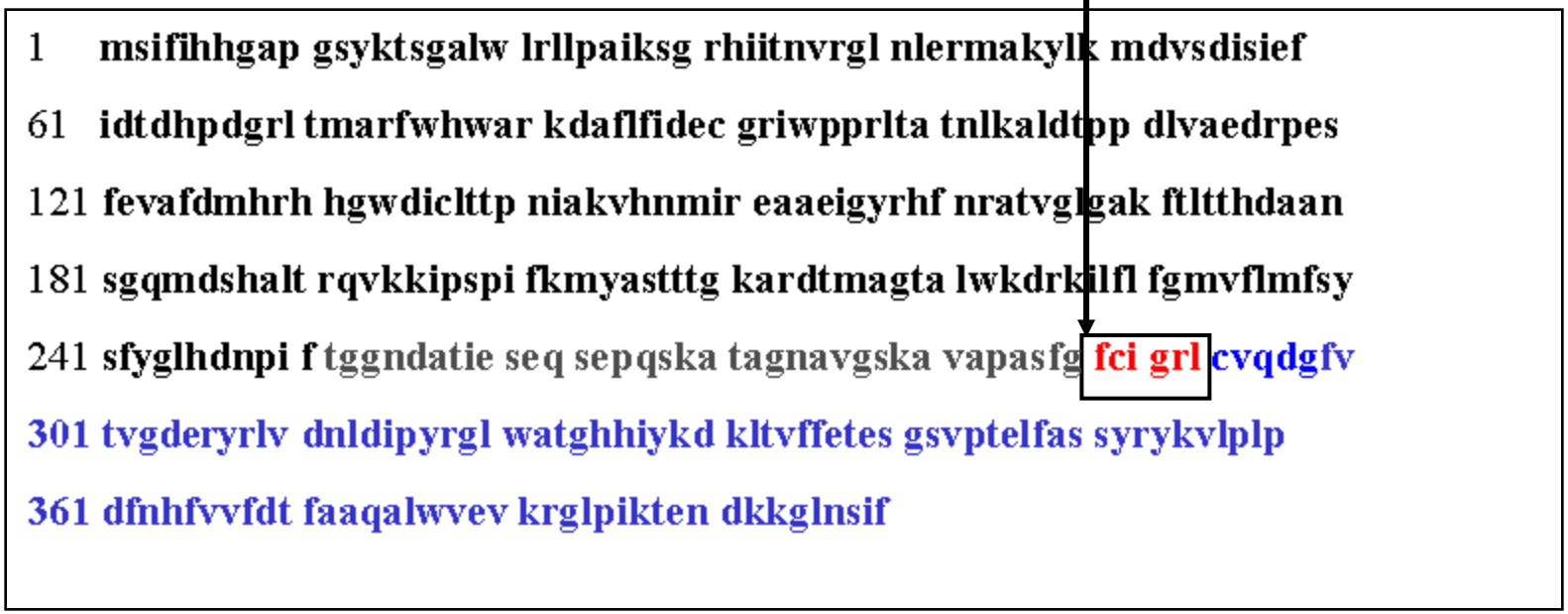
Background

V.cholerae cleavage site



As we have previously reported, Zonula occludens toxin (Zot) elaborated by *Vibrio cholerae* anchors to the bacterial outer membrane and undergoes a *Vibrio*-specific cleavage at amino acid residue 288, with subsequent release of its C-terminal fragment in the intestinal micromilieau. The N-terminus of the cleaved Zot fragment contains a conserved 6-mer protease activated receptor (PAR)-activating peptide (AP) motif. We synthesized the 6-mer and named it AT1002.

