Discovery of Novel Immunopharmacological Ligands Targeting the IL-17 Inflammatory Pathway

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Abstract

Interleukin 17 (IL-17) is a proinflammatory cytokine that acts as an immune checkpoint for several autoimmune diseases. Therapeutic neutralizing antibodies that target this cytokine have demonstrated clinical efficacy in psoriasis. However, biologics have limitations such as their high cost and their lack of oral bioavailability. Thus, it is necessary to expand the therapeutic options for this IL-17A/IL-17RA pathway, applying novel drug discovery methods to find effective small molecules. In this work, we combined biophysical and cell-based assays with structure-based docking to find novel ligands that target this pathway. First, a virtual screening of our chemical library of 60 000 compounds was used to identify 67 potential ligands of IL-17A and IL-17RA. We developed a biophysical label-free binding assay to determine interactions with the extracellular domain of IL-17RA. Two molecules (CBG040591 and CBG060392) with quinazolinone and pyrrolidinedione chemical scaffolds, respectively, were confirmed as ligands of IL-17RA with micromolar affinity. The anti-inflammatory activity of these ligands as cytokine-release inhibitors was evaluated in human keratinocytes. Both ligands inhibited the release of chemokines mediated by IL-17A, with an IC_{50} of 20.9 ± 12.6 μM and 23.6 ± 11.8 μM for CCL20 and an IC_{50} of 26.7 ± 13.1 μM and 45.3 ± 13.0 μM for CXCL8. Hence, they blocked IL-17A proinflammatory activity, which is consistent with the inhibition of the signalling of the IL-17A receptor by ligand CBG060392. Therefore, we identified two novel immunopharmacological ligands targeting the IL-17A/IL-17RA pathway with antiinflammatory efficacy that can be promising tools for a drug discovery program for psoriasis.

25 **Key words**

26 Interleukin-17, interleukin-17 receptor, label-free, ligand, inflammation, keratinocytes

1. Introduction

The interleukin-17 (IL-17) family has become a relevant pharmacological target for inflammatory autoimmune diseases [1,2]. IL-17A, one of the six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F), is a therapy cornerstone after the EMA and FDA approval of IL-17-blocking antibodies for the treatment of plaque psoriasis [3].

The IL-17 receptor is a dimer constituted of two the five subunits: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. All of them have a SEFIR domain (similar expression to fibroblast growth factor genes) in the cytoplasmic region, critical for functional signalling [4]. IL-17A is a homodimer that binds to a heterodimeric receptor with subunits A and C. This cytokine induces the expression of proinflammatory cytokines (such as IL-6 and G-CSF), chemokines (e.g. CXCL1, CXCL8 or IL-8, CXCL5 or CCL20), matrix metalloproteinases (MMPs) and antimicrobial proteins (AMPs) upon a number of physiopathological conditions. Several of these inflammatory mediators are chemoattractants that recruit neutrophils, lymphocytes and monocytes [5–9].

The IL-17/IL-17R signalling pathway is initiated by the recruitment of Act1 by the SEFIR domain of the receptor. Act1 acts as an E3 ubiquitin ligase and ubiquitinates TRAF6, that activates NF-κB and MAPK cascades, for a consequent inflammatory gene transcription turn on [2,9–11]. Additionally, the signalling cascade can activate C/EBPβ and C/EBPδ, which mediate the transcription of specific target genes [9,12]. There is also a critical post-transcriptional signalling of IL-17, where different RNA-binding proteins, such as Regnase-1 (or ZC3H12A), increase or decrease the expression of IL-17 target mRNAs. This post-transcriptional pathway not only regulates the stability of inflammatory mediators but also the proliferation of primary keratinocytes, which is relevant in the psoriasis pathogenesis [9,13,14].

In psoriasis, dysregulation of Th17 cells and high levels of IL-17 promote that keratinocytes release proinflammatory cytokines such as IL-1β, TNFα, IL-6 and chemokines such as IL-8, CXCL1 and CCL20, leading to the characteristic lesions of this pathology [15,16]. Chronic response mediated by IL-17A plays a central role in the pathogenesis of autoimmune diseases, as it was demonstrated by the clinical efficacy of its blockade with mild adverse events in patients with psoriasis [8,17]. Three neutralizing antibodies targeting IL-17 or its receptor are available for clinical use since 2016: secukinumab and ixekizumab inhibit IL-17A; and brodalumab, which inhibits the IL-17 receptor A. They block the detrimental inflammatory state in the skin of patients with psoriasis and psoriatic arthritis [18,19].

Although effective, antibodies blocking IL-17 might not be sufficient to address the unmet clinical needs of patients with autoimmune diseases. Antibodies may generate immunogenicity, inducing the formation of anti-drug antibodies (ADAs) that eventually result in the loss of the response of the patient to treatment. The prevalence of patients treated with secukinumab was < 1% across in a long-term safety study of 21 pooled clinical trials [20]. However, although IL-17 inhibitors have been reported to produce ADAs, only ixekizumab showed a decrease in efficacy and a wide range of clinical immunogenicity incidence rates [21,22]. Brodalumab also show variable incidences, from 1.8% to 11% [21]. Moreover, there are a significant proportion of patients that do not respond to any of the available biological drugs. While biologics are generally safe, a few patients develop adverse effects, including a mild increase in the risk of infections such as *Candida albicans* [23–26].

One of the reasons for developing new therapeutic agents that inhibit the IL-17 pathway is to progress into oral bioavailable drugs [27]. Oral delivery is the most popular administration route for patients, due to the ease of ingestion and the absence of pain in the process of administration. Also, oral delivery systems are less expensive to

manufacture than injectable ones, as they do not require sterile conditions. On the other hand, small molecule antagonists may address side effects inherent to biologics. Recently, several synthetic antagonist macrocycles were discovered, indicating that the IL-17 pathway may be druggable with oral bioavailable, lower cost and more effective treatments [28,29]. They were previously identified using approaches such as DNA-encoded chemical libraries screening, and design and co-crystallization, but they have not progressed into clinical trials [28–30].

For this, we reasoned that methodologies that enable the discovery of novel ligands targeting IL-17A/IL-17RA might demonstrate the feasibility of developing treatments with small molecules for psoriasis, in which this pathway is relevant. Thus, we decided to evaluate ligands in human keratinocytes, as inducing inflammation in these skin cells would be relevant in the study of efficacy of early drug discovery compounds for psoriasis.

We aim to identify novel immunopharmacological ligands for the IL-17A/IL-17RA proinflammatory pathway by using a combination of *in silico* screening, biophysical and cell-based methodologies that can set the basis for drug discovery programs.

