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Structure–activity relationship studies of permeability modulating peptide AT-1002

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ABSTRACT

AT-1002 a 6-mer synthetic peptide belongs to an emerging novel class of compounds that reversibly increase paracellular transport of molecules across the epithelial barrier. The aim of this project was to elaborate on the structure–activity relationship of this peptide with the specific goal to replace the P2 cysteine amino acid. Herein, we report the discovery of peptides that exhibit reversible permeability enhancement properties with an increased stability profile.

Innovative drug delivery approaches have been developed to administer molecules with suboptimal absorption profile.[1] However, the utility of the paracellular transport mechanism for drug delivery has remained unsatisfactory. This is mainly due to limited understanding of tight junction (TJ) physiology and more specifically to lack of access to reagents capable of increasing TJ permeability without irreversibly compromising barrier integrity and function. Recent work by Fasano and coworkers on Zonula occludens toxin (ZOT) has shed light on the intricate mechanism involved in the regulation of TJ permeability.[2] ZOT, a 45-kDa protein derived from *Vibrio cholerae*, has been shown to reversibly increase TJ permeability in a dose-dependent manner.[3] Abundant evidence suggests that ZOT is a modulator of TJ permeability.[4] Recent structure activity relationship studies using ZOT resulted in a small 12-kDa fragment (ΔG) that exhibits similar activity in vitro and in vivo when compared to ZOT.[5] ΔG has proven to be a versatile reagent useful for drug delivery in a variety of in vivo models including intranasal, intra-duodenal and oral delivery.[6] Additional advances in structure–activity relationship studies have led to identification of **AT-1002** as the active fragment of ΔG with minimum structure requirement for in vitro and in vivo permeability modulation activity.[7]

During our recent studies with **AT-1002**, we discovered that this peptide can undergo Cys–Cys dimerization in a variety of in vitro and in vivo conditions, which may hamper the utility of this compound.

In further investigations we discovered a dose- and time-dependent dimerization in a variety of biologically relevant conditions (Fig. 1). Hence, data obtained from biological experiments may not accurately reflect the activity and the mechanism of action of **AT-1002**. Therefore, it was imperative to identify more stable non-dimerizing analogs of this peptide to allow additional proof

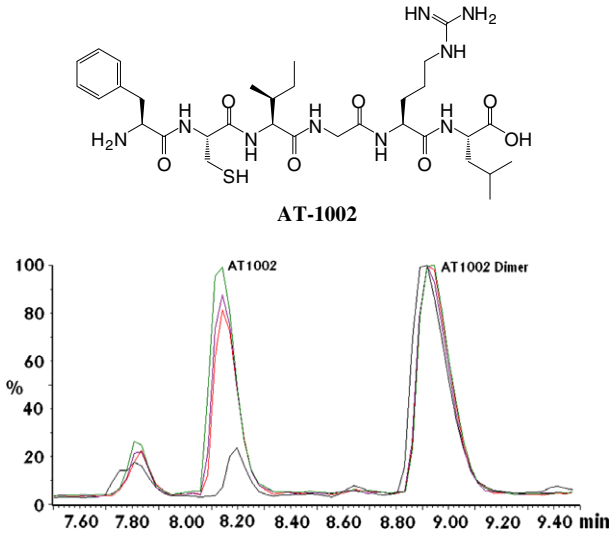

Figure 1. Structure of **AT-1002** and its overlay HPLC profile at different time intervals: 1.0 h (green), 2.0 h (purple), 3.0 h (pink), and 8.0 h (dark).

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of concept studies. Herein, we report a focused structure–activity relationship study that led to discovery of new generation non-dimerizable permeability enhancers. Compounds were prepared using solid phase peptide chemistry. Individual steps such as coupling, deprotection, and cleavage were performed following a reported protocol[8] as depicted in Scheme 1.

