PHA-739358, a potent inhibitor of Aurora kinases with a selective target inhibition profile relevant to cancer

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Abstract

PHA-739358 is a small-molecule 3-aminopyrazole derivative with strong activity against Aurora kinases and cross-reactivities with some receptor tyrosine kinases relevant for cancer. PHA-739358 inhibits all Aurora kinase family members and shows a dominant Aurora B kinase inhibition—related cellular phenotype and mechanism of action in cells in vitro and in vivo. p53 status—dependent endoreduplication is observed upon treatment of cells with PHA-739358, and phosphorylation of histone H3 in Ser10 is inhibited. The compound has significant antitumor activity in different xenografts and spontaneous and transgenic animal tumor models and shows a favorable pharmacokinetic and safety profile. In vivo target modulation is observed as assessed by the inhibition of the phosphorylation of histone H3, which has been validated preclinically as a candidate biomarker for the clinical phase. Pharmacokinetics/pharmacodynamics modeling was used to define drug potency and to support the prediction of active clinical doses and schedules. We conclude that PHA-739358, which is currently tested in clinical trials, has great therapeutic potential in anticancer therapy in a wide range of cancers. [Mol Cancer Ther 2007;6(12):3158–68]

Introduction

A number of potential molecular targets for the identification of novel anticancer drugs interfere with the cell cycle (1, 2). Protein kinases play important roles in promoting or retarding transitions between different stages of the cell cycle and act as or regulate cell cycle checkpoints. In mammals, a closely related subgroup of three serine/threonine protein kinases, the Aurora kinases, are believed to play a key role in protein phosphorylation in mitosis and have been shown to contribute in the development and progression of cancer. The human Aurora kinases display distinct roles during mitosis, which are reflected in their subcellular locations (reviewed in refs. 3, 4). Significant interest for Aurora kinases inhibition as an anticancer strategy was generated because some Aurora kinase family members are overexpressed in a variety of human cancers, and elevated expression has been correlated with chromosomal instability and clinically aggressive disease in some instances (5–9). Ectopic overexpression of one member of the family, Aurora A, was shown to induce oncogenic transformation in cells (10). Although an oncogenic activity of Aurora B has not been shown, elevated Aurora B activity promotes Ras-mediated transformation by enhancing oncogenic signaling and by inducing aneuploidy (11). Aurora A was also reported as a candidate low-penetration tumor susceptibility gene. A polymorphism (F31I) has been observed in the Aurora A gene and reported to be correlated with the degree of aneuploidy in human colon tumors and several other cancers (12).

Several small-molecule inhibitors of Aurora kinases have now been reported. For example, ZM447439 (13), Hesperadin (14), and MK0457/VX-680 (15) all induce similar phenotypes when tested in cell-based assays, and MK0457/VX-680 exhibited efficacy against several human tumor xenografts in athymic mice. More recently, inhibitors of Aurora kinases have entered clinical development, including MK0457/VX-680, MLN8054 (16), a selective Aurora A inhibitor, and AZD1152 (17), a selective Aurora B inhibitor (reviewed in ref. 18).

Previously, we described the identification of a new chemical class of Aurora kinase inhibitors (19) and the pharmacologic profile of an initial preclinical candidate (20). A novel series of Aurora inhibitors had been identified from the combinatorial expansion of the 1,4,5,6-tetrahydropyrazole bi-cycle, a versatile scaffold designed to target the ATP pocket of protein kinases. The development of the pyrrolopyrazole sub-series resulted in the identification of PHA-739358 (21), which shows higher potency, better drug-like properties, and most important, inhibitory activity on other cancer-related targets without affecting the preclinical safety profile. PHA-739358 inhibits some tyrosine
kinases, most importantly Abl, including the T315I mutant (22), as well as Ret, Trk-A, and fibroblast growth factor receptor-1 (FGFR-1), which are involved in a number of malignancies, including chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), and thyroid, prostate, and breast carcinoma (23–26). Here, we describe the in vitro and in vivo pharmacologic profile of PHA-739358, a compound currently in clinical trials in phase II.

Materials and Methods
Chemicals
PHA-739358, N-[5-(2-methoxy-2-phenyl-acetyl)-1,4,5,6-tetrahydro-pyrrolo [3,4-c]pyrazol-3-yl]-4-(4-methyl-piperazine-1-yl)-benzamide, was synthesized at Nerviano Medical Sciences, S.r.l. The synthesis of PHA-739358 has been reported previously (21). DMSO was purchased from Sigma.

