Antimetastatic Effect of a Small-Molecule Vacuolar H\textsuperscript{+}-ATPase Inhibitor in in Vitro and in Vivo Preclinical Studies

Rosanna Supino, Giovanna Petrangolini, Graziella Pratesi, Monica Tortoreto, Enrica Favini, Laura Dal Bo, Patrizia Casalini, Enrico Radaelli, Anna Cleta Croce, Giovanni Bottirol, Paola Misiano, Carlo Farina, and Franco Zunino

Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Istituto Nazionale Tumori, Milan, Italy (R.S., G.P., G.P., M.T., E.F., L.D.B., P.C., F.Z.); Facolta Medicina Veterinaria Universita degli Studi di Milano, Milan, Italy (E.R.); Consiglio Nazionale delle Ricerche, Universita di Pavia, Pavia, Italy (A.C.C., G.B.); and Nikem Research, Baranzate, Milan, Italy (P.M., C.F.)

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ABSTRACT

On the basis of the evidence that vacuolar H\textsuperscript{+}-ATPase is implicated in the development of the metastatic phenotype, we have explored the possibility to target the enzyme function in an attempt to control the metastatic behavior of tumor cells. In this study, we used an indole derivative, NiK-12192 [4-(5,6-dichloro-1\textsuperscript{n}-indol-2-yl)-3-ethoxy-N-(2,2,6,6-tetramethyl-piperidin-4-yl)-benzamide], recently identified as an effective inhibitor of vacuolar H\textsuperscript{+}-ATPase, as a potential antimetastatic agent in the treatment of NSCLC H460 xenograft, which is able to induce lung metastases in mice. Oral administration of NiK-12192 caused a significant inhibition of formation of spontaneous metastases. In contrast, the drug exhibited a negligible effect on the development of artificial metastases (i.e., after i.v. injection of tumor cells), thus supporting that the drug affects the early events of the metastatic process (e.g., migration and invasion). Cellular effects are consistent with this interpretation. In conclusion, the available results show for the first time that a vacuolar H\textsuperscript{+}-ATPase inhibitor is effective in modulation of the metastatic behavior of a lung carcinoma, supporting its potential therapeutic interest as a novel treatment approach.

The metastatic process occurs through a cascade of events involving a complex series of interactions with the host tissues allowing migration and invasion of tumor cells (Liotta and Stetler-Stevenson, 1993). The acidic extracellular microenvironment, which characterizes the solid tumors, is recognized to be a critical factor in stimulating tumor cell motility and invasive behavior (Harguindey et al., 2005). Indeed, the acidification of the hypoxic extracellular microenvironment facilitates the action of the acidic proteases, as well as cell locomotion (Rozhin et al., 1994; Martinez-Zaguilan et al., 1996; Stock et al., 2005; Rofstad et al., 2006). Acidification of extracellular microenvironment reflects homeostatic mechanisms regulated by proton exchangers or proton pumps. Proton transporters implicated in pH regulatory mechanisms include the Na\textsuperscript{+}/H\textsuperscript{+}-exchanger, Na\textsuperscript{+}/K\textsuperscript{+}-ATPase pump, vacuolar H\textsuperscript{+}-ATPases, H\textsuperscript{+}/Cl\textsuperscript{−} symporter and proton lactate symporters (Putnam, 2001; Harguindey et al., 2005). The vacuolar H\textsuperscript{+}-ATPase is expressed in plasma membrane and cytoplasmic organelles and can contribute to the metastatic potential of many tumor cells (Sennoune et al., 2004a,b; Martinez-Zaguilan et al., 1993, 1998). On the basis of its function, it is conceivable that inhibition of vacuolar H\textsuperscript{+}-ATPase function/expression may affect the invasive behavior of tumor cells. Indeed, the knocking down of ATP6L, the 16-kDa subunit of vacuolar H\textsuperscript{+}-ATPase, in a human hepatocellular carcinoma cell line resulted in inhibition of growth and metastases in nude mice (Lu et al., 2005). In clinical specimens, vacuolar H\textsuperscript{+}-ATPase was detected in invasive pancreatic carcinomas (Ohta et al., 1996).