2. Materials and methods

2.1 Virtual screen

BioFarma chemical library was employed. It owns 60000 drug-like rationally selected compounds with structural and biological diversity. This library contains different sets of compounds that can be applied to different early drug discovery projects: repurposing

drugs, with almost 5000 drugs that were clinically tested, including drugs approved by the FDA and the EMA; focused chemical libraries to different therapeutic targets, as kinases, GPCRs or ionic channels; academic compounds added after the agreement of different public research groups; natural compounds; compounds from the National Cancer Institute chemical library; and selected commercial compounds that cover as much chemical space as possible.

In order to develop the virtual screen, first a SDF file was generated with the list of ligands and then split into single files to transform the 2D molecules into a Protein Data Bank (PDB) 3D format by means of Babel software. They were then used in PDBQT format and imported to AutoDock Vina software suite 1.1.2 (May 11, 2011) [31].

Target protein structures were downloaded in PDB (3D) format. The multiple molecular configurations were split and the protein part was extracted. Protein was transformed into Vina PDBQT format. The structures of PDB target proteins employed were 2VXS, 4HR9, 4HSA, 4QHU, 5HHV, 5HHX, 5HI3, 5HI4 and 5HI5 (available at the time of the study). They correspond to IL-17A alone or bound to IL-17RA or to different ligands. Subsequently, a folder for each ligand-target pair was generated. Files with cluster jobs were created and then separated into groups and sent to the BioCAI HPC cluster queue from which the affinity energies (kcal/mol) for each ligand-target pair were obtained as a result list.

2.2 Protein expression and purification

Full length human IL-17A was cloned into a C-terminal His-tagged plasmid (pcDNA3.1). The vector was transfected into HEK 293 mammalian cells with polyethylenimine (PEI from Sigma Aldrich, USA) (DNA/PEI ratio was 1:4), and cells were cultured in Freestyle 293 Expression Medium (Gibco, UK). After 48 h of incubation, supernatant was collected.

Subsequently, it was purified with an affinity chromatography on Ni-Sepharose Fast Flow column with ÄKTA™ start chromatography system (GE Healthcare, Uppsala, Sweden). Binding and wash buffer used for the purification was 20 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.4; and elution buffer was 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4 (Sigma Aldrich, Germany). An IL-17A-enriched fraction was obtained.

2.3 Label-free dynamic mass redistribution assay (DMR)

EnSpire LFB high sensitivity plates (PerkinElmer 6057460, USA) were activated with 400 mM EDC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride) (Sigma Aldrich, UK) and 100 mM sulfo-NHS (sulfo-*N*-hydroxysulfosuccinimide) (Thermo Fisher, USA) diluted in ultrapure water for 30 min at room temperature. Microplates were subsequently washed with ultrapure water. The extracellular domain of the IL-17 human receptor A (IL-17RA ECD Fc) (Sino Biological, China) was immobilized (25 µg/ml) in 20 mM sodium acetate buffer, pH 5.5. After an overnight incubation at 4 °C, the microplate was washed with PBS with 0.005 % Tween-20, pH 7.4 (assay buffer). Baseline was read after a three-hour equilibration inside the EnSpire™ 2300 Multilabel Plate Reader (PerkinElmer, Singapore). Ligands identified by virtual screening (20 µM) were added to the microplate, incubated for 1 hour and the final read was performed in the EnSpire™ 2300 Multilabel Plate Reader. Recombinant human IL-17A preparation and assay buffer were employed as a positive and a negative control, respectively.

Hit compounds identified in the screening were characterized by means of concentration-response curves with concentrations between 100 µM and 0.4 µM (1:3 serial dilutions in triplicates), following the protocol described above. Response (pm) was obtained by subtracting the baseline measurement from the final read.

2.4 Surface plasmon resonance assay (SPR)

SPR assay was performed in a Biacore™ S200 SPR instrument (GE Healthcare, Buckinghamshire, UK) [32]. IL-17RA ECD Fc (25 μg/ml) was captured on a Series S CM5 sensor using amine coupling (GE Healthcare, USA) to achieve 10000 RU (response units) of immobilized protein. The SPR immobilization buffer was 10 mM sodium acetate, pH 5. ECD and sulfo-NHS (both from Sigma Aldrich, USA) were employed to activate the chemistry of the chip, prior to the addition of the protein. The SPR running buffer used was 20 mM HEPES, 200 mM NaCl, 0.05% Tween-20, pH 7.4. Compounds were injected at a flow rate of 10 μl/min (15 °C). Different concentrations were tested (0.078 to 50 μM, 1:3 serial dilutions in triplicates). Data were analysed with Biacore GE Software to calculate the binding constants.

2.5 Cell culture of human keratinocytes (HaCaT) and CCL20 and IL-8 quantification by Luminex®

HaCaT cell line was purchased from ADDEXBIO (#T0020001) and was cultured in DMEM GlutaMAX® (Gibco, Netherlands) supplemented with 10 % fetal bovine serum (Sigma Aldrich, Switzerland) and penicillin-streptomycin (Sigma Aldrich, Israel). 1.25×10^4 cells per well were seeded in a 96-well plate and were incubated for 24 hours at 37 °C and 5 % CO_2. Then, cells were treated with medium containing 100 ng/ml of human recombinant IL-17A (Sigma Aldrich, USA) and/or 10 ng/ml of human recombinant TNFα (Sigma Aldrich, USA) in the presence or absence of serial dilutions of the anti-IL-17A peptide (IHVTIPADLWDWINK) [33], CBG040591, CBG060392 or DMSO only (which is the vehicle for all of the three treatments). The anti-IL-17A peptide was employed as a

positive control of inhibition, between 15.6 and 1000 nM (1:2 serial dilutions), as literature indicated that its IC_{50} is 253 nM for the inhibition of CCL20, and 151 nM for IL-8 [33]. Both of the small molecules were employed between 1.56 and 100 μM (1:2 serial dilutions), following the data of their binding constants, in the micromolar range.

After 24 hours, supernatants were collected and frozen at -80°C. Supernatants were employed to determine human CCL20 and IL-8 concentrations by Human ProcartaPlex® Simplex kit (EPX01A-12128-901 and EPX01A-10204-901) (Invitrogen, Netherlands) according to the manufacturer protocol [34]. MAGPIX® Luminex® instrument (Luminex Corporation, Austin, Texas, USA) was used to perform the measurement of the quantity of CCL20 and IL-8 on the samples.

