Structure-based design of small bicyclic peptide inhibitors of Cripto-1 activity

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**Running title:** Bicyclic peptides as Cripto-1 inhibitors
ABSTRACT

Bicyclic peptides assembled around small organic scaffolds are gaining an increasing interest as new potent, stable and highly selective therapeutics because of their uncommon ability to specifically recognize protein targets, of their small size that favor tissue penetration and of the versatility and easiness of the synthesis. We have here rationally designed bicyclic peptides assembled around a common tri-bromo-methylbenzene moiety in order to mimic the structure of the CFC domain of the oncogene Cripto-1 and, more specifically, to orient in the most fruitful way the hot spot residues H120 and W123. Through the CFC domain, Cripto-1 binds the ALK4 receptor and other protein partners supporting uncontrolled cell growth and proliferation. Soluble variants of CFC have the potential to inhibit these interactions suppressing the protein activity. A CFC analogue named B3 binds ALK4 in vitro with an affinity in the nanomolar range. Structural analyses in solution via NMR and CD show that B3 has rather flexible conformations, like the parent CFC domain. The functional effects of B3 on the Cripto-1-positive NTERA cancer cell line have been evaluated showing that both CFC and B3 are cytotoxic for the cells and block the Cripto-1 intracellular signaling. Altogether, the data suggest that administration of the soluble CFC and of the structurally related analogue has the potential to inhibit tumor growth.
INTRODUCTION

Synthetic peptides are gaining an increasing role as a new class of drugs (1). They possess a number of versatile features, including high affinity and selectivity that stem from a generally high flexibility and the capacity to adapt to and complement the surfaces of targets. Their flexibility is also one major drawback because it causes a poor stability toward endogenous proteases especially in presence of natural amino acids in their sequence, and a reduced ability to recognize small and well-defined pockets. The scarce absorption in tissues and the difficulty to cross cell membranes is an additional shortcoming for these molecules. Cyclic or even multicyclic peptides are emerging as suitable alternatives with greater potential compared to linear ones, also by virtue of their increased chemical and enzymatic stability, their receptor selectivity, and improved PK/PD properties (high binding affinity, target selectivity and low toxicity) (2). For these reasons, bicyclic peptides are becoming a new generation of peptide therapeutics. They have loop-like structures with multiple domains that can mimic discontinuous binding sites on proteins. They also exhibit sufficiently large surface areas and a limited conformational flexibility, giving rise to relatively big and rigid structures. Such structural features reduce the entropic gap during binding, leading to higher affinity and selectivity compared to linear peptides and to small molecules. Due to the small size, bicyclic peptides can also penetrate better into tissues and have a faster clearance. A higher activity per mass and a wider choice of administration routes has been invoked for bicyclic peptides compared to “big biomolecules”, such as antibodies or other recombinant therapeutic proteins (3, 4), paving the way to their use as alternative small biopharmaceuticals (5, 6).

Several high-affinity bicyclic peptide ligands have been identified by screening huge molecular libraries generated by display techniques such as phage, yeast surface, ribosome and mRNA (2, 6). The peptide components of these libraries are engineered to form the bicyclic structures (4). Bicyclic peptides have been also designed by using computational approaches, chemically prepared as single entities and tested in parallel for binding to target and for functional activity in in vitro assays (4). One very elegant and effective way to achieve bicyclic peptides was originally proposed by Timmermann and coworkers (7), whereby linear peptides containing three cysteines are rapidly and quantitatively converted to bicyclic structures by using tris-bromo-methyl-benzene (TBMB).

We have applied here this technology to the design of bicyclic peptides that target the ALK4 receptor of Cripto-1. Bicyclic peptides have been designed starting from the known structure of one of the protein domain, the CFC, with the aim of generating smaller and more compact mimics that may prevent the binding between the two proteins acting as soluble inhibitors.
Cripto-1 is the founding member of Epidermal Growth Factor/Cripto/FRL-1/Cryptic (EGF-CFC) gene superfamily and works as an onco-developmental protein with multiple biological functions. Physiologically, it plays an essential role in embryonic development and stem-cells differentiation whereas its expression is very low in adult tissue. Post-natally, its over-expression is associated with tumour growth, progression and metastasis in a variety of human solid cancers. It is also expressed on the surface of cancer stem cells (CSC) subpopulations, representing an attractive candidate for the diagnosis, prognosis and therapy of cancer cells with stem-like properties within aggressive tumours. For these properties, Cripto-1 is regarded as an ideal onco-diagnostic target present on the membranes of cancer cells or as a soluble extracellular ligand released following the processing by GPI-phospholipase D. As extensively reported, Cripto-1 mainly exerts its pro-tumorigenic activity through the modulation of the Smad-2/Smad-3 and Src/MAPK/AKT signal transductions in several solid tumors (8). An additional role of the protein has also been extensively documented in the differentiation of stem cells to various cell types (8, 9). The protein (118 residues) is composed of a N-terminal signal sequence, a modified epidermal growth factor (EGF)-like motif, a conserved cysteine-rich motif (CFC region) and a short hydrophobic C-terminus that bears a GPI (glycosylphosphatidylinositol) moiety needed for the anchorage to cell membrane within lipid rafts microdomains. Cripto-1 triggers its various signaling pathways through interactions of the EGF-like and/or CFC domains with several molecular partners on the cell membrane, like the TGFβ family members Nodal and some GDFs, the ALK receptors and Glypican-1 or with other proteins like GRP78 and Activins. Of interest, the Cripto-1/Nodal/ALK4 and Cripto-1/GRP78 complexes have been reported as relevant protein-protein interactions nodes that mediate the Cripto-1 tumorigenic and CSC-related activity. The CFC domain of Cripto-1 mediates the interactions with both the ALK4 receptor in the ternary complex with Nodal, with Activins (10) and with the chaperone GRP78 protein (11-13). Experimental data have been reported regarding the residues involved in the CFC/ALK4 recognition (14, 15), while only theoretical studies have described the potential interactions surface underlying the CFC/GRP78 recognition (16, 17).

