The Integrin Inhibitor Cilengitide Affects Meningioma Cell Motility and Invasion

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Abstract

Purpose: Meningiomas are frequent intracranial or spinal neoplasms, which recur frequently and can show aggressive clinical behaviour. We elucidated the impact of the integrin inhibitor cilengitide on migration, proliferation, and radiosensitization of meningioma cells.

Experimental Design: We analyzed integrin expression in tissue microarrays of human meningiomas and the antimeningioma properties of cilengitide in cell cultures, subcutaneous and intracranial nude mouse models by measuring tumor volumes and survival times.

Results: αvβ5 was the predominantly expressed integrin heterodimer in meningiomas, whereas αvβ3 was mainly detected in tumor blood vessels. Application of up to 100 μg/ml cilengitide resulted in only mildly reduced proliferation/survival of meningioma cell lines. Effects on cell survival could be enhanced by irradiation. One μg/ml cilengitide was sufficient to significantly inhibit meningioma cell migration and invasion in vitro. A daily dosage of 75 mg/kg did neither affect tumor volumes nor overall survival (P = 0.813, log-rank test), but suppressed brain invasion in a significant fraction of treated animals. A combination of 75 mg/kg cilengitide daily and irradiation (2 × 5 Gy) led to a 67% reduction of MRI-estimated tumor volumes in the intracranial model (P < 0.01), whereas the corresponding reduction reached by irradiation alone was only 55% (P < 0.05).

Conclusions: These data show that a monotherapy with cilengitide is not likely to achieve major responses in rapidly growing malignant meningiomas, although brain invasion may be reduced because of the strong antimigratory properties of the drug. The combination with radiotherapy warrants further attention. Clin Cancer Res; 19(19); 5402–12. ©2013 AACR.

Introduction

Integrins are cell surface adhesion molecules important for many cellular features, including proliferation, survival, and migration (1). Cilengitide is a pentapeptide that targets αvβ3, αvβ5, and αvβ1 integrins by mimicking the Arg-Gly-Asp (RGD)-binding site (2, 3). Cilengitide inhibits proliferation and differentiation of endothelial progenitor cells, which play an important role in tumor neoangiogenesis (4). Brain tumors are known to express integrins, which besides their role in tumor angiogenesis are key players in the setting of diffuse brain infiltration by gliomas (5). The biologic roles of integrins in meningiomas have not been extensively studied, but integrin expression has been shown in meningiomas of all World Health Organization (WHO) grades (6–8).

Meningiomas have incidence rates comparable with gliomas (9). The vast majority belongs to WHO grade I and can usually be cured by neurosurgery. About 5% of these tumors belong to the more aggressive atypical (grade 2) or anaplastic subtypes (grade 3) with poor overall survival (reviewed in ref. 10). However, even among WHO grade I meningiomas, the rate of local recurrences is high. Furthermore, atypical and especially malignant meningiomas show in some cases an intriguing capacity to invade brain tissue, further reducing surgical treatment options. Despite additional irradiation, they recur frequently (11). Chemotherapeutic agents, such as hydroxyurea, and targeted cancer drugs have been used in these cases with only marginal efficacy (reviewed in refs. 12, 13). For instance, a recent study using the EGF receptor-targeting drug erlotinib has shown only modest effects (14). This failure of establishing efficient therapy approaches is, at least partly, based on the still limited knowledge about basic molecular alterations operating in meningioma development, progression, and invasion.
Cilengitide in Meningiomas

Translational Relevance
Meningiomas are frequent intracranial tumors without clear effective pharmaceutical treatment options thus far. Brain invasion by meningiomas occurs frequently and has negative prognostic impact. We show that the integrin inhibitor, cilengitide, affects meningioma cells especially by inhibiting motility and invasion, while largely not influencing cell proliferation and viability. In an orthotopic meningioma xenograft mouse model, we show that cilengitide as a single agent has no major impact on tumor growth and overall survival, despite an observed inhibition of brain invasion. Simultaneous treatment of meningiomas with the integrin inhibitor and radiation reduced tumor growth more effectively than radiation alone. Our data suggest that a cilengitide monotherapy has limited efficacy in meningiomas, whereas cilengitide treatment of irradiated meningioma warrants further attention.