Biochemical Kinase Assays
The method for obtaining biochemical IC50 values for PHA-739358 against its targets, as well as other selected kinases, was determined as described previously (20). For each enzyme, the Km values for ATP and the specific substrate were initially determined, and each assay was then run at optimized ATP (2Km) and substrate (5Km) concentrations. This setting enabled direct comparison with compound and incubated for 72 h at 37°C. After 24 h, cells were treated with PHA-739358 at the indicated concentrations and times, starting from a stock solution of 10 mmol/L in PBS/C02. IC50 values for ATP and the specific substrate were initially determined, and each assay was then run at optimized ATP (2Km) and substrate (5Km) concentrations. This setting enabled direct comparison with compound and incubated for 72 h at 37°C. After 24 h, cells were treated with PHA-739358 at the indicated concentrations and times, starting from a stock solution of 10 mmol/L in PBS/C02.

Analysis of Cell Proliferation
Analysis was done as previously described (20). Briefly, cells were seeded at different densities in 24-well plates with the appropriate medium. After 24 h, cells were treated with compound and incubated for 72 h at 37°C in 5% CO2 atmosphere. At the end of the incubation time, cells were detached from each plate and counted using a cell counter (Coulter Counter-Beckman). IC50 values were calculated using percent of growth versus untreated control.

Cell Cycle Analysis
Analysis was done as previously described (20). Samples were analyzed by flow cytometry with a FACScan (Becton Dickinson). Data were analyzed with ModFit LT software (Verity Software House).

Cell-Based Assays
HeLa cells were treated for 1 h with PHA-739358 at the indicated concentrations, and then the cells were collected and analyzed by Western blot for Aurora A autophosphorylation (Thr288) and Aurora B-mediated phosphorylation of histone H3 (Ser10) with specific antibodies.

For cellular tyrosine kinase experiments, TT cells were serum starved for 24 h and then treated with vehicle (DMSO) or increasing concentrations of the compound for 2 h. After the treatment, the cells were collected and lysed. Total cell lysates were immunoblotted with rabbit polyclonal anti-phospho-Ret (Tyr490) antibody. The same membrane was then stripped and re-probed with rabbit polyclonal anti-Ret antibody. PC-12 cells were serum starved for 24 h and pretreated with different doses of the compound for 2 h before nerve growth factor (NGF; 100 ng/mL) stimulation for 5 min. After the treatment, the cells were collected and lysed. Total cell lysates were immunoblotted with rabbit polyclonal anti-phospho-Trk-A (Tyr490) antibody. The same membrane was stripped and re-probed with rabbit polyclonal anti-total Trk-A antibody. NIH-3T3 cells were serum starved for 24 h, and then the cells were incubated with the compound for 1 h. Then, the cells were stimulated with 100 ng/mL FGF-1 or epidermal growth factor (EGF) for 20 min. After the treatment, the cells were collected and lysed. Total cell lysates were immunoblotted with rabbit polyclonal anti-phospho-extracellular signal-regulated kinase 1/2 (anti-phospho-ERK1/2) antibody. A rabbit polyclonal anti-AKT antibody was used for loading normalization. Cell lysates and immunoblotting were done as previously described (20). The following antibodies were used: anti-phospho-histone H3 Ser10 (Upstate Biotech), anti-histone H3 (Abcam), anti-α-tubulin (Sigma), anti-p21 (BD Biosciences), P-Ret (Tyr905), P-Trk-A (Tyr490), and P-Aurora (Thr288) from Cell Signaling Technology, Inc. p53 and Trk-A antibodies were purchased from Santa Cruz Biotechnology, Inc., and Calbiochem, respectively. The enhanced chemiluminescence kit (GE Healthcare Amersham) was used for detection.

Immunohistochemistry
Tumors were excised, formalin fixed, and paraffin embedded. Analysis was done as previously described (20). The antibodies used were rabbit polyclonal phospho-histone H3 Ser10 antibody (Upstate); rabbit polyclonal anti-p53 antibody (Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-p21 antibody (BD Biosciences); rabbit polyclonal anti-active caspase-3 antibody (Cell Signaling Technology, Inc.). After primary antibody staining, sections were incubated for 30 min at room temperature with Envision Labeled Polymer-HRP anti-mouse or rabbit (DAKO) and then rinsed and incubated with DAB Plus (DAKO). The sections were then counterstained with hematoxylin and dehydrated, and coverslips were added for permanent mounting. For bromodeoxyuridine (BrdU) staining, slides were treated with 4 N HCl in PBS + 0.1% Triton X-100, followed by 20 mg/mL proteinase-K (Roche).
BrdU mouse monoclonal antibody (Becton Dickinson) was coupled with biotinylated mouse immunoglobulin G in vitro, followed by incubation with ARK-streptavidin (DAKO).

