Targeting Aurora Kinases with Danusertib (PHA-739358) Inhibits Growth of Liver Metastases from Gastroenteropancreatic Neuroendocrine Tumors in an Orthotopic Xenograft Model

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Abstract

Purpose: Aurora kinases play a crucial role in cell-cycle control. Uncontrolled expression of aurora kinases causes aneuploidy and tumor growth. As conservative treatment options for advanced gastroenteropancreatic neuroendocrine tumors (GEP-NET) are disappointing, aurora kinases may be an interesting target for novel therapeutic strategies.

Experimental Design: Human GEP-NETs were tested for aurora kinase expression. The efficacy of the new aurora kinase inhibitor danusertib was evaluated in two human GEP-NET cell lines (BON1 and QGP) in vitro and in vivo.

Results: The majority of ten insulinomas and all 33 nonfunctional pancreatic or midgut GEP-NETs expressed aurora A despite a mostly high degree of cell differentiation. Both human GEP-NET cell lines expressed aurora kinase A and B, and high Ser10 phosphorylation of histone H3 revealed increased aurora B activity. Remarkably, danusertib led to cell-cycle arrest and completely inhibited cell proliferation of the GEP-NET cells in vitro. Decreased phosphorylation of histone H3 indicated effective aurora B inhibition. In a subcutaneous murine xenograft model, danusertib significantly reduced tumor growth in vivo compared with controls or mice treated with streptozotocine/5-fluorouracil. As a consequence, decreased levels of tumor marker chromogranin A were found in mouse serum samples. In a newly developed orthotopic model for GEP-NET liver metastases by intrasplenic tumor cell transplantation, dynamic MRI proved significant growth inhibition of BON1- and QGP-derived liver metastases.

Conclusions: These results show that danusertib may impose a new therapeutic strategy for aurora kinase expressing metastasized GEP-NETs. Clin Cancer Res; 18(17); 4621–32. ©2012 AACR.

Introduction

The incidence of neuroendocrine tumors of the gastroenteropancreatic system (GEP-NET) is increasing and more than 80% of patients are diagnosed in advanced stages, including the presence of liver metastases (1). Despite recent therapeutic improvements such as biotherapeutic regimens, targeted radiotherapy, and locally ablative treatments (2–7), definitive cure in metastatic disease remains a rare event. Results from recent phase III studies using the mTOR inhibitor everolimus or the multitargeted tyrosine kinase inhibitor sunitinib have shown for the first time that molecular targeted therapy can successfully be applied in metastatic GEP-NETs (8). However, the clinical benefits attained in these studies were only moderate. Thus, novel therapeutic approaches for advanced stages of disease are clearly required.

Danusertib (formerly PHA-739358) is a small molecule, pan-Aurora kinase inhibitor (AKI; ref. 9). Aurora kinases are key regulators of protein phosphorylation during mitosis (10). These serin/threonine kinases interact with other proteins to control chromosome assembly and segregation. The aurora family of kinases, which consists of 3 members, aurora A, B, and C, differs in subcellular localization, kinetics of activation and function. Aurora kinases are frequently overexpressed in hematologic malignancies and solid tumors (10, 11). Therefore, several AKIs have been developed and showed variable efficacy in different preclinical models (12, 13). The most advanced clinical
compound is danusertib, a highly effective pan-AKI that inhibits aurora kinases A, B, and C at nanomolar concentrations (9). Off-target effects of danusertib include inhibition of receptor tyrosine kinases such as Abl, Ret, FGFR-1, and TrkA (9, 14). Phase II clinical trials for patients with hematologic malignancies and solid tumors are currently being carried out with this inhibitor.

The impact of aurora kinases for the tumorigenesis of GEP-NETs is largely unknown. First promising results were yielded by ZM447439, a selective aurora B kinase inhibitor used in vitro at high concentrations (15). To further elucidate the applicability of aurora kinases in GEP-NETs as a potential target for anticancer therapy, we examined aurora A expression in a series of GEP-NET tissue samples. The potential impact of aurora kinase inhibition in the treatment of GEP-NETs was evaluated using the novel small-molecule AKI danusertib. Its antiproliferative features and possible therapeutic mechanisms were examined in vitro and in a subcutaneous xenograft model. On the basis of the positive results, we developed a novel animal model for stable generation of GEP-NET liver metastases and could show in vivo efficacy of danusertib in this orthotopic tumor model.

