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Structure-based discovery of a small non-peptidic Neuropilins antagonist exerting *in vitro* and *in vivo* anti-tumor activity on breast cancer model

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ABSTRACT

Neuropilin-1/-2 (+33 NRPs), VEGF-A_{165} co-receptors, are over-expressed during cancer progression. Thus, NRPs targeted drug development is challenged using a multistep *in silico/in vitro* screening procedure. The first fully non-peptidic VEGF-A_{165}/NRPs protein–protein interaction antagonist (IC_{50} = 34 μM) without effect on pro-angiogenic kinases has been identified (compound-1). This hit showed breast cancer cells anti-proliferative activity (IC_{50} = 0.60 μM). Compound-1 treated NOG-xenografted mice significantly exerted tumor growth inhibition, which is correlated with Ki-67^{low} expression and apoptosis. Furthermore, $CD31^{+}$/$CD34^{+}$ vessels are reduced in accordance with HUVEC-tube formation inhibition (IC_{50} = 0.20 μM). Taking together, compound-1 is the first fully organic inhibitor targeting NRPs.
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Introduction

Neuropilins (NRPs) are 120–130 kDa transmembrane glycoproteins, non-tyrosine kinase receptors, initially described in neuronal guidance [1]. Two homologs, NRP-1 and NRP-2, which share 44% amino acid sequence identity and have identical domain structures, have been described. NRPs bind VEGF-A in association with tyrosine kinase receptors (VEGF-R1/-R2/-R3) and modulate their functions. Therefore, they are considered as essential regulators in angiogenesis. In cancer, over-expression of NRPs is known to promote tumor cell migration and survival (see for review) [2] and their inhibition leads to tumor cell apoptosis [3]. Clinically, NRP over-expression also correlates with metastatic potential and poor prognosis in different cancer types (see for review) [4,5].

Thus, NRPs fulfill the criteria of a promising pharmacological target in cancer. Anti-NRP antibodies have been shown to inhibit VEGF-A_{165}/NRP-induced angiogenesis [6]. In a mouse model, anti-NRP-1 antibodies were successfully used in association with bevacizumab to reduce tumor growth [6,7]. Otherwise, a monoclonal antibody targeting NRP-2 was shown to block VEGF-C binding and to disrupt VEGF-C-induced lymphatic endothelial cell migration [8]. It also reduces metastasis to sentinel lymph nodes and distant organs [8]. Nevertheless, therapeutic monoclonal antibody development is very costly compared with small molecules, and recently, peptide and pseudo-peptide antagonists have been developed to inhibit VEGF-A_{165}/NRP-1 protein–protein interaction, the human pro-angiogenic VEGF spliced form overexpressed in cancer [9]. A phage display screening identified the heptapeptide ATWLPPR [10,11] used by Barberi-Heyob to design sugar-based peptidomimetics [12]. An alternative approach was based on the structure of tuftsin, the tetrapeptide TKPR mimicking the

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C-terminal tail of VEGF-A_{165} (exon eight), which displays a high affinity for NRP-1 and competes with VEGF-A_{165} binding [13]. Thus, in cell-free assay, the bicyclic peptide EG3287 and the compound EG00229 including an arginine moiety and a peptide bond, have been designed by Zachary's group [14,15]. Interestingly, EG00229 reduces the viability of human lung carcinoma A549 cells and strengthens the potency of anti-cancer drugs.

Peptidic and/or pseudopeptidic compounds are easily cleavable by proteases and rarely provide pharmacological agents. Thus, we aimed in the present work to identify new small organic and fully non-peptidic VEGF-A_{165}/NRP-1 protein–protein interaction antagonists which development is very challenging. Usually, the interface between the two partners is large and flat. Nevertheless, in this study, the reported first-generation peptidic-antagonists (EG00229), which follow an arginine C-end rule [16,17], and the X-ray structure of tuftsin bound to the NRP-1 b1-domain [18], suggest the existence of a promising "hot spot" in NRP-1.

