1521-0103/12/3433-617–627$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 343, No. 3
197087/3804403

JPET 343:617–627, 2012

Preclinical Characterization of ABT-348, a Kinase Inhibitor Targeting the Aurora, Vascular Endothelial Growth Factor Receptor/Platelet-Derived Growth Factor Receptor, and Src Kinase Families

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Received May 31, 2012; accepted August 21, 2012

ABSTRACT

ABT-348 [1-(4-(4-amino-7-(1-(2-hydroxyethyl)-1*H*-pyrazol-4-yl)thieno[3,2-*c*]pyridin-3-yl)phenyl)-3-(3-fluorophenyl)urea] is a novel ATP-competitive multitargeted kinase inhibitor with nanomolar potency (IC_{50}) for inhibiting binding and cellular autophosphorylation of Aurora B (7 and 13 nM), C (1 and 13 nM), and A (120 and 189 nM). Cellular activity against Aurora B is reflected by inhibition of phosphorylation of histone H3, induction of polyploidy, and inhibition of proliferation of a variety of leukemia, lymphoma, and solid tumor cell lines (IC_{50} = 0.3–21 nM). In vivo inhibition of Aurora B was confirmed in an engrafted leukemia model by observing a decrease in phosphorylation of histone H3 that persisted in a dose-dependent manner for 8 h and correlated with plasma concentration of ABT-348. Evaluation of ABT-348 across a panel of 128 kinases revealed additional potent binding activity (K_i < 30 nM) against vascular endothelial growth factor receptor (VEGFR)/platelet-derived growth factor receptor (PDGFR) families and the Src family of cytoplasmic tyrosine kinases. VEGFR/PDGFR binding activity correlated with inhibition of autophosphorylation in cells and inhibition of vascular endothelial growth factor (VEGF)-stimulated endothelial cell proliferation ($IC_{50} \leq 0.3$ nM). Evidence of on-target activity in vivo was provided by the potency for blocking VEGF-mediated vascular permeability and inducing plasma placental growth factor. Activity against the Src kinase family was evident in antiproliferative activity against BCR-ABL chronic myeloid leukemia cells and cells expressing the gleevec-resistant BCR-ABL T315I mutation. On the basis of its unique spectrum of activity, ABT-348 was evaluated and found effective in representative solid tumor [HT1080 and pancreatic carcinoma (MiaPaCa), tumor stasis] and hematological malignancy (RS4;11, regression) xenografts. These results provide the rationale for clinical assessment of ABT-348 as a therapeutic agent in the treatment of cancer.

Introduction

Targeting kinase-mediated signaling pathways is a proven approach in the search for cancer therapies (Clark et al., 2005; Rugo et al., 2005; Baka et al., 2006; Motzer et al., 2006). Notable among the cancer-relevant kinases that have been successfully targeted are the VEGF and PDGF receptor tyrosine kinases, which have been implicated in tumor progression through multiple distinct mechanisms, including angiogenesis (Fong et al., 1995; Rafii et al., 2002; Ferrara et al., 2003; Peters et al., 2005), lymphangiogenesis (Lyden et al.,

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
http://dx.doi.org/10.1124/jpet.112.197087.

ABBREVIATIONS: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CSF-1R, colony-stimulating factor 1 receptor; DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging; ELISA, enzyme-linked immunosorbent assay; ABT-348, 1-(4-(4-amino-7-(1-(2-hydroxyethyl)-1*H*-pyrazol-4-yl)thieno[3,2-*c*]pyridin-3-yl)phenyl)-3-(3-fluorophenyl)urea; ABT-869, 1-[4-(3-amino-1*H*-indazol-4-yl)phenyl]-3-(2-fluoro-5-methylphenyl)urea; A-993352, 4-amino-*N*-(3-((3-(4-chloro-2-fluorophenyl)ureido)methyl)phenyl)thieno[2,3-*d*]pyrimidine-5-carboxamide; AZD1152, barasertib; PHA-739358, danusertib; PPHA-739358, (*R*)-*N*-(5-(2-methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-*c*]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide; ENMD-2076, (*E*)-*N*-(5-methyl-1*H*-pyrazol-3-yl)-6-(4-methylpiperazin-1-yl)-2-styrylpyrimidin-4-amine; R763, (1*S*,2*S*,3*R*,4*R*)-3-((5-fluoro-2-((3-methyl-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)bicyclo[2.2.1]hept-5-ene-2-carboxamide; APC, allophycocyanin; HUVEC, human umbilical vein endothelial cells; DMSO, dimethyl sulfoxide; MDR, multidrug resistance protein; NSCLC, non–small-cell lung carcinoma; PBS, phosphate-buffered saline; KIT, mast/stem cell growth factor receptor; FBS, fetal bovine serum; PLGF, placental growth factor; MWF, Monday, Wednesday, Friday; FLT, fms-like tyrosine kinase.

2001; Alitalo et al., 2005), and host-tumor cell interactions (Viloria-Petit et al., 2001; Kunz-Schughart and Knuechel, 2002; Cheng and Weiner, 2003; Dong et al., 2004; Pollard, 2004; Lewis and Pollard, 2006). This group of kinases has been validated as cancer targets by the clinical success of a number of small-molecule inhibitors (Slevin et al., 2008; Soltau and Drevs, 2009; Bhargava and Robinson, 2011). The Aurora kinases are another family of kinases that have been linked to cancer based on amplification (Aurora A) or overexpression (Aurora B and C) in tumors (Bischoff et al., 1998; Kimura et al., 1999; Kurai et al., 2005; Smith et al., 2005; Sorrentino et al., 2005; Hienonen et al., 2006) and the ability to potentiate the transforming activity of H-Ras (Kanda et al., 2005). The Aurora kinase family mediates multiple essential events in cell division (Andrews, 2005; Giet et al., 2005; Fu et al., 2007), providing the rationale for developing small-molecule inhibitors as antimitotic agents (Brave et al., 2008). In addition, members of the Src family of tyrosine kinases have been implicated in solid tumors as well as hematologic malignancies, such as chronic myelogenous leukemia and acute lymphoblastic leukemia (Johnson and Gallick, 2007; Li, 2008).

Agents that target either VEGF/PDGF or Aurora kinase signaling pathways have provided clinical benefit (for reviews, see Bhargava and Robinson, 2011; Farag, 2011). However, targeting these pathways separately may have limitations. Although the inhibition of VEGF/PDGF signaling slows tumor progression, the overall survival of patients with solid tumors is not always extended, suggesting the emergence of resistance (Ramalingam and Belani, 2011; Roodink and Leenders, 2011). Therapy with experimental Aurora inhibitors has also resulted in clinical benefit in some hematological cancers, but to date, therapy with Aurora-selective inhibitors has not proven effective with patients with solid tumors (Kelly et al., 2011). An approach to further exploit the therapeutic utility of kinase inhibition while possibly overcoming the apparent shortcomings of relatively selective agents is to use multitargeted therapy that inhibits both angiogenesis and proliferation associated with tumor growth. To that end, we embarked on a medicinal chemistry-directed search and discovered ABT-348 [1-(4-(4-amino-7-(1-(2-hydroxyethyl)-1*H*-pyrazol-4-yl)thieno[3,2-*c*]pyridin-3-yl)phenyl)-3-(3-fluorophenyl)urea], a novel kinase inhibitor with activity toward Aurora kinases (Aurora A, B, and C), the VEGF and PDGF kinase families, and the Scr family of kinases. The preclinical pharmacology of ABT-348 is described herein.

Methods and Materials

The following compounds were synthesized at Abbott Laboratories (Abbott Park, IL): ABT-348 (Curtin et al., 2012); ABT-869 [1-[4-(3-amino-1*H*-indazol-4-yl)phenyl]-3-(2-fluoro-5-methylphenyl)urea] (Dai et al., 2007); and A-993352 [4-amino-*N*-(3-((3-(4-chloro-2-fluorophenyl)ureido)methyl) phenyl)thieno[2,3-*d*]pyrimidine-5-carboxamide] (McClellan et al., 2011). AZD1152 (barasertib) and PHA-739358 (danusertib) were obtained commercially (Selleckchem, Houston, TX).

Cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and were cultured by following the recommendations of the supplier. HUVEC (pooled donors of mixed gender) were obtained from Lonza Inc. (Allendale, NJ).

Kinase Assays. Potencies (IC_{50} values) were determined by assays of active kinase domains cloned and expressed in baculovirus using the FastBac baculovirus expression system (Invitrogen, Carlsbad, CA) or obtained commercially. For tyrosine kinase and Aurora kinase assays, a biotinylated peptide substrate was used with 1 mM ATP, an Eu-cryptate-labeled antiphosphotyrosine or antiphosphopeptide antibody, and streptavidin-APC in a homogeneous time-resolved fluorescence assay (Albert et al., 2006). Ser/Thr kinases were assayed using 5 μM ATP, [^{33}P]ATP, and a biotinylated peptide substrate with peptide capture and incorporation of ^{33}P determined using a SA-Flashplate (PerkinElmer Life and Analytical Sciences, Waltham, MA) (Luo et al., 2004). For kinase and cellular assays described below, the concentration resulting in 50% inhibition (IC_{50}) of activity and 95% confidence limits were calculated using nonlinear regression analysis of the concentration response data (Symyx Assay Explorer; Accelrys, Inc., San Diego, CA). Mutant Aurora B (Y156H) was produced by site-directed mutagenesis of the Aurora B kinase cDNA, expressed in baculovirus, and assayed in the homogeneous time-resolved fluorescence format as described above.

Receptor Phosphorylation. An ELISA-based screen in 3T3 murine fibroblasts engineered to express human VEGFR2 was used to evaluate receptor phosphorylation and is described in detail elsewhere (Guo et al., 2006). Functional aspects of plasma protein binding were evaluated in the VEGFR2 cell assay by including 50% plasma in the medium (plasma purchased from Bioreclamation, Westbury, NY). NIH3T3 cells engineered to express a VEGFR2:KIT chimera (VEGFR2 extracellular domain fused to KIT transmembrane and intracellular domains) were used to quantify the inhibition of KIT. PDGFRα and PDGFRβ phosphorylation was performed in MG63 osteosarcoma cells stimulated with PDGFAA or PDGFRBB, respectively. fms-like tyrosine kinase (FLT)-3 phosphorylation was determined in the SEM cell line (acute lymphocytic leukemia), where autocrine phosphorylation of FLT-3 occurs. In brief, autocrine or stimulated (with appropriate ligand) cells were incubated with inhibitor for 20 min and stimulated for 10 min with ligand. For autocrine phosphorylation (FLT-3), cells were incubated with compound for 2 h before lysate preparation. Lysates were prepared and added to precoated ELISA plates and processed according to the manufacturer's protocol. For CSF-1R phosphorylation, NIH3T3 cells engineered to express human CSF-1R were stimulated for 10 min with macrophage-CSF (200 ng/ml), lysed, and processed for Western blot by immunoprecipitation with anti-CSF-1R antibody (EMD Oncogene Research Products, San Diego, CA) or probed directly with anti-PY-721 CSF-1R antibody (Cell Signaling Technologies, Danvers, MA). Human embryonic kidney 293 cells expressing full-length human VEGFR3 were stimulated with VEGF-C for 10 min before lysate preparation and Western blot analysis as described for CSF-1R. The antiphosphotyrosine bands were normalized using the total receptor bands, and the percentage of inhibition was calculated at each concentration of inhibitor. BaF3/VEGFR1 (VEGFR1 catalytic domain: TEL fusion) cells were purchased from Advanced Cellular Dynamics (San Diego, CA), cells were incubated with compound for 24 h and assayed for VEGFR1-dependent proliferation as a surrogate cellular assay for VEGFR1 activity.

Aurora Phosphorylation. Autophosphorylation of Aurora A was determined in HeLa cells, and that of Aurora B and C was determined in HCT-116 cells. Cells were treated with nocodazole (Sigma-Aldrich, St. Louis, MO) at 1 μg/ml for 16 h (overnight). Cells were treated with compound for 1 h (0.1% DMSO final), and cell lysates were prepared for Western blot. Cell lysates were electrophoresed and transferred to polyvinylidene difluoride membranes according to the manufacturer's instructions. Membranes were probed with antiphospho-Aurora A, B, and C (Cell Signaling Technologies) and then visualized with Alexa Fluor 680 goat anti-rabbit antibody (Invitrogen on the Odyssey Li-Cor imaging system (LI-COR Biosciences, Lincoln, NE). Fluorescence was digitized, and appropriate bands were quantified to determine percentage inhibition of control signal.

Three-Day Proliferation. Carcinoma cells (2500 cells/well) were plated overnight in full-growth medium (containing 10% FBS). Compound was added to the cells in full-growth medium and incubated for 72 h at 37°C in a CO_2 incubator. For leukemia cells, generally 50,000 cells/well were plated in full-growth medium, drug was added, and they were incubated for 72 h. The effects on proliferation were determined by the addition of alamarBlue (final solution 10%; BioSource International, Camarillo, CA), incubation for 4 h, and analysis in a fluorescence plate reader (excitation 544; emission 590), or alternatively, medium was removed and replaced with 200 μl of Cell TiterGlo reagent (Promega, Madison, WI) and analyzed for luminescence. Noncycling primary HUVEC were used to assess the effect of ABT-348 on nonproliferating cells (Hardwicke et al., 2009). Cells (35,000/well) were seeded in growth medium in a 96-well tissue culture plate, and after 2 days, the medium was changed and the cells were treated with ABT-348. After an additional 3 days, cell viability was measured with Cell TiterGlo reagent.

Colony Formation Assay. For colony formation assays, 500 cells/well were seeded into six-well plates in drug-free medium. Twenty-four hours later, compounds were added to the cells, which were cultured for 7 to 10 days in a CO_2 incubator. Cells were then fixed and stained with 0.2% crystal violet to visualize and count colonies.

Induction of Polyploidy by Cell Cycle Analysis. Cell cycle analysis of polyploidy was performed by fluorescence-activated cell sorting using H1299 or H460 carcinoma cells. After 24-h exposure to compound in complete growth medium (containing 10% FBS), the cells were stained with nuclear isolation and staining solution for analysis of nuclear DNA content as described previously(Wilkinson et al., 2007).

Phosphorylation of Histone H3. HCT-116 cells plated at 100,000 cells/well were treated overnight (16 h) with nocodazole (Sigma-Aldrich) at 1 μg/ml in complete growth medium (Dulbecco's modified Eagle's medium with 10% FBS). Medium was removed from each well, and 100 μl of complete growth medium with compound (0.1% DMSO final) was added for 1 h. The medium was removed, and 100 μl of lysis buffer (Cell Signaling Technologies) was added to each well and incubated with shaking for 30 min at 4°C. Cell lysate (80 μl) was added to each well of the PathScan P-histone H3 (Ser_{10}) Sandwich ELISA plate (Cell Signaling Technologies) and processed according to the manufacturer's instructions. IC_{50} values were determined by nonlinear regression analysis of the concentration response curves. Western blot analysis was used for pharmacokinetic studies in solid tumors. Frozen solid tumor samples were pulverized and homogenized in 2 ml of lysis buffer (Invitrogen) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). The samples were sonicated and clarified by centrifugation, and 20 μl of the supernatant was loaded onto NuPAGE 4 to 12% Bis-Tris gel for Western blotting. Antiphospho-histone H3 and histone H3 antibodies (p-Ser^{10}, Cell Signaling Technologies) were used for detection.