Materials and Methods

Materials

Antibodies were for Ki-67, chromogranin A, (CgA; Dako), liver sinusoidal endothelial cells (LSEC; Miltenyi), CD31 (BD Biosciences), aurora-A, aurora-B (Abcam), phosphorylated histone H3 (Ser10), PARP (Cell Signaling Technology), and p21, p27, p53, proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Inc.). Danusertib was provided by Nerviano Medical Sciences and dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration for all in vitro assays was 0.1%. For in vivo experiments, an in situ salt was mixed as described (16).

Immunohistochemistry of human GEP-NET

Formalin-fixed, paraffin-embedded tissue samples of surgically resected human insulinomas, nonfunctional pancreatic NETs, and midgut NETs from small intestine were pretreated, immunostained, and analyzed as described (12, 16).

Human GEP-NET cells and culture

Serotonin secreting human pancreatic carcinoid cell line BON1 and human nonfunctioning pancreatic islet cell tumor line QGP (17) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37°C in a 5% CO2 atmosphere.

Cell analysis, immunostaining, and flow cytometry

Cells were seeded in DMEM, fixed and immunostained for aurora-A, aurora-B, and histone H3 phosphorylation, as described (12). For analysis of cell proliferation, increasing concentrations of danusertib (dissolvent control; 5 nmol/L–5 μmol/L) were added after 24 hours of cell culture. Forty-eight to 120 hours later, the number of viable cells was determined (12). Analysis of DNA content and H3 phosphorylation were carried out by flow cytometry as described (12, 13, 18).

Western blot

Tumors resulting after subcutaneous transplantation of BON1 and QGP cells were shredded in Tissue Protein Extraction Reagent (Pierce Biotechnology, Inc.) followed by homogenization with 3 × 5 s sonication. Total proteins from treated cells and untreated controls were extracted as described (12). Nuclear extracts were prepared by sequential cell lysis using the nondenaturing detergent Igepal (Sigma), followed by centrifugation and lysis of the nuclei-containing pellet with a hypertonic buffer containing 0.4 mol/L NaCl. A total of 30 μg protein extract was loaded on SDS-PAGE (12% gel) and electrotransferred. Immunodetection was carried out as described (19), using the following antibodies: PARP, phospho-AKT, phospho-JNK, p53, p21, p27, Actin, p-Erk.

Animals

The Special Animal Core of the Hamburg University Medical Center provided 6- to 10-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. All animal experiments were approved by the local authorities in accordance with the NIH Guide for Care and Use of Laboratory animals.

Subcutaneous tumor xenografts

To generate subcutaneous tumors, 5 × 10⁶ BON1 or QGP cells in 0.1 mL DMEM containing 2% FCS were mixed with extracellular matrix gel (12) and injected into flanks of...
NOD/SCID mice. Treatment was initiated when tumor volumes reached median sizes of 22 to 126 mm³. Determination of tumor size, assignment of mice to treatment groups, termination criteria, and tumor sampling has been described previously (12).

Orthotopic model of GEP-NET liver metastases

To generate GEP-NET liver metastases, the spleen was exposed by a small flank laparotomy. A total of 0.5 x 10⁶ BON1 or QGP cells were injected in 0.2 mL DMEM containing 2% FCS using 27-gauge needles. Hemostasis was secured by ligature of the splenic pole. Splenectomy was carried out 10 minutes following cell injection to prevent growth of tumor cells inside the spleen. In pilot experiments for biodistribution of tumor cells, direct cell transplantation into the portal vein was also used. MRI of transplanted mice was carried out weekly to detect liver metastases following tumor cell transplantation.

Identification of human cells in mice

To identify transplanted GEP-NET cells in organs, liver lobes, lungs, kidneys, heart, and intestine were collected 2 hours after cell injection and DNA-PCR for human-specific centromere regions was done (20). Transplanted human cells were identified with in situ hybridization (digoxigenin-labeled pancentromeric probe) as described previously (20), with the following modification: to visualize hybridization signals, sections were incubated with fluorescein isothiocyanate-conjugated anti-digoxigenin Fab fragments for 1 hour at room temperature. Immunohistochemistry of xenograft tumors was carried out as described previously (12), with the following primary antibodies: aurora-A and aurora-B (1:250), CgA (1:400), Ki-67 (1:50), PCNA (1:500), CD31 (1:50), and LSEC (1:500).

