BAY 12-9566, A Novel Inhibitor of Matrix Metalloproteinases with Antiangiogenic Activity

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ABSTRACT
Matrix metalloproteinases (MMPs) have been implicated in tumor cell invasion, metastasis, and angiogenesis. BAY 12-9566, a novel, non-peptidic biphenyl MMP inhibitor, has shown preclinical activity on a broad range of tumor models and is currently in clinical development. The purpose of this study was to investigate the antiangiogenic activity of BAY 12-9566. In vitro, BAY 12-9566 prevented matrix invasion by endothelial cells in a concentration-dependent manner (IC_{50} = 8.4 \times 10^{-7} \text{ M}), without affecting cell proliferation. In vivo, oral daily administration of BAY 12-9566 (50–200 mg/kg) inhibited angiogenesis induced by basic fibroblast growth factor in the Matrigel plug assay, reducing the hemoglobin content of the pellets. Histological analysis showed a reduction in the amount of functional vessels within the Matrigel. We conclude that the MMP inhibitor BAY 12-9566 inhibits angiogenesis, a property that further supports its clinical development as an antimetastatic agent.

INTRODUCTION

Angiogenesis, the process that leads to the formation of new capillaries from existing blood vessels, is essential for solid tumor growth and metastatic spread (1). A critical step in angiogenesis is the degradation of the extracellular matrix by invading enzymes by invading endothelial cells and is blocked by inhibitors (3). A large body of evidence suggests that MMPs play a fundamental role in this event (4–6). MMPs consist of a large family of zinc-dependent endopeptidases that function at a neutral pH (4–6). In particular, gelatinase A (MMP-2) and gelatinase B (MMP-9) are key regulators of angiogenesis (7–10). The prevention of matrix degradation through the inhibition of MMP activity has been shown to be a promising therapeutic approach to block the invasive processes that occur during angiogenesis and tumor progression (6, 11–13). A number of preclinical studies have shown that MMPs can inhibit angiogenesis, tumor growth, and local tumor cell invasion and metastasis (5, 11, 14). The majority of these compounds (some of which have entered clinical trials) are hydroxamate derivatives with inhibitory activity against a broad spectrum of MMPs. BAY 12-9566 is a non-peptidic biphenyl MMP inhibitor with a Zn-binding carboxyl group. BAY 12-9566 has a high substrate selectivity, particularly for MMP-2 and MMP-9, and is p.o. bioavailable (15–17). This compound has shown anti-invasive and antimetastatic activity in several experimental tumor models, including the human colon carcinoma HCT 116 (15), mammary carcinoma MDA-MB-435 (18), murine B16-F10 melanoma, and Lewis lung carcinoma (16). Because MMPs can exert their antitumor effect through inhibition of tumor cell dissemination as well as inhibition of angiogenesis, we have designed the studies reported here to investigate the antiangiogenic potential of BAY 12-9566 in in vitro, and in vivo models of angiogenesis.

MATERIALS AND METHODS

Reagents. BAY 12-9566 (M, 410.95), 4-[4–4-(chlorophenyl)phenyl]-4-oxo-2S-(phenethylmethyl)butanoic acid [obtained from Dr. Harold Kluender (Bayer Corp., West Haven, CT)], is an inhibitor of MMP-2, MMP-3, MMP-9, and MMP-13 (K_i = 11, 134, 301, and 1470 nm, respectively), but not of MMP-1 (K_i > 5000 nm) (16). For the in vitro experiments, a 1000× stock solution in absolute ethanol was freshly prepared and then diluted in test medium (M199 medium supplemented with 20% calf serum for the proliferation assay and DMEM with 0.1% BSA for the invasion assay) immediately before the assay. For oral administration, a suspension of the compound in 0.5% carboxymethylcellulose and 0.2% Tween-80 was prepared. Matrigel was obtained from Becton Dickinson (Bedford, MA). All culture reagents were from Life Technologies, Inc. (Paisley, Scotland).

Endothelial Cells. HUVECs were grown in M199 medium supplemented with 10% FCS, 10% newborn calf serum,
50 μg/ml endothelial cell growth supplement (crude extract from bovine brain), 100 μg/ml heparin, and 20 mM HEPES (19, 20). Cells were used between the third and fifth passage of their isolation.