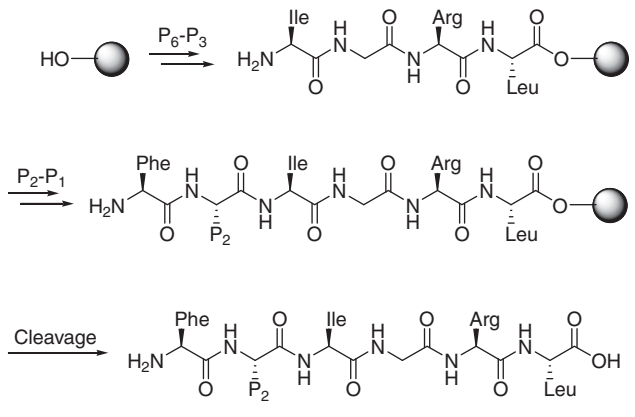
Little is known about the role of the P2 cysteine in **AT-1002** permeability modulating function. Therefore, a variety of amino acids were incorporated into the P2 position to search for the best cysteine surrogate(s) (Table 1).

Examples include not only naturally occurring amino acids, but also examples of unnatural amino acids. For comparison and control purposes, **AT-1002** disulfide dimer and its 'all-carbon' analog were synthesized as depicted in Scheme 2.

In order to screen compounds for their functional permeability activity, a versatile in vitro permeability assay was implemented using Caco-2 cell monolayers as previously described.[9] Table 1 depicts permeability data as the enhancement fold of LY permeability from the apical to basolateral direction in the presence of the peptide compared to untreated control cells. TEER data are presented as the percent TEER of control compared to untreated control cells.

Briefly, Lucifer yellow (LY) flux was measured across Caco-2 cell monolayers in the presence of peptides (7.0 mM) introduced from the apical compartment in triplicates. Permeability experiments were conducted in a humidified atmosphere of 37 °C while maintaining sink conditions. Samples were collected from the receiver chamber at $t = 60$, 120, and 180 min. Cell monolayer integrity was monitored using an epithelial voltohmmeter to measure transepithelial resistance (TEER) at $t = 0$, 60, 120, and 180 min. TEER measurements were used as an indicator of cell monolayer integrity during compound treatment, and TEER values typically reduce as paracellular permeability increases. The following discussion on structure-activity relationship is based on the enhancement of LY permeability. Compounds that enhanced LY permeability more than 3-fold are considered active.

As predicted, synthetically prepared disulfide dimer (**33**) and all-carbon version (**34**) were inactive in our assay. Table 1 depicts truncated peptide analogs of the parent structure (**AT-1002**). Removal of Leu (**1**) did not affect the peptide activity when compared to the parent compound. However, further deletion of amino acids from the C-terminus (**2**) or N-terminus (**4**) rendered these peptides inactive. For the purpose of this study, we sought to explore cysteine modifications with the 6-mer parent structure.

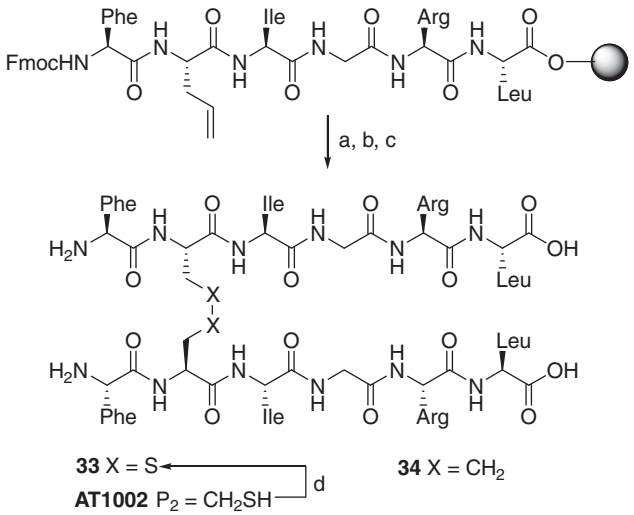


Scheme 1. Synthesis of **AT-1002** analogs. Reagents and conditions: coupling: resin (1.0 equiv), amino acids (3.0 equiv), HBTU (3.0 equiv), HOBt (3.0 equiv), DIEA (6.0 equiv), DMF, rt, 3 h; Deprotection: piperidine:DMF (20:80, v/v), rt, 1 h; cleavage: TFA/TES/H_2O (95:2.5:2.5, v/v/v), rt, 2 h.