Because previous immunohistochemical studies have shown that meningiomas do express αvβ3 and αvβ5 integrins (6), cilengitide may be an option for their treatment. Here, we characterized basic effects of cilengitide on meningioma cell viability, radiation sensitization, and tumor cell migration in benign and malignant human meningioma cells with special emphasis on the relation to the NF2 gene status. In addition, we analyzed the impact of the drug to inhibit malignant meningioma growth with and without irradiation in mice.

Materials and Methods
Cell cultures, inhibitors, and confocal microscopic analysis
The cell line BenMen-1 (15) was delivered by Prof. Werner Paulus (University of Münster, Münster, Germany) and HBL-52 by Cell lines service. Both were derived from WHO grade I meningiomas. Malignant IOMM-Lee cells were provided by Prof. H. Gutmann (Department of Neurology, Washington University School of Medicine, St. Louis, MO). Men and Men-shNF2 (further referred as Men-NF2) cells, as well as KT21 malignant meningioma cells were a kind gift from Dr. Anita Lal (University of California, San Francisco, CA; ref. 16). The primary meningioma culture was established from a WHO grade I meningioma in our laboratory. All cells were cultured in high-glucose Dulbecco’s modified Eagle medium with 10% fetal calf serum and penicillin/streptomycin. Their identity was analyzed using the AmpFSTR kit and GeneMapper ID v3.2 from Applied Biosystems.

For the evaluation of morphologic changes under cilengitide, cells were grown on chamber slides until 70% to 80% confluence was reached and treated with 1 μg/mL cilengitide (24 hours). Cells were fixed for 10 minutes in 4% paraformaldehyde/PBS and washed three times in PBS. Slides were submerged (5 minutes) in 0.15% Triton X-100/PBS before blocking in 1% bovine serum albumin/PBS (10 minutes). They were stained with a dilution (1:25) of Alexa-Fluor-546–labeled phalloidin (Invitrogen) for 1 hour at room temperature. After washing, slides were postfixed with 4% paraformaldehyde/PBS (10 minutes), washed again, embedded with Vectashield (Vector Laboratories), and covered. Slides were evaluated under a TCS-LC confocal microscope (Leica Mikrosystems), and quantitative morphometric analyses of cell protrusions were conducted using ImageJ (NIH). A total of 10 cells for each condition were evaluated.

Integrin expression analyses
Immunohistochemistry was conducted using a tissue microarray (TMA) containing samples from 46 meningiomas (29 WHO grade I, 11 atypical WHO grade II, and 6 anaplastic WHO grade III tumors). Rabbit monoclonal antibodies against αvβ3 and αvβ5, developed by the company Merck were used. Negative controls included omission of the primary antibody and substitution by an irrelevant antibody. Immunoperoxidase was graded semiquantitatively using the following score: – no staining, + weak staining, ++ moderate staining, +++ strong staining. For integrin immunofluorescence of cultured cells, the same antibodies were used, as applied for fluorescence-activated cell sorting (FACS) analysis of integrin expression (see below). Nuclei were visualized in addition by 4′, 6-diamidino-2-phenylindole staining.

For quantitative PCR analyses of integrin expression, 48 meningioma cases (42 benign WHO grade I, 4 atypical meningioma WHO grade II, and 2 anaplastic meningiomas WHO grade III), as well as tumor-free control meningiomas, were analyzed. Total RNA from frozen tissues or meningioma cells was isolated by Trizol reagent (Invitrogen) and transcribed into cDNA. Real-time PCR was conducted on an ABI-Prism-7000SDS using a SYBR Green PCR master mix (ABI). Primers were: β2-microglobulin (MG): CCAGCA-GAGAAAAGTC and GATGCTGCTTGTCTGC, bcl2: GATGACTGAGAACG and CAGGGCGAAAGACG, bax: CCAGCTCTGAGCAGATCATG and CAATCATCCTCTGC-5GAGAAAAGTC and GATGCTGCTTGTCTGC, bcl2: GATGACTGAGAACG and CAGGGCGAAAGACG, bax: CCAGCTCTGAGCAGATCATG and CAATCATCCTCTGC-AGCTCC, Integrin αv: CAAGCTATTTCTGGCAAGGC and ATAGATGGAACTGGCCTGG, Integrin β3: GGCTACAAA-CAGTGTCGAC and CTTCCGTCATGCTAATGAT, Integrin β5: ATGGAGAAGTGTCTGACTT and CAATCCACC-TGTTGTCATC. Expression levels were normalized to β2-microglobulin.

