UPARANT: A Urokinase Receptor–Derived Peptide Inhibitor of VEGF-Driven Angiogenesis with Enhanced Stability and In Vitro and In Vivo Potency

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Abstract

This work is based on previous evidence showing that chemotactic sequence of the urokinase receptor (uPAR88-92) drives angiogenesis in vitro and in vivo in a protease-independent manner, and that the peptide Ac-Arg-Glu-Arg-Phe-NH₂ (RERF) prevents both uPAR88-92- and VEGF-induced angiogenesis. New N-acetylated and C-amidated peptide analogues containing α-methyl α-amino acids were designed and synthesized to optimize the biochemical properties for therapeutic applications. Among these, Ac-ι-Arg-Alb-ι-Arg-ι-Cot(Me) Phe-NH₂, named UPARANT, adopts in solution a turned conformation similar to that found for RERF, is stable to sterilization in 3 mg/mL sealed vials in autoclave for 20 minutes at 120°C, is stable in blood, and displays a long-time resistance to enzymatic proteolysis. UPARANT competes with N-formyl-Met-Leu-Phe (fMLF) for binding to the formyl-peptide receptor, inhibits VEGF-directed endothelial cell migration, and prevents cytoskeletal organization and αvβ3 activation in endothelial cells exposed to VEGF. In vitro, UPARANT inhibits VEGF-dependent tube formation of endothelial cells at a 100× lower concentration than RERF. In vivo, UPARANT reduces to the basal level VEGF-dependent capillary sprouts originating from the host vessels that invaded Matrigel sponges implanted in mice, and completely prevents neovascularization induced by subcorneal implantation of pellets containing VEGF in rabbits. Both excellent stability and potency position UPARANT as a promising new therapeutic agent for the control of diseases fueled by excessive angiogenesis, such as cancer and inflammation. Mol Cancer Ther; 13(5); 1092–104. ©2014 AACR.

Introduction

Angiogenesis is a complex multistep process leading to the formation of new blood vessels from a preexisting vascular network. During new vessel formation, endothelial cells degrade their basement membrane, migrate into the interstitial matrix, and proliferate. An imbalance in this process contributes to numerous malignant, inflammatory, ischemic, infectious, and immune disorders (1).

The receptor for urokinase-type plasminogen activator (uPAR) plays an important role in controlling cell migration (2, 3). uPAR is a glycosylated glycosyl-phosphatidyl-inositol–anchored protein (4) formed by 3 domains (DI, DII, and DIII) connected by short linker regions (5). Besides being responsible for focalizing uPA-mediated plasminogen activation on cell surface (6), uPAR also promotes intracellular signaling, thus regulating physiologic processes such as wound healing, immune responses, and stem cell mobilization, as well as pathologic conditions such as inflammation and tumor progression (7–10). The role of uPAR in angiogenesis is well documented. uPAR is able to focus urokinase proteolytic activity on cell surface and to modulate cell migration (11, 12). We found that soluble forms of uPAR, containing the Ser-Arg-Ser-Arg-Tyr² sequence (uPAR88-92), as well as the synthetic peptide Ser-Arg-Ser-Arg-Tyr (SRSRY), stimulates in vitro and in vivo angiogenesis in a protease-independent manner (13). The uPAR88-92 sequence interacts with the formyl peptide receptors (FPR) type 1 and 2, henceforth inducing cell migration in an integrin-dependent manner (9, 14, 15). Upon binding to FPR, the synthetic peptide SRSRY causes FPR internalization and triggers vitronectin receptor activation with an inside–outside type of mechanism (16).
Recently, we found that the residue Ser\textsuperscript{90} of uPAR is critical for uPAR signaling, and that S90P and S90E single amino acid substitutions exert opposite effects on uPAR activities. Cells expressing membrane-associated uPAR carrying Ser\textsuperscript{90} substituted with Glu exhibit a reduced binding to and a decreased adhesion onto vitronectin, an impaired agonist-induced FPR internalization, and a dramatic reduction of \textit{in vitro} and \textit{in vivo} cell migration and invasion (17). To inhibit uPAR functions, we developed, a family of penta-peptides carrying the S90E substitution in the uPAR\textsubscript{ss88-92} sequence. These peptides block uPAR-dependent cell signaling by interfering with the complex cross-talk involving uPAR, FPR, and integrins. The peptide containing the N-terminal pyro-glutamic acid (pGlu)-Arg-Glu-Arg-Tyr-NH\textsubscript{2} (p\textit{E}RE\textit{R}Y-NH\textsubscript{2}) shares the same binding site with SRS\textsubscript{RY} and competes with fMLF for binding to FPR, thus preventing uPAR/FPR interaction. p\textit{E}RE\textit{R}Y-NH\textsubscript{2} inhibits migration of various tumor cell lines in culture (18). Subsequently, new tetra-peptides having the general formula Ac-Arg-Glu-Arg-X-NH\textsubscript{2} (X = Phe, Tyr, Trp) were synthesized, and the peptide Ac-Arg-Glu-Arg-Phe-NH\textsubscript{2} (denoted RERF) was selected for its ability to potently prevent \textit{in vitro} and \textit{in vivo} cell migration and invasion (19). Recently, we found that RERF also behaves as an antiangiogenic agent. It inhibits \textit{in vitro} and \textit{in vivo} responses promoted either by uPAR\textsubscript{ss88-92} or by VEGF. RERF also prevents cytoskeletal organization and the recruitment of αβ3 integrin at the focal adhesions in endothelial cells exposed to VEGF, by forcing αβ3 in an inactive state either directly or indirectly, through FPR (20).

