



**Celtarys**  
RESEARCH



## **Case Study**

# **Development of a C5aR fluorescent ligand for screening assays**

January 2023

# C5aR: need for new research tools

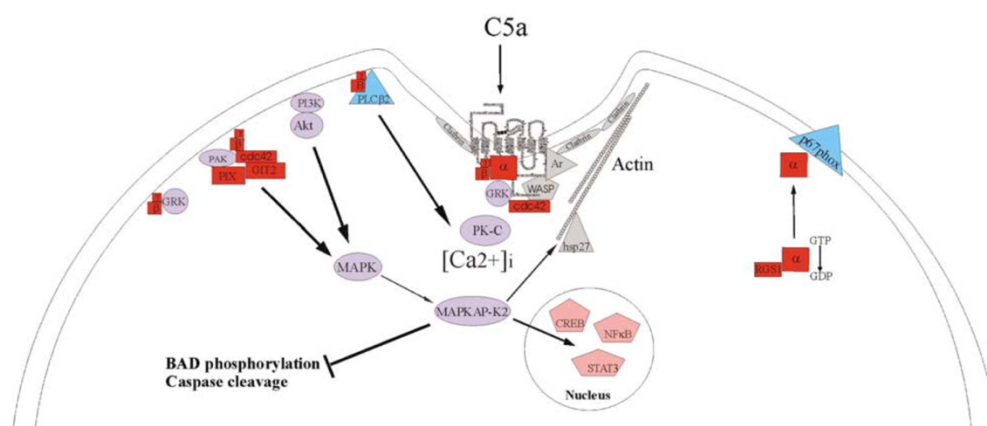
Twist Bioscience is a synthetic biology company based in South San Francisco, California. The company has developed a proprietary silicon-based manufacturing process for the production of synthetic DNA.

Twist Bioscience serves Life Science researchers who are changing the world for the better. Coming from diverse fields of medicine, agriculture, industrial chemicals and data storage, scientists use their synthetic genes, oligo pools, and NGS target enrichment to better lives and improve the sustainability of the planet. Twist Bioscience technology overcomes inefficiencies and enables cost-effective, rapid, precise, high-throughput DNA synthesis and sequencing.

Twist Bioscience is studying the C5a Receptor, a member of the large family of G-protein-coupled receptors (GPCRs). GPCRs are considered the most important family of therapeutic targets for the pharmaceutical industry, for two reasons: they are involved in a wide spectrum of pathologies -immunological, vascular, neurological, degenerative, oncological, metabolic...- and around 33 % of currently marketed drugs exert their therapeutic activity through at least one GPCR.<sup>1</sup>

The C5a anaphylatoxin chemotactic receptor 1 (C5aR, also known as CD88) belongs to the rhodopsin family of GPCRs and has been a topic of interest in the last decades due to its relevance in several inflammatory pathologies, such as asthma, arthritis, sepsis, and more recently Alzheimer's disease and cancer.<sup>2</sup>

Activation of C5aR triggers intracellular signalling pathways which control a variety of immunological responses in vivo, such as chemotaxis, cell activation, and inflammatory signalling<sup>3</sup> (figure 1). Despite the fact that the interaction between C5a and C5aR is of high therapeutic value, their molecular binding mechanism remains elusive, and several efforts are ongoing to find new drugs acting on C5aR.



**Figure 1.** C5aR intracellular signalling. C5aR interacts directly or indirectly with kinases (purple), GTP binding/regulatory proteins (red), transcription factors (pink), other signalling enzymes (blue) or structural proteins (grey). Internalization of C5aR is mediated by clathrin, which associates with receptor-bound  $\beta$ -arrestin (Ar) and the actin cytoskeleton. Proteins, such as hsp27, phosphorylated by MAP kinase-activated protein kinase 2 (MAPKAP-K2), may regulate the actin cytoskeleton. MAPKAP-K2 is itself activated by the mitogen-activated kinase (MAPK/ERK/ JNK) cascade, in turn activated by kinase Akt (also known as PK-B) or by p21-associated protein kinase (PAK) complexed with Rac/Cdc42 guanine nucleotide exchange factor PIX $\alpha$ , cdc42 and G-protein-coupled receptor kinase-interactor 2 (GIT2). G-protein  $\alpha$ -subunits are deactivated by regulator of G-protein signalling 1 (RGS1) that stimulates GTP conversion to GDP; in the GDP-bound state,  $\alpha$ -subunits can bind to and modulate the activity of the NADPH-oxidase component p67<sup>phox</sup>.  $\beta\gamma$ -subunits directly