MRI

MR measurements of xenografted mice were carried out on 4-day intervals for volumetric analysis. Mice were imaged with a 3 Tesla Scanner (Intera, Philips Medical Systems) using a custom-made small solenoid animal coil (Philips Research Laboratories). We applied a T2-weighted 2D turbo spin echo sequence [TR/TE 3820/98 ms, 30 x 30 mm field of view (FoV), matrix 144 x 140, 20 slices, slice thickness 1.0 mm, effective voxel volume (0.20 mm³), acquisition time 2:32 minutes], in transversal adjustment. During MRI procedures, animals were anesthetized with Ketamine/Xylazin. Volumetric quantification of liver metastases was carried out by computer-aided software (Image 1.37; NIH).

Experimental protocol

Mice bearing BON1 or QGP tumors were divided into a treatment group receiving danusertib intraperitoneally at a dose of 2 x 15 mg/kg/d or a control group receiving the same volume of vehicle solution (5% dextrose). Streptozotocine and 5-fluorouracil were applied intraperitoneally at a dose of 1 x 15 mg/kg/d.

Chromogranin A serum levels

For CgA serum analysis, blood was collected at the end of each BON-1 experiment. CgA serum levels were measured with an ELISA (Maier Analytik) according to the manufacturer's instructions.

Statistical methods

Where applicable (see below), significance of differences was analyzed by χ² tests, Fisher exact test, or 2-tailed Student t tests. P values less than 0.05 were considered significant.

Results

Expression of aurora kinases and phospho-H3S10

A total of 43 human GEP-NET samples were available for analysis of aurora kinase expression (10 insulinomas, 13 nonfunctional pancreatic NETs, and 20 midgut NETs from small intestine), most of which were grade 1 and 2 (Table 1). Aurora-A was expressed in 8 of 10 insulinomas, and in all nonfunctional pancreatic and midgut NETs (Fig. 1A, Table 1), whereas surrounding nonmalignant tissue and controls (normal pancreas) were negative (data not shown). Aurora-B was not expressed in insulinomas, whereas 2 of 13 nonfunctional pancreatic NETs (1x G1, 1x G2) and 3 of 20 midgut NETs (2x G1, 1x G2) expressed aurora B (Fig. 1B, Table 1). Four of 5 aurora-B–positive GEP-NETs also expressed downstream target histone H3, which is phosphorylated at Ser10 (phospho-H3S10) by aurora-B during mitosis (Table 1).

Serotonin secreting human pancreatic carcinoid cell line BON1 and human nonfunctioning pancreatic neuroendocrine tumor line QGP were tested positive for aurora-A and aurora-B by Western blot (Fig. 1C). Differential cell lysis revealed primarily nuclear localization. The overall expression of both aurora kinases was higher in BON1 compared with QGP cells, which is in line with higher expression of the proliferation marker PCNA (Fig. 1C). Aurora kinase expression was confirmed in subcutaneous tumors after xenotransplantation of both human cell lines into immunodeficient mice (Fig. 1D and E).

Phospho-H3S10 was expressed in both GEP-NET cell lines: quantitative analysis of phospho-H3S10 by flow cytometry revealed a cell fraction of 1.60 ± 0.04% in BON1 and 1.49 ± 0.04% in QGP cells, indicating aurora-B activity in both human GEP-NET cell lines. Phospho-H3S10 expression was also confirmed by immunostaining of subcutaneous xenograft tumors (Fig. 1F).

