Porphyran Analogues as Novel Antagonists of Fibroblast Growth Factor and Vascular Endothelial Growth Factor Receptor Binding That Inhibit Endothelial Cell Proliferation, Tumor Progression, and Metastasis

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ABSTRACT

Fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) play a pivotal role in the multistep pathway of tumor progression, metastasis, and angiogenesis. We have identified a porphyrin analogue, 5,10,15,20-tetrakis(methyl-4-pyridyl)-21H,23H-porphine-tetrap-tosyloxy salt (TMPP), as a potent inhibitor of FGF2 and VEGF receptor binding and activation. TMPP demonstrated potent inhibition of binding of soluble FGF receptor 1 (FGFR1) to FGF2 immobilized on heparin at submicromolar concentrations. TMPP inhibits binding of radiolabeled FGF2 to FGFR in a cell-free system as well as to cells genetically engineered to express FGFR1. Furthermore, TMPP also inhibits the binding of VEGF to its tyrosine kinase receptor in a dose-dependent manner. In an in vitro angiogenic assay measuring the extent of endothelial cell growth, tube formation, and sprouting, TMPP dramatically reduced the extent of the FGF2-induced endothelial cell outgrowth and differentiation. In a Lewis lung carcinoma model, mice receiving TMPP showed a marked inhibition of both primary tumor progression and lung metastases development, with nearly total inhibition of the metastatic phenotype upon alternate daily injections of TMPP at 25 μg/kg of body mass. Finally, novel meso-pyridyl-substituted, nonsymmetric porphyrins, as well as a novel corrole-based derivative, with >50-fold increase in activity in vitro, had a significantly improved efficacy in blocking tumor progression and metastasis in vivo.

INTRODUCTION

FGFs1 and VEGF act in concert to enhance tumor angiogenesis and metastasis (1). In adults, FGF2 (basic FGF) is highly abundant in tumors, whereas the oncogenes FGF-4 (hst/fgf4) and FGF-3 (int-2) are found in tumors such as stomach cancer, Kaposi sarcoma, melanoma, and breast cancer (2). FGFR genes are also frequently amplified and overexpressed in a variety of cancers such as breast cancer, gastric adenocarcinomas, melanoma, pancreatic cancers, and many others (3–6). Recently, activating mutations in FGFRs were also found to be frequently expressed in both cervical and bladder carcinomas (7).

A most potent and selective angiogenic factor is VEGF. VEGF is a multifunctional cytokine that plays a key role in physiological and pathological angiogenesis in vivo by stimulating endothelial cell proliferation and vessel hyperpermeability (8). VEGF binds to Flt-1 and Flk-1/KDR cell membrane receptors that, similar to FGFRs, are members of the tyrosine kinase receptor superfamily (9). The association between the growth factor ligands and their respective receptors stimulates tyrosine kinase activity as one of the initial biochemical events leading to DNA synthesis and cell division. Therefore, compounds that inhibit ligand-mediated signal transduction pathways may be useful for the treatment of cellular proliferative disorders.

FGF2 and VEGF have synergistic activities on endothelial cell activation and differentiation (10). Both FGF2 and VEGF require a cooperative interaction with heparan sulfate proteoglycans to form functional growth factor-receptor complexes that are essential for high-affinity binding and activation of their cognate receptors (11–13) and angiogenic activity (14). On the basis of the crucial role of growth factor-heparin interaction, we designed a high throughput screening system using FGF ligands immobilized on heparin and tagged soluble receptors to identify molecules that can modulate heparin-FGFR interactions. The screening process has identified several potent compounds, among which is TMPP, a member of the porphyrin molecule family.

Porphyrans have been of interest to chemists and medical scientists for over a century. It has been known for many years that porphyrins interact with neoplastic tumors (15), and the fact that porphyrins demonstrate high affinity to tumorigenic cells in vitro and solid tumors in vivo is well established (16, 17). Moreover, porphyrin derivatives have been used for the treatment of tumors and malignant tissues in combination with electromagnetic radiation or radioactive emissions. Because they strongly absorb light, many porphyrins are still being used as photosensitizers in photodynamic therapy (17).

In this study, we demonstrate that TMPP (M, 1363) is capable of directly inhibiting FGF2 and VEGF receptor binding. TMPP inhibits endothelial cell proliferation and differentiation in an in vitro angiogenesis model and dramatically reduces primary tumor growth and metastatic spread of Lewis lung carcinoma tumors in mice. A series of novel, rationally designed porphyrin analogues demonstrated significantly improved potency in inhibiting FGFR binding in vitro and improved efficacy in blocking tumor progression and metastasis in vivo.

