

Enhancement of Oral Drug Absorption - Effect of Lipid and Glycoside Conjugation on the Enzymatic Stability and Intestinal Permeability of IM862

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Introduction

The dipeptide L-Glu-L-Trp-OH, also known as IM862 or Thymogen[®], is a naturally occurring thymic dipeptide which has been shown to possess immuno-modulating and anti-angiogenic properties [1] and is currently under development for potential treatments of certain cancers and immuno-deficiency disorders. Its intranasal administration in a clinical trial for the treatment of AIDS-related Kaposi's sarcoma showed a large volume of distribution and high bioavailability (71%) [2]. However, due to its highly hydrophilic character, IM862 demonstrates low permeability across the gastro-intestinal track, which makes it not orally available.

In recent years, the conjugation of glycosyl and lipid moieties has been thoroughly investigated as a means to enhance the stability towards enzymatic degradation and the permeability across biological membranes of peptides, either by passive diffusion or by targeting specific membrane receptors [3-5]. In this prospect, a library of eight novel derivatives of the dipeptide IM862 was designed and synthesized by conjugating dodecanoic α -amino acid (C₁₂) and/or β -D-glucuronic acid (GlcA), 1-amino- β -D-glucuronic acid (GlcAN) and N - β -D-glucosamine succinamic acid (GlcNS) residues to the Glu-Trp peptide scaffold, using a 9-Fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) strategy on 2-chlorotrityl resin (Fig. 1 and 2). A ninth conjugate containing a D-cellobiose residue on the N-terminus was also prepared, as the use of

disaccharides has previously been reported to potentially enhance the enzymatic stability and membrane permeability of peptides [6]. All derivatives were then evaluated *in vitro* for stability and permeability improvements using Caco-2 cell models. The ability of one of the designed prodrugs to release the original dipeptide was also investigated, and followed by an *in vivo* testing in rats.

Results and Discussion

With the exception of the cellobiose conjugate of IM862, all peptides were synthesized by a step-wise SPPS process. An Fmoc strategy was preferred to a tertbutyloxy carbonyl (Boc) approach as it required less hazardous cleavage conditions and also proved in this study to yield greater amounts of purer products. Glutamic acid and tryptophan were used side-chain protected, respectively Trp(Boc) and Glu(OtBu). The C₁₂ lipoamino acid was prepared from its bromide precursor [7] and N^{α} -protected with either Boc or N -1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-ethyl (Dde) group. Glycosides were peracetylated and, when destined to be C-conjugated, were derived into 1-azides to provide an amino attachment point after reduction. The protected residues were successively loaded onto a 2-chlorotrityl resin after *in situ* neutralization and activation with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and N,N -diisopropyl ethylamine (DIPEA) in dimethyl formamide (DMF). Upon removal of the N^{α} -protecting group (Fmoc with 20% piperidine in DMF, Dde with 2% hydrazine in DMF), the synthesis was pursued until the peptide scaffold was

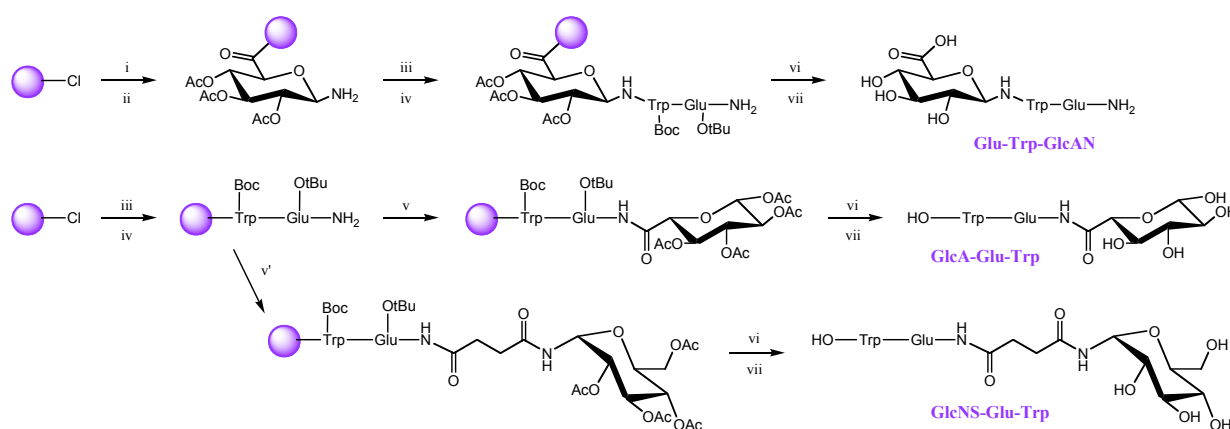


Fig 1. Solid-phase synthesis of glycosyl derivatives of the dipeptide L-Glu-L-Trp-OH (IM862)