To conduct pharmacodynamic studies with circulating tumor cells, blood (120 μl) was obtained by jaw bleeding from mice 4 to 5 weeks after engraftment with RS4;11 cells and mixed with 4 ml of Lyse/Fix buffer (BD Biosciences, San Jose, CA). After 30 min at room temperature, the cellular fraction was separated by centrifugation (1800 rpm for 8 min), washed with PBS, resuspended in 0.5 ml of PBS containing 1% FBS in PBS, and stored at 4°C for up to 3 days. On the day of analysis, cell fractions were washed twice with PBS and then incubated for 20 min with 10 μl of human IgG and 20 μl of mouse serum (Sigma-Aldrich) to block Fc receptor expression. The cells were then washed with PBS and lysed by adding, with vortex mixing, 0.5 ml of ice-cold Phospho Flow Perm buffer II (BD Biosciences). After 30 min on ice, the cells were washed first with PBS and then with staining buffer (BD Biosciences). For antibody staining, 20 μl of APC-antihuman CD45 (BD Biosciences) and 20 μl of fluorescein isothiocyanate-human phosphohistone H3 (Cell Signaling Technologies) were added to the cells, and the suspension was incubated at room temperature in the dark for 60 to 120 min. The cells were then washed with PBS and resuspended in 400 μl of staining buffer. The proportion of human-CD45-positive cells that were positive for human phosphohistone H3 was determined by two-color fluorescence-activated cell sorting analysis (BD LSR II; Invitrogen) counting 150,000 events/sample (forward scatter 358; side scatter 330; fluorescein isothiocyanate Alexa, 290; APC Alexa, 420).

Plasma Concentration of ABT-348. Plasma samples were dispensed into 96-well plates, and proteins were precipitated using acidified methanol. Supernatants were stored at −20°C. Sample analyses were performed by liquid chromotography-mass spectrometry using a Shimadzu 10A-VP chromatography system with a Waters YMC-AQ 5-cm column. The mobile phase consisted of 30% acetonitrile and 0.1% acetic acid in water, flow rate 0.4 ml/min. Mass detection was accomplished with an, electrospray ionization-equipped LCQ-Duo by Thermo Fisher Scientific (Waltham, MA). External standards were prepared from spiked control plasma and used to generate a response factor for every study. Limits of detection were between 20 and 50 nM.

Uterine Edema. Dosing solutions of ABT-869 and ABT-348 were prepared using the following formulation: 2.5% DMSO, 10% Solutol HS-15 (BASF, Florham Park, NJ), and 87.5% 25 mM tartaric acid. To initiate the study, BALB/c mice (Harlan, Indianapolis, IN) at least 12 weeks of age were dosed with 10 IU of pregnant mare serum gonadotropin (Calbiochem, San Diego, CA) 72 and 24 h before compound administration. Mice were then randomized, weighed, separated into groups ($n = 6$) and given either a test compound or vehicle by intravenous administration. Thirty minutes later, the vehicle and test groups received an intraperitoneal injection of 25 μg of 17 β-estradiol (Sigma-Aldrich). Sham-control animals received an injection of sterile water. Mice were euthanized 3 h after compound and vehicle administration. Uteri were excised, fat and connective tissue were removed, and uteri were weighed. Mean increase in weight above sham controls was computed and used to calculate the percentage of inhibition of vehicle-treated control.

PLGF. PLGF was measured using a murine PLGF-2 ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's instructions with the following exception: detection was performed using SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific) to increase assay sensitivity.

Human Tumor Xenografts. All in vivo studies were conducted in compliance with Abbott's Institutional Animal Care and Use Committee and the National Institutes of Health *Guide for Care and Use of Laboratory Animals* guidelines in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The DCE-MRI study in an orthotopic rat glioma model was conducted as described previously (Luo et al., 2012). For flank xenograft models, cells were suspended in PBS, mixed with Matrigel (phenol red free; BD Biosciences) in a ratio of 1:4 (v/v), and inoculated into the flank of female SCID/beige mice (5 million cells per site). Inoculated mice were randomized into groups of 10, and treatment was initiated when mean tumor volume was approximately 0.4 cm^3 (HT1080) or 0.5 cm^3 (MiaPaCa, RS4;11). Tumor growth in the flank was assessed by measuring tumor size with calipers and calculating volume using the formula ($L \times W^2/2$). Study groups were terminated before tumor volume reached 3 cm^3. Inhibition of tumor growth was assessed at the time the vehicle-treated group was terminated by calculating the ratio of the mean volume of the test drug group to the mean volume of the untreated (control) group (T/C) and calculating the percentage of inhibition of control [$(1 - T/C) \times 100$]. The dosing formulation of test agents was prepared by stepwise addition, with mixing, of the following reagents: EtOH, Tween 80, polyethylene glycol 400, and 2% hydroxypropyl methylcellulose (2:5:20:73, v/v). Dosing volume was 10 ml/kg.

For engraftment studies, female NOD/SCID mice (22–26 g) were irradiated (250 rad/mouse) 1 day before engraftment. On the day of engraftment, the mice were inoculated (intravenously, tail vein) with 10 million RS4;11 human leukemia cells in 0.2 ml of phosphate-

buffered saline, pH 7.4. Treatment with test compound or vehicle was initiated 21 days after engraftment (10 mice per group). Survival time was monitored daily and defined as duration to expiration or morbidity. Signs of morbidity (e.g., ≥20% body weight loss, lethargy, tachypnea) were assessed using a stress scoring system that monitored appearance, hydration, natural behavior, provoked behavior, and respiratory signs. Survival data were analyzed using JMP software (SAS Institute Inc., Cary, NC).

Results

In Vivo Proof of Concept. The search for a molecule with properties of ABT-348 (Fig. 1) was spurred by the hypothesis that combining in a single molecule potent anti-VEGFR and PGFR activity with the antimitotic activity of pan Aurora inhibition would lead to favorable antitumor activity. To validate this hypothesis in the absence of such a molecule, initial studies were conducted in a human fibrosarcoma xenograft model (HT1080) using available selective inhibitors to confirm the benefit of combining Aurora inhibition with antiangiogenic activity. This study used a small-molecule inhibitor (ABT-869) that has been previously shown to selectively target the VEGF/PDGF axes [enzyme IC_{50} = 4 and 66 nM (Albert et al., 2006)] and a selective tool inhibitor of Aurora kinases (A-993352), representative of the thienopyrimidine class [IC_{50} = 193 and 3 nM for inhibition Aurora A and B (McClellan et al., 2011)]. When given at their maximally efficacious dose (100 mg/kg p.o. and 20 mg/kg i.p., respectively), both of these agents significantly inhibited growth of the xenograft (Fig. 2). However, combining the two inhibitors led to efficacy greater than that achievable with either inhibitor alone. It is noteworthy that the increased combination efficacy was not associated with tolerability issues (e.g., no increase in weight loss with combination treatment). These results supported the concept that an inhibitor targeting the Aurora, VEGFR, and PDGFR kinase families will have greater efficacy than selective agents and prompted the medicinal chemistry effort to identify a kinase inhibitor that targets these kinase families (Curtin et al., 2012).

Kinase Inhibitory Activity and Selectivity. The potency of ABT-348 for inhibiting a panel of cancer-relevant kinases is presented in Table 1. ABT-348 proved to be a potent inhibitor of all three Aurora kinases, although it has greater activity versus Aurora B and C than Aurora A. The activity against wild-type Aurora B extends to mutant forms

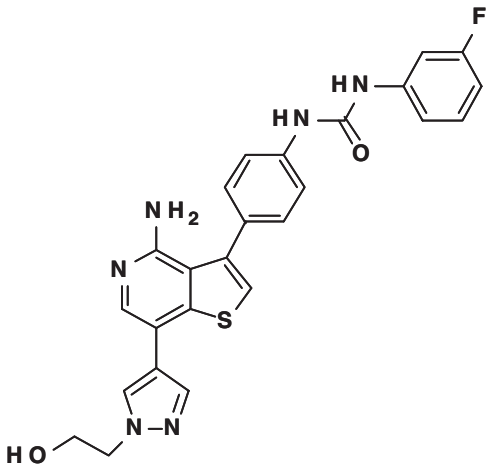

Fig. 1. Structure of ABT-348.

