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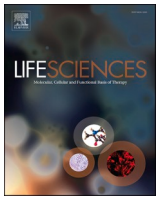

# Substituted 2-pyrrolinone compounds as inhibitors of B-cell activating factor (BAFF) for autoimmune diseases treatment

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ABSTRACT

B-cell activating factor (BAFF) is a cytokine that plays a critical role in the proliferation and differentiation of B cells. We have previously demonstrated that its inherited overexpression is associated with increased circulating B cell and immunoglobulin levels, correlating with increased risk of multiple sclerosis and systemic lupus erythematosus. These findings suggest that enhanced BAFF expression may be involved in the causal biology of these disorders, thus supporting the rationale for therapeutic inhibition of this cytokine. However, to date, no small-molecule modulator capable of inhibiting BAFF is available. We therefore employed a virtual screening approach to analyze a library of 275,561 small molecules, followed by in vitro validation of 218 selected compounds with potential to disrupt the interaction between BAFF and its receptor BAFFR. Our results identified two promising small molecules, C45 and C145, belonging to the substituted 2-pyrrolinone family, which effectively inhibited BAFF activity. These compounds warrant further investigation as potential therapeutic agents for BAFF-driven autoimmune diseases.

## 1. Introduction

Approaches that combine genome wide association studies (GWAS) and functional genomics experiments have proven to be effective in identifying novel susceptibility risk genes and more specific drug targets for human disease. Indeed, drug targets supported by genetic studies are more likely to lead to the identification of new treatments [1,2] enhancing the chances of successful drug discovery programs [3–6].

We previously demonstrated, through a genetic approach, that a genetic variant in the TNFSF13B gene, which encodes the B-cell-activating factor (BAFF) cytokine, is associated with an increased risk of multiple sclerosis (MS) and systemic lupus erythematosus (SLE) [7]. By searching for coincident associations of genetic variants affecting the risk of autoimmune disease and quantitative immune variables, we established that the same causal variant, through increased circulating levels of soluble BAFF (sBAFF), is associated with up-regulated humoral immunity, B lymphocytes and immunoglobulins [7,8]. These findings support the primary role of sBAFF overexpression (i.e. gain of function) and the resulting increase in B cells and immunoglobulins levels in the pathogenesis of MS and SLE.

BAFF is a membrane-bound type II transmembrane protein (mBAFF) mainly expressed on the surface of myeloid cells [9]. It can be released upon proteolytic processing at furin consensus site to produce sBAFF. As a pro-survival factor, BAFF maintains B cell maturation and homeostasis at various B cell differentiation stages [10] through the binding with three receptors, mainly expressed on B-lymphocytes: BAFFR (BR3), Transmembrane activator and CAML interactor (TACI), and B cell maturation antigen (BCMA). Interestingly, each receptor has different binding affinities for BAFF. BAFFR binds only sBAFF [11] and with a greater affinity than the other two receptors. Since 1999, several BAFF modulators have entered clinical trials, but some have been rapidly discontinued (Table 1). To date, BAFF inhibitor – belimumab – has been approved for treatment of SLE and Lupus Nephritis and other autoimmune conditions, by many different regulatory agencies. Belimumab was also under development for the treatment of other condition affecting B cell functions, including MS (NCT04767698); Addition of belimumab to B-cell Depletion in Relapsing-remitting Multiple Sclerosis), a study interrupted in 2023 and, systemic sclerosis associated interstitial lung disease (NCT05878717), have been approved for generalized myasthenia gravis as a result of positive outcome from a

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**Table 1**
Drugs targeting the BAFF.

| Generic Drug Name | Global Status | Development Status | Drug Disease | Mechanism Of Action |
|---|---|---|---|---|
| AB-001, AKSO Biopharmaceutical | Preclinical | Active | Berger's disease; Cancer, lymphoma, B-cell; Colitis, ulcerative; Irritable bowel syndrome; Lupus erythematosus, systemic | APRIL inhibitor; B-cell activating factor inhibitor; Immuno-oncology therapy |
| anti-BAFF MAb, Dana | No Development Reported | Ceased | Multiple myeloma | B-cell activating factor inhibitor |
| Ardenermin | No Development Reported | Ceased | IgA deficiency | B-cell activating factor inhibitor; B-cell activating factor stimulant; DNA inhibitor |
| Atacicept | Phase III Clinical Trial | Active | Renal Glomerulosclerosis, Primary membranous nephropathy, Rheumatoid Arthritis, Chronic Lymphocytic Leukaemia, B-cell lymphoma, Non-Hodgkin's lymphoma, Multiple myeloma, Waldenstrom's hypergammaglobulinaemia, Berger's disease, Systemic Lupus Erythematosus, Multiple sclerosis relapsing-remitting, Lupus Nephritis | APRIL inhibitor; B-cell activating factor inhibitor; B-cell stimulant |
| AUR-200 | Phase I Clinical Trial | Active | Renal failure, Systemic Lupus Erythematosus | APRIL inhibitor; B-cell activating factor inhibitor |
| BAFF inhibitors, Xencor | No Development Reported | Ceased | Immunological disease, unspecified | B-cell activating factor inhibitor |
| BAFF-R/CD3 bispecific antibody, Pepromene Bio | Preclinical | Active | Cancer, unspecified | B-cell activator factor receptor antagonist; CD3 agonist; Immuno-oncology therapy |
| Belimumab | Launched | Active | Lupus erythematosus, systemic, Nephritis, lupus, Systemic sclerosis-associated interstitial lung disease, Connective tissue disease-associated interstitial lung disease, Arthritis, rheumatoid, Inflammation, vascular, Microscopic polyangiitis, Multiple sclerosis, unspecified, Myasthenia gravis, Nephrotic syndrome, Scleroderma, Sjogren's disease, Thrombocytopenia, unspecified, Transplant rejection, organ, Waldenstrom's hypergammaglobulinaemia, Wegener's granulomatosis | B-cell activating factor inhibitor |
| Blisibimod | Discontinued | Ceased | Berger's disease, Chronic lymphocytic leukaemia, Multiple myeloma, Vascular inflammation, Systemic lupus erythematosus, Idiopathic thrombocytopenic purpura | B-cell activating factor inhibitor |
| BLyS radiolabelled, HGS | No Development Reported | Ceased | Marginal zone B-cell lymphoma, Non-Hodgkin's lymphoma, Multiple myeloma | B-cell inhibitor; DNA inhibitor |
| Briobacept | No Development Reported | Ceased | Rheumatoid arthritis, Sjögren's disease | B-cell activating factor inhibitor |
| CD19-BAFF CAR-T cell therapy, Yake Biotechnology | Phase I Clinical Trial | Active | Dermatomyositis, Systemic lupus erythematosus, Nephritis (unspecified), Neuromyelitis optica, Scleroderma | Immuno-oncology therapy; T cell stimulant |
| ESG-206 | Phase II Clinical Trial | Active | Idiopathic thrombocytopenic purpura, B-cell lymphoma, B-cell lymphoma | B-cell activating factor inhibitor; B-cell activator factor receptor antagonist; Tumour necrosis factor ligand antagonist |
| Ianalumab | Phase III Clinical Trial | Active | Autoimmune haemolytic anaemia, Non-infectious hepatitis, Systemic lupus erythematosus, Lupus nephritis, Sjögren's syndrome, Idiopathic thrombocytopenic purpura, Rheumatoid arthritis, Hidradenitis suppurativa, Scleroderma, Chronic lymphocytic leukaemia, Non-Hodgkin B-cell lymphoma, Idiopathic pulmonary fibrosis, Relapsing-remitting multiple sclerosis, Pemphigus | B-cell activating factor inhibitor; B-cell activator factor receptor antagonist; Immunoglobulin inhibitor; Tumour necrosis factor ligand antagonist |
| LMY-920 | Phase I Clinical Trial | Active | Non-Hodgkin's lymphoma, Myeloma, Systemic lupus erythematosus, B-cell lymphoma, Unspecified lupus erythematosus, Mantle cell lymphoma, Scleroderma, Sjögren's syndrome | B-cell activating factor inhibitor; Immuno-oncology therapy; T cell stimulant |
| LMY-921 | Preclinical | Active | B-cell lymphoma, Mantle cell lymphoma | Immuno-oncology therapy; T cell stimulant |
| LMY-922 | Preclinical | Active | Lupus erythematosus<br>Scleroderma | T cell stimulant |
| LY-4152199 | Preclinical | Active | Non-Hodgkin's lymphoma | B-cell activator factor receptor antagonist; CD3 agonist; Immuno-oncology therapy; T cell stimulant |
| OSX-100 | No Development Reported | Ceased | Inflammation, muscle | B-cell activator factor receptor antagonist |
| OSX-110 | No Development Reported | Ceased | Multiple sclerosis | B-cell activating factor inhibitor |
| PMB-101 | Phase I Clinical Trial | Active | Acute lymphocytic leukaemia, Follicular lymphoma, Mantle cell lymphoma | CD3 antagonist; Immuno-oncology therapy; T cell stimulant |