Following a bioinformatic study, we have designed a library of small bicyclic peptides that can potentially work as CFC mimics. In these peptides we have fixed as “hot spots” the H120 and W123 residues which are directly involved in the CFC domain/ALK4 binding (14, 15). Accordingly, a set of CFC-like bicyclic peptide has been prepared by chemical synthesis using TBMB as “folding” unit, screened by direct binding to the ALK4 receptor through label-free assays
and characterized in solution by CD and NMR analyses. The best binder has been further tested in \textit{in vitro} functional studies evaluating its potential anti-tumor activity.

**MATERIALS AND METHODS**

All chemicals for peptide synthesis were from commercial sources and used without further purification unless otherwise stated. Solvents, including acetonitrile (CH$_3$CN), dimethylformamide (DMF) were purchased from Sigma Aldrich, Milan, Italy. Acetic acid, N,N-Diisopropylethylamine (DIPEA), piperidine, and TFA were also from Sigma-Aldrich, Milan, Italy. Protected amino acids and the coupling agents used for peptide synthesis were from IRIS Biotech GmbH (Marktrethewitz, Germany). The sources of other materials are reported in the sections where they are described.

**Molecular design and docking studies**

The bicyclic structures were obtained by cyclizing linear peptides containing three cysteine residues with tris-(bromomethyl) benzene (TBMB) (7), following the format Cys-(Xaa)$_m$-Cys-(Xaa)$_n$-Cys. (Xaa) represent any amino acid and $m$ and $n$ are equal to either 4 or 6. Different sizes of the two rings were therefore taken into consideration, with rings containing both four amino acids (abbreviated as 4 x 4), both six amino acids (6 x 6), or six and four amino acids (6 x 4), respectively (3, 18). The various bicyclic peptides were designed to simulate the binding residues of the CFC domain of human Cripto-1 (hCFC) to the extra-cellular domain (ECD) of ALK4 type I receptor. They thereby contain the two key aromatic residues of hCFC, Trp120 and His123, directly involved in the ALK4 receptor recognition, properly spaced by a glycine residue at 6 Å, \textit{i.e.} the distance observed in the molecular complex (15). Furthermore, for a correct positioning of the ligand onto the receptor domain, the bicyclic peptides were equipped with positively charged residues (Arg, Lys) at the C-terminal side and polar/charged residues at the N-terminal side in order to match the negatively charged and polar regions of the ALK4 binding surface. The central TBMB group used to form the bicyclic structure was properly positioned to contribute to the binding to ALK4 by hydrophobic interactions. By using these criteria, over 50 different bicyclic peptide structures were built with Weblab Viewer Pro program v. 3.7 and the geometry of residues adjusted with PyMol software program, v. 1.74. Subsequently, docking simulations of the bicyclic peptides onto the homology model of ALK4 were performed by Autodock Vina program (19). A docking grid around the ALK4 model with a size of 104 Å x 82 Å x 114 Å was used. For each ligand/receptor couple, 800 docking solutions were obtained, which were different in terms of poses (\textit{i.e.} the ALK4 region where the peptide docks), were differently ranked on the basis of their binding affinity as calculated
by Autodock Vina (18). For each ligand, we chose to select and analyze the first 200 ranked poses. Three bicyclic structures (namely, B1, B2 and B3, **Table 1, entries 1-3**) best performing in terms of matching to the ALK4 binding surface, as resulted by PIC (20) and COCOMAPS (21) web server analysis, were selected for synthesis and further studies. Since B1 and B2 showed insolubility, the attention was given to B3. In order to investigate the contribution of single amino acids to the binding with ALK4, we designed a set of point-mutated B3 analogues, where residues where replaced by alanine and basic residues by glutamic acid. B3 peptide and its analogues were synthesized and tested in binding assays. The best ligand B3 wild type was characterized in solution by NMR and its molecular models was used for docking simulations to the homology model of ALK4 by Autodock Vina program.

**Chemical Synthesis**

All the linear precursors of the bicyclic peptides (**Table 1**) were obtained by stepwise solid phase synthesis as acetylated and amidated molecules following standard Fmoc/tBu chemistry protocol by using a Rink-amide MBHA resin, (0.56 mmol/g). Amino acid derivatives with standard protections were used for their assembly onto the resin. Syntheses were performed under canonical conditions of coupling (HATU/DIEA pre-activation, 5-fold excess of Fmoc-protected amino acids) and deprotection (Piperidine 40% in DMF). Cysteines residues were introduced as Tryt (Trt) derivatives, generating after cleavage free thiol groups. Finally, N-terminal acetylation was performed on the resin using acetic anhydride at 0.5 M in DMF with 5% DIEA, 30 min at room temperature. The N-terminally biotinylated variant of B3 (entry n°16, in **Table 1**) was prepared by adding a β-alanine and biotin, introduced as 6-Biotinylamino-hexanoic acid (Iris Biotech GmbH). β-alanine served as spacer to favor the subsequent cyclization. After removal from the solid support by treatment with TFA/H2O/tri-isopropylsilane (TIS) (90:5:5, v/v/v) for 3 hours at room temperature (1.0 ml mixture/100 mg resin) and lyophilization, the crude peptides were reduced by treatment with 100 mM DTT (1,4-dithiothreitol, Sigma Aldrich, Milan, Italy) for 90 min at room temperature. Purifications of linear peptides were carried out by reversed-phase HPLC using an X-Bridge Prep C18 Column (19 x 150 mm ID, Waters, Milan Italy) applying a linear gradient of solvent B (0.1% TFA in CH3CN) over solvent A (0.1% TFA in H2O) from 5% to 70 % over 15 min (flow rate 15 mL/min) using a Waters HPLC system. Cyclization reactions, also termed here as refolding, were achieved in 20 mM bicarbonate buffer, 5 mM EDTA, pH 8.0 at a final peptide concentration of 0.5 mM. The solution was maintained at 30 °C, then TBMB (tris-bromo-methylbenzene, Sigma Aldrich, Milan, Italy) dissolved in CH3CN was added drop-wise until
reaching the final concentration of 1.0 mM. The solution was stirred under nitrogen. For each peptide, refolding required at least 36 hours. Refolding reactions were monitored by RP-HPLC using an ONYX monolithic C18 column (50 x 2 mm ID; Phenomenex, Castel Maggiore, Italy) applying a linear gradient of 0.05% TFA in CH₃CN (solvent B) over Solvent A (0.05% TFA in H₂O) from 5% to 70% over 15 min (flow rate 0.6 mL/min). All linear and cyclized peptides were characterized by LC-MS analyses using either a Bruker ESI Ion Trap mass spectrometer connected to an Alliance HPLC (Waters) or a 6230 ESI-TOF mass spectrometer coupled to a 1290 Infinity LC System (Agilent, Cernusco sul Naviglio, Italy). The LC module made by a binary solvent pump degasser, a column heater and an autosampler was also coupled with a PDA detector. Binary gradients were made using solvent A, 0.01% TFA/H₂O (v/v) and B, 0.01% TFA/CH₃CN (v/v).

