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Multivalent Interactions: Synthesis and Evaluation of Melanotropin Multimers – Tools for Melanoma Targeting

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Abstract

In order to develop agents for early detection and selective treatment of melanomas, high affinity and high specificity molecular tools are required. Enhanced specificity may be obtained by simultaneously binding to multiple cell surface targets *via* the use of multimeric analogs of naturally occurring ligands. Trimers targeting overexpressed melanocortin receptors have been found to be potential candidates for this purpose. In the present letter, we describe the synthesis and study of multimers based on a dendrimer-like scaffold. The binding affinity and activity results revealed that dendrimers promote multivalent interactions via statistical and/or cooperative effects on binding. Moreover, viability studies showed no significant toxicity at micromolar concentrations, which will allow these molecular complexes to be used *in vivo*. Finally, imaging studies showed effective internalization for all the molecules confirming their potential as delivery agents.

Keywords

Multivalent interactions; multimers; peptides; dendrimers; cancer; melanoma; targeted therapy; delivery

Melanoma is a type of skin cancer, which develops in melanocytes and accounts for 80% of skin cancer deaths. Once, the tumor metastases and spread through the body, the chances of survival are poor with only 14 % of patient survival in 5 years. Metastatic melanoma being one of the most virulent types of cancer, meaningful tools for its early detection and effective treatment are key to improve chances of patient survival and recovery^{1,2}. To provide such molecular entities, tools possessing a high affinity and selectivity toward the cancer cells are desirable. In order to obtain selectivity, specific characteristics of the cancer

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ASSOCIATED CONTENT

Supporting Information. Experimental details for synthesis, characterization of compounds and the experimental procedures for biological studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

cell must be identified then targeted. G-protein coupled receptors (GPCRs) are involved in a myriad of biological responses within the body and their responses are altered in many diseases³. In cancer, some specific GPCRs are found to be overexpressed at the cancer cell surface^{4, 5}. Therefore, targeting receptor overexpression *via* the use of multivalent interactions provides an alternative way to enhance selectivity toward these cancer cells. Indeed, multivalent interactions, arising from synergy of binding (or cooperative effect) are known to be much more specific than the corresponding monovalent ligands⁶⁻¹³. In the case of metastatic melanoma, melanocortin 1 receptors (MC1-R) are known to be overexpressed at the melanoma cell surface¹⁴⁻¹⁸. Thus, targeting these receptors *via* the use of synthetic agents composed of multiple copies of a low affinity melanocortin ligand should allow enhancement in affinity and specificity toward metastatic melanoma cells due to the creation of cooperative multivalent interactions. In a previous paper¹⁹, we described the design, synthesis and study of trimers bearing copies of the pharmacophore for melanocortin receptors, MSH(4) ligand (His-D-Phe-Arg-Trp-CONH₂), on a cancer cell model overexpressing MC4R as a substitute for MC1R. The enhancement in affinity was shown to correlate with the valency and the lead compound, trimer NB341 (designated here as compound B) showed more than 300-fold increases in affinity compared to its monovalent version, therefore resulting in the creation of a 15 nM affinity trimer from a 4.9 μM monovalent ligand. It can also be noted that an order of magnitude in binding affinity was afforded per ligand added, providing evidence for the creation and efficiency of multivalent interactions.

Encouraged by these results, the development of higher order constructs based on dendrimers-like scaffolds was undertaken. Indeed, knowing that dendrimers usually provide statistical binding due to the close proximity of ligands, we were interested in determining their effect on binding and the resulting biological properties of multimer combinations. Also, the highly branched and globular structure of dendrimers makes them attractive for their delivery properties as well as for their tumor passive targeting via enhanced permeability and retention effect (EPR effect) which consists of the accumulation of macromolecules within tumor tissues²⁰⁻²². Thus, in the present letter, we report the synthesis and *in vitro* analysis of multimers resulting from the combination of trimers on an optimal established scaffold in order to determine if these macromolecules can promote multivalent interactions.

The multimers investigated in this study are represented in Figure 1 and were synthesized following the procedures described in the Supporting Information. The scaffolds previously described¹⁹ were attached to the resin followed by deprotection and coupling of azido acetic acid to prepare the precursor for the click chemistry reaction. Upon peptide attachment, cleavage and purification were performed to provide the desired compounds.

