Non-peptidic Thrombospondin-1 Mimics as Fibroblast Growth Factor-2 Inhibitors

AN INTEGRATED STRATEGY FOR THE DEVELOPMENT OF NEW ANTIANGIOGENIC COMPOUNDS

Giorgio Colombo§, Barbara Margosio†, Laura Ragona§, Marco Neves§, Silvia Bonifacio§, Douglas S. Annis§, Matteo Stravalaci**, Simona Tomaselli§, Raffaella Giavazzi§, Marco Rusnati†‡, Marco Presta‡‡, Lucia Zetta§, Deane F. Mosher§, Domenico Ribatti§§, Marco Gobbi***, and Giulia Taraboletti****

From the §Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, Milan 20131, Italy, the †Department of Oncology, Maria Negri Institute for Pharmacological Research, Bergamo 24125, Italy, the **Istituto per lo Studio delle Macromolecole, Consiglio Nazionale delle Ricerche, Milan 20133, Italy, the ††Departments of Biomolecular Chemistry and Biotechnology, School of Medicine, University of Brescia, Brescia 25123, Italy, and the §§Department of Human Anatomy and Histology, University of Bari, Bari 70124, Italy.

Endogenous inhibitors of angiogenesis, such as thrombospondin-1 (TSP-1), are promising sources of therapeutic agents to treat angiogenesis-driven diseases, including cancer. TSP-1 regulates angiogenesis through different mechanisms, including binding and sequestration of the angiogenic factor fibroblast growth factor-2 (FGF-2), through a site located in the calcium binding type III repeats. We hypothesized that the FGF-2 binding sequence of TSP-1 might serve as a template for the development of inhibitors of angiogenesis. Using a peptidic array approach followed by binding assays with synthetic peptides and recombinant proteins, we identified a FGF-2 binding sequence of TSP-1 in the 15-mer sequence DDDDDNDKIPD-DRDN. Molecular dynamics simulations, taking the full flexibility of the ligand and receptor into account, and nuclear magnetic resonance identified the relevant residues and conformational determinants for the peptide–FGF interaction. This information was translated into a pharmacophore model used to screen the NCI2003 small molecule databases, leading to the identification of three small molecules that bound FGF-2 with affinity in the submicromolar range. The lead compounds inhibited FGF-2-induced endothelial cell proliferation in vitro and affected angiogenesis induced by FGF-2 in the chicken chorioallantoic membrane assay. These small molecules, therefore, represent promising leads for the development of antiangiogenic agents. Altogether, this study demonstrates that new biological insights obtained by integrated multidisciplinary approaches can be used to develop small molecule mimics of endogenous proteins as therapeutic agents.

Inhibitors of angiogenesis, aimed at preventing the deregulated formation of new blood vessels, are emerging as a successful approach to treat an array of diseases, including cancer (1). Antiangiogenic strategies are designed to reestablish the balance between angiogenic factors and endogenous inhibitors, restoring the physiologically quiescent condition of the vasculature (2). A strategy to achieve this is to exploit the activity of endogenous inhibitors of angiogenesis (3).

Thrombospondin-1 (TSP-1)2 was the first endogenous inhibitor of angiogenesis identified (4, 5). Of the five members that constitute the TSP family in mammals, the homotrimeric TSP-1 and TSP-2 share domain organization and the ability to inhibit angiogenesis. Like other thrombospondins, TSP-1 is a multi-modular protein. Each monomer consists of an N-terminal globular domain followed by the coiled-coil oligomerization domain, a von Willebrand factor type C repeat, three properdin-like type I repeats, two TSP-type epidermal growth factor (EGF)-like or type II repeats, and a signature domain comprising a third EGF-like repeat, the calcium binding wire or type III repeats, and the lectin-like C-terminal globular domain (6).

TSP-1 has been classified functionally as a matricellular protein, i.e. an extracellular protein that acts to regulate cell interactions with the environment (7). TSP-1 binds to a variety of proteins on the cell surface and in the extracellular milieu. Each TSP-1 domain has a distinct complement of binding molecules, and TSP-1 can elicit different functions, depending on which domain is active and able to engage its specific cell receptors and extracellular ligands. It is, therefore, not surprising that TSP-1 can exert its antiangiogenic activity through multiple mechanisms involving different active sequences in different domains (8, 9).

The main antiangiogenic site of TSP-1 has been identified in the type I repeats and includes adjacent sequences that interact

2 The abbreviations used are: TSP-1, thrombospondin-1; FGF-2, fibroblast growth factor-2; HSPG, heparan sulfate proteoglycans; EGF, epidermal growth factor; MD, molecular dynamics; SPR, surface plasmon resonance; RU, resonance units; STD, saturation transfer difference.
with the CD36 receptor, transforming growth factor-β and heparan sulfate proteoglycans (HSPGs). Other sequences in different domains of TSP-1 influence angiogenesis through binding to angiogenic growth factors, integrins, CD47, HSPG, proteases, and low density lipoprotein receptor-related proteins (for review, see Refs. 10 and 11). Recently, using recombinant portions of TSP-1, we identified a previously undescribed antiangiogenic site in the type III repeats of TSP-1 and demonstrated that binding of the angiogenic factor FGF-2 to this site inhibits angiogenesis by sequestration of FGF-2 (12). Binding of FGF-2 to TSP-1 and its type III repeats is of high affinity and involves a yet unidentified sequence sensitive to calcium and heparin (12–14). Each antiangiogenic TSP-1 sequence offers a potential tool for the design of new angiogenesis inhibitors. The starting sequence GVITRIR in the second type I repeat that was exemplified by ABT-510, a synthetic nonapeptide based on the sequence GVITRIR in the second type I repeat that was the first TSP-1-based antiangiogenic compound to reach clinical testing (16).