FACS analysis of integrins
Antibodies against αvβ5 (dilution 1:1000) and αvβ3 (dilution 1:25) from Millipore were used to label 1 million cells in a total volume of 200 μL PBS. After washing twice with PBS, a fluorescently labeled secondary antibody (fluorescein isothiocyanate or Cy3) was applied. Excess of unbound secondary antibody was removed by two additional PBS washes. Labeled cells were then analysed on a FACS Calibur (Becton Dickinson Biosciences) with CellQuest Pro (Becton Dickinson).
Clinical Cancer Research

Western blot analysis
To monitor merlin, expression lysates of MEN/MEN-NF2 cells were analyzed using an anti-merlin antibody (sc-331, Santa Cruz Biotechnology Inc.). Vinculin levels were assessed for loading control (Santa Cruz).

Cell viability assays
Cell survival was assessed using standard MTT assay. Cells were seeded in microtiter plates (2,000 per well). For the experiments with different cell densities, 1,000, 2,000, and 4,000 cells were plated to obtain 30%, 60%, or 90% confluence, respectively. After overnight incubation, plates were treated with 1, 10, or 100 μg/mL cilengitide for 24 or 48 hours. Control wells received culture medium. For the experiments designed to test a possible synergism between the drug and irradiation, plates received 2, 5, or 8 Gy X-rays in a Gulmay-D3225 machine (Gulmay Inc.) 2 hours after drug addition to the wells. After 48 hours, medium was removed and MTT (0.75 mg/mL medium) added. The solution was discarded after 2 hours (37°C), and 100 μL dimethyl sulfoxide were added to each well. Formazane absorbance at 562 nm was measured in a microplate reader (Tecan). The percentage of viable cells was defined as optical density relative to that of untreated controls.

Cell-cycle analysis
Two lakh cells were seeded in T25 cell culture flasks and incubated overnight. Cilengitide was then added to a final concentration of 1, 10, or 100 μg/mL for 24 hours. Adherent cells were then trypsinized and harvested together with floating cells by centrifugation, fixed with cold 70% ethanol, and treated with RNase A (100 μg/mL) for 10 minutes at 37°C. Cells were resuspended in propidium iodide (50 μg/mL in PBS). DNA content was determined by flow cytometry using FACs calibur and CellQuest Pro (BD Biosciences). Debris and damaged cells were excluded by gating on a forward and sideward scatter dot plot. DNA was recorded in the FL2 channel using linear amplification. Data obtained were evaluated with FACScan to calculate the percentage of cells in each phase.

Caspase assay
Meningioma cells were plated in T25 flasks and grown overnight. Ten flasks per concentration were treated with 0, 1, 10, and 100 μg/mL cilengitide. After 48 hours, cells were harvested by trypsin and PBS washing, lysed with one volume share lysis buffer, supplied with the CCP32/caspase-3 fluorimetric protease assay kit (Chemicon), and stored on ice for 10 minutes. Protein was quantified using the Bio-Rad Dc assay (Bio-Rad). The fluorimetric caspase-3 reaction was conducted according to the manufacturer’s instructions. Fluorescence was measured in a Safire reader (Tecan) at excitation/emission wavelengths of 400/505 nm.

Cell invasion and migration assay
The upper and lower compartments of a 24-well Transwell chamber (Corning) were separated by polycarbonate filters with 8-μm pore size. Before the assays, the polycarbonate filters were coated with 100 ng Matrigel. A total of 10^4 cells/well were seeded onto the filters and incubated for 48 hours in medium containing 0, 1, 10, and 100 μg/mL cilengitide. As control, the same number of cells was seeded into normal 24-well plates with the same cilengitide concentrations. Cells passing the filters and attaching to the lower sides of the Matrigel-coated membranes (migrated cells) were harvested using trypsin/EDTA. The cell number was quantified by a Neubauer chamber. The percentage of migrating cells was calculated in relation to controls grown under identical conditions without filters (in triplicate).