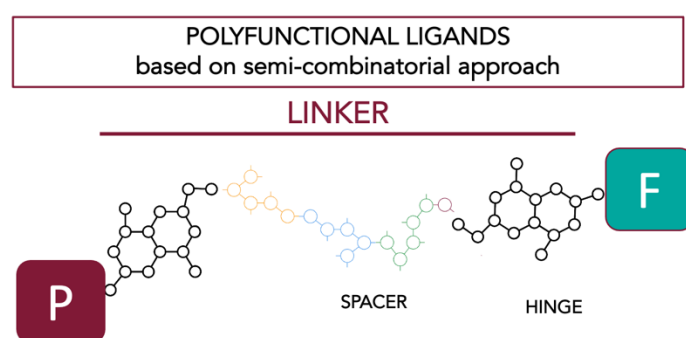
activate PAK and indirectly activate PK-C $\beta$  by increasing diacylglycerol and intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) through phospholipase C $\beta$  (PLC $\beta$ ).  $\beta\gamma$  may be sequestered by G-protein-coupled receptor kinase (GRK), which also phosphorylates C5aR along with PK-C $\beta$ . Transcription factors signal transducer and activator of transcription 3 (STAT3), cAMP responsive element binding protein (CREB) and nuclear factor (NF)- $\kappa$ B are activated at the convergence of the kinase pathways, and apoptosis inhibited by phosphorylation of Bcl-associated death promoter (BAD) and upregulation of caspase degradation. JNK, c-Jun N-terminal kinase; NADPH, nicotinamide adenine dinucleotide phosphate.<sup>3</sup>

One promising way of speeding up the identification of potential drugs acting on C5aR (being small molecules or biologicals as antibodies), is using fluorescence-based assays, like flow cytometry or fluorescence polarization. This kind of assays can be adapted to medium and high throughput screening with a read out easy to scale up. However, there is a major bottleneck in this approach: the availability of optimal fluorescent probes. In particular, for the C5aR there are no fluorescent probes in the market, preventing the development of fluorescence-based assays to study this promising target.

## Celtarys conjugation technology

Celtarys proprietary conjugation technology is suitable to develop different kinds of polyfunctional ligands. Indeed, it has been extensively validated for the identification of fluorescent ligands with optimal pharmacological and photophysical properties for several GPCRs.<sup>4-6</sup>

This conjugation platform relies on a semi-combinatorial format in which components belonging to different ready-to-use toolboxes previously synthesized by us (e.g pharmacophores, fluorophores, spacers and hinges, see figure 2) are combined convergently.

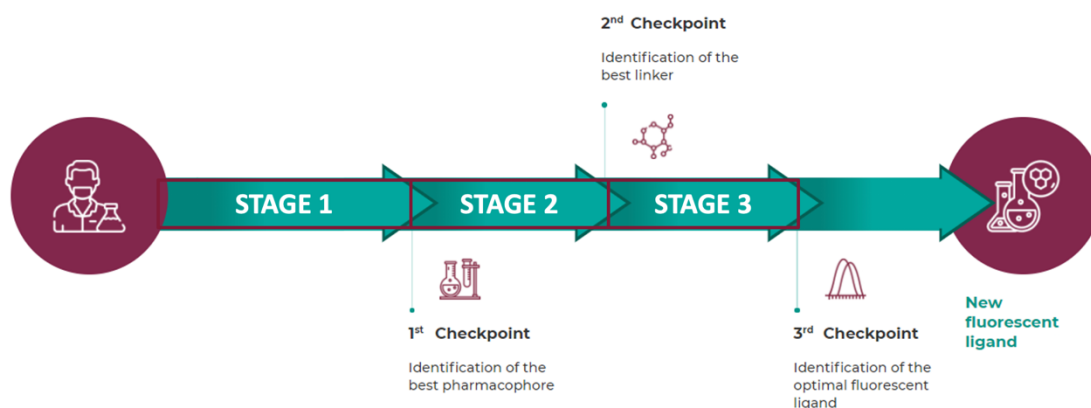


**Figure 2.** General structure of ligands architecture obtained by Celtarys Technology.

- (I) Firstly, we start with the identification of the appropriate pharmacophore for each target, integrating structure-based and ligand-based drug discovery approaches for compound selection. A joint *in silico*/experimental structure-activity relationship study enables the identification of the optimal region for pharmacophore functionalization and linker conjugation. The optimal pharmacophore(s) are typically detected among a set of 3-5 different chemical scaffolds.
- (II) Then, the functionalized pharmacophore(s) showing the best physicochemical and pharmacological profile are conjugated to different

spacers and hinges from our toolboxes, obtaining a library of intermediates (pharmacophore + linker). The biological evaluation of these compounds allows to identify the optimal linker for the target of interest.