2.6 Analysis of expression by qRT-PCR

Proinflammatory chemokine (CCL20 and CXCL8), C/EBPβ and Regnase-1 (ZC3H12A) expression was detected through a TaqMan® assay and quantified using the comparative method C_T ($\Delta\Delta C_T$) [35].

5×10^5 HaCaT cells per well were seeded in a 6 well plate in DMEM GlutaMAX® medium (Gibco, Netherlands) supplemented with 10 % fetal bovine serum (Sigma Aldrich, Switzerland) and penicillin-streptomycin (Sigma Aldrich, Israel). They were incubated for 24 hours at 37 °C y 5 % de CO_2. Then, cells were stimulated: 100 ng/ml IL-17A (Sigma Aldrich, USA) and 10 ng/ml de TNFα (Sigma Aldrich, USA); 100 ng/ml IL-17A; 10 ng/ml TNFα; and an untreated condition. HaCaT cells treated with 100 ng/ml IL-17A and 10 ng/ml TNFα were also stimulated with the anti-IL-17A peptide IHVTIPADLWDWINK (1 μM) [33], CBG040591 (100 μM) or CBG060392 (100 μM). After 24 hours, cells were collected and RNA was isolated according to the manufacturer protocol of the RNeasy Mini Kit (Qiagen, Denmark).

In order to quantify *CCL20*, *CXCL8, CEBPB* and *ZC3H12A* expression of the treated HaCaT cell line the EXPRESS One-Step SuperScript® qRT-PCR (Invitrogen, Netherlands) was employed. TaqMan® probes used for *CCL20* (Hs00355476_m1), *CXCL8* (Hs00174103_m1), *CEBPB* (Hs00270923_s1) and *ZC3H12A* (Hs00962356_m1) with a FAM tag were from Thermo Fisher, and the VIC™ probe for the reference human gene was *36B4*. This is a gene that encodes a constitutive protein of the large ribosomal subunit [36,37]. qRT-PCR was performed for 40 cycles in QuantStudio® 12K Flex Real-Time PCR (Life Technologies, Singapore).

2.7 Western blotting

HaCaT cells (DMEM GlutaMAX® medium (Gibco, Netherlands) supplemented with 10 % fetal bovine serum (Sigma Aldrich, Switzerland) and penicillin-streptomycin (Sigma Aldrich, Israel)) were stimulated with 100 ng/ml IL-17A (Sigma Aldrich, USA) and 10 ng/ml de TNFα (Sigma Aldrich, USA) in the absence or presence of CBG040591 or CBG060392 (100 μM) for 24 hours. Subsequently, lysates were obtained with RIPA buffer (Santa Cruz Biotechnology, Germany) and phosphatase inhibitors (PhosSTOP™ from Roche, Germany). Total protein was quantified with DC™ kit of Bio-Rad.

Samples were denatured at 95 °C and a Bolt™ 4-12 % Bis-Tris Plus gel (Invitrogen, Netherlands) was used for the SDS-PAGE. Afterwards, they were blotted to the Immun-Blot® PVDF Membrane (Bio-Rad, USA) (transference buffer: 20 % methanol, 20 mM Tris and 150 mM glycine, pH 8). Membrane was blocked with 5 % BSA in TBS with 0.1 % Tween 20 (Bovine serum albumin). Primary monoclonal antibodies were used at 1: 1000 dilution and incubated overnight at 4 °C: NF-κB p65 (#D14E12) rabbit, phospho-NF-κB p65 (Ser536) (#93H1) rabbit, IκBα (#L35A5) mouse, or phospho-IκBα (Ser32) (#14D4) rabbit, all of them from Cell Signaling Technology, USA. Secondary antibody was

an anti-rabbit or an anti-mouse HPR linked (both from Cell Signaling Technology) (1: 5000), and it was incubated for 1 hour. Amersham ECL Prime Western Blotting Detection Reagent was used to detect the blot, from GE Healthcare (UK). ImageJ software (version 1.52p, December 2019, USA) was employed for the density quantification of the bands.

2.8 Statistical analysis

Data analysis and processing was performed with GraphPad Prism® software (version 6.0e, March 2014) (GraphPad Software, San Diego, CA). Values are expressed as the mean ± SEM of at least three independent experiments in biological duplicates or triplicates. For chemokine release assays we performed at least four independent experiments, as well as for western blotting. For gene expression assays we performed three independent experiments. Comparison of differences among groups was determined by the analysis of variance (ANOVA) followed by Bonferroni's test. $P < 0.05$ was determined as significant in all statistical analysis. For the dynamic mass redistribution screening, we determined the Z' factor, coefficient of variation (CV) values and signal-to-background (S/B) ratio. An excellent assay, which has a wide separation between the distributions of positive and negative controls, would have a Z' value between 0.5 and 1; while a moderate separation would be defined by a Z' value between 0 and 0.5, which is considered acceptable [38,39].

3. Results

3.1 Identification and binding of ligands of IL-17A/IL-17RA

3.1.1 Identification of IL-17A/IL-17A receptor ligands by molecular docking

The first step in the identification of small molecule IL-17 ligands was a target-based *in silico* screening. 60 000 compounds from the BioFarma chemical library were used to set up molecular docking studies with the 2VXS, 4HR9, 4HSA, 4QHU, 5HHV, 5HHX, 5HI3, 5HI4 and 5HI5, corresponding to IL-17 receptor A and IL-17A structures available in Protein Data Bank. A total of 67 hits were obtained by establishing -8.8 kcal/mol as the affinity energy threshold. These hits were considered as potential ligands for the interaction with the cytokine receptor.

3.1.2 Ligand-target binding studies by biophysical assays

In order to confirm the potential binding of ligands to IL-17A/IL-17RA, a label-free DMR assay was developed using the human fusion protein IL-17RA ECD Fc. Immobilization of the target on the amine coupling surface of the plate was efficiently achieved, obtaining response signals over 1500 pm at 25 μg/ml, using a 20 mM sodium acetate buffer solution at pH 5.5. IL-17A-enriched fraction showed binding to the IL-17RA ECD Fc receptor with a K_D of 0.91 ± 0.21 μM, and therefore was established as a positive binding control (**Figure 1A**). Next, the 67 hits obtained from the virtual screening were tested in the label-free binding assay at a concentration of 20 μM (**Figure 1C**). The statistical parameters for the screening assay, including Z' factor, signal to background and coefficient of variation were calculated and considered acceptable for screening purposes, as it was defined in section

2.8 (**Table 1**) (**Figure 1B**). The binding threshold to select hits was fixed as the mean of the response of the group of the compounds plus two standard deviations. Subsequently, two different chemical structures of quinazolinone and pyrrolidine-2,5-dione families were identified as potential ligands for the pair IL-17A/IL-17RA with a percentage of binding over 50 % compared to our IL-17A control.

