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Synthesis and evaluation of cholecystokinin trimers: A multivalent approach to pancreatic cancer detection and treatment

Nabila Brabez^{a,b,c,d}, Kevin L. Nguyen^d, Kara Saunders^d, Ryan Lacy^d, Liping Xu^e, Robert J. Gillies^e, Ronald M. Lynch^f, Gerard Chassaing^{a,b,c}, Solange Lavielle^{a,b,c}, and Victor J. Hruby^{d,*}

^aUPMC Paris 06, UMR 7203, Laboratoire des BioMolécules, Université P. et M. Curie, 75005 Paris, France

^bCNRS, UMR 7203, France

^cENS, UMR 7203, Département de Chimie, Ecole Normale Supérieure, 75005 Paris, France

^dUniversity of Arizona, Department of Chemistry and Biochemistry, Tucson, AZ 85721, USA

^eH. Lee Moffitt Cancer Center and Research Institute, Department of Cancer Imaging and Metabolism, Tampa, FL 33612, USA

^fUniversity of Arizona, Department of Physiology, Tucson, AZ 85721, USA

Abstract

In the quest for novel tools for early detection and treatment of cancer, we propose the use of multimers targeting overexpressed receptors at the cancer cell surface. Indeed, multimers are prone to create multivalent interactions, more potent and specific than their corresponding monovalent versions, thus enabling the potential for early detection. There is a lack of tools for early detection of pancreatic cancer, one of the deadliest forms of cancer, but CCK2-R overexpression on pancreatic cancer cells makes CCK based multimers potential markers for these cells. In this Letter, we describe the synthesis and evaluation of CCK trimers targeting overexpressed CCK2-R.

For the last decade, our group and others have investigated the use of multivalent interactions to enhance ligand–receptor binding properties.^{1,2} Indeed, multivalent interactions are higher in affinity and more specific than monovalent ones. It is our belief that by targeting the appropriate cancer cell surface receptor combinations, cancer cells could be selectively targeted with a high sensitivity thanks to the creation of such multivalent interactions.³

We previously described an efficient synthetic strategy to afford libraries of multivalent molecules and successfully generated MSH multimer libraries for melanoma targeting.⁴ These molecules have shown interesting properties and binding affinities were enhanced up to 350-times that of the monovalent ligand, resulting in the successful creation of powerful multivalent interactions with a dendrimer-like structure.

Pancreatic cancer is one of the most virulent and lethal cancers with a poor prognosis with less than 5% five year survival and on average six months of survival after diagnosis.^{5,6} In the case of pancreatic cancer, a lack of evident symptoms is the source of such prognosis. Therefore, novel and sensitive detection methods are required in order to afford early detection and treatment to increase the chances of recovery and survival.

Cholecystokinin (or CCK, a gastrin-like peptide) by binding to its cognate receptors CCK1-R and CCK2-R, is involved in multiple physiological responses, especially in the gastrointestinal tract, where it regulates pancreatic secretion, gastric function and gall bladder contraction.⁷⁻⁹ In cancer, the CCK ligand–receptor system is known to be involved in gastrointestinal tract tumor growth via autocrine and paracrine functions resulting in receptor overexpression such as in pancreatic cancer, in which the CCK2-R is over expressed.¹⁰⁻¹³ This receptor overexpression constitutes an ideal target for the creation of molecular markers specific to pancreatic cancer cells via the formation of multivalent interactions. Thus, we decided to apply our new strategy and synthesize multimers targeting CCK2-R. In this Letter, we describe the synthesis and evaluation of multimers bearing CCK ligands for CCK-2R targeting.

Our molecules are based on a trimeric template which upon modification with the desired ligand results in the multimer of interest.⁴ In our previous studies, we established that the use of the minimal pharmacophores as ligands was optimal due to their short size (easy synthesis) and their relative weak binding affinities (micromolar). However, CCK1-R and CCK2-R share the same pharmacophore: Trp-Met-Asp-Phe. Fortunately, SAR studies provided ways to differentiate the two subtypes and the following CCK(4) agonist peptide: Trp-NMeNle-Asp-Phe, known to be 6000 times more selective for the CCK2-R,^{14,15} was selected as our ligand. This sequence can be modified on its N-terminus but has to be a carboxamide on its C-terminus. A lysine bearing a triple bond was added on the N-terminus of the selected ligand for ligation purposes. The desired protected ligand (Fig. 1) was synthesized on a Sieber amide resin using a Fmoc strategy, and cleavage was performed using 1% TFA to afford the desired protected CCK(4) sequence. Because the linker length is a key factor in the formation of multivalent interactions, we decided to incorporate the same linkers as in our previous paper.⁴ Trimers possessing β -Ala or Pro-Gly linkers were synthesized as shown in Scheme 1 with ligand spacing ranging from 30 to 60 Å following the established synthetic procedure.⁴

Shortly, the scaffold **A** was coupled to an MBHA resin and the linkers composing the multimers were attached following a Boc strategy procedure. Upon linker attachment, azido acetic acid was coupled and click chemistry was performed using the CCK(4) protected ligand. Cleavage was performed using HF and the resulting trimers were purified by HPLC to afford the final compounds for which analytical data is provided in Table 1.