Starting from these pieces of biologic information, we intended to generate peptide analogues with optimized properties for therapeutic applications. We synthesized new peptides containing α-methyl-α-amino acids, and they were characterized from a biologic point of view. All these new analogues are N-acetylated and C-amidated, as being a common way of the N- and C-terminal modifications to enhance the stability to exopeptidase-mediated proteolysis (21). Among the series of peptides, Ac-L-Arg-Aib-L-Arg-D-C(\textit{Me})Phe-NH\textsubscript{2} showed the best desired activity in preliminary experiments of VEGF-directed endothelial cell migration.

It adopts in solution a turned conformation, it is quite stable to trypsin and chymotrypsin digestion, in mice and rats blood. It is also stable to sterilization in 3 mg/mL sealed vials in autoclave for 20 minutes at 120°C. One sample was immediately analyzed by liquid chromatography/mass spectrometry (LC/MS) to ascertain the lack of any modified compound. The remaining samples were kept in a thermostat at 25°C. Three vials were opened every month and analyzed by LC/MS.

Blood stability

Blood stability studies were performed at IRBM Science Park. Technical details are reported in the Supplementary Materials and Methods.

Enzymatic digestion of peptides

The peptides were subjected to digestion by trypsin at a peptide:trypsin ratio of 1,000:1 (w:w) for RERF and Icm\textsubscript{25}, whereas a 10:1 (w:w) peptide:trypsin ratio was used for Icm\textsubscript{25B} and UPAR\textsubscript{ANT}. The trypsin concentration is 0.6 mg/L. The peptides were also subjected to digestion by chymotrypsin at a peptide:chymotrypsin ratio of 10:1 (w:w). Icm\textsubscript{25}-4 was not studied for enzyme digestion. Digestions were followed for 1 hour at 25°C in 0.1 M Tris-HCl buffer pH 8.5. The peptide consumption was followed by RP-HPLC measuring the area peaks at different times between 0 and 60 minutes. Quantification of the intact peptide peak over time indicated that its disappearance seemed to follow first-order kinetics (data not shown), and the \textit{t}_{1/2} was estimated.

Nuclear magnetic resonance spectroscopy and structure calculations

Nuclear magnetic resonance (NMR) experiments were performed on Bruker Avance 600 MHz spectrometer, equipped with triple resonance cryo-probe. NMR characterization was performed in H\textsubscript{2}O/D\textsubscript{2}O 90:10 (v/v) and in H\textsubscript{2}O/CF\textsubscript{3}CD\textsubscript{2}OD 70:30 (v/v) at 298 K. Details are reported in the Supplementary Materials and Methods. Restrained molecular dynamic (RMD) simulations were carried out using a hand-built starting model with structural data from NMR measurements (e.g., interproton distances from Nuclear Overhauser Effect (NOE) value).
Details on structure calculations are reported in the Supplementary Materials and Methods.

Cell cultures
Human umbilical vein endothelial cells (HUVEC) were purchased by Lonza (C2519A, Lot No. 0000115425), which provided a certificate of analysis for each cell lot. This guarantees the expression of CD31/105 and von Willebrand Factor through 15 population doublings. HUVEC, used between the third to the seventh passage according to Arnaoutova and colleagues, (22), were grown in Eagle basal medium (EBM) supplemented with 4% FBS, 0.1% gentamicin, 1 µg/mL hydrocortisone, 10 µg/mL EGF, and 12 µg/mL bovine brain extract (Cambrex).

Migration assay
Cell migration of HUVEC was performed in Boyden chambers, using 8-µm-pore size polycarbonate-free-poly carbonate filters at 37°C, 5% CO2 as previously described (13). Briefly, 7 × 10^4 viable cells suspended in serum-free EBM were allowed to migrate for 4 hours toward EBM or 40 ng/mL VEGF165 (Pepro Tech), mixed with diluents or the indicated peptides at 37°C in a 5% CO2. At the end of assays, cells on the lower filter surface were fixed, stained with hematoxylin, and 10 random fields/fielder were counted at 200 magnification.

Binding assays
HUVECs (5 × 10^5 cells/sample) were preincubated with diluents or the indicated effectors for 60 minutes at 4°C, extensively rinsed with PBS and then exposed to 10 nM/L N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FITC-fMLF) purchased from Molecular Probes for 60 minutes at 4°C. Quantification of cell-associated fluorescence was assessed by reading endothelial cell lysates with a fluorescence plate reader Victor 3 (Perkin Elmer) using 485 nm excitation and 535 nm emission filters. An aliquot of each sample was separated on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat dry milk and probed with anti-phospho-Akt (Ser473; p-AKT) Ab or anti-p-ERK1/2 (Thr202/204) Ab (Cell Signaling) and then with anti-AKT mAb (R&D Systems) and expressed as a cell index value.