The affinity of the multimers was evaluated using a time resolved fluorescence (TRF) binding assay²³⁻²⁵. The multimers were competed against Eu-NDP-α-MSH (previously described¹⁹) and were evaluated on a cancer cell model overexpressing the melanocortin 4 receptor (MC4-R), constituted of HEK293 cells transfected with the MC4R. These multimers were able to efficiently displace the Eu-NDP-α-MSH analog revealing their potency; the results are summarized in Table 1. The multimers **C** and **D** exhibit a 100-fold enhancement in affinity compared to the monomer. However, the trimer **B** exhibits the lowest IC₅₀ although the multimer IC₅₀ values remain within the same order of magnitude as the trimer. The similarity of IC₅₀ values for the higher order multimers compared to the trimer suggest that some limit to the effect of multivalent interactions on apparent binding affinity is reached at the trimer stage. The trimer is considered to bind by a cluster/chelate mechanism where each ligand binds to a different receptor resulting in cooperative binding. This is supported by our previous studies¹⁹ which showed a correlation between valency and

enhanced binding affinity and functional activity. On the other hand, the limit on binding of the higher order dendrimers may result from a mixture of statistical binding and cluster/chelate binding, or limits placed on binding of individual recognition elements due to steric effects.

Even though increasing the valency above three did not result in further affinity enhancement, the macromolecules still possess nanomolar affinity and remain interesting tools for targeting especially due to their larger size and higher branches which theoretically should increase the EPR effect.

Functional activity measurements were also performed using a chemiluminescent immunoassay. The melanocortin receptors activate cAMP production upon stimulation by an agonist ligand. As observed in Figure 2, the larger molecules activate cAMP production, and therefore remain functional agonists. Interestingly, the same correlation observed for the binding is also observed for activity in that the larger multimers produce cAMP less efficiently when compared to the trimer. Also, the multimer **D**, possessing nine MSH(4) binding sequences, activates cAMP production less efficiently than **C**, possessing six ligands, lending evidence for the presence of a statistical effect with higher ligand density.

To further investigate the potential use of these compounds for *in vivo* studies, the toxicity of the multimers was investigated using a cell viability assay. Cell viability was measured at three different concentrations over three different periods of time. The results are illustrated in Figure 3. By comparison to NDP- α -MSH, a well-known melanotropin ligand used as a drug in some countries, no significant toxicity was observed for all the multimeric constructs at 1 μ M and up to 48 hrs. Although no toxicity was observed at 10 μ M after two days for the smaller constructs, partial toxicity was observed for the largest molecule bearing nine peptides at 10 μ M. However, since these compounds possess high nanomolar affinity, such high concentrations would not be necessary for *in vivo* applications. Therefore, the constructs show a low cytotoxicity in the range of interest, which supports their potential as candidates for efficient *in vivo* use.

The significant size of the multimeric agonist molecules may preclude normal processing of the ligand-receptor complex. To study if these compounds internalize, a fluorescent tag was added to facilitate high-resolution imaging. The chosen tag, composed of a triarginine linker and a Cy5 NIR dye, was added to the *C*-terminus of the multimers (See Supporting information for structure and synthesis). Cy5 was selected for its commercial availability as an NHS ester and the low autofluorescence observed at its emission wavelength. Even though the multimers were soluble in water, a triarginine linker was chosen to increase the length between the fluorescent probe and the molecules as well as to prevent the use of costly PEG linkers. A model molecule was synthesized and evaluated by TRF assay, and confirmed that the linker addition did not affect the compound binding properties (See Supporting information for details.). Images were recorded using an epifluorescence microscope. The labeled multimers (3M*, 6M* and 9M*) were evaluated as well as a Cy5 labeled NDP- α -MSH molecule (NDP*).

At first, a time course study was performed to evaluate initial processing. All compounds initially localized to the cell surface at three minutes, then began to localize within small punctate structures within five minutes (See Supporting information). To confirm internalization, membrane staining was performed by treatment with FM1-43. The data shown in Figure 4 demonstrate that no ligand was localized at the membrane after 90 minutes. Therefore, these compounds do internalize and potentially could be used for drug delivery purposes. In summary, these studies revealed the applicability of our established

synthetic scheme to the creation of a new generation of higher order constructs using dendrimers as scaffolds.