Chromatographic analyses of bicyclic peptides were performed using an X-Bridge reverse phase C18 column (Waters, Milan, Italy) applying a linear gradient of solvent B from 5% to 70% in 30 min. The column flow rate was kept at 0.2 mL/min with the heater at a constant temperature of 20 °C. ESI MS analyses of the peptides were conducted in positive ion mode under standard conditions, as reported previously (22). The synthetic CFC[112-150] was prepared and refolded as described elsewhere (23). The linear peptide CFC[115-128] was obtained as the Acm-alkylated derivative. The cyclic variant of this last peptide was obtained using the canonical Fmoc-L-Cys(Trt)-OH derivative and the linear peptide (entry n° 17, in Table 1) was obtained by spontaneous oxidation in a slightly alkaline buffer at a concentration of 0.1 mM. CFC[112-150] (hereafter only CFC), was prepared as previously described (23).

**NMR analysis**

An NMR conformational analysis was performed on the B3 bicyclic peptide. NMR spectra were acquired at 298 K by using a Varian Inova spectrometer operating at the proton frequency of 600 MHz. Peptide samples were typically 3 mM in H₂O/D₂O 90/10 (v/v). 1D ¹H-NMR and 2D ¹H-NMR, TOCSY (mixing time τm 70 ms), NOESY (τm 300, 400 ms) ROESY (τm 300 ms) and DQF-COSY, were recorded at 298 K. The water resonance was suppressed by use of gradients. Chemical shifts were referred to internal sodium 3-(trimethylsilyl) propionate 2,2,3,3-d₄ (TSP, Sigma Aldrich, Milan, Italy). NMR spectra were analyzed by using the CARA program (http://cara.nmr.ch/doku.php/home). Proton sequential assignment of amino acid spin systems and temperature coefficients (Δδ/ΔT ppm/K) of the amide NH protons were assigned and used for calculating the peptide structure. NOE intensities, evaluated by integration of cross-peaks in the 300
ms NOESY spectra, were converted into inter-proton distances by use of the CALIBA program (24). Geminal protons were chosen as reference with a distance of 2.2 Å. Structure calculations started from 100 randomized conformers and was performed using the standard CYANA simulated annealing schedule with 20000 torsion angle dynamics steps per conformer (25). Three-dimensional structures were obtained by using inter-proton distances evaluated from NOEs as upper limits, without use of stereospecific assignments. All the conformers showed a fairly agreement with experimental constraints showing no violations. The first ten CYANA structures with the lowest final CYANA target function (TF) were chosen as representative of the conformational space accessible to the peptide.

Circular Dichroism (CD) analysis

CD analysis was carried out using a Jasco spectropolarimeter, J-710 model (Jasco, Easton, MD, USA), equipped with a Peltier system to control the temperature. Samples were dissolved in water and analyzed at the concentration of 150 µM. Spectra were collected within the wavelength range of 260-190 nm in the far-UV region at a scan rate of 50 nm/min, with a data pitch of 0.1 nm, a band-width of 1.0 nm and a response time of 4 seconds. For each sample, four independent spectra were recorded, averaged and smoothed using the Spectra Manager software, version 1.53 (Easton, MD, USA). Buffer scans were recorded under the same conditions and subtracted.

Surface Plasmon Resonance (SPR) binding assays

In order to assess the recognition of the bicyclic peptides toward the ALK4 receptor, real time binding assays were performed by SPR technique on a Biacore 3000 (GE Healthcare, Milan, Italy). The Fc-fused recombinant ectodomain of Activin-Like Receptor 4 (rhECD-ALK4, R&D System) and an Fc fragment used as blank (produced from the commercial antibody Herceptin in our lab, as reported in Selis et al., (26)) were immobilized on a CM5 sensor chip (GE Healthcare) by standard amine coupling procedure according to the manufacturer’s instructions. Briefly, the carboxylic surface was activated by injection of a solution containing 200 mM EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and 50 mM NHS (N-hydroxysuccinimide), both from GE Healthcare, for 7 minutes, followed by injection of rhECD-ALK4 protein (10 µg/mL solution in 10 mM sodium acetate, pH 4.0 for 120 sec) achieving an immobilization level of about 8500 RU. Capping of unreacted sites was achieved by injecting 1.0 M ethanolamine (GE Healthcare), pH 8.5, for 5 minutes. A blank surface was obtained by derivatization with an Fc fragment (10 µg/mL solution in 10 mM sodium acetate, pH 4.5 for 90 sec) achieving an immobilization level of about
5300 RU. The surface functionalization procedures and binding analyses were carried out in HBS-EP buffer (Hepes 10 mM pH 7.4, NaCl 150 mM, EDTA 3 mM, P20 surfactant 0.005%; GE Healthcare). Binding assays were carried out by injecting solutions of peptides at increasing concentrations ranging from 1.0 µM to 20 µM. Regeneration steps with NaOH (from 5.0 to 20 mM, pulse 10 µL) were performed depending on the strength of the ligand-analyte interactions. Binding data were collected in triplicate for each bicyclic peptide. SPR technique was also used to evaluate the ability of the B3 bicyclic peptide and of the linear and cyclic peptides corresponding to CFC region [115-128] (used as controls) to recognize the anti-CFC monoclonal antibody 1B4 (27). Briefly, a 1B4-functionalized CM5 sensor chip was efficiently prepared as previous reported by Focà et al (27). Dose-dependent assays were performed injecting the peptides at increasing concentrations. For every single analysis experimental sensorgrams were aligned and subtracted of blank signal. All mathematical manipulations and fittings were performed using the BIAevaluation software, version 4.1 from GE Healthcare-Biacore AB (Uppsala, Sweden).