We have recently reported that the vacuolar H\textsuperscript{+}-ATPase inhibitor NiK-12192, a lead compound of a novel series of substituted indole derivatives (Farina et al., 2001), is able to potentiate the antitumor activity of the camptothecin topotecan at well tolerated doses (Petrangolini et al., 2006). Although NiK-12192 exhibits a marginal effect on the s.c. growth of tumor xenografts, the intra-/extracellular modulation of pH is expected to influence spreading of tumor cells and metastasis development. The present study was based on the hypothesis that inhibition of vacuolar H\textsuperscript{+}-ATPase, which has
a prominent role in modulating the pH gradient, may influence the metastatic behavior of tumor cells. Therefore, the aim of the study was to investigate the role of NiK-12192 on the growth and the metastatic spreading of a human lung tumor, H460, which is able to form spontaneous lung metastases when implanted s.c. in nude mice (Corti et al., 1996). The results indicated that oral treatment with NiK-12192 resulted in a relevant antimetastatic effect in animal models. Moreover, in vitro studies confirmed the inhibitory activity of the compound in various assays designed to investigate cellular effects relevant to the metastatic behavior.

Materials and Methods

Drugs

NiK-12192 and topotecan used in this study are products of Nikem Research (Milan, Italy) and Glaxo SmithKline (Verona, Italy), respectively. Bafilomycin A1 is provided by Sigma-Aldrich (St. Louis, MO).

For in vitro studies, stock solutions (1 mg/ml) were stored in DMSO for NiK-12192 and diluted before use in saline solution. In experiments, the final concentration of DMSO in the medium was 0.1%, which has been verified to be nontoxic in our cell system. For in vivo studies, NiK-12192 was dissolved by adding absolute ethanol and Cremophor ELP (both 5% of the final volume) and diluted (just before use) by adding 0.9% NaCl. Topotecan was dissolved in sterile distilled water.

H460 Human Tumor Model System

The NCI-H460 nonsmall cell lung tumor cell line (hereafter named H460) was used in the in vitro studies. For animal experiments, H460 tumor cells were adapted to grow as ascitic in nude mice and maintained in vivo by i.p. passages (5 × 10⁶ cells/mouse in 0.5 ml of PBS). In brief, cells were collected from the donor mice approximately 7 days after inoculum, and, after washing, cell number and viability were determined by trypan blue exclusion. Cell suspension was injected s.c. or i.v. to achieve spontaneous or experimental metastases (Cassinelli et al., 2006).

In Vitro Studies

Intracellular pH Determination. Cells grown on coverslips were incubated for 40 min with SNARF-4F 5- (and -6) carboxylic acid, acetoxymethyl ester, acetate, stock solution (80 mM in DMSO); Invitrogen, Eugene, OR) at the final concentration of 8 μM. Dilutions were performed directly in the medium deprived of serum to prevent the hydrolysis of the fluorogenic substrate. After incubation, the coverslips were rinsed with PBS and mounted upside down on the microscope slides. SNARF-4F incubation was performed on control (untreated) cells and on cells pretreated with NiK-12192 (2 μM). After 24 h of drug exposure, samples were incubated with the FITC-secondary anti-rabbit antibody (Invitrogen) and mounted with Mowiol (Mowiol 4,88; Calbiochem, San Diego, CA) and observed by fluorescence microscope DMRB (Leitz). Images were taken by a 50× immersion oil objective, with an excitation light provided by a 100-W Hg lamp and an excitation filter at 450 to 490. Images were recorded with a Spot Insight digital camera (Delta Sistemi, Rome, Italy) equipped with a system of image analysis (IAS 2000; Delta Sistemi).