**Image Analysis**
Three random pictures, chosen in viable tumor areas were collected at 10× magnification for each immunostaining, and an image analyzer was used to count the positive cells per field. Data reported are obtained counting about 5,000 cells per tumor. Total cells per picture were counted in representative tumors for each group and the mean used to calculate the percentage of positive cells. Statistical analysis was done using the Mann-Whitney test.

**Animal Efficacy Studies**
Female Hsd, athymic nu/nu mice, 5 to 6 weeks of age (average weight, 20–22 g), and female severe combined immunodeficiency (SCID) mice were obtained from Harlan. MMTV-v-Ha-ras transgenic mice were obtained from Charles River Breeding Laboratories and maintained in a FVB mouse strain background. All animal studies were carried out in compliance with Italian Legislative Decree no. 116 dated January 27, 1992, and the European Communities Council Directive no. 86/609/EEC concerning the protection of animals used for experimental or other scientific purposes and according to institutional policy regarding the care and use of laboratory animals. A2780 human ovarian carcinoma and HCT116 colon carcinoma cell lines were transplanted s.c. in athymic mice. Mice bearing a palpable tumor (100–200 mm³) were selected and randomized into control and treated groups. Treatments started 1 day after randomization at doses and schedules as indicated in the figure legends. In the HL-60 study, female SCID mice were injected s.c. with 5 × 10⁶ leukemia cells. When tumors were 200 to 250 mm³ in size, treatments were started. Dimensions of the tumors were measured regularly by caliper during the experiments, and the tumor volume was calculated according to the formula

\[ \text{tumor volume} = \frac{d_1 \times d_2 \times d_3}{2} \]

where \( d \) is the minimum diameter and \( D \) the maximum diameter.

**Treatment of Rats with Established 7,12-Dimethylbenz[a]anthracene–Induced Mammary Tumors**
Female 50-day-old Sprague-Dawley rats were given a single intragastric dose of 20 mg of 7,12-dimethylbenz[a]-anthracene (DMBA; Sigma-Aldrich) in 1.0 mL of sesame oil. After 40 days, animals were examined weekly by palpation. When at least one mammary tumor measuring 0.5 cm in diameter was reached, the rats were placed randomly into two groups and treated i.v. bd with 25 mg/kg of PHA-739358 or vehicle. The tumor volume was measured weekly (using Vernier calipers) for the duration of the treatment.

**The Transgenic Adenocarcinoma of the Mouse Prostate Model**
The Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) males (28) were screened for prostate tumor onset, and the tumor growth was followed by MRI every 2 weeks starting at the age of 14 weeks (29). Animals were inserted in the study when prostate tumors reached a tumor volume between 50 and 500 mm³ and treated with PHA-739358 at 30 mg/kg bd i.v. for 5 consecutive days. A Bruker Pharmascan instrument operating at 7.0 T was used for the study. MRI was done at day 0 (before treatment), day 7, day 11, day 15, and for some animals, days 22 and 32. Sixteen adjacent 0.4-mm-thick slices were acquired across the tumor; slice thickness was increased for bigger tumors to cover the whole mass.

**Pharmacokinetics**
The PK of PHA-739358 was investigated in an ancillary group of three tumor-bearing mice. Blood samples for the pharmacokinetic assessment were collected, and the drug was assayed in plasma using liquid chromatography tandem mass spectrometry techniques as described previously (27).

**Data Analysis**
Simultaneous fitting of the average tumor growth curves observed in control and treated animals were done. Winnonlin Program (Pharsight) was used both for PK and PK/PD analysis.

**Results**

**Cellular Kinase Inhibition**
PHA-739358 is a pyrrolo-pyrazole (Fig. 1A), which inhibits Aurora kinases in a biochemical assay with IC₅₀ of 13, 79, and 61 nmol/L for Aurora A, B, and C, respectively (Fig. 1B; ref. 21). High activity was also observed against kinases known to be mutated or overexpressed in different cancers such as Ret, Trk-A, and Abl (2-fold higher IC₅₀ compared with Aurora A). Cross-reactivity with FGFR-1 was seen at a 4-fold higher IC₅₀ compared with Aurora A, whereas 28 other kinases displayed at least a 10-fold selectivity as reported by Fancelli et al. (21). Based on these data, PHA-739358 can be defined as a spectrum-selective kinase inhibitor for cancer-related kinases.