We report in this article an original "hit" identification procedure divided into three successive parts. First, structure-based virtual ligand screening was performed on a large commercial compound library to retain a smaller sub-library containing unproven VEGF-A_{165}/NRP antagonists. Second, these molecules were submitted to *in vitro* tests (cellular and molecular), in order to identify a hit compound. Third, the potency of this "hit" was compared *in vitro* with the organo–peptidic EG00229 compound [15]. Overall, compound-1 exhibited more potent anti-angiogenic activities than previously reported pseudo-peptide VEGF-A_{165} antagonist EG00229. Then, compound-1 was evaluated *in vivo* for its anti-tumor and anti-angiogenic potential activities in MDA-MB-231-NOG xenografted mice. As expected, compound-1 exerted a tubulogenesis inhibition capacity *in vitro* and *in vivo*. Moreover, it was also efficient to block both *in vitro* and *in vivo* tumor growth. Taken together, this compound constitutes a different approach on anti-cancer drug development when targeting NRPs.

Materials and methods

Molecular modeling and virtual screening

The binding site has been defined at 4 Å around the co-crystallized tuftsin bound to NRP-1 (PDB code 2ORZ) [18]. Hydrogen atoms were added using Chimera [26]. ChemBridge compound collection was retrieved from www.hit2lead.com. FAF-drugs2 was used as ADME–tox filterin [27]. Consensus molecular docking was performed using Surflex-dock v2.5. and ICM-VLS-v3.4. Surflex-dock is based on a modified Hammerhead fragmentation/reconstruction algorithm to dock compounds flexibly into the binding site [19]. The query molecule is decomposed into rigid fragments that are superimposed on the Surflex-Protomol, i.e., molecular fragments covering the entire binding site. The docking poses were evaluated by an empirical scoring function. ICM is based on Monte Carlo simulations in internal coordinates to optimize the position of molecules using a stochastic global optimization procedure combined with pseudo-Brownian positional/torsional steps and fast local gradient minimization [20]. The docking poses were evaluated using the ICM-VLS empirical scoring function. The top 3000 consensus scoring compounds were visually inspected, and a resulting consensus hit list (1317 compounds) emerged.

Cell culture and proliferation assay

HUVECs (Lonza, Belgium) used between passages 3-4, were cultured in the presence of EGM-2 medium complemented with EBM-2 (growth factor mix) and 2% fetal bovine serum (FBS, Lonza). HUVECs (3×10^3 cells), MDA-MB-231 (6×10^3 cells, from the ATCC (France)), were plated in 200 µL/well in 96-well plates. After 24 h, they were treated with different compounds dissolved in DMSO (final concentration < 0.05%). Following 48–72 h, WST-1 (Roche®, France) was added for 1–2 h. Then Optical Density was analyzed with a microplate reader (Microplate Manager 5.2, Bio-Rad) at 490 nm. For each compound, the IC_{50} value was determined from a sigmoid dose-response curve using Graph-Pad Prism (GraphPad Software, San Diego, USA).

Binding inhibition platform

Binding inhibition assay was performed as previously described [28] in 96-well plate surface coated overnight at 4 °C with PBS buffer containing 0.2 µg/mL of NRP-1 or NRP-2 (R&D Systems, France). After 3 washes with PBS 0.5% Tween 20 (buffer-A) and saturation with PBS 0.5% BSA (buffer-B) (Sigma–Aldrich, France) 2 h at 37 °C, plates were incubated with different concentrations of each inhibitor in DMSO (final concentrations between 0.1% and 0.5%) for 1 h at 37 °C, then 50 µL of bt-VEGF-A_{165} (200 µg/mL for NRP-1, 800 µg/mL for NRP-2) (R&D-Systems, France) supplemented with 4 µg/mL heparin (Sigma Aldrich) were added during 2 h at 37 °C. After 3 washes with buffer-A, plates were saturated 2 h at 37 °C with buffer-B. After washing three times with buffer-A, 100 µL of AMDEX streptavidin-horseradish peroxidase (Amersham, USA) in buffer-A and 0.3% BSA was added to each well. After 45 min incubation in darkness at 25 °C, plates were washed 5-times with buffer-A and 100 µL of SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) was added. Chemiluminescence was quantified with an EnVision™-2101 Multilabel reader (Perkin Elmer, USA). Data were analyzed using the nonlinear regression function in Prism (Version-4.03, GraphPad Software, USA).