Migration and Invasion Assays. H460 tumor cells were seeded in 24-well plates at 4.5 × 10⁴ cells/well in growth medium. A single scratch wound was created on confluent monolayers by using a micropipette tip (Liang et al., 2007). Cells were washed with PBS to remove cell debris, supplemented with serum-free medium containing NiK-12192, and monitored. Images were captured by a Spot Insight digital camera (Delta Sistemi) using a ×40 contrast objective at 0 and 48 h postwounding, and wound distance (micrometers) was measured by system of image analysis (IAS 2000). Three different regions for each wound were considered. The experiment was performed two times.

Wound Healing Assay. H460 tumor cells were seeded onto 24-well plates at 4.5 × 10⁵ cells/well in growth medium. A single scratch wound was created on confluent monolayers by using a micropipette tip (Liang et al., 2007). Cells were washed with PBS to remove cell debris, supplemented with serum-free medium containing NiK-12192, and monitored. Images were captured by a Spot Insight digital camera (Delta Sistemi) using a ×40 contrast objective at 0 and 48 h postwounding, and wound distance (micrometers) was measured by system of image analysis (IAS 2000). Three different regions for each wound were considered. The experiment was performed two times.

αvβ3 Expression and Cellular Localization. Cells, seeded on coverslips in six multiwell plates, were treated with NiK-12192 (2 μM). After 24 h of drug exposure, samples were incubated with a 1:400 solution of FITC-conjugated mouse anti-human-αvβ3 monoclonal antibody (Mab1976F; Chemicon International, Temecula, CA) for 1 h, mounted with Mowiol, and observed by fluorescence microscope as reported above.

Tubulin and Actin Microscopic Analysis. For confocal experiments, cells were grown on coverslips, treated with NiK-12192 (2 μM) for 24 h, fixed with 4% paraformaldehyde for 30 min, and then washed and added with 70% ethanol for 20 min at −20°C. After washing with PBS containing 1% bovine serum albumin, cells were incubated with phallolidin, fluorescein-isothiocyanate-labeled 1:200 (Sigma-Aldrich), and monoclonal anti-β-tubulin 1:2000 antibody (clone SDL3D10; Sigma-Aldrich) for 1 h at 37°C. Samples were then incubated with Alexa 594-conjugated anti-mouse IgG antibody (Invitrogen) for 30 min, mounted with Prolong Gold Antifade reagent (Invitrogen) and analyzed by confocal microscopy (Microphoresis 2000; Bio-Rad Laboratories, Inc., Hercules, CA) equipped with Ar (488 nm) and HeNe (543 nm) lasers. Images (1024 × 1024 pixels)
were obtained using a 60× oil immersion lens and analyzed using Lasersharp 2000 software. Reported images represent a single z-section of the samples with 0.5-μm step. The pinhole diameter was regulated according to the value suggested by the acquisition software to obtain the maximum resolution power.

**Three-Dimensional Cell Culture.** H460 tumor cells (0.8 × 10⁶ cells/plate) were cultured in bacteriological plates (5-cm diameter) in complete culture medium. After 6 days, single spheroids were transferred to 96-well plates in culture medium containing NiK-12192 at different concentrations. Measures of spheroid diameters were taken by a micrometer lens in the inverted microscope at 0, 24, 48, and 72 h. Values are reported as mean percentage of the diameter length over that of time 0 (100%). Six spheroids for each point were considered.

**In Vivo Studies.**

**Animals.** All experiments were carried out using female athymic nude CD-1 mice, 8 to 11 weeks old (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethic Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy), according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (Workman et al., 1998).