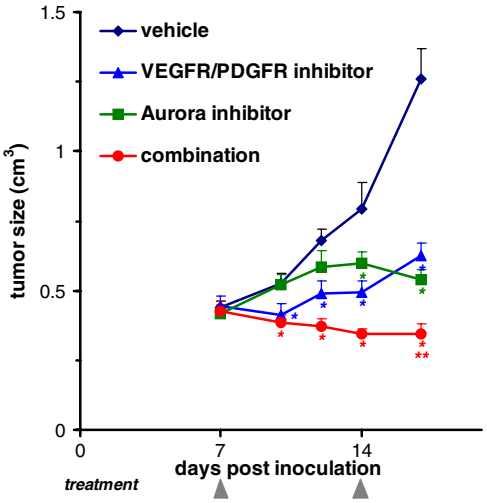

Fig. 2. An effect of selective agents as monotherapy and in combination in a fibrosarcoma flank xenograft (HT1080) given at their maximal efficacious dose, once weekly. Tumor volumes are expressed as mean ± S.E.M., n = 10 per group.*, $P < 0.05$ versus control; **, $P < 0.05$ vs. monotherapies.

TABLE 1
ABT-348 enzyme and cellular pharmacodynamic potency

Kinase	Biochemical IC_{50}, nM[a] (95% Confidence Limits)	Cellular PD Marker IC_{50}, nM (95% Confidence Limits)
Aurora A	120 (117–123)	189 (153–233)[b]
Aurora B	7 (2–14)	13 (5–27)[b] 21 (11–42)[c]
Aurora B (Y156H)[d]	12 (11–17)	N.D.
Aurora C	1 (1–2)	13 (5–27)[b]
VEGFR1	1 (0.6–2)	0.3 (0.1–0.4)[e]
VEGFR2	2 (1–3)	5 (4–7)[e]
VEGFR3	43 (18–93)	2 (0.1–23)[e]
FLT-3	1 (0.9–2)	2 (2–3)[e]
CSF-1R	3 (2–4)	3 (0.8–8)[e]
c-KIT	20 (6–25)	45 (33–64)[e]
PDGFR-α	11 (6–21)	16 (6–19)[e]
PDGFR-β	13 (1–46)	11 (4–28)[e]

N.D., not done.
[a] Enzyme assays were conducted in HTRF format using 1 mM ATP.
[b] Aurora A, B, and C autophosphorylation was performed in nocodazole-arrested HeLa cells by Western analysis using phospho-A, -B, and -C-specific antibodies.
[c] Phosphorylation of histone H3.
[d] Aurora B kinase Y156H mutant.
[e] Cellular phosphorylation assays for VEGFR2, CSF-1R, KIT, PDGFR-α, and PDGFR-β were ligand stimulated for 5 to 20 min for optimal phosphorylation depending on receptor type. FLT-3 was constitutively phosphorylated in SEM cells, inhibition was determined after 60 min of exposure to the compound. VEGFR1 activity was determined in BaF3 cells expressing the TEL:VEGFR1 catalytic domain fusion using proliferation as a surrogate readout.

of the enzyme, including the Y156H catalytic site mutation, which has been reported to interfere with binding by small-molecule inhibitors (Girdler et al., 2008). The ability of ABT-348 to retain activity against the mutant form may lower the probability of the emergence of resistance associated with more selective agents.

The rank order of potency of ABT-348 to inhibit the Aurora kinases (Aurora B ≈ Aurora C > Aurora A) was also observed in the molecule's ability to inhibit autophosphorylation of Aurora kinases in nocodazole-arrested cancer cells (Table 1). Taken together with the enzyme activity, these results help to confirm on-target inhibition of Aurora kinases in a cellular setting.

In addition to targeting Aurora kinases, ABT-348 is a potent inhibitor of the VEGFR and PDGFR kinase families and, to a lesser extent, the Src family of cytoplasmic tyrosine

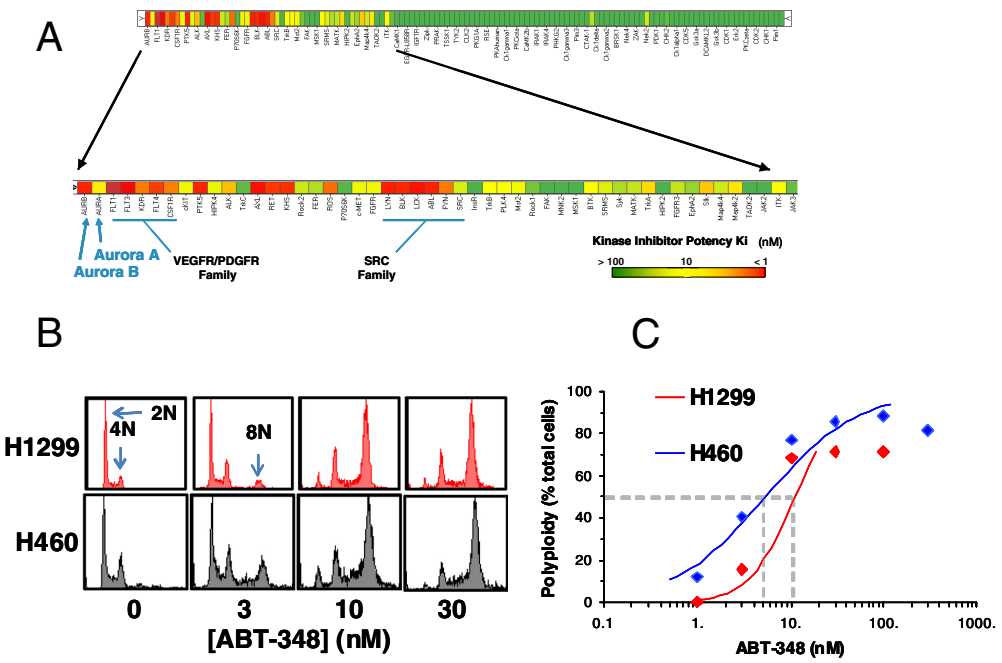

Fig. 3. A, kinome inhibition profile of ABT-348. B, flow cytometric analysis of the induction of polyploidy (8N) in NSCLC cell lines incubated with ABT-348 for 24 h. C, concentration dependence of polyploidy induction after exposure to ABT-348 for 24 h.

kinases. The activity is evident in enzyme assays performed at an ATP concentration (1 mM) approximating that in cancer cells (Table 1). As was the case with the Aurora kinase activity, inhibition of VEGFR/PDGFR kinases in the biochemical assays correlated with inhibition of autophosphorylation of targeted kinases in cells for VEGFR2, FLT-3, CSF-1R, KIT, PDGFRα, and PDGFRβ.

An overview of the activity of ABT-348 across a broader range of kinases (128) is provided in Fig. 3A. The potent Aurora, VEGFR/PDGFR, and Src activity evident in the kinome profiling provides a unique "signature" inherent to ABT-348 that has the potential to distinguish it from the other Aurora kinase inhibitors.

Functional Cellular Activity and Evidence of On-Target Inhibition. The nanomolar potency of ABT-348 against Aurora B is reflected in cellular responses, including the induction of polyploidy, a hallmark of the essential role of Aurora B in cytokinesis. As illustrated in Fig. 3B for two NSCLC cell lines, ABT-348 induces a concentration-dependent increase in the extent and number of cells exhibiting polyploidy. The potency for inducing this response (EC_{50} = 5 and 10 nM; Fig. 3C) correlates well with cellular potency for inhibiting Aurora B and provides further support of mechanism-based activity. Inhibition of Aurora B activity in a cellular setting was further confirmed by measuring phosphorylation of histone H3, an Aurora B substrate. The potency for inhibiting this response (IC_{50} = 21 nM; Table 1) is in close agreement with potency for inhibiting autophosphorylation of Aurora B (13 nM; Table 1).