Bio-layer interferometry (BLI) measurements

BLI measurements (28) were performed using a BLItz system and Streptavidin (SA)-modified biosensors (ForteBio Inc). Assays were set up in a configuration suitable for evaluating both the B3 ability to recognize ALK4 in a dose-dependent manner and the mutually exclusive binding of soluble CFC to verify that they bind ALK4 on the same site. In this configuration, direct binding assays were performed immobilizing biotinylated B3 (b-B3) on the sensorchip and exposing it to soluble ALK4 at increasing concentrations. Competition assays were performed exposing the sensorchip to ALK4 pre-incubated with soluble CFC. Experiments were performed following the manufacturer’s instructions using a 4.0 µL sample cuvette. After pre-hydratation for 10 min in PBS buffer, the SA tips were efficiently functionalized with b-B3 at 0.2 mg/mL in PBS for 180 sec achieving an average immobilization level of 2.7 nm; reference biosensors were similarly prepared by exposing the tips to biocytin (Thermo Fisher) solutions at 0.2 mg/mL in PBS for 180 sec. An average 0.8 nm signal change was recorded during this step. Similar assays were performed to assess the specificity of binding of B3 to ALK4, using as control proteins the ECD of recombinant human TGFBR1, also known as ALK5, (rhECD-TGFBR1/ALK5, R&D System; UNIPROT: P36897) and the ECD of recombinant human ALK7 (rhECD-ALK7 (Sinobiological; UNIPROT: Q8NER5). ALK4 (UNIPROT P36896), ALK5 and ALK7 belong to the same family of receptors and share a sequence identity of 67.51% (ALK4/ALK5) and 63.63% (ALK4/ALK7), respectively (source: https://www.uniprot.org/uniprot/?query=human+alk&sort=score). The three proteins were
tested at the same concentrations. Competition assays were carried out using rhECD-ALK4 pre-incubated with soluble CFC domain at molar ratios of 1:0.5, 1:1 and 1:2. Since preliminary experiments confirmed no interaction with BSA (Bovine Serum Albumin, Sigma Aldrich) all binding experiments with the proteins were performed in PBS containing 0.0002% Tween-20, and 0.1 mg/mL BSA. Each single assay was performed following the following steps: I) exposure to running buffer to acquire the initial baseline (baseline, exposure time 30 sec). II) exposure to protein solutions (association, volume 4.0 µL, exposure time 120 sec). II) exposure to running buffer (dissociation, exposure time 120 sec). III) exposure to 10 mM NaOH (regeneration, exposure time 2 x 30 sec). The shaker speed was set to 2000 rpm, according to the manufacturer’s instructions. Proper controls were recorded using the reference sensors treated with biocytin. Final interferograms were obtained by subtraction of the reference signals. Data were exported from the BLItz Pro 1.2 software and re-plotted with GraphPad Prism, vers. 5.00, GraphPad Software (San Diego, California). Plateau values of binding as reflected by changes in optical thickness (nm) at 140 sec were used to calculate the affinity constant (K_D) by applying a non-linear curve fitting and one binding site hyperbola as model (GraphPad Prism).

**Cell viability assay**

A standard MTT colorimetric assay (G4000, Promega, Madison, WI) was used to determine the effect of B3 and of synthetic CFC used as control on the viability of NTERA (human embryonal carcinoma) cells. The C8161 cells, a Cripto-1 low expression human melanoma cell line (29), were used to evaluate off target cytotoxicity effects. Briefly, after adding 5.0 µM solutions of either B3, CFC or PBS to NTERA and C8161 cells, these were incubated at 37 °C for 24 hours. After treatment, a Tetrazolium salt dye solution was added and allowed to react for 4 hours at 37 °C. The MTT reaction was then interrupted by adding a stop/solubilization buffer to solubilize the formazan crystals from the MTT reaction for 1 hour at 37 °C. The plates were then read using a DTX 880 Multimode Detector (Beckman Coulter Brea, CA, USA) to determine optical density (OD) at 570 nm. Mean OD values were calculated from quadruplicate wells. T-test was used to determine statistical significance between conditions if the P value was less than 0.05.

**Cell signaling analyses**

Western blotting was used to evaluate cell signaling in NTERA cells treated with 5.0 µM solutions of either B3, CFC or PBS control. Whole cell lysates were prepared and quantified as previously described (30). SDS-PAGE gel electrophoresis and protein transfer to PVDF membranes was
performed using standard techniques. Membranes were then blocked in 5% BSA for 1hr at RT and incubated overnight at 4 °C with the following antibodies, all at 1:1000 dilution: rabbit anti-p-ERK1/2 (Cell Signaling, Beverley, MA); rabbit anti-ERK1/2 (Life Technologies, Grand Island, NY); rabbit anti-p-SRC (Invitrogen, Waltham, MA); rabbit anti-SRC (Invitrogen, Waltham, MA); rabbit anti-α-Tubulin (Cell Signaling, Beverley, MA). After three washes with TBS-T, the membrane was incubated with the appropriate secondary antibody for 1 hour at RT, then washed thrice with TBS-T and finally incubated with the chemiluminescent reagent Clarity Western ECL (BioRad, Hercules, CA). Signal was detected using the ChemiDoc MP imaging system (BioRad). Intensity of bands was then quantified using ImageJ analysis software.

**Cell proliferation assays**

For cell proliferation assays, approximately 1x10^6 NTERA and C8161 cells growing in 12-well plates were treated with 5.0 μM solutions of either B3, CFC or PBS for control. A first cell count was obtained after 24 hours of treatment. Cell counting was performed manually using standard Trypan Blue staining using a hemocytometer. A second cell count was obtained at 48 hours after the initial media was replaced with fresh media again containing 5.0 μM solutions of either B3, CFC or PBS for control cells. Mean values for 24 and 48 hours cell counts were calculated from quadruplicate wells. T-test was used to determine statistical significance between conditions if the P value was less than 0.05.

**Cell cycle analyses**

For cell cycle analysis, approximately 1x10^6 of NTERA cells were treated daily with 5.0 μM solutions of either B3, CFC or PBS control and harvested after 72 hours. Cells were washed several times with ice-cold PBS and then fixed in ice-cold 80% ethanol overnight at 4 °C. Cells were stored up to 48 hours at 4 °C until staining was performed. After washing with ice cold PBS, the cells were stained with a Propidium Iodide (50 mg/mL final concentration) RNase containing solution in 0.1% Triton X. Analysis was performed using the CytoFLEX flow cytometer system (Beckman Coulter, Brea, CA, USA).