**Antitumor and Antimetastatic Activities.** The effects of NiK-12192, topotecan, or combination of them on the growth of primary tumors and spontaneous lung metastases were tested in mice inoculated s.c. in the right flank with H460 ascitic tumor cells (2 × 10⁶/mouse). Each drug or control-treated group included 9 to 11 mice. The growth of the s.c. tumors was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor weight (TW) and TW inhibition percentage in treated over control mice were calculated as previously reported (Cassinelli et al., 2006). Drug treatment was delivered p.o., by gavage, daily for 9 weeks (weekends excluded), starting the day after cell injection (day 1). NiK-12192 was delivered at the dose of 30 mg/kg, topotecan at the dose of 1 mg/kg. In the mice treated with the combination, NiK-12192 was delivered approximately 1 h after topotecan. At day 63, tumor-bearing mice were sacrificed by cervical dislocation, and their lungs were removed. Lung lobes were split between two glass slides, and the metastatic nodules were macroscopically counted against a bright light. Reading of metastases was performed by two independent observers, unaware of the experimental group, with an interobserver reproducibility >95%. The metastatic nature of these areas was confirmed by histological analysis.

To induce experimental lung metastases, mice (seven to eight per group) were injected i.v. with ascitic H460 cells (2 × 10⁶/mouse) (Cassinelli et al., 2006) and then treated p.o. daily with NiK-12192 (30 mg/kg), and/or topotecan (1 mg/kg) for 7 weeks (weekends excluded), from day 1. At day 83, all mice were sacrificed for lung observation as described above. In the presence of confluent metastatic nodules, lungs were defined as “tumor invaded,” and tumor nodules were not counted. The number of 500 metastases was ascribed. For ethical reasons, mice presenting signs of suffering were sacrificed before day 83.

For histological observation, s.c. implanted tumor-bearing mice (three to four mice per group) were treated with NiK-12192 (30 mg/kg) alone or in combination with topotecan (1 mg/kg), daily for 2 weeks, starting at day 1. The day after the last treatment, mice were sacrificed, and their tumors were excised and fixed in 10% buffered formalin for hematoxylin-eosin staining. Angiogenesis was analyzed by detection of microvessel density (MVD). Number of blood vessels was assessed in three fields, at 400×, within non-necrotic areas. Only vascular units containing erythrocytes were considered (Belotti et al., 1996).

**Statistical Analysis.** The Student’s t test was used to compare TW in control and treated mice. The number of metastases and microvessels in control and treated mice was compared by the Mann-Whitney test. All analyses were performed using GraphPad Prism software (GraphPad Prism Inc., San Diego, CA).

**Results**

**Modulation of Intracellular pH.** NiK-12192 was able to decrease the intracellular pH, as assessed by means of the pH-dependent shift of the SNARF-4F emission band through the microspectrofluorometric analysis. Indeed, as shown in Fig. 1, in NiK-12192-treated cells, the pH probe exhibited a shift to shorter wavelengths consistent with a more acidic pH environment with respect to the control cells. The emission peak position of control cells was constant at approximately 650 nm, whereas those of the cells treated for 24 h with NiK-12192 (2 μM) were always comprised between 640 and 630 nm. The estimation of the intracellular pH value was performed exclusively on the base of the blue shift of the SNARF-4F longer emission band and indicated a decrease from approximately 7.2 for the control to a pH range of approximately 6.2 for NiK-12192-treated cells. The increase in the emission band centered at approximately 560 nm was not considered because it can be due both to the conversion of SNARF-4F emission at acidic pH and to a shoulder of the NiK-12192 emission. Although the level of vacuolar H⁺-ATPase expression was not modified by treatment with the compound (Fig. 2A), NiK-12192 produced changes in intracellular localization (Fig. 2B). In control samples, vacuolar H⁺-ATPase localized in the cytoplasm and was evident as spots in most cells. Treatment with the inhibitor caused a more homogeneous and diffuse distribution of the enzyme.

**Migration, Invasion, and Wound Healing.** The effect of NiK-12192 on H460 cell migration and invasion was investigated after 24 h of drug exposure to concentrations not affecting cell adhesion (Fig. 3). All cells (control or NiK-12192-treated) were typyran blue negative. Under these conditions, NiK-12192 caused a concentration-dependent inhibition of cell migration and invasion.