To evaluate the migratory properties, a gap assay was conducted using chamber-inserts from Ibidi. A cell suspension (5 × 10^5 cells/mL) was prepared. Seventy microliters were filled into each well and incubated at 37°C for 6 hours before cilengitide was added for 24 hours. The culture insert was removed to create the gap of 500 to 600 μm. Migration was measured under an inverted microscope at various time intervals between 0 hour and 29 hours. With Axiovision (Carl Zeiss AG), the distances between the outermost cells were measured on twenty marker lines cutting the gap square. The assay was conducted in the absence or presence of 1 μg/mL cilengitide.

Cilengitide treatment and irradiation of tumor-bearing nude mice. Experiments were done in accordance with the regulations of animal protection. Fifteen 8- to 10-week-old Swiss Nude mice (Charles River) were injected subcutaneously on both flanks with 3 × 10^5 IOMM-Lee cells in 100 μL PBS/Matrigel. Beginning at day 3, 8 mice were intraperitoneally injected daily (5 days per week) with 8 mg/kg cilengitide in PBS for 17 days. Seven PBS-treated tumor-bearing mice served as a control. The tumor volume (V) was estimated weekly by measurement (caliper rule) of two perpendicular axes according to V = π/6 a × b^2 (a > b). After killing the mice, tumors were resected and weighed.

In MRI studies, thirty-nine 8- to 10-week old mice were used for subarachnoidal inoculation with IOMM-Lee cells. Animals were anesthetized intraperitoneally (Rompun/Ketamin) and stabilized in a stereotactic head holder. Two holes were drilled 2 mm anterior of the bregma and 1.5 mm left and right from the sagittal suture, just deep enough to penetrate bone and meninges with minimal alteration of the neocortex. Approximately 2.5 × 10^6 cells in 5 μL PBS were slowly (1 minute) injected per hole to a depth of 1 mm with a Hamilton syringe. In one set of experiments, 8 mice received a daily dose of 8 mg/kg cilengitide intraperitoneally (beginning at day 3), whereas 7 mice received PBS. In another set of experiments, 7 mice were treated with 75 mg/kg cilengitide daily, 6 mice with 75 mg/kg and brain X-irradiation (5 Gy, Gulmay-3225) at days 3 and 6, whereas 5 mice received irradiation only and 6 animals served as completely untreated controls (PBS). To measure a potential effect of cilengitide monotherapy (75 mg/kg) in another malignant meningioma cell line with similar dose-dependent in vitro response to cilengitide in MTT assays as compared with IOMM-Lee cells (P ≤ 0.01, data not shown), the above described orthotopic xenograft experiment was repeated with malignant KT21 cells in 10 mice, of which received cilengitide.
MRI scanning of intracranial tumor growth. Meningioma growth was monitored in isofurane-anesthetized mice by magnetic resonance imaging at days 4 and 10 after inoculation of IOMM-Lee cells or at day 21 after inoculation of the slowly growing KT21 cells using a Bruker Biospec 47/20 scanner (4.7 T, Bruker BioSpin GmbH) equipped with a BGA09 (400 mT/m) gradient system. A 25-mm Lizcage system (DotScientific Inc.) was used for RF excitation and signal reception. T2-weighted images were acquired by a rapid acquisition relaxation enhanced (RARE) sequence with the following parameters: TR 4,000 ms, TE 15 ms, slice thickness 800 μm, field of view 25.6 × 25.6 mm, matrix 256 × 256 (i.e., nominal in plane resolution 100 × 100 μm), RARE factor 8, 6 averages, total scanning time for one direction 12 minutes 48 seconds. ImageJ (http://rsb.info.nih.gov/ij/) was used to calculate the tumor volume at day 10. For this volumetric analysis, the tumor was segmented manually in each section and the determined area multiplied by the slice thickness (0.8 mm). Tumor tissue grown from both injection holes per mouse were regarded as a single tumor for statistical analysis.

Determination of overall survival. Twelve mice were inoculated intracranially with IOMM-Lee cells and 6 were treated with 75 mg/kg cilengitide or PBS. Tumors were allowed to grow until ethical criteria for killing were reached. The period between inoculation and killing was defined as survival time.