Fluorescence microscopy
HUVEC grown on glass slides to semiconfluence were starved for 60 minutes in EBM. Then, cells were exposed to EBM alone, 40 ng/mL VEGF165 with/without 10 nmol/L UPARANT at 37°C in humidified air with 5% CO2. At the indicated times, cells were lysed in radioimmuno-precipitation assay buffer (10 mmol/L Tris pH 7.5, 140 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 0.5% NP40) containing 5 mmol/L Na3VO4 and a protease inhibitor mixture (Sigma-Aldrich). Cell lysate protein content was measured by a colorimetric assay (Bio-Rad). Western blot analysis was performed as previously described (17, 20). Briefly, 50 µg proteins were separated on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% nonfat dry milk and probed with anti-phospho-Akt (Ser473; p-AKT) Ab or anti-p-ERK1/2 (Thr202/204) Ab (Cell Signaling) and then with anti-AKT mAb (R&D System) or anti-ERK1/2 mAb (Millipore). In all cases, washed filters were incubated with horseradish peroxidase–conjugated antimouse or anti-rabbit antibody and detected by Enhanced Chemiluminescence Kit (GE-Healthcare).

Cell adhesion assay
Cell adhesion assays were performed using 16-well plates coated with 5 µg/mL vitronectin, diluted in PBS and the xCELLigence technology (Roche Diagnostics) as described (23). HUVEC (5 × 10^5 cells/well) were plated in each coated well, in serum-free EBM in the presence or the absence of 40 ng/mL VEGF165 with/without 10 nmol/L C-terminal amidated Ac-Glu-Arg-Phe-Arg-NH2 control peptide (ERFR), 10 nmol/L RERF or 10 nmol/L UPAR-ANT and allowed to adhere for 4 hours at 37°C, 5% CO2.

Cell viability
For the MTS assay, the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega) was used following the manufacturer’s instruction. Briefly, HUVEC (2 × 10^3/well) were seeded in 96-well tissue culture plates and left to
adhere in complete media for 3 hours, then rinsed twice with PBS followed by the incubation with EBM mixed to 40 ng/mL VEGF_{165} with or without peptides at the indicated concentration. Medium was replaced every 24 hours. At the indicated times, suspended cells were removed and 10 μL of the MTS reagent was added into each well and cells were incubated at 37°C for 3 hours. The absorbance was detected at 490 nm with a Microplate Reader (Bio-Rad). With a similar experimental design, endothelial cell proliferation was assessed using 16-well plates and the Real Time Cell Analysis xCELLigence technology. The impedance value of each well was automatically monitored and expressed as a cell index.

**Endothelial cell tube formation assay**

The formation of vascular-like structures was assessed on Matrigel as described (13). HUVEC were suspended in 300 μL prewarmed EBM. Diluents or effectors were added to the cell suspension before seeding cells (4 × 10^5 cells/well) on plates coated with 300 μL/well growth factor reduced Matrigel (Becton Dickinson). When indicated, cells were preincubated for 30 minutes at 37°C with 1 μg/mL anti-human VEGF Ab, 2 μg/mL LM609 anti-αvβ3 mAbs, or nonimmune serum. Complete capillary tube networks were assessed within a low-magnification field observed under light microscopy after 6-hour incubation at 37°C in humidified air with 5% CO₂. To quantify tube formation, 5 random areas/well at ×100 magnification were imaged and the number of tubes formed by cord-like structures exceeding 100 μm in length (24) were visualized using Axiovision 4.8 software (Carl Zeiss) and counted.

**Matrigel sponge angiogenesis assay**

Twelve 6- to 8-week-old C57BL/6j male mice (Charles River Laboratories) of 23 to 25 g 6 to 8 weeks old, were maintained in accordance with institutional guidelines complying with national and international laws and policies. Briefly, VEGF_{165} (100 ng/mL) and heparin (50 U/mL) were diluted in PBS, with or without 75 or 150 μg/kg UPARANT, were added to unpolymerized liquid Matrigel at 4°C, to a final volume of 500 μL. The Matrigel suspension was slowly injected subcutaneously into the flanks of mice, using a cold syringe, where it quickly polymerizes to form a solid gel. Matrigel with buffer alone was used as negative control. Five days after injection, the animals were killed and the gels were removed, minced, and then diluted in water for hemoglobin content measurement with a Drabkin Reagent Kit (Sigma). The final hemoglobin concentration was normalized to 100 mg of recovered gel and calculated from a standard calibration curve after spectrophotometric analysis at 540 nm.

**Corneal pocket assay**

Corneal pocket assays were performed at PRIMM. Twelve female New Zealand White rabbits (Charles River) weighing 2.5 to 3.0 kg were anesthetized by intramuscular injection of acepromazine (1 mg/kg), ketamine hydrochloride (35 mg/kg), and xylazine hydrochloride (5 mg/kg) and 3 to 4 drops of 0.4% ophthalmic chlorohydrate solution were topically applied to the eye before micropocket surgery. A wire speculum was positioned in the eye, and a sucralfate-hydrate suspension containing PBS, 5 μg UPARANT, or 180 ng VEGF with/without 5 μg UPARANT were implanted into the cornea after making a micropocket in the stroma, using standard surgical tools (25, 26). Tobradex (0.3% tobramycin–0.1% dexamethasone) was applied to the surface of the cornea after gel implantation to prevent infection. Observation and quantification of the angiogenic responses were performed by a slit-lamp stereomicroscope. The angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries. A density value of 1 corresponded to 0 to 25 vessels per cornea; 2 from 25 to 50, 3 from 50 to 75, 4 from 75 to 100, and 5 for >100 vessels.