**Invasion Assay.** Invasion was assessed as described using Boyden chambers and polycarbonate Nucleopore filters (8-μm pore size) (19, 20). The filters were coated with an even layer of Matrigel (0.5 mg/ml). The supernatant of NIH-3T3 cells was used as the attractant and added to the lower compartment of the Boyden chamber. Endothelial cells were detached, washed in DMEM with 0.1% BSA, resuspended in the same medium at a concentration of 0.5–1 × 10^6 cells/ml, and added to the upper compartment of the Boyden chamber. BAY 12-9566 was added to the endothelial cells at the concentration indicated in “Results” and incubated throughout the assay (6 h). Filters were then stained with Diff-Quik (Marz-Dade, Dudingen, Switzerland), and the migrated cells were counted in 10 high-power fields. Data are expressed as the percentage of control migration (vehicle-treated cells). The IC50 was calculated from the plotted data.

**Proliferation Assay.** Endothelial cell proliferation was assessed as described previously (20). Briefly, 4 × 10^3 cells were plated in each well of a 96-well plate. After 24 h, BAY 12-9566 was added at the concentration indicated in “Results” and incubated for 6 h. The drug was then removed, and the cells were washed and incubated with fresh medium for an additional 72 h. The plate was then stained with 0.5% crystal violet in 20% methanol, rinsed with water, and air dried. The stain was eluted with a 1:1 solution of ethanol:0.1 m sodium citrate, and absorbance at 540 nm was measured with a Multiscan MC Titertek (Flow Laboratories, Milan, Italy). Data are expressed as the percentage of control proliferation (vehicle-treated cells). The IC50 was calculated from the plotted data.

**Angiogenesis in Vivo Assay.** The method described by Passaniti et al. (21), modified as described previously (20), was used for the in vivo angiogenesis assay. Briefly, bFGF (150 ng/pellet) was embedded in a pellet of Matrigel (12.5 mg/ml; 0.5 ml) and injected s.c. into C57BL/6N mice (Charles River, Calco, Italy). Mice received BAY 12-9566 (50–200 mg/kg) p.o. immediately before and for 6 days after the injection of the pellet of Matrigel. Control mice received the same volume of vehicle. In one experiment, BAY 12-9566 was added directly to the Matrigel plug together with bFGF. After 7 days, the pellet was removed, and the hemoglobin content was measured using the Drabkin’s procedure (20). For histological analysis, the Matrigel pellets, in combination with surrounding skin and soft tissue, were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Five-μm sections were then stained with H&E following standard procedures. The angiogenesis response was evaluated following the classification proposed by Belotti et al. (20). We evaluated the presence of cords and tubules within the pellet (scored as follows: 0; none; 1; occasional; 2; moderate; and 3, prominent) and the presence of blood-filled channels and/or lacunae containing RBCs (scored as follows: 0; absence of RBCs; 1; presence of RBCs in 5% of blood-filled channels; 2, presence of RBCs in 5–50% of blood-filled channels; and 3, presence of RBCs in more than 50% of blood-filled channels). For each sample, the resulting sum was recorded as the final score.

**RESULTS AND DISCUSSION**

BAY 12-9566 is a novel, non-peptidic byphenyl inhibitor of MMPs that inhibits the activities of MMP-2 and MMP-9 (16). In this study, we show that this compound affects endothelial cell invasion, a key process of angiogenesis mediated by MMP, and the angiogenesis response.

BAY 12-9566 inhibited HUVEC invasiveness through a layer of reconstituted basement membrane (Matrigel) by a maximum of 89% (Fig. 1). The effect was dose dependent, with an IC50 of 8.4 × 10^{-7} M. The same concentrations of BAY 12-9566 that inhibited HUVEC invasion did not affect the motility (chemotaxis) of endothelial cells (data not shown), a process that does not require matrix degradation by MMPs (20). The observed inhibition of invasion is consistent with the hypothesis that MMPs prevent the process of matrix degradation and cell invasion. To assess whether the inhibitory activity of the compound could be due to a possible cytotoxic effect, the proliferation of endothelial cells was assessed in the presence of BAY 12-9566. Under experimental conditions comparable to those of the invasion assay (6-h exposure to the drug), BAY 12-9566 had only a marginal antiproliferative effect on endothelial cells only at the highest concentration tested (maximal inhibition was 22.6% at 1 × 10^{-5} M; Fig. 1). Even when cells were continuously exposed to the compound for longer periods of time (72 or 144 h), no significant inhibition of their proliferation was observed (data not shown). These findings thus indicate that the anti-invasive activity of BAY 12-9566 is independent from a direct cytotoxic effect toward endothelial cells.