Table 1
Lucifer yellow (LY) permeability and TEER assay of **AT-1002** and its analogs **1–33**

Peptide	Sequence or P2 modification	Enhancement fold LY Permeability	% of control TEER
AT-1002	FCIGRL	52 ± 12	11 ± 0.6
1	FCIGR	31 ± 8.7	21 ± 0.9
2	FCIG	2.2 ± 1.3	103 ± 5.2
3	CIGRL	NT	NT
4	IGRL	0.9 ± 0.2	70 ± 2.0
5	3-Me-Phe	0.6 ± 0.1	101 ± 7.1
6	4-CN-Phe	0.3 ± 0.004	118 ± 0.2
7	*t*-Bu-Gly	6.7 ± 1.0	35 ± 1.6
8	Tyr	0.6 ± 0.2	118 ± 0.2
9	Arg	2.2 ± 0.2	102 ± 21
10	Lys	0.4 ± 0.2	110 ± 3.6
11	Asp	0.7 ± 0.4	148 ± 25
12	Glu	1.3 ± 0.7	104 ± 26
13	His	1.0 ± 0.2	102 ± 2.8
14	Dab	2.1 ± 1.5	94 ± 16
15	Thi	1.4 ± 0.6	119 ± 22
16	Ser	2.1 ± 1.5	94 ± 16
17	Thr	3.9 ± 2.8	66 ± 27
18	Abu	1.1 ± 0.1	96 ± 3.0
19	Allyl-Gly	103 ± 26	20 ± 5.2
20	Asn	1.5 ± 0.5	83 ± 11
21	Gln	1.5 ± 0.4	112 ± 6.0
22	Val	5.7 ± 4.5	50 ± 19
23	Leu	1.0 ± 0.05	121 ± 4.5
24	Cha	2.1 ± 1.4	68 ± 40
25	Met(O)	1.1 ± 0.1	106 ± 0.3
26	$Met(O)_2$	0.7 ± 0.1	110 ± 2.6
27	Styryl-Gly	1.0 ± 0.2	113 ± 1.3
28	Cyclopropyl-Ala	0.6 ± 0.05	111 ± 0.4
29	Nva	1.6 ± 0.7	98 ± 22
30	2-Pyridyl-Ala	0.5 ± 0.01	148 ± 39
31	4,5-Dehyro-Leu	1.9 ± 0.5	115 ± 4.8
32	(D)-Allyl-Gly	0.8 ± 0.01	130 ± 10
33	Disulfide	0.7 ± 0.1	137 ± 4.5

Dab, 2,4-diaminobutyric acid; **Thi**, 2-thienylalanine; **Abu**, 2-aminobutyric acid; **Cha**, 3-cyclohexylalanine; **Met(O)**, methionine sulfoxide; **$Met(O)_2$**, methionine sulfone; **Nva**, 2-aminopentanoic acid. NT indicates the compound was not tested.



Scheme 2. Synthesis of **AT-1002** dimers. Reagents and conditions: (a) second generation Grubb's reagent, CH_2Cl_2, reflux, 24 h; (b) H_2, $Pd(OH)_2$, MeOH, rt, overnight; (c) deprotection and cleavage, 12% (four steps overall); (d) 1% H_2O_2, CH_3CN/H_2O (50:50, v/v), rt, 2 h, 100%.