Protein-protein interactions are generally difficult to inhibit with small molecules because of their large, flexible surfaces, which pose a fundamentally different problem from targeting small, well-defined concave active sites (17, 18). The use of a biologically relevant, functional peptide sequence as an entry point for lead development represents a potential powerful approach in drug discovery (19–22). The starting sequence can elucidate the roles of key interactions (hot spots) involved in the regulation/inhibition of important protein-protein interactions that a synthetic molecule must mimic. The knowledge of relevant interactions and their space relationships in the stabilization of protein-protein complexes may thereby boost our ability to interfere with specific protein-protein interactions, providing attractive therapeutic opportunities and extending medicinal chemistry to new classes of compounds. Different rational approaches are in fact based on the identification of synthetic molecules that mimic the hot spot interactions in relevant macromolecular complexes (20, 23, 24). Computational biology methods based on all-atom molecular dynamics (MD) have recently proved successful in identifying the functional and conformational determinants of molecular recognition and translating these models into novel antagonists with specific activities, thus expanding the molecular diversity space of mimics of interacting active sequences (25–29). This study aimed at identifying the FGF-2-binding sequence of the TSP-1 type III repeats and used an approach integrating computational biology, biophysics, biochemistry, and functional biology to design new, non-peptidic inhibitors of angiogenesis based on this sequence.

**EXPERIMENTAL PROCEDURES**

Reagents—Sequences of peptides used in the study are indicated in Table 1. Peptides (biotin-labeled or acetylated at the N terminus) were obtained from Pepscan Presto BV (Lelystad, The Netherlands) or were synthesized by R. Longhi (Consiglio Nazionale delle Ricerche, Milan, Italy). At least two batches of the peptides DD15, DIDG16, AQT19, and DD35 were tested. The small molecules were obtained from the Developmental Therapeutics Program, NCI, National Institutes of Health (Rockville, MD). Preparation of recombinant human TSP-1 fragments (30) is described in the supplemental Methods. Human recombinant FGF-2 (R&D Systems, Minneapolis, MN) was obtained through the NCI-Biological Resources Branch (Frederick, MD). For the NMR studies, human recombinant FGF-2 was kindly provided by A. Bikfalvi (University of Bordeaux). Proteins were labeled with biotin or the lanthanide europium, as described in the supplemental Methods.

**Peptide Array Analysis—**A peptide array was designed based on the sequence of the type III repeats. Peptides were synthesized and screened by Pepscan Presto as described previously (31). Briefly, 237 20-mer peptides with partially overlapping sequences (19-amino acid overlaps) were synthesized and covalently linked to polystyrene cards. The binding of biotinylated FGF-2 (10 μg/ml) to the peptides was tested. Bound FGF-2 was detected with peroxidase-conjugated streptavidin and the peroxidase substrate 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS). Color development was quantified with a CCD camera (Sony CCD Video Camera XC-77RR) and the image processing software Optimas 6.5 (Media Cybernetics, Silver Spring, MD). Data are presented as the relative signal value, calculated by normalizing each value $S$ to an arbitrarily set background value $S_{\text{bckg}}$ and to the maximal signal $S_{\text{max}}$: 

$$\frac{(S_{\text{on}} - S_{\text{bckg}})}{(S_{\text{max}} - S_{\text{bckg}})}$$

**Binding Assays—**The peptide-FGF-2 interaction was assessed as direct binding of the biotin-labeled peptides to microtiter plates coated with FGF-2, as described in the supplemental Methods. Bound peptide was detected by mouse anti-biotin antibody and mouse IgG Elite VECTASTAIN ABC kit (Vector Laboratories) followed by 1,2-phenylenediamine dihydrochloride (Dako, Glostrup, Denmark) as a chromogen. A similar procedure was used to assess the binding of biotin-labeled FGF-2 to recombinant TSP-1 domains and to analyze the ability of small molecules to inhibit the binding of the biotin-labeled recombinant type III domain to FGF-2. See the supplemental Methods for detailed protocols.

Kinetic binding constants and affinity values were obtained by using surface plasmon resonance (SPR), carried out with the ProteOn XPR36 Protein Interaction Array system (Bio-Rad) (32). FGF-2 was covalently immobilized on the sensorchip (Proteon GLC Bio-Rad) by amine coupling chemistry, with final immobilization levels of 3500–4000 resonance units (RU; 1 RU = 1 pg of protein/mm²). A reference surface was always prepared in parallel with no ligand immobilized. Peptides and small molecules were then injected over immobilized FGF-2 at a rate of 30 μl/min for 3 min (association phase), whereas dissociation was measured in the following 10–15 min. The running buffer, also used to dilute analytes, was phosphate-buffered saline, pH 7.4, 0.005% Tween 20 (Bio-Rad). All these assays were done at 25 °C.

The sensorgrams (time course of the SPR signal in RUs) were normalized to a base-line value of 0. The signal observed in the surfaces immobilizing FGF-2 was corrected by subtracting the nonspecific response observed in the reference surface. The resulting sensorgrams were fitted by the simplest 1:1 interaction model (Langmuir model, ProteOn analysis software) to obtain the corresponding association and dissociation rate constants ($K_{\text{on}}$ and $K_{\text{off}}$) and the equilibrium dissociation constant.
\( K_d \) (\( K_{\text{off}}/K_{\text{on}} \)). Details are provided in the supplemental Methods.