Inhibition of tumor cell proliferation by danusertib

Cell counting analysis with trypan blue exclusion assay using different concentrations of the compound showed that danusertib, starting from 50 nmol/L, led to a complete stop of GEP-NET cell proliferation in BON1 cells and to a decrease of cell numbers in QGP cells (Fig. 2A and B; P < 0.001 for both cell lines at 96 hours). Flow cytometric cell-cycle analysis revealed a dose-dependent effect of danusertib (Fig. 2C). Significant reduction of cells in S-phase started from 50 nmol/L, leading to endoreduplication of cells with
a substantial increase of the DNA content (4N and >4N fractions) and to a complete block of mitosis with loss of the 2N fraction, at higher concentrations (Fig. 2C). Phosphorylation of the aurora-B downstream target H3S10 was effectively inhibited in both GEP-NET cell lines following treatment with danusertib (Fig. 2D for BON1). However, as observed in other cell lines (12), a significant inhibition of H3S10 phosphorylation started at concentrations 10-fold higher than the dose inhibiting proliferation (phospho-H3S10 cell fraction at 500 nmol/L: BON1: 0.05% vs. 1.63% in controls, \( P < 0.01; n = 3 \); QGP: 0.03% vs. 1.49%, \( P < 0.01; n = 3 \)). Western blot of cell lysates treated for 48 hours with danusertib at various concentrations showed an increase of PARP-1 cleavage upon treatment, indicating induction of apoptosis (Fig 2E). Maximal PARP-1 cleavage was observed at 50 nmol/L in both cell lines, whereas higher doses resulted in a gradual decrease down to baseline at the highest dose of 5 \( \mu \)mol/L. There was no apparent effect of the treatment on either p53 accumulation or p21 expression; similarly, we did not observe an increase in the G1 transition cell-cycle regulator p27 (data not shown). To rule out any off-target effects of danusertib on other mitogenic signaling pathways, the activation of PI3K/Akt and mitogen-activated protein kinase pathways was analyzed. However, danusertib did not affect basal activities or FCS-induced activation of Akt, Erk, and JNK in both cell lines (data not shown).

Danusertib treatment inhibits growth of subcutaneous GEP-NET xenografts and lowers serum chromogranin levels

Treatment of tumor-bearing mice with danusertib at a dose of 2 \( \times \) 15 mg/kg/d decreased growth of BON1 and QGP.

Table 1. Expression of aurora kinases and phospho-H3S10 in human GEP-NETs

<table>
<thead>
<tr>
<th>Localization</th>
<th>Grading</th>
<th>Ki-67 (%)</th>
<th>Aurora A</th>
<th>Aurora B</th>
<th>Phospho-H3S10</th>
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Abbreviation: n.a., not available
Danusertib in GEP-NETs

Figure 1. Aurora kinase expression in human GEP-NET tissues and cells. A and B, immunohistochemistry of a G1 midgut NET with nuclear and cytoplasmatic aurora-A expression (A) and aurora-B expression in 2 nuclei (B, arrows), one of which shows a mitotic figure. C, Western blot after differential cell lysis with aurora-A and aurora-B expression in nuclei and cytoplasms of human GEP-NET cell lines BON1 and QGP. D and E, immunohistochemistry of subcutaneous BON1 xenograft tumor with expression of aurora A (D) and aurora B (E) in vivo. F, positive immunostaining for pH3S10 indicating aurora-B activity in BON1 xenograft tumors. Magnification: A, B, D, E × 400; F × 200.

xenografts (Fig. 3A and B). BON1 tumor growth was significantly inhibited from day 4 \((P < 0.001)\) until the end of the experiment \((P < 0.001)\). The mean absolute tumor volume was reduced by 88.2%, and the mean percental tumor growth was also reduced by 88.2%, compared with vehicle-treated controls (Fig. 3A, Table 2). In mice with QGP xenografts, treatment with danusertib led to a virtual shrinkage of QGP tumors from day 4 \((P < 0.001)\), until the end of the experiment \((P < 0.001)\). The final tumor volume was only 6.3 ± 8.2% of the original tumor volume (Fig. 3B, Table 2).

Mean absolute and percental tumor growth in danusertib-treated mice was reduced by 98.4% and 98.6%, respectively, compared with vehicle-treated controls. When compared with treatment with streptozotocine/5-fluoruracil (STZ/5-FU), which is a frequently used cytostatic therapy for GEP-NETs, the antiproliferative effect of danusertib was significantly higher from day 12 \((P < 0.001)\), in both BON1 and QGP tumors (Fig. 3A and B, Table 2).