Aims

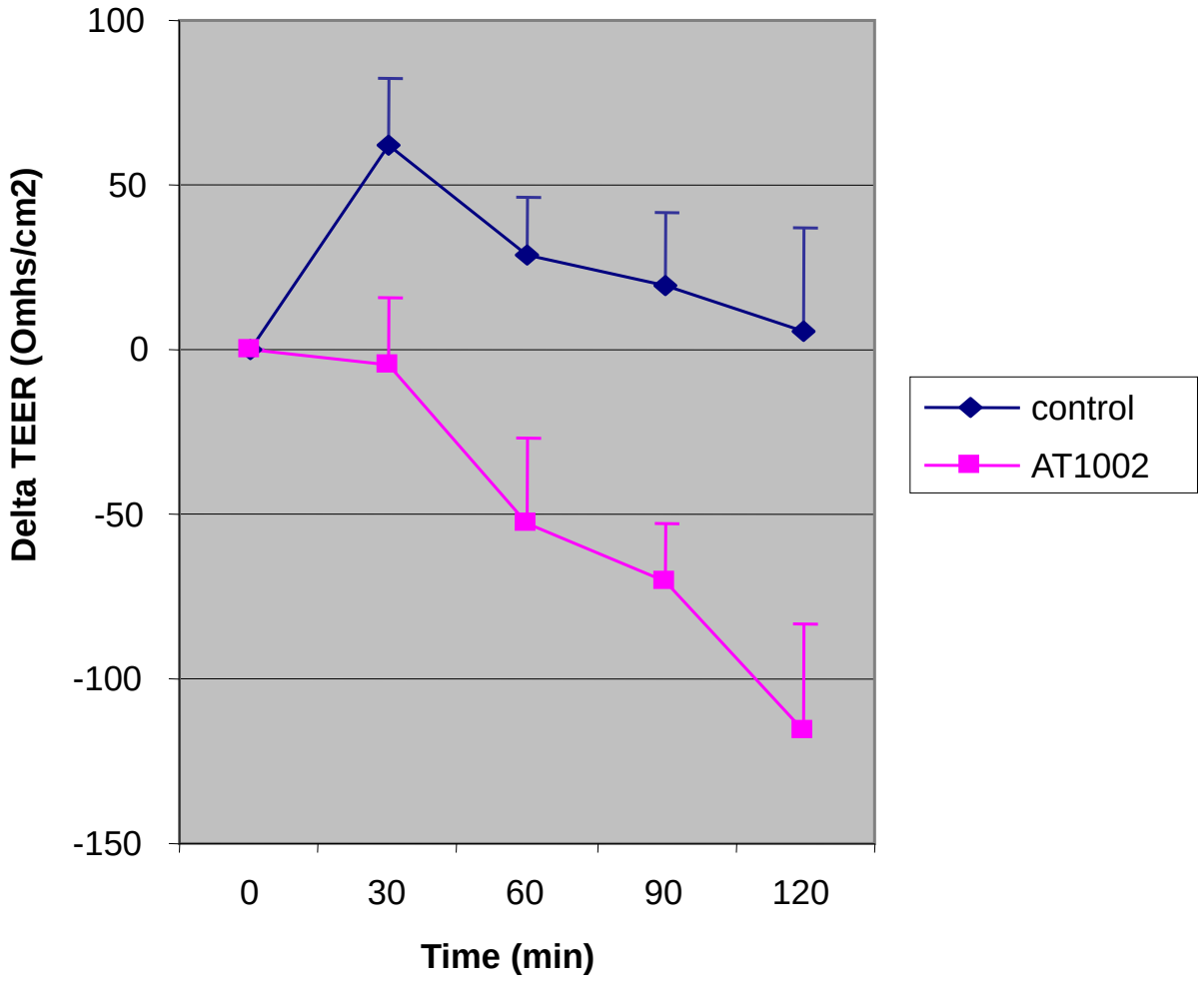
1. To determine whether AT1002 modulates tj both *in vivo* and *in vitro*.
2. To establish whether AT1002 signaling affect ZO-1 phosphorylation.
3. To study ZO-1 interaction with partner and scaffolding proteins in presence of AT1002.