- (III) Eventually, in the last stage of the process, we combine the previous identified precursors with fluorophores suitable for the kind of assay of interest. The activity of the final molecules, measured in a binding or functional assay, allows us to select the best one(s) (figure 3).



**Figure 3.** Development process of fluorescent probes using Celtarys technology and its stages.

**Attending Twist Bioscience request, we set out to apply our conjugation strategy to the development of the first C5aR small-molecule fluorescent ligand to be used in flow cytometry screening assays.**

## C5aR fluorescent ligand development

The first step of the project was a detailed analysis of the orthosteric ligands of the C5a receptor published in literature, with a special interest in antagonistic functional activity. Indeed, for competition binding screening, fluorescent antagonists are preferred since they show the same affinity for both receptor conformations of the receptor (active and inactive) and they will not trigger any mechanism of internalisation, which could impair the reproducibility of the assay.

3 scaffolds of pharmacophores (**P1**, **P2** and **P3**) were selected following specific criteria: activity range, structure-activity information available, chemical scaffold and synthetic accessibility.

To date, C5aR has been crystallised with only one orthosteric ligand, the cyclopeptidic antagonist PMX53, together with 2 allosteric ligands, NDT9513727 and Avacopan. Therefore, information of the possible fitting in the binding pocket of the selected pharmacophores was obtained using suitable computational models together with SAR studies performed by the chemical functionalization of the pharmacophores and their biological evaluation.

## Calcium functional assays

Several modifications of **P1**, **P2** and **P3** were designed using structure-based and ligand-based approaches and synthesized during Stage 1 of the project. 4 promising functionalised structure of **P1** showed  $K_B < 100$  nM (table 1 in green, measured in a Calcium flux assay, Ready-to-Assay™ C5aR Anaphylotoxin Receptor Frozen Cells from Eurofins). Because of the good activity, these 4 structures were selected for linker assembly (see the corresponding pharmacophore+linker Stage 2 columns).

SCAFFOLD	PRECURSORS						FLUORESCENT LIGANDS (STAGE 3)				
	Pharmacophore (STAGE 1)			Pharmacophore+linker (STAGE 2)			Biological Data				
	Pharmacophore functionalization	Code	Biological Data		Code	Biological Data		Code	Biological Data		
Calcium assay ( $K_B$ nM)			cAMP assay $IC_{50}$ (nM)	Calcium assay ( $K_B$ nM or % of inhibition at 1 $\mu$ M)		cAMP assay $IC_{50}$ (nM)	Calcium assay ( $K_B$ nM or % of inhibition at 1 $\mu$ M)		cAMP assay $IC_{50}$ (nM)	Affinity binding $EC_{50}$	
P1	Unmodified pharmacophore <sup>a</sup>	MFLV5	22.85	4.62							
	A	MFLV6 SG11	543.09 938.31								
	B	MFLV10 SG12	94.56 72.76		SG43 SG45	10% 17%					
	C	MFLV17 SG8	25.36 33.05		SG33 SG36	21% 14%		SG40	15%	> 10 $\mu$ M <sup>b</sup>	
	D	MFLV18	195.329					CELT-58	5788	Inactive	30.38 nM
P3	A				MFLV50 MFLV66	1379 34%	> 10 $\mu$ M 10.27	SG65 CELT-68	14% 10%	Inactive 0.05	24.89 nM > 10 $\mu$ M <sup>b</sup>

**Table 1.** Biological activity of the most representative compounds synthesized in C5aR fluorescent ligand development project. <sup>a</sup> In addition to the W-54011, the unmodified pharmacophore 1 was used as internal control for further assay validation; <sup>b</sup> The  $EC_{50}$  is not accurate since the saturation curve did not reach the plateau (figure 5).

As mentioned above, thanks to our technology we can assemble the linker combining suitable functionalized pharmacophores (or any molecules of interest), hinges and spacers in a dynamic fashion, having access to hundreds of possible combinations. In this stage the accessible linker structures are filtered, basing on the desired physicochemical properties for the specific application on which the project is focused.