Histochemical analyses of meningiomas grown in nude mice. The skulls were decalcified, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) and MIB-1/Ki67 antibody (Dako) to assess microscopically brain invasion and to count the percentage of Ki67-positive nuclei (proliferation). To assess the level of apoptosis, the percentage of TUNEL-positive nuclei was counted after TUNEL staining with the In Situ Cell Death Detection kit (Roche). For quantification of Ki67 and TUNEL indices, 100 nuclei were analyzed in three microscopic slices per tumor. The degree of tumor cell invasion was assessed in H&E-stained slices by calculating the ratio between the contour area of tongue-like infiltrating tumor cells within the brain and the length of the baseline of this area at the brain–tumor border using a 10× microscope objective and the Cell-D software (Olympus), as previously described (Kliese and colleagues, 2012).

Statistical analysis
Multiple comparisons were conducted by ANOVA followed by Tukey post hoc test. Differences of the means between two groups were analyzed by a t test. All figures show mean ± SD. Cumulative survival was depicted as Kaplan–Meier curves and analyzed by log-rank test. Calculations were conducted using SPSS, release 21. Significance was assumed for P ≤ 0.05.

Results
Integrins are differentially expressed in meningiomas and meningioma cell lines
It has been previously shown that meningiomas express αvβ3 and αvβ5 integrin (6), two targets of the integrin inhibitor cilengitide (17). We first re-evaluated immunoprojection patterns of αvβ3 and αvβ5 using a TMA containing meningiomas of different malignancy and found that αvβ5 was strongly expressed in the majority of tumors, whereas αvβ3 was detected only in blood vessels within the tumors (Fig. 1A). Normal leptomeningeal tissue also showed some αvβ5 staining (Fig. 1A, a). Semi-quantitative analysis of αvβ5 immunoexpression revealed no major differences between different grades of malignancy (Table 1).

Next we determined the mRNA expression level of the three integrin monomers. Expression of all three proteins was reduced in meningiomas compared with non-neoplastic meninges, with statistically significant reduction for αv (Fig. 1B). We found no significant differences in the integrin mRNAs between tumor grades (data not shown). Analysis of various meningioma cell lines (Supplementary Fig. S1) showed high expression of the αv subunit in IOMM-Lee and HBL52 cells, whereas the β5 and β3 subunits were less abundant. In BenMen-1 cells, all subunits were expressed at lower levels. By FACS analysis of meningioma cell lines, we confirmed the predominance of the αvβ5 heterodimer in IOMM-Lee, BenMen-1, and HBL-52 cells. Figure 1C shows an example for a typical fluorescence distribution. Only low expression of αvβ3 was seen in all the three cell lines (data not shown). In most experiments, the immunofluorescence of αvβ3 could not clearly be distinguished from controls without primary antibody. Visualization of αvβ5 in meningioma cells by confocal imaging is shown in Fig. 1D [comparable data were obtained for IOMM-Lee cells (not shown)].

Effects of cilengitide on meningioma cell survival
Cilengitide (1, 10, and 100 μg/mL) was added to IOMM-Lee, HBL52, and Ben-Men1 cultures. Morphologic changes were monitored over 24 hours. In all three meningioma lines, cells started to round up and detach from the flask in a concentration-dependent manner, showing that cilengitide decreases cell adhesion (data not shown). Quantification of cell viability after 24 hours, cilengitide treatment showed in all three cell lines a highly significant dose dependent but rather mild decline of viable cells, without major differences between cell lines (Fig. 2A). IC50 values could not be reached, even with higher concentrations or longer incubation times (data not shown). To study a potential role of meningioma cell confluency before cilengitide administration, MITT assays were done with IOMM-Lee cells at 30%, 60%, or 90% confluency. Figure 2B shows that cilengitide exhibited the highest efficacy at low cell density.

MITT assay measures the viable cell number, which is often used to quantify the reaction of tumor cells to cytotoxic drugs, but it does not discriminate between cytotoxic and antiproliferative effects. Therefore, we analyzed potential cilengitide-induced changes of cell-cycle progression. In two analyzed cell lines (BenMen-1, IOMM-Lee), no cell-cycle arrest was induced by cilengitide and no increase of the sub-G0 fraction of dead cells was seen (Supplementary
Moreover, application of cilengitide did not change caspase-3 activity significantly in both cell lines compared with untreated cells (not shown). No significant alterations of the mRNA levels of the apoptotic regulators, bax and bcl-2, were found for various cilengitide concentrations (data not shown). Even under hypoxia (0.1% O2 for 24 hours before RNA isolation), no increase of bax or decrease of bcl2 mRNA could be seen in the cilengitide-treated samples (data not shown).