In serum analyses of BON1 tumor-bearing mice, we detected elevated levels of the clinically applied GEP-NET tumor marker CgA. The average serum level was 23 ± 15 nmol/L after 2 weeks (untransplanted controls <4 nmol/L) and rose to 47.3 ± 22.7 nmol/L in untreated animals at the end of experiments \((n = 16; P < 0.001)\). CgA levels were significantly lower in danusertib-treated animals \((12.0 ± 12.4 \text{ nmol/L})\) compared with vehicle-treated controls \((P < 0.001, n = 9)\) and mice treated with STZ/5-FU \((32.0 ± 12.9 \text{ nmol/L}, P < 0.05, n = 6; \text{Fig. 3C})\). Despite low-level expression of CgA in QGP cells by Western blotting, we were not able to detect CgA in the serum of QGP tumor-bearing mice.

Biodistribution of GEP-NET cells in intact animals for generation of an orthotopic model of liver metastases

On the basis of successful inhibition of tumor growth in subcutaneous xenografts, we next aimed to generate an orthotopic in vivo model to evaluate the effects of danusertib on GEP-NET liver metastases. To identify differences in organ-specific targeting, we injected BON1 or QGP cells into the portal vein or the spleen, followed by sequential splenectomy in NOD-SCID mice. Both injection routes led to rapid translocation of cells into the liver within 2 hours, as verified by human-specific CMT1 PCR. A faint PCR signal was also found in the lungs and the intestine, whereas the human sequence was absent in kidneys and heart (Fig. 4A).

After 4 weeks, multiple tumor nodules were detected in the liver and a massive hepatomegaly had occurred (Fig. 4B). To visualize GEP-NET cells in tissues, we used FISH for human-specific sequences, which verified that tumor nodules in the liver comprised cells of human origin (Fig. 4C). By in situ hybridization, we found no evidence of transplanted human tumor cells in other organs, including the lungs. Histology of livers showed multiple tumors composed of small cells with a necrotic area in the center of the nodule (Fig. 4D).

Characterization of liver metastases from GEP-NET cells

BON1 liver metastases showed strong expression of CgA by immunohistochemistry (Fig. 5A), indicating that cells retained their in vitro characteristics of neuroendocrine differentiation. BON1 cells showed extensive proliferation in vivo, as shown by immunohistochemistry for Ki-67 and PCNA (Fig. 5B and C). Tumors derived from nonfunctioning pancreatic islet cell tumor–derived QGP cells were negative for CgA, but liver metastases from these cells retained typical NET morphology; for example, small cells with a high nucleus/cytoplasm ratio (Fig. 5D). Human origin of these metastases was verified by in situ hybridization (Fig. 5E). Although QGP cells proliferated less extensively, the proliferative index...
in both cell lines was more than 20% in metastases in all animals (data not shown). Capillary vessels in human GEP-NET metastases were of murine origin, as verified by immunohistochemistry using mouse-specific antibodies for CD31 or liver sinusoidal endothelial cells, indicating tumor angiogenesis from the host liver (Fig. 5F).

Treatment of GEP-NET liver metastases with danusertib
To identify the time course of metastatic tumor growth, we carried out MRI of recipient mice and tumor volumetry. Two to 3 weeks after cell transplantation, small metastatic tumor nodules (diameter 100–120 µm) in the liver became detectable with a metastatic tumor take rate of approximately 80% for both transplanted cell lines. BON1 metastases grew more diffusely from small originating tumor nodules, whereas QGP metastases showed a different growth pattern dominated by singular tumor nodules (Fig. 6A and B).