(*continued on next page*)

**Table 1** (*continued*)

| Generic Drug Name | Global Status | Development Status | Drug Disease | Mechanism Of Action |
|---|---|---|---|---|
| Povetacicept | Phase III Clinical Trial | Active | Berger's disease, Autoimmune haemolytic anaemia, Cold agglutinin disease, Lupus nephritis, Primary membranous nephropathy, Idiopathic thrombocytopenic purpura, Unspecified autoimmune disease, Systemic lupus erythematosus, Sjogren's disease, Unspecified blood/clotting disease, Unspecified dermatological disease, Renal failure | APRIL inhibitor; B-cell activating factor inhibitor |
| Rozibafusp alfa | No Development Reported | Ceased | Rheumatoid arthritis, Systemic lupus erythematosus | B-cell activating factor inhibitor; Immune checkpoint stimulant; Inducible T-cell costimulator ligand inhibitor |
| SBI-3150 | Phase I Clinical Trial | Active | Systemic lupus erythematosus, Nephritis | B-cell inhibitor; Immunosuppressant; Interferon beta antagonist |
| Tabalumab | Discontinued | Ceased | Rheumatoid arthritis, Myeloma cancer, Chronic renal failure, Systemic lupus erythematosus, Relapsing-remitting multiple sclerosis | B-cell activating factor inhibitor |
| TE-2324 | No Development Reported | Ceased | Lupus erythematosus, systemic<br>Sjogren's disease | B-cell activating factor inhibitor; Collagen inhibitor |
| Telitacicept | Launched | Active | Rheumatoid arthritis, Systemic lupus erythematosus, Myasthenia gravis, Berger's disease, Neuromyelitis optica, Sjogren's disease, Relapsing-remitting multiple sclerosis, Unspecified multiple sclerosis, Lupus nephritis | APRIL inhibitor; B-cell activating factor inhibitor |
| Tibulizumab | Phase II Clinical Trial | Active | Hidradenitis suppurativa, Scleroderma, Rheumatoid arthritis, Systemic lupus erythematosus, Sjogren's disease | B-cell activating factor inhibitor; Interleukin 17 antagonist; Interleukin 17 A antagonist; Tumour necrosis factor ligand antagonist |
| UBP-1213 | Phase I Clinical Trial | Active | Systemic lupus erythematosus, Autoimmune disease, unspecified, Inflammatory disease, unspecified | B-cell activating factor inhibitor |
| VT-109 | Preclinical | Active | Immunological disease, unspecified | Angiotensin I converting enzyme inhibitor; B-cell activator factor receptor antagonist |

phase three clinical trial (NCT05737160).

Overall, even if a growing body of evidence now suggests that B cells play an active role in the pathogenesis not only of SLE but also of MS [12] many details of the disease processes, as well as their similarities and differences in these two conditions, remain unclear. The specific receptor(s) through which increased levels of the cytokine sBAFF exert a disease predisposing role, as well as the downstream mechanisms involved in MS and SLE, remain a subject of debate. Similarly, the specific B cell subtypes, and their products primarily involved in the pathogenesis of the two diseases and the mechanisms underlying they action, remain unknown.

Nevertheless, additional studies are needed to better understand these aspects and no approved small-molecule modulator is currently available to inhibit sBAFF.

Here we therefore decided to perform a screening to identify small molecules able to block sBAFF functions. Small molecules are organic compounds with low molecular weight and a well-defined chemical structure, characterized by clear and stable pharmacokinetics. They are generally suitable for oral administration and are typically non-immunogenic [13]. Due to their small size, they are also more likely to cross the blood–brain barrier (BBB), which is particularly important in diseases such as MS, where a dysregulated immune system can impair brain function [14]. We performed a comprehensive *in-silico* Virtual Screening (VS) to identify small molecules potentially capable of inhibiting sBAFF functions. Starting from the crystallographic structure of the complex, we developed a structure-based pharmacophore model to screen commercial compound virtual libraries. Hits were then refined through molecular docking and *in-silico* pharmacokinetics profiling. The top-ranked candidates were subsequently validated using in vitro assays. Our results support substituted 2-pyrrolinone (specifically compounds C45 and C145) as promising leads for the generation of novel BAFF inhibitors.

## 2. Materials and methods

### 2.1. In-silico virtual screening

The VS pipeline used in our study was implemented as follows.

*Database generation for screening*. A reference library composed by the following commercial databases has been generated: Asinex Gold (205,883 compounds), Asinex BioDesign (48,291 compounds), Asinex protein-protein interactions (11,387 compound) and Sigma Aldrich Diverse Collection (10,000 compound) [15,16]. All the possible stereoisomers and tautomers have been generated using the LigPrep program from the Schrodinger software [17].

*Generation of Pharmacophore model.* The crystal structure of BAFF in complex with BAFFR was retrieved from the Protein Data Bank (PDB ID: 1OQE) [18,19]. The complex was solvated in a TIP3P water box, with an ionic strength of 0.15 M, and the system charge was neutralized. Energy minimization (2500 steps) and equilibration (1 ns) were performed using NAMD 2.9 [20]. Molecular dynamics (MD) simulations lasting 100 ns were conducted using ACEMD [21] on an NVIDIA GTX580 GPU. Frames from the simulations were clustered using the SPICKER tool [22], and the most populated cluster was used to generate the pharmacophore. PHASE, a pharmacophore modelling solution for ligand- and structure-based drug design (Schrödinger Release 2013–3), was used to construct the pharmacophore model.

*Molecular Docking studies*. The Autodock Tool (ADT) was used for docking studies [23]. Initially, Autogrid4, as implemented in the Autodock4 software package, was used to generate grid maps. The Lamarckian genetic algorithm (LGA) was then employed to generate orientations/conformations of the ligand within the binding site. For each compound, 250 independent runs and a maximum number of 1,000,000 operations were performed with a population size of 500 individuals.

*ADME-Tox Studies*. To predict *in-silico* absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox), each selected molecule was processed using the QuikProp tool, an advanced tool for predicting pharmacokinetic and physicochemical (ADME-Tox) properties of small molecules (Schrödinger Release 2013–3).

### 2.2. In silico modelling and analysis of APRIL

The structure of APRIL was obtained from the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/entry/O75888) and prepared using the Protein Preparation Wizard (Schrödinger Suite 2024–3). Molecular docking was performed using AutoDock Tools (ADT), and binding energies were refined using MM-GBSA calculations in Prime (Schrödinger Suite 2024–3). All parameters and procedures were identical to those used for BAFF.

### 2.3. MM-GBSA calculations

The binding free energy of the complexes was estimated using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach. Calculations were performed with the Prime module (Schrödinger Release 2024–3). The VSGB solvation model [24] was applied to account for solvation effects, and the OPLS4 force field [25] was used for energy calculations.

### 2.4. Cell culture

COS-1 cells (a fibroblast-like cell line), Ramos (Burkitt's Lymphoma cells), MM.1S (B lymphoblast cell) and Raji cells (Burkitt's lymphoma cells) were purchased from ATCC.

COS-1 cells were cultured in DMEM; Ramos, MM.1S and Raji cells were cultured in RPMI-1640 Medium, each supplemented with 10 % fetal bovine serum and antibiotics; All reagents used for cell culture were purchased from Gibco (Uxbridge, UK). Cells were cultured in a humidified incubator at 37 °C/5 % CO2.

### 2.5. Small molecules and MTT assay

The 218 small molecules identified through VS were purchased from Asinex. Molecules were diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA) to create a stock solution of 100 mM and stored at −20 °C. The presence of crystal and colour was annotated for each molecule (Supplemental Table S1).

Cell proliferation was assessed by monitoring the conversion of 3-(4, 5-dimethylthiazolyl−2) − 2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, USA) to formazan, as previously described by Berridge et al. [26]. Briefly, Raji cells ($1 \times 10^4$ cells/mL; 90 μL/well) which express BAFFR at both mRNA level and on the cell surface as demonstrated by RT-qPCR and Flow Cytometry respectively (Supplemental Fig. S1), were seeded into 96-well plates and incubated for 12 h in incubator to allow recovery. The day after, test compounds were added to each well at a final concentration of 30 μM in the presence of recombinant soluble BAFF (rsBAFF, 200 ng/mL) to assess the ability of the compounds to inhibit BAFF-induced cells proliferation; recombinant human BAFF was purchased from DBA (Ames, USA). To ensure a balance between assay sensitivity and specificity, we used a concentration of 30 μM, which falls within the range (10–30 μM) commonly applied in high-throughput screening (HTS) assays. This concentration is sufficiently high to detect early hit activity while minimizing cytotoxic effects. It is also compatible with the DMSO tolerance of the assay system used and is in line with established HTS practices [27,28].