**Simulation Set Up and Analysis**—Simulations for DD15 were started from a fully extended conformation of the peptide to eliminate possible conformational biases. An initial representative conformation for the peptide was obtained by conformational search using the Systematic Unbounded Multiple Minimum (SUMM) routine implemented in MACROMODEL Version 8.1 with the AMBER force field and the Polak-Ribiere Conjugate Gradient (PRCG) minimization method with a maximum of 700 minimization steps and an energy convergence criterion of 0.05 \( \text{kJ/mol} \). Solvent effects were taken into account by using the Generalized Born/Surface Area (GB/SA) explicit solvent model with a dielectric constant \( \epsilon \) of 78 (33). The global minimum conformation obtained from this preliminary calculation was then subjected to 60 ns of MD refinement in explicit water solvent using the same protocol as described in the next paragraph for MD simulations of the complexes. The resulting trajectories were analyzed by the structural clustering method described by Daura et al. (34). The most representative structures of DD15 obtained after cluster analysis of the trajectory were subjected to multiple docking runs on the surface of FGF-2 (x-ray structure downloaded from the PDB code 1eq9) using the program AUTODOCK (35) essentially as described in Meli et al. (25) and Plescia et al. (36). The representative structure of the most populated cluster obtained from the docking runs, corresponding also to the free energy minimum, was used for successive MD refinement. The structural evolution of DD15 in isolation and of the complex DD15-FGF-2 was studied with long time scale MD simulations in explicit water solvent. DD15 or the DD15-FGF-2 complex was first solvated with water in a periodic-truncated octahedron large enough to contain the peptide or complexes and 0.8 nm of solvent on all sides. The protonation and charge states of the side chains of the peptides and the receptor were chosen to be consistent with the solution conditions of the experiments; amine groups were considered as having a +1 charge, and carboxylic groups were considered as having a −1 charge. The FGF/DD15 system was found to have a total charge of +4 (FGF +11/DDD −7). Four \( \text{Cl}^- \) counter ions were added to the system to ensure electroneutrality. The system was initially energy-minimized with a steepest descent method for 5000 steps. The temperature was maintained close to the intended value of 300 K by weak coupling to an external temperature bath. The GROMOS96 force field and the simple point charge water model were used. The calculation of electrostatic forces utilized the particle mesh Ewald implementation of the Ewald summation method. Molecular Dynamics simulations were run at constant number of particles, volume, and temperature (NVT ensemble) and were 60 ns long. All the MD runs and the analysis of the trajectories were performed using the GROMACS software package. Configurations of the receptor-ligand complex were saved every 4 ps for subsequent statistical analysis.

More complete descriptions of the simulation and docking methodologies are detailed in the supplemental Methods. For the sake of simplicity the numbering of FGF-2 starts from 1 (residue 16 in the original Protein Data Bank file).

**Nuclear Magnetic Resonance (NMR) Spectroscopy**—A set of two-dimensional NMR experiments (TOCSY, ROESY, NOESY) was used to assign the proton chemical shifts of DD15 dissolved at 0.7 mM peptide concentration in 30 mM water phosphate buffer, 50 mM NaCl, pH 7. NMR spectra were recorded on a Bruker DMX 500 MHz NMR spectrometer. \( T_1 \) measured in the presence of FGF-2, were found to be rather constant along the peptide side chains in a range of 0.5–0.8 s, except for a longer \( T_1 \) value measured for methyl protons of acetyl group (1.3 s).

The DD15 chemical groups involved in the direct contact with FGF-2 were identified by saturation transfer difference (STD) methods. One-dimensional STD pulse sequence incorporating a \( T_{1\alpha} \) filter to remove disturbing protein signals was used (37). On-resonance irradiations were performed at different frequencies in the methyl (−236 Hz) and aromatic (3,220 Hz) regions, and off resonance irradiation was performed at −15,000 Hz using a series of Gaussian pulses with a 1% truncation and 50-ms duration to give different total saturation times of 2, 2.5, 3, 4, and 4.5 s. See the supplemental Methods for details.

**Pharmacophore Generation**—The central structure of the most populated cluster for the DD15-FFG-2 complex obtained from MD simulations was used as a template to direct pharmacophore design. The relative distances and orientations (dihedral angles) among the different groups of DD15 identified as responsible for the most persistent interactions with FGF-2 from MD were used to define the pharmacophoric points. Catalyst software (Accelrys Catalyst, San Diego CA, 2005) was used for pharmacophore design. The models created were used to screen the NCI, National Institutes of Health data base. The list of compounds retrieved was post-processed using drug-likeness filters. See the supplemental Methods for details.

**Biological Assays**—The biological activity of the small molecules was analyzed as the ability to prevent the binding of europium-labeled FGF-2 to bovine aortic endothelial cells, measuring bound FGF-2 by time-resolved fluorescence using a Victor multilabel plate reader (PerkinElmer Life Sciences). Proliferation of endothelial cells exposed to 5 ng/ml FGF-2 in the presence of the small molecules was assessed with a colorimetric assay (14). One small molecule (sm27) was also tested for ability to affect angiogenesis in the chicken embryo chorioallantoic membrane assay using fertilized White Leghorn chicken eggs, where angiogenesis is induced by sterilized gelatin sponges loaded with FGF-2 with or without the small molecule (38). Details are described in the supplemental Methods.