**Statistical analysis**

The results are expressed as the mean ± SD of the number of the indicated determinations. Data were analyzed by one-way analysis of variance and post hoc Bonferroni modified t test for multiple comparisons. P < 0.001 was accepted as significant.

**Ethics statement**

The research work with mouse model has been approved by Institutional Ethical Committee of Istituto Nazionale Tumori “Fondazione G. Pascale”-IRCCS, Naples, Italy (protocol no. 09, December 20, 2010).

**Results**

**Development and synthesis of RERF analogues**

Previously, we reported the NMR structure of RERF in solution (19). RERF, although displaying some conformational flexibility, preferentially adopts an α-turn (type I-α_{hel}) conformation (27) in water/trifluoroethanol (TFE) mixed solvent. Cα-methyl-α-amino acids with well-defined stereo chemical properties impose local restrictions on backbone conformation, thus conferring structural stability (28–29). Aib residue (α-amino isobutyric acid) is the prototype of Cα-methyl-α-amino acids, and is well known to be characterized by a restricted conformational freedom (30) in the 3_{10} -α-helical region of the Ramachandran plot. Other Cα-methyl-α-amino acids, including Cα-methyl-phe-nylalanine (31) are β-turn and helix inducers, much stronger than the unmethylated, phenyl-containing, protein amino acid Phe. Cα-methy-α-amino acid, when incorporated into peptide sequences containing coded residues, strongly facilitates and stabilizes type II β-turns or helicogenic α-turns as previously described (32, 33). In addition, Cα-methyl-α-amino acid substitutions could provide enhanced binding to a molecular target, resistance to proteolytic degradation and longer persistence in circulation. On the basis of this information, we have synthesized 4 peptide analogues of RERF
containing commercially available Cα-methyl-α-amino acids, namely:

Icm25-4       Ac-Arg-Aib-Arg-Phe-NH₂
Icm25       Ac-Aib-Aib-Aib-Arg-Phe-NH₂
Icm25B       Ac-Aib-Aib-Aib-Arg-α-Cu(Me)Phe-NH₂
Icm25-4c       Ac-Aib-Aib-Arg-α-Cu(Me)Phe-NH₂

Icm25-4 and Icm25-4c are both tetrapeptides analogues of RERF in which the Glu residue has been replaced by Aib. Icm25-4c also contains a second substitution of the terminal Phe residue with D-Cα(Me)Phe (S-configuration). Icm25 and Icm25B are both pentapeptides corresponding to N-terminal elongation of Icm25-4 and Icm25-4c with a second Aib residue.

**Enzymatic digestion**

The peptides were subjected to digestion by trypsin and chymotrypsin at different peptide:enzyme ratios, depending on the digestion rates. The enzyme concentration is 0.6 mg/L, and is similar to trypsin concentration determined in plasma 117 to 637 μg/L (34). Icm25-4 was not studied for enzyme digestion. The peptide consumption was determined by measuring the HPLC area peaks during digestion. It seemed to follow first-order kinetics (data not shown), and the t1/2 were estimated. We found that RERF and Icm25 at a peptide:trypsin ratio of 1,000:1 are rapidly digested, the t1/2 being 3.05 and 6.19 minutes, respectively. No digestion of Icm25B and Icm25-4c was detected at a peptide:trypsin ratio of 1,000:1, whereas at a peptide:trypsin ratio of 10:1 the t1/2 were 37.5 and 11.4 minutes for Icm25B and Icm25-4c, respectively. Both RERF and Icm25, containing the natural Phe residue at the C-terminus, were digested by chymotrypsin with t1/2 being 23.6 and 20.0 minutes, respectively. Conversely, no digestion of Icm25B and Icm25-4c, both containing the unnatural C-terminal Cα(Me)Phe, was detected within 60 minutes.

**Screening of the peptides as inhibitors of VEGF-dependent endothelial cell migration**

RERF has been shown to inhibit endothelial cell migration driven by VEGF (20). We compared the effects of RERF and Aib containing peptides in inhibiting endothelial cell migration directed toward 40 ng/mL VEGF 165. By conventional Boyden chamber assays, we found that 40 ng/mL VEGF 165 elicited endothelial cell migration up to 235% of the basal cell migration. When used as chemoattractant at 10 nmol/L concentration, Aib containing peptides, as well as RERF, slightly reduced basal cell migration, whereas the control peptides ARARY or ERFR were ineffective (Table 1). As expected, a 57% and 54%