Experimental controls included human recombinant BAFFR (hrBAFFR) at a final concentration of 4 ng/mL (Vinci-Biochem, Italy), which competes with the endogenous BAFF receptor (BAFFR) expressed on Raji cells and thereby inhibits BAFF signalling, and 0.1 % DMSO, which served as a negative control. Cell viability was assessed after 48 h by adding 5 mg/mL of MTT to each well avoiding light exposure. Following 2 h incubation at 37 °C formazan crystals were solubilized by adding solubilization solution (10 % Triton-X100, 0,1 N HCL in isopropanol). Absorbance measurements were performed using the Tecan Sunrise™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 570 nm. The XFLUOR4 software program was used to read the results.

### 2.6. Toxicity assay

Cell cytotoxicity of the 5 best compounds was assessed in Raji cells using CellTox™ green cytotoxicity assay (Promega, Madison, USA). Cells were seeded at a density of $1 \times 10^4$ cells/well in 96 well plates in presence of the selected compounds for 48 h. After this incubation period, cells were treated with 100 μL CellToxTM Green reagent (2× prepared as a 1:500 dilution of CellToxTM Green Dye in Assay Buffer). The resulting solution was incubated at room temperature for 15 min (min) and fluorescence then measured at 495/519 nm (λex/λem). CellToxTM green signals were normalized by setting the untreated control as 0 % of the toxicity.

### 2.7. Cloning of the hybrid receptor (HR), stable cell line generation and characterization

Based on the work of Hu et al. [29], a hybrid receptor (HR) was generated, comprising the extracellular BAFF-binding domain of BAFFR (nt 1–195 containing characteristically spaced cysteine residues essential for binding sBAFF) and the intracellular signalling domain of TNFR1, which is critical for transducing the signal within the cell and includes the TNFR1-associated death domain. This hybrid receptor (HR = BAFFR–TNFR1) enables the transduction of the sBAFF signal via the fast-acting canonical NF-κB pathway, thereby allowing TNF-specific downstream signalling in response to BAFF stimulation.

- PCR amplification and cloning.

To generate the HR we initially performed a polymerase chain reaction (PCR) to amplify: (i) the extracellular domain of BAFFR (nt 1–195) and (ii) the cytoplasmic domain of TNFR1 (nt 230–499). Amplification was carried out using DreamTaq Polymerase (Invitrogen, Waltham, USA) with the following primers: BAFFR (FW: ACCATGAGGCGAGGGCCC RV: GGCAGCGCCGCCTCGCCGGC), TNFR FW: GGCACTGAGGACTCAGGCACCA RV: GCCTCATCTGAGGAAGACTG). The amplified fragments were then cloned in pGEM-T Easy (Promega, Madison, USA) vector generating the constructs: pGEMteasy-BAFFR and pGEMteasy-TNFR.

To extract the respective inserts, pGEMteasy-BAFFR was digested with *Kpn*I and MaeI, and pGEMteasy-TNFR1 with MaeI and BamHI. The BAFFR and TNFR1 fragments were gel-purified and ligated into the mammalian expression vector pcDNA3.1/V5-His A, previously digested with KpnI and BamHI, generating the final construct pcDNA3.1-BAFFR/TNFR1 (HR). All restriction enzymes were purchased from New England Biolabs (Ipswich, USA). An empty vector control (pcDNA3.1-EV) was also prepared and used as a control. All plasmid constructs were sequence-verified by Sanger sequencing.

- Generation of stable cell lines and evaluation of the generated stable line.

The expression vector pcDNA3.1-BAFFR/TNFR1 and the empty vector pCDNA3.1-EV were digested and linearized with the *Bgl*II restriction enzyme. A total of 20 μg of linearized DNA was transfected into $2.5 \times 10^6$ COS-1 cells using Lipofectamine (LTX) (Invitrogen, Waltham, USA) following the manufacturer's protocol. 24 h later cells were transferred into 6 well plates and selection of positive clones using 600 μg/mL of geneticin (G418) started.

To assess the level of endogenous BAFFR protein on the membrane of Raji cells and the efficiency of the stable line generated, cells were analysed by flow cytometry using unconjugated anti-BAFFR antibody (ABCAM, Cambridge, UK). Briefly, $1 \times 10^5$ cells were incubated with anti-BAFFR antibody for 20 min at 4 °C and then washed with phosphate-buffered saline in 5 % BSA (Bovine serum albumin).

Following incubation with secondary antibody (Goat anti-rabbit Alexa Fluor 488, Thermo Fisher Scientific, Invitrogen, Waltham, USA)) cells were washed, re-suspended in FACSFlow solution (BD-Bioscience, San Jose, USA) and finally analysed using FACSCanto cytometer (BD-Bioscience, San Jose, USA). Data analysis was performed using FACS-Diva software Version 6.1.3 and Flowjo v10.8.1 (BD-Bioscience, San Jose, USA). To evaluate the functionality of the stable cell lines, COS-1 cells expressing the HR were stimulated with recombinant soluble BAFF (rsBAFF) and IL-8 production in the culture supernatant was measured using an ELISA assay. IL-8 levels served as a readout for NF-κB activation and were used to identify the most active and responsive clones.

### 2.8. ELISA assay

IL-8 production was measured in supernatants of COS-1 cells transfected with the HR and EV and treated as follows: cells were treated for 48 h with the selected compounds at a single concentration of 30uM or with 0.1 % DMSO (negative control) in presence of rsBAFF (200 ng/mL). hrBAFFR (1 mg/mL) was used to neutralise rsBAFF and function as positive control. After treatment, 50 μL of supernatant from each well were removed, and the level of IL-8 expression was quantified using a human IL-8 ELISA kit (R&D Systems, Minneapolis, USA)), following the manufacturer's instructions. All reagents and samples were equilibrated to room temperature prior to use, and all standards, controls, and samples were assayed in duplicate. Briefly, 100 μL of assay diluent was added to each well, followed by 50 μL of standard, control, or sample. After a 2 h incubation at room temperature, wells were washed four times and incubated with 100 μL of IL-8 conjugate for 1 h. Following additional washes, 200 μL of substrate solution was added and incubated for 30 min in the dark. The reaction was stopped with 50 μL of stop solution, and absorbance was measured at 450 nm with correction at 540 nm.

### 2.9. Dose response assay

The inhibitory activity of selected compounds was investigated in a dose response manner using COS-1 cells stably transfected with the HR and the selected small molecules (C45 and C145) at the concentrations of 10, 1, 0.1 and 0.01 μM. The inhibition of the proliferation activity was assessed in terms of the concentration capable of reducing the measured IL-8 production as described above.

### 2.10. RNA extraction e RT-qPCR

Total RNA was extracted from Raji, Ramos and MM.1S cells using the TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) and following the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically. 100 ng of total RNA were then reverse transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) and random hexamers and analysed by quantitative (q)PCR analysis using SYBR Green mix (Kapa Biosystems) and BAFFR specific primers (FW: GGAA-GACCCAGGAACCAC; RV: AAGGCAAGCACACCAAA). Gene expression levels were normalized to GAPDH (FW: TGCACCACCAACTGCTTAGC; RV: GGCATGGACTGTGGTCATGAG), and the relative mRNA expression levels were calculated by the ΔΔCt method.

### 2.11. Structure-activity relationship (SAR) analysis

Analogous compounds were identified in the MolPort database of commercial compounds (https://www.molport.com/shop/index) by searching for analogues of the two active compounds, C45 and C145, with a Tanimoto similarity index greater than 0.75.

### 2.12. Statistical analyses

R-package software was used to analyze the experimental data. The statistical significance of experimental differences was determined by *t*-test, and $p$-value$<0.05$ was statistically significant.

## 3. Results

### 3.1. In Silico identification of small molecules targeting the BAFF–BAFFR interface

Virtual screening (VS) is an in silico method designed to explore large libraries of compounds efficiently. Compared to traditional high-throughput screening (HTS), VS typically yields a higher hit rate employing a series of advanced computational filters to predict the binding affinity of ligands to biological targets, thereby significantly reducing the number of compounds that need to be tested experimentally.

To identify novel compounds interacting with sBAFF and possibly inhibiting its activity, we implemented a VS protocol (Fig. 1) using four commercial compound databases (Asinex and Sigma Aldrich Diverse Collection) [19].

The VS protocol was performed through sequential steps here summarized (Fig. 1):

a) Database generation for screening. Starting from 275,561 compounds (Asinex and Sigma Aldrich Diverse collection), molecules were processed to generate all the possible stereoisomers, tautomers and ionisation states, resulting in the creation of a 3D database for screening.
b) Generation of Pharmacophore model. We first used a molecular dynamics (MD) simulation to explore the most relevant amino acids residues implicated in BAFF-BAFFR interaction. Next based on the structural insights obtained, a structure-based pharmacophore model was generated to identify and exclude compounds lacking basic chemical, steric, and electronic characteristics for the interaction with the target (sBAFF).

Briefly, the starting point of the MD simulation was the X-ray crystal structure of BAFF in complex with the receptor BAFF-R [19]. All structure frames resulting from the MD simulation were clustered, and the most populated frame was chosen as the reference. All possible interactions at the binding site were tracked throughout the MD simulation.

