Tetrahedron 68 (2012) 4406–4412

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Cell-penetrating γ-peptide/antimicrobial undecapeptide conjugates with anticancer activity

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ARTICLE INFO

Article history:
Received 30 September 2011
Received in revised form 26 January 2012
Accepted 3 February 2012
Available online 9 February 2012

Keywords:
γ-Peptides
Anticancer
Antimicrobial peptides
Cell-penetrating peptides
Foldamers

ABSTRACT

In this study, we combined a cell-penetrating γ-peptide, **PEG-1**, with antimicrobial undecapeptides in order to provide compounds with anticancer properties against MDA-MB-231 human breast cancer cells. We demonstrated that the conjugates were more cytotoxic than **Ac-PEG-1** and the parent undecapeptides. We also evaluated the toxicity of the conjugates against non-malignant cells. The peptide conjugate with the best biological profile was **BP77-PEG-1**, which, at 10 μM, showed a 71% growth inhibition in MDA-MB-231 cells and only a 17% inhibition in non-malignant cells. Therefore, this study suggests that **PEG-1** mediated the undecapeptide delivery into cancer cells and that these conjugates are the proof-of-concept of this strategy to generate improved anticancer drugs based on peptides.

1. Introduction

In spite of great advances in cancer therapy, there is considerable current interest in developing anticancer agents with a new mode of action due to the development of resistance by cancer cells toward current anticancer drugs.[1] A growing number of studies have shown that some antimicrobial peptides, which are toxic to bacteria but not to normal mammalian cells, exhibit a broad spectrum of cytotoxic activity against cancer cells.[2]

Although antimicrobial peptides display a wide structural diversity, many of them are short, cationically charged, and able to form amphipathic secondary structures.[3] Their exact mode of action is not completely understood. However, there is a consensus that antimicrobial peptides act by disrupting negatively charged bacterial and cancer cell membranes to which they are electrostatically attracted, leading to cell lysis and death,[1b,3d] unlike currently available conventional drugs, which typically interact with a specific target protein. Upon binding, they disrupt cell membranes, possibly by transient pore formation or disruption of lipid packing.[1b,3d] Based on this mode of action, these peptides are unlikely to cause rapid emergence of resistance because it would require significant alteration of membrane composition, which is difficult to occur.[3g,4] In addition, there is increasing evidence that apart from membrane damage, other mechanisms may be involved including intracellular targets.[1b,2b,3b,d] Some of these AMPs showed remarkable selectivity to cancer cells versus untransformed proliferating cells.[1b,3a,b,d]

Despite the excellent properties displayed by antimicrobial peptides, the development of peptide-based drugs is hampered by their limited access to the intracellular space. This obstacle has been tried to overcome by using diverse methods to promote the cellular uptake of exogenous molecules. Cell-penetrating peptides (CPPs) have become one of the most efficient and explored transporters for achieving intracellular access.[5] CPPs are usually short cationic sequences and may be derived from natural sequences (i.e., TAT peptide[6]) or be de novo designed peptides (polyarginine[7] or transportan[8]). The conjugation of biologically active peptides to CPPs offers advantages, such as low toxicity and selective and controlled

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0040-4020/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2012.02.003

cell delivery compared with other administration vectors,[5] being considered a promising strategy for the design of anticancer agents. A drawback of this strategy is the low stability of these CPPs to proteases that it can prevent the drug to reach its target, forcing an increment of the doses to keep the activity and consequently generating an increase in the toxicity. Some alternatives with good protease stability have been described keeping internalizing properties, like diverse oligomers with foldamer properties, such as peptoids,[9] arylamide oligomers,[10] β-[11] and γ-peptides.[12] Some of these foldamers have not only been explored as potential CPPs, but also as antimicrobial compounds with highly remarkable results.[13] Generally both applications derived in similar sequence pattern that combine hydrophobic/aromatic residues with cationic groups. In 2004, Farrera-Sinfreu and co-workers described a new family of γ-peptides hexamers based on the trifunctional amino acid *cis* 4-aminoproline that adopt a C9 ribbon in H_2O.[12a] This adopted secondary structure gives to these peptides a tridimensional pattern with high amphipaticity character that contribute to their internalization properties. Series of these γ-peptide diversely functionalized on the N^{α} of *cis* 4-aminoproline were synthesized and have proven capacity for cellular uptake.[12b] These peptides also showed low cytotoxicity and high resistance to proteases, properties that convert them in a promising class of CPPs, specially peptide **1** that showed a 40% of internalization capacity compared to TAT peptide (Fig. 1) considered a gold standard in the field.