A first library of **P1**+linker conjugates was synthesized using the previously identified functionalized derivatives of **P1**. Despite the good activity of the precursors, none of the new conjugates showed  $K_B < 100$  nM in Calcium assays (table 1), which led us to test further combinations of **P1**+linkers and to work on the functionalization of pharmacophores 3 (**P3**), which had shown moderate activity ( $K_B > 100$  nM) in calcium assay during Stage 1. In the end of this iterative process, a library of more than 30 compounds was synthesized. The best activity in **calcium assays** was obtained in the case of MFLV50, a **P3**+linker conjugate (table 1 in blue).

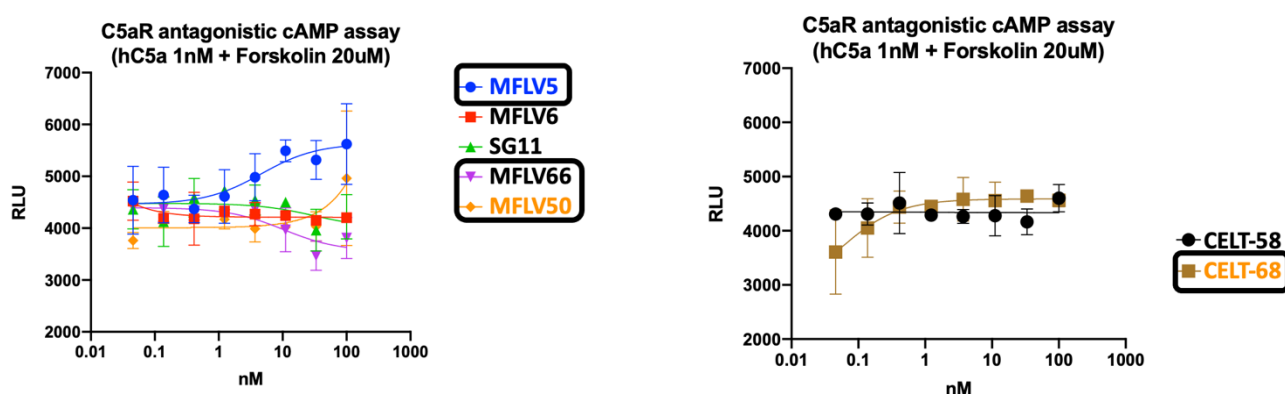
Even though a moderate activity was observed, we decided to move on to Stage 3, labelling with a red-emitting fluorophore (Cy5) the most promising derivatives of **P1** and **P3** identified in Stages 1 and 2. The main reason is the discrepancy between the biological data observed in calcium functional assays and the expected activity predicted by a rigorous study of the SAR and docking studies using the crystal structure of C5aR in complex with the cyclic peptide PMX53<sup>7</sup> for the receptor configuration set up. Indeed, promising and reliable binding modes had been predicted for **P1** and **P3**, for instance for MFLV18 (table 1 in blue), a derivative of pharmacophore 1, able to establish an intramolecular hydrogen bond, presenting a bioactive configuration that resembles the fold of PMX53.

The fluorescently labelled **P1** and **P3** synthesized did not show outstanding activity in calcium functional assays. The best compound was **CELT-58** which was obtained by the direct conjugation of pharmacophore **MFLV18** with Cy5, showing a  $K_B$  of **5788 nM** (table 1 compound in red).

In order to perform an extensive biological characterization of the fluorescent ligands synthesized, a selection of 7 fluorescently labelled compounds (**P1** and **P3**) were tested in Twist Bioscience facilities. The assays performed were C5aR binding assay by flow cytometry in both C5aR-HEK (Multispan) and C5aR-Chem1 (DiscoverX) transfected cell lines and cAMP Hunter™ eXpress C5aR CHO-K1 GPCR Assay.