The reference structure was then used to generate a structure-based pharmacophore model, that is a 3D model representing the key steric and electronic features necessary for molecular recognition by the biological target. The generated BAFF pharmacophore included 7 features: 3 positive ionizable, 2 hydrophobic, 1 negative ionizable, and 1H-bond acceptor (Fig. 2). This model was subsequently applied to screen the previously described 3D compound database in order to identify small molecules that matched the pharmacophoric criteria.

c) Docking studies. The 6200 compounds that passed the initial pharmacophore-based screening were subjected to molecular docking simulations [23] to evaluate their ability to bind to the active site of the BAFF protein. These simulations were performed to predict the preferred binding orientations of each compound within the active site of the target and to identify specific interactions between the ligand functional groups and amino acid residues in the designed binding site. Docking analysis enabled the elimination of compounds that failed to interact meaningfully or lacked specificity for the binding site. Key parameters used in the docking evaluation included predicted binding energy, number and population of clusters, binding mode analysis, and characterization of molecular interactions.

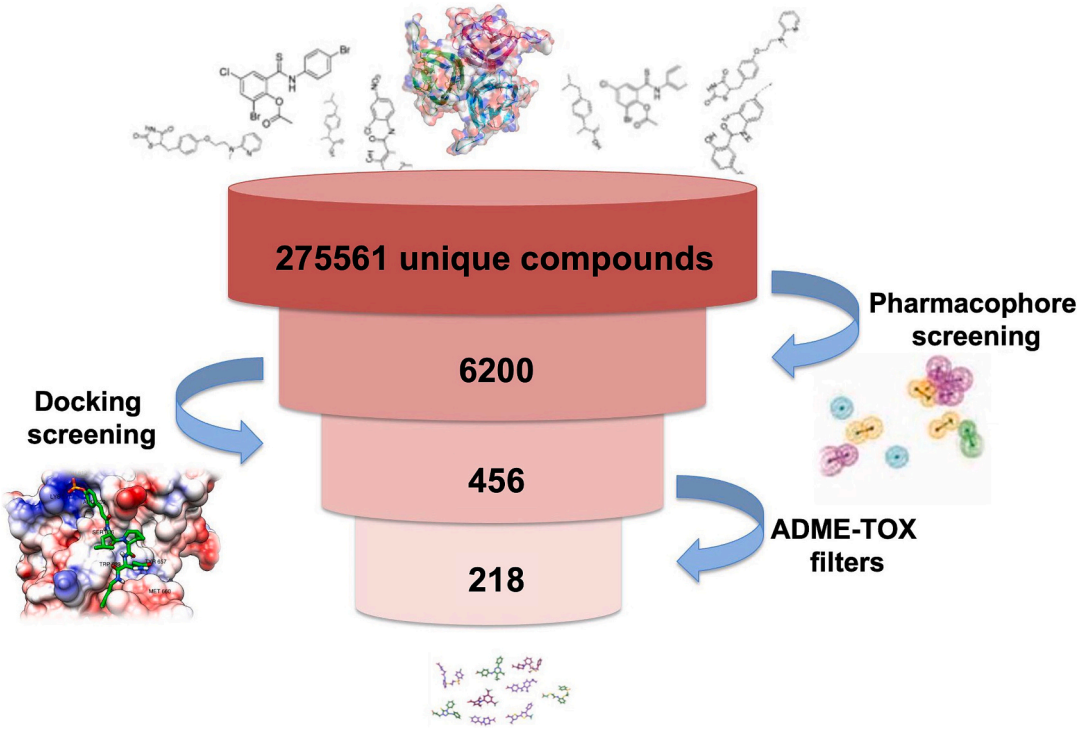



**Fig. 1.** Virtual Screening. The screening workflow that was applied to discern novel BAFF-BAFFR inhibitors.

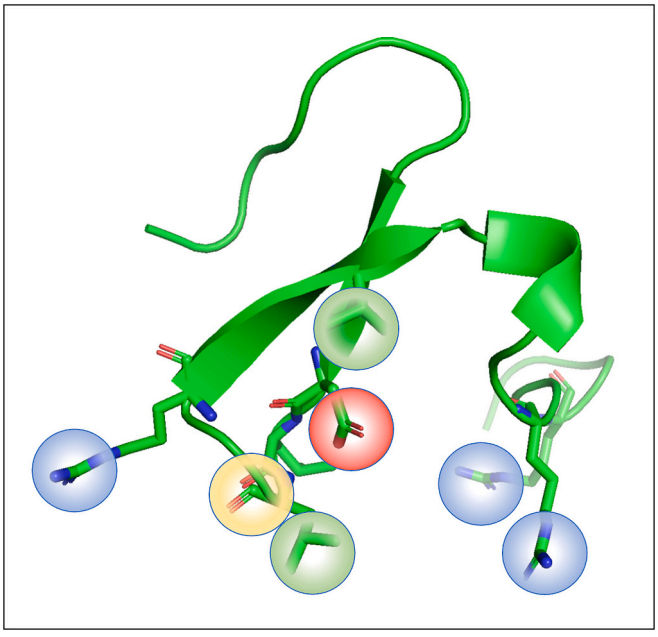


**Fig. 2.** Pharmacophore model. Pharmacophore model illustrating functional features here represented as a sphere: 3 positive ionizable (blue), 2 hydrophobic (green), 1 negative ionizable (red), and 1 H-bond acceptor (yellow).

d) ADME-Tox Studies. Finally for the remaining 456 unique compounds, we performed an in silico evaluation of ADME-Tox properties. Compounds that did not meet established thresholds for absorption, distribution, metabolism, excretion, or toxicity—particularly those inconsistent with oral drug-likeness—were excluded.

Ultimately, 218 compounds satisfied all virtual screening criteria and were selected for purchase and subsequent in vitro validation (Fig. 1).

### *3.2. Primary screening and follow-up assays identifies five inhibitors of sBAFF-Induced proliferation*

The top 218 hits identified through VS and confirmed to be commercially available were purchased for primary in vitro testing. Each compound was tested at a single concentration of 30 μM in duplicate on Raji cells cultured in 96-well plates. DMSO was used as the negative control, and a recombinant BAFF receptor (rsBAFFR) served as the positive control, as described in the Materials and Methods section. After 48 h of incubation, cell proliferation was assessed by measuring metabolic activity using the MTT assay (Fig. 3A).

Inhibition of cell proliferation was observed in approximately 50 % of the treated compounds (data not shown). Notably, inhibitory effects were detected in both conditions: cells treated with the compound alone (light blue bars) and cells co-treated with the compound and rsBAFF (dark blue bars), as exemplified by the 5 best-performing compounds shown in Fig. 3B. Based on these results, the five compounds - C32, C45, C72, C92, and C145- were selected for follow-up validation. Initially, an additional MTT assay, performed under the same experimental conditions, confirmed that all five compounds were capable of inhibiting rsBAFF-induced proliferation by approximately 50 % (Fig. 4A).

To evaluate potential cytotoxic effects, Raji cells were subjected to Cytotoxicity Assay following 48 h treatment with each compound at concentrations of 120–60–30-15-7.5–3.25 μM. No significant cytotoxicity was observed at the concentration of 30 μM and lower for any of the molecules tested (Fig. 4B and Supplemental Fig. S2). Notably, C92 and C145 did not show toxic effects even at higher concentrations of 60 and 120 μM.

In summary, the primary screening and subsequent validation identified five small molecules capable of selectively inhibiting BAFF-induced cell proliferation without exhibiting cytotoxic effects in Raji cells.