Irradiation enhances cilengitide response of meningioma cells

Because inaccessible residual tumor tissue and malignant meningiomas are often irradiated, we wondered whether irradiation might affect the sensitivity of meningioma cells toward cilengitide. We combined various doses of X-rays (2, 5, or 8 Gy) with various cilengitide concentrations in IOMM-Lee and BenMen-1 cells, applying X-rays 2 hours after the initiation of drug treatment. As shown in Fig. 2C, significant and dose-dependent

Table 1. Immunoexpression of αvβ5 in human meningiomas

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<th>WHO Grade</th>
<th>αvβ5</th>
<th>N (%)</th>
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<tr>
<td>I (N = 29)</td>
<td>–</td>
<td>3 (10)</td>
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<td></td>
<td>+</td>
<td>9 (31)</td>
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<td></td>
<td>++</td>
<td>5 (17)</td>
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<td></td>
<td>+++</td>
<td>12 (41)</td>
</tr>
<tr>
<td>II (N = 11)</td>
<td>–</td>
<td>1 (9)</td>
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<td>+</td>
<td>2 (18)</td>
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cytostatic effects of cilengitide occurred under all irradiation regimens. The surviving cell fractions for a given drug concentration relative to solely irradiated samples were rather similar among the irradiation regimens, and indicated mild synergistic effects in this assay.

**Cilengitide effects on meningioma cells are NF2 dependent**

A significant proportion of human meningiomas carries alterations at the NF2 gene, which encodes the "merlin" protein. Using meningioma cells with stable short hairpin RNA (shRNA)-mediated knockdown of NF2 (Fig. 3A), we first observed that the level of αvβ5 integrin expression was reduced in the merlin-deficient cells (Fig. 3B). Confocal imaging showed not only reduced expression, but also a change in the integrin distribution within the cell, from a more generalized distribution to a perinuclear expression in the merlin-deficient cell line MEN-NF2 (Fig. 3C). MEN-NF2 cells were more susceptible to cilengitide treatment as compared with MEN cells in MTT-assays, as long as no concomitant radiation was applied (Fig. 3D). Surprisingly, we observed that this difference disappeared under irradiation.

**Cilengitide reduces migration and invasion of meningioma cells**

Tumor cell invasion of meningioma cells in surrounding structures like bone or brain constitutes a significant event. Because cilengitide inhibits integrins, which are responsible for cell adhesion, we next wanted to analyze effects on meningioma cell invasion. First, we used a Transwell assay to investigate the invasion of IOMM-Lee, HBL-52, and primary tumor cells derived from a WHO grade I meningioma. As shown in Fig. 4, cilengitide clearly inhibited the invasiveness of the malignant cell line IOMM-Lee (A) and the primary culture of benign meningioma cells (B) in a dose-dependent manner. In contrast, invasiveness of the benign cell line, HBL-52, was not suppressed by cilengitide (A). Already 1 µg/mL of cilengitide decreased the invasive ability of IOMM-Lee and primary cells by about 50% ($P < 0.05$). To further support this result, we conducted gap assays using IOMM-Lee cells to evaluate the effects of the drug on meningioma cell migration. Figure 4C shows that already 1 µg/mL of cilengitide is best in inhibiting the migration of IOMM-Lee cells. After 29 hours, the remaining gap width for treated cells was 45% compared with 25% for untreated cells. Because cilengitide
detaches adherent cells, this experiment could not be carried out with higher concentrations. Confocal imaging of IOMM-Lee cells stained with phal-loidin (Fig. 4D) showed reduced spike number and length (arrows) in cells treated with a low cilengitide concentration (1 µg/mL).

**Cilengitide monotherapy does not inhibit tumor growth but brain invasion in mice, and may be beneficial in combination with irradiation**

In a first set of *in vivo* experiments, we tested the ability of a low-dose cilengitide monotherapy (8 mg/kg daily) to inhibit the subcutaneous meningioma growth (IOMM-Lee cells) in nude mice. No significant impact of the integrin inhibitor was observed. The mean tumor volume (±SD) of treated mice on day 17, as estimated by external calliper rule measurement in living animals, was 2,042 ± 569 mm³, as compared with 1,964 ± 352 mm³ in controls (difference n.s.). Accordingly, the weights of explanted tumors (651 ± 249 mg vs. 547 ± 183 mg) showed no significant difference between the groups. Treating the orthotopic meningioma mouse model with the same dosage did not yield a significantly reduced tumor volume 10 days after inoculation (36.1 ± 11.9 mm³) in comparison with PBS controls (42.1 ± 19 mm³), as estimated by MRI scans.