The effect of BAY 12-9566 on angiogenesis in vivo was evaluated in the Matrigel plug assay, in which angiogenesis is induced by bFGF embedded in a pellet of Matrigel implanted
Table 1  Effect of BAY 12-9566 on in vivo angiogenesis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Stimulus(\text{a})</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>Hemoglobin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>bFGF</td>
<td>Vehicle</td>
<td>p.o. once a day</td>
<td>0.017 ± 0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>Vehicle</td>
<td>p.o. once a day</td>
<td>0.060 ± 0.017(^{b})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>BAY 12-9566</td>
<td>200</td>
<td>p.o. once a day</td>
<td>0.036 ± 0.023(^{c})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAY 12-9566</td>
<td>100</td>
<td>p.o. twice a day</td>
<td>0.043 ± 0.020(^{c})</td>
</tr>
<tr>
<td>2</td>
<td>bFGF</td>
<td>Vehicle</td>
<td>p.o. once a day</td>
<td>0.017 ± 0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>Vehicle</td>
<td>Once into pellet</td>
<td>0.043 ± 0.015(^{b})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 12-9566</td>
<td>200</td>
<td>Once into pellet</td>
<td>0.025 ± 0.016(^{c})</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>bFGF</td>
<td>Vehicle</td>
<td>p.o. once a day</td>
<td>0.039 ± 0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>Vehicle</td>
<td>p.o. once a day</td>
<td>0.088 ± 0.040(^{b})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 12-9566</td>
<td>25</td>
<td>p.o. once a day</td>
<td>0.060 ± 0.029</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 12-9566</td>
<td>50</td>
<td>p.o. once a day</td>
<td>0.031 ± 0.014(^{c})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAY 12-9566</td>
<td>200</td>
<td>p.o. once a day</td>
<td>0.044 ± 0.018(^{c})</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}\) bFGF (150 ng) was embedded in a pellet of Matrigel and implanted s.c. in C57BL/6 mice, and the angiogenic response was evaluated 7 days later as the hemoglobin content of the pellet (mean ± SD, n = 7–10).

\(\text{b}\) P ≤ 0.05 compared to negative control, without stimulus (Mann-Whitney U test).

\(\text{c}\) P ≤ 0.05 compared to positive control, bFGF-containing pellets in vehicle-treated mice (Mann-Whitney U test).

In vivo

s.c. in mice (20). In 7 days, bFGF induced a massive angiogenic response, with cells infiltrating the Matrigel support and the formation of blood-containing vessels. In parallel, there was a significant increase in the hemoglobin content of the pellets as compared with Matrigel pellets without the stimulus (Table 1 and Fig. 2A and B).

Oral daily treatment with BAY 12-9566 significantly prevented the angiogenic response induced by bFGF, as evaluated by the hemoglobin content of the pellets (Table 1). In the first experiment (Table 1, experiment 1), treatment with BAY 12-9566 (dose, 200 mg/kg) immediately before and for 6 days after the injection of the pellet resulted in a comparable inhibitory activity, regardless of whether it was administered in one dose or split into two daily doses (100 mg/kg each). This indicates that one daily treatment with the compound is sufficient for activity. In a parallel experiment (Table 1, experiment 2), BAY 12-9566 was added directly to the bFGF-containing Matrigel pellet. A significant reduction in the angiogenic response was observed. Similar results were obtained in preliminary experiments in which angiogenesis in the Matrigel was driven by tumor cell supernatant (Ref. 20; data not shown). A third experiment showed that inhibition of angiogenesis occurred at BAY 12-9566 doses of ≥50 mg/kg and that inhibition was dose dependent, with no significant effect at a dose of 25 mg/kg (Table 1, experiment 3).