**RESULTS**

**Identification of a FGF-2 Binding Sequence of TSP-1**—The peptide array technology was used to identify linear sequences involved in the interaction of FGF-2 with the type III repeats of TSP-1. The array consisted of 237 partially overlapping 20-mers peptides spanning the entire sequence of the type III repeats (residues 692–945). The immobilized peptides were tested for their ability to bind biotinylated FGF-2. Three potentially active sequences were identified: DDDDDNDKIPD-DRDN (residues 739–753; hereafter named DD15), AQYDY-DRDD (761–769; AQ9), and DIDGDG (residues 800–805, DIDG6, Fig. 1a). Biotin-labeled peptides corresponding to the three candidate sequences were then synthesized and tested for
Non-peptidic TSP-1 Mimics as FGF-2 Inhibitors

**FIGURE 1. Identification of the FGF-2 interacting sequence of TSP-1.** a, shown is peptide array analysis. Binding of biotin-labeled FGF-2 to the 237 peptides was analyzed and expressed as described under “Experimental Procedures.” Shown below the graph is a schematic representation of the structure of the type III repeats, organized in 13 C- or N-type motifs, and of the recombinant fragments Tr1–4 used in the above results, no binding was observed with AQ19 peptide and the dissociation rate (Koff) of 28.0 ± 1.8 s⁻¹ (Table 2). In agreement with the computational model, accounting for about 80% of structures visited, was chosen for multiple blind docking experiments with the protein, we used four recombinant TSP-1 constructs starting from the third EGF-like repeat (residue 648) and covering progressively longer segments of the type III repeats (Fig. 1a). Consistent with the experiments with synthetic peptides, Tr3 (the shortest construct that includes the DD15 sequence) bound FGF-2, whereas the shorter proteins Tr1 and Tr2, lacking this sequence, did not (Fig. 1d). Tr4 (residues 648–776), comprising both sequences of DD15 and AQ19, had even higher binding activity, suggesting co-involvement of the two sequences. In agreement with this, the synthetic peptide DD35 (residues 739–773) that extended the DD15 sequence to include AQ19 (Table 1) showed higher affinity for FGF-2 due to a lower dissociation rate than DD15 (Table 2). The QQ19 sequence, although not directly able to bind FGF-2, might have a stabilizing or supportive effect on DD15 binding to FGF-2.

**MD Analysis of DD15 and the DD15-FGF-2 Complex, Characterization of the Binding Interface, and Identification of Critical Residues—** The conformational dynamics of DD15 in isolation in solution was studied by 60-ns-long all-atom, explicit solvent MD simulations. The dominant structure for the peptide was extended, maximizing the interactions between the large number of charged residues and the water solvent. This conformation, accounting for about 80% of structures visited, was chosen for multiple blind docking experiments with the DD15 and the DD15-FGF-2 complex.
mental competition binding assays showed that heparin prevented DD15 peptide binding to FGF-2 (supplemental Fig. 1), confirming that the peptide and heparin compete for the same binding site on FGF-2. The large number of Asp residues was crucial in the recognition of FGF-2 and the orientations of the segment Lys-746-Asp-750, recapitulated in Fig. 3b. Addi- 

mental Fig. 2). The conformation of DD15 and the orientations of some of the unassigned aspartic residues cannot be ruled out. Altogether the NMR data point to specific contacts mainly included Asp-750, interacting with FGF-2-Tyr-9, Asp-741, interacting with FGF-2-Arg-105 and FGF-2-Asn-12, Asp-749, interacting with FGF-2-Tyr-9, and Asp-740, interacting with FGF-2-Thr-106 (supplemental Table 2).

To define hydrophobic/aromatic interactions, we monitored the contacts involving non-polar DD15 side chains, Ile-747 and Pro-748, and the FGF-2 protein. These residues interact with various amino acids on the surface of FGF-2, namely FGF-2-Tyr-9, FGF-2-Cys-10, FGF-2-Lys-11, FGF-2-Arg-29, and the turn spanning FGF-2-Asn-14—Gly-16 during most of the production run (supplemental Fig. 2). Solvent-accessible surface analysis revealed that, despite being involved in a large number of contacts with the protein, DD15-Ile-747 was more exposed to the solvent, whereas DD15-Pro-748 fit nicely into an FGF-2 pocket, resulting in much less exposure to the solvent.

NMR Analysis of DD15-FGF-2 Interaction—The validity of the atomic resolution computational model of DD15-FGF-2 complex was verified experimentally by NMR-based interaction studies. Complete 1H resonance assignment of DD15 was possible for Asp-745—Arg-751 region (supplemental Table 3), but unambiguous assignment of the N-terminal segment was hampered by the large numbers of aspartic and asparagine residues and the extensive signal overlap in the NMR spectra. STD NMR methods were recently successful in characterizing the interaction between FGF-2 and antiangiogenic peptides derived from natural FGF-2 binders (41–43). The same approach was used to investigate the structural basis of DD15-FGF-2 interactions and map the peptide residues making direct contact with the protein. STD spectra prove that H\textsubscript{e} of Lys-746 and methyl H\textsubscript{a} and H\textsubscript{b} protons of Ile-747 receive saturation from the protein (Fig. 3a). The STD signals at 2.70 and 2.83 ppm suggest that DD15 interacts with FGF-2 through the side-chain protons of aspartic residues, possibly Asp-741. In particular, beta protons of Asp-750 correspond to the signal observed at 2.70 ppm, although the contribution to the interaction of some of the unassigned aspartic residues cannot be ruled out. Altogether the NMR data point to specific contacts mainly established by the C-terminal region of DD15 peptide involving the segment Lys-746-Asp-750, recapitulated in Fig. 3b. Additional stabilizing contacts could be established by electrostatic interactions involving other aspartic side chains of DD15.