Methods

1. Transepithelial electrical resistance (TEER) was monitored either in presence or absence of AT1002 added to the mucosal aspect of rat small intestine mounted in Ussing chambers.
2. The *in vivo* intestinal permeability of mouse intestine was studied by dual sugar test with HPLC technique.
3. Phosphorylation of ZO-1 induced by AT1002 was analyzed by Western immunoblotting.
4. The effect of AT1002 on protein-protein interaction of ZO-1 with partner proteins and scaffold proteins were investigated by co-immunoprecipitation analysis.

Results 1

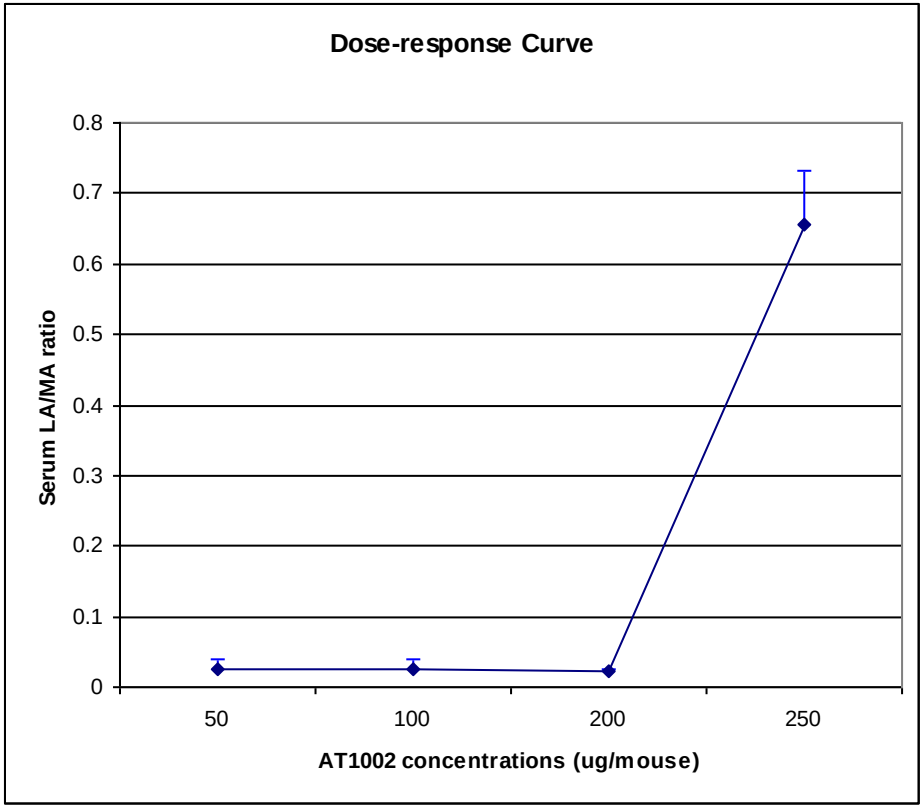
Effect of AT1002 in vitro



AT1002 50 μM added to the mucosal aspect of rat small intestine mounted on a modified snap well, induced a significant decrease in TEER. This effect was reversible upon AT1002 removal (data not shown).

Results 2

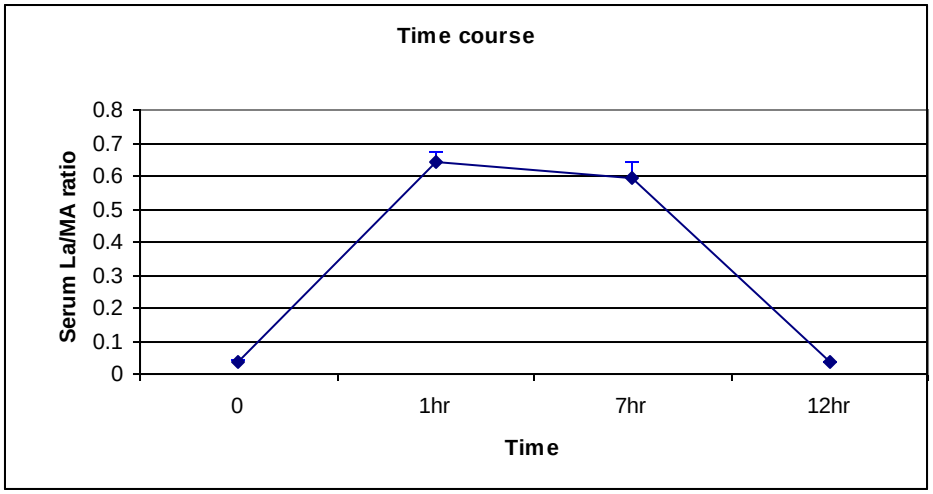
Effect of AT1002 in Vivo (Dose response)