3.1.3 Affinity calculation by DMR and SPR binding assays

Two ligands identified by DMR screening (CBG040591 and CBG060392) were characterized by means of a concentration-response curve employing the label-free assay. CBG040591 and CBG060392 were defined as ligands of the extracellular domain of IL-17RA with a B_{max} of 103 ± 19.2 % and 74.8 ± 17.7 %; and with a K_D of 40.4 ± 16.4 μM and 19.4 ± 13.0 μM, respectively (**Figure 1D-E**). However, both ligands showed a certain degree of unspecific binding at high micromolar concentrations that imply that their dissociation constants (K_D) might not be determined accurately using this experimental setup.

SPR technology was employed as a confirmatory biophysical assay in order to evaluate the affinity of the hits. The protocol used for this assay was similar to the label-free DMR assay. Briefly, the IL-17RA ECD-Fc receptor was immobilized, the interaction with the compounds was analysed, and the affinity binding constants were determined. Consistent with the DMR assay, both compounds showed specific binding to the receptor, although analogously to the DMR determination, higher micromolar concentrations of compounds showed unspecific binding (**Figure 1F-G**). As a result, the saturation curve did not reach maximal stability signals and, therefore, their binding constants (K_D) are estimated to be 34.6 μM and 22.6 μM for CBG040591 and CBG060392, respectively. Hence, compounds

CBG040591 and CBG060392 were identified as ligands with micromolar potency for the extracellular domain of the IL-17RA.

3.1.4 Model of ligand-IL-17A/IL-17RA interaction by molecular docking

CBG040591 and CBG060392 have quinazolinone and pyrrolidine-2,5-dione chemical scaffold, respectively (**Figure 2**). The affinity energy for the PDB structure 4HSA exceeds the established threshold to identify hits by the virtual screen in both ligands. The 4HSA structure comprises two complexes of IL-17A homodimer (chains A, B, D and E) with the extracellular domain of IL-17RA (chains C and F), showing the interaction and the conformational change of the cytokine-receptor complex. Affinity energies obtained were -10.5 kcal/mol for CBG040591 and -10.8 kcal/mol for CBG060392, indicating these quinazolinone and pyrrolidine-2,5-dione scaffolds may bind such IL-17A/IL-17RA complexes (**Figure 2**).

Interactions between quinazolinone CBG040591 and the dimer complex involved 26 residues: 20 of the C chain of the complex 4HSA, which is the extracellular domain of IL-17RA; and 6 of them with the B chain of the complex, which represents one of the monomers of the IL-17A dimer. Additionally, 4 hydrogen bonds interacted between the molecule and the target proteins (**Supplementary Table 1**). Interactions between CBG060392 pyrrolidine-2,5-dione and the complex IL-17A/IL-17RA included 23 residues: 15 of the F chain of the complex 4HSA, which is the extracellular domain of IL-17RA; and 8 of them with the E chain of the complex, which represents one of the monomers of the IL-17A homodimer. Additionally, 3 hydrogen bonds interacted between the molecule and the target proteins (**Supplementary Table 2**).

Compound CBG040591 and compound CBG060392 do not share the same core chemical structure, but our model predicted that both might interact within the same areas of the

IL-17A/IL-17RA complex. 10 of the residues that bind the molecules (both from IL-17RA and also from IL-17A) are common to both ligands (**Table 2**).

3.2 Chemokine release inhibition and anti-inflammatory activity of IL-17RA ligands

3.2.1 Evaluation of the inhibition of CCL20 and IL-8 release mediated by IL-17A in human keratinocytes

A CCL20 and IL-8 release assay was used to test the inhibitory activity of ligands of the IL-17A-mediated chemokine release in HaCaT human keratinocytes. Cells were stimulated with crescent concentrations of IL-17A and in the presence of 10 ng/ml of TNFα. 100 ng/ml IL-17A with 10 ng/ml TNFα were selected as a sufficient dose to stimulate cells and allow the quantification of an inhibitory activity (**Figure 3A**). The anti-IL-17A peptide (IHVTIPADLWDWINK) was employed as a positive control for the inhibition of the release of CCL20 and IL-8 after the IL-17A and TNFα synergic stimulation. The anti-IL-17A peptide inhibitory activity was calculated by a dose-response curve (concentrations between 15.6 and 1000 nM) and the IC_{50} was determined: 736 ± 115 nM and 378 ± 125 nM for IL-8 and CCL20 release blocking, respectively (**Figure 3B, Table 3**).

The potential blockade of the CCL20 and IL-8 release due to IL-17A and TNFα was also defined for both ligands, obtaining an inhibitory activity dependent on concentration (range of concentrations between 1.56 and 100 μM) (**Figure 3C-D, Table 3**). The inhibition of the production of CCL20 and IL-8 was partially blocked, with a percentage of reduction of the maximum IL-17A and TNFα response to 25 % for CCL20 for both ligands. The reduction in the release of IL-8 was different for both compounds: CBG060392

reduced the response up to 25.2 %, while the response for CBG040591 only reached 51.9 %. Therefore the small molecules are partially blocking the IL-17 aberrant inflammatory response existing in psoriatic lesions.

We also measured IL-6 and IL-1β release mediated by IL-17A and TNFα in HaCaT human keratinocytes (**Suppl Figure 1S**). Cells were stimulated with crescent concentrations of IL-17A and in the presence of 10 ng/ml of TNFα (**Suppl Figure 1S**). This cytokine combination induced a robust release of IL-6 (with a 3-fold difference between maximum to minimum signals, which are similar to those observed for CCL20 and IL-8), and to a lesser extent, it induced the release of IL-1β (where signal differences are 1.5 fold). In relation to the ligands, CBG060392 partially blocks the release of IL-6 induced by IL-17A and TNFα to an approximately 50%. This indicates that this ligand attenuates the inflammatory effect of this cytokines on IL-6 (**Suppl Figure S1 B**). CBG040591 ligand does not have an effect on IL-6 release, which is consistent with the potency and selectivity profile observed. On the other hand, the assay window for IL-1β release is limited to robustly observe differences. Thus, CBG060392 does not block significantly the release of this cytokine, while CBG040591 ligand may have an effect at only 25 μM (**Supp Figure S1 D**).