The cellular effects of ABT-348 on Aurora activity are in line with the molecule's ability to inhibit tumor cell growth, as proven by the effect on proliferation of leukemia and lymphoma cell lines or on the extent of colony formation by solid tumor cell lines (Table 2). Leukemia cells in general responded rapidly to the induction of polyploidization, usually within 72 h, after which the potency value (IC_{50}) remained relatively constant. In contrast, epithelial carcinoma cell lines were much more resistant in the shorter time frame

TABLE 2
Potency of ABT-348-antiproliferative effects

Cell Line	Cell Type	IC_{50}, nM (95% Confidence Limits)
DoHH2	Follicular lymphoma	4 (2–9)[a]
MV-4-11[c]	AML:FLT-3-ITD	0.3 (0.2–0.4)[a]
RS4;11	ALL	3 (1–8)[a]
SEM	ALL	1 (0.4–4)[a]
HNT-34[d]	AML:Bcr-Abl	68 (36–91)[a]
Loucy	ALL	2 (0.3–11)[a]
HSB2[e]	ALL:LCK-TCRB	3 (2–5)[a]
SUP B15[d]	ALL	4 (3–5)[a]
K562[d]	CML:Bcr-Abl	103 (14–759)[a]
BaF3-Bcr-Abl wt[f]	Bcr-Abl wild type	47 (8–212)[a]
BaF3-Bcr-Abl T315I[f]	Bcr-Abl T315I mutation	260 (38–315)[a]
HCT-15	Colorectal	6 (0.1–456)[b]
SW620	Colorectal	6 (3–15)[b]
H1299	NSCLC	2 (0.6–4)[b]
H460	NSCLC	2 (1–57)[b]
MiaPaCa	Pancreatic	4 (2–6)[b]
OVCAR5	Ovarian	7 (1–389)[b]
HT1080	Fibrosarcoma	5 (2–11)[b]
HUVEC	Primary endothelial	
	VEGF-stimulated	≤0.3 (89 ± 13%, 0.3 nM)[a,g]
	Nonproliferating	≥1000 (47 ± 1%, 1000 nM)[h]

[a] Cell proliferation determined in a 3-day assay.
[b] Cell proliferation determined in a 7-day colony formation assay.
[c] AML cell line harboring the FLT-3-internal tandem duplication (ITD) mutation.
[d] Ph^+ cells harboring the BCR-ABL fusion protein.
[e] Cell line harboring the t(1;7)(p34;q34) LCK-p T-cell receptor gene fusion.
[f] BaF3 murine interleukin-3-dependent B cells transfected with wild-type (wt) BCR-ABL or BCR-ABL T315I gleevec-resistant mutant.
[g] Primary HUVEC stimulated with human VEGF for 3 days in the absence of added serum.
[h] Primary HUVEC maintained under growth arrest conditions for 3 days with ABT-348.

(24–27 h) to the development of polyploidization. For longer incubation times (7–10 days), time-course studies demonstrated that a colony-forming assay format provided stable IC_{50} values for most adherent cell lines that were evaluated, which correlated with the biochemical SAR against the Aurora B kinase. The potency for growth inhibition was somewhat cell line-dependent, ranging from 0.3 (MV-4-11) to 103 nM (K562). However, it is noteworthy that there was no loss

in potency when ABT-348 was evaluated against HCT-15, a colorectal cancer cell line with a multidrug resistance (MDR) phenotype (Guo et al., 2009). Activity against the Src kinase family may contribute to the antiproliferative activity of ABT-348 against BCR-ABL expressing CML cells and cells expressing the gleevec-resistant BCR-ABL T315I mutation (IC_{50} = 47 and 260 nM).

Demonstrating the cellular response to VEGFR/PDGFR inhibition, beyond receptor phosphorylation, is more of a challenge considering that the proliferation and survival of most tumor cells does not depend on VEGFR/PDGFR signaling. However, the proliferation of endothelial cells associated with angiogenesis that occurs within tumors is highly dependent on VEGF signaling through its receptor, VEGFR2 (Neufeld et al., 1999). ABT-348 proved to be a potent inhibitor of VEGF-stimulated endothelial cell proliferation ($IC_{50} \leq$ 0.3 nM; Table 2). By comparison, a pan Aurora inhibitor devoid of VEGFR2 activity [PPHA-739358; IC_{50} = 13, 79, and 61 nM for Aurora A, B, and C, respectively (Carpinelli et al., 2007)] was ineffective as an inhibitor of this response (8% inhibition at 100 nM). Thus, the potent inhibition of VEGF-stimulated proliferation observed with ABT-348 is consistent with its VEGFR2 kinase inhibition activity and provides support for on-target inhibition of VEGFR2 in a cellular setting. In contrast to endothelial cells proliferating in response to VEGF, HUVEC in a nonproliferating state were >1000-fold less sensitive to ABT-348 (Table 2), thereby confirming the specificity of ABT-348 for cycling cells.

In Vivo Modulation of Pharmacodynamic Markers of Aurora and VEGFR Inhibition. Inhibition of Aurora B activity in vivo by ABT-348 was confirmed by measuring phosphorylation of histone H3, an Aurora B substrate, in circulating tumor cells obtained from an engrafted leukemia model. As shown in Fig. 4A, administration of ABT-348 led to an inhibition of histone H3 phosphorylation 4 h after dosing that persisted in a dose-dependent manner for at least 8 h. The extent of inhibition at 4 h from these and other studies was related to the plasma concentration of ABT-348 (IC_{50} = 3.2 μM; Fig. 4B). The potency for inhibiting this response in vivo is in close agreement with the value determined for inhibition of histone H3 phosphorylation in cells in the presence of mouse plasma (3.3 μM). This shift in potency in the presence of plasma corresponds to a relatively high level (>99%) of protein binding observed for ABT-348 in mouse blood (data not shown).

The pharmacokinetic effect of ABT-348 for Aurora inhibition in solid tumors is illustrated in Fig. 4C. Administration of ABT-348 (25 mg/kg for 24 h) via subcutaneous minipump resulted in inhibition of histone H3 phosphorylation that was evident by the end of the drug administration period and persisted, in parallel with the level of drug in tumor, throughout the length of the study (Fig. 4D). Total histone