explore its anticancer activity. Moreover, taking profit of the cell-uptake properties offered by γ-peptide **1** (see Fig. 1), we decided to design new γ-peptide **1**/CECMEL11 undecapeptide conjugates. The conjugation of these antimicrobial peptides to **1** can promote its internalization, which can interact with intracellular targets increasing the cytotoxic effect, i.e., by disruption of mitochondrial membranes.[15]

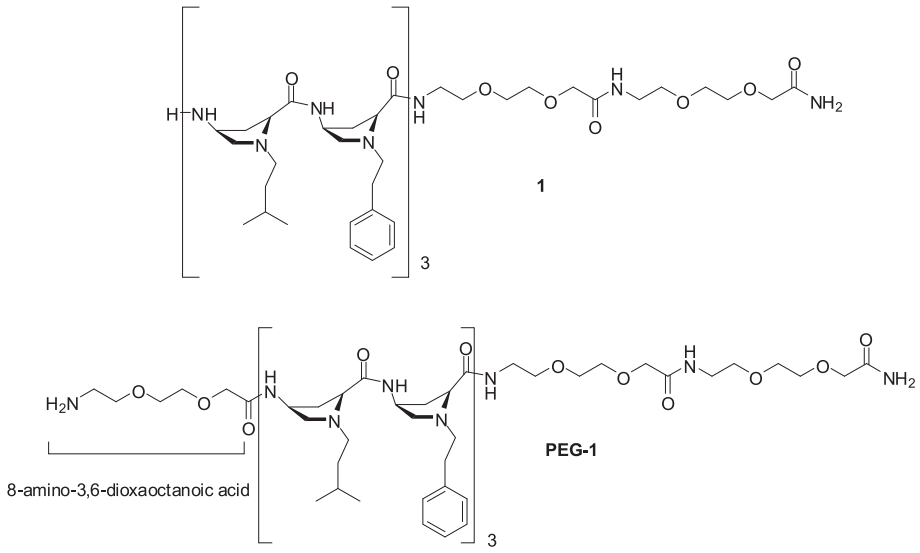
As a linker between the CECMEL11 undecapeptides and γ-peptide **1**, it was decided to use a short monodisperse bifunctional polyethylene glycol (PEG) unit (8-amino-3,6-dioxaoctanoic acid), generating the peptide **PEG-1**. Polyethylene glycol has many properties that make it an ideal carrier for peptides, such as high water solubility, high mobility in solution and low immunogenicity. PEG is often attached to the N or C termini of peptides.[16] Besides increasing overall size, this modification also protects peptides from exopeptidases and therefore increases their overall stability in vivo.

In this report, we describe the design and synthesis of **PEG-1**/antimicrobial undecapeptide conjugates. The influence of the γ-peptide on the anticancer activity is also discussed.

2. Results and discussion

2.1. Design and synthesis

The undecapeptides were representative examples of the 125-member CECMEL11 library.[14] The selected peptides showed



Peptide:		Conjugate:
BP15	KKLFKKILKVL	BP15-PEG-1
BP33	LKLFKKILKVL	BP33-PEG-1
BP76	KKLFKKILKFL	BP76-PEG-1
BP77	Ac-KKLFKKILKFL	BP77-PEG-1
BP101	Ac-KKLFKKILKYL	BP101-PEG-1
BP125	Ts-KKLFKKILKKL	BP125-PEG-1
BP126	Bz-KKLFKKILKKL	BP126-PEG-1

Fig. 1. Structures of the peptide conjugates.

Recently, Badosa and co-workers designed linear undecapeptides (CECMEL11) to be used in plant protection.[14] The general structure of this library was R-X^1KLFKKILKX^{10}L-NH_2, where X^1 and X^{10} corresponded to amino acids with various degrees of hydrophobicity and hydrophilicity (Leu, Lys, Phe, Trp, Tyr, Val) and R included different N-terminal derivatizations (H, Ac, Ts, Bz, Bn). The antimicrobial evaluation of the CECMEL11 library led to the identification of peptides with high antibacterial activity (MIC<7.5 μM) and with low hemolysis (2–6% at 50 μM and 8–40% at 150 μM). Based on these properties, these peptides can be considered as good candidates to a good balance between antimicrobial and hemolytic activities. Their sequences differ from the N-terminal derivatization (H, Ac, Ts or Bz) and the residues at positions 1 (Lys or Leu) and 10 (Val, Phe, Tyr or Lys). Peptide conjugates were designed by linking the corresponding antimicrobial undecapeptide to the cell-penetrating peptide **PEG-1** (Fig. 1).

The parent CECMEL11 undecapeptides **BP15**, **BP33**, **BP76**, **BP77**, **BP101**, **BP125**, and **BP126** were prepared using a Fmoc-Rink-MBHA resin following a standard Fmoc/*t*-Bu strategy.[14] After the synthesis, peptides were cleaved from the resin by treatment with TFA/