We assessed small intestinal permeability by means of dual sugar test with HPLC technique in mice. Lactulose and L-rhamnose were used as sugar molecular probes for paracellular and transcellular pathways, respectively, and the results were expressed by L/R ratio (n.v.≤0.04). The mice were orally given100 µl of solution containing 12 mg Lactulose and 8 mg L-rhamnose. The L/R ratio increased significantly at 250 µg/mouse compared to lower concentrations.

Results 3

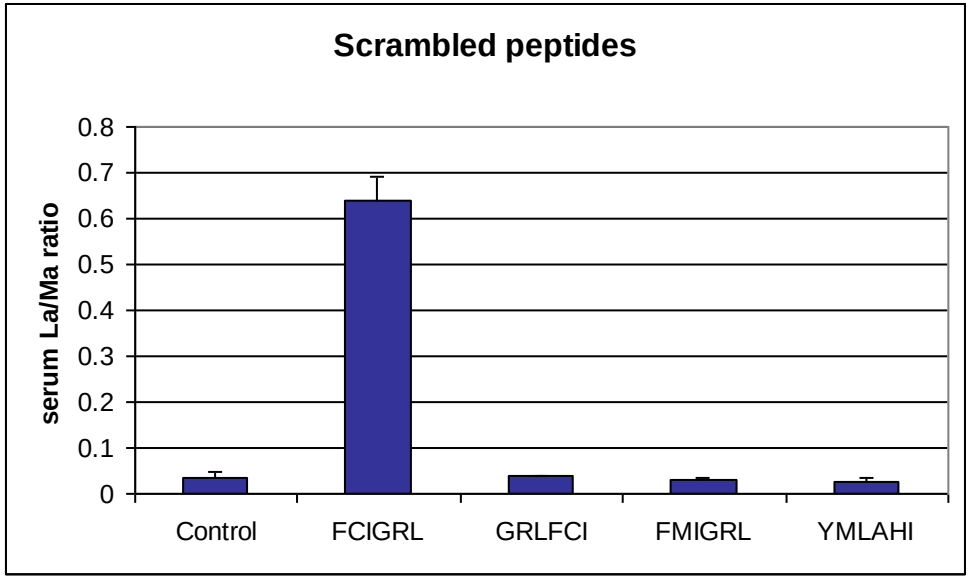
Effect of AT1002 in Vivo (Time response)



At 250 µg/mouse concentration of AT1002, the serum levels of sugars increased at 1hr and 7 hr compared to control and spontaneously returned to baseline after 12 hrs.