In a new set of experiments, we intended to test a high daily dosage of cilengitide (75 mg/kg) as a monotherapy or combined with irradiation in the orthotopic mouse model. Again, the cilengitide-treated group exhibited no statistically significant difference to PBS controls. Irradiation alone (2 × 5 Gy) led to a more than 2-fold reduction of the tumor volume at day 10 (*P < 0.05*), whereas the combination of irradiation and cilengitide resulted in a more than 4-fold (*P < 0.01*) decrease (Fig. 5A). On the basis of the used number of animals, the difference between irradiation alone and combined therapy became not statistically significant. Immunohistochemical analyses of paraffin-embedded tumor sections revealed high proliferation rates and moderate levels of apoptosis in all groups, as shown by a mean Ki67 index of 17.1% (±5.1%) and a mean TUNEL
index of 2.7% (±1.7%). A high variability was observed among the mice of identical treatment, and no significant effect of cilengitide on these parameters could be detected. On the other hand, a significant reduction of tongue-like brain invasion (P < 0.01) could be observed in tumors of mice treated with either cilengitide alone (35% decrease) or with cilengitide and irradiation (35.5% decrease).

To verify the nonresponsiveness with a second cell line, an MRI-based comparison was conducted between mice carrying intracranial xenografts of slowly growing KT21 tumors and treated either with high-dosage cilengitide (75 mg/kg) or PBS. Even after a prolonged intracranial growth period (21 days), the tumor volumes remained small as compared with IOMM-Lee–induced meningiomas. The MRI estimated mean tumor volumes in treated and control mice were highly similar (difference < 5%, n.s.).

These data suggested that rapidly growing meningiomas, as represented by IOMM-Lee xenografts, have no survival benefit from cilengitide monotherapy, despite its antimigratory properties. This hypothesis was proven in the orthotopic model by measuring cumulative survival (Supplementary Fig. S3) in 6 cilengitide-treated animals (75 mg/kg) versus 6 controls. A log-rank test revealed no significant impact of cilengitide on overall survival.
Because these tumors had grown for longer periods (mean survival 15.1 days), the invasion status was histologically examined again. Only 2 treated animals exhibited brain invasion, whereas all 6 untreated mice showed clear tongue-like brain invasion of meningioma cells (Fig. 5B; Table 2).

Discussion

Cilengitide is a promising new drug against human tumor diseases, which is currently tested in clinical studies. Because of its impact on tumor vascularization, it became a candidate for targeted tumor therapy. Furthermore, cilengitide has been shown to affect tumor cell invasion and migration (18). Clinical activity with low side effects was observed in phase I and II glioma studies (19, 20), and a first phase III trial with the drug has been carried out (21). Although meningiomas are common intracranial tumors, they nevertheless receive less attention due to their benign biologic behaviour in the majority of the patients. Therefore, no data of cilengitide sensitivity are available, despite the need for a supportive cytostatic therapy for incompletely removed, atypical, or anaplastic tumors.

Integrin αvβ5 and αvβ3 expression in meningiomas

The expression of these two heterodimers, which are targeted by cilengitide, had already been described in tumor cells and neoplastic vasculature of meningiomas of all WHO grades (6). These results were basically confirmed by the present study, although in our series we found that αvβ5 integrin was highly expressed in the tumor cells, whereas αvβ3 was only present in blood vessels. In contrast with Bello and colleagues (6), we also analyzed normal meninges and observed that integrin expression was reduced in the tumor tissues compared with tumor-free meninges. This might have an impact on the effectiveness of cilengitide in tumor cells.