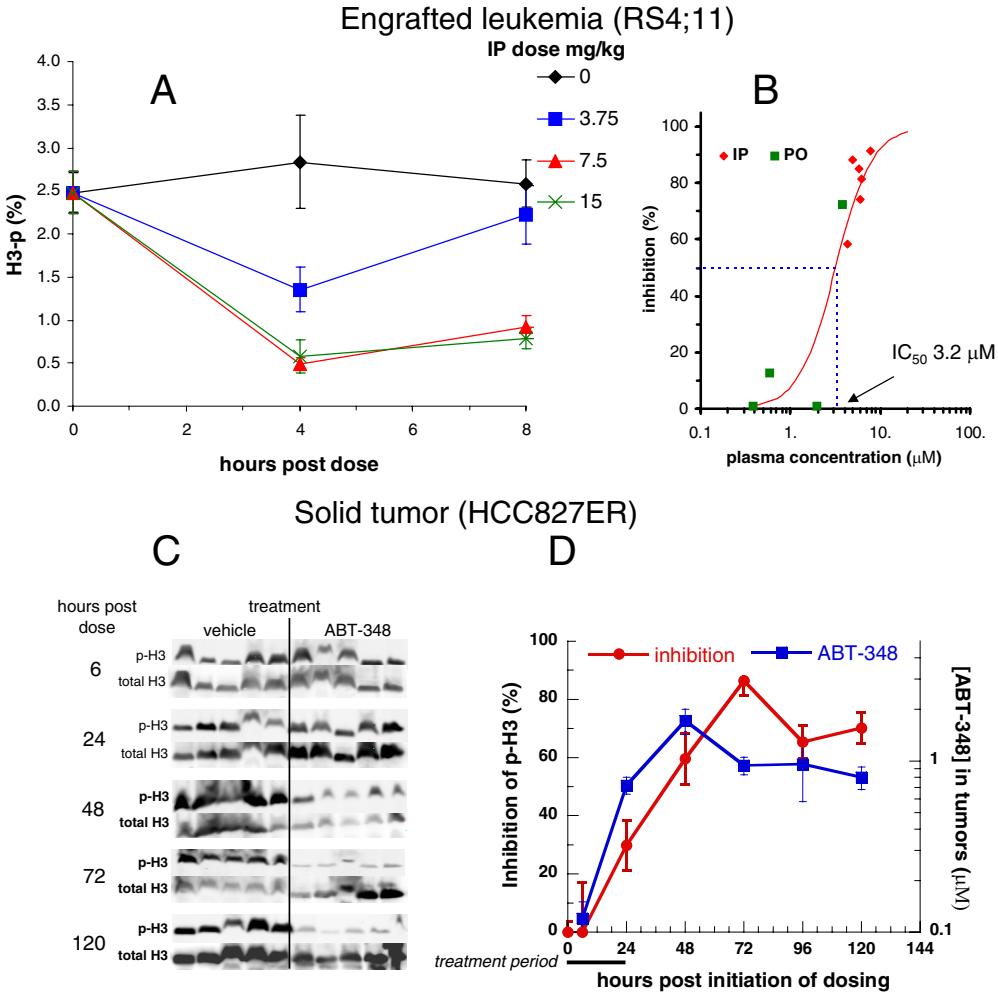

Fig. 4. A, time- and dose-dependent inhibition of histone H3 phosphorylation in blood-borne tumor cells. Blood was collected at the indicated time after administering ABT-348 to mice (n = 4–5 per group) engrafted with human RS4;11 leukemia cells for the assessment of H3 phosphorylation in human cells by flow cytometry (expressed as the mean ± S.E.M.). B, concentration-dependent inhibition of histone H3 phosphorylation in blood taken 4 h after dose. IP, intraperitoneal; PO, orally. C, inhibition of histone H3 phosphorylation in solid tumors (HCC827ER). ABT-348 (25 mg/kg) was administered subcutaneously to tumor-bearing mice using implanted osmotic minipumps for 24 h. Tumor samples were obtained at the indicated time after implantation for Western blot analysis of pan (p)-histone H3. Each lane represents a tumor sample obtained from a different animal (n = 5). D, relationship between histone H3 phosphorylation and tumor drug concentration determined by liquid chromatography-mass spectrometry. Values are expressed as mean ± S.E.M., n = 5 per group.

H3 was not consistently effected, although there did seem to be a diminution at 48 h. The tumor drug concentration associated with 50% inhibition of histone H3 phosphorylation, which occurred between 24 and 48 h, was 1 to 2 μM, ~2-fold less than that observed in the leukemia model (Fig. 4D).

Evidence that ABT-348 blocks VEGF-mediated responses in vivo was provided using a uterine edema model that is based on estradiol-induced up-regulation of VEGF expression. The vascular permeability associated with this model is a hallmark of VEGF-mediated responses (Albert et al., 2006). As shown in Fig. 5A, ABT-348 inhibited the VEGF response with a potency (ED_{50} = 0.2 mg/kg i.v.) that is comparable with another potent anti-VEGF agent, ABT-869, which has intrinsic VEGFR2 potency similar to ABT-348 (IC_{50} = 4 and 3 nM, respectively) (Albert et al., 2006). On-target VEGF receptor inhibition was also implicated by the observation that administration of ABT-348 to tumor-bearing mice resulted in increased plasma levels of the proangiogenic PLGF (Fig. 5B). Dose-proportional elevation of PLGF has been observed in responses to therapy with antiangiogenic agents, leading to the incorporation of the assay for this protein as a biomarker into the clinical development-multitargeted tyrosine kinase inhibitors (Bass et al, 2010).

Additional evidence of the anti-VEGF activity of ABT-348 via VEGFR2 inhibition in tumor growth models was obtained by observing effects on tumor vascular permeability using DCE-MRI. It has been well established that treatment with antiangiogenic agents results in an acute change in tumor perfusion evidenced by a decrease in uptake of tumor-associated contrast agent (Wong et al., 2009; Koh et al., 2011; Luo et al., 2012). We have recently reported that administration of the VEGFR/PDGFR inhibitor ABT-869 in an orthotopic brain tumor model results in a 37% reduction in tumor perfusion (measured by decreases in K^{trans}) as early as 2 h after administration and a 72% reduction after chronic administration (4 days of dosing) (Luo et al., 2012). By use of the same model, acute changes in the MRI signal were observed during treatment with ABT-348 (Fig. 5C). After a sharp decrease in K^{trans} that was apparent within 24 h after the first treatment cycle of ABT-348, the MRI signal returned to

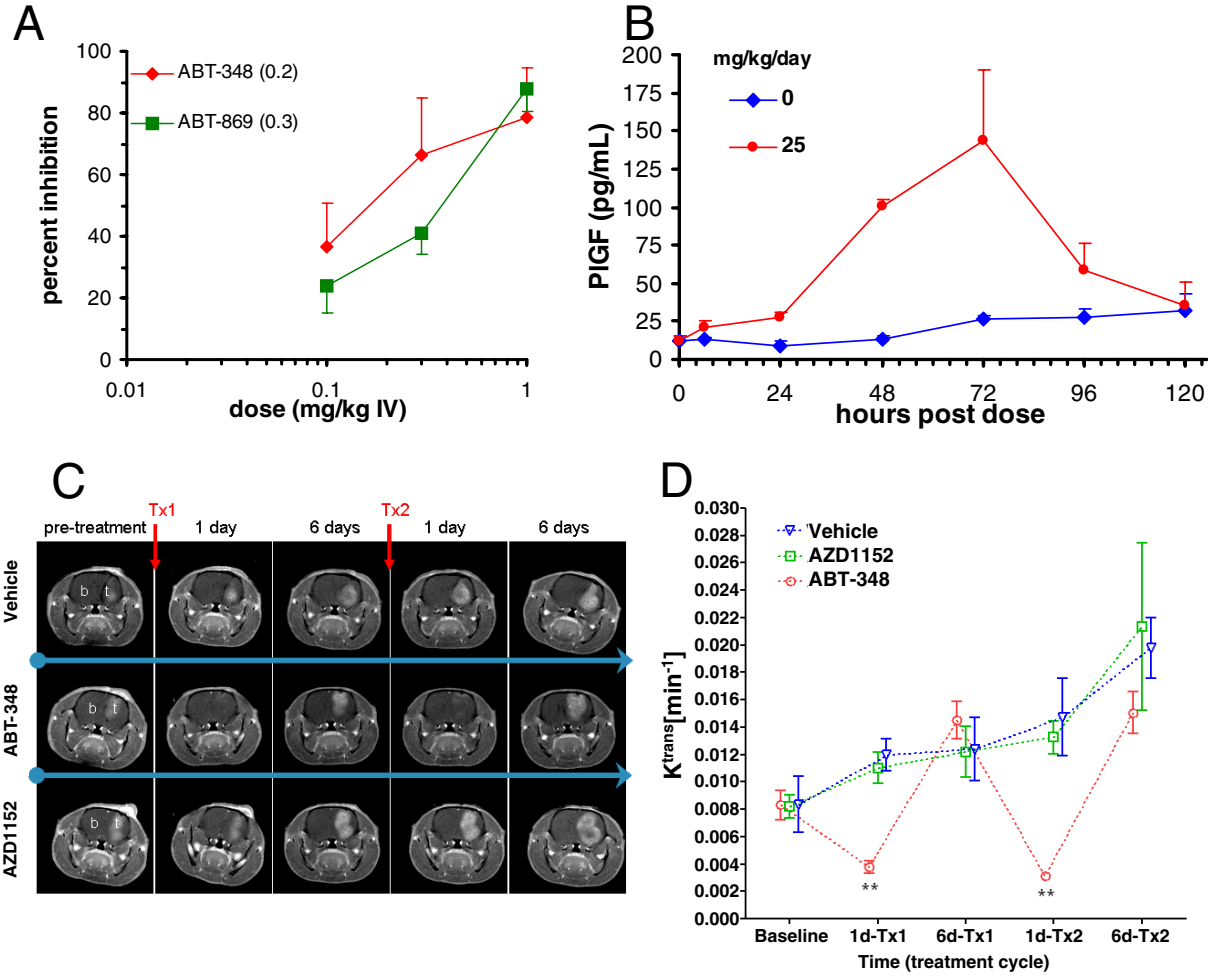

Fig. 5. A, inhibition of VEGF-mediated uterine edema. Compounds were administered intravenously at the indicated dose 30 min before estradiol challenge. Uterine edema was assessed 2 h thereafter. Inhibition >35% of the response was significantly different from vehicle-treated group ($P < 0.05$). ED_{50} (milligrams per kilogram) is shown within parentheses. Values are expressed as mean ± S.E.M., n = 6 per group. IV, intravenously. B, induction of plasma PLGF after treatment with ABT-348. Mice-bearing tumors derived from a human NSCLC cell line (HCC827ER) were treated with 25 mg/kg ABT-348 via subcutaneous osmotic minipump. At the indicated time, plasma samples were obtained and assayed for murine PLGF. Values shown are the mean ± S.E. (n = 5 per group). C, representative longitudinal MRI images showing gadolinium contrast enhancement in a rat glioma model with treatment with vehicle, ABT-348 (6.25 mg/kg i.p. b.i.d., every 7 days; two treatment cycles on days 11 and 18 after inoculation), or AZD1152 (25 mg/kg i.v., every 4 days; two treatment cycles commencing on days 11 and 18 after inoculation). b, normal brain; t, tumor, Tx1, first treatment cycle; Tx2, second treatment cycle. D, K^{trans} as a function of treatment cycle. Values represent the mean ± S.E.M., n = 12 per group. **, $P < 0.01$ vs. vehicle.