MATERIALS AND METHODS

Materials

Human recombinant FGF2 and EGF were purchased from R&D systems. Human recombinant VEGF was generated by H. Weiss (GBF, Braunschwig, Germany). Heparin-coated plates were prepared by Carmeda (Sweden). FRAP was prepared as described (18). EGF receptor immunoglobulin fusion protein was generated by Y. Yarden as described (19). CHO cells expressing FGFR1 were generated as described (11). DMEM and F12 growth mediums, bovine calf serum, and glutamine were made by Biological Industries (Bet Haemek, Israel).
Compound Synthesis

Preparation of TMPP
A mixture of 5.7 ml (60 mmol, 4 equivalents) of 4-pyridinecarboxaldehyde and 4.15 ml (60 mol, 4 equivalents) pyrrole was dissolved in 300 ml of propionic acid, and the mixture was heated to reflux for 2 h. After cooling to room temperature, the solvent was evaporated to dryness, and the oily residue was washed by hot water, neutralized by aqueous ammonia (25%), and washed again with hot water. The purple solids obtained by this procedure were filtered and dried. The dry solid material was treated with three portions of 50 ml of dichloromethane, each followed by filtration. The resulting crystals from mixtures of methanol and EtoAc.

Preparation of Compound P1012
The noncharged intermediate, 5,10,15,20-tetakis (3-pyridyl)porphyrin (chemical abstract registry no. 40882-83-5), was alkylated with methyl tolulene sulfonate. 1H NMR (CDCl3, δ): 9.45 (s, 4H), 9.06 (d, J = 5.5 Hz, 4H), 8.85 (s, 8H), 8.52 (d, J = 7.8 Hz, 4H), 7.7 (dd, J = 7.8 Hz, 4H), −2.86 (s, 2H).

Preparation of Compound P1016 and Related Porphyrins

(a) Preparation of the Intermediate Compounds (Condensation Step).
A mixture of 4.3 ml (45 mmol, 3 equivalents) of 4-pyridinecarboxaldehyde, 2.06 ml (16.5 mmol, 1 equivalent) of pentafluorobenzaldehyde, and 4.15 ml (60 mmol, 4 equivalents) of pyrrole was dissolved in 300 ml of acetic acid, and the mixture was heated to reflux for 2 h. After cooling to room temperature, the solvent was evaporated to dryness by vacuum, and the oily residue was washed by hot water, neutralized by aqueous ammonia (25%), and washed again with hot water. The purple solids obtained by this procedure were filtered and dried. The dry solid material was treated with three portions of 50 ml of dichloromethane, each followed by filtration. To the combined organic phases, 10 g of basic alumina (activity II) were added, and the solvent was evaporated to dryness. Separation of 5,10,15,20-tetra(4-pyridyl)porphyrin was achieved by column chromatography. Rf (2% ethanol in CH2Cl2) = 0.18; 1H NMR (CDCl3, δ): 9.04 (d, J = 5.5 Hz, 8H), 8.85 (s, 8H), 8.14 (d, J = 5.5 Hz, 8H), −2.95 (2H). Two hundred mg (0.3 mmol) of 5,10,15,20-tetra(4-pyridyl)porphyrin were dissolved in 30 ml of dry DMF, and 9 ml of CH2I2 were added in one portion. The reaction was stirred at room temperature for 10 h, after which the reaction mixture was evaporated to dryness by high vacuum at room temperature. The resulting crystals were recrystallized from mixtures of methanol and EtoAc.