**Pharmacophoric Hypothesis, Small Molecule Identification, and Binding Analysis**—MD and NMR analyses yielded a consistent identification of DD15 residues mostly involved in the recognition of FGF-2 and stabilization of the complex. This prompted us to search for small molecule mimics, with functional and structural features directly related to the DD15 recognition properties at the FGF-2 surface (25). In principle, these molecules should be able to interfere with FGF-2 functions at the cellular level.

A pharmacophoric hypothesis (HYP-DD15) was built based on the results of the MD simulation, validated by NMR data, which indicated that both hydrophobic and electrostatic contributions are relevant to the interaction (Fig. 3b and supplemental Fig. 2). The conformation of DD15 and the orientations

### TABLE 1
Peptides used in the study

| Peptide | Residues | Sequence |
|---------|----------|----------|
| AQ19    | 755-773  | PPFYPAQDYDVRD0GDR |
| AQ29    | 761-786  | AWQYDYDROD |
| DD15    | 739-753  | DDDDDNKIPDRD0N |
| DD35    | 739-773  | DDDDDNKIPDRD0NCPFHYPAQDYDVRD0GDR |

### TABLE 2
FGF-2 binding parameters of peptides and small molecules

| Peptide | n | K\textsubscript{on} | K\textsubscript{off} | K\textsubscript{d} |
|---------|---|----------------|----------------|----------------|
| DD15    | 6 | 19.7 ± 2.0 | 5.5 ± 0.8 \times 10^{-4} | 28.0 ± 4.0 |
| AQ29    | ND | ND | ND | ND |
| DD35    | 4 | 10.1 ± 0.2 | 1.2 ± 0.2 \times 10^{-4} | 12.0 ± 2.0 |

| Small molecule | n | K\textsubscript{on} | K\textsubscript{off} | K\textsubscript{d} |
|----------------|---|----------------|----------------|----------------|
| sm27 (NSC-37204) | 4 | 917.0 ± 140.0 | 3.2 ± 0.2 \times 10^{-4} | 0.3 ± 0.1 |
| sm8 (NSC-1698) | 4 | 434.0 ± 18.0 | 1.9 ± 0.1 \times 10^{-4} | 0.4 ± 0.1 |
| sm10 (NSC-63983) | 8 | 15.8 ± 1.1 | 5.2 ± 0.6 \times 10^{-4} | 33.8 ± 3.5 |
of its side-chain functional groups in the most populated structural cluster from the MD trajectory of the complex were used as a structural template (Fig. 3c). The distributions of dihedral values and distances between critical functionalities were used to define the upper and lower boundaries for geometric constraints. Three pharmacophoric points were considered; two negative ionizable functionalities mapped over the carboxyl groups of Asp-741 and Asp-750 and one five- or six-member ring moiety mapped on the position of the corresponding ring of Pro-748, to mimic the peptide hydrophobic patch defined by I747-P748 and provide structural rigidity to hits (Figs. 2a and 3b). The ring moiety was allowed to be either aliphatic or aromatic and contain carbon, oxygen, nitrogen, or sulfur atoms.

The pharmacophore was used to screen the NCI2003 data base of molecules (containing ~300,000 compounds) (Developmental Therapeutics Program, NCI/ National Institutes of Health (nci.nih.gov) (44). This search yielded 258 compounds. Subsequent filtering based on bioavailability rules and maximum number of rotatable bonds (RB ≤ 6) reduced the number of hits to 130 and 42 molecules, respectively. Nineteen of them, made available from the NCI, National Institutes of Health were subjected to experimental analysis of FGF-2 binding.

In preliminary screening, all the molecules were tested at a high concentration (50 μM) for their ability to compete for the binding of the labeled type III repeats to FGF-2 (Fig. 4a). Three molecules (sm8, sm27, and to a lesser extent sm10, Fig. 4b) showed significant competition activity and were tested further. In a dose-response analysis, sm27 was more potent than sm8 in preventing the binding of the TSP-1 domain to FGF-2 (with IC_{50} of 1.3 and 8.2 μM respectively; not shown). Analyzed by SPR, both sm27 and sm8 gave significant binding signals despite their low molecular weight (Fig. 4c). The sensograms indicated submicromolar affinity (K_{d} ~ 400 nM) with faster association rates and slower dissociation rates than DD15 (Table 2). Sm10 showed much lower affinity (K_{d} 34 μM).

Biological, Antiangiogenic Activity of the New Leads in Vitro and in Vivo—The high affinity interaction of lead molecules with FGF-2 suggested they might retain the antiangiogenic activity of the entire TSP-1 and the type III repeats (12, 14). Indeed, both sm27 and sm8, although not sm10, inhibited the binding of FGF-2 to endothelial cells (Fig. 5a). The effect was dose-dependent, sm27 being more potent than sm8 (mean IC_{50} 24.0 ± 6.9 and 79.9 ± 28.1 μM, respectively). The binding assay measures total bound FGF-2, mainly FGF-2 bound to HSPG. The finding, therefore, indicates an inhibitory effect on FGF-2 binding to cell surface HSPG. At the same concentrations, the two molecules also inhibited FGF-2-induced endothelial cell proliferation (Fig. 5b), with only a marginal effect on proliferation induced by serum (not shown). Again, the most active was sm27 (mean IC_{50} 20.3 ± 5.9 μM) followed by sm8 (IC_{50} 67.8 ± 15.9 μM), whereas sm10 had only a modest inhibitory effect. FGF-2-induced proliferation of fibroblasts (NIH-3T3 cells), not inhibited by TSP-1, was also not inhibited by sm27 (not shown). The most active molecule, sm27, was tested in vivo in the chorioallantoic membrane assay where angiogenesis is induced by FGF-2 embedded in gelatin sponges. Sponges loaded with FGF-2 alone induced a macroscopic angiogenic response that was not
observed with sponges loaded with FGF-2 and sm27 (Fig. 5). The presence of the small molecule (0.5 mg) reduced the mean number of blood vessels entering the sponge from 26 ± 4 (FGF-2 alone) to 10 ± 2 (FGF-2 + sm27, p < 0.001, n = 10). No inhibition was observed with a lower dose of sm27 (0.15 mg) nor with the DD15 peptide (not shown), in agreement with the lack of activity of the peptide in vitro. Interestingly, angiogenesis induced by vascular endothelial growth factor was not affected by sm27 (not shown). These findings confirm that small molecules mimetic of the FGF-2 binding sequence of TSP-1 bind and sequester FGF-2, inhibiting its angiogenic activity.