Kinase autophosphorylation or substrate phosphorylation as a measure for inhibition of the specific targets was examined in cells after compound treatments. PHA-739358 has strong activity on Aurora A, as determined by inhibition of autophosphorylation in position Thr288 (30) and also on Aurora B as determined by inhibition of its substrate histone H3 in position Ser10 (31). In HeLa cells, inhibition for both Aurora kinases starts to be seen from about 0.1 μmol/L concentrations upward (Fig. 2A).
Inhibition of Abl, Ret, and Trk-A by PHA-739358 was assessed using cell lines where these proteins are relevant for growth or survival. The Ret and Trk-A kinase-inhibitory activities of PHA-739358 were evaluated for a dose-related inhibition of receptor autophosphorylation in appropriate cell-based assays using TT and PC-12 cells, respectively. TT cells were derived from a tumor of a patient with sporadic medullary thyroid carcinoma. These cells contain a Ret allele with a constitutively activating mutation (C634W) in the extracellular domain (32). The cells were starved for 24 h and treated for 2 h with several doses of PHA-739358. Inhibition of ligand-induced Trk-A phosphorylation by PHA-739358 was examined in PC-12 cells, a NGF-responsive cell line established from a rat pheochromocytoma. PC-12 cells were serum starved and pretreated with the compound at different doses for 2 h before NGF stimulation for 5 min. Both Ret and Trk-A kinases were inhibited at low micromolar concentrations of PHA-739358 (Fig. 2B and C) in agreement with the inhibitory activity observed in the biochemical assay. The compound also inhibits Abl autophosphorylation and its downstream pathway (data not shown), as well as histone H3 phosphorylation. When examining the effect of PHA-739358 on the cell cycle profile of K562, a BCR-Abl−positive cell line, the dominant cellular phenotype is clearly related to the inhibition of Aurora kinases (4N, some endoreduplication) and less to inhibition of Abl (G1 block, apoptosis) as shown in Supplementary Fig. S1, although contributions of Abl inhibition might be relevant to increase log killing of tumor cells. This dominant effect was also seen when imatinib, an inhibitor of Abl with no activity on Aurora kinases, was used simultaneously with PHA-739358 (data not shown).

Mitogen-activated protein kinase (MAPK) activation upon FGF or EGF stimulation was analyzed by Western blot in NIH-3T3 cells treated with the compound. PHA-739358 has an effect on MAPK activation by FGF and not by EGF, as assessed by a reduction in phosphorylation of MAPK (ERK1/2), demonstrating selectivity for inhibition of the FGF receptor pathway, but not the epidermal growth factor receptor (EGFR) pathway in these cells (Fig. 2D).

**Effect on Tumor Cell Proliferation and Mechanism of Action**

The antiproliferative effect of PHA-739358 was evaluated in several tumor cell lines covering different histotypes, including colon, breast, prostate, lung, and ovary. The exponentially growing cells were exposed to PHA-739358 for 72 h, and cell viability was measured by cell counting. All cell lines examined were highly sensitive to the compound in the range of 28 nmol/L (A2780) up to 300 nmol/L (U2OS) as reported in Supplementary Table S1. Treated A2780 cells showed a substantial increase in DNA content (4N) and >4N sub-populations, indicating that the dominant phenotype is related to Aurora B inhibition (Fig. 3A). Although >4N sub-populations were seen in most cell lines at variable degrees, some cell lines showed rather a 4N accumulation or a high level of apoptosis. An explanation for cells responding differentially to an Aurora kinase inhibitor has been recently suggested to be depending on the integrity of the p53-p21-dependent postmitotic checkpoint as assessed in different tumor cell lines (33). Several reports have shown a p53-dependent post-mitotic checkpoint function (34). To address the question whether p53 status could be the reason for the different cellular phenotype observed, we examined the impact of loss of p53 on PHA-739358–induced polyploidy formation. After treatment of wild-type and p53-deficient MEFs with PHA-739358, the wild-type cells underwent an arrest in mitosis (4N) that is maintained also at 48 h, whereas the p53-deficient cells did not arrest with a 4N DNA content, but continued through additional rounds of DNA synthesis to become >8N (Fig. 3B). It has been shown that p53 is post-translationally stabilized after cell stress and is a direct substrate for Aurora kinase A (35, 36). Therefore, we investigated the effect of PHA-739358 on p53 stability and its downstream pathway in HCT-116 cells. Treatment with PHA-739358 led to an increase in p53 protein levels and an associated increase in p21 protein (Fig. 3C), which is supplemented by Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
known to be transcriptionally regulated by p53. Although HCT-116 cells are expressing wild-type p53, other checkpoint defects might be responsible for the endoreduplication phenotype observed in these cells.