(b) Chromatographic Separation. Separation and purification of the compounds obtained in step a was achieved by column chromatography, in which the polarity of the eluents was gradually increased from dichloromethane to mixtures of dichloromethane and 2–10% ethanol. The order of elution (the Rf values are for silica with 2% ethanol in CH2Cl2) and the chemical yields were as follows: 5,10,15,20-tetra(4-pyridyl)porphyrin (1a) = P2, traces; Rf = 0.95; 5,10,15-tris(2,3,4,5,6-pentafluorophenyl)-20-(4-pyridyl)porphyrin (1b, 1.1%; Rf = 0.66); 5,15-bis(2,3,4,5,6-pentafluorophenyl)-10,20-bis(4-pyridyl)porphyrin (1c; Rf = 0.60); 5,10-bis(2,3,4,5,6-pentafluorophenyl)-15,20-bis(4-pyridyl)porphyrin (1d; Rf = 0.54); 5,15,10-tris(4-pyridyl)porphyrin (1e; Rf = 0.45); and 5,10,15,20-tetra(4-pyridyl)porphyrin (1f; Rf = 0.18). The combined yield of compounds 1c and 1d was 13.4%. Their separation required an additional column in which the eluant was 2% ethanol in dichloromethane. Spectroscopic characteristics of the compounds (1a and 1f are known compounds): 1a. UV-vis (CHCl3) λmax nm: 414, 508, 582; 1H NMR (CDCl3, δ): 8.91 (s, 8H), −2.93 (s, 2H); 13C NMR (CDCl3, δ): −136.9 (dd, J1 = 22.8 Hz, J2 = 7.0 Hz, 8F), −151.6 (t, J = 20.7 Hz, 4F), −161.7 (d, J = 22.4 Hz, J2 = 5.8 Hz, 8F); 1b. UV-vis (CHCl3) λmax nm: 414, 506, 582; 1H NMR (CDCl3, δ): 9.06 (d, J = 4.3 Hz, 4H), 8.89 (s, 6H), 8.16 (d, J = 4.2 Hz, 2H), 8.15 (s, 2H), −2.92 (s, 2H); 13C NMR (CDCl3, δ): 137.0 (m, 6F), −151.8 (m, 2 overlaying 4F), −161.8 (m, 6F); MS m/z: 886.1 (M+, 100%); MS m/z: 884.6 (M−, 40%), (M− + 2H, 60%); 1c. UV-vis (CHCl3) λmax nm: 412, 508, 584; 1H NMR (CDCl3, δ): 9.06 (d, J = 4.4 Hz, 4H), 8.89 (s, 4H), 8.05 (s, 4H), 8.15 (d, J = 4.5 Hz, 4H), −2.94 (s, 2H); 13C NMR (CDCl3, δ): −137.2 (dd, J = 23.2 Hz, J2 = 7.2 Hz, 4F), −152.0 (t, J = 20.9 Hz, 2F), −161.9 (J1 = 22.8 Hz, J2 = 7.3 Hz, 4F); MS m/z: 797.4 (M+, 100%); MS m/z: 794.9 (M− + 2H).
mixture was stirred for 1 h at 78°C and then hydrolyzed with saturated aqueous boric acid solution. The layers were separated, the aqueous layer was washed with ether, and the combined ether extracts were dried and evaporated to a solid residue. The product was purified by column chromatography on silica gel (1:1 EtOAc:hexane) and recrystallized from CHCl₃:hexane to give 13 mg (35% yield) of the pure product as violet crystals.

**Binding of Soluble FGFR Alkaline Phosphatase Fusion Protein to Immobilized FGF2**

FGF2 (100 ng/ml) was incubated on 96-well plates to which heparin had been covalently attached (Carmeda). Subsequently, 200 μl of FRAP condition medium and TMPP were added and incubated together for 2 h. After three cycles of washing with HNTG (20 mM HEPEs, pH 7.5), 150 mM NaCl, 1% Triton X-100, and 10% glycerol, alkaline phosphatase substrate (Sigma; 15 mM) was added, and catalysis of the chromogenic product was measured by spectrophotometry at 405 nm.

**Binding of 125I-Labeled FGF2 to Soluble FGFR**

Conditioned medium from NIH 3T3 cells secreting FGFR1-FRAP was incubated for 45 min at room temperature with rabbit antihuman placental AP antibodies prebound to agarose-protein A beads (Pierce). The FRAP-coupled beads were washed three times with 1 ml of HNTG and incubated with 2 ng/ml of 125I-labeled FGF2, 1 μg/ml heparin, and TMPP at different concentrations for 1 h at room temperature. High-affinity-bound 125I-labeled FGF2 was determined by counting the tubes in a gamma counter.

**Binding of 125I-Labeled VEGF to Cells**

Confluent cultures of CHO cells expressing FGFR1 (11) in 24-well plates (Falcon) were precooled and washed twice with cold DMEM supplemented with 20 mM HEPEs (pH 7.5) and 0.1% BSA (DMEM/BSA). They were then incubated for 1.5 h at 4°C with 125I-labeled VEGF (2 ng/ml) and increasing concentrations of TMPP. The binding medium was discarded, and the cells were washed once with ice-cold DMEM/BSA and twice with cold PBS (pH 7.5) containing 1.6 mM NaCl. High-affinity, receptor-bound FGF2 was determined by extraction of the cells with 20 mM sodium acetate (pH 4.0) containing 2.0 mM NaCl. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled FGF2.