3.2.2 Evaluation of the selectivity of ligands in the inhibition of chemokine release in human keratinocytes

In order to evaluate whether the inhibitory response of ligands CBG040591 and CBG060392 was specific for the release of IL-8 and CCL20 mediated by IL-17A, keratinocytes were stimulated with 10 ng/ml TNFα in the absence of IL-17A. Previously, we obtained a dose-response curve for TNFα as a way to establish the maximal response achieved for this inflammatory stimulus (**Figure 3E**).

IL-8 and CCL20 release was quantified after stimulating the cell line with 10 ng/ml TNFα in the presence of 6,25 μM, 25 μM and 100 μM of ligands (**Figure 3F-G**). CBG040591 inhibits the release of both IL-8 and CCL20 at higher concentrations: CCL20 is inhibited with at least 25 μM; and IL-8 is inhibited only at 100 μM. On the other hand, CBG060392 does not inhibit the release of IL-8 nor CCL20 induced by TNFα. Therefore, CCL20 and IL-8 release elicited by TNFα was partially inhibited only by the quinazoline, indicating that this scaffold might not be selective at blocking only the IL-17A inflammatory response related to psoriasis. On the other hand, CBG060392 did not inhibit the release of IL-8 and CCL20 induced by TNFα, which suggests that IL-17A is required for its inhibitory activity.

3.3 Intracellular activity and signalling of IL-17A/IL-17RA inhibitors

3.3.1 Evaluation of the activity of ligands in *CCL20* and *CXCL8* expression mediated by IL-17A in human keratinocytes

A *CCL20* and *CXCL8* expression assay was set up in human keratinocytes, which were stimulated with 100 ng/ml IL-17A and 10 ng/ml of TNFα, 100 ng/ml IL-17A or 10 ng/ml TNFα for 24 hours (**Figure 4A**). An anti-IL-17A peptide was employed as a control for the inhibition of *CCL20* and *CXCL8* expression after an IL-17A and TNFα stimulation. A significant decrease in the expression of both chemokines was observed upon exposure to 1 μM of peptide (**Figure 4B**).

We evaluated the activity of both CBG040591 and CBG060392 in the inhibition of the expression of *CCL20* and *CXCL8* in HaCaT cells. For this, *CCL20* and *CXCL8* expression was obtained after the stimulation of the cell line with IL-17A and TNFα in the presence of 100 μM of each compound (**Figure 4C**). Compound CBG060392 showed a significant

391 inhibition of the expression of both *CCL20* and *CXCL8* in keratinocytes. On the other hand,
392 CBG040591 did not show a significant inhibition of the expression of any of the target
393 cytokines (**Figure 4C**). The repression of the gene transcription is consistent with the
394 inhibitory profile observed in chemokine release experiments, in which CCL20 and IL-8
395 release is partially blocked, and the inflammatory response blocked.

396

397 ### 3.3.2 Effect of the ligands on the IL-17A-induced NF-κB cascade in human
398 keratinocytes

399 We evaluated the effect of CBG040591 and CBG060392 on the proinflammatory signalling
400 mediated by IL-17A. For this purpose, expression of total and phosphorylated NF-κB p65
401 and IκBα was determined after the stimulation of HaCaT cells with 100 ng/ml IL-17A and
402 10 ng/ml TNFα for 24 hours, in the presence or absence of ligands.

403 The level of NF-κB p65 expression was similar both in cells non-stimulated and stimulated
404 with IL-17A and TNFα. The same applies in the presence of ligands (**Figure 5A**).
405 Proinflammatory stimulation with IL-17A and TNFα produced a significant increase in the
406 phosphorylation of NF-κB p65. This increase in phospho-NF-κB p65 was not attenuated by
407 the presence of CBG040591 nor CBG060392, which therefore did not have an effect on
408 p65 (**Figure 5B**).

409 The level of IκBα expression was similar both in cells non-stimulated and stimulated with
410 IL-17A and TNFα. Expression levels were the same for the cells treated with CBG040591
411 or CBG060392 (**Figure 5C**). Proinflammatory stimulation produced a significant increase
412 on the phosphorylation of IκBα. This increase was maintained in the presence of
413 CBG040591. Interestingly, with regard to CBG060392, a significant decrease of phospho-
414 IκBα (Ser32) was observed. This indicates that CBG060392 ligand partially inhibits the

proinflammatory signalling of IL-17A and TNFα at IκBα, which may result in the attenuation of canonical NF-κB inflammatory pathway (**Figure 5D**).

3.3.3 Effect of the ligands on the IL-17A inflammatory signalling in human keratinocytes

A *CEBPB* and *ZC3H12A* expression assay was set up in human keratinocytes with analogous conditions to the chemokine expression assay. Cells were stimulated with 100 ng/ml IL-17A and 10 ng/ml of TNFα, 100 ng/ml IL-17A or 10 ng/ml TNFα for 24 hours. The anti-IL-17A peptide (1 μM) was employed as a control for inhibition, because of its disruption of the interaction between IL-17A/IL-17RA. Therefore, both *CEBPB* and *ZC3H12A* expression were induced with IL-17A and TNFα, and significantly blocked with the use of the peptide (**Figure 6A-B**).

We then evaluated the activity of CBG040591 and CBG060392 in the expression of *CEBPB* in HaCaT cells. Keratinocytes were stimulated with IL-17A and TNFα in the presence of 100 μM of each compound. Both compounds showed a significant inhibition of the expression of *CEBPB*, indicating that they are preventing the transduction of the IL-17A receptor cascade (**Figure 6A**).

Furthermore, CBG040591 and CBG060392 were employed in the same conditions of stimulation as *CEBPB* expression assay in order to quantify *ZC3H12A* expression. CBG060392 showed a significant inhibition of the expression of *ZC3H12A* keratinocytes. On the contrary, the reduction of the expression of *ZC3H12A* by CBG040591 was not significant (**Figure 6B**).

4. Discussion

In this work, we have shown that it is feasible to use small molecules to block the IL-17A inflammatory activity in keratinocytes. The clinical success of the neutralizing antibodies that target interleukin-17A and the IL-17 receptor A for the treatment of psoriasis have sparked interest in this inflammatory cytokine pathway in autoimmune diseases [8,17,18]. The disrupted balance of proinflammatory and anti-inflammatory cytokines that occurs in the skin of patients with psoriasis is pharmacologically modulated and the clinical manifestations are improved with IL-17 inhibition. Nevertheless, there are still drawbacks on the development of antibodies as drugs, including not only their high cost of manufacture due to their complexity or the low drug survival in long-term treatments (only shown for secukinumab, but not for ixekizumab or brodalumab) but also their lack of oral bioavailability, the preferred administration route for patients, who are generally reluctant to use injectables [25,26]. Therefore, oral administration may improve the adherence and patient satisfaction with their chronic treatments [40]. Dosing regimen is another point to consider, since biologics do not need to be taken daily in psoriasis management, while optimal administration of small molecules would be defined by ADME, pharmacokinetics/pharmacodynamics and half-life time data to improve the effectiveness of treatment by maximizing drug efficacy and minimize toxicity.