**RESULTS**

**Structure-based design of bicyclic CFC-like peptides**
De novo designed bicyclic CFC-like peptides, aimed at simulating the CFC Cripto-1 site involved in the binding with the ALK4 receptor (here also referred as “CFC epitope”), were designed starting from the analysis of the complex Cripto-1/ALK4 previously obtained by our group by computational studies (14, 15, 31). In the process of design and optimization of the bicyclic peptide binding properties, in silico docking experiments were iteratively performed. A first series of fifty bicyclic peptides, different for composition and/or for the size of the two rings, but all containing the two CFC residues crucial for the receptor binding (Trp120 and His123), were computationally generated (see Table SM1). Once analyzed by docking simulations, the different peptides showed different ability in silico to dock the ALK4 binding epitope. Among these, the peptides named B1, B2 and B3 (Table 1, entries 1-3) were found as the best ALK4 ligands with the best docking scores. Indeed, their complexes correctly reproduce the interface of the model of Cripto-1/ALK4, with Trp120 and His123 of CFC well interacting with the ALK4 receptor. Based on these observations, B1, B2 and B3 were selected for chemical synthesis and experimental binding assays. While synthetic B1 and B2 could not be experimentally tested due to their high insolubility, synthetic B3 was used for binding assays (See below). In order to identify residues within B3 relevant for ALK4 binding, a second set of in silico docking experiments was performed using mutated variants of the original peptide. The list of new B3 peptides designed and analyzed in this study is also reported in Table 1 (entries from 4 to 15). A more simple variant of the CFC domain reproducing the region spanning residues 115-128 containing H120 and W123, was also designed and prepared as linear and cyclic variant (See Table 1, entry 17). The peptide was designed as the simplest variant of the CFC region containing the Cripto-1 epitope involved in the binding with ALK4 and was used as control to assess that the recognition properties of the bicyclic peptides were not due to the mere presence of these residues but to the three dimensional organization of the molecule.

**Synthesis, purification and characterization of CFC-like peptides**

All peptides reported in Table 1 were synthesized as linear precursors following the standard Fmoc procedure. Crude products were extensively reduced using DTT and subjected to reverse phase purification. Linear peptides with three free cysteines were bi-cyclized using tris-(bromomethyl)benzene (TBMB) as reported in (32) slightly modifying the protocol as reported in the section of Methods. The reactions were monitored by RP-HPLC, following the backward shift of the peaks during refolding. In Fig. 1, an exemplary overlay of chromatograms showing the refolding of B3 is reported. The bicyclic and cyclic peptides were again purified at homogeneity.
achieving a final recovery ranging between 7% and 30%. Purity and identity of final products were confirmed by ESI-TOF LC-MS analyses (Table 1).

**Fig. 1**

**Binding of CFC–like bicyclic peptides to ALK4**

*Dose-dependent binding assays of B3. rhEDC-ALK4 receptor was covalently bound on a CM5 dextran sensor chip surface reaching an immobilization level of around 8500 RU; a control surface was functionalized with an Fc fragment accounting 5300 RU immobilization level. Among the three *ad hoc* designed bicycles peptides (Table 1), only B3 was initially tested due to the extended insolubility of B1 and B2. As shown in Fig. 2, B3 bound the immobilized rhEDC-ALK4 in a dose-dependent manner at concentrations starting from 125 nM, exhibiting very fast association (*k*_on = 3.0*10^4 M^-1 s^-1) and dissociation rates (*K*_off = 1.8*10^-3 s^-1) (Table SM2). Fitting the data an affinity constant value of 168 nM was initially estimated, not at the steady state. B3 has therefore a much stronger affinity for rhEDC-ALK4 compared to the parent synthetic CFC domain which has a reported *K*_D of about 13 µM (about 78 fold increased) (15). B3 instead shows a reduced affinity (around 40 fold) compared to that of full-length Cripto-1, estimated to be about 4.3 nM (8). Remarkably, B3 recognized in a dose dependent fashion also the 1B4 anti-CFC monoclonal antibody (Fig. SM1, Table SM3), whereas no significant binding to the same antibody was recorded by testing the cyclic and linear variants of CFC [115- 128] (Fig. SM2), a peptide containing the two crucial hot spots underpinning the binding with ALK4. Taken together these data suggest that the bicyclic synthetic B3 peptide is a mimetic of the CFC domain with an improved ability to bind the target receptor ALK4.

**Fig. 2**

**Screening of B3 mutants.** In order to improve the affinity of B3, structural modifications were investigated through single and double amino acid substitutions. The basic residues (R4, R10, R11, K12) were replaced with glutamic acid to confirm their involvement in the ALK4 receptor recognition. Other residues in the peptide sequence were systematically substituted with alanine to evaluate their contribution to the binding and to the stability of the complex. Particularly, the arginine at position 4 was replaced with polar threonine and tyrosine residues. The threonine on position 9 was replaced with a hydrophobic isoleucine residue. A doubly mutated variant was
obtained by replacing asparagine at position 3 with an alanine and threonine on position 9 with an isoleucine. Furthermore, a B3 variant (B3-ctrl) in which both key residues W5 (W123 in CFC) and H7 (H120 in CFC) were replaced with alanine, was also prepared and tested as negative control. Overall, a set of 13 B3 bicyclic analogues (Table 1, entries 4-15) was prepared by chemical synthesis and screened for binding to ALK4 by SPR at the single concentration of 1.0 μM.

As shown in Fig. 3, all mutations drastically reduced the binding of the molecule to ALK4 as compared to B3. The substitution of the polar residue Asn3 with alanine in B3, peptide N3A, shows only a slightly decreased SPR response in comparison to B3. Instead replacing Arg4 with glutamic acid, B3_R4E, tyrosine (B3_R4Y) or threonine (B3_R4T) almost abolished the binding. The replacement of the other basic residues R10, R11 and K12 with glutamic acid also severely decreases the binding. A significant binding reduction of about 70% was detected also with the mutant B3_G6A where glycine on position 6 was replaced with alanine, likely due to a reduced flexibility of the peptide. As expected, the single (B3_W5A and B3_H7A) and double (B3_WHAA) substitution of the two key residues strongly impaired the recognition. In particular, replacement of W5 with alanine reduced the binding of about 90%, substitution of H7 decreased the binding by more than 50% while the double mutation lead to a 95% reduction of the binding. These results confirmed the relevance of aromatic interactions in these positions for the stability of the complex and indicated the very important role played by the basic residues.

