Proceedings of the 37th European Peptide Symposium
Michal Lebl, Editor
European Peptide Society, 2024 *http://doi.org/10.17952/37EPS.2024.P2224*

Synthesis and characterization of nucleolin receptor recognizing F3 peptide fragment-drug conjugates

Bettina Basa[1, 2, 3], Diána Vári-Mező[3, 4, 5], Kata Nóra Enyedi[1, 3], József Tóvári[5], Szilvia Bősze[3, 6], Krisztina Kiss[3], Gábor Mező[1, 3]

[1]ELTE Eötvös Loránd University, Faculty of Science, Institute of Chemistry, Department of Organic Chemistry, Budapest, 1117 Hungary, [2]ELTE Hevesy György PhD School of Chemistry, Budapest, 1117 Hungary, [3]HUN-REN-ELTE Research Group of Peptide Chemistry, Budapest, 1117 Hungary, [4]School of Ph.D. Studies, Doctoral School of Pathological Sciences, Semmelweis University, Budapest, 1085 Hungary; [5]Department of Experimental Pharmacology and the National Tumor Biology Laboratory, National Institute of Oncology, Budapest, 1122 Hungary; [6]Department of Genetics, Cell- and Immunobiology, Faculty of Medicine, Semmelweis University, Budapest, 1089 Hungary

Introduction

Chemotherapy remains the most widely used approach in cancer treatment. Despite its widespread use, the frequency of tumor relapse, and the associated severe toxic side effects, require continuous methodological development. Targeted tumor therapy has emerged as a promising route for this development. This strategy involves conjugating drugs with targeting molecules that exhibit high specificity and selectivity towards tumor-selective proteins or receptors. By doing so, the drug delivery becomes tailored to tumor cells, minimizing the impact on healthy cells [1]. Nevertheless, a pervasive challenge in targeted therapeutic methods is the limited number and types of tumor-specific markers. This is particularly true for tumor subtypes, called tumor stem-like cells (CSCs) [2], where the lack of overexpression of common tumor markers impedes methodological progress. In addition, because of their high self-renewal properties, CSCs are also implicated in cancer relapse. Thus, the development of effective compounds for targeted tumor therapy is necessary that can recognize not only the conventional tumor cells but the resistant CSCs, too. Nucleolin is a protein that is expressed both on sensitive cells and resistant CSCs, which makes it a suitable target in tumor therapy. The 31-mer peptide (F3), selectively binds to the nucleolin receptor, offering a novel approach for targeted tumor therapy [3]. This research endeavors to optimize the F3 peptide structure for the development of a more cost-effective drug candidate.

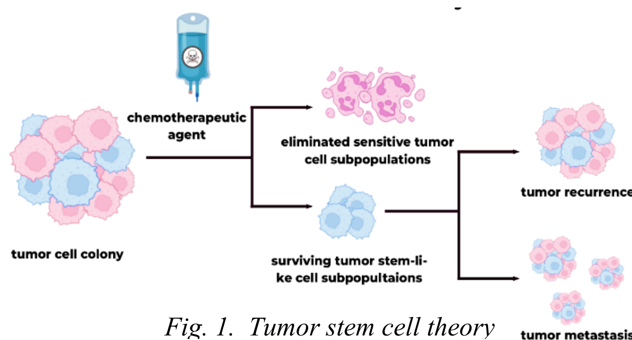

Fig. 1. Tumor stem cell theory

Results and Discussion

In this study, we aimed to develop peptide-daunomycin conjugates with enhanced targeting activity. To ensure uniform drug release we inserted the cathepsin-B enzyme labile GFLG spacer between the peptide and daunomycin. Daunomycin was selected as the chemotherapeutic agent due to its intrinsic autofluorescence, which allowed for simultaneous evaluation of cellular uptake and cytostatic effect. The efficacy of the conjugates was measured using MTT assays on human melanoma (A2058) and human colorectal adenocarcinoma (HT-29) cell lines. To optimize the peptide sequence for nucleolin receptor binding, we synthesized eight overlapping decapeptides that cover the entire F3 peptide. The *in vitro* cytostatic activity studies demonstrated that the peptide-daunomycin conjugate, that contains the first 10 amino acids of the N-terminal of the F3 peptide, exhibited higher antitumor activity compared to the full-length conjugate. To identify the shortest, most effective targeting sequence we synthesized peptides containing the first 7,13, and 20 amino acids of the N-terminal. The cytotoxicity results indicated, that shortening the sequence reduced the efficacy of the conjugate, while elongating the sequence did not improve the effectiveness.