Clear impact of cilengitide on meningioma cell migration

Because migration and invasion of high-grade meningioma cells into the brain tissue play a significant role for disease outcome (22), we analyzed the influence of cilengitide toward meningioma cell invasion and migration in Transwell and gap assays. We found dose-dependent inhibition in both assays, which did however not occur with all cell lines. In previously published studies of glioma cell lines, the invasiveness of LN-308 was significantly reduced by cilengitide, whereas U87MG or LNT-229 did not show this effect (23). This underlines that even among different cell lines of the same tumor entity variable migratory responses toward cilengitide can be observed.
Only moderate cytostatic effects in vitro

MTT assays showed only limited dose-dependent cytostatic effects of cilengitide in the meningioma cell lines tested. The IC_{50} could not be reached even after 48-hour incubation. This result was confirmed by propidium iodide FACS analysis, which showed no growth arrest and no increase of the sub-G_{0} fraction of dead cells, even with 100 \mu{g}/ml cilengitide. This is in accordance with the reports, which showed only moderate effects of cilengitide on cell viability in glioma cell lines (23), not reaching IC_{50} with 1,000 \mu{mol}/L of the drug within 72 hours.

Because patients with high-grade or recurrent meningiomas often receive radiation therapy, we tested whether there is a synergistic effect of cilengitide treatment and irradiation using MTT assays. No major synergistic effect could be seen. Other studies show that cilengitide amplifies the toxicity of irradiation only mildly in the U251 glioma cell line, but HUVECs are strongly affected (24). However, this effect was seen only 5 days after irradiation. In vivo cotreatment with cilengitide and irradiation prolonged the survival of U251 tumor-bearing rats two-fold compared with irradiation alone (24).

In clinical studies, the intravenous administration of 600 mg/ml cilengitide resulted in plasma concentrations of about 250 \mu{g}/ml which decreased very fast in a logarithmic manner, yielding only 1 \mu{g}/ml after approximately 8 hours (19, 25). For our experiments, we used 0, 1, 10, and 100 \mu{g}/ml cilengitide for 24 or 48 hours, which should be a relevant range, compared with the plasma concentrations reached in clinical studies.

Potential role of the NF2 gene

We observed a relation between the NF2 gene status of meningioma cells, and both \alpha v \beta 5 expression, and sensitivity to cilengitide treatment. A large proportion of meningiomas harbor NF2 gene alterations (26), but the interaction between chemotherapy and NF2 gene status, as well as treatment response has not been studied thus far. It was shown recently that merlin interacts with integrins via the src/Fak pathway (27). Integrons may also control the mTORC1 signaling pathway through merlin (28). However, the detailed relation between NF2/merlin and integrin action in the regulation of tumor cell invasion and migration is not well studied.

Inhibition of brain invasion and trend for improvement of radiotherapy in mouse models

To assess a potential impact of cilengitide to inhibit meningioma development in vivo and to assess a potential synergism with irradiation in vivo, as described for glioma-bearing animals (24, 29), we used subcutaneous and intracranial meningioma mouse models. We first chose a daily dosage of 8 mg/kg, which was similar to a regimen exhibiting excellent growth suppression in slowly growing rat gliomas (29). The regimen did not yield any effect in both meningioma models. An extremely high daily dosage (75 mg/kg) was then tested, which was known to inhibit the progression of breast cancer bone metastases in rats (30). It did not decelerate intracranial meningioma growth, but clearly suppressed brain invasion. In view of the rapid volume increment, suppressed invasion was not translated into prolonged survival. These results are compatible with a recent phase III study looking for an additional benefit of cilengitide in glioblastoma treatment, if added to the current standard regimen of irradiation plus temozolomide. Although brain infiltration is a hallmark of glioblastomas, the drug did not improve overall survival (ASCO 2013 abstract LBA2009).

However, cilengitide may enhance the efficacy of irradiation (compare Fig. 5A), although a synergism could not yet be statistically proven on the basis of the number of mice used. This point warrants a more detailed analysis with variation of both, dosage of cilengitide and irradiation.

In summary, the present study showed no activity of cilengitide on meningioma apoptosis, mild cytostatic synergistic effects with irradiation, and inhibition of cell migration and invasion in some cell lines, primary cultures and in vivo. The study does not suggest any benefit of a monotherapy with the inhibitor in malignant meningiomas. The observed effect of a combined therapy with cilengitide and irradiation in meningioma-bearing mice warrants further attention. If a radiosensitization can be established in mouse models, a benefit of the drug in meningioma therapy may still be possible.

Disclosure of Potential Conflicts of Interest

C. Mawrin has a commercial research grant and is a consultant/advisory board member of Merck.

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