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Peptide Inhibitors Based on the *C*-Terminal Tail of Connexin43

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Introduction

Connexin43 (Cx43), the most abundantly expressed member of the connexin protein family in ventricular cardiomyocytes, oligomerizes into hemichannels (HCs), forming thereafter intercellular channels known as gap junctions (GJ) channels [1]. While GJs are involved in 'healthy' physiological intercellular communication, HCs activities are linked to various pathological states, and therefore hemichannel inhibition might offer a potential therapeutical tool in the treatment of heart diseases. In particular, a segment of amino acids 10 residues long at the *C*-terminal domain (CT) of Cx43 is known to be involved in both intramolecular interactions within Cx43, as well as intermolecular interactions with other proteins, resulting in effects on Cx43 HC trafficking, insertion in GJs and regulation of GJ channel and HC activity [1,2]. Mimetic peptides based on this fragment (*e.g.*, αCT1 [3], which mimics the last 9 amino acids of the CT linked to a cell-penetrating motif) have revealed to be promising therapeutic agents preserving left ventricular cardiac function after ischemia/reperfusion injury [4]. Therefore, in order to pharmacologically improve existing lead sequences, a set of conformationally and proteolytically stabilized peptide inhibitors was designed and tested for their inhibitory capacity by *in vitro* assays. The results constitute a starting point for the development of a set of peptidomimetics targeted against Cx43 hemichannels with improved bioactivity.

Results and Discussion

A peptide mimicking the last 10 amino acids of the Cx43 CT, **CT10** (Figure 1), has been shown to interact with the L2 region (aa 119-144) of the cytoplasmic loop of the protein. This "loop-tail interaction" is critical in regulating Cx43 HC activity. [3,6,7] In the current work, a set of peptides based on the **CT10** sequence (Figure 2) was rationally designed to investigate the role of individual amino acids in the binding process, to examine the importance of the *N*- and *C*- termini of the peptide sequence and to provide proteolytically more stable analogues to be used as pharmacological tools. As such, a structure-activity relationship (SAR) study based on an Ala-scan was performed to first assess the importance of the positively and negatively charged residues in the sequence. Secondly, the proline residues were replaced in an attempt to elucidate the importance of these amino acids in the bioactivity of the peptide.

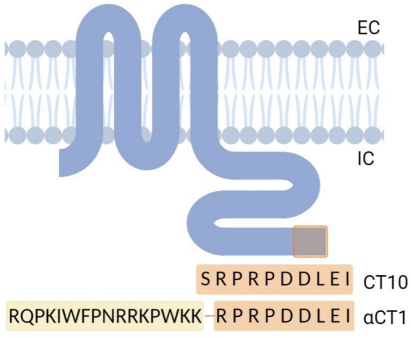

Fig. 1. Topology of a Cx43 monomer. The extra- and intracellular domains are abbreviated EC and IC, respectively. The reference sequences of CT10 and αCT1 are shown, with the mimicked region of the C-tail highlighted in orange.

Additionally, four cyclic peptides were designed as *N*-terminal acetylated and *C*- terminal carboxamides. In one analogue, a proline residue was replaced by a (4*S*)-4-azidoproline residue, while the *C*- terminal Ile was replaced by a propargylglycine residue, in order to constrain the peptide sequence through the formation of a triazole ring via a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. The length of the covalent tether was also changed, to provide macrocyclized analogues bridged by (*i*, *i+5*) to (*i*, *i+3*) linkages. Finally, the tethering Pro residue was also replaced by propargylglycine (Pra) and azidolysine (Azk) residues, respectively, and the triazole ring was moved closer to the *N*-terminal side of the peptide (Figure 2).

Next, the peptides were tested *in vitro* for their inhibitory capacity. In the applied assay, ATP release was used as an indicator of hemichannel activity. In the presence of an adequate trigger (in this case, low extracellular Ca^{2+} levels) the channels open and release ATP, while in the presence of an inhibitor the release of ATP is decreased. A non-specific small molecule inhibitor of Cx43, carbenoxolone (Cbx) was used as a control, as well as the aforementioned **αCT1** and **CT10** peptides. None of the substitutions on the linear sequence led to a significant decrease in inhibitory activity compared to the native **CT10** sequence, while all cyclic analogues show an inhibitory capacity comparable to that of **αCT1**, but without bearing a cell-penetrating motif.

In order to evaluate the effect of the macrocyclization on the proteolytic resistance of the peptides, *in vitro* plasma stability experiments were performed. For **αCT1**, presumed plasma protein binding was observed which prevented the half-life to be experimentally determined. For **CT10**, a half-life of 75.86 (± 4.01) minutes was determined in concordance with literature [8,9], while the stapled analogues displayed high stability (half-life > 24 hours). Additionally, circular dichroism (CD) spectroscopy experiments were performed on the linear **CT10** compound and the cyclic analogues to determine whether they adopt some form of secondary structure. However, most peptides were largely unstructured (*i.e.* random coil) even in the presence of the stabilizing agent trifluoroethanol (TFE).

In conclusion, a set of novel peptidomimetic inhibitors of Cx43 HC were identified. Several analogues show a similar bioactivity *in vitro* as the most established inhibitor of Cx43 hemichannels **αCT1**, with the advantage of a shorter, chemically and proteolytically stabilized sequence. These promising results constitute a starting point for the development of potent and stable Cx43 HC inhibitors.

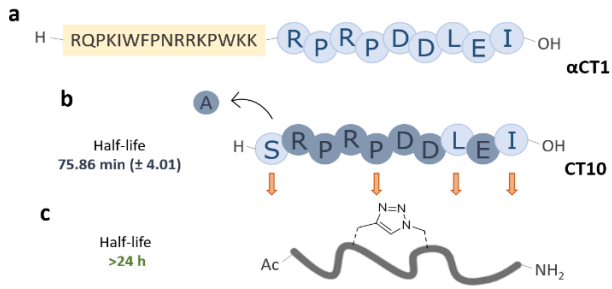

*Fig. 2. a) Sequence of the **αCT1** peptide. b) Sequence of the **CT10** peptide. The residues that were replaced by Ala are indicated in grey. The residues that were replaced by (4S)-4-azidoproline, propargylglycine and/or azidolysine are indicated by arrows. c) Generic structure of the macrocyclic peptides presented in this work.*

Acknowledgments

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