Tube formation assay

The underlying collagen gel was prepared as previously described [28]. After 30 min at 37 °C in a 5% CO_2 atmosphere, HUVECs in the absence or presence of antagonist (compound-1 or EG00229) were seeded on collagen with EBM2 medium supplemented with 1% FBS. After confluent monolayer formation, a second collagen gel was added over the apical cell surface. After 24 h, tube formation was observed. Wells were photographed using a SPOT camera attached to a Nikon Eclipse TE2000-S inverted microscope at 200× magnification. Resulting images were optimized by enhancing edge detection and by inverting the background using Gimp software, and the tubes were then quantified using ImageJ, plug-in: angiogenesis analyzer from Gilles Carpentier (http://rsb.info.nih.gov/ij/).

In vivo xenografted-tumor mouse model

Protocol was approved by the INSERM Institutional Care and Use Committee according to the European Communities Council Directive. NOD/scid/IL-2Rγ−/− (NOG) female mice were bred and housed in pathogen-free conditions in accordance with the Federation of European Animal Associations (FELSA) guidelines. MDA-MB-231 cells were washed twice in PBS and resuspended in DMEM. Subsequently, cells were injected subcutaneously into NOG mice (6–7 weeks old) at the concentration of 2×10^6 cells/200 µL. When tumors grew to approximately 100 mm^3, mice were randomly divided into different groups (8 mice/group). Mice then received using force feeding compound-1 (10 mg/kg or 50 mg/kg, respectively) or vehicle every three days for 35 days. Tumor growth and body weight were measured every three days during the treatment. Mice were weighed regularly to assess the toxicity of the treatment and the tumors were measured with calipers (width × width × length × Pi/6) to determine growth. Mouse survival was assessed during the study. 5-weeks after the injection, surviving mice were euthanized.

Immunohistochemistry

Tumor tissues from mice from each group (untreated, treated) were frozen in OCT (optimal cutting temperature) and sections (7 µm) were fixed in acetone. Endothelial cells within the blood vessels supplying human breast cancer in NOG mice were analyzed by double color immunofluorescence microscopy using anti-mouse CD31 (BD Pharmingen, USA) revealed using a goat anti-rat IgG(H + L)-Dylight-549 (Jackson, England) and anti-CD34-Dylight-488 (Novus, UK). In addition, representative tumor sections from each group were stained with anti-human Ki-67-Alexa-555 (BD Pharmingen) to assess cell proliferation.

TUNEL assay

TUNEL staining protocol was done as the recommendation of the manufacturer (*in situ* cell death detection kit, fluorescein) (Roche®, France).

Apoptosis and cell cycle assays

HUVEC were incubated for 72 h in the presence of medium alone, DMSO or compound-1 (IC_{50}). Cells were then harvested, centrifuged and pellets were resuspended and incubated for 10 min at 4 °C with FITC-conjugated-annexin-V and propidium iodide (PI) (Immunotech, France), and fluorescence intensity was analyzed by a FACS-calibur (Becton Dickinson, USA) to detect apoptosis. Cell cycle of pelleted cells were assessed using PI/triton buffer after 15 min incubation at 37 °C, then cells were acquired by flow cytometer and cell cycle were analyzed using Dean-Jett-Fox methods.

Statistical analysis

Data are expressed as the arithmetic mean ± SD of at least three different experiments. The statistical significance of results was evaluated by ANOVA, with probability values $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, being considered as significant.

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