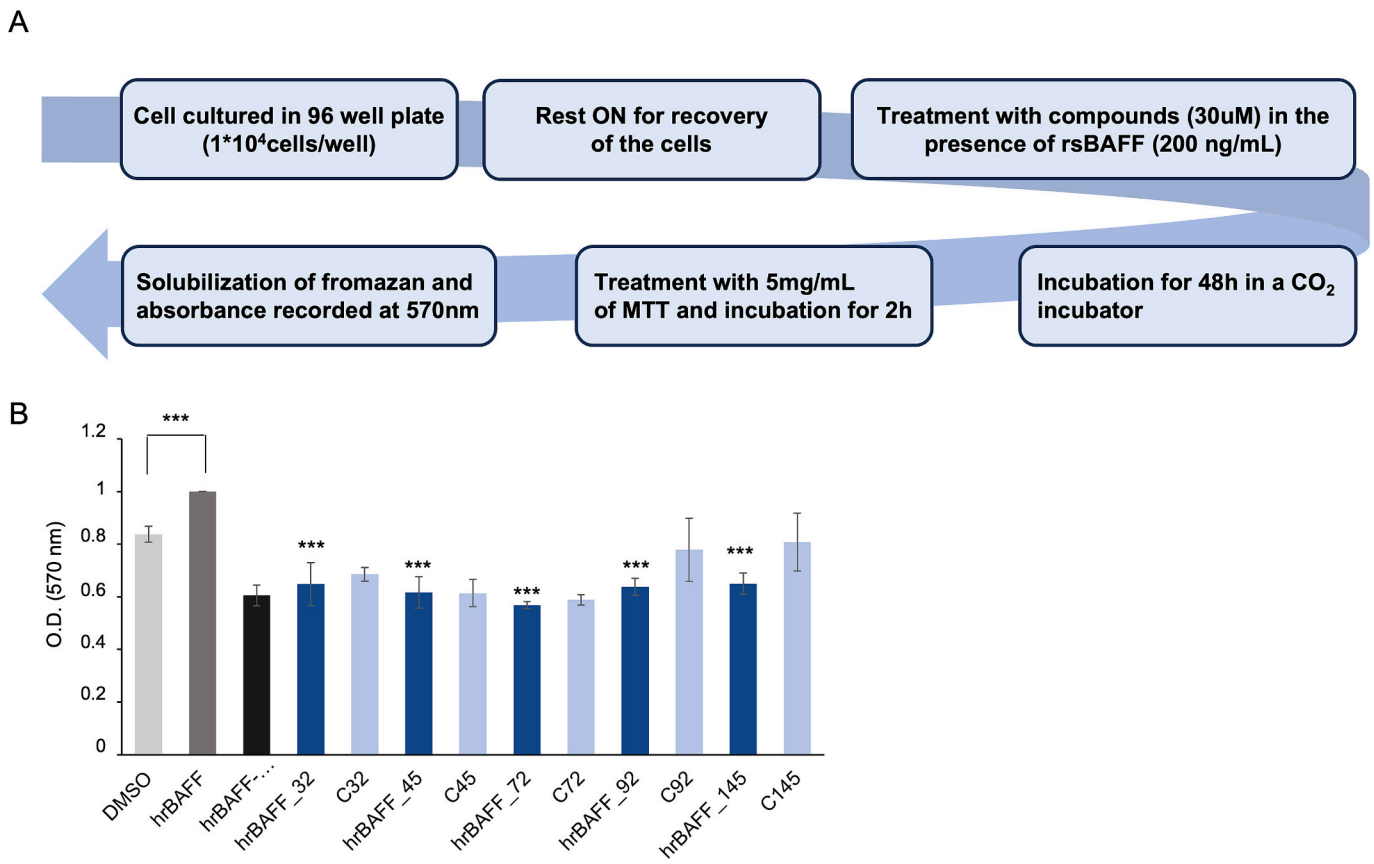



**Fig. 3.** Primary MTT assay to evaluate reduction in sBAFF cell-induced proliferation driven by selected the compounds. (A) Schematic representation of MTT assay protocol. Raji cells were cultured and treated in duplicate with 30 μM of the 218 small molecule inhibitors selected using the VS in presence of soluble BAFF for 48 h. The MTT assay was used to assess the drug's effect on cell proliferation. (B) Results for the 5 compounds selected for further studies are shown. Data are presented as mean ± SD. Statistical significance was assumed for P-values $< 0.05$ (***$P < 0.001$).

### *3.3. C45 and C145 inhibit BAFF-induced IL-8 production in a HR cell-based assay*

Subsequently, we validated five candidate compounds using a stable cell line expressing the hybrid receptor (HR), composed of the extracellular domain of human BAFFR fused to the intracellular domain of TNFR1 (see Material and Methods). The HR consists of the BAFFR extracellular domain, which binds soluble BAFF (sBAFF), fused to the TNFR1 intracellular domain, which activates the canonical NF-κB pathway and induces IL-8 production. In contrast to the native BAFF–BAFFR axis, which primarily involves the non-canonical pathway, this artificial system provides a rapid, quantifiable IL-8 readout upon sBAFF stimulation, offering a clean and controlled system to assess BAFF–BAFFR interactions.

After cloning, the newly generated pcDNA3.1-BAFFR-TNFR1 vector was checked to evaluate the correctness of the open reading frame sequence using Sanger sequencing (Fig. 5A).

Subsequently, pcDNA3.1-BAFFR-TNFR1 and the empty vector control (pcDNA3.1-EV) were stably transfected into COS-1 cells. Next, both cell lines were analysed by flow cytofluorimetry (a powerful technique that can be used to analyze receptor expression on cell surface) using antibody anti-BAFFR to assess the presence of the HR. Indeed, flow cytofluorimetry analysis confirmed the cell-surface expression of the HR with 60 % of BAFFR positive cells in pcDNA3.1-BAFFR-TNFR1 as compared to the EV control (Fig. 5B).

Subsequently, to optimize rsBAFF stimulation conditions, COS-1-HR and COS-1-EV cells were treated with varying concentrations of rsBAFF at different time points; 200 ng/mL for 48 h was selected as the optimal condition for maximal response (data not shown).

Using the newly generated cell line, we tested the ability of compounds C32, C45, C72, C92, and C145 to inhibit rsBAFF-induced cell activation using IL-8 levels as read out. As described above DMSO and human recombinant BAFFR were used as negative and positive controls, respectively (Fig. 5C). As expected, rsBAFF stimulation induced IL-8 production, which was significantly reduced by hrBAFFR through neutralization of rsBAFF activity. Interestingly, compounds C32, C72 and C92 did not show consistent results while compounds C45 and C145 significantly and consistently inhibited rsBAFF-induced IL-8 secretion, in line with prior MTT assay results. C45 and C145 were selected for further analysis.

### *3.4. Evaluation of BAFF inhibition by C45 and C145 in multiple B cell lines and dose-response assessment*

Initially, we performed an additional MTT assay using two other B cell lines, Ramos and MM.1S, which express BAFFR at comparable levels to Raji cells, as assessed by RT-qPCR (Supplemental Fig. S1). The results from these cell lines were consistent with our previous findings in Raji cells and support the ability of compounds C45 and C145 to inhibit BAFF-induced cell proliferation. These data reinforce our observations and strengthen the evidence that C45 and C145 can modulate BAFF activity across multiple B cell models expressing BAFFR (Supplemental Fig. S3A-B). Next, to better characterize the inhibitory activity of C45 and C145, a dose–response analysis was conducted using decreasing concentrations of the compounds (10, 1, 0.1, 0.01 μM), in the presence of a fixed amount of rsBAFF. Both compounds demonstrated a concentration-dependent suppression of IL-8 production, supporting their capacity to counteract HR activation. While the response was not