Results 4

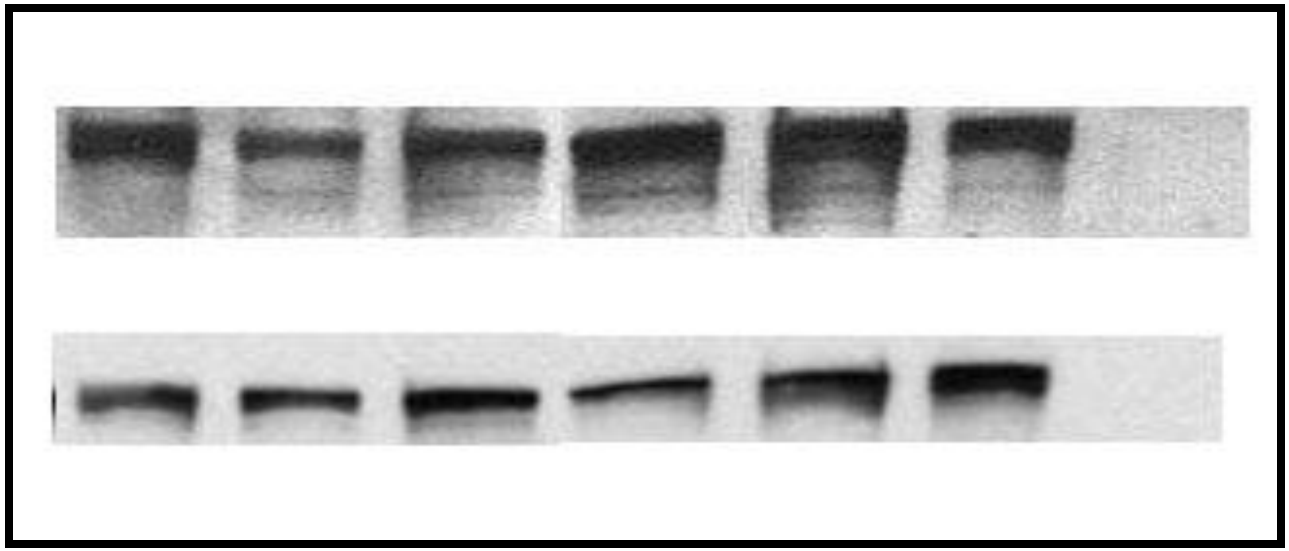
Effect of Scrambled peptides *in vivo*



To establish whether the effect of AT1002 (FCIGRL) on intestinal permeability was specific, we copmared its permeating activity with a series of scrambled peptides. AT1002 250 µg/mouse caused the expected changes in intestinnal permeability. Conversely, reversing the first three amino acids, changing the cystine in position 2 with a methionine, or generating an irrelevant peptide caused the loss of the permeating activity.

Results 5

AT1002-induced ZO-1 serine phosphorylation: Dose-response curve



Phosphoserine

ZO-1

0 1 10 50 100 200 Ab

μM

The total protein from IEC6 cell lines was subjected to ZO-1 immunoprecipitation after exposure to increasing AT1002 concentrations and then immunoblotted using anti-phosphoserine antibodies. The filter was stripped and re-probed with anti-ZO-1 antibodies to control for equal loading. The phosphoserine western blot shows increase in ZO-1 phosphorylation with increasing dose of AT1002.