Histological analysis of Matrigel pellets without angiogenic stimuli presented only a few infiltrating stromal cells (Fig. 2A). In vehicle-treated mice, pellets containing the angiogenic stimulus (bFGF) presented a high degree of infiltrating cells, many of which were organized into thin cords, tubules, and blood-containing vessels (Fig. 2B). These structures showed endothelial cells positive for CD31 (data not shown). In mice treated with BAY 12-9566, the cellularity surrounding and infiltrating the pellets appeared to be only partially reduced; however, single cells remained scattered throughout the Matrigel and formed a reduced number of cords, tubules, or blood-containing vessels compared to the pellets of vehicle-treated mice (Fig. 2C). The histological score was a median of 0 (range, 0–1) for negative control pellets, 5 (range, 2–6) for positive control pellets, and 3 (range, 1–5) for pellets in BAY 12-9566-treated mice (P < 0.05 compared to vehicle-treated mice). This finding might provide a clue to the mechanism of angiogenesis inhibition by BAY 12-9566. The presence of single cells scattered throughout the Matrigel might be the consequence of two different actions of the MMPI. BAY 12-9566 might prevent the invasion of endothelial cells into the Matrigel support, in agreement with the observed inhibition of invasiveness in vitro.

Interestingly, a recent study reported that MMPIs can selectively prevent the invasion of endothelial cells, but not of other cell types, within a fibrin substrate (22). In support of this hypothesis, we observed a reduced number of cells positive for the endothelial marker CD31 in pellets of mice treated with BAY 12-9566 compared with pellets of mice treated with vehicle (data not shown). Alternatively, BAY 12-9566, as well as other agents that alter the process of matrix remodeling by cells, might affect the complex endothelial cell-matrix interactions that eventually lead to the formation of functional, blood-containing vessels (7, 9). Several endothelial cell functions might be affected by an altered interaction with the extracellular matrix, such as cell proliferation (which is not inhibited by BAY 12-9566) and survival, as well as the organization of capillary structures (2). The presence of single cells within the Matrigel might therefore be the result of an inhibitory effect of BAY 12-9566 on the tridimensional organization of endothelial cells to form mature, functional structures. Moreover, MMPIs might prevent complex activities of the proteolytic enzymes, such as the release of angiogenic factors stored within the matrix, protein activation, or the cleavage or shedding of ectodomains of plasma proteins (6).

We reported previously (19) that another MMPI batimastat, which prevented endothelial cell invasion in vitro and angiogenesis in vivo, also inhibited experimental tumor growth and metastatic spread. Treatment with BAY 12-9566 has demonstrated significant antitumor activity in preclinical models (15, 16, 18). Here we show that BAY 12-9566 also has antian- giogenic activity. Pharmacokinetic studies show that the oral absorption of BAY 12-9566 is rapid, with high bioavailability (23). In Phase I clinical trials, BAY 12-9566 has been reported
to be well tolerated, inducing only mild and reversible adverse effects, with a remarkable lack of the musculoskeletal effects observed with other MMPIs (17, 24). At the recommended dose of 800 mg p.o. twice daily, steady-state plasma levels sufficient to demonstrate preclinical activity were achieved (24).

In conclusion, the antiangiogenic activity of BAY 12-9566 observed in the present study indicates that the compound might affect tumor growth not only by inhibiting its dissemination, but also by inhibiting angiogenesis, thus opening up new possibilities for its clinical development.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 2  Histological analysis (H&E staining) of the effect of BAY 12-9566 on in vivo angiogenesis. A (negative control), Matrigel containing no angiogenic stimulus showed few infiltrating cells (×300). B (positive control), Matrigel containing bFGF in vehicle-treated mice demonstrated a high degree of cellularity and the presence of blood-containing vessels (×300). Arrows, examples of structures referred to as blood-containing vessels. C, BAY 12-9566-treated mice. The bFGF-containing Matrigel pellets presented a reduced number of blood-containing vessels (×300). D (positive control), magnification of tube-like structures (×600) containing RBCs (arrow). Similar findings were observed in two independent experiments.


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