(i) 1-azido-per(OAc)GlcAN, DIPEA, HBTU, DMF, RT (30 min); (ii) Et₃N / propane-1,3-dithiol (1:1), RT (16 h); (iii) Fmoc-Trp(Boc)-OH, DIPEA, HBTU, DMF, RT (30 min), followed by removal of the Fmoc group with 20 % piperidine in DMF; (iv) Fmoc-Glu(OtBu)-OH, DIPEA, HBTU, DMF, RT (30 min), followed by removal of the Fmoc group with 20 % piperidine in DMF; (v) per(OAc)GlcNS, DIPEA, HBTU, DMF, RT (30 min); (v') per(OAc)GlcA, DIPEA, HBTU, DMF, RT (30 min); (vi): hydrazine-MeOH; (vii) TFA - scavengers (water, TIPS, EDT, thioanisol, phenol), cold Et₂O

complete. The acetyl groups of the glycosides were converted back into hydroxyls with 12.5% hydrazine and other protecting groups (Boc, OtBu) were removed during the final cleavage using trifluoroacetic acid (TFA) and appropriate scavengers.

The disaccharide conjugate of IM862 was obtained by reacting D-cellobiose with L-Glu-L-Trp in the presence of sodium cyanoborohydride in a phosphate buffer (pH 7). All peptides were finally isolated in 38-84% yield and characterized by reversed phase high-performance liquid chromatography and mass spectrometry.

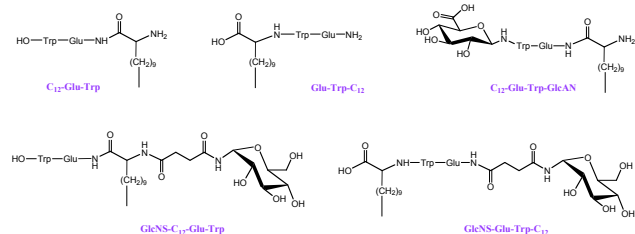


Fig 2. Lipid and glycolipid conjugates of IM862

IM862 is a known substrate for aminopeptidase W, an enzyme present in the renal and intestinal brush border membranes [8], and also expressed by colon cancer-derived Caco-2 cells. To assess the effect of lipid and/or glycoside conjugation on the enzymatic stability of IM862, the synthesized conjugates were tested in Caco-2 homogenates at 37°C. All of them showed a greater resistance to enzymatic degradation than IM862, with remaining amounts of peptides after 1 hour ranging from 32% to 89% (compared to 10% for L-Glu-L-Trp). After 3 hours, while the concentration of IM862 was virtually nil, 8 out of the 9 conjugates were still present in appreciable amounts (15-45%). The most stable compounds appeared to be the lipid derivatives, with a lipid chain alone or along with a glycosyl moiety.

Prior to evaluating the membrane permeability of the conjugates in Caco-2 monolayers, a toxicity study was performed in rat red blood cells (RBCs) and the percentage

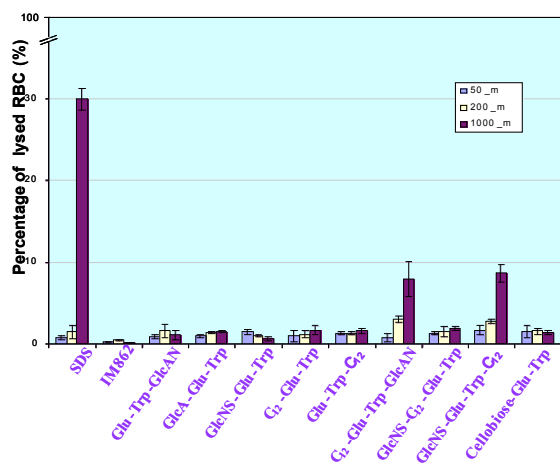


Fig 3. Haemolytic assay of IM862 and its conjugates – % release of haemoglobin in rat RBCs after 60 min at 37°C (n = 3).

of haemoglobin release measured at three different

concentrations (50 μM, 200 μM and 1000 μM) after a 60 minute incubation at 37°C, with reference to sodium dodecyl sulfate (SDS) as a positive control (Fig. 3). None of the conjugates displayed significant toxicity at 200 μM, a concentration often used for *in vitro* assays in Caco-2 cells. At 1000 μM, most peptides yielded to less than 2% haemoglobin release, with the exception of C₁₂-Glu-Trp-GlcAN and GlcNS-Glu-Trp-C₁₂, which percentage values reached 8% haemoglobin release on average. This could be explained by the surfactant properties borne by the functional groups of these two glycolipid conjugates, which might have caused a disruption of the RBCs membranes. Nevertheless, the toxicity level of the synthesized peptides was well below that of SDS, indicating that the compounds were safe to be tested *in vitro* in Caco-2 cells.