**Antitumor Activity In vivo**

To assess the antitumor activity of PHA-739358 in vivo, several human tumor xenograft models in nude mice were evaluated. The preclinical models chosen included also syngenic models such as transgenic and carcinogen-induced tumor models to exclude species-related effects on efficacy or toxicity. Usually, PHA-739358 was given i.v. at the maximally tolerated doses (MTD) of 60 mg/kg/day for 5 days or for 10 days at the doses of 30 and 45 mg/kg/day (MTD for this schedule). When PHA-739358 was evaluated in the different xenograft models implanted in mice, significant TGI was observed in the range from 66% to 98%, with a good safety profile in terms of loss of body weight and mild myelosuppression, both of which are transient effects with full recovery after the end of the treatment. A summary of efficacy experiments done is shown in Table 1. In HL-60 xenografts, the compound, upon bd i.v. administration for 5 days, showed a TGI of up to 98% at the highest dose, and full TGI was still visible 1 week after the end of treatment. There was also evidence of tumor regression and occasional cures (2/8). We extended our study to a different animal species, a rat model consisting of DMBA-induced primary mammary carcinomas. This model displays many similarities with human breast cancer. Administration of 25 mg/kg PHA-739358 bd i.v. to these rats resulted in 75% inhibition of tumor growth with complete regression in one animal. The efficacy was similar to that achieved in the xenograft mouse models and shows the effectiveness of the compound also in spontaneous tumors in a different species. To evaluate the efficacy of PHA-739358 in prostate cancer, we used the TRAMP transgenic mouse prostate cancer model, which exhibits many similarities to human prostate cancer, including epithelial origin, progression from the prostate intraepithelial neoplasia (PIN) stage to adenocarcinoma, and metastasis (28). A representative example is shown in Fig. 4. The tumors typically doubled their volumes in about 5 to 7 days. When mice were treated for 5 days bd i.v. with 30 mg/kg of PHA-739358, 3 out of 16 mice showed tumor regression up to 80%. In 10 mice, tumors were stabilized, whereas in three mice, tumors continued growth as assessed by noninvasive imaging, such as MRI (29).

**In vivo Biomarker Modulation**

To determine if inhibition of Aurora kinases signaling correlates with suppression of tumor growth, we examined histone H3 phosphorylation and protein levels of p53 and p21 in lysates from A2780 tumor xenografts after treatment with PHA-739358. Tumor-bearing mice were treated with PHA-739358 at 30 mg/kg bd i.v., and for the mechanism of action studies, tumors were collected on day 5, 2 h after the last administration of the compound or vehicle. Figure 5A shows that after repeated treatment, the compound inhibited histone H3 phosphorylation and induced an accumulation of p53 and its downstream target p21 in s.s., serum starved.

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**Figure 2.** Inhibition of cellular pathways by PHA-739358. A, HeLa cells were treated for 1 h with PHA-739358 at the indicated concentrations. Western blot analysis of the activity on Aurora A autophosphorylation (Thr288) and Aurora B–mediated phosphorylation on histone H3 (Ser10) with specific antibodies. B, effect of PHA-739358 on Ret phosphorylation. TT cells were starved and treated with different doses of the compound for 2 h. Total cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti phospho Ret (Tyr905) antibody. The same membrane was then stripped and re-probed with rabbit polyclonal anti-Ret antibody. C, effect of PHA-739358 on Trk-A phosphorylation. PC-12 cells were starved and pretreated with different doses of the compound for 2 h before NGF stimulation for 5 min. Total cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti – phospho-Trk-A (Tyr490) antibody. The same membrane was then stripped and re-probed with rabbit polyclonal anti-total Trk-A antibody. D, effect of PHA-739358 on FGFR1 downstream pathway. NIH-3T3 cells were serum starved for 24 h and then incubated with the compound for 1 h. Then, the cells were stimulated with 100 ng/mL FGF-1 or EGF for 20 min. After the treatment, the cells were collected and lysed. Total cell lysates were immunoblotted with rabbit polyclonal anti – phospho-ERK1/2 antibody. A rabbit polyclonal anti-AKT antibody was used for loading normalization.
A2780 tumors. Parallel histologic evaluation of treated tumors revealed an increase in cellular and nuclear size and the presence of multinucleated cells. Good modulation of several proliferation-associated markers was observed (Fig. 5B): proliferation was strongly inhibited as shown by the reduction of BrdUrd incorporation from 38% in controls compared with 10% in treated tumors. In parallel, phospho-histone H3–positive cells decreased from 4% in control tumors to 0.3% in treated tumors. p53- and p21-positive cells had a strong increase, rising from 7% and 8% in controls to 51% and 43%, respectively, in treated tumors. All modulations were quantified and showed high statistical significance (Fig. 5C). Active caspase-3 as an indicator for apoptosis induction was only slightly increased in treated tumors. Interestingly, the number of macrophages (that could have been attracted by apoptotic cells) was also increased in the same tumors (data not shown). Overall, these data show that PHA-739358 in vivo results in biomarker modulation accompanied by tumor growth inhibition compatible with an expected mechanism of action of Aurora kinase inhibition.