<table>
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<tr>
<th>Effector</th>
<th>Chemoattractants</th>
<th>Cell migration (%)</th>
<th>Inhibition (%)</th>
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<tr>
<td>EBM</td>
<td>CTL</td>
<td>100</td>
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<td></td>
<td>40 ng/mL VEGF</td>
<td>235 ± 14</td>
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<tr>
<td></td>
<td>10 nmol/L ERFR</td>
<td>101 ± 2a</td>
<td>57</td>
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<tr>
<td></td>
<td>10 nmol/L ARARY</td>
<td>110 ± 4b</td>
<td>57</td>
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<td></td>
<td>10 nmol/L RERF</td>
<td>99 ± 5b</td>
<td>57</td>
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<td></td>
<td>10 nmol/L Icm25</td>
<td>95 ± 9b</td>
<td>57</td>
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<td></td>
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<td>94 ± 9b</td>
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<td>105 ± 12b</td>
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<td>89 ± 6b</td>
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<tr>
<td>1 μg/mL VEGF Ab</td>
<td>EBM</td>
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<td>40 ng/mL VEGF</td>
<td>102 ± 2b</td>
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<td>231 ± 9</td>
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<td>40 nmol/L Icm25-4</td>
<td>121 ± 8a</td>
<td>49</td>
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NOTE: HUVEC (4 × 10⁴ cells/well) were allowed to migrate toward chemoattractants, in the presence of the indicated effectors for 4 hours at 37°C, 5% CO₂. Quantitative analysis of cell migration was calculated as a percentage of cell migration assessed in the absence of any effector or chemoattractant (CTL). The inhibitory effect of each peptide was reported relative to the extent of cell migration toward VEGF (taken as 100%). Data are the mean ± SD of 2 independent experiments, performed in duplicate. They were analyzed by one-way ANOVA and post hoc Bonferroni modified t test for multiple comparisons. Statistical significance with P values was calculated against 40 ng/mL VEGF.

*aStatistical significance with P < 0.001.

*bStatistical significance with P < 0.0001.
inhibition of VEGF-directed endothelial cell migration was elicited by anti-VEGF Ab and by RERF, respectively. The combination of VEGF165 with 10 nmol/L concentration of Icm25, Icm25B, Icm25-4, or Icm25-4c reduced endothelial cell migration by 59%, 62%, 49%, and 68%, respectively (Table 1). Because the peptide Icm25-4c showed a long t1/2 in enzyme digestions, and exerted the strongest inhibition of endothelial cell migration, it was named UPARANT, and further characterized for its biochemical properties, conformational preferences and biologic activity.

**Stability of UPARANT**

We examined the stability of UPARANT during standard autoclave sterilization. Ninety sealed vials containing 0.5 mL of UPARANT in PBS at a concentration of 3 mg/mL were autoclaved for 20 minutes at 120°C. One sample was immediately analyzed by LC/MS to ascertain the lack of any modified compound. The remaining samples were kept in a thermostat at 25°C. Three vials were opened every month and analyzed by LC/MS. As a result, no degradation after autoclaving and standing at 25°C in sealed vials for 2 years was observed (data not shown).

The stability of UPARANT in physiologic solution, in mice and rats blood was also studied at 0.3 and 1 μg/mL concentrations. Blood stability was followed for 2 hours. UPARANT resulted undegraded as shown in Supplementary Tables S1 and S2.

**NMR analysis of UPARANT**

We investigated the conformational preferences of UPARANT in water and in water/TFE by NMR spectroscopy (Supplementary Tables S3 and S4 and Fig. S1). ROESY spectra in water and in water/TFE essentially display the same pattern of sequential and medium-range NOE effects (see Supplementary Materials and Methods), indicating that the peptide has a quite similar conformational behavior in both solvent systems. However, detailed analysis was performed in water/TFE solution only, because of more dispersed amide signals and fewer resonance overlaps. The pattern of NOEs and their relative intensities are summarized in Fig. 1A. RMD calculations, using the NMR experimental data as conformational restraints, gave an ensemble of conformers quite similar in terms of backbone conformation, underlining the presence of one predominant and stable structure for UPARANT. Figure 1B reports the backbone atoms superposition of the ensemble of conformers along the MD trajectory. Figure 1C reports the average backbone torsion angles of UPARANT as obtained from RMD simulation in vacuo at 300 K.

The peptide conformation corresponds to an incipient 310-helix characterized by 2 consecutive type III β-turn, followed by a type I β-turn. This structure is stabilized by the typical CO→HN hydrogen bonds (see Fig. 1C): a weak interaction Ac-CO→NH-Arg3, a H-bond Arg1-CO→NH-Cu(Me)Phe3, and Aib2-CO→NH-amide. Along the trajectory of the RMD simulation, the Arg1 and Arg2 guanidine groups experience intrasidue H-bonds, and the Arg1 guanidine group is also H-bonded to Cu(Me)Phe3-CO group.

UPARANT competes with fMLF for binding to the formyl-peptide-receptor

We have previously documented that RERF competes with fMLF for binding to FPR, being FPR the main binding site of RERF (19). To test whether similar to RERF (20), UPARANT competes with fMLF for binding to FPR, HUVEC were preincubated at 4°C (to avoid receptor internalization) with diluents, 100 nmol/L...
concentration of fMLF, RERF, or UPARANT for 60 minutes at 4°C, and then exposed to 10 nmol/L N-formyl-Nle-or Leu-Phe-Nle-Tyr-Lys-fluorescein (FITC-fMLF) for additional 60 minutes at 4°C. Fluorimetric measurement of cell-associated fluorescence was assessed using 485 nm excitation and 535 nm emission filters. Data, expressed as a percentage of the fluorescence associated to cells exposed to FITC-fMLF (None = 100%), represent mean ± SD from an experiment performed in triplicate, representative of 2 replicates. Inset, Western blot analysis for the α-tubulin content on an aliquot of each cell lysate. B–E, representative images of HUVEC incubated with diluents (None), 100 nmol/L fMLF, 100 nmol/L RERF, or 100 nmol/L UPARANT for 30 minutes at 37°C, exposed to 10 nmol/L FITC-fMLF (B and C) or 10 nmol/L FITC-UPARANT (D and E) for 30 minutes at 37°C and then visualized using a Zeiss 510 META LSM microscope. Z-series images represent focal planes corresponding to 0.38 μm vertical interval of HUVEC incubated with FITC-fMLF (C) or FITC-UPARANT (E) alone. Scale bar, 10 μm. Original magnifications, ×630.
by unlabeled fMLF, RERF, as well as by UPARANT (Fig. 2D and E). All together, these findings suggest that uPAR-ANT competes with fMLF for binding to FPR on cell surface and inhibits FPR activation by preventing its internalization.