These new ligands also provided new insight into the mechanism by which these macromolecules bind to cells via multivalent interactions. Moreover, we have reported novel tools that possess a high affinity for the targeted receptor, no toxicity and which could be used as a prodrug for targeted cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GPCR	G-protein coupled receptor
MC1-R	melanocortin receptor 1
EPR effect	enhanced permeation and retention effect
TRF	time resolved fluorescence
MC4-R	melanocortin receptor 4
Eu-NDP-α-MSH	Eu labeled [Nle ⁷ -D-Phe ⁷]- α -melanocyte stimulating hormone
cAMP	cyclic adenosine monophosphate
Cy5	Cyanine dye 5
NIR	Near infrared
NHS	N-hydroxy succinimide
PEG	polyethylene glycol
FM 1-43	Fey Mao dye 1-43

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Peptide = MSH(4) = His-D-Phe-Arg-Trp-CONH₂

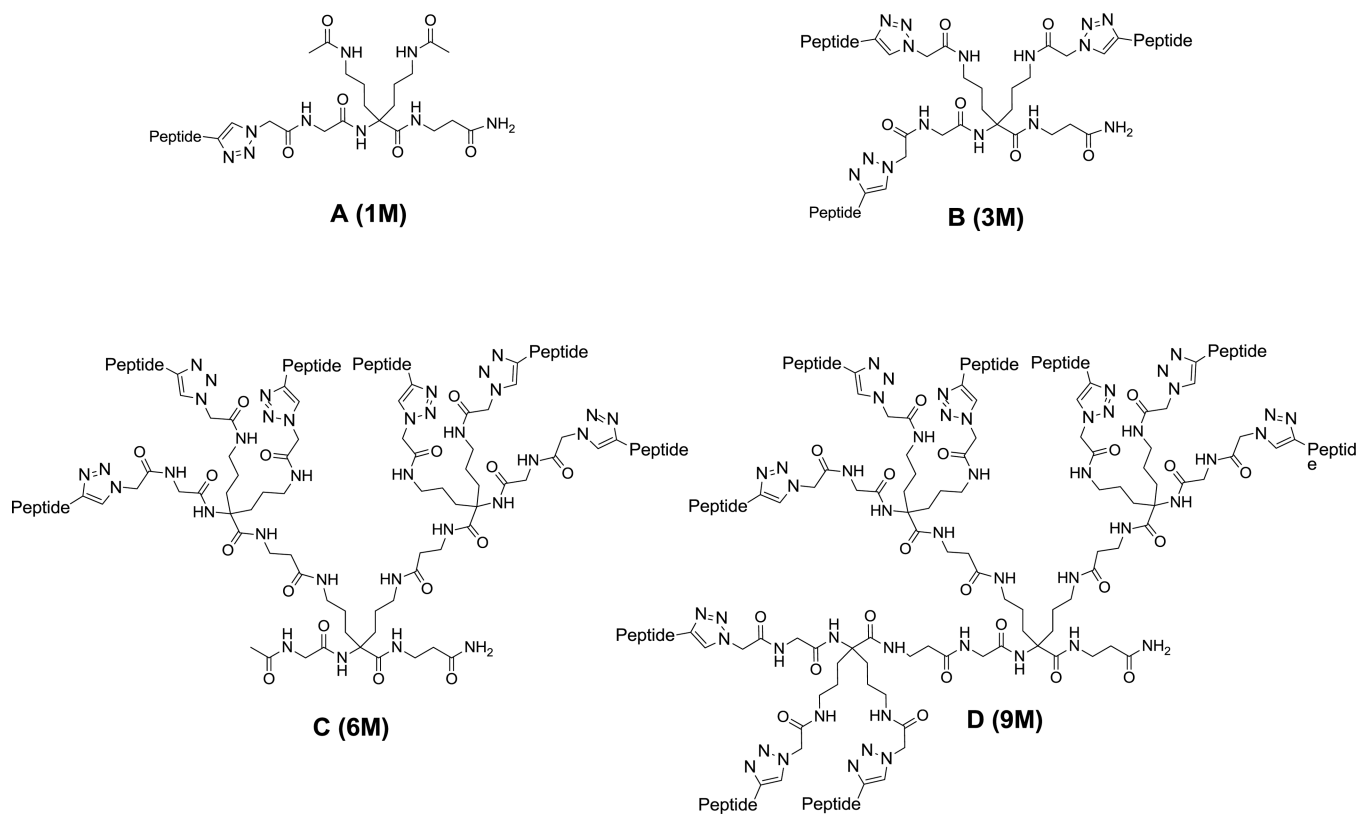


Figure 1.
Sequences of multimers investigated.

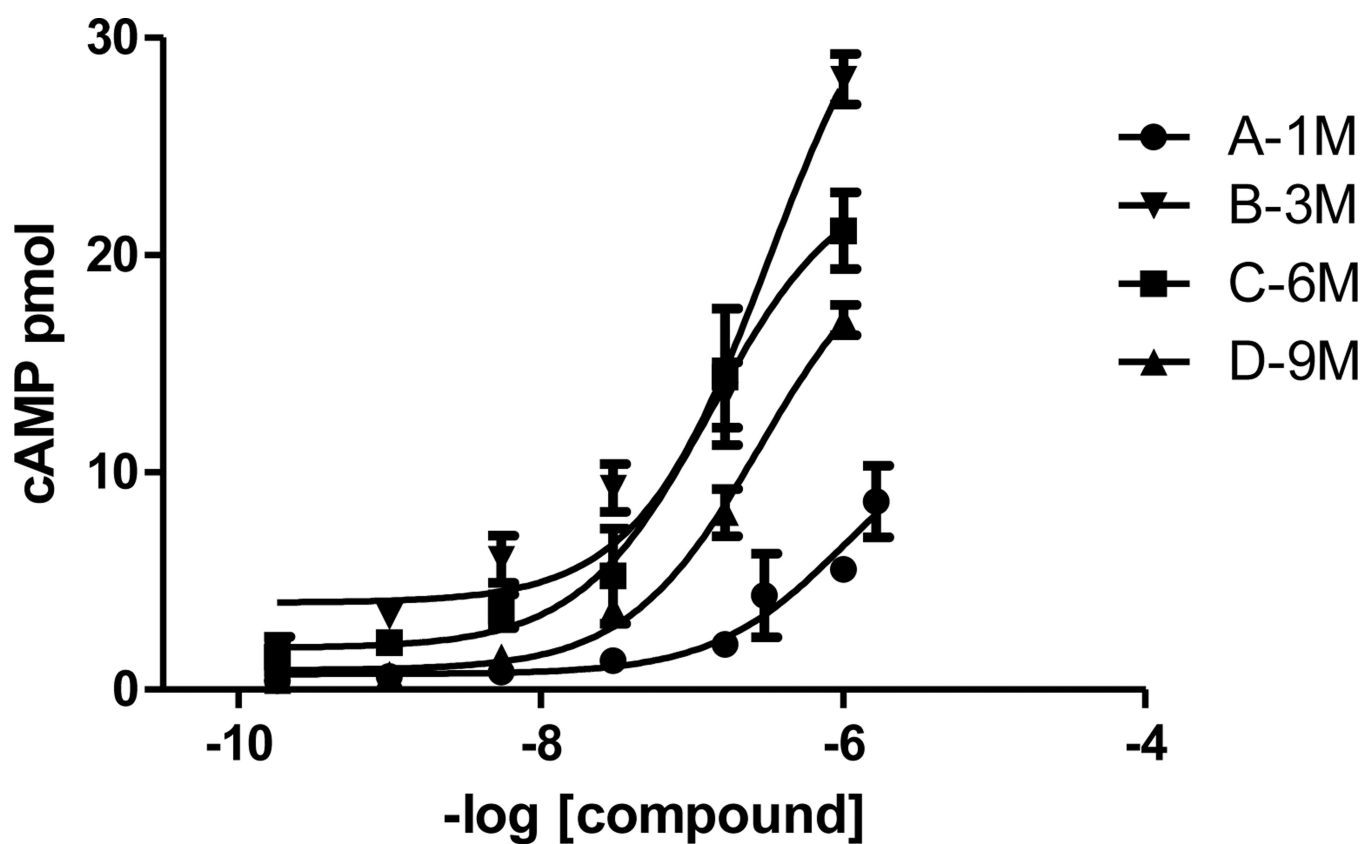


Figure 2. cAMP accumulation in response to dendrimers. cAMP accumulation in hMC4R cells in response to multimers was quantified using a chemiluminescent immunoassay described on the Supporting information.