**DISCUSSION**

Pathological angiogenesis underlies a wide range of diseases, including cancer, autoimmune diseases, atherosclerosis, and rheumatoid arthritis. Endogenous inhibitors of angiogenesis are a powerful source of tools for developing therapies for these diseases. Among them, TSP-1 is an attractive, not yet fully exploited model for potential new compounds.

We report the identification of an FGF-2 binding sequence of TSP-1 within the type III repeats and the discovery of small molecule mimics of this sequence that retain the ability to bind FGF-2 and inhibit angiogenesis. To achieve this goal, we developed a multidisciplinary strategy integrating molecular and cell biology, peptide array, computational biology, NMR spectroscopy, and drug discovery to develop a molecular understanding of the TSP-1-FGF-2 interaction and use this information to search for small molecule leads. This strategy identified three small molecules as possible starting points for the development of novel anticancer agents.

To locate the FGF-2 binding sequence within the TSP-1 type III repeats, we used peptide array technology, widely employed...
to map ligand-interacting linear sequences. Our finding of a peculiar pattern of repetitive active peaks is consistent with the repetitive nature of this TSP-1 domain. Of the three potentially active sequences, only DD15 retained the ability to bind FGF-2 in experimental binding assays, as confirmed by SPR and NMR analysis. Interestingly, this sequence is different from other active sites mapped in the type III repeats, such as the integrin interacting RGD, binding sequences for cathepsin G, neutrophil elastase, collagen V, and the attachment sites for neutrophils and sickle red blood cells (45–48). The type III repeats comprise 13 aspartate-rich calcium-binding repeats of the N-type or C-type (6, 48). The DD15 sequence is located in the third, C-type calcium binding wire repeat (supplemental Fig. 3) and is conserved in TSP-2 and cartilage oligomeric matrix protein. Calcium inhibits the binding of TSP-1 and its type III repeats to FGF-2 (12). In a crystal structure that includes calcium-replete type III repeats of TSP-2 (49), the sequence homologous to DD15 follows a tortuous path that contributes to coordination with four calcium atoms through the side chains of residues homologous to Asp-739, Asp-741, Asp-742, Asp-743, Asn-744, Asp-745, Asp-749, and Asp-752 and the main chain carbonyls of Asp-739, Asp-741, and Ile-747 (supplemental Fig. 3). Thus, when the calcium binding sites are occupied, the sequence would not be expected to adopt the highly negatively charged, extended conformation that interacts with FGF-2. However, the cysteine residues that flank the DD15 sequence are disulfide-bonded not to one another but to other cysteines, N-terminal or C-terminal. Therefore, the DD15 sequence is not constrained by a disulfide and has the potential to extend into the active conformation when calcium is removed.

The synthetic peptide DD15 bound FGF-2 with lower affinity ($K_d 28 \mu M$) than the whole TSP-1 ($K_d 11 nM$) or the type III repeats ($K_d 314 nM$) (12, 13). The biotin used to label the peptides might partly account for that. Indeed, an unlabeled DD15 peptide had higher affinity for FGF-2 than the biotin-labeled peptide ($K_d 3.6$ and $28 \mu M$, respectively; not shown), indicating a possible underestimation of the affinity values calculated with biotin-labeled peptides. Nonetheless, the progressive drop in affinity from the entire molecule to the fragment and to the small peptide suggests a contribution of other residues to FGF-2 binding. This is supported by the increased affinity of larger recombinant domains (see Fig. 1d) or extended synthetic peptides (such as DD35). Adjacent TSP-1 regions might contribute to FGF-2 binding by providing contacts that cooperatively increase affinity or inducing structural changes that favor the exposure and correct conformation of the active site. It is well known that the conformation and activity of the type III repeats are particularly sensitive to the influence of other TSP domains (50).

Associated with the decreased affinity, the synthetic peptides had no biological activity in terms of preventing FGF-2 binding to cells or inhibiting endothelial cell proliferation (not shown). Despite this drawback, the experimental demonstration of DD15:FGF-2 binding provided important information on the possible physicochemical determinants of FGF-2 recognition by TSP-1.

The computational model of DD15:FGF-2 complex predicted the binding of DD15 at the heparin-binding site of FGF-2. This was confirmed by experimental competition experiments and is in agreement with our previous observation...
Non-peptidic TSP-1 Mimics as FGF-2 Inhibitors

FIGURE 5. Antiangiogenic activity of small molecules mimetic of the FGF-2-binding sequence of TSP-1. a, shown is binding of FGF-2 to endothelial cells. BAEC were incubated with labeled FGF-2 in the presence of the small molecule (1–100 μM). The cell-bound FGF-2 is expressed as a percentage of control (in the absence of small molecules). b, shown is endothelial cell proliferation. BAEC were exposed to 5 ng/ml FGF-2 with the small molecule (6–100 μM) and incubated for 3 days. Proliferation is expressed as a percentage of control (in the absence of small molecules). c, shown is FGF-2-induced angiogenesis in the chorioallantoic membrane assay. FGF-2 (200 ng) was administered in the absence or presence of sm27 (0.5 μg) on day 8. A representative picture taken 4 days later is shown. Original magnification, 50×.

that intact TSP-1 as well as a recombinant fragment comprising the type III repeats inhibited the binding of FGF-2 to HSPG on the surface of endothelial cells and in the extracellular matrix (12–14). The biological importance of this interaction in FGF-2 bioavailability and activity suggests that DD15 mimics might be developed as inhibitors of FGF-2 biological functions.