To further support the ability of NiK-12192 to modulate cell migration, we examined the drug effect in wound healing, because this assay provides information on oriented mi-
migration involving cell polarization. As shown in Fig. 4, during 48-h incubation, control cells were able to repair approximately 50% of the wound. NiK-12192, at a low subtoxic concentration (0.5 μM), caused a reduction in the wound healing.

**Modulation of Factors Related to Cell-Cell Interactions.** Because the αvβ3 integrin is highly expressed in lung carcinoma cells (Albert et al., 2006) and associations between integrins and vacuolar H⁺-ATPase have been already reported (Lee et al., 2004), the intracellular localization of αvβ3 was investigated in H460 cells after treatment. As shown in Fig. 5, in untreated cells, the integrin was found mainly polarized in the cell cytoplasm, whereas in treated cells, αvβ3 was more homogeneously diffused in the cytoplasm, thus suggesting a different behavior of treated cells in cell-cell and cell-matrix interactions.

The effect of the vacuolar H⁺-ATPase inhibitor on proteins of cytoskeleton was examined by confocal microscopy (Fig. 6). In this analysis, actin was evidenced as green filaments and tubulin as red filaments. In control cells, long and regular fibers of tubulin in the cell cytoplasm and filaments of actin forming pseudopodia were found. Marked modifications were found in H460 cells exposed to NiK-12192 (2 μM) for 24 h. In treated cells, actin fibers were broken, and spots of aggregation were evident. Moreover, no pseudopodia and lack of regular structure for actin filaments could be observed.

The drug ability to interfere with three-dimensional growth of H460 cells was examined on agar substratum because this effect may have relevance in the metastatic growth. Spheroids with a diameter of 100 to 150 μm were treated with subtoxic concentrations of the drug, and their size was then determined at various exposure times (Fig. 7). In spheroids treated with NiK-12192, a concentration-dependent inhibition growth was found already at 24 h, also with the low concentration (0.5 μM).

**Antitumor and Antimetastatic Activity of NiK-12192.** To determine whether the inhibitory activity on invasion/
The results presented in the study indicated that the vacuolar H+-ATPase inhibitor NiK-12192 was able to reduce the migration/invasion of H460 cells in vitro and significantly reduce the number of spontaneous metastases in the lung of nude mice s.c. implanted with a human lung carcinoma. The ability of our model to produce spontaneous metastases, which is a unique feature among s.c. implanted human tumor xenografts, is of particular relevance, because it may represent a behavior more closely related to the clinical dissemination of tumor cells. It has been shown that some steps of the complex process leading to the formation of spontaneous metastases are dependent on extra and intracellular pH, including the secretion of acid-dependent proteases and the local degradation of basement membranes and extracellular matrix (Rozbin et al., 1994; Martinez-Zaguilan et al., 1996; Stock et al., 2005; Roftstad et al., 2006). Indeed, the acidic pH of extracellular microenvironment of solid tumors has been shown to be a critical factor in stimulating tumor cells motility, invasion, and angiogenesis (Harguindey et al., 2005).

Despite the significant inhibition of the metastatic potential of s.c. growing H460 tumor, NiK-12192 exhibited a marginal effect on the development of artificial metastases (i.e., after i.v. injection of the same tumor cells). The poor effect shown by NiK-12192 against artificial lung metastases and primary tumor supports the hypothesis that the compound was able to inhibit the early steps of the metastatic process, rather than the tumor cell growth in the target organ.