Caco-2 cell monolayers have been extensively studied as a model for predicting the transport of pharmaceuticals across the intestinal epithelium as they express most structural and functional characteristics of the human small intestine, including enzymes and several transporters. In this study, 200 μM solutions of each conjugate in Hank's Balanced Salt Solution (HBSS) were added to the apical side of Caco-2 monolayers and samples were taken from the basolateral side over a two-hour period. The integrity of the monolayers was assessed prior to, and after each assay by measuring the transepithelial electrical resistance (TEER) of the cells, and ¹⁴C-mannitol was used as a negative marker during the assay. Concentrations of peptides were determined by Electron Spray Ionization Liquid Chromatography - Mass Spectrometry tandem and

Compound	$P_{app} \cdot 10^6$ (cm ² /s)
IM862	nd
C ₁₂ Glu-Trp	3.5 ± 0.52 ^(a) / 2.94 ± 1.9 ^(b)
GlcA-Glu-Trp	1.9 ± 0.19
GlcNS-Glu-Trp	0.85 ± 0.17
GlcNS-C ₁₂ Glu-Trp	3.66 ± 0.69
Glu-Trp-C ₁₂	4.33 ± 1.18
GlcNS-Glu-Trp-C ₁₂	6.7 ± 0.28 ^(a) / 6.1 ± 1.1 ^(b)
Glu-Trp-GlcAN	nd
C ₁₂ Glu-Trp-GlcAN	1.25 ± 0.16
CellobioseGlu-Trp	0.50 ± 0.05
¹⁴ C-mannitol	2.6 ± -

Table 1. Apparent permeability coefficients (P_{app}) for 200 μM solutions of IM862 and its conjugates (values are given as mean ± SD; n ≥ 3; 'nd' = non detectable; (a)/(b) where diastereomers were isolated).

apparent permeability coefficients (P_{app}) were calculated by $P_{app} = (\Delta Q / \Delta t) \times 1 / (A \cdot C_0)$, where ($\Delta Q / \Delta t$) represents the permeability rate, A the monolayer surface area and C₀ the initial apical peptide concentration (Table 1).

The permeability of IM862 alone could not be determined as its basolateral concentration was found to be below the limit of quantification (0.01 μM), hence confirming the poor oral absorption of the dipeptide. In comparison, nearly all the synthesized lipid and glycoside conjugates displayed greater P_{app} values. The increase was most significant when a lipid moiety was added to the peptide scaffold,

either alone or along with a glycoside. The best candidate was found to be GlcNS-Glu-Trp-C₁₂, which apparent permeability coefficient exceeded 6·10⁻⁷ cm/s, i.e. over sixty-fold compared to IM862. Yet, the values measured in the Caco-2 assay all averaged that of ¹⁴C-mannitol, denoting that the overall permeability of the synthesized conjugates was still low.

Interestingly though, further investigations by mass spectrometry showed that, in a separate assay, one of the C₁₂ conjugates of IM862, C₁₂-Glu-Trp, was able to release the initial dipeptide in the basolateral compartment after permeating the Caco-2 monolayer, with a ratio of 1:7 C₁₂-Glu-Trp / Glu-Trp (as given by peak area of each of the two ions detected), hence acting as a prodrug for IM862. No such result was however observed in the case of GlucA-Glu-Trp, also tested.

Following these results, a preliminary *in vivo* examination of the *N*-terminus C₁₂ conjugate of IM862 was conducted in male Wistar rats using radiolabelling (via tritium-acetylation). Rats (300-350 g) were administered an oral formulation of ³H-Ac-C₁₂-Glu-Trp and ³H-Ac-Glu-Trp (5 mg/kg in phosphate buffer) by oral gavage under light anesthesia (50/50% O₂/CO₂) and blood samples were taken over a 6 hour period from the femoral artery *via* an intra-arterial cannula surgically inserted prior to the experiment. The beta-radioactivity of the samples was then quantified on a liquid scintillation counter. The two tested compounds showed very similar absorption profiles with an average of 25 ng/mL of material present in the blood stream after 30 minutes and up to 6 hours. Despite an enhanced *in vitro* stability and permeability profile, the C₁₂ conjugate of IM862 did not seem to exhibit greater oral absorption. It is unknown however whether this was due to enzymatic degradation or to a low penetration of the intestinal epithelium.

Conclusion

A series of eight novel derivatives of dipeptide IM862 incorporating lipid and/or glycosyl moieties was designed to potentially provide accrued resistance to enzymatic degradation and improve the intestinal permeability of this immunomodulator. A synthetic solid-phase peptide synthesis method was developed to yield the desired compounds in relatively high purity. The *in vitro* evaluation of the conjugates using Caco-2 monolayers and enzymatic extracts revealed that nearly all conjugates offered an enhanced resistance to enzymes and permeability through the intestinal-like epithelium.

Although the apparent permeability values remained low (< 10⁶ cm/s), the conjugation of lipid and/or glycosyl residues to the dipeptide scaffold proved to be an efficient way of increasing the enzymatic stability and intestinal permeability of IM862, without causing any significant toxicity. Furthermore, it was shown that the C₁₂ *N*-terminal conjugate of IM862, C₁₂-Glu-Trp, was able to act as a prodrug and release the original dipeptide as it penetrates the Caco-2 monolayer. Preliminary *in vivo* assays also revealed the capacity of C₁₂-Glu-Trp to cross the intestinal barrier upon oral administration, although no significant improvement in the absorption of the peptide compared to IM862 was noticed.

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