**UPARANT inhibits cytoskeletal organization, αvβ3 recruitment at focal adhesions, and adhesion onto vitronectin of endothelial cells stimulated with VEGF**

VEGF-triggered endothelial cell motility is the result of marked cytoskeletal reorganization and accumulation of stress fibers associated with new actin polymerization, and rapid formation of focal adhesions, which do not occur in the presence of RERF (20). To analyze the effects of UPARANT on cytoskeletal organization induced by VEGF, HUVEC were exposed to 40 ng/mL VEGF165 in the presence/absence of 10 nmol/L UPARANT, and then double stained for vinculin and F-actin. As expected, VEGF induced a marked reorganization of actin into stress fibers spanning the length of the cells, most of which colocalized with vinculin-positive focal adhesions, that was prevented by 10 nmol/L RERF (Fig. 3A). A total of 10 nmol/L UPARANT fully abrogated VEGF-induced effects on cytoskeleton. In the latter case, endothelial cells had a condensed, rounded morphology; similar to untreated cells, the F-actin was condensed into fewer fibers and was completely absent from the leading edges of the cells (Fig. 3A). It is known that αvβ3 integrin has a prominent role in the activity of VEGF. In response to VEGF, β3 integrin regulates integrin-dependent actin reorganization, thus leading αvβ3-VEGFR2 complexes to localize at new formed focal adhesions (35). UPARANT caused disappearance of αvβ3 at focal adhesions and the appearance of thin, αvβ3-positive linings at the cell edge, similar to untreated cells, whereas the addition of 10 nmol/L ERFR did not modify VEGF-dependent integrin redistribution. (Fig. 3B). RERF has been proven to inhibit VEGF-dependent signaling by affecting αvβ3 integrin-dependent cell adhesion onto vitronectin (20). We investigated whether αvβ3 integrin is indeed involved in the
inhibitory activity of UPARANT. HUVEC suspended in EBM mixed to diluents or 40 ng/mL VEGF165 with/without 10 nmol/L ERFR, 10 nmol/L RERF, or 10 nmol/L UPARANT, were allowed to adhere for 4 hours at 37°C on 16-well plates coated with vitronectin. We found that RERF and UPARANT reduced endothelial cell adhesion onto vitronectin at a similar extent, either in the presence or in the absence of VEGF, whereas the control peptide ERFR was almost ineffective (Fig. 3C). All together, these findings show that UPARANT prevents VEGF-driven αvβ3 relocalization and ligand-dependent αvβ3 activation.

**UPARANT inhibits VEGF-dependent migration and intracellular protein phosphorylation of endothelial cells without affecting cell proliferation**

To better characterize the inhibitory effect of UPARANT on VEGF-dependent cell migration, HUVEC were allowed to migrate in Boyden chambers toward 40 ng/mL VEGF165 plus diluents (None) or increasing concentrations of the indicated peptides. The extent of cell migration was expressed as a percentage of the net VEGF165-dependent cell migration, considered as 100%. Data, mean ± SD of 3 independent experiments, performed in duplicate. Statistical significance with P values was calculated against 40 ng/mL VEGF (None). *, statistical significance with P < 0.0001; ***, statistical significance with P < 0.001. B, HUVEC grown on glass slides to semiconfluence were exposed to 40 ng/mL VEGF165 with/without 10 nmol/L UPARANT at 37°C in humidified air with 5% CO2 for the indicated times. Whole cell lysates (50 μg/sample) immunoblotted with anti-phospho-Akt (pAKT) or anti-phospho-ERK1/2 (pERK1/2) Abs and then with total anti-Akt (AKT) Ab or total anti-ERK1/2 (ERK) mAb. An experiment representative of 2 replicates is shown. C and D, HUVEC (2 × 10^5/well) were seeded in 96-well tissue culture (C) or 16-well plates (D), left to adhere in complete media for 3 hours at 37°C 5% CO2, rinsed twice with PBS, and then allowed to grow in EBM mixed to 40 ng/mL VEGF165 with or without the indicated peptides for 72 hours at 37°C 5% CO2. Medium was replaced every 24 hours. C, absorbance of adherent cells was assayed by MTT assay. Plot represents the mean ± SD of 2 independent experiments, each performed in quadruplicate. D, the impedance value of each well was automatically monitored by the xCELLigence system and expressed as cell index. Data, mean ± SD from a quadruplicate experiment representative of 2 replicates.
automatically monitored with the xCELLigence system (Fig. 4D). This is in agreement with our previous data showing that 10 mmol/L RERF does not modify the growth of endothelial cells. Although we have not identified the exact molecular mechanism by which RERF and UPARANT inhibit VEGF-triggered signaling, our data suggest that RERF and UPARANT impinge on common downstream signaling mediators.