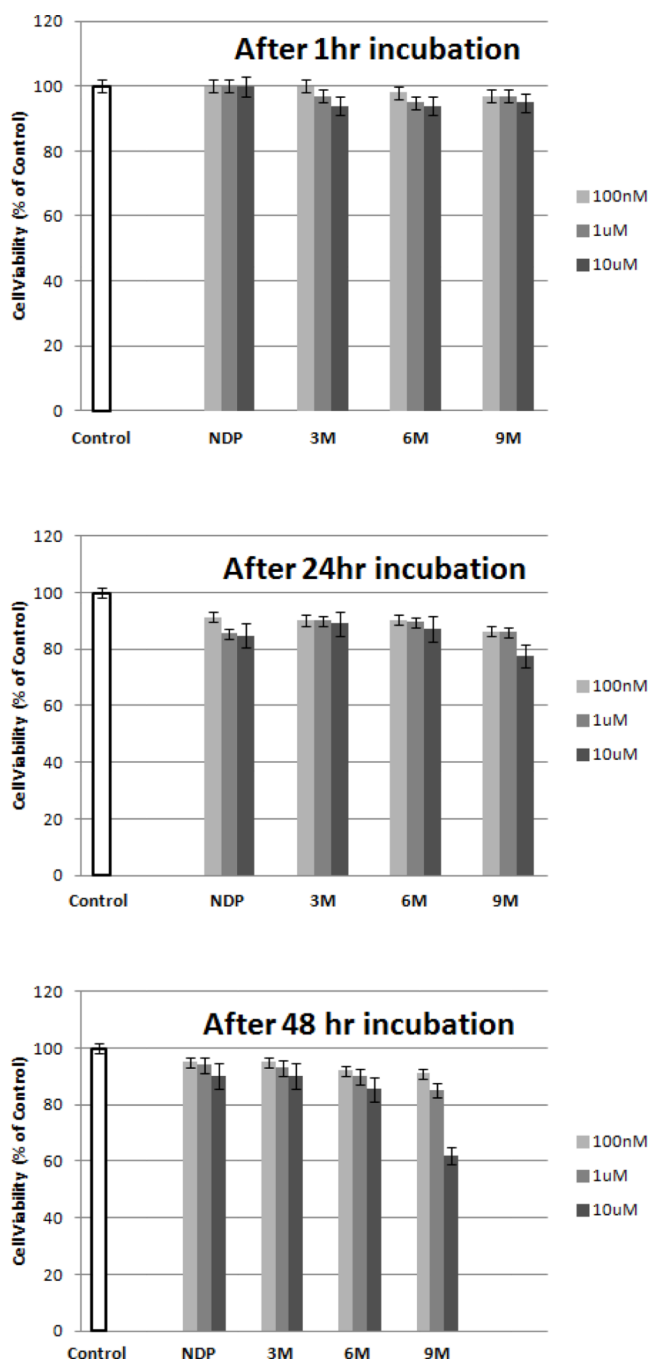


Figure 3. Toxicity studies. Cell viability was measured and represented as a % of control. The results are an average of three independent experiments done in triplicates.