Results 6

100μM ofAT1002-induced ZO-1 serine phosphorylation: Time-response curve

phosphoserine

ZO-1

-	+	-	+	-	+

AT1002

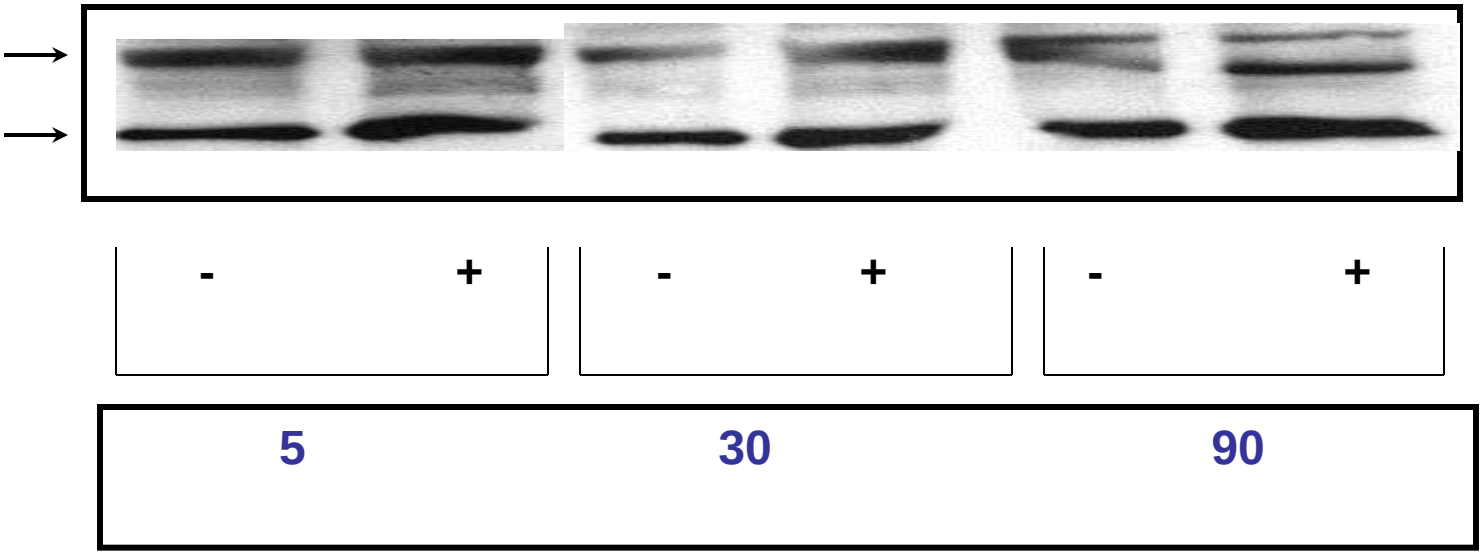
5	30	90

Minutes

AT1002 was used at a concentration of 100 mM. Immunoprecipitation was performed on IEC6 total protein using anti ZO-1 antibodies. The filter was stripped and re-probed with anti-ZO-1 antibodiues to control for equal loading. The phosphoserine Western immunoblot shows an increase in phosphorylation of ZO-1 at 30 minutes onwards compared to the control. No change in the phosphorylation was seen at 5 minutes time point.

Results 7

Phosphoralyted 110Kda Protein Co-immunoprecipitates with ZO-1



AT1002 was used at a concentration of 100 mM. Immunoprecipitation was performed on IEC6 total protein using anti ZO-1 antibodies. The phosphoserine Western immunoblot showed an increase in phosphorylation of a ~110 KDa protein starting at 5 minutes post-AT1002 incubation. In order to identify the protein, MS/MS spectroscopic analysis was performed.

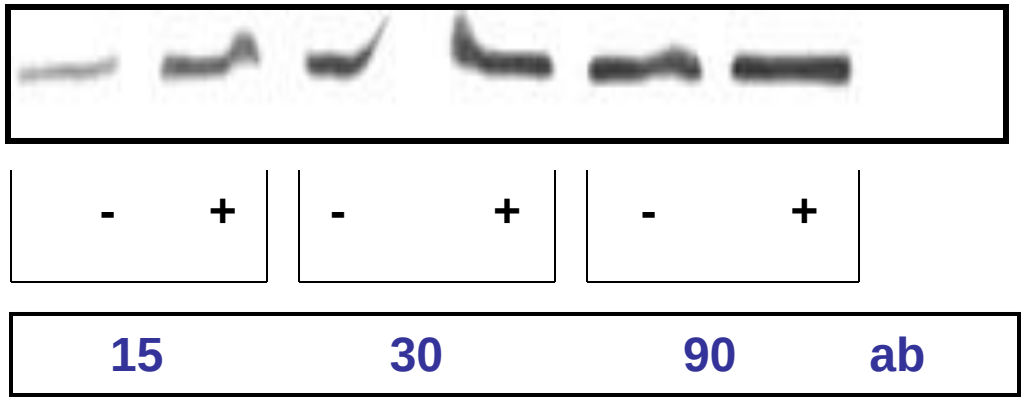
Results 8

MS/MS of 110Kda protein co-immunoprecipitated with ZO-1 identified the protein as myosin 1 beta

Observed Mass	End Sequence
1001.468	LGTEEISPR
1033.5106	NGLAVVAPR
1054.5081	KYEAFLQR
1058.5562	LLGETTLR
1062.4844	TLRNDNSSR
1075.4733	DNYPQSVPR
1075.4733	WHQNHGER
1108.4991	YLGLMENLR
1176.5614	RPETVATQFK
1192.532	YMDVQFDFK