UPARANT inhibits tube formation of endothelial cells exposed to VEGF in vitro

To investigate the ability of UPARANT to inhibit VEGF-triggered angiogenesis in vitro, endothelial cells were plated on Matrigel in the presence of 40 ng/mL VEGF165 mixed to diluents or increasing concentrations of RERF, ERFR, or UPARANT. As expected, endothelial cells exposed to VEGF, formed a 3-dimensional network of tubes resembling capillary-like structures, which reached at 6 hours the 257 ± 10% of branches counted in the absence of any angiogenic stimulus (Fig. 5A and B). Unlike ERFR control peptide or nonimmune serum, endothelial cell incubation with anti-VEGF Ab or anti-αvβ3 mAb reduced VEGF-dependent tube formation to the basal level (Fig. 5B). UPARANT reduced the extent of VEGF-dependent tube formation in a dose-dependent manner. Similar to RERF, the inhibitory effect of UPARANT starts in the femtomolar concentration range and levels off in the nanomolar range. Remarkably, UPARANT caused an overall 75% inhibition of VEGF-dependent tube formation above 10 nmol/L, when compared with RERF, which caused a 54% inhibition. Also, the IC50 of UPARANT seems to be 100-fold lower as compared with RERF (Fig. 5C), indicating again that UPARANT inhibits VEGF signaling more efficiently than RERF.

UPARANT inhibits angiogenesis in vivo

To assess the effect of UPARANT on angiogenesis in vivo, first a quantitative Matrigel sponge assay was
performed. A cocktail of VEGF$_{165}$ (100 ng/mL) and heparin (50 U/mL) promoted a hemorrhagic vascularization of the Matrigel sponge, which was clearly detectable at 5 days postimplantation (Fig. 5D). The presence in the sponge of 75 or 150 μg UPARANT produced a marked reduction of capillary sprouts originating from the host vessels that invaded Matrigel sponges as detected by visual inspection of the gels and quantification of hemoglobin recovered from sponges (Fig. 5D). A 64% and 72% reduction of hemoglobin was found in sponges loaded with 75 and 150 μg, respectively.

Furthermore, antiangiogenic activity of UPARANT was investigated by a corneal pocket assay in rabbits. Neovascular growth was evaluated in 12 rabbits upon subcorneal implantation of slow release pellets containing PBS, 5 μg UPARANT, or 180 ng VEGF with/without 5 μg UPARANT. Implantation of pellets containing 180 ng VEGF stimulated ingrowth of blood vessels in the rabbit corneas starting from day 2, and continuing to grow progressively up to 15 days before regressing. At day 15, rabbit corneas probed with pellets impregnated with VEGF exhibited higher angiogenic score than the average score of those impregnated with vehicle only (PBS; 3.3 ± 0.6 vs. 0.1 ± 0.1 with P < 0.0001; Fig. 5E and F). When UPARANT was tested at 5 μg/pellet alone, it did not elicit inflammatory response. Conversely, UPARANT elicited a 75% inhibition of VEGF-induced vascularization (0.84 ± 0.4 vs. 3.3 ± 0.6 with P < 0.0001; Fig. 5E and F). Taken together, our findings indicate that UPARANT behaves as an antiangiogenic factor, which prevents VEGF-induced angiogenesis in vitro more effectively than RERF.

**Discussion**

This work is based on previous evidence showing that the chemotactic sequence of the human urokinase receptor (uPAR$_{88-92}$) drives in vitro and in vivo angiogenesis in a protease-independent manner (13). A few years ago we developed the peptide Ac-Arg-Glu-Arg-Phe-NH$_2$ namely RERF, that prevents, in vitro and in vivo, migration and invasion of tumor cells by inhibiting the uPAR$_{88-92}$-dependent signals (19). More recently, we have documented that RERF prevents both uPAR$_{88-92}$ and VEGF-induced angiogenesis in vitro and in vivo (13). However, RERF is expected to have some pharmacokinetic drawbacks as drug candidate, which are common to peptides: they are usually very susceptible to proteolytic degradation in vivo and are rapidly cleared from the circulation in minutes (36). To overcome these drawbacks, we have generated a series of peptide analogues of RERF containing Cα-methyl-α-amino acids, a structural modification used in the past to overcome these limitations (37). Among the enormous scenario of possible peptide modifications developed in the last 50 years, we have chosen Cα-methyl-α-amino acid substitutions on the basis of the previously reported NMR solution structure of RERF. In fact, when it is necessary to modify the peptide composition to improve the pharmacologic profile, it is also mandatory to keep, and possibly reinforce, the bioactive conformation, and henceforth the pharmacophore orientation as in the original bioactive peptide. The NMR solution structure of RERF revealed that this peptide, although displaying some conformational flexibility, preferentially adopts a turned structure, therefore Cα-methyl-α-amino acid substitutions were selected for their well-known propensity to strongly induce turned structures. Among the series of newly synthesized analogues (Icm25, Icm25B, Icm25-4, and UPARANT), UPARANT displayed in a preliminary evaluation of the biologic activity the strongest inhibition of endothelial cell migration and showed a long $t_{1/2}$ in enzyme digestions. UPARANT was first characterized from a structural point of view by NMR spectroscopy.