**Binding of 125I-Labeled VEGF to Cells**

Confluent cultures of bovine aortic endothelial cells in 24-well plates (Falcon) were precooled and washed twice with cold DMEM supplemented with 20 mM HEPEs (pH 7.5) and 0.1% BSA (DMEM/BSA). The cells were then incubated for 1.5 h at 4°C with 125I-labeled VEGF (2 ng/ml) and increasing concentrations of TMPP. The binding medium was discarded, and the cells were washed once with ice-cold DMEM/BSA and twice with cold PBS (pH 7.5) containing 1.6 mM NaCl. High-affinity, receptor-bound FGF2 was determined by extraction of the cells with 20 mM sodium acetate (pH 4.0) containing 2.0 mM NaCl. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled VEGF.

**Binding of 125I-Labeled EGF to EGF Receptor**

Confluent cultures of A431 cells in 24-well plates (Falcon) were precooled and washed twice with cold DMEM supplemented with 20 mM HEPEs (pH 7.5) and 0.1% BSA (DMEM/BSA). The cells were then incubated for 1.5 h at 4°C with 125I-labeled EGF (2 ng/ml) and increasing concentrations of TMPP (19). Binding of radiolabeled EGF to soluble receptors was performed by incubating conditioned medium from 293 cells secreting EGF receptor-Fc immunoglobulin fusion protein for 45 min at room temperature with agarose-protein A beads (Pierce). The EGF receptor-coupled beads were washed three times with 1 ml of HNTG and incubated with 2 ng/ml of 125I-labeled EGF and TMPP at 1 h at room temperature as described by Tzahat et al. (19). High affinity-bound, 125I-labeled EGF was determined by counting the tubes in a gamma counter.

**Rat Aorta in Vitro Angiogenesis Assay**

Type I collagen was prepared from the tail tendons of adult Sprague Dawley rats (21). The collagen matrix gel was obtained by simultaneously raising the pH and ionic strength of the collagen solution (22). Thoracic aortas were obtained from 2-month-old Sprague Dawley rats. The fibroadipose tissue was carefully removed under a dissecting microscope, and aortic rings were sectioned (1-mm long) and placed on top of a 0.2-ml collagen gel in 16-mm culture wells. Collagen solution (0.4 ml) was carefully poured on top of the ring. After the gel was formed, 0.4 ml of serum-free endothelial growth medium was added and replaced every other day by fresh medium containing FGF2 (2 ng/ml). TMPP was added to the growth medium twice a week. Microvessel outgrowth was visualized by phase microscopy, and the number of capillary vessels was counted throughout the course of the experiment. After 14 days, the cultures were fixed with 4% formaldehyde, embedded in paraffin, and sectioned at 5 μm, and the extent of microvascular endothelial tube outgrowth was measured under a light microscope.

**Lewis Lung Carcinoma Tumor Assay**

Murine Lewis lung carcinoma D122 cells (2 × 10⁵ cells/50 ml PBS) were injected into the foot pads of 10-week-old C57 black mice (23). Twenty-five μg/g body mass of porphyrins dissolved in PBS were injected i.p. twice a week in the treated group, and tumor size was measured periodically to follow primary tumor formation. To evaluate inhibition of lung metastasis by TMPP, the primary tumors were allowed to develop over a period of 4 weeks to a volume of ~8 mm³, after which the tumors were removed by amputation, and metastases were allowed to develop for an additional 4 weeks, during which the treated group received 25 μg/g body mass of porphyrin i.p. twice a week. Subsequently, the mice were sacrificed and dissected, and the lungs were removed and photographed. The extent of lung metastasis was measured by weighing the lungs. C57/black mice were maintained on lab chow and tap water and were housed with a 12-h day-night cycle.

**RESULTS**

**TMPP Inhibits the Binding of FGF2 to the FGF Receptor.** A high throughput screening system composed of a heparin matrix, FGF2, and a FGFR1 tagged by alkaline phosphatase (FRAP) was designed using 96-well plates to which heparin had been covalently attached. FGF2 binding to the immobilized heparin is then followed by the addition of FRAP, and the compounds were to be screened for their ability to modulate heparin-FGFR, receptor-heparin, and receptor-FGF interactions. The end point of the assay measures enzymatically the formation of FGF-receptor complexes quantitated by the specifically associated alkaline phosphatase-catalyzed chromogenic product. This, a lowered alkaline phosphatase activity would indicate inhibition at one or more of the three levels of interactions required for the formation of the FGF-FGFR-heparin ternary complex. The screen of the chemical synthetic library has identified several compounds for their capacity to inhibit soluble FGFR binding. One of the most potent ones was TMPP.