Developing strategies that allow the identification of new ligands with anti-IL-17A activity may help to overcome the unmet clinical needs in autoimmune diseases such as psoriasis. In this work, we outlined a combination of *in silico* binding, biophysical and cell-based methodologies for the identification and validation of small molecules targeting the IL-17A/IL-17RA pathway. Both computational and biophysical approaches are broadly employed for hit identification in early drug discovery projects. Furthermore, along with structural biology, they are a common approach for the assessement of target druggability and for identifying binding pockets and ligands that disrupt protein-protein interactions

[41–46]. The surface of interaction between IL-17A and IL-17 receptor A is extensive and flat, occupying an area of approximately 2200 $Å^2$ [47]. This protein-protein interaction surface is difficult to target, and likely the main reason why there are no small molecules in development yet [33,41,43].

First, we carried out a target-based *in silico* screening with the BioFarma chemical library, which owns a degree of structural and biological diversity useful for the identification of original molecules. Molecules included in this library were rationally selected in order to meet standards of broad screening network initiatives such as EU-OPENSCREEN high-capacity screening platforms [48]. Indeed, physicochemical properties, drug-likeness, and maximal coverage of chemical space were among the main selection criteria to ensure the identification of hits for a wide spectrum of targets, including cytokines [49].

Once identified 67 small molecules as potential ligands, they were tested in a biophysical dynamic mass redistribution assay. 28 compounds (41.8 % of the total amount of molecules) exceeded the 25 % of IL-17A binding, the positive control for the assay, and only two hits exceeded 50 %. These binding activities allowed us to confirm that our DMR assay was efficient to identify ligands. Next, we characterized the binding of the two hits (CBG040591 and CBG060392) to the receptor IL-17RA by means of two biophysical assays: DMR and SPR. These label-free assays avoid interferences as any of the partners of the binding need to be labelled. Also, target proteins can be studied in their native conformation and non-invasively [50]. In particular, DMR was used in a biochemical assay format (where immobilized proteins and potential ligands are tested in solution), which has proven usefulness in defining the binding constants of biologically active peptides [51,52].

Both small molecules identified as low-potency ligands for IL-17RA ECD are novel structures not biologically annotated before. They have quinazolinone and pyrrolidine-2,5-dione chemical scaffolds, respectively. These structures have biological and

pharmacological significance, since a number of approved synthetic drugs contain similar scaffolds, including protein kinase inhibitors (e.g. gefitinib, lapatinib, erlotinib and afatinib) and anticonvulsants (e.g. levetiracetam, brivaracetam, ethosuximide and methsuximide). Although CBG040591 and CBG060392 do not share the same core chemical structure, our docking model shows that they interact within the same areas of the IL-17A/IL-17RA protein complex. This specific area is circumscribed to the interaction of one of the IL-17A monomers and the ECD of IL-17RA, which indicates that both ligands may be able to disrupt the protein-protein interaction. Interactions at hydrogen bonds and at 10 of the residues are common binding motifs between both ligands and, therefore, may constitute anchor residues for the rationale design and optimization of ligands targeting this pathway. Even if both molecules are structurally different, they share a common aromatic ring presenting two (CBG060392) and three (CBG040591) hydroxyl groups. In fact, both phenolic rings are oriented towards the same amino acid residues: Arg101, Gly140, Ile137 and Pro136 (Figure 2). Therefore, polyphenol interactions focused on strong hydrogen bonding may occur. In the case of CBG060392, hydrogen bonding between the Lys135-containing amide backbone and one of the carbonyl groups of the pyrrolidine-2,5-dione may occur. Finally, CBG040591 is chemically more rigid than CBG060392. The quinazolinone bicycle is a planar double ring conjugated system. CBG060392 has chemical fragments connected by rotatable bonds. This may explain a slightly better adaptability to the binding pocket, and the slightly better affinity for the IL-17A-IL-17RA complex.

Some limitations for further development of CBG040591 and CBG060392 as future drugs may be related to their structure and their binding activity. Both of them present low potency, which may limit their selectivity, as high doses would be required to achieve biological activity. Also, they have a phenolic structure that could influence *in vivo* toxicity and bioavailability because of their physicochemical properties. Small molecules whose scaffolds include phenols are considered to have a structural alert since phenols can

evolve into quinones, highly reactive metabolites [53,54]. However, sometimes the presence of phenols cannot be avoided, because they are widely used as building blocks that provide pharmacological activity and current drugs with phenols are safe in humans [53,55]. Given the absence of small molecules that antagonize IL-17A or its receptor, further optimisation and characterisation of CBG040591 and CBG060392 may provide pharmacological tools to modulate the IL-17A/IL-17RA pair in target tissues. However, medicinal chemistry and hit optimization in preclinical drug discovery are time-consuming and require an enormous effort. Assessing the efficacy of preliminary candidates might be a way to enhance the efficiency of these steps in the drug discovery process.

With this purpose, we decided to evaluate the efficacy of CBG040591 and CBG060392 as neutralizing molecules in an inflammatory cellular context related to psoriasis. For this, we employed HaCaT cells (human keratinocytes), which constitutively express the IL-17A receptor and represent a target for IL-17-mediated skin conditions, such as psoriasis [56–59]. In this regard, we used this model to evaluate the effect of the ligands on the neutralization of CCL20 and IL-8 release mediated by IL-17A in the presence of TNFα. Similar *in vitro* neutralization assays were used to determine the functional inhibition of IL-17A by antibodies such as ixekizumab, and were required for its development and subsequent clinical approval [60]. The use of TNFα, and not only IL-17A, is justified due to the fact that it synergizes with IL-17A to produce a measurable inflammatory response in keratinocytes. Either of them used independently also generates a response, but together they enhance the inflammatory cytokine release [61–65]. This integrative inflammatory cytokine release event is especially relevant as a trigger in the psoriasis pathogenesis [64,66]. Keratinocytes are affected by the excessive presence of IL-17A in psoriasis, where the role of IL-17A has been demonstrated central, which was further established by the success of therapeutic neutralizing antibodies [67–70]. An anti-IL-17A peptide was employed as a positive control to test the efficacy of CBG040591 and CBG060392 ligands,

as we confirmed that potently and completely inhibits the proinflammatory activity of IL-17A in HaCaT cells, similarly to what was previously described [33]. In fact, we obtained comparable IC_{50} values for the inhibition of the release of CCL20 and IL-8 mediated by IL-17A and TNFα.