This prompted us to search for small molecule mimics of DD15. We developed pharmacophore models based on ensembles of conformations obtained from long MD simulations of the DD15-FGF-2 complex and validated by NMR measurements. The dynamic pharmacophoric approach had already been used successfully to develop chaperone and aggregation small molecule inhibitors from peptide antagonists (25, 26, 36). The inclusion of dynamics in the small molecule discovery process is particularly important in the case of protein-peptide and protein-protein interaction inhibitors. Conformational adaptation and selection are intimately related to the recognition between flexible and exposed surfaces. Including receptor and ligand flexibility provides a more realistic physical understanding of the process with respect to the use of static models, allowing the ligand to undergo conformational changes on the protein surface to find optimal interaction networks.

The present computational biology studies provided an atomic resolution model of the DD15-FGF-2 interaction and its dynamic properties that were subsequently benchmarked through NMR-STD experiments. Computational and experimental data provided a consistent view of the molecular interaction determinants of DD15-FGF-2 binding, including both hydrophobic and electrostatic moieties. This information was then translated into pharmacophore models that enabled us to search for new small molecule inhibitors of FGF-2 independently of any predetermined chemical scaffold or structure, resulting in three new molecular candidates for future development. In contrast to the original TSP-1 sequence, active only in the absence of calcium, these small molecules are modeled to mimic the active conformation of the sequence and, therefore, are independent of the presence of calcium ions in the environment. The small molecules are characterized by a symmetric structure, with two negatively charged sulfonated groups flanking a central aromatic core, reminiscent of the suramin-like FGF-2-antagonists, which bind to the heparin-binding site of FGF-2 (51). Moreover, these molecules meet the stereochemical rules, obtained through a totally different approach, proposed to develop naphthalene sulfonates as FGF inhibitors (52). The hits discovered here have much lower molecular weight than suramin, making them interesting for pharmacological development.

The most active molecules, sm8 and sm27, bound FGF-2 with high affinity (0.3 μM) and retained the ability of the entire TSP-1 (as well as a recombinant fragment containing the type III repeats) to inhibit FGF-2 biological activity; that is, binding to endothelial cells, induction of endothelial cell proliferation in vitro, and induction of angiogenesis in vivo in the chorioallantoic membrane assay. These activities are consistent with the ability of the small molecules and of the entire TSP-1 and the type III repeats to sequester FGF-2, preventing its interaction with HSPG (12–14). The HSPG-FGF interaction has a fundamental role in regulating the bioavailability, signaling, and activity of numerous FGFs. The possibility that TSP-1, peptide DD15, and sm27 target other FGFs besides FGF-2 warrants further investigation.

The clinical experience with inhibitors of angiogenesis, primarily targeting vascular endothelial growth factor, has revealed important limitations, such as the phenomenon of acquired, evasive resistance, associated with the production of different angiogenic factors including FGF-2 (53). The development of new agents targeting angiogenic factors other than vascular endothelial growth factor has, therefore, become a priority. In particular, FGF-2 is emerging as an attractive target for new antiangiogenic therapies (54). Our study shows that it is possible to rationally expand the molecular diversity space of FGF-2 antagonists, with much smaller and chemically “tractable” molecules than the ones known so far. Clearly, the leads...
Non-peptidic TSP-1 Mimics as FGF-2 Inhibitors

identified here cannot yet be considered real drug candidates, but they do offer a solid base for the extension of traditional medicinal chemistry efforts to develop non-traditional strategies. In view of their structure, it may be possible to further derivatize the leads with combinatorial chemistry and improve on their drug-like properties while maintaining the FGF-2 targeting properties.

Acknowledgments—We thank R. Longhi (Consiglio Nazionale delle Ricerche) and A. Bikfalvi (University of Bordeaux, France) for kindly providing reagents.