We have previously shown, either in cellular and in vivo systems, that NiK-12192 is able to potentiate topotecan efficacy against human colon carcinomas, possibly by a cytoplasmic redistribution of the cytotoxic drug and by modulating the expression of membrane-associated transport systems (Petrangolini et al., 2006). In the present study, we showed that the combination topotecan + NiK-12192 resulted in a significant reduction of spontaneous metastasis incidence over that achieved by each single drug. The mechanisms of antimetastatic effects of NiK-12192 and topotecan are substantially different. The effects of topotecan were probably related to its cytotoxic activity because both spontaneous and artificial metastases were inhibited by the topotecan treat-
however, the antimetastatic effect of topotecan could reflect multiple events including a direct tumor growth inhibition and an early antiangiogenic effect, which could account for the topotecan efficacy in reducing both spontaneous and artificial metastases. The antiangiogenic effects of topotecan in primary tumor could at least in part account for the synergistic inhibition of spontaneous metastasis by the topotecan + NiK-12192 combination. The angiogenic effects of camptothecins have been already described and are more evident when the drugs are delivered by the daily low-dose schedule (Petrangolini et al., 2003). The effect of NiK-12192 on microvessel density, which may be expected on the basis of the knowledge that a significant number of angiogenic factors acts closely related to intracellular H⁺ dynamics (Orive et al., 2003), was marginal compared with that achieved by the cytotoxic drug, suggesting that the antiangiogenic effect is not a primary mechanism of antimetastatic activity.

In the previous study, an increased toxicity was achieved by the combination topotecan + NiK-12192, when the topotecan maximum tolerated dose (every 4th day for three times) was delivered (Petrangolini et al., 2006). Thus, a different regimen was investigated in the present study, i.e., a daily low-dose for long time. Such regimen was well tolerated, and, despite the prolonged time of treatment (up to 9 weeks), no signs of toxicity (nor lethal toxicity, nor body weight loss) were observed in mice treated with topotecan + NiK-12192, thus indicating that the toxicity of the combination can be modulated in this regimen.

The ability of NiK-12192 to inhibit vacuolar H⁺-ATPase activity appears to influence a number of cellular functions, which may be relevant to the observed antimetastatic efficacy in the in vivo studies. Indeed, the results achieved in the in vitro assays support the ability of NiK-12192 to modulate the intracellular pH and to inhibit a series of metastasis-associated events. Our results indicated that in H460 cells, NiK-12192 was able to cause cytoskeleton modifications, including aggregation of actin and tubulin microfilament as well as reduction of cell pseudopodia. In addition, it was able to modulate the subcellular distribution of adhesion molecules such as αvβ3 integrin. The reduced cell motility was reflected in the inhibition of cell migration, invasion, and likely in wound healing. Several lines of evidence have indicated a relation among cellular functions involving transport systems, cytoskeleton, and adhesion molecules (Vitavska et al., 2003; Zuo et al., 2006). These functions, which may be modulated by intracellular and extracellular pH, play a critical role in cellular interactions with the extracellular matrix. A lot of evidences suggested important interactions among vacuolar H⁺-ATPase and cytoskeleton; the aminoterminal domain of the vacuolar H⁺-ATPase subunit B has been
already demonstrated to contain a filamentous actin binding site (Holliday et al., 2000; Zuo et al., 2006), and the vacuolar H^+\textsubscript{-}ATPase subunit C can bind both F- and G-actin (Vitavska et al., 2005) by controlling the dynamics of the actin cytoskeleton. A modification of the cytoskeleton has been in fact observed in our model after a 24-h treatment with NiK-12192, resulting in a lack of regular structure for actin filaments together with the absence of pseudopodia.

Vacuolar H\textsuperscript{+}-ATPases are implicated in multiple diseases, including osteoporosis, deafness, and cancer, and their role as drug target is under study (Bowman and Bowman, 2005). A recent study reported the efficacy of another vacuolar H\textsuperscript{+}-ATPase inhibitor, FR202126, in reducing osteolysis induced by a metastatic breast cancer in a syngenic mouse model. However, the compound was unable to prevent lung or liver metastasis (Niikura, 2007). It is unclear whether the drug specificity for various vacuolar H\textsuperscript{+}-ATPase isoforms expressed in different cells (including osteoclasts) may be responsible for the different effects of these inhibitors. The ability of the natural macrolide vacuolar H\textsuperscript{+}-ATPase inhibitor bafilomycin A1 to decrease in vitro both invasion and migration of human breast metastatic cells (Sennoune et al., 2004a) strongly supports the key role of vacuolar H\textsuperscript{+}-ATPase in these two fundamental processes of tumor malignancy. The implication of the enzyme in modulation of the metastatic phenotype is supported by the observation that cells with a low metastatic potential are characterized by a widespread distribution of the enzyme in cytoplasm. Relevant to this point is the NiK-12192-induced modulation of intracellular distribution of the enzyme.