The replacement of polar threonine residue on position 9 with alanine (B3_T9A) and isoleucine (B3_T9I) also resulted in a considerable loss of binding, leading to a residual 35% and 16% binding compared to B3. Altogether, the data show that the bicyclic peptide B3 is an optimized binder of the ALK4 receptor that at most recapitulates the structural properties of the synthetic soluble CFC domain of Cripto-1.

**Fig. 3**

**BLI-based binding and competition assays**

In order to validate the interaction between B3 bicyclic peptide and ALK4 receptor and also to investigate its binding specificity and selectivity, we took advantage of the BLI technique (28) implemented in the BLItz instrument which allows binding analyses of biomolecular interactions using small amounts of material. Firstly, to confirm the binding of B3 to ALK4 we performed dose-response binding assays of rhECD-ALK4 to SA biosensors opportunely functionalized with b-B3. The resulting dose-dependent binding response is shown in Fig. 4. By data fitting a $K_D = 61.2 \pm$
19.0 nM ($R^2 = 0.9759$) was estimated in partial agreement with the SPR result (168 nM). Using a similar experiment, we also investigated whether B3 shared the same binding site on ALK4 with CFC. To this purpose we performed binding competition experiments using of rhECD-ALK4 at 250 nM pre-incubated with CFC at different molar ratios. As shown in Fig. 5AB, the presence of CFC reduced (1:0.5 and 1:1) or fully abolished (1:2) the binding of rhECD-ALK4 to B3, strongly suggesting that the two peptides bind the receptor at the same site and with high specificity. To further investigate the selectivity of the interaction between B3 and rhECD-ALK4 we finally evaluated the binding with other ALK receptors, such as TGFBR1/ALK5 and ALK7, which share with ALK4 high sequence homology. Remarkably, no binding was detected with the immobilized B3 even at the highest concentration tested (500 nM, Fig. SM3).

**Fig. 4**

**Fig. 5AB**

### Structural characterization of the B3 bicyclic peptide

NMR analysis, performed in water by classical 2D spectra, provided chemical shifts (Tables SM4 of the Supplementary Material) and NOE effects. A set of 100 (83 intra-residual, 16 sequential and 1 long-range) experimental NOE constraints were measured for the B3 bicyclic peptide and a set of tridimensional models were building using CYANA program (Fig. SM4). Statistical data collected on the 40 CYANA structures are reported in Table SM5. The molecular models show that this peptide, despite the presence of two bridges, has still a large conformational flexibility in accordance with the CD result (Fig. SM6). The best ten NMR-CYANA structures were selected to perform docking simulations with ALK4.

### Docking simulations of selected B3 structures with ALK4

To gain insights on the atomic details of the binding with ALK4 receptor of the best ligands B3, we used the NMR experimental structures of the ligands for docking simulations onto the ALK4 model. To account for ligand flexibility, we performed AutoDock Vina blind simulations by using the ensemble of the 10 best NMR structures of the peptides. The resulting 200 docking solutions were structurally analyzed and compared to the native interactions found in the CFC/ALK4 complex. We found that the most of B3 poses were located in correspondence of the native epitope
of ALK4. Furthermore, in at least 30% of poses, the key residues W5 and H7, appeared positioned as in the Cripto-1/ALK4 complex. The AutoDock data suggest that B3 is potentially able to interact with the receptor with an orientation similar to that previously described for CFC, supporting its experimentally verified binding ability. In the B3/ALK4 docking complex (Fig. 4) the key residue W5 shows favorable aromatic interactions with the Y56 residue of the ALK4 receptor. The other B3 key residue, H7, is hosted in the ALK4 pocket formed by residues P44, V46, L48, V49, A51, Y56 and forms a hydrogen bond with Y56 residue. Electrostatic interactions are observed between basic residues of B3 and acid residues of ALK4, as R4 - D62, R10 - E47, R11 - D22, K12 - E20, K12 - D22.

Fig. 6

On the grounds of the docking results, the interactions of W5 and H7 are confirmed as fundamental for the correct positioning of the peptide onto ALK4 binding surface. This observation is in line with SPR data that show low or no binding for the analogues bearing mutations at positions 5 and 7. It is worth to note that a very low affinity is observed also for the G6A analogue. We guess that this result can be explained by considering that G6, the residue chosen in our design as a spacer between W5 and H7, confers the flexibility necessary for the accommodation of W5 and H7 side chains in the receptor pockets. G6 substitution likely drastically influences the flexibility and thus the peptide binding properties. By docking studies it also turned out that the electrostatic interactions established by basic residues (R4, R10, R11 and K12) are crucial for complex stabilization. This finding justifies the loss and/or reduction of binding abilities of all the analogues whenever these charges are removed.

**B3 and CFC reduce cell viability and signaling in NTERA cells**

For a comparative analysis, cellular assays were carried out using the selected B3 bicyclic peptide and the parental CFC synthetic domain. Peptides were used at the same concentration on the NTERA cell line expressing Cripto-1. After 24 hours of treatments with 5.0 μM of either B3 or CFC, the viability of NTERA cells was reduced by 27% (P < 0.01) as assessed through MTT assays (Fig. 7). No significant toxicity was detected on the C8161 cells line (expressing low Cripto-1) (Fig. SM7). Furthermore, western blot analyses of the corresponding cell lysates (Fig. 8A) showed a reduction of the phosphorylated forms of ERK1/2 and Src compared to control (buffer). These results demonstrated the inhibitory activity of B3 and CFC on Cripto-1 associated cell signaling.
The densitometric analyses confirmed the reduced levels of p-ERK1/2 and p-Src in treated NTERA cells and clearly showed a more pronounced inhibitory effect on ERK1/2 and Src signaling in NTERA cells treated with CFC compared to NTERA cells treated with B3. In particular, we observed that while B3 afforded, respectively, 31% and 37% reduction of p-ERK1/2 and p-Src compared to control, CFC induced 86% (p-ERK1/2) and 70% (p-Src) decrease under the same conditions (Fig. 8B).

