

BAY 80-6946 Is a Highly Selective Intravenous PI3K Inhibitor with Potent p110 α and p110 δ Activities in Tumor Cell Lines and Xenograft Models

Ningshu Liu, Bruce R. Rowley[†], Cathy O. Bull, Claudia Schneider, Andrea Haegebarth, Christoph A. Schatz, Paul R. Fracasso, Dean P. Wilkie, Martin Hentemann, Scott M. Wilhelm, William J. Scott, Dominik Mumberg, and Karl Ziegelbauer

Abstract

Because of the complexity derived from the existence of various phosphoinositide 3-kinase (PI3K) isoforms and their differential roles in cancers, development of PI3K inhibitors with differential pharmacologic and pharmacokinetic profiles would allow best exploration in different indications, combinations, and dosing regimens. Here, we report BAY 80-6946, a highly selective and potent pan-class I PI3K inhibitor with subnanomolar IC₅₀s against PI3K α and PI3K δ . BAY 80-6946 exhibited preferential inhibition (about 10-fold) of AKT phosphorylation by PI3K α compared with PI3K β in cells. BAY 80-6946 showed superior antitumor activity (>40-fold) in *PIK3CA* mutant and/or HER2 overexpression as compared with HER2-negative and wild-type *PIK3CA* breast cancer cell lines. In addition, BAY 80-6946 revealed potent activity to induce apoptosis in a subset of tumor cells with aberrant activation of PI3K as a single agent. *In vivo*, single intravenous administration of BAY 80-6946 exhibited higher exposure and prolonged inhibition of pAKT levels in tumors versus plasma. BAY 80-6946 is efficacious in tumors with activated PI3K when dosed either continuously or intermittently. Thus, BAY 80-6946 induced 100% complete tumor regression when dosed as a single agent every second day in rats bearing HER2-amplified and *PIK3CA*-mutated KPL4 breast tumors. In combination with paclitaxel, weekly dosing of BAY 80-6946 is sufficient to reach sustained response in all animals bearing patient-derived non-small cell lung cancer xenografts, despite a short plasma elimination half-life (1 hour) in mice. Thus, BAY 80-6946 is a promising agent with differential pharmacologic and pharmacokinetic properties for the treatment of PI3K-dependent human tumors. *Mol Cancer Ther*; 12(11); 2319–30. ©2013 AACR.

Introduction

The phosphoinositide 3-kinase (PI3K) family generates 3'-phosphoinositides that activates a variety of cellular targets important for cell proliferation, survival, differentiation, and migration (1, 2). The class I PI3Ks transmit signals from receptor tyrosine kinases (RTK) and from the oncogenic protein Ras via the PI3K α , β , or δ isoforms and from G protein-coupled receptors (GPCR) via the PI3K β or γ isoforms (3–5). Activation of PI3K has been directly linked to cancer through somatic mutations or amplification of *PIK3CA* (6–8), and loss of function of tumor suppressor PTEN (9). The loss or inactivation of PTEN leads to increased

PI3K signaling and tumor growth that seems to be mediated via PI3K β isoform (10–12). In addition, overexpression or mutational activation of RTKs occurs in multiple tumor types, leading to increased PI3K signaling (13, 14).

Aberrant activation of class I PI3Ks has been associated with both intrinsic and acquired resistance to targeted agents, such as imatinib and trastuzumab (15–17), as well as to traditional chemo- and radiotherapy (18–21). The prototype PI3K inhibitors (e.g., LY294002) did not enter clinical development due to their lack of specificity and therapeutic window (22). A number of second generation selective PI3K or PI3K/mTOR inhibitors have entered early clinical development. However, clinical benefit has not yet been clearly shown in solid tumors. This is largely due to the complexity derived from the existence of various PI3K isoforms and their differential roles in cancer pathology and compensatory feedback activation of oncogenic pathways (23). As such, development of PI3K inhibitors with differential pharmacologic and pharmacokinetic profiles would allow best exploration in different indications, combinations, and dosing regimens to maximize the tumor-killing effects of PI3K inhibitors.

The present report explores the pharmacologic and pharmacokinetic profile of the intravenous PI3K inhibitor

Authors' Affiliation: Bayer HealthCare Pharmaceuticals, Global Drug Discovery, Berlin, Germany

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[†]Deceased.

Corresponding Author: Ningshu Liu, Bayer HealthCare Pharmaceuticals, Muellerstr 178, Berlin 13342, Germany. Phone: 49-30-468193340; Fax: 49-30-46818069; E-mail: ningshu.liu@bayer.com

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BAY 80-6946, which preferentially inhibits PI3K α and PI3K δ and is highly potent in *in vitro* and *in vivo* preclinical tumor models with activated PI3K.

Materials and Methods

Reagents and tumor cell lines

BAY 80-6946 [2-amino-N-[7-methoxy-8-(3-morpholinopropoxy)-2,3-dihydroimidazo[1,2-c]quinazolin]; Fig. 1A] was identified and synthesized at Bayer HealthCare

Pharmaceuticals. The information about the reference compounds was described in Supplementary Fig. S1. For *in vitro* studies, 5 mmol/L stock solution of BAY 80-6946 (in dimethyl sulfoxide with 10 mmol/L trifluoroacetic acid) was used. All tumor cell lines, except KPL4 cells, were obtained from the American Type Culture Collection; KPL4 cells were obtained from the laboratory of Dr. Junichi Kurebayashi (Kawasaki Medical School, Okayama, Japan). All cell lines were authenticated by