### cAMP functional assays

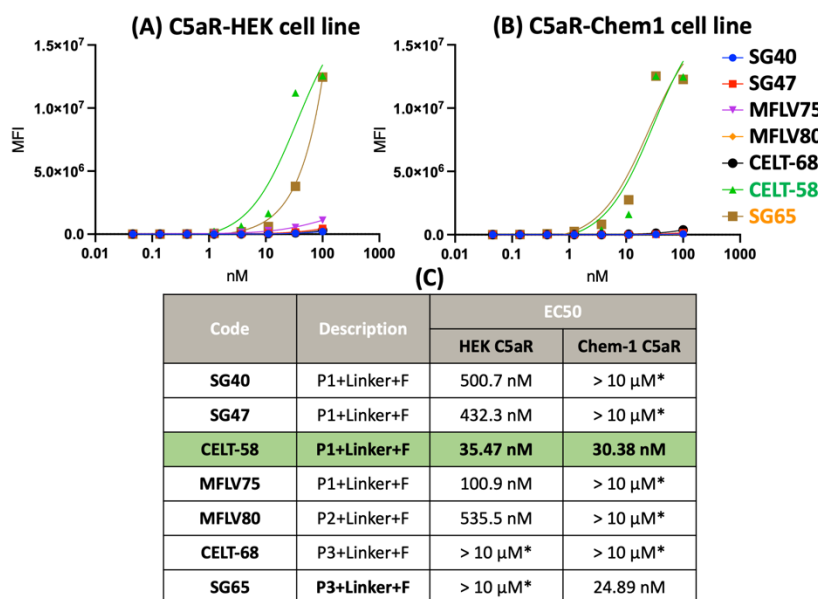
Only the reference compound MFLV5 (**P1**), the **P3**+linker conjugates MFLV50 and MFLV66 and the fluorescent ligand **CELT-68** (**P3**) showed activity in cAMP assays (figure 4 and table 1).



**Figure 4.** cAMP functional assays performed on representative precursors and final fluorescent probes.

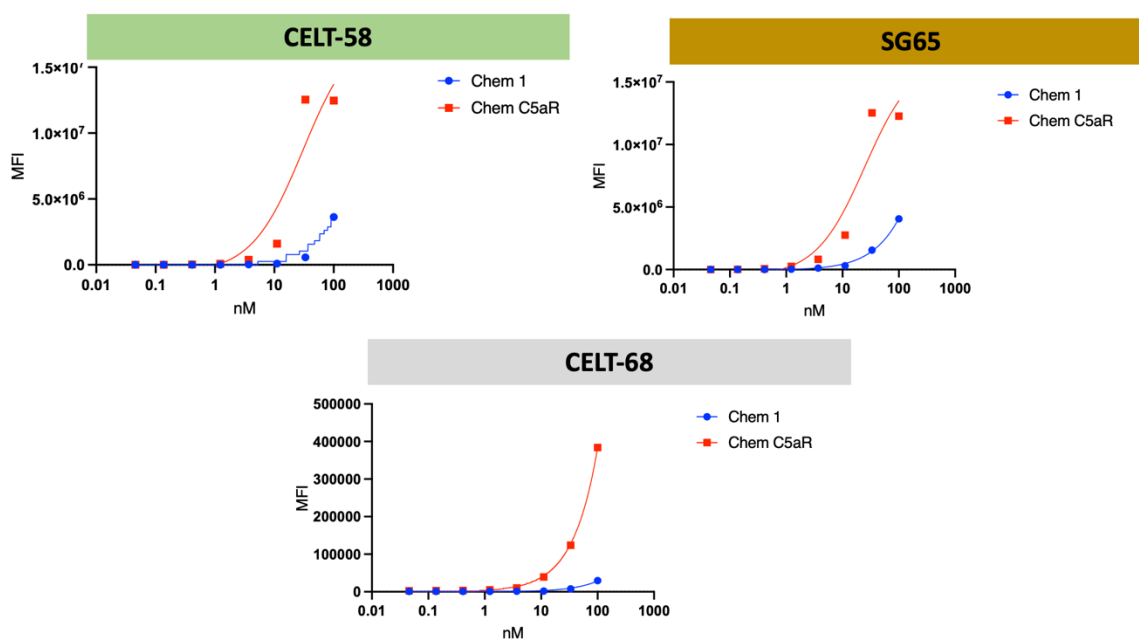
### Flow cytometry binding assays

The binding of the 7 fluorescent probes was studied in flow cytometry saturation binding assays using two different cell lines: C5aR-Chem1 transfected cells and C5aR-HEK transfected cells (Figure 5). Comparing the saturation curves of the 7 fluorescent ligands obtained using transfected and parental cell lines, the best results were observed in C5aR-Chem1 transfected cells, with high specific binding (figure 6).