Pharmacokinetics

The pharmacokinetic properties of PHA-739358 have been investigated in the mouse and rat. In both species, the compound showed moderate to high systemic clearance and a high volume of distribution, suggesting extensive tissue distribution. Figure 6A reports an example of pharmacokinetics of PHA-739358 evaluated using a two-compartment model after the last administration of 45 mg/kg of PHA-739358 given once a day for 10 days in an efficacy study of A2780-xenografted mice. The fitting was confirming the bi-exponential behavior of the compound with a terminal half-life of about 3.5 h. Clearance was low (1.7 L/h/kg), and the volume of distribution higher than the total body water $V_{ss} = 2.7$ L/kg.

Upon administration by i.v. bolus or infusion PHA-739358, plasma levels increased largely in proportion to dose, and data from repeated-dose studies indicated that the pharmacokinetic parameters were comparable to those observed after single administration and did not show important differences when different treatment schedules were used.
A pharmacokinetic and pharmacodynamic approach, based on a previously published model (27), was applied to the efficacy data obtained with this compound in different xenograft models using different doses. This methodology links the plasma concentrations of the anticancer compounds to the effect on the tumor growth curves observed in the treated animals and provides quantitative estimates of the potency of the compounds through the evaluation of two model parameters: $K_2$ and $C_t$. $K_2$ is the proportionality factor linking the plasma concentration to the effect and can be regarded as a drug-specific measurement of the potency of the compound. The $C_t$ value provides an estimate of the steady-state drug concentration in plasma to be maintained for observing tumor regression and eventually tumor eradication ($C_{ss} > C_t$). For each experiment, a simultaneous fitting of tumor growth in control and treated animals was done, and quality of fit was evaluated comparing predicted versus

### Table 1. In vivo activity of PHA-739358

<table>
<thead>
<tr>
<th>Model</th>
<th>Dose and schedule</th>
<th>Maximal TGI, % (day)</th>
<th>Maximal weight loss, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780 human ovarian carcinoma xenograft in nude mice</td>
<td>30 mg/kg i.v. qD, D1–10</td>
<td>80 (19)</td>
<td>18</td>
</tr>
<tr>
<td>HCT-116 human colon carcinoma xenograft in nude mice</td>
<td>30 mg/kg i.v. bd, D1–5</td>
<td>66 (17)</td>
<td>22</td>
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<tr>
<td>HL-60 human acute myelogenous leukemia xenograft in SCID mice</td>
<td>30 mg/kg i.v. bd, D1–5</td>
<td>98 (22)*</td>
<td>16</td>
</tr>
<tr>
<td>DMBA-induced mammary carcinoma in rat</td>
<td>25 mg/kg i.v. bd, D1–3 q wk × 2</td>
<td>75 (10)†</td>
<td>10</td>
</tr>
<tr>
<td>MMTV-RAS transgenic mammary carcinoma in mice</td>
<td>30 mg/kg i.v. bd, D1–3 q wk × 2</td>
<td>68 (23)</td>
<td>15</td>
</tr>
<tr>
<td>TRAMP transgenic prostate carcinoma in mice</td>
<td>30 mg/kg i.v. bd, D1–5</td>
<td>3/16 PR</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10/16 SD</td>
<td>3 progressions</td>
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NOTE: $n = 8–10$ animals/study. The TGI (%) was calculated according to the equation $\% \text{TGI} = 100 \times (\text{mean tumor weight of treated group}/\text{mean tumor weight of control group}) - 100$.