TMPP demonstrated potent inhibition at submicromolar concentrations, with a distinct dose-dependent inhibition pattern (Fig. 1A). To evaluate the capacity of TMPP to inhibit FGF2 receptor binding, we measured the binding of radiolabeled FGF2 to FGFR1 in two independent experimental systems. In the first experiment, we used a cell-free system, measuring the binding of radiolabeled FGF2 to a dimeric soluble FGFR1 fused to alkaline phosphatase (18). The soluble FRAP was immobilized using an anti-alkaline phosphatase antibody prebound to agarose-protein A beads. As a second experimental model, we used heparan sulfate-deficient CHO cells, genetically engineered to express FGFR1 (11). TMPP inhibited binding of 125I-labeled FGF2 to the FGFR in both experimental systems. Fig. 1B illustrates that TMPP is capable of profoundly inhibiting FGF2-FGFR1 binding in the soluble receptor assay with an IC₅₀ of ~1 μM (Fig. 1B). In the cellular receptor system, TMPP was capable of inhibiting FGF2 binding with an IC₅₀ of ~2.5 μM (Fig. 1C). The slightly higher concentrations of TMPP required for inhibition of cellular FGF2 binding in the cellular assay result most likely from the
inherent reduced affinity of the soluble FGFR to the FGF ligand compared with that of cell-associated receptors (18).

Affinity Labeling of Cells by 125I-Labeled FGF2 Is Inhibited by TMPP. To determine the specificity of this effect, chemical cross-linking of 125I-labeled FGF2 to cells was carried out in the absence and presence of increasing concentrations of TMPP. As shown in Fig. 3A, there is complete inhibition of the formation of a typical FGF2-receptor complex at TMPP concentrations as low as 5 μM, in agreement with the direct binding data (Fig. 1C).

TMPP Inhibits the Binding of VEGF to the VEGF Receptor. Because FGF and VEGF share several similar characteristics and may play synergistic role in tumor angiogenesis (2, 8, 10), we examined the capacity of TMPP to inhibit VEGF binding to its receptor. Fig. 3A demonstrates that TMPP efficiently inhibits VEGF binding to human umbilical vein endothelial cells that express the Flk-1/KDR VEGF receptor and with high potency. TMPP also inhibits VEGF binding to bovine aortic endothelial cells expressing VEGF receptors (data not shown) in a manner similar to the Flk-1/KDR-transfected cells.

TMPP Does Not Inhibit the Binding of EGF to the EGF Receptor. When TMPP was tested for its capacity to inhibit the binding of radiolabeled EGF to the EGF receptor on A431 cells, no inhibition of binding was noted, even at mM concentrations of TMPP (data not shown). To unequivocally determine the specificity of this effect, chemical cross-linking of 125I-labeled FGF2 to FGFR1-expressing cells or 125I-labeled EGF to EGF receptor (19) was carried out in the absence or presence of 10 μg/ml TMPP. As shown in Fig. 3B, TMPP as expected inhibits FGF2 receptor binding and the formation of a FGF-receptor complex but had no effect on the binding of 125I-labeled EGF to the EGF receptor, in agreement with the results of the binding experiment on A431 cells.

Inhibition of in Vitro Angiogenesis by TMPP. To establish the biological effect of TMPP, we examined the compound for its effects on an in vitro angiogenic assay using rat aorta sections embedded in a collagen type I gel (21, 22, 24). The assay measures the extent of endothelial cell growth and microvascular tubules sprouting from the vessel tissue embedded in the gel. Basal tubule formation can be detected, even when no additional factors were added. The addition of
the VEGF receptor (Flk-1/KDR) were incubated with 125I-labeled VEGF (2 ng/ml) for 90 min at 4°C in the presence of increasing concentrations of TMPP. The binding medium was discarded, and the cells were washed with ice-cold DMEM/BSA. To determine the degree of receptor-bound 125I-labeled VEGF, the cells were incubated in cold PBS (pH 4) containing 1.6% NaCl and 25 mM HEPES. The cell extracts were counted in a gamma counter. Nonspecific binding was determined in the presence of increasing concentrations of unlabeled ligand and did not exceed 20% of the total bound ligand. TMPP does not inhibit covalent cross-linking of 125I-labeled EGF to EGF receptor. Binding of 125I-labeled EGF to soluble EGF receptor-Fc immunoglobulin fusion protein was performed as described by Tzahar et al. (19). Binding of 125I-labeled FGF2 to confluent monolayers of FGFR1-expressing cells was performed as described above. Both binding experiments were performed in the presence or absence of 10 μg/ml TMPP. After 90 min, chemical cross-linking was performed by the addition of disuccinimidyl suberate (0.15 mM in PBS). The protein complexes were separated by electrophoresis on a 7.5% SDS polyacrylamide gel and analyzed on X-ray film.