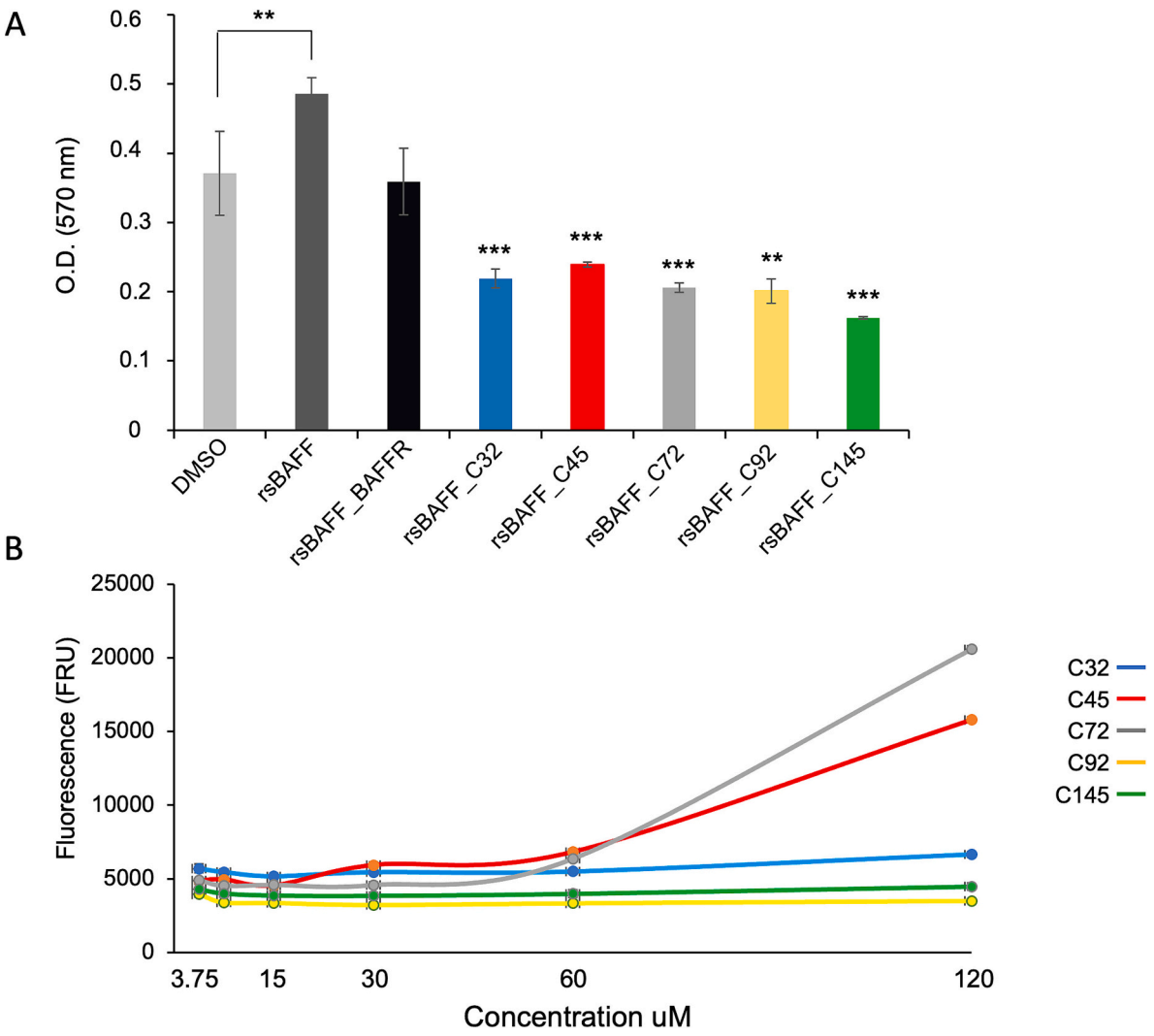



**Fig. 4.** MTT and cell toxicity assays to validate the best compounds identified with the primary assay. Raji cells were cultured and treated with the five best compounds identified in the primary assay at a single concentration of 30 μM. (A) Cell proliferation was assessed using an MTT assay; (B) cell toxicity using CellTox™ green cytotoxicity assay. Results are represented as a mean ± SD from at least three independent experiments. Statistical significance was assumed for P-values $< 0.05$ (**P $< 0.01$, ***P $< 0.001$).

strictly linear across all concentrations, a clear trend of reduced cytokine secretion with increasing compound potency was observed, supporting the potential of C45 and C145 as effective BAFF pathway inhibitors (Fig. 6).

### 3.5. *In-silico prediction of chemical-physical properties of C45 and C145*

Next, we evaluated the pharmacokinetic and drug-like properties of C45 and C145 to determine their suitability as starting points for further optimization. To this end, the ADME-Tox properties were predicted using QikProp. The main pharmacokinetic parameters relevant to the two active compounds identified in the assays are summarized in Table 2. Specifically, we evaluated lipophilicity (expressed as the n-octanol/water partition coefficient, LogP), cellular permeability through the Caco-2 model (predictive of intestinal absorption) and MDCK permeability (associated with CNS penetration), predicted oral absorption, the number of potential metabolites, and estimated central nervous system (CNS) activity. We also assessed drug-likeness parameters, including any violations of Lipinski's Rule of Five and Jorgensen's Rule, the number of properties falling outside the 95 % confidence interval for known drugs (#stars), and the presence of reactive functional groups (#rtvFG). Both compounds displayed acceptable pharmacokinetic properties and drug-like characteristics; however, further optimization will be required, particularly to enhance cellular permeability. Although C45 exhibited higher permeability than C145, the overall permeability of both compounds remains below the desired threshold and will be a key focus in future development. Neither compound is predicted to penetrate the CNS, but both showed favourable oral absorption, supporting their potential as promising starting points for subsequent optimization.

### 3.6. *Binding mode and selectivity of C45 and C145*

To gain structural insights into the potential mode of action and selectivity of C45 and C145, we carried out molecular docking and comparative binding analyses against BAFF and its closest homolog, APRIL. Molecular docking analyses suggest that both C45 and C145 bind at the BAFF–BAFFR interaction interface, occupying a pocket involved in receptor recognition (Fig. 7). The predicted binding poses reveal favourable interactions, including hydrogen bonds and hydrophobic contacts with residues critical for BAFF–receptor engagement.

To assess the selectivity of these compounds, next we performed comparative docking studies using AutoDock on both BAFF and APRIL. APRIL was chosen for this analysis because it is the most homologous

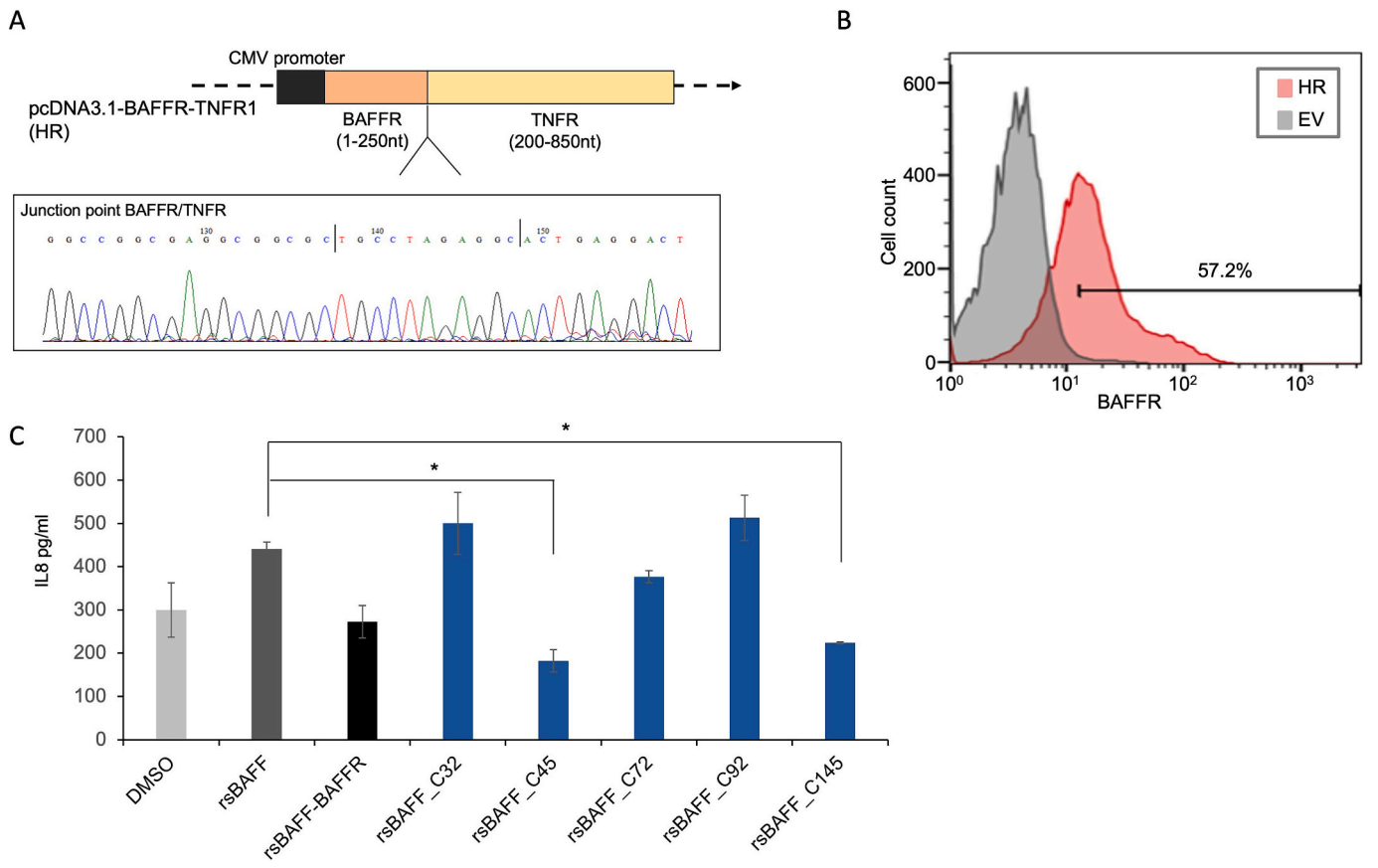



**Fig. 5.** Validation and functional characterization of the Hybrid BAFFR-TNFR1 Receptor in COS-1 Cells. (A) Electropherograms from direct sequencing of the hybrid receptor (HR) generated, comprising the extracellular BAFF-binding domain of BAFFR (nt 1-195) that contain the characteristically spaced cysteine residues essential for binding sBAFF, and the intracellular signaling domain of TNFR. (B) Flow cytometry using unconjugated anti-BAFFR was performed to assess the expression of the HR on COS-1 cells after stable transfection. Data analysis was performed using FACSDiva software Version 6.1.3 and Flowjo v10.8.1. (C) To further validate the five best compounds, we used the stably generated cell lines expressing the HR. Cells were stimulated with recombinant soluble BAFF (rsBAFF) in presence of C32, C45, C72, C 96 and C145. IL-8 production in supernatant was used as readout and levels measured using an ELISA assay. In C results are represented as mean ± SD from at least three independent experiments. Statistical significance was assumed for P-values $< 0.05$ (*$P < 0.05$).

TNF family member to BAFF and shares two receptors—BCMA and TACI—with it. Docking scores were more favourable for BAFF in both cases: C45 showed a binding energy of −7.28 kcal/mol for BAFF versus −5.50 kcal/mol for APRIL, while C145 showed −7.03 kcal/mol for BAFF versus −4.97 kcal/mol for APRIL. These results indicate a preferential interaction of C45 and C145 with BAFF.