Hydrophobic amino acids at the P2 position (**5**–**8**) with exception of *t*-Butyl-Gly (**7**) were inactive when compared to

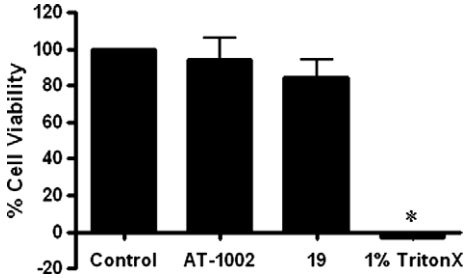


Figure 2. Cell viability in using Caco-2 cells. *Significant reduction in cell viability ($p < 0.05$). Results are reported as means ± standard deviation (SD) ($n = 3$).

AT-1002. Neither the basic (**9**, **10**, and **13**) nor the acidic amino acid (**11** and **12**) surrogates exhibited activity in our assay. Surprisingly, compounds **14** and **16**, which bear cysteine isosteres Dab and Ser, respectively, were also inactive. However, Thr (**17**) and Val (**22**) analogs did induce an increase in LY flux. Compound **19** was the most potent peptide among all the synthesized analogs (Table 1). Encouraged by this finding, multiple P2 derivatives of **19** were prepared and evaluated for permeability modulation effect in this assay. Surprisingly, none of the additional analogs of this parent structure such as, Nva (**30**), terminal olefin rigidified analogs **28**, **30**, and **31** exhibited activity in our assay including (D)-Ally-Gly (**32**). The data obtained to-date (Table 1) unambiguously suggests that the P2 terminal olefin function is an imperative structure unit for maintaining or enhancing the tight junction modulating activity. Potential dimerization of compound **19** was evaluated using HPLC analysis.[10] As predicted, this molecule is void of dimerization and is stable as a monomer.

The effect of **AT-1002** and compound **19** on cell viability was measured using the CellTiter-Glo® cell viability assay. After 3 h of treatment, **AT-1002** and Compound **19** did not significantly reduce cell viability compared to untreated control cells (Fig. 2). These data suggests that these compounds enhance LY permeability due to their permeability modulation activity and not due to cell viability reduction.

The data reported herein suggest that P2 functionalization can be exploited to generate potent and stable analogs of **AT-1002**. Application of the P2 single mutation approach described herein has resulted in compounds that are void of undesirable dimerization. In general, peptides reported in this study do not significantly reduce cell viability. Also, the effects are reversible as shown by compound removal experiments (data not shown). The incorporation of allylglycine (**19**) delivers a promising permeability modulator whose activity and non-dimerizing property warrants further studies as a potential new agent. Additional studies including in vivo testing are underway with compound **19** in our group and will be reported in due course.

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10. Each peptide sample was analyzed using LC–MS with a Waters 2695 HPLC system interfaced with a Waters Micromass ZQ mass spectrometer. An analytical separation was carried out using a Phenomenex Luna-C18 column (150 × 4.6 mm, 5.0 μm) and a dual solvent system consisting of A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). A 15 min gradient from 0% to 40% B at a flow rate of 1.0 mL/min was employed for general analysis purpose. The MS measurement was performed using a positive electrospray ionization mode and the cone voltage was optimized for the presence of maximum precursor ion signal $[M+H]^+$. The purity was evaluated by using LC–MS and comparing the UV absorbance of the sample solution with the solvent blank using a Waters 2695 HPLC equipped with a Waters 2996 diodearray detector at different wavelengths. The physicochemical data of **AT-1002**, compounds **35** and **19** are as follows: **AT-1002**: $R_t = 8.1$ min, $[M+H]^+$ 708.2 (Calcd 708.3); compound **35**: $R_t = 8.9$ min, $[(M+2H)/2]^+$ 707.6 (Calcd 707.3); compound **19**: $R_t = 7.8$ min, $[M+H]^+$ 702.2 (Calcd 702.4).