FGF2 (2 ng/ml) dramatically increased the degree of cell growth and vascularization. However, the addition of FGF2 together with 10 μM TMPP dramatically reduced the extent of endothelial cell growth and differentiation. When 100 μM TMPP was added in the presence of FGF2, complete inhibition of microvascular tubule sprouting was achieved, and no endothelial cell growth was observed (Fig. 4).

**TMPP Inhibits Primary Tumor Progression in a Lewis Lung Carcinoma Tumor Model.** Lewis lung carcinoma D122 cells (200,000 cells/mouse) were injected into the foot pads of 10-week-old C57 black mice according to O’Reilly et al. (23). Mice that received the TMPP (25 μg/g of body mass) by i.p. injections twice a week for 5 weeks showed a marked inhibition in primary tumor growth in comparison with the control group (Fig. 5). The experiment was repeated five times, and the findings were reproducible and statistically established. These results indicated that TMPP is not only active in vitro but is capable of inhibiting tumor growth in vivo as well.

**TMPP Inhibits Lung Metastasis in the Lewis Lung Carcinoma Model.** Mice were injected with Lewis lung carcinoma cells (2 × 10^5 cells/mouse) into the foot pad, and primary tumors were allowed to develop over a period of ~3 weeks to a volume of ~8 mm^3. Subsequently, primary tumors were removed through amputation, and lung metastases were allowed to develop for 4 weeks before the mice were sacrificed. The extent of lung metastasis at this point was examined by gross morphological examination and by determining the gain in lung weight. Aggressive metastasis formation is noted in the lungs of control mice (those not treated with TMPP). In mice treated with TMPP (25 mg/kg body mass), the lungs were significantly less affected (Fig. 6) and in some cases indifferent from those of the noninjected control mice weighing ~200 mg, similar to the lungs from the noninjected control mice (Fig. 6).

**Novel Porphyrin Derivatives Demonstrate Improved in Vitro and in Vivo Activity.** To elucidate the structural requirements needed to achieve FGF and VEGF inhibitory activity, we have synthesized and examined a series of porphyrin analogues. It became clear that only cationic charged porphyrins, but not neutral or anionic charged derivatives, are active. On the basis of the structure of TMPP, we have synthesized novel meso-pyridinium-substituted porphyrins in which the position of the N-methyl (the positive charge) was varied from the para position to ortho and meta, as well as porphyrins with fewer than 10 atoms of nitrogen in the position of the meso-chlorine (the positive charge).

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**Fig. 3.** A, effect of TMPP on the binding of 125I-labeled VEGF to endothelial cells transfected with the VEGF receptor. Confluent monolayers of endothelial cells expressing the VEGF receptor (Flk-1/KDR) were incubated with 125I-labeled VEGF (2 ng/ml) for 90 min at 4°C in the presence of increasing concentrations of TMPP. The binding medium was discarded, and the cells were washed with ice-cold DMEM/BSA. To determine the degree of receptor-bound 125I-labeled VEGF, the cells were incubated in cold PBS (pH 4) containing 1.6% NaCl and 25 mM HEPES. The cell extracts were counted in a gamma counter. Nonspecific binding was determined in the presence of increasing concentrations of unlabeled ligand and did not exceed 20% of the total bound ligand. B, TMPP Inhibits Lung Metastasis in the Lewis Lung Carcinoma Model. Lewis lung carcinoma cells were injected into the foot pads of 10-week-old C57 black mice according to O’Reilly et al. (20,000 cells/mouse) and examined a series of porphyrin analogues. It became clear that only cationic charged porphyrins, but not neutral or anionic charged derivatives, are active. On the basis of the structure of TMPP, we have synthesized novel meso-pyridinium-substituted porphyrins in which the position of the N-methyl (the positive charge) was varied from the para position to ortho and meta, as well as porphyrins with fewer than 10 atoms of nitrogen in the position of the meso-chlorine (the positive charge).