To further support these findings, we subsequently carried out MM-GBSA binding free energy calculations using the Prime module. Consistently, these results confirmed preferential binding to BAFF: for C45, the predicted binding free energy was −38.64 kcal/mol for BAFF and − 37.20 kcal/mol for APRIL; for C145, −43.93 kcal/mol for BAFF and − 39.85 kcal/mol for APRIL.

Taken together, these results indicate that both compounds exhibit higher predicted affinity for BAFF than for APRIL, suggesting a degree of selectivity. However, as C45 and C145 are still hit compounds and further optimization will be necessary to improve their binding affinity and enhance their selectivity in the lead development phase.

### *3.7. Structure–activity relationship study identifies active analogues of C45 and C145*

Compounds C45 and C145 are structural analogues (Table 3), which prompted us to expand the pool of active candidates and perform a structure-activity relationship (SAR) study. Using a SAR approach based on Tanimoto similarity, nine additional analogues (designated AN1–AN9) were selected for their similarity to C45 and C145, as summarized in Table 3. Specifically, two of these compounds are close analogues of C45, while the remaining seven are analogues of C145. To evaluate their biological activity in vitro, COS-1 cells stably expressing HR were treated with each analogue at a single concentration (10 μM) in the presence or absence of recombinant soluble BAFF (rsBAFF) for 48 h. IL-8 production was then measured by ELISA as previously described. Among the nine tested analogues, four compounds demonstrated significant activity at 10 μM: AN6 and AN8 (analogues of C45), as well as AN2 (analogues of C145), all of which effectively inhibited rsBAFF-induced receptor activation (Supplemental Fig. S4). By contrast AN5 showed a significative upregulation of the rsBAFF - induced BAFF proliferation.

These results confirm the potential of these analogues, in particular the C45 group which showed more consistent results, as promising leads for further optimization and detailed pharmacological characterization.

## 4. Discussion

Genetics is playing an increasingly important role in the research and development of new therapies by revealing key molecules and mechanisms underlying diseases through ‘experiments of nature’. This allows robust therapeutic hypotheses based on causal biology to be formulated. In this context, target identification and validation are critical in drug development, where the high failure rate, particularly during clinical trials, emphasizes the need for selecting biologically relevant targets [30,31]. Thus, to improve clinical success, focusing on targets supported

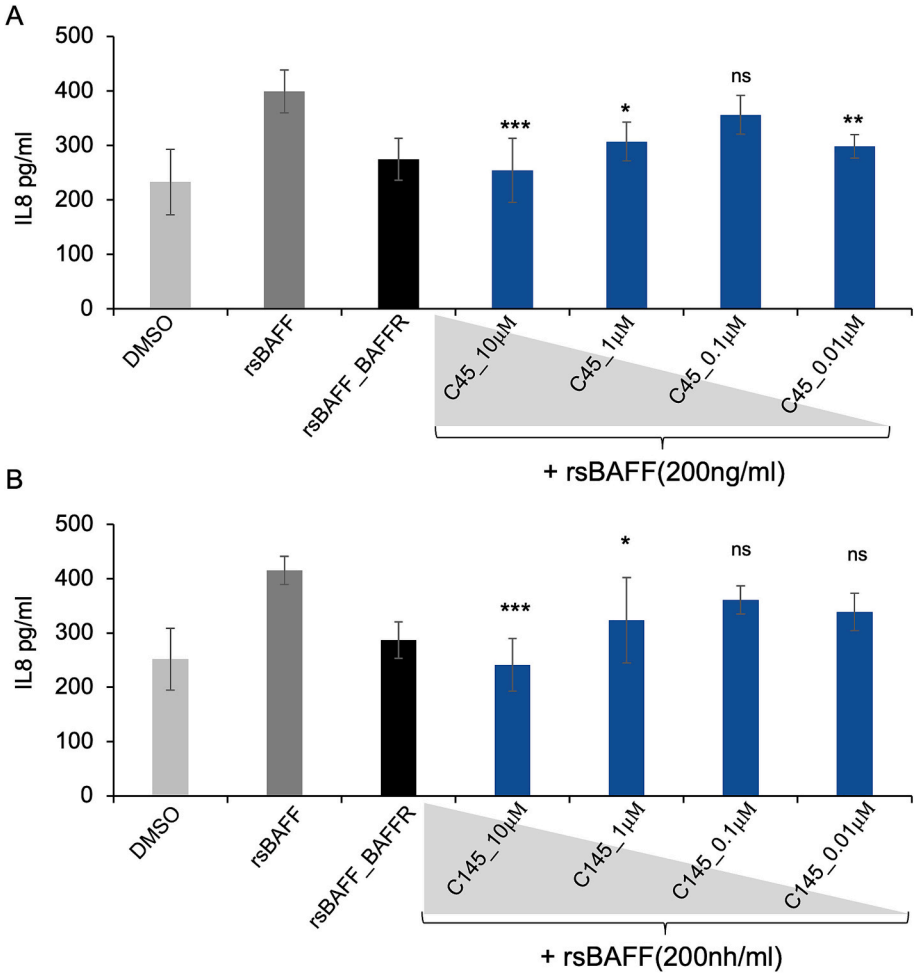



**Fig. 6.** Dose response curves for the active compounds. COS-1 cells stably expressing the HR were treated for 48 h with C45 and C145 at the following concentrations 10-1-0.1-0.001 μM for 48 h in the presence of rsBAFF. IL-8 production in supernatant was used as readout and levels were measured using an ELISA assay. Data are shown as mean ± SD from at least three independent experiments. Statistical significance was assumed for P-values $< 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

**Table 2**
In silico pharmacokinetic parameters for C45 and C145.

| Principal Descriptor | Range 95 % of Drugs | C45 | C145 |
|---|---|---|---|
| MW | 130 / 725 | 410.42 | 457.91 |
| #starts | 0 to 5 | 0 | 0 |
| #rtvFG | 0 to 2 | 0 | 0 |
| Predicted CNS Activity | a − 2 (inactive) to +2 (active) | -2 | -2 |
| Apparent MDCK Permeability (nm/s) | (<25 po or, >500 great) | 56.6 | 24.1 |
| Apparent Caco-2 Permeability (nm/s) | (<25 po or, >500 great) | 134.5 | 21.6 |
| QP logP for octanol/water | (−2.0 / 6.5) | 2.9 | 3.8 |
| No. of Primary Metabolites | (1.0 / 8.0) | 5 | 5 |
| Human Oral Absorption | 1 low, 2 medium, 3 high | 3 | 2 |
| % Human Oral Absorption in GI | (<25 % is poor) | 84 % | 76 % |
| Lipinski Rule of 5 Violations | (maximum is 4) | 0 | 0 |
| Jorgensen Rule of 3 Violations | (maximum is 3) | 0 | 0 |

#starts: Number of property or descriptor values that fall outside the 95 % range of similar values for known drugs.
#rtvFG: Number of reactive functional groups.

by genetic evidence is crucial [5]. Here we decided to identify novel small molecules that can disrupt the interaction between BAFF and its receptor, BAFFR, and based the selection of the candidate target BAFF based on our previous published data [7] and state of art literature supporting the relevance of BAFF in autoimmune diseases [32,33].

BAFF is a cytokine which belongs to the TNF ligand superfamily, that is essential for B-cell maturation, survival, and differentiation. It binds to three receptors—BAFFR, TACI, and BCMA—but interacts uniquely with BAFFR activating the non-canonical NF-κB pathway, a key process in immune regulation. Unlike the rapid and transient canonical NF-κB pathway, the non-canonical pathway is slower and more sustained, playing a critical role in regulating genes involved in B-cell function [34].

Elevated BAFF levels are implicated in autoimmune diseases like MS and SLE, as well as malignancies such as multiple myeloma and lymphoma [7,35,36]. In autoimmune diseases, BAFF overexpression predominantly affects humoral immunity, aligning with the observed efficacy of B-cell depletion therapies (add references on ocrelizumab etc). Moreover, supported by our previous results we hypothesised that patients stratified by BAFF-var variant status may show varying responses to anti-BAFF therapies, potentially due to a faster recurrence of memory B cells [7]. It is important also to underline that B cells contribute to autoimmune pathogenesis not only through autoantibody production but also by antigen presentation, cytokine secretion, and formation of tertiary lymphoid structures, which support the efficacy of B cell depletion therapies in diseases such as rheumatoid arthritis and MS despite limited effects on autoantibody levels [37–39]. CD20, expressed on B cells from pre-B to memory stages but absent on plasma cells, is the main target of therapies like rituximab, which depletes $CD20^{+}$ and prevents the Ab-independent B cell function, resulting in