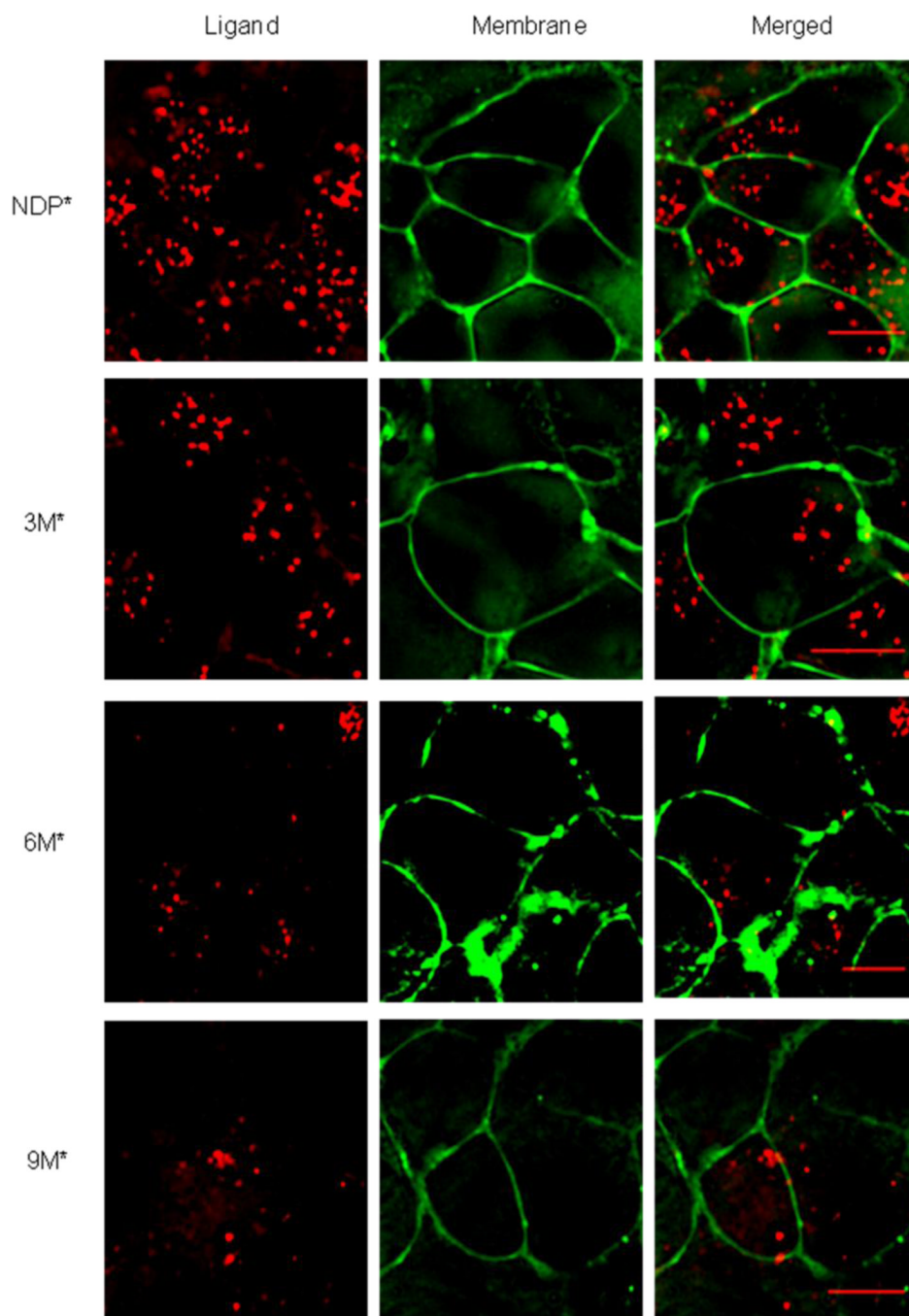


Figure 4. Localization of Cy5 labeled molecules (Red) at the membrane 90 minutes after initiation of incubation. The membrane probe, FM 1-43 (Green) was added two minutes prior to image acquisition (Scale bar, 10 μ m).

Table 1

Multimer binding affinities. Competition experiments were performed using a time resolved fluorescence based assay^{23–25}. Ligands were competed against Eu-NDP- α -MSH on HEK293 cells overexpressing the MC4R and IC₅₀'s values are averaged from four experiments performed in quadruplicate.

Name	Valency	IC ₅₀ (nM)	Relative potency to A
A (1M)	1	4900 ± 760	-
B (3M)	3	14 ± 2	350
C (6M)	6	46 ± 8	104
D (9M)	9	49 ± 9	98