Abbreviations: ND, not determined; qD, once a day.

*Two of eight animals showed complete regression.

†One of 10 animals showed complete regression; 1/10 death at day 5.

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Figure 4. PHA-739358 treatment results in the inhibition of tumor growth in TRAMP mice as assessed by MRI evaluation. Animals with a developed prostate tumor reaching a tumor volume between 50 and 500 mm$^3$ were treated with PHA-739358 at 30 mg/kg bd i.v. for 5 d. Magnetic resonance images were acquired before (day 0) and after the end of the treatment (days 7, 11, and 22) and showed clear tumor shrinkage after the treatment with PHA-739358 (right).
observed curves, plot of residuals, and order of magnitude of parameter coefficients of variation. Some examples of the fittings are reported. The estimated $C_t$ value in the A2780 model was about 1.5 μmol/L, and the potency $K_2$ was between 0.0055 and 0.0096 h/μmol/L, independently of the tumor size at the start of treatment. For comparison, these values for paclitaxel are 0.545 μmol/L ($C_t$) and 0.022 h/μmol/L ($K_2$; ref. 27). Similar values were obtained in three different models, indicating the robustness of the obtained values (Fig. 6B, D, and E). No dependency of dose and schedule was observed, and the activity was maintained with the same potency as when the compound was given to tumors in more advanced stages (Fig. 6C). These parameters were helpful to guide schedules in clinical trials and predictions of active doses and target plasma levels.

Discussion

Aurora kinases play distinct roles during mitosis, with Aurora A being essential for centrosome maturation and spindle assembly and Aurora B functioning at kinetochores in chromosome attachment and at the end of mitosis in cytokinesis (reviewed in refs. 3, 4).

Inhibition of Aurora kinases is emerging as a new strategy for anticancer therapy, and several small-molecule inhibitors have entered clinical trials. In this report, we describe the biochemical and pharmacologic activity profile of PHA-739358, a spectrum-selective small-molecule kinase inhibitor, which was identified during a screen for inhibitors of Aurora kinases. In most of the cell lines tested, cells fail to divide, resulting in polyploidy without a strong impact on the timing of mitosis (data not shown).
and finally leading to a reduction in viability. In some cell lines, an increased apoptosis was seen. This speaks for a dominant Aurora B kinase-related mechanism of action for inhibition because Aurora A inhibition would rather result in a G2-M block (16). A similar effect is shown by another study (37), whereby small interfering RNA experiments targeting simultaneously Aurora A and B resulted in a phenotype comparable with Aurora B siRNA alone, supporting the idea that inactivation of Aurora B bypasses Aurora A functions in mitosis. Based on the selectivity profile of the reported Aurora kinase inhibitors in clinical development, PHA-739358 is most similar to VX-680/MK0457, which is a pan-Aurora inhibitor, and also, there seems to be an overlap in the cross-reactivity profile, for example, both compounds hit wild-type and mutant Abl (15, 22, 38). In contrast, MLN-8054 inhibits preferentially Aurora A, and AZD-1152 inhibits preferentially Aurora B (16, 17).

It should be noted that, in some cell lines, the accumulation of cells with >4N DNA content after treatment with PHA-739358 could be attributed to the integrity of the p53-dependent post-mitotic checkpoint as evidenced by the increased endoreduplication in MEFs after abrogation of p53 function. These findings suggest that the polyploidy phenotype seen in cells lacking a functional p53 checkpoint arises through a combination of defective cytokinesis and the inability of cells to prevent further cell cycle progression. Although the p53 status might contribute to the variations in sensitivity of different cell lines to PHA-739358, additional genetic defects or cell-specific transporter protein profiles could be responsible for the observed differences. A direct link between Aurora A and p53 has been shown because phosphorylation of p53 by Aurora A leads to the destabilization of p53 (35). As expected from the inhibition of Aurora A, we observe p53 stabilization and induction of its downstream transcriptionally regulated target, p21. An alternative explanation for p53 induction could be the activation of a p53-dependent G1-S checkpoint in response to the failed cytokinesis upon inhibition of Aurora B, although there is less evidence for this possibility.

The compound has good PK properties and a strong antitumor activity observed in a broad range of in vivo tumor models with minor and transient toxicity signs. Overall, these are important attributes for clinically viable drugs.