Results 9

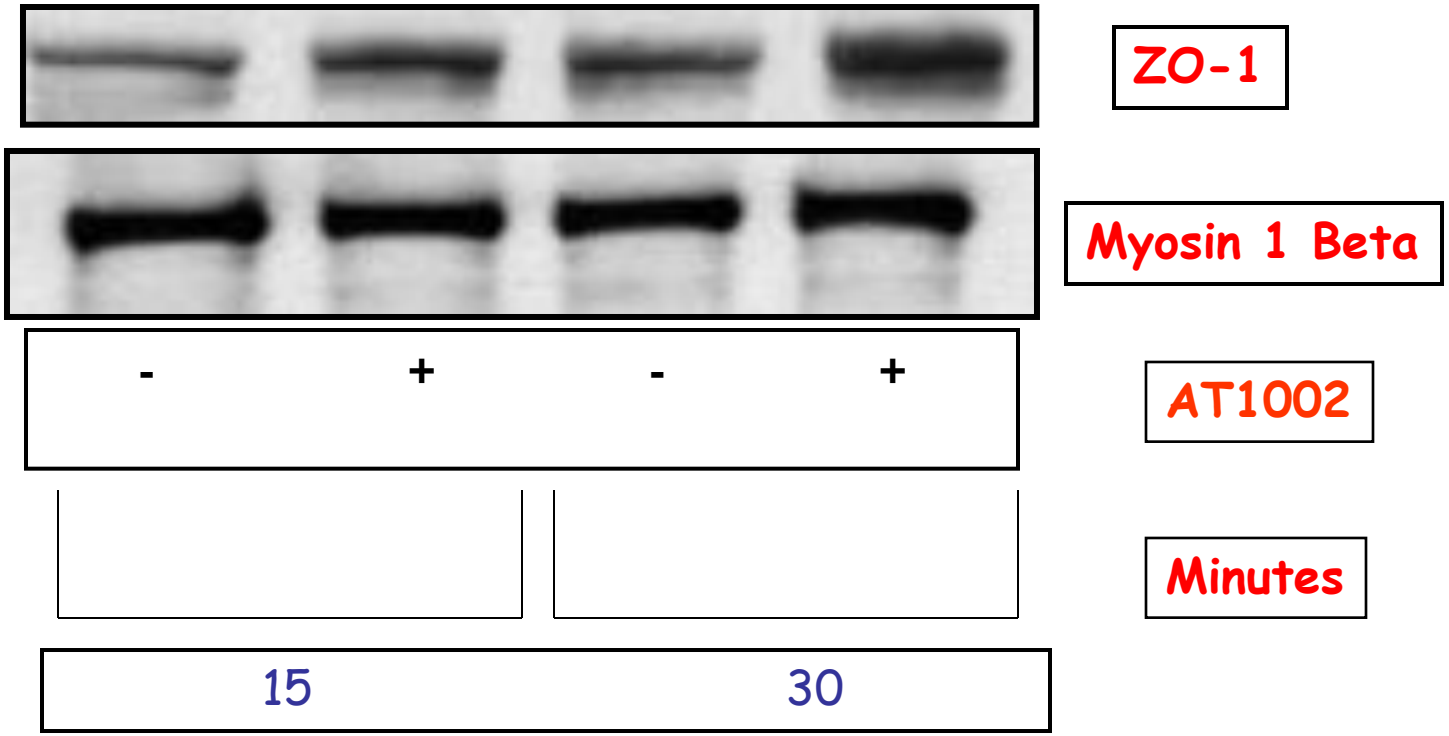
Confirmation of Myosin 1 Beta



AT1002 was used at a concentration of 100 mM. Immunoprecipitation was performed on IEC6 total protein lysate using anti ZO-1 antibodies. The myosin 1 beta Western immunoblot confirmed the MS/MS spectroscopic results about the nature of the 110 KDa as myosin 1 beta.