Table 1. – Length optimization of the targeting sequence

Sequence	*Cytostatic effect IC_{50} (µM)*	
	A2058	*HT-29*
Dau-Aoa-GFLG-^{1}KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK^{31}-NH_2	2,99 ± 0,37	11,78 ± 0,12
Dau-Aoa-GFLG-^{1}KDEPQRRSARLSAKPAPPKP^{20}-NH_2	3,35 ± 0,12	87,85 ± 0,13
Dau-Aoa-GFLG-^{1}KDEPQRRSARLSA^{13}-NH_2	3,60 ± 0,09	90,91 ± 0,15
Dau-Aoa-GFLG-^{1}KDEPQRRSAR^{10}-NH_2	**3,78 ± 0,12**	**28,32 ± 0,09**
Dau-Aoa-GFLG-^{1}KDEPQRR^{7}-NH_2	17,15 ± 0,10	> 100

Further optimization was carried out using alanine scanning, in which each amino acid was systematically substituted with apolar alanine, to evaluate its role in the conjugate's activity. This approach identified critical residues, like the DEPQ sequence and the C-terminal arginine, whose modification significantly diminished the conjugate's efficacy, which means these amino acids could be important in the attachment to the nucleoline receptor. Conversely, substitutions at the N-terminal lysine, and the 8th position serine improved the conjugate effectiveness, suggesting that these positions tolerate modifications.

Fig. 2. Results of the alanine scan

Table 2. – Alanine scan

Sequence	Cytostatic effect IC_{50} (μM)	
	A2058	*HT-29*
Dau-Aoa-GFLG-[1]KDEPQRRSAA[10]-NH2	32,0 ± 3,4	> 50
Dau-Aoa-GFLG-[1]KDEPQRRAAR[10]-NH2	**11,0 ± 1,2**	**17,2 ± 9,1**
Dau-Aoa-GFLG-[1]KDEPQRASAR[10]-NH2	22,8 ± 2,8	26,7 ± 3,6
Dau-Aoa-GFLG-[1]KDEPQARSAR[10]-NH2	15,1 ± 1,0	33,0 ± 16,9
Dau-Aoa-GFLG-[1]KDEPARRSAR[10]-NH2	> 50	> 50
Dau-Aoa-GFLG-[1]KDEAQRRSAR[10]-NH2	> 50	> 50
Dau-Aoa-GFLG-[1]KDAPQRRSAR[10]-NH2	39,1 ± 8,4	> 50
Dau-Aoa-GFLG-[1]KAEPQRRSAR[10]-NH2	> 50	> 50
Dau-Aoa-GFLG-[1]ADEPQRRSAR[10]-NH2	**3,3 ± 0,3**	**9,7 ± 3,4**

Additionally, to improve both the efficacy and serum stability of the conjugates, non-natural amino acids and intramolecular cyclization were introduced. Although the incorporation of a disulfide bridge slightly reduced the cytotoxicity of the peptide-drug conjugate, it could increase the serum stability, ultimately enhancing the overall therapeutic potential of the conjugate. The preliminary in vivo experiments confirmed that the shorter peptides with optimized sequences might be better drug conjugates than the F3-drug conjugate.

Table 3. – Incorporation of non-natural amino acids and intracellular rings

Sequence	Cytostatic effect IC_{50} (μM)	
	A2058	*HT-29*
Dau-Aoa-GFLG-[1]KDEPQRCitSAR[10]-NH2	19,70 ± 0,10	> 50
Dau-Aoa-GFLG-[1]KDEPQCitRSAR[10]-NH2	18,20 ± 0,14	> 50
Dau-Aoa-GFLG-[1]NleDEPQRRSAR[10]-NH2	20,30 ± 0,07	> 50
Dau-Aoa-GFLG-[1]KDEPQF(pCl)RSAR[10]-NH2	**10,13 ± 0,15**	**> 50**
Dau-Aoa-GFLG-[1]CDEPARRCAR[10]-NH2 (-S---------S-)	**8,17 ± 0,11**	**30,23 ± 0,07**
Dau-Aoa-GFLG-[1]KDEAQRRCAR[10]-NH2 (HN-CO-CH2-S)	12,94 ± 0,12	> 50

Acknowledgments

The research was supported by the National Research, Development and Innovation Office under grants NKFIH K146039.

References

1. G. Mező, M. Manea - Receptor-mediated tumor targeting based on peptide hormones - Expert Opinion in Drug Delivery, 2010; 7; 79-96; https://doi.org/10.1517/17425240903418410
2. L. S. Carvalho, N. Grançalves, N. A. Fonseca, J. N. Moreira: Cancer Stem Cells and Nucleolin as Drivers of Carcinogenesis - Pharmaceuticals - 2021; 14(1); 60; 1-21; https://doi.org/10.3390/ph14010060
3. K. Prokka, P. Laakkonen, J. A. Hoffman, M. Bernsconi, E.Ruoslathi. - A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells in vivo - Proceedings of the National Academy of Sciences of the United States of America, Biochemistry, 2002, 99, 7444-7449 https://doi.org/10.1073/pnas.062189599