**Fig. 7**

**Fig. 8AB**

**B3 and CFC reduce proliferation and negatively affect cell cycle in NTERA cells**

The inhibitory effects on Cripto-1 functions of both peptides were confirmed through their anti-proliferative effect on NTERA cells and on cell cycle. Cell counts showed a significant reduction (P < 0.05) in number of NTERA cells after treatment for 48 hours with 5.0 μM B3 or CFC peptides, compared to control (Fig. 9A), demonstrating the negative effect of both peptides on NTERA cell proliferation. Furthermore, after 72 hours, B3 or CFC treated NTERA cells still showed reduced mitotic activity as evidenced by results from cell cycle analysis (Fig. 9B), which demonstrates an increased percentage of NTERA cells in S phase (+ 3.9% for B3 and + 6% for CFC, compared to control) and a corresponding decrease of cells in G2/M phase (-3.6% for B3 and -6.1% for CFC, compared to control).

**Fig. 9AB**

**DISCUSSION**

Bicyclic peptides are becoming a new class of drugs with a number of advantages compared to linear molecules, including increased activity and improved PD/PK properties. Currently, the first bicycle peptide conjugated to DM1 (BT1718) and targeting MT1-MMP (Membrane Type 1 Matrix Metalloproteinase also known as MMP-14) is under phase I/IIa clinical trials for testing in patients with MT1-MMP positive solid tumors (33). Other bicyclic peptides have been developed as potential therapeutics, imaging/diagnostic probes and research tools against different molecular targets (3, 18, 34-41). Taking advantage of the properties of these molecules we have attempted to generate bicyclic peptides able to block the activity of Cripto-1, a growth factor involved in tumor
development, progression and relapse, we have already previously targeted using a multimeric peptide named Cripto Binding Peptide (CBP) (42) and monoclonal antibodies (8, 27). The principally targeted site of the protein (43) is the CFC domain that mediates the interaction with different receptors or soluble ligands involved in tumor-promoting signal transduction. Through the CFC Cripto-1 interacts with the membrane-bound ALK4, GRP78 and with soluble Activins, modulating the pro-survival activity of the Smad2/3 and Src/MAPK/PI3K axes. CBP works by binding Cripto-1, putatively on the CFC domain, with an estimated affinity of about 400 nM. It was selected from the screening of a synthetic combinatorial library and so far has been only employed as a stem cell specification agent capable of antagonizing the Cripto-1/ALK4 signaling, switching in vivo the differentiation of mouse ES cells from cardiomyocytes to neurons (42). Its efficacy as potential anti-tumoral agent has never been tested. The mAb 1B4 also binds Cripto-1 on the CFC domain with an affinity in the picomolar range and blocks its signalling in tumor cell lines (27). Given these properties, the isolated soluble CFC (15, 23, 44) has the potential to bind the target proteins and to inhibit the Cripto-1 activity. The use as Cripto-1 antagonists of the CFC domain or of synthetic analogues has never been reported.

Along these lines, starting from a previously published molecular model of the CFC/ALK4 complex, we have designed, prepared and tested bicyclic CFC-like peptides containing the two hot spot residues H120 and W123 in different conformations and with different exposure. Supported by molecular docking analyses, we have optimized distances and geometries between the main chemical groups present on these residues and designed scaffolds with different ring sizes to hold them in place at predetermined positions in the attempt to improve surface complementarity, thus affinity and selectivity. The precursor linear peptides have been cyclized around the TBMB scaffold and tested for their ability to interact with ALK4 receptor. Compared to the soluble folded domain, the B3 peptide emerged from this study, is smaller in size, more compact, thereby with the potential of being metabolically much more stable, and also more potent in terms of affinity toward the target ALK4 receptor, exhibiting a K_D much lower compared to that of the parent domain (between 62 and 168 nM versus 14 μM). The data demonstrate the success of the rational design based on the use of W120 and H123 as fixed hot-spot positions to generate smaller size (2.5 fold) molecules mimicking the CFC-domain. At further support of the successful design, B3 was also recognized by the anti-CFC conformation mAb 1B4 while the same antibody could not bind a linear or monocyclic epitope containing the same target residues. Furthermore, the binding of B3 to ALK4 was highly selective as suggested by the lack of interactions with ALK5 and ALK7 that belong to the same family of receptors. The binding of this peptide with the receptor is much less efficient
compared to full length Cripto-1 since the protein expectedly provides a greater juxtaposition of residues, a higher conformational stability of the entire structure/surface involved in the binding and the contribution of other residues (15). The potential antagonistic effect of the synthetic CFC domain and of the related B3 peptide have been evaluated in a set of functional assays using Cripto-1-expressing NTERA cells. By comparative analyses, we found that both peptides exert an overall comparable cytotoxicity and anti-proliferative effect while are ineffective on a cell line expressing low levels of the protein. Of interest, the treatment with the CFC domain resulted in a more pronounced inhibitory effect on ERK1/2 and Src signaling compared to that achieved with B3 (p-ERK1/2, 31% reduction with B3 and 86% reduction with CFC; p-Src, 37% reduction with B3 and 70% reduction with CFC). While this result could be unexpected on one side, we can speculate that it may derive from the ability of the CFC domain to interact with others Cripto-1 partners, especially GRP78, using also other residues not present or not structurally optimized in B3. The interaction of CFC with the other Cripto-1 receptors not recognized by B3 could in some way explain the higher activity of the native domain.

From a structural point of view, despite its constrained bicyclic structure, B3 do not exhibit a very compact conformation, a feature shared with the CFC domain that notwithstanding the presence of three disulfide bridges also behaves like a quite flexible module. It is plausible that with further rounds of design, synthesis and testing, also the rigidity and affinity of B3 toward ALK4 be improved, as demonstrated for example with the bicyclic inhibitors of FXIIa, whose affinity was similarly increased, passing from 1.2 μM (36) to 840 pM (45) introducing non natural amino acids.

In conclusion, we have evaluated for the first time the option of using the soluble synthetic CFC domain and one rationally designed bicyclic analogue as potential Cripto-1 inhibitors that work by specifically binding the ALK4 receptor. Both molecules, with different abilities, are capable of affecting the proliferation of Cripto-1-positive cancer cell lines blocking the relevant signalling activated by the oncoprotein. Given the small size of B3, the increased affinity compared to the parent molecule, the high selectivity and the straightforward synthesis, this molecule can be seen as a valid precursor for the preparation of tumor-penetrating agents, also conjugated to cytotoxic drugs, to be employed as anti-tumor tools in Cripto-1-positive tumors.