In transplanted mice, treatment with danusertib or vehicle was commenced when liver metastases became clearly detectable and were eligible for volumetric analysis, which was after 2 to 3 weeks following cell transplantation. Average size of BON1 metastases (single nodules) before treatment was 2.9 ± 1.3 mm³ (n = 20). Although liver metastases of BON1 tumors in vehicle-treated controls continued to grow until large areas of the liver were replaced by tumor tissue, treatment with danusertib significantly inhibited growth of liver metastases (day 12 after start of treatment: 55.9 ± 39.7 mm³; n = 10 vs. 5.2 ± 3.8 mm³; n = 10; P < 0.01; Fig. 6A and C, Table 3). This corresponds to a relative tumor volume compared with day 0 (100%) of 1,495.3 ± 1,277.9% in controls versus 164.5 ± 50.2% in danusertib-treated mice (P<0.01). Mean absolute and percental tumor
growth in danusertib-treated mice was reduced by 90.7% and 89.0%, respectively, compared with vehicle-treated controls. Average size of QGP metastases in the liver before treatment was $6.8 \pm 3.7 \text{mm}^3$ ($n = 9$). Danusertib entirely inhibited growth of QGP metastases, although no shrinkage was observed, as in subcutaneous tumors (control: day 12, $51.7 \pm 35.0 \text{mm}^3$; $n = 4$ vs. danusertib $6.1 \pm 4.3 \text{mm}^3$; $n = 5$, $P < 0.05$; Fig. 6B and C, Table 3). This corresponds to a relative tumor volume of $810.7 \pm 355.4\%$ versus $108.7 \pm 58.9\%$, $P < 0.01$. The mean absolute tumor volume was reduced by 88.2%, and the mean per cental tumor growth was reduced by 86.6%, compared with vehicle-treated controls.

**Discussion**

Metastatic GEP-NETs are currently managed with standard chemotherapies, such as STZ/5-FU for differentiated tumors of pancreatic origin and platinum-based protocols for undifferentiated GEP-NETs with poor efficacy. Although 2 recent landmark studies have shown, for the first time, that targeted therapy is effective in GEP-NETs (21, 22), the overall effects remain moderate and non-response to the substances emphasizes the demand for alternative strategies.

Overexpression of aurora kinases has been shown in many tumors. Thus, the inhibition of aurora kinases moved into the focus of anticancer therapy (23–26). Because their role has never been assessed in GEP-NETs, we first screened expression in human insulinomas, nonfunctional pancreatic NETs and midgut NETs. Although aurora-A was highly abundant in these tumors, aurora-B expression was enhanced in only 5 cases. In contrast, in both human GEP-NET cell lines, with high proliferative indices in xenograft models, both aurora A and aurora B were expressed. This may be because of the differentiation of human GEP-NETs analyzed and may not reflect the situation in higher proliferative metastatic tumors. This also points out limitations of existing models as all available human GEP-NET cell lines have high proliferative indices. Alteration of aurora-B controlled cellular mechanisms leads to aneuploidy, one of the main features and driving forces of cancer progression. Furthermore, overexpression of aurora-B has been observed in several tumor types and has been linked with poor prognosis of cancer patients (25). Only one of our 43 GEP-NETs was a highly proliferative grade 3 tumor. Although this tumor was indeed positive for aurora-B and histone H3, the other 4 NETs with these properties were grade 1 or 2. Therefore, whether aurora-B expression is also related to malignant progression of GEP-NETs into higher proliferative tumors needs to be investigated with a larger number of clinical tumor specimens of various grades and proliferative indices. On the other hand, we detected a strong antiproliferative effect of danusertib already at low concentrations, whereas aurora-B inhibition—revealed by decreased phosphorylation of histone H3—only appeared at higher concentrations. Therefore, the effect of cell-cycle inhibition and endoreduplication at low concentrations without measurably affecting histone H3 most likely results from successful aurora-A inhibition with aurora-A–regulated spindle apparatus being the primary target. This effect has recently been observed in hepatocellular carcinoma models and is in accordance with quantitative kinase inhibition assays showing higher sensitivity of aurora-A for danusertib (12, 27).

Recently, Georgieva and colleagues reported first promising *in vitro* results using high doses of aurora-B kinase inhibitor ZM447439 (15). To our knowledge, no clinical trial activity is being pursued with this compound. In...
contrast, pan-AKI danusertib is in advanced clinical development. Thus, a phase I clinical trial evaluating danusertib in 50 patients with metastatic solid tumors mostly derived from the gastrointestinal tract has been completed in 2009 (28). Stable disease was observed in 24% of patients. In ongoing phase II clinical trials, the efficacy of danusertib is evaluated in patients suffering from tumor entities resistant to first- or second-line chemotherapy (29–31).