**Fig. 4.** In vitro angiogenic assay using a rat aorta section embedded in a collagen gel. Sections of rat aorta were immobilized in a collagen gel. After the addition of FGF2 to the medium, the presence or absence of TMPP, the extent of endothelial cell growth and microvascular tubules sprouting from the vessel tissue embedded in the gel was measured. Basal tubule formation can be detected even when no additional factors were added. The results are expressed as the percentage of microvascular tubules sprouting in comparison with the control experiment where FGF2 alone was added. Bars, SE.

**Fig. 5.** Inhibition of primary tumor growth in the Lewis lung carcinoma murine tumor model by TMPP. Lewis lung carcinoma cells were injected into the foot pads of 10-week-old C57 black mice. TMPP (25 μg/g of body mass) was injected i.p. twice a week over a period of 7 weeks, during which time the primary tumor volume was monitored. Bars, SE.
four 4-pyridylium substituents. In this series, the most beneficial effect, as judged by the inhibition of FGF2 binding (Fig. 7A), was obtained with P1016, a nonsymmetric porphyrin with three positive charges. The activity of P1016 in vitro was ~50 times higher than that of TMPP, as can be seen in Fig. 7A. Another derivative, P1020, which contains four positive charges at more remote positions, was also significantly more active than TMPP (Fig. 7A). In contrast, P1012 was 10-fold less active in inhibiting FGF2 binding, with an IC50 of 10 μM (data not shown). In the Lewis lung carcinoma tumor model, however, only P1020, but not P1016, was more active than TMPP and P1012, which demonstrated only residual capacity to inhibit FGF2 binding and had no effect whatsoever in vivo (Fig. 7B).

Finally, we tested a novel water-soluble corrole analogue of TMPP (P1021; M1311.3) for its activity for both in vitro inhibition of FGF2 binding and for the inhibition of tumor growth. Indeed, this derivative, having three positive charges as in P1016 but with the same side groups as in P1020, displayed the best of both P1016 and P1020 characteristics, because it was ~10-fold more active than TMPP in vitro and 5-fold more potent in the in vivo tumor models, inhibiting lung metastasis formation in vivo at a concentration of only 5 mg/kg body weight (Fig. 7B). These results suggest that rationally modified porphyrin analogues can serve as highly potent inhibitors of growth factor activity in vitro and in vivo.

DISCUSSION

We have identified TMPP, a member of the porphyrin family, as a potent inhibitor of FGF2 and VEGF receptor binding in cells and cell-free systems. TMPP also dramatically reduced the extent of FGF2-induced endothelial cell growth and differentiation in an in vitro angiogenesis model and efficiently blocks Lewis lung carcinoma murine primary tumor growth and lung metastasis. Novel, rationally designed TMPP porphyrin analogues demonstrate improved potency in inhibiting receptor binding in vitro and tumor progression and metastasis in vivo. Taken together, we have identified TMPP and its analogues as a novel class of potent inhibitors of FGF2 and VEGF activity in vitro and in vivo.

The exact mechanism by which TMPP and other related porphyrin-like molecules inhibit growth factor-receptor binding and activation is not clear. However, preliminary results suggest that TMPP interferes with the formation of the trimolecular complex of growth factor-heparin and the tyrosine kinase receptor (11), thus abrogating receptor signaling. It is interesting to note that TMPP does not inhibit the binding of EGF, which is not a heparin-binding or heparin-dependent growth factor (25), to its high-affinity tyrosine kinase receptor, suggesting that interfering with heparin binding may play a key role in the inhibitory effect of TMPP. Both the FGF ligand and the FGFR contain heparin-binding domains critical for FGF activation (26–29), and several heparin mimetics have been described as potent inhibitors of FGFR binding and activation (24, 30). TMPP, however, does not resemble in its structure any of the known heparin mimetics. Nevertheless, the requirement for positively charged groups and their spatial distribution may mimic a restricted highly sulfated domain in heparin, thus serving as a heparin mimic. Several other inhibitors of FGF and VEGF that have been shown to inhibit angiogenesis were designed to inhibit

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Fig. 6. Effect of TMPP on lung metastasis in the Lewis lung carcinoma murine tumor model. Lewis lung carcinoma cells were injected into the foot pads of 10-week-old C57 black mice, and primary tumors were allowed to develop over a period of 4 weeks to a volume of ~8 mm3. Subsequently, the primary tumors were removed by amputation, and metastases were allowed to develop for an additional 4 weeks before mice were sacrificed. Twenty-five μg/g body mass TMPP were injected i.p. twice a week during the 4-week period. The mice were sacrificed and dissected, and the lungs were removed. The average extent of lung metastasis was measured by weighing the lungs for gain of mass. Bars, SE.