Results 10

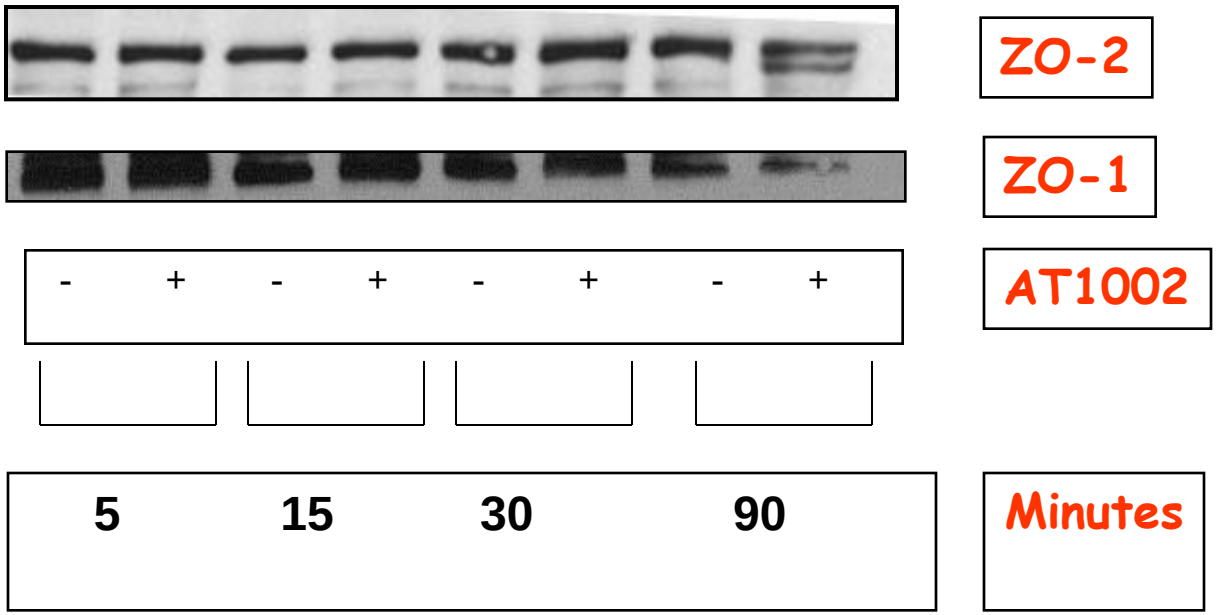
Co-immunoprecipitation of ZO-1 with Myosin 1 Beta



AT1002 was used at a concentration of 100 uM. Immunoprecipitation was performed on IEC6 total protein using anti myosin 1 beta antibodies. The ZO-1 Western immunoblot shows an increase in association of ZO-1 and myosin 1 beta at 15 minutes onwards compared to the control. The same filter was stripped and re-probed with anti myosin 1 beta antibodies to control for equal loading.

Results 11

Co-immunoprecipitation of ZO-2 with ZO-1



AT1002 was used at a concentration of 100 µM. Immunoprecipitation was performed on IEC6 total protein using anti ZO-1 antibodies. The ZO-2 Western immunoblot shows an increase in association of ZO-1 and ZO-2 at 90 minutes compared to the other time points. The same filter was stripped and re-probed with anti ZO-1 antibodies to control for equal loading.

Conclusions

1. AT1002 caused the tight junctions disassembly as shown by decrease in TEER in rat tissues mounted in Ussing chambers.
2. AT1002 was biologically active both *in vivo* and *in vitro* as established by experiments performed on intestinal tissues in Ussing chambers and dual sugar test permeability test.
3. AT1002 induced ZO-1 serine phosphorylation that temporarily preceded tight junction disassembly.
4. AT1002 increased serine phosphoralytion of myosin 1 beta and increased association of myosin 1 beta with ZO-1.
5. 6. AT1002 didn't alter the association of ZO-1 and ZO-2.