The CCK trimers were tested for their binding affinities by competitive binding assay using time resolved fluorescence (TRF)^{4,16–18} The ligands were evaluated by displacement of the potent Eu-DTPA-CCK(8) as previously described.¹⁹ As shown by the binding results in Table 2, the constructs remain within nM range so no negative cooperativity was observed. However, no significant fold increase was observed either, independent of the linker length when comparing to the monovalent free CCK(4) sequence. Therefore, only a weak statistical interaction is observed, which surprisingly did not correlate with our previous results with MSH trimers targeting melanocortin receptors.

However, one parameter which differs between the previous and current studies, is the affinity for the target of the monomeric pharmacophore. Indeed, in this case the monovalent CCK possesses nM affinity for the target ($IC_{50} = 16 \pm 1.5$ nM) while the monovalent MSH possessed only a μ M one ($IC_{50} = 4600 \pm 790$ μ M). In order to confirm that the results observed were due to the high affinity of the monovalent ligand, we decided to synthesize a trimer with MSH(7), a ligand with nM affinity for the melanocortin receptors ($IC_{50} = 40 \pm 3.2$ nM). We observed the same insignificant fold increase of threefold for the trimeric MSH(7) construct ($IC_{50} = 12 \pm 1$ nM), supporting the fact that the approach was not the issue, but that the monovalent ligand needs to be chosen carefully in order to afford efficient multivalent interactions (see Supplementary data for binding assay details).

To conclude, we were able to demonstrate the modularity of the synthetic approach by applying it to another target. Even though no negative cooperativity was observed, trimers bearing the high affinity CCK2-R selective pharmacophore did not seem to effectively enhance the affinity for the target and show no or poor multivalent interactions. Our previous studies covering MSH analogs showed increase in binding potency ranging from 100 to 300-fold. In theory, such an increase should also be accessible and should provide pM affinities trimers from nM affinities monovalent ligands. However, the present study showed that it was not the case for the CCK analogs and confirmed the importance of selecting low affinity monovalent ligands. Indeed, in order for the three different ligands composing the multimers to bind, the first binding event needs to possess a quick dissociation rate (poor affinity) to allow for the other binding events to occur rapidly, at least prior to any signaling response such as internalization. Thus, the use of antagonists, which would prevent cellular response, such as internalization, might allow these high affinity multivalent interactions to occur. To investigate if the presence of cellular responses was an issue to afford high affinity multimers from high affinity ligands, studies with high affinity antagonists are ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CCK	cholecystokinin ligand
CCK1-R	cholecystokinin 1 receptor
CCK2-R	cholecystokinin 2 receptor
MSH	melanotropin ligand
SAR	structure–activity relationship
Trp	tryptophan
Met	methionine
Asp	aspartic acid
Phe	phenyl alanine
Nle	norleucine
TFA	trifluoroacetic acid

Ala	alanine
βAla	3-aminopropanoic acid
Pro-Gly	proline-glycine
HF	hydrogen fluoride
MeCN	acetonitrile
TRF	time-resolved fluorescence

Figure. 1.
Sequence for selective monovalent ligand for CCK2-R targeting.

Scheme 1.
Synthesis of CCK(4) trimers.

Table 1

Analytical data for synthesized molecules

Name	Linker	Mass Spectrum ^a		HPLC ^b , <i>t_R</i> (min)
		Ion	Calcd	Obsd
CCK(4)	–	(M+Na) ¹⁺	865.4	865.4 22.1
NB310	Gly-(Pro-Gly) ₃	(M+Na) ⁺	4673.2	4673.3 25.4
NB309	Gly-(Pro-Gly) ₂	(M+Na) ⁺	4212.5	4212.5 24.7
NB308	Gly-(Pro-Gly) ₁	(M+Na) ⁺	3748.8	3748.7 24.6
NB312	(–Ala) ₂	(M+Na) ⁺	3541.7	3541.3 23.8
NB311	(–Ala) ₁	(M+Na) ⁺	3328.6	3328.6 24.8
NB306	None	(M+Na) ⁺	3115.5	3115.5 22.6

^a Maldi-TOF.^b Peptides were eluted with a linear gradient from 0% to 100% MeCN containing 0.1% TFA in 30 min at 1 mL/min. The purity of all constructs determined by HPLC was >95%.

Table 2
Binding data for the CCK(4) trimers library

Compound	Linker	Estimated length between ligands ^a (Å)	IC ₅₀ ^b (nM)	Relative potency to CCK(4)
CCK(4)	–	–	16 ± 1.5	–
NB310	Gly-(Pro-Gly) ₃	60 ± 20	6 ± 1.5	2.5
NB309	Gly-(Pro-Gly) ₂	52 ± 20	8 ± 5	2
NB308	Gly-(Pro-Gly) ₁	45 ± 20	6 ± 1.5	2.5
NB312	(β-Ala) ₂	48 ± 10	8.5 ± 2	2
NB311	β-Ala	43 ± 10	5.2 ± 0.6	3
NB306	None	35 ± 10	12 ± 3	1.5

^aLength was estimated from an energy-minimized structure using Macromodel.

^bCompetition experiments were carried out using TRF assay.^{16–18} The compounds were competed against Eu-DTPA-CCK(8). The IC₅₀ values are the average of three experiments each done in quadruplicate.