CBG040591 and CBG060392 partially blocked the release of CCL20 and IL-8 produced by IL-17A stimulation in the presence of TNFα. Particularly, CBG060392 achieved the reduction of the maximum IL-17A and TNFα response to 25 % for the release of both chemokines. This inhibitory effect can be explained because of their binding to the interaction between IL-17A and the extracellular domain of IL-17RA, as we reported in our docking binding model. Thus, the molecules are not only disrupting this interaction but also inhibiting the inflammatory chemokine release generated by IL-17A in the presence of TNFα in human keratinocytes. In order to evaluate whether this inhibitory response of CBG040591 and CBG060392 was specific for the chemokine release mediated by IL-17A, cells were stimulated with TNFα in the absence of IL-17A. While CBG040591 inhibits the release of both IL-8 and CCL20 at high concentrations, CBG060392 does not inhibit the release of IL-8 nor CCL20 induced by TNFα and, therefore, it can be considered as a selective inhibitor of the IL-17A ligand-receptor interaction. This difference between both of the small molecules may be explained because of their different chemical scaffolds (quinazolinone and pyrrolidinedione, respectively). This might be further investigated by means of structure-activity relationship studies, in which a series of synthetic ligands could be tested using the biophysical and cell-based methodologies already developed in this study.

We then decided to evaluate whether the gene expression of *CCL20* and *CXCL8* induced with IL-17A in the presence of TNFα was altered by CBG040591 or CBG060392. Analogously to the synergy of both IL-17A and TNFα in the release of inflammatory mediators, the expression of these target genes is the result of the ability of IL-17A to

stabilize the mRNA transcripts induced by TNFα [71,72]. The interruption of the protein-protein interaction by CBG040591 and CBG060392 resulted in reduced expression of this target genes, in correlation with the chemokine release data. Only the pyrrolidinedione CBG060392 showed a significant inhibition of the expression of *CCL20* and *CXCL8* in keratinocytes, which suggests that it is blocking the expression of the proinflammatory target genes for IL-17A.

The anti-inflammatory activity of ligands was further evaluated in relation to their effect on the canonical NF-κB pathway. Although IL-17A only has a mild effect on triggering NF-κB, this cascade is also activated by TNFα [9,65,73]. We measured the effect on p65 (also named RelA) and IκBα, being the first one of the transcription factors of the NF-κB family [74]. When IκBα is phosphorylated it is tagged for ubiquitination and degradation, this allows the activation of the NF-κB complex, which is then translocated into the nucleus [75]. We observed a significant increase of phospho-NF-κB p65 due to the stimulation with IL-17A and TNFα after a 24-hour treatment that appeared not to be attenuated by the presence of CBG040591 nor CBG060392. Interestingly, CBG060392 inhibited the phosphorylation of IκBα at serine 32, the initial event that targets IκBα for degradation. This IκBα inhibition may be the result of the blockade of the IL-17A/IL-17RA interaction by CBG060392, and therefore, the IL-17RA signal transduction pathway NF-κB. This partial anti-inflammatory activity in IL-17A is consistent with the selectivity of CBG060392 in preventing the expression of the proinflammatory target genes and their resulting chemokine release, which suggests that this ligand might be a promising starting point for an early drug discovery program to target this pathway.

The anti-inflammatory activity of CBG040591 and CBG060392 was also assessed in relation to their intracellular activity in other IL-17R signalling pathways. We evaluated the anti-inflammatory activity of CBG040591 and CBG060392 in the expression of C/EBPβ. This transcription factor is phosphorylated after ERK1/2 and GSK3β signalling

pathways, whose activation derives from IL-17R transduction triggered by IL-17A [9,12]. IL-17A and TNFα functional cooperation in inflammation is also mediated by the activation of C/EBP family [76]. The interruption of the protein-protein interaction by CBG040591 and CBG060392 resulted in a significant decrease in the expression of *CEBPB*, preventing the transduction of the IL-17A receptor cascade. C/EBPβ regulates the activation and expression of IL-17 target genes; therefore, this inhibitory activity of CBG040591 and CBG060392 is consistent with the results in chemokine expression and release. We also evaluated the activity of CBG040591 and CBG060392 in the expression of Regnase-1 (ZC3H12A). It is a CCCH zinc finger protein, which has RNAse activity and, therefore, Regnase-1 can directly degrade the mRNA of IL-17 target genes, inhibiting the IL-17 response. Thus, this post-transcriptional mechanism stops the IL-17 proinflammatory signalling that may be potentially destructive [8]. Regnase-1 expression is induced by IL-17 via NF-κB [14,77], which was observed in our keratinocyte model. Although CBG040591 reduced the expression of *ZC3H12A*, only CBG060392 showed a significant inhibition of *ZC3H12A* in human keratinocytes. This suggests that the pyrrolidinedione scaffold is preventing the activation of the IL-17A receptor transduction. Consequently, negative postranscriptional regulation of IL-17A, that is, *ZC3H12A* expression, it is also reduced.

Collectively, our data show that a combination of binding, biophysical and cell-based assays can be used to identify novel small molecules targeting the IL-17A/IL-17RA pathway. These molecules are immunopharmacological ligands for the IL-17 receptor A and have a robust inhibitory effect of the production of inflammatory chemokines in human keratinocytes, potentially working as modulators of the disrupted immune response in the skin.

Figures

Figure 1. Screening and affinity determination of ligands of the extracellular domain of IL-17RA by biophysical assays. (**A**) Binding potency of IL-17A fraction to IL-17RA ECD Fc (25 μg/ml) in a dynamic mass redistribution (DMR) assay. Mean ± SEM of three independent experiments. (**B** and **C**) Screening of 67 small molecules (20 μM) in a DMR assay. The binding threshold was fixed as mean + 2 × SD. Positive control (IL-17A) is represented as a triangle and negative control (assay buffer) as a square. ****P <0,0001 (Student's t test) (**D** and **E**) Binding potency of CBG040591 and CBG060392 to IL-17RA ECD Fc (25 μg/ml) in a DMR assay. Mean ± SEM of three independent experiments. (**F** and **G**) Binding of CBG040591 and CBG060392 to immobilized IL-17RA ECD Fc in a surface plasmon resonance assay. Response units (RU) shown are different in each axis. Mean of two independent experiments.