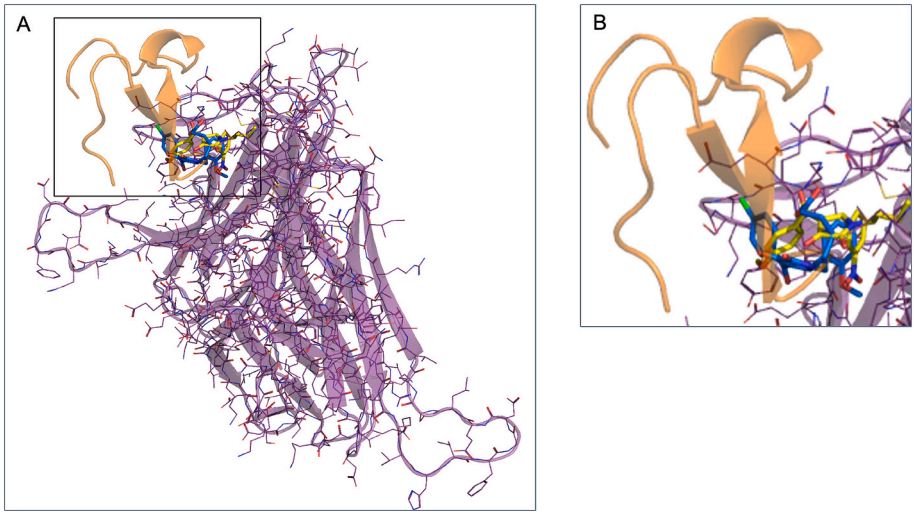



**Fig. 7.** Predicted binding mode of compounds C45 and C145 on BAFF. The docking poses show both compounds, C45 in yellow and C145 in blue, occupying the BAFF–BAFFR interaction interface, suggesting potential interference with receptor binding. BAFF is shown in purple and BAFF-R in orange. (A) Overall view of the entire BAFF monomer with BAFFR and the ligands C45 and C145. (B) Close-up of the binding region.

attenuation of autoimmune inflammation [40]. Among the primary therapies available are biological drugs that target BAFF (Table 1), such as belimumab; it is a complete human monoclonal antibody approved [41,42] for SLE that inhibits BAFF, reducing B-cell proliferation and survival. It is also being evaluated in combination with ocrelizumab, an anti-CD20 monoclonal antibody, for MS to enhance B-cell modulation (NCT04767698); the trial has been discontinued for MS due to registered adverse effects on patients. Povetacicept (ALPN-303; TACI vTD-Fc) a crystallizable fragment fusion protein of an engineered TACI domain which mediates a potent inhibitory activity on BAFF and April [43], and AUR200 a dual inhibitor of BAFF and APRIL based on BCMA-IgG4 Fc fusion protein are also available but secondary to efforts focused on specific and selective [44]. Additionally, anti-BAFF therapies have encountered challenges; for example, tabalumab (LY 2127399) was withdrawn after failing to meet primary endpoints in its phase III for SLE.

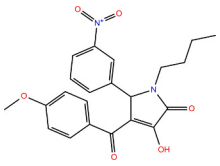
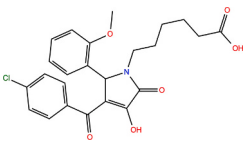
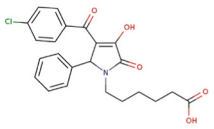
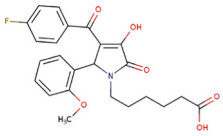
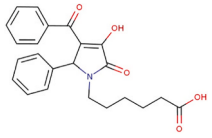
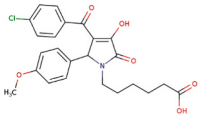
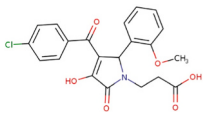
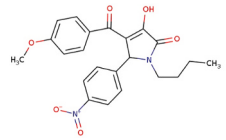
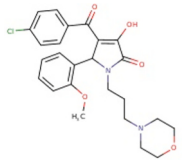
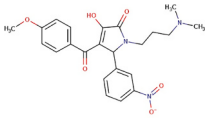
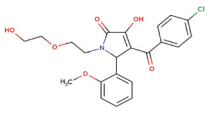
This highlights the complexity and the ongoing difficulties in developing effective therapies targeting the BAFF pathway. Indeed, disrupting the BAFF-BAFFR interaction with small molecules that are inhibitors of the BAFF activate pathway could modulate B-cell activity and improve disease outcomes. Small molecules are low molecular weight organic compounds with well-defined chemical structures and predictable pharmacokinetic properties. Their compact size and chemical stability make them ideal for oral administration and typically reduce the risk of immunogenic responses [13]. One of the key advantages of small molecules is their ability to cross biological barriers, including the blood–brain barrier (BBB), which is especially relevant in neurological autoimmune diseases such as MS [45]. In such conditions, small molecules can modulate immune responses directly within the central nervous system, providing therapeutic benefits not always achievable with larger biologics or monoclonal antibodies. This pharmacological versatility has contributed to their continued relevance in drug discovery and the treatment of complex immune-mediated diseases.

Our study employed a comprehensive virtual screening (VS) approach, informed by genetic evidence linking BAFF to autoimmune conditions [7]. We developed a pharmacophore model based on MD simulations of the BAFF-BAFFR interaction, identifying key structural features necessary for effective binding. This model guided our search for compounds likely to disrupt this interaction. Following pharmacophore-based screening, we conducted molecular docking to evaluate binding affinities and spatial interactions of candidate compounds with BAFF, selecting only those capable of effectively interacting with the protein. This step further refined the list of potential inhibitors and provided detailed insights into their binding modes. This refined our list of potential inhibitors and provided insights into their binding activity. To assess drug-like properties, we performed *in-silico* ADME-Tox predictions, which helped select compounds with favourable pharmacokinetic and toxicological profiles [46]. In vitro validation confirmed the biological activity of two identified compounds, C45 and C145, which effectively inhibited BAFF in lymphoblast-like Raji cells naturally expressing the BAFFR receptor and COS-1 cells transfected with a Hybrid Receptor (HR). The HR was engineered to specifically respond to BAFF and, via the extracellular domain of BAFFR fused to the intracellular domain of TNFR1, activate the canonical NF-κB pathway. This design enables a measurable functional readout—IL-8 production—allowing precise assessment of BAFF inhibition [29].

This validation underscores the effectiveness of our VS approach and the potential of these compounds as therapeutic agents. Additionally, in silico selectivity assessment of C45 and C145 against the homologous protein APRIL showed higher selectivity toward BAFF, supporting their specificity as potential inhibitors of BAFF. Finally, structural analogues were also analysed to expand our candidate pool. By examining the structure-activity relationship (SAR), we identified more active compounds, providing further insights for optimizing BAFF inhibitors. Of note, C45 and C145 are structural analogues belonging to the class of substituted 2-pyrrolinones, a group of compounds previously investigated as HIV-1 integrase inhibitors [47], thus supporting the versatility of this class of small molecules.

In conclusion, the present study was prompted by a genetics-driven approach that identified the BAFF–BAFFR interaction as a critical pathway in the pathogenesis of autoimmune diseases, subsequently leading to the discovery of novel small-molecule inhibitors targeting this interaction. Indeed, supported by strong genetic and clinical evidence linking BAFF to B cell-mediated autoimmunity [7,48], we developed a pharmacophore model based on molecular dynamics simulations of the receptor-ligand interface, and applied a comprehensive virtual screening approach. This approach led to the identification of two promising compounds, C45 and C145 which belong to the substituted 2-pyrrolinone and demonstrated functional inhibition of BAFF signalling in vitro. Moreover, SAR-based analogue screening expanded our compound library and provided insights into key features to be used for future optimization. While these compounds represent promising initial hits, further optimization is essential to enhance their pharmacokinetic

**Table 3**
Identified analogue compounds tested at single concentration.

| Reference compounds | | |
|---|---|---|
| C45  | C145  | |
| Structure of the Analogues | Name | Analogue of |
|  | AN1 | C145 |
|  | AN2 | C145 |
|  | AN3 | C145 |
|  | AN4 | C145 |
|  | AN5 | C145 |
|  | AN6 | C45 |
|  | AN7 | C145 |
|  | AN8 | C45 |
|  | AN9 | C145 |

profiles and selectivity, ultimately enabling their advancement toward lead candidates suitable for comprehensive preclinical evaluation.

Given the limitations of existing biologics such as rituximab and belimumab (such as lack of effect on long-lived plasma cells or CNS penetration), the development of small molecules represents a valuable alternative. Additionally, advantages in terms of oral bioavailability, pharmacokinetic predictability, and BBB permeability [49,50] may significantly expand therapeutic opportunities in diseases like SLE and MS. Overall, this work highlights the potential of integrating genetic evidence with rational drug design to discover next-generation immunomodulatory therapies for autoimmune disorders.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2025.123938.

## CRediT authorship contribution statement

**Stefania Olla:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Maria Laura Idda:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Manila Deiana:** Methodology. **Valeria Lodde:** Writing – review & editing, Methodology. **Giuseppe Delogu:** Methodology. **Antonio Cristian Caria:** Methodology. **Matteo Floris:** Writing – review & editing, Methodology, Investigation. **Francesco Cucca:** Writing – review & editing, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors report no conflict of interest.

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## Data availability

Data will be made available on request.

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