The solution structures of UPARANT and RERF reveals common features as can be seen by comparison of Fig. 6A–C. The backbone atoms superimposition for all residues of RERF and UPARANT gave root-mean-square deviation (RMSD) values of 0.76 Å, which underlines their structural similarity. Both RERF and UPARANT adopt in solution a helicogenic-turned structures; an α-turn (type I-αRS) is found for RERF and an incipient 310-helix for UPARANT (Fig. 1C). However, UPARANT shows a more stable and compact turned structure with respect to RERF. As anticipated from the design, the presence, in the tetrapeptide UPARANT, of Cα-methyl-α-amino acids, such as Aib and Cα(Me)Phe, induces a significant stabilization of the turned structure. UPARANT and RERF share a common structural motif: the aromatic ring of the Phe residue is flanked by 2 positively

**Figure 6.** Comparison of the solution structures of UPARANT and RERF. A, simplified representation with backbone atoms in ribbon drawing of UPARANT average structure. B, average molecular conformation of RERF as derived from NMR analysis in water/TFE solution, backbone atoms are in ribbon drawing. C, backbone atoms superposition of RERF (purple) and UPARANT (cyan).
charged residues. A major difference regards the orientation of Arg1 side chain. In the structure of RERF, the guanidinium group is involved in ion pairing with Glu7, whereas in UPARANT the same group is involved in hydrogen bonding with the amide terminal CO group. In addition, it is worth mentioning that RERF and UPARANT were both studied by NMR spectroscopy in 2 solvent systems, water and water/TFE, for the well-known secondary structure inducing effect of TFE. RERF is poorly structured in water and its turned structure clearly appears in the presence of TFE only. Conversely, UPARANT is well structured in both solvent systems. We have foreseen that stabilization of the turned conformation for UPARANT, similar to that found for RERF, would have beneficial effects on biologic activity. We found that UPARANT inhibits VEGF-driven angiogenesis in vitro and in vivo more efficiently as compared with RERF. In vitro, UPARANT inhibits VEGF-dependent tube formation of endothelial cells at a 100× lower concentration than RERF. Remarkably, a 75% reduction of tubes formed by endothelial cells exposed to VEGF is achieved by UPARANT at 10 nmol/L concentration whereas only a 54% inhibition has been observed in the presence of 10 nmol/L RERF. In vivo, UPARANT reduces to the basal level VEGF-dependent capillary sprouts originating from the host vessels that invaded Matrigel sponges implanted in mice and prevents neovascularization induced by subcorneal implantation of pellets containing VEGF in rabbits.

Our previous work has documented that RERF prevents uPAR/FPR and fMLF/FPR interactions, being FPR the main binding site of RERF (19). Although we have not investigated UPARANT/FPR association and dissociation process, we found that an excess of UPARANT prevents fMLF binding to FPR, suggesting that fMLF and UPARANT share the same binding site. The pivotal role of αβ3 in mediating VEGF-dependent proangiogenic stimulus is well established (35). The involvement of αβ3 integrin in the UPARANT inhibitory effect is supported by the findings that cell adhesion to vitronectin is greatly reduced in the presence of UPARANT. Taken together, our results confirm the involvement of FPR in binding and mediating inhibition by UPARANT and suggest a mechanism by which αβ3 is forced into an inactive state by UPARANT and, consequently, VEGF downstream signaling mediators are not activated.

Our findings unravel a complex picture of the mechanistic effects of UPARANT on endothelial cells and, more generally, highlight the role of uPAR, integrins, and VEGF-induced signaling events in the regulation of endothelial cell functions. At molecular level, inhibitory effect of UPARANT on VEGF signaling is showed by the decreased amount of phospho-AKT and phospho-ERK1/2 in VEGF-treated endothelial cells.

Pharmacologic control of angiogenesis is considered one of the most promising approaches for the treatment of diseases sustained by excessive angiogenesis, such as cancer and inflammation. However, several side effects have been ascribed to antiangiogenic drugs such as bevacizumab that significantly increases the risk of cardiac ischemic events in cancer patients (38). With respect to this, UPARANT may be considered a good drug by virtue of its ability to selectively inhibit FPR unlike bevacizumab, indicating that UPARANT behaves differently from other VEGF inhibitors. Mechanistically, UPARANT blocks VEGF-triggered signaling by preventing FPR and αβ3 integrin activities without affecting cell survival. In this respect, although we have not ascertain whether a direct interaction between UPARANT and VEGF occurs, it is possible to assume that UPARANT blocks integrin αβ3 activity, thus inhibiting only a part of the VEGF/VEGFR signaling. This suggests a potentially wider, but still target-specific activity for UPARANT.

Furthermore, unlike RERF, UPARANT is stable to sterilization, is stable in blood, and displays a long-time resistance to enzymatic digestion. Peptide-based therapeutics is one of the fastest growing class of new drugs (39). Peptides have unique advantages as therapeutic agents. They have high activity per unit mass, and low manufacturing costs. They also offer great potency, selectivity, and specificity, have low off-target toxicity and low drug–drug interaction potential. In this frame, UPARANT could be considered a promising therapeutic agent for the control of diseases fueled by excessive angiogenesis, such as cancer and inflammation.

Disclosure of Potential Conflicts of Interest

M.V. Carriero has ownership interest (including patents) in Pharmaphelix s.r.l. V. Pavone is the President of Pharmaphelix s.r.l. and also has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Maglio
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