In this study, we show, for the first time, that aurora kinases can successfully be targeted in GEP-NETs in vivo. Efficacy of danusertib was documented in human GEP-NET cells and tumor xenografts and resulted in inhibition of proliferation and tumor growth. Mechanisms of action included cell-cycle arrest and apoptosis. However, only in QGP tumors a reduction of tumor size could be attained, although the size of BON1 tumors size remained stable during treatment. Interestingly, the selective aurora B inhibitor ZM447439 showed strong induction of apoptosis in the same cell lines (15). This may be due to the high concentrations at micromolar ranges applied in the in vitro experiments. Most probably, these concentrations cannot be reached in vivo. However, as no in vivo studies were carried out with ZM447439, currently no statement with regard to the effect of the inhibitor at lower concentrations can be made (15). The reason that apoptosis induction was shown mainly at lower concentrations of danusertib may be related to the ongoing cell-cycle activity shown by detectable S-phase at 50 nmol/L. In contrast, we observed a massive reduction in S-phase at higher doses, consecutively leading to reduced apoptosis. It is therefore likely that danusertib-induced apoptosis is dependent on actively dividing cells. Treatment with concentrations of 500 nmol/L resulted in a significantly reduced phosphorylation of histone H3, resulting in a complete block of mitosis and loss of the

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number</th>
<th>Initial (mm³) mean ± SD</th>
<th>Final (mm³) mean ± SD</th>
<th>Final (% growth) median ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BON1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>52.3 ± 22.6</td>
<td>356.9 ± 224.6</td>
<td>627.2 ± 395.4</td>
</tr>
<tr>
<td>STZ/5-FU</td>
<td>29</td>
<td>44.7 ± 19.8</td>
<td>121.1 ± 72.7a</td>
<td>238.4 ± 194.3a</td>
</tr>
<tr>
<td>Danusertib</td>
<td>35</td>
<td>47.7 ± 22.4</td>
<td>42.0 ± 33.2.5ab</td>
<td>73.9 ± 79.9a</td>
</tr>
<tr>
<td>STZ/5-FU</td>
<td>25</td>
<td>54.2 ± 23.9</td>
<td>75.3 ± 51.2a</td>
<td>130.7 ± 86.2a</td>
</tr>
<tr>
<td>Danusertib</td>
<td>22</td>
<td>45.8 ± 24.3</td>
<td>4.6 ± 9.2ab</td>
<td>6.3 ± 8.2ab</td>
</tr>
</tbody>
</table>

aP < 0.001 vs. controls.
bP < 0.001 vs. STZ/5-FU.

Table 2. Effects of therapy with danusertib on the growth of subcutaneous human GEP-NET xenografts in NOD/SCID mice

Figure 4. Biodistribution of GEP-NET cells after intrasplenic injection. A, two hours after BON1 cell transplantation, human PCR signals were detectable almost exclusively in the liver. B, massive hepatomegaly with multiple tumor nodules, 5 weeks after intraportal BON1 cell transplantation. C, FISH using a digoxigenin-labeled alphoid-DNA probe verifies human origin of GEP-NET liver metastases in mice. D, hematoxylin/eosin staining showing nodular BON1 liver metastases (arrows), some with central necrosis (N). Magnification: C, ×400; D, ×100.
2N fraction. Remarkably, such serum concentrations are also achievable in humans (32).

Whereas BON1 tumors remained stable under danusertib treatment, subcutaneous QGP tumor sizes decreased. These in vivo results reflect in vitro experiments with decreased cell numbers only in QGP cells, although apoptosis was observed in both cell lines. This may be due to the higher proliferation rate in BON1 cells leading to a compensatory increase in cell numbers. Interestingly, reduced cell proliferation and tumor reduction occurred early in the treatment phase and remained stable thereafter. Cells with normal 2N chromosome numbers may be more susceptible to apoptosis than cells with higher >2N chromosome numbers after endoreplication. Alternative mechanisms such as differences in cell-cycle analyses or the p53/p21 axis could not be detected. Because various receptor tyrosine kinases that might have been influenced by danusertib share the same mitogenic signaling cascades as a mechanism of action, off-target effects of the compound were ruled out by showing unaltered Erk (downstream target of Ras/Raf signaling, FGR, src), JNK (several growth factors, cell surface receptors, src) and Akt (PI-3 Kinase, FGFR).