pretreatment levels by 6 days when reassessed longitudinally, which was reflective of the Q7D-dosing sequence. The same pattern of response was observed on days 1 and 6 after a second treatment cycle with ABT-348 so that, after two cycles of dosing (1d-Tx2; Fig. 5D), the reduction in K^{trans} (75%) was similar in magnitude to that previously reported for the selective VEGFR/PDGFR inhibitor. In contrast to the results obtained with inhibitors possessing VEGFR/PDGFR inhibitory activity, AZD1152, an Aurora inhibitor devoid of VEGFR2 activity, had no effect on MRI signal. Thus, the decrease in VEGF-mediated tumor perfusion observed in this study serves as further evidence of mechanism-based activity and supports the potential to distinguish ABT-348 from Aurora-selective agents.

Efficacy in Tumor Models. ABT-348 has demonstrated efficacy in xenograft models derived from solid and hematologic tumor cell lines. Administration of ABT-348 three times a week to tumor-bearing mice resulted in a dose-dependent inhibition of tumors derived from the HT1080 fibrosarcoma cell line (Fig. 6A). Both dose levels resulted in highly significant ($P < 0.001$) reductions in tumor size relative to the vehicle-treated group. However, the higher dose level (20 mg/kg/day, MWF) resulted in increased mortality and consequently was viewed as not tolerated. The lower dose tested (10 mg/kg/day, MWF) also caused weight loss but did not increase mortality relative to the vehicle treated. Therefore, it was considered marginally tolerated (weight loss <25%). In another tumor model, ABT-348 given once a week (20

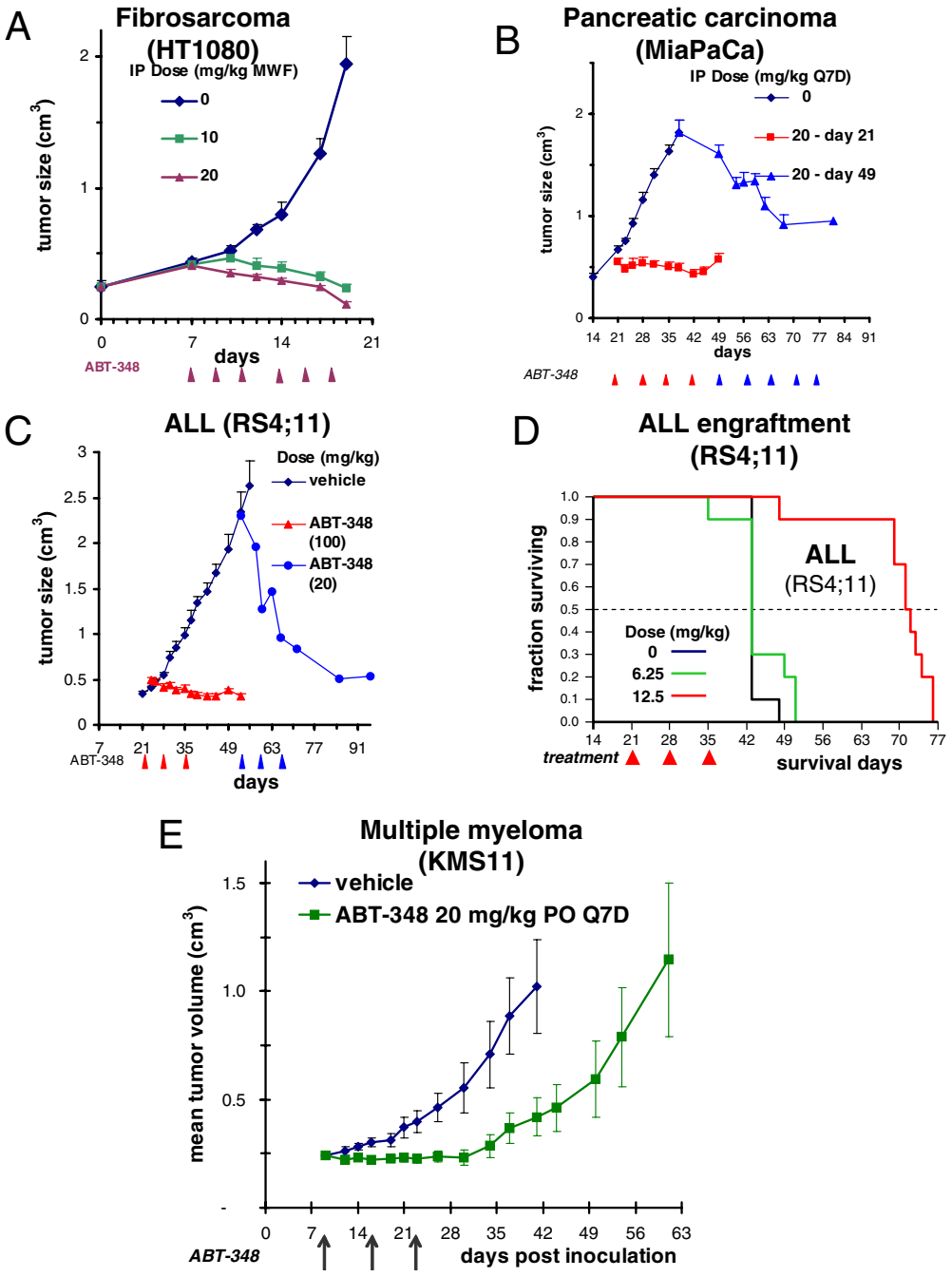

Fig. 6. Efficacy of ABT-348 in human xenograft models. A, fibrosarcoma (HT1080). Dosing [intraperitoneally (IP)] of ABT 348 or vehicle was initiated 7 days after inoculation and continued three times a week (MWF) Tumor size is represented as mean ± S.E. (n = 8–10). B, pancreatic carcinoma (MiaPaCa). Dosing (IP) of ABT 348 or vehicle was initiated 21 or 49 days after inoculation and continued once a week (mean ± S.E., n = 10). C, ALL (RS4;11, flank). Dosing (IP) of ABT 348 or vehicle was initiated 21 or 49 days after inoculation and continued once a week. D, effect of ABT-348 on survival in an engraftment model of ALL (RS4;11). SCID/nod mice (10 per group) were irradiated and engrafted with RS4;11 cells on day 0. Osmotic minipumps containing vehicle or ABT 348 were implanted subcutaneously on the indicated day after engraftment and then removed 24 h later. E, tumor growth delay in a xenograft model of multiple myeloma (KMS11). Oral administration (PO) was conducted on the indicated day. Tumor volumes represented as the mean ± S.E., n = 10.

mg/kg i.p.) inhibited the growth of pancreatic carcinoma tumors in a highly significant manner ($P < 0.001$) in the absence of increased weight loss (Fig. 6B). The same dose level was also effective in regressing and stabilizing advanced tumors (>1.5 cm^3) to 39% of the initial volume.