Author contribution
E.I. performed the in silico study and the synthesis of all peptides. L.C. performed the in silico study and the NMR investigation. G.U and L.S. performed the cells-based assays and contributed to write the manuscript. L.F. and G.D.A performed the in silico study, the NMR investigation and
contributed to write the manuscript. D.L. and J.S provided mAbs and performed binding studies. A.S. performed binding studies, provided the mAbs, analyzed the data and wrote the manuscript. M.R. conceived and designed the study, analyzed the data and wrote the manuscript.

FUNDINGS
Authors acknowledge the support from Regione Campania for the projects: i) “Fighting Cancer resistance: Multidisciplinary integrated Platform for a technological Innovative Approach to Oncotherapies (Campania Oncotherapies)”; ii) “Development of novel therapeutic approaches for treatment-resistant neoplastic diseases (SATIN)”; iii) NANOCAN, NANOfotonica per la lotta al CANcro. Also, support from MIUR for project PRIN n°20155ACHBN to AS and 2015783N45 to MR is gratefully acknowledged.

ACKNOWLEDGMENTS
Authors also acknowledge the technical advice and support for mass spectrometry by Maurizio Amendola and by Leopoldo Zona for NMR analyses.
The authors declare no conflicts of interests.
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### Table 1. Analytical data of the set of *ad hoc* designed CFC-like cyclic peptides. Histidine and Tryptophan corresponding to H120 and W123 in the native protein are in bold; mutations are underlined.

| Name      | Sequence                     | MW, Theor. amu | MW, Exp. amu | % RECOVERY |
|-----------|------------------------------|----------------|--------------|------------|
| 1 B1      | Ac-C\(^1\)YWGHCIQRWC\(^{11}\)-NH\(_2\) | 1608.80        | 1608.65      | 12         |
| 2 B2      | Ac-C\(^1\)AYTWGHCIQRWC\(^{11}\)-NH\(_2\) | 1780.88        | 1780.74      | 19         |
| 3 B3      | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1744.93        | 1744.90      | 33         |
| 4 B3\(_N3A\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1701.94        | 1701.76      | 20         |
| 5 B3\(_R4E\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1717.88        | 1717.72      | 29         |
| 6 B3\(_R4Y\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1751.90        | 1751.73      | 10         |
| 7 B3\(_W5A\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1629.89        | 1629.73      | 19         |
| 8 B3\(_G6A\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1758.95        | 1758.79      | 30         |
| 9 B3\(_H7A\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1678.91        | 1678.75      | 20         |
| 10 B3\(_T9I\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1756.97        | 1756.95      | 17         |
| 11 B3\(_T9A\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1714.92        | 1714.73      | 12         |
| 12 B3\(_R10E\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1717.88        | 1717.71      | 15         |
| 13 B3\(_R11E\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1717.88        | 1717.71      | 13         |
| 14 B3\(_K12E\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1745.88        | 175.72       | 7.0        |
| 15 B3\(_ctrl\) | Ac-C\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 1563.87        | 1563.82      | 9.1        |
| 16 b-B3    | Biotin-βAC\(^1\)ANRWGHCTRRKC\(^{11}\)-NH\(_2\) | 2000.05        | 2000.89      | 30         |
| 17 CFC\([115\text{-}128]\) | Ac-C\(^{115}(\text{GSVP})\)HDTWLPPKCC\(^{128}\)-NH\(_2\) | 1608.76        | 1608.79      | 45         |
FIGURE LEGENDS

Fig. 1. Overlay of chromatograms obtained during the refolding of B3 to form the bicyclic structure. Chromatograms were obtained at t = 0 (*) and t = 16h (**).

Fig. 2. SPR based dose-response binding assay of B3 to ALK4 immobilized rhECD-ALK4 receptor. The overlay of sensorgrams obtained at concentrations ranging between 125 nM and 1.0 µM in HBS is shown.

Fig. 3: Bar graph reporting the normalized SPR binding data of B3 and the mutated bicyclic variants to ALK4 at 1.0 µM. SPR signals are expressed as percentage of RUmax obtained for each peptide analogue compared to that of B3 assumed as 100%.

Fig.4: BLI dose-response binding measurements showing the binding of rhECD-ALK4 receptor to immobilized biotinylated_B3 bicyclic peptide at the reported concentrations, fitted with 1:1 binding model, using GraphPad Prism 5.0.

Fig. 5: BLI measurements showing the binding of rhECD-ALK4 receptor at 250 nM to immobilized biotinylated B3 bicyclic peptide in absence and in presence of different amounts of synthetic CFC domain (1:0.5, 1:1 and 1:2 molar ratio) (A). Bar graph reporting the normalized BLI based measurements recorded in the competition assays showing the % residual binding of rhECD-ALK4 (B).

Fig. 6: Docking solutions for B3/ALK4 complex. ALK4 is shown with blue/white surface, with residues that interact coloured by type (acid: red, polar: yellow, hydrophobic: grey). The peptide is shown as sticks.

Fig. 7: Reduced viability of NTERA cells treated with B3 or CFC. Results from MTT assays show a significant reduction in viability of NTERA cells treated for 24 hours with 5.0 µM B3 or CFC compared to control PBS treated cells (* P < 0.01).

Fig. 8: Reduced cell signaling in NTERA cells treated with B3 or CFC. A. Western blot results show that 24 hours treatment of NTERA cells with 5.0 µM B3 or CFC results in reduced expression of p-ERK1/2 and p-Src compared to control cells. B. Densitometric analyses of Western blot bands revealed that the reduction of ERK1/2 and Src signaling in the NTERA cells is more pronounced with CFC treatment compared to B3 treatment.

Fig. 9: Reduced proliferation and cell cycle in NTERA cells treated with B3 or CFC. A. Treatment of NTERA cells for 48 hours with 5.0 µM B3 or CFC resulted in a significant reduction of cell numbers, compared to control cells (* = P < 0.05) (A). B. Results from cell cycle analysis show that after 72 hours NTERA cells treated with 5.0 µM B3 or CFC have increased population of cells stalled in S phase and a reduced population of cells in G2/M phase, compared to control.