Fig. 7. A, inhibition of FGF2 binding to the FGFR by porphyrin analogues. Effect of porphyrin analogues on the binding of radiolabeled FGF2 to soluble FGFR. 125I-Labeled FGF2 (2 ng/ml) was incubated (90 min at 4°C) with immobilized FGFR1. Incubations were performed in the presence of 100 ng/ml heparin and increasing concentrations of porphyrin analogues TMPP, P1016, P1020, and P1021. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled FGF2 and did not exceed 10% of the total binding. Results represent the means in one of at least two independent experiments. B, inhibition of metastasis growth in the Lewis lung carcinoma tumor model by porphyrin analogues. Lewis lung carcinoma cells were injected into the foot pads of 10-week-old C57 black mice, and primary tumors were allowed to develop over a period of 4 weeks to a volume of ~8 mm3. After the formation of primary tumors, they were removed by amputation, and metastases were allowed to develop for 4 weeks before mice were sacrificed. Porphyrin analogues TMPP, P1012, P1016, P1020, and P1021 were injected i.p. twice a week. The mice were sacrificed and dissected, and the lungs were removed. The average extent of lung metastasis was measured by weighing the lungs. The graph demonstrates the concentration (mg/kg body mass) of porphyrin analogue required to achieve inhibition of metastasis growth. Bars, SE.

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the intrinsic tyrosine kinase activity of the FGF and VEGF growth factor receptors (31, 32). This novel class of FGF and VEGF inhibitors, however, most likely works via a different molecular mechanism involving direct interference with growth factor receptor interaction, thus inhibiting their biological responses.

Porphyrin derivatives are widely used for the treatment of tumors and malignant tissues in combination with electromagnetic radiation or radioactive emissions. Because they strongly absorb light in the 690–880 nm region, many porphyrins were suggested for use as photosensitizers in photodynamic therapy (16). Some porphyrin derivatives are used in combination with electromagnetic radiation or radioactive emissions for inhibiting angiogenesis (33). It has been suggested that the activity of porphyrin derivatives as antitumor agents in the absence of electromagnetic radiation or radioactive emission may be based on their ability to cleave DNA because of their capacity to bind to DNA and because they must always include an excitable central Fe or Mn metal atom. Here we find that porphyrin-like compounds that do not contain a metal atom can directly interfere with growth factor receptor tyrosine kinase interactions.

The potent antiproliferative effect of TMPP is of potential clinical application not only in blocking growth factor-mediated tumor proliferation but also in other processes of pathological proliferation such as restenosis, accelerated atherosclerosis, and pathological angiogenesis as in diabetic retinopathy and arthritis. In support is the observation that TMPP markedly inhibited the outgrowth of microvessels from aortic rings embedded in a collagen gel. Furthermore, TMPP and its analogues are potent inhibitors of vascular smooth muscle cell proliferation in vitro. Studies are under way to elucidate the inhibitory effect of TMPP on angiogenesis and restenosis in experimental animal models.

The fact that we were able to improve the potency of the TMPP lead compound for its FGF and VEGF inhibitory activity, by rationally modifying specific groups on the porphin backbone, is of great importance. On the basis of the TMPP blueprint, we found that only cationic charged porphyrins, but not neutral or anionic charged derivatives, were active. When meso-pyridyl-substituted porphyrins, as well as porphyrins with fewer than four pyridyl substituents, were synthesized and tested, the most beneficial effect, as judged by the inhibition of FGF2 binding (Fig. 7A), was obtained with P1016, a nonsymmetric porphyrin with three positive charges. Another derivative, P1020, which contains four positive charges at more remote positions, was also significantly more active (Fig. 7A). The correlate (P1021), which contains three positive charges as in P1016 but with the same side groups as in P1020, displayed the best of both P1016 and P1020 characteristics. Furthermore, P1021 synthesis takes a much more straightforward approach than P1016 (20). The key to developing highly potent and specific antitumor agents relies on the ability to perform chemical modifications along the course of the development process. The vast knowledge accumulated with regard to the biological and chemical properties of porphyrins is therefore of great advantage for any potential medicinal chemistry approach. This fact, along with their capacity to block growth factor-mediated tumor progression and angiogenesis, makes these porphyrins highly attractive candidates for the development of anticancer drugs in the future.

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