Figure 2. Model of ligand-IL-17A/IL-17RA interaction by molecular docking. Molecular structures and model of binding between the small molecules CBG040591 (**A**) or CBG060392 (**B**) and the PDB structure 4HSA: a complex formed by two IL-17A homodimers and the extracellular domain of IL-17RA. Residues that participate in the binding are shown.

Figure 3. Evaluation of the ligand inhibition of CCL20 and IL-8 release mediated by IL-17A in human keratinocytes. (**A**) Release of CCL20 and IL-8 in response to increasing concentrations of IL-17A and IL-17A with 10 ng/ml TNFα in HaCaT cell line. Mean of median fluorescence intensity (MFI) ± SEM of two independent experiments. Inhibition of CCL20 and IL-8 release produced by IL-17A and TNFα in response to increasing concentrations of anti-IL-17A peptide (**B**), CBG040591 (**C**) and CBG060392 (**D**) in HaCaT cell line. MFI ± SEM of four independent experiments. (**E**) Release of CCL20 and IL-8 in response to increasing concentrations of TNFα in HaCaT cell line. MFI ± SEM of two

independent experiments. Inhibition of CCL20 and IL-8 release produced by TNFα (10 ng/ml) in response to CBG040591 (**F**) and CBG060392 (**G**) in HaCaT. MFI ± SEM of four independent experiments. *P <0,05; ** P <0,01; *** P <0,001 (ANOVA).

Figure 4. Evaluation of the inhibitory activity of ligands in *CCL20* and *CXCL8* expression mediated by IL-17A in human keratinocytes. (**A**) Expression of *CCL20* and CXCL8 in response to 100 ng/ml IL-17A and 10 ng/ml TNFα, 100 ng/ml IL-17A or 10 ng/ml TNFα in HaCaT cell line. Mean of expression ± SEM of three independent experiments. * P <0,05; ** P <0,01; *** P <0,001 (ANOVA). Expression of *CCL20* and *CXCL8* in response to 100 ng/ml IL-17A and 10 ng/ml TNFα inhibited by the anti-IL-17A peptide (**B**), CBG040591 (**C**) and CBG060392 (**C**) in HaCaT cell line. Axes are different for each cytokine. Mean of expression ± SEM of three independent experiments. * P <0,05; ** P <0,01 (ANOVA).

Figure 5. Effect of the ligands on the NF-κB pathway in human keratinocytes. Western blot of NF-κB p65 (**A**) and phospho-NF-κB p65 (Ser536) (**B**) in HaCaT cells treated with 100 ng/ml IL-17A and 10 ng/ml TNFα in the absence or presence of 100 μM CBG040591 or CBG060392. A representative image of six independent experiments is shown. Mean of NF-κB p65 and phospho-NF-κB p65 intensity ± SEM of six independent experiments. Western blot of IκBα (**C**) and phospho-IκBα (Ser32) (**D**) in HaCaT cells treated with 100 ng/ml IL-17A and 10 ng/ml TNFα in the absence or presence of 100 μM CBG040591 or CBG060392. A representative image of five independent experiments is shown. Mean of IκBα and phospho-IκBα intensity ± SEM of five independent experiments. * P <0,05; ** P <0,01; *** P <0,001 (ANOVA).

Figure 6. Effect of the ligands on the IL-17A inflammatory signalling in human keratinocytes. (**A**) Expression of *CEBPB* in response to 100 ng/ml IL-17A and 10 ng/ml TNFα, 100 ng/ml IL-17A or 10 ng/ml TNFα inhibited by the anti-IL-17A peptide, CBG040591 or CBG060392 in HaCaT cell line. Mean of expression ± SEM of three

674 independent experiments (**B**) Expression of *ZC3H12A* in response to 100 ng/ml IL-17A
675 and 10 ng/ml TNFα, 100 ng/ml IL-17A or 10 ng/ml TNFα inhibited by the anti-IL-17A
676 peptide, CBG040591 or CBG060392 in HaCaT cell line. Mean of expression ± SEM of three
677 independent experiments. * P <0,05; ** P <0,01; *** P <0,001; **** P <0,0001 (ANOVA).

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Tables

Table 1. Dynamic redistribution assay parameters.

Level of IL-17RA ECD Fc immobilization	1546 pm
CV IL-17A (positive control)	19.2 %
CV assay buffer (negative control)	19.8 %
Signal to background ratio	5.13
Z'	0.47

Table 2. Common residues to ligands CBG040591 and CBG060392 and the IL-17A/IL-17RA complex of PDB structure 4HSA.

Protein chain	Amino acid	Atom
Interactions atom – residue		
IL-17A	Pro59	CB
IL-17RA	Ile137	O
IL-17RA	Pro138	CA
IL-17RA	Pro136	CG
IL-17RA	Lys135	HZ2
IL-17RA	Gly140	C
IL-17RA	His131	NE2
IL-17A	Arg101	NH1
IL-17A	Ser64	HG
IL-17RA	Ser84	HG

Table 3. Inhibitory response of anti-IL-17A peptide, CBG040591 and CBG060392 (IC_{50} and percentage of the reduction of the maximum IL-17A and TNFα response) of the IL-8 and CCL20 release in HaCaT cell line normalized to the release produced by IL-17A and TNFα. Mean ± SEM of four independent experiments.

	CCL20 IC_{50} (μM)	**CCL20** Reduction of the max response (%)	**IL-8** IC_{50} (μM)	**IL-8** Reduction of the max response (%)
Anti-IL-17A peptide	0.378 ± 0.125	-4.9 ± 11.8	0.736 ± 0.115	1.9 ± 8.2
CBG040591	20.9 ± 12.6	28.0 ± 6.8	26.7 ± 13.1	51.9 ± 6.6
CBG060392	23.6 ± 11.8	16.5 ± 12.06	45.3 ± 13.0	25.2 ± 7.0

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CRediT authorship contribution statement

Elia Álvarez-Coiradas: Methodology, Conceptualization, Investigation, Formal analysis, Visualization, Writing - Original Draft. **Cristian R. Munteanu:** Methodology, Software, Formal analysis. **Laura Díaz-Sáez:** Methodology, Investigation. **Alejandro Pazos:** Writing - Review & Editing, Supervision. **Killian V. M. Huber:** Supervision, Resources. **María Isabel Loza:** Writing - Review & Editing, Supervision. **Eduardo Domínguez:** Conceptualization, Visualization, Project administration, Writing - Review & Editing, Supervision.

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Declaration of Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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