In conclusion, we have shown for the first time that a vacuolar H\textsuperscript{+}-ATPase inhibitor, NiK-12192, has a strong antimetastatic effect in a preclinical model of spontaneous lung metastases. Moreover, the effect was further increased by the

### TABLE 1

<table>
<thead>
<tr>
<th>Drug\textsuperscript{a}</th>
<th>Weeks of Treatment\textsuperscript{b}</th>
<th>Animals with Metastases/Total No. of Animals</th>
<th>No. of Spontaneous Metastases\textsuperscript{c}</th>
<th>No. of Artificial Metastases\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>9</td>
<td>11/11</td>
<td>26</td>
<td>338</td>
</tr>
<tr>
<td>NiK-12192</td>
<td>7</td>
<td>6/6</td>
<td>15\textsuperscript{***}</td>
<td>300</td>
</tr>
<tr>
<td>Topotecan</td>
<td>9</td>
<td>10/10</td>
<td>11\textsuperscript{***}</td>
<td>42</td>
</tr>
<tr>
<td>Topotecan + NiK-12192</td>
<td>7</td>
<td>7/7</td>
<td>5\textsuperscript{***}</td>
<td>81</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Topotecan and NiK-12192 were delivered p.o. by gavage. When combined, topotecan was delivered about 1 h before NiK-12192.

\textsuperscript{b} Drugs were administered daily five times a week.

\textsuperscript{c} Counted in the lungs when mice were sacrificed, i.e. day 63 for spontaneous metastases and day 83 for artificial metastases.

\textsuperscript{d} Inhibition percentage compared with solvent-treated mice.

\textsuperscript{e} One lung lobe totally invaded was calculated as 100 metastases. Totally invaded lungs are reported as 500.

\textsuperscript{f} \textit{P}<0.05 vs. single-agent-treated mice, by Mann-Whitney rank test.

\textsuperscript{g} \textit{P}<0.05 vs. control.

\textsuperscript{h} \textit{P}<0.02 vs. control.

\textsuperscript{i} \textit{P}<0.01 vs. control.

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**Fig. 8.** Effect of NiK-12192 on the growth of primary H460 tumor. Mice were implanted s.c. with H460 lung cancer cells (2 × 10\textsuperscript{6} cells/mouse) and treated p.o. with solvent (\(\times\)), 1 mg/kg topotecan (TPT) (\(\bullet\)), 30 mg/kg NiK-12192 (\(\square\)), or TPT + NiK-12192 (\(\diamondsuit\)). Treatments were delivered daily from day 1, 5 days/week, for 9 weeks. TPT was delivered approximately 1 h before NiK-12192. Means of 9 to 11 tumors/group are reported.

**Fig. 9.** MVD in H460 tumor xenografts. Mice implanted s.c. with H460 lung cancer cells (2 × 10\textsuperscript{6} cells/mouse) were treated p.o. with solvent (control) or TPT (1 mg/kg) or NiK-12192 (30 mg/kg) or TPT + NiK-12192. Treatments were delivered daily, from day 1, for 2 weeks. Topotecan was delivered approximately 1 h before NiK-12192. The day after the last treatment, tumors were excised and fixed in 10% buffered formalin for hematoxylin-eosin staining. MVD was assessed in three fields, at 400×, within non-necrotic areas. Means of three to four tumors/group (S.D.) are reported. *\textit{P}<0.01; **\textit{P}<0.005 versus control.
combination with a cytotoxic drug, thus supporting a potential interest for vascular H+-ATPase inhibitors in the therapy of cancer.

References