REFERENCES

1. Folkman, J. (2007) *Nat. Rev. Drug Discov.* 6, 273–286
2. Carmeliet, P. (2005) *Nature* 438, 932–936
3. Nyberg, P., Xie, L., and Kalluri, R. (2005) *Cancer Res.* 65, 3967–3979
4. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouch, N. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 6624–6628
5. Taraboletti, G., Roberts, D., Liotta, L. A., and Giavazzi, R. (1990) *J. Cell Biol.* 111, 765–772
6. Carlson, C. B., Lawler, J., and Mosher, D. F. (2008) *Cell Mol. Life Sci.* 65, 672–686
7. Bornstein, P., and Lawler, E. H. (2002) *Curr. Opin. Cell Biol.* 14, 608–616
8. Ragona, L., Tomaselli, S., Vacca, A., and Presta, M. (2009) *Chem. Biodivers.* 1, 505–519
9. Leali, D., Alessi, P., Coltrini, D., Rusnati, M., Zetta, L., and Presta, M. (2006) *J. Cell. Mol. Med.* in press
10. Leali, D., Bianchi, R., Bugatti, A., Nicola, S., Mitola, S., Ragona, L., Tomasselli, S., Gallo, G., Catello, S., Rivieccio, V., Zetta, L., and Presta, M. (2009) *J. Cell. Mol. Med.* in press
11. Folkman, J. (2007) *Nat. Rev. Drug Discov.* 6, 273–286
12. Whitty, A., and Kumaravel, G. (2005) *Nature* 438, 122–118
13. Wells, J. A., and McClendon, C. L. (2007) *Nature* 450, 1001–1009
14. Robinson, J. A., Demarco, S., Gombert, F., Moehe, K., and Obrecht, D. (2008) *Drug Discov. Today* 13, 944–951
15. Murray, I. K., and Fellman, S. H. (2007) *Biopolymers* 88, 657–686
16. Sulochana, K. N., and Ge, R. (2007) *Curr. Pharm. Des.* 13, 2074–2086
17. Clackson, T., and Wells, J. A. (1995) *Science* 267, 383–386
18. Thanos, C. D., DeLano, W. L., and Wells, J. A. (2006) *Proc. Natl. Acad. Sci. U.S.A.* 103, 15422–15427
19. Meli, M., Pennati, M., Martino, A., Daidone, M. G., Plescia, J., Toba, S., Altieri, D. C., Zaffaroni, N., and Colombo, G. (2006) *J. Med. Chem.* 49, 7721–7730
20. Esteras-Chopo, A., Morra, G., Moroni, E., Serrano, L., Lopez de la Paz, M., and Colombo, G. (2008) *J. Mol. Biol.* 383, 266–280
21. Murray, J. K., and Gellman, S. H. (2007) *J. Chem. Inf. Model.* 47, 2358–2365
22. Carlson, H. A., Smith, R. D., Khazanov, N. A., Kirchhoff, P. D., Dunbar, J. B., Jr., and Benson, M. L. (2008) *J. Med. Chem.* 51, 6432–6441
23. Frembgen-Kesner, T., and Elcock, A. H. (2006) *J. Mol. Biol.* 359, 202–214
24. Müller, M., Sallenfehr, M., and Mosher, D. F. (2004) *J. Biol. Chem.* 279, 51915–51922
25. Sloatstra, J. W., Puijck, W. C., Ligtvoet, G. J., Langeveld, J. P., and Meloen, R. H. (1996) *Mol. Divers* 1, 87–96
26. Bravman, T., Bronner, V., Lavie, K., Notovich, A., Papalia, G. A., and Mysza, D. G. (2006) *Anal. Biochem.* 358, 281–288
27. Still, W. D., Tempczyk, A., Hawley, R. C., and Hendrickson, T. (1990) *J. Am. Chem. Soc.* 112, 6127–6129
28. Dauri, X., Gademann, K., Jaun, B., Seebach, D., van Gunsteren, W. F., and Mark, A. E. (1999) *Angew. Chem. Int. Ed. Engl.* 38, 236–240
29. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) *J. Comput. Chem.* 19, 1639–1662
30. Plescia, J., Salz, W., Xia, F., Pennati, M., Zaffaroni, N., Daidone, M. G., Meli, M., Dohi, T., Fortugno, P., Nefedova, Y., Gabrilovich, D. I., Colombo, G., and Altieri, D. C. (2005) *Cancer Cell* 7, 457–468
31. Mayer, M., and Meyer, B. (2001) *J. Am. Chem. Soc.* 123, 6108–6117
32. Ribatti, D., Nico, B., Vaccia, A., and Presta, M. (2006) *Nat. Protoc.* 1, 85–91
33. Calero, S., Lago, S., van Gunsteren, W. F., and Daura, X. (2004) *Chem. Biodivers.* 1, 505–519
34. Gyurkocza, B., Plescia, J., Raskett, C. M., Garlick, D. S., Lowry, P. A., Carter, B. Z., Andreuff, M., Meli, M., Colombo, G., and Altieri, D. C. (2006) *J. Natl. Cancer Inst.* 98, 1068–1077
35. Leali, D., Bianchi, R., Bugatti, A., Nicola, S., Mitola, S., Ragona, L., Tomasselli, S., Gallo, G., Catello, S., Rivieccio, V., Zetta, L., and Presta, M. (2009) *J. Cell. Mol. Med.*, in press
36. Leali, D., Tomasselli, S., Quezener, C., Zetta, L., and Bikfalvi, A. (2009) *Biochim. Biophys. Res. Commun.* 382, 26–29
37. Leali, D., Alessi, P., Coltrini, D., Pennati, M., Zetta, L., and Presta, M. (2009) *Curr. Pharm. Des.* 15, 3577–3589
38. Milne, G. W., Nicklaus, M. C., Driscoll, J. S., Wang, S., and Zaharevitz, D. V. (1994) *J. Chem. Inf. Comput. Sci.* 34, 1219–1224
39. Hogg, P. J. (1994) *Thromb. Haemost.* 72, 787–792
40. Esteras-Chopo, A., Botta, M., Corelli, F., Mongelli, N., Biasoli, G., Borgia, A. L., and Ciomei, M. (1998) *Bioorg. Med. Chem.* 6, 947–958
41. Fernández-Tornero, C., Lozano, M. R., Redondo-Horcajo, M., Gómez, A. M., López, J. C., Quesada, E., Uriel, C., Valverde, S., Cuevas, P., Rossi, I., and Giménez-Gallego, G. (2003) *J. Biol. Chem.* 278, 21774–21781
42. Bergers, G., and Hanahan, D. (2008) *Nat. Rev. Cancer* 8, 592–603
43. Beenken, A., and Mohammadi, M. (2009) *Nat. Rev. Drug Discov.* 8, 235–253