Although we were able to show reductions in tumor growth after treatment with danusertib, the use of a heterotopic in vivo system may not reflect the influence of the relevant tumor microenvironment, an important factor in tumor growth and metastasis (33, 34). Because liver metastases constitute the main clinical problem in GEP-NETs, this study extends the established knowledge of antitumor effects and mechanisms of danusertib in an orthotopic model of metastatic GEP-NETs. In accordance, an interesting orthotopic tumor model has recently been established to combine somatostatin receptor scintigraphy with MRI after injection of rat-derived pancreatic tumor cells into mouse pancreas (35). In our model, liver metastases derived from human GEP-NET cell lines retained characteristics of the GEP-NETs, that is, CgA expression and secretion as well as stimulation of angiogenesis. Generation of liver metastases was achieved by techniques used in therapeutic liver cell transplantation, for example, intrasplenic or intraportal injection (16, 36). In line with the metastatic spread observed in gastrointestinal cancer, tumor cells enter the liver sinusoids via the portal venous blood. With regard to the increasing knowledge about the importance of tumor–stroma interactions (33), this enables analysis of treatment efficacy on tumor cell growth under similar conditions as in human disease. Furthermore, the understanding of tissue-specific pharmacologic properties of danusertib may play an important role to predict the response of metastases at similar anatomical sites in humans (34, 37). Thus, tumor shrinkage of QGP cells occurred in the subcutaneous tumor model, whereas in the orthotopic model, only stable disease was obtained. We also observed a more rapid tumor growth of liver metastases compared with the subcutaneous model. This may be due to a stimulatory effect of the liver microenvironment, for example, the presence of growth factors such as IGF1 (38). Therefore, this new model offers options for translational research with potential clinical relevance. Furthermore, we were recently able to show that BON1 cells are amenable for multicolor labeling by simultaneous lentiviral transduction with 3 fluorescence proteins, which remained stable after xenografting (39). This technique, together with intrahepatic formation of liver metastases for serial MRI will offer new opportunities to study clonality of GEP-NETs and mechanisms of chemotherapy resistance.

As shown by several studies, circulating CgA correlates with tumor burden and prognosis in humans (40–42) and has a high diagnostic accuracy (43). We observed significantly decreased CgA levels in danusertib-treated mice compared with controls and standard therapy with STZ/5-FU. However, in line with a previous study, we observed a large variety of CgA levels in tumor-bearing animals before treatment (44). Furthermore, CgA levels did not correlate with the reduction in tumor mass observed in treated mice. Thus, CgA levels may not be a mere reflection of tumor mass, but other mechanisms regulating CgA release from...
tumor cells may play a role in different treatment modalities. Whether CgA could act as a biomarker for therapy monitoring and outcome needs to be studied further. It also needs to be evaluated whether AKI have the same positive effect on pancreatic NETs as the recently approved everolimus and sunitinib, which had been shown to delay tumor growth using a murine transgenic pancreatic islet cell tumor model (21, 22).

In conclusion, this study supports aurora kinase targeting using potent small-molecule inhibitors such as danusertib for the treatment of GEP-NETs. Our study advances previous work by showing strong antitumor activity in a new

<table>
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<th>Table 3. Effects of danusertib treatment on BON1 and QGP liver metastasis in the orthotopic tumor model</th>
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<tbody>
<tr>
<td><strong>Experiment</strong></td>
</tr>
<tr>
<td><strong>BON1</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Danusertib</td>
</tr>
<tr>
<td><strong>QGP</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Danusertib</td>
</tr>
</tbody>
</table>

$^a$P < 0.01 vs. controls.

$^b$P < 0.05 vs. controls.
orthotopic tumor model of GEP-NET liver metastases. The potent effects on cell-cycle interference and apoptosis induction provide rationales for forthcoming clinical trials targeting aurora kinases in GEP-NETs.

Disclosure of Potential Conflicts of Interest

J. Moll is a former employee of Novo Nordisk Medical Sciences, the manufacturer of danusertib. D. Horsch is a consultant and advisory board member of Novartis Pharma. No potential conflicts of interest were disclosed by the other authors.

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Katharina Fraedrich, Jörg Schrader, Harald Ittrich, et al.


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