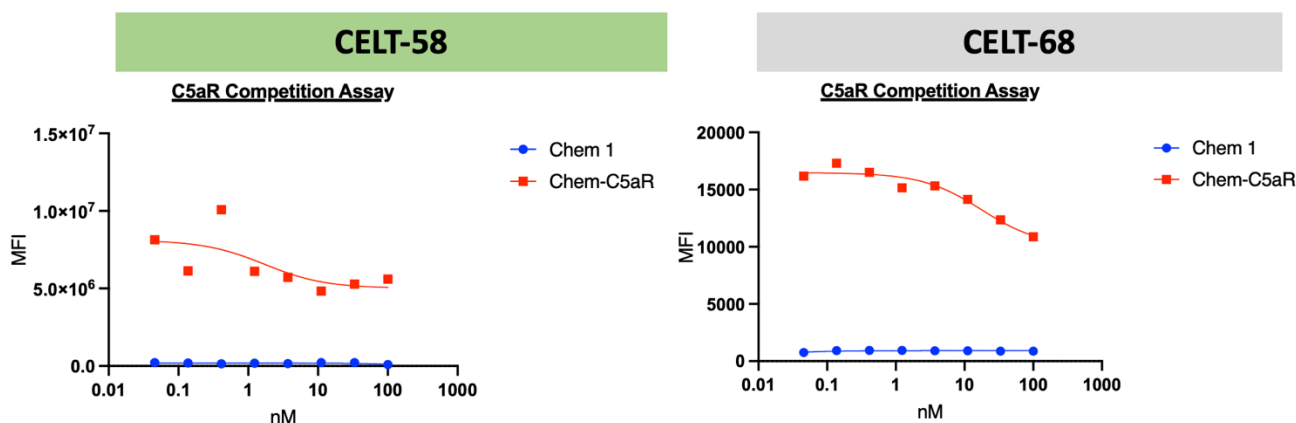
**Figure 5.** Dose response curves obtained by flow cytometry saturation binding experiments of the 7 fluorescent ligands in C5aR-HEK (A) and C5aR-Chem1 (B) transfected cells. C) EC<sub>50</sub> affinities values. \*The EC<sub>50</sub> is not accurate since the saturation curve did not reach the plateau (figure 5).

In both cell lines, **CELT-58** showed strong binding properties (figure 5).



**Figure 6.** Specific binding of the most promising fluorescent antagonists in C5aR-Chem 1 cell lines. The signal in C5aR transfected Chem-1 is compared with the untransfected parent cell line to study fluorescent probe specific binding.

The next step was the study of fluorescent ligands competition at EC<sub>50</sub> concentration with the endogenous peptidic ligand C5a (figure 7). This time the best results were shown by **CELT-58** (high affinity binder EC<sub>50</sub>=30.38 nM) and **CELT-68** (high activity in cAMP). For CELT-58, the concentration used was 30 nM which corresponds to its EC<sub>50</sub> in saturation binding experiments. For CELT-68, since the EC<sub>50</sub> obtained was not accurate (the saturation curve did not reach the plateau), the EC<sub>50</sub> of the closer curve to it (SG47 EC<sub>50</sub>=432.3 nM) was used as reference concentration.



**Figure 7.** C5aR competition binding of CELT-58 and CELT-68 with the endogenous ligand C5a by flow cytometry.

## Discussion

In this project, to develop a C5aR fluorescent ligand, more than 50 different molecules were synthesized following a three stages process (Celtarys technology), leading to the identification of 2 optimal fluorescent tools for C5aR screening. The efficiency, versatility and convergence of our conjugation strategy make possible the design and synthesis of such a large number of compounds in a short time, in order to explore a wide chemical space and increase success rate.

The good biological activity observed (Calcium flux assay) in Stage 1 for functionalised derivatives of P1 (low nanomolar range) was followed by a reduction of the activity in Stage 2, which was not in accordance with the predicted activity. This led us to an iteration of Stage 2 and the sequential labelling (Stage 3) of moderate activity conjugates.

The fluorescent ligands synthesized of P1 and P3 were submitted to additional biological characterization. As a result, **CELT-58** and **CELT-68** were identified as optimal ligands to perform competition binding studies by flow cytometry.

These results highlighted the high variability of the biological data depending on the type of functional assay (signalling pathway monitored: Calcium, cAMP,  $\beta$  arrestin, etc) performed and the loss of information it can generate if the interaction of the molecule with the target (binding) wants to be studied.

## Conclusions

Applying Celtarys chemical conjugation technology, we have designed and synthesized two optimal fluorescent ligands for C5aR, **CELT-58** and **CELT-68**. Both ligands show high specific binding to C5a Receptor in saturation binding assays (figure 5) and good competition with the endogenous ligand C5a by flow cytometry in C5aR-Chem1 cell lines (Figure 7). Both are orthosteric ligands with antagonistic activity in Calcium and cAMP assays respectively (table 1).

**These two fluorescent probes have proven to be optimal tools to perform fluorescence-based assays to unlock the therapeutic potential of this important receptor.**

## References

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