A similar pattern of efficacy and tolerability when dosed once a week was observed in hematologic tumor xenografts. As shown in Fig. 6C, ABT-348 inhibited the growth of established tumors (~0.5 cm^3) and caused regression of advanced tumors (>2 cm^3) resulting from leukemia cells implanted in the flank. ABT-348 was also highly effective in an engraftment model of leukemia. Mice engrafted with human ALL cells (RS4;11) began to succumb to the burden of disseminated leukemia within 6 weeks after engraftment (Fig. 6D). Intermittent treatment with ABT 348 (12.5 mg/kg) via osmotic minipumps implanted subcutaneously for 24 h once a week prolonged survival, extending the median survival by >65% from 43 to 72 days ($P < 0.001$ vs. vehicle). The lower dose of ABT-348 (6.25 mg/kg) was not effective. Potent antitumor activity of ABT-348 was also evident from the results of a xenograft model of multiple myeloma in which ABT-348 (20 mg/kg) was administered orally once weekly (Fig. 6E).

The results with intermittent dosing in solid and hematologic xenografts presented herein demonstrate that continuous systemic exposure to ABT-348 is not required for antitumor activity and that ABT-348 can be administered via oral or systemic routes. A more systematic evaluation of optimal dosing frequency and route of administration has been conducted and will be presented elsewhere (Y. Wang, L. Kaleta, L.E. Rodriguez, P. A. Ellis, G. Bukofzer, J. Clarin, S. Schlessinger, J. Li, K. Glaser, M. Michaelides, D. H. Albert, C. Tse, J. P. Palma, and C. K. Donawho, manuscript in preparation).

Discussion

ABT-348 is a novel ATP-competitive inhibitor of Aurora A, B, and C. The potency for inhibiting Aurora kinases is consistent with the ability of ABT-348 to optimally bind, based on modeling, into the extended-hinge region of the enzyme active site (Curtin et al., 2012). In addition to inhibiting the Aurora kinases, ABT-348 is a potent inhibitor of all members of the VEGF and PDGF family of receptor tyrosine kinases. The inhibition activity established in kinase assays correlated with activity in cellular phosphorylation and proliferation assays and was confirmed by the in vivo functional assays PLGF release and inhibition of VEGF-mediated permeability.

The potent activity of ABT-348 against the Aurora kinases and its unique kinase selectivity profile, including inhibition of VEGF and PDGF tyrosine kinase receptors, engender the ability to block multiple mechanisms of tumor progression. Previously identified Aurora inhibitors reported to exhibit VEGFR/PDGFR activity (e.g., ENMD2076 and R763) are, based on literature values, less potent than ABT-348 in both enzyme and cellular assays (Farag, 2011). Therefore, the VEGFR/PDGFR activity of ABT-348 constitutes a distinguishing factor relative to other Aurora inhibitors (Carpinelli and Moll, 2008). The activities of ABT-348 also extend into the Src family of cytoplasmic tyrosine kinases. Inhibition of members of the Src family has translated into therapeutics, such as dasatinib, for the treatment of gleevec-resistant CML (Brave et al., 2008). This unique spectrum of kinase inhibitory activity inherent in ABT-348 has the potential to distinguish it from the other Aurora kinase inhibitors by simultaneously affecting both the tumor cell and the tumor microenvironment.

In terms of selectivity relative to normal cells, Aurora B inhibitors in general are antimitotic and therefore are expected to target proliferating cells whether they are transformed or "normal." ABT-348 shares that property and has added inhibitory activity that allows it to target survival pathways driven by growth factors, such as VEGFR and PDGFR ligands. As a consequence, in contrast to selective Aurora kinases inhibitors, ABT-348 is a potent inhibitor of VEGF-induced proliferation of certain normal cell populations, such as endothelial cells. However, although ABT-348 has not been evaluated in vitro against a wide range of normal cell populations, the molecule does not seem to exhibit nonspecific (i.e., non–cell cycle- or nongrowth factor-mediated) cytotoxicity. This is proven by the lack of an acute effect (<3 days) of ABT-348 on the viability of adherent nonproliferating endothelial cells in culture and is further suggested by the ability to achieve an acceptable therapeutic index with this molecule in tumor models.

The initial evaluation of in vivo antitumor activity reported here indicates that ABT-348 is efficacious in xenograft models of both solid and hematological tumor types, with complete inhibition or regression observed by a once-weekly dosing regimen. Generation of additional efficacy data to further guide the design of potential clinical studies requires assessment of activity in models representing a broader range of tumor types, the subject of a separate communication (Y. Wang, L. Kaleta, L.E. Rodriguez, P. A. Ellis, G. Bukofzer, J. Clarin, S. Schlessinger, J. Li, K. Glaser, M. Michaelides, D. H. Albert, C. Tse, J. P. Palma, and C. K. Donawho, manuscript in preparation). Nonetheless, the current results demonstrate the effectiveness of ABT-348 given either once or three times a week; however, the latter dosing frequency leads to a diminished therapeutic index (increased weight loss and mortality) that will necessitate careful monitoring of toxicity during any subsequent studies done at this or greater dosing frequency.

Presently, there is no compelling rationale to accurately predict sensitivity to Aurora inhibitor therapy. All tumor cell lines examined in the current article exhibit roughly similar sensitivity to the antiproliferative effects of ABT-348 in cell culture. Furthermore, Aurora A and B expression levels have not correlated with cellular responsiveness to ABT-348 (data not shown). Studies are currently underway to evaluate broader gene expression profiles in an attempt to establish a signature that is predictive to assist in identifying potential patient populations. Characterization of these and other cell lines with respect to tissue of origin, cell doubling time, amplification/overexpression of Aurora A, overexpression of Aurora B, and p53 status has not proven useful to date in predicting the antiproliferative activity of ABT-348 or other Aurora inhibitors. It is worth noting that, as reported here, ABT-348 is effective against a cell line with a MDR phenotype (HCT-15). Subsequent work has demonstrated a lack of MDR-mediated resistance to ABT-348 in cell lines and within vivo tumor models that overexpress the major drug transporters MDR1 and breast cancer resistance protein (J. Guo, M. L. Curtin, R. Heyman, M. G. Anderson, P. A. Marcotte, P.

Tapang, J. Palma, L. E. Rodriguez, A. Niquette, J. J. Bouska, D. H. Albert, C. K. Donawho, R. R. Frey, M. Michaelides, C. Tse, K. B. Glaser, and O. J. Shah, manuscript in preparation). This lack of cross-resistance in MDR cell lines indicates that ABT-348 is not a substrate for MDR or breast cancer resistance protein transporters and that MDR expression will not be a determinate of sensitivity to ABT-348.

Although predictive indicators suitable for patient selection are not currently available, biomarkers of on-target activity are clinically assessable. The modulation of PLGF demonstrated preclinically for ABT-348 in this article has been observed previously in responses to therapy with antiangiogenic agents, leading to the incorporation of the assay for this protein as a biomarker into the clinical development multitargeted kinase inhibitors (Bass et al., 2010). Inhibition of histone H3 phosphorylation, as reported in the current study, has been used as a clinical biomarker of Aurora inhibition (Schöffski et al., 2011). Taken together, these assays should provide useful tools to help guide future clinical assessment of ABT-348 with the goal of defining its therapeutic utility and tolerability.

In summary, ABT-348 is a potent inhibitor of the Aurora, VEGFR, and PDGFR family of kinases. Targeting this array of cancer-relevant kinases results in notable efficacy in tumor xenograft models. Whether or not this preclinical profile translates into therapeutic utility with acceptable tolerability needs to be determined from the results of early-phase clinical studies in solid and hematological cancers.

Authorship Contributions

Participated in research design: Glaser, Marcotte, Donawho, Michaelides, Tse, Davidsen, and Albert.

Conducted experiments: Li, Marcotte, Magoc, Guo, Reuter, Tapang, Wei, Pease, Bui, Chen, Johnson, Osterling, Olson, Bouska, and Luo.

Contributed new reagents or analytic tools: Frey and Curtin.

Performed data analysis: Chen and Johnson.

Wrote or contributed to the writing of the manuscript: Glaser, Tse, and Albert.

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