Most clinically relevant kinase-targeting drugs, which originally were screened for a single target, have shown to

Figure 6. Pharmacokinetic profile and PK/PD modelling in different xenograft models. A, the fitting of the pharmacokinetic data of PHA-739358 after the last administration of 45 mg/kg PHA-739358 given once a day for 10 d to mice bearing A2780 tumor. The parameters of this fitting were used for the pharmacokinetic-pharmacodynamic modeling. Observed and model-fitted tumor growth curves obtained in nude mice bearing A2780 xenograft tumor treated i.v. with the vehicle or PHA-739358 at 30 mg/kg bd for 5 d from day 8. C, as in B with the exception that large tumors were treated i.v. with the vehicle or PHA-739358 at 15 and 30 mg/kg bd for 5 days from day 13. Mice bearing HCT-116 (D) or HL-60 (E) xenograft tumors were treated i.v. with the vehicle or PHA-739358 at 7.5, 15, and 30 mg/kg bd for 5 d from day 8. K2 (h/mol/L) and C0 (µmol/L) values are reported for each experiment in the panels. V1, volume of central compartment (mL/kg). K10, K12, K21, micro-rate constants (1/h).
have a wider spectrum of kinase targets than originally anticipated (39). In some cases, the therapeutic success is based on their opportunistic cross-reactivity profile such as the inhibition of Kit by the Abl inhibitor Imatinib, which is used in the treatment of GIST patients (40). Additional examples of multi-targeted drugs are Sorafenib (BAY 43-9006) or Sunitinib (SU11248). Sorafenib has been developed as a Raf kinase inhibitor, but among its molecular targets, there are several receptor tyrosine kinases involved in angiogenesis [vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptor-β (PDGFRβ)] and tumorigenesis (Flt-3, c-Kit, and Ret) that might account for its antitumor effects in the clinic (41, 42). Sunitinib is an oral multi-targeted tyrosine kinase inhibitor with antitumor and antiangiogenic activities through targeting PDGFRβ, VEGFRs, c-Kit, and Flt-3 (43). Promising clinical activity in the treatment of advanced solid tumors as well as in hematologic malignancies has been shown (44, 45). Such success stories have raised the interest in additional agents hitting multiple targets to achieve better single-agent efficacy in a broader range of tumors and a lower probability of resistance.

In addition to the inhibition of Aurora kinases, PHA-739358 shows also activity against c-Abl, Ret, and Trk-A. These additional molecular targets might contribute to its activity in particular in tumor cells, which are highly dependent on these kinases for growth or survival. Gain-of-function mutations in the Ret proto-oncogene are responsible for the development of medullary and papillary thyroid carcinoma, the most common form of endocrine tumor in humans, and make it a potential target for molecular cancer therapy (46). In the same indication, Trk-A was found to be constitutively activated due to a gene rearrangement (24). In addition, Trk-A is expressed in other tumors such as prostatic and pancreatic carcinoma (25). Interestingly, Aurora B levels are correlated to the stage of malignant progression of thyroid carcinoma, and the block of the kinase activity reduces the growth of thyroid carcinoma cells (47). An inhibitor targeting simultaneously three relevant kinases important for tumor growth and/or progression might develop into a treatment option for advanced thyroid carcinoma.

PHA-739358 is able to inhibit wild-type Abl as well as the most clinically frequent Imatinib-resistant Abl mutants, including the T315I mutant (22). Inhibition is due to the ability of PHA-739358 to bind with high affinity to the Abl kinase domain as revealed by the co-crystal structure. Interestingly, at least two other Aurora kinase inhibitors (VX-680/MK-0457 and AT-9283) are also active against Abl, including the T315I mutant, and this might translate into a therapeutic value for the treatment of CML patients as has been described recently for VX-680/MK0457 (48).

Moreover, the application of a PK/PD model for predicting the TGI in mice (27) successfully describes the tumor growth in our preclinical efficacy studies, providing relevant parameters to support clinical development. Several Aurora inhibitors with different selectivity profiles have entered clinical trial, and others are under way. The true value for inhibition of Aurora kinases as a potent anticancer strategy still needs to be proven in the clinic, but many currently ongoing clinical trials will give this answer in the very near future, including the question of which Aurora kinase is the better cancer target. Hitting simultaneously more than one mitosis-relevant kinase might overcome point mutations as a mechanism of resistance as experienced, for example, in Bcr-Abl–bearing CML patients treated with Imatinib (49). The favorable preclinical profile of PHA-739358, which is currently in phase II clinical trials, makes the compound a strong candidate to contribute answers to these questions.

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Molecular Cancer Therapeutics

PHA-739358, a potent inhibitor of Aurora kinases with a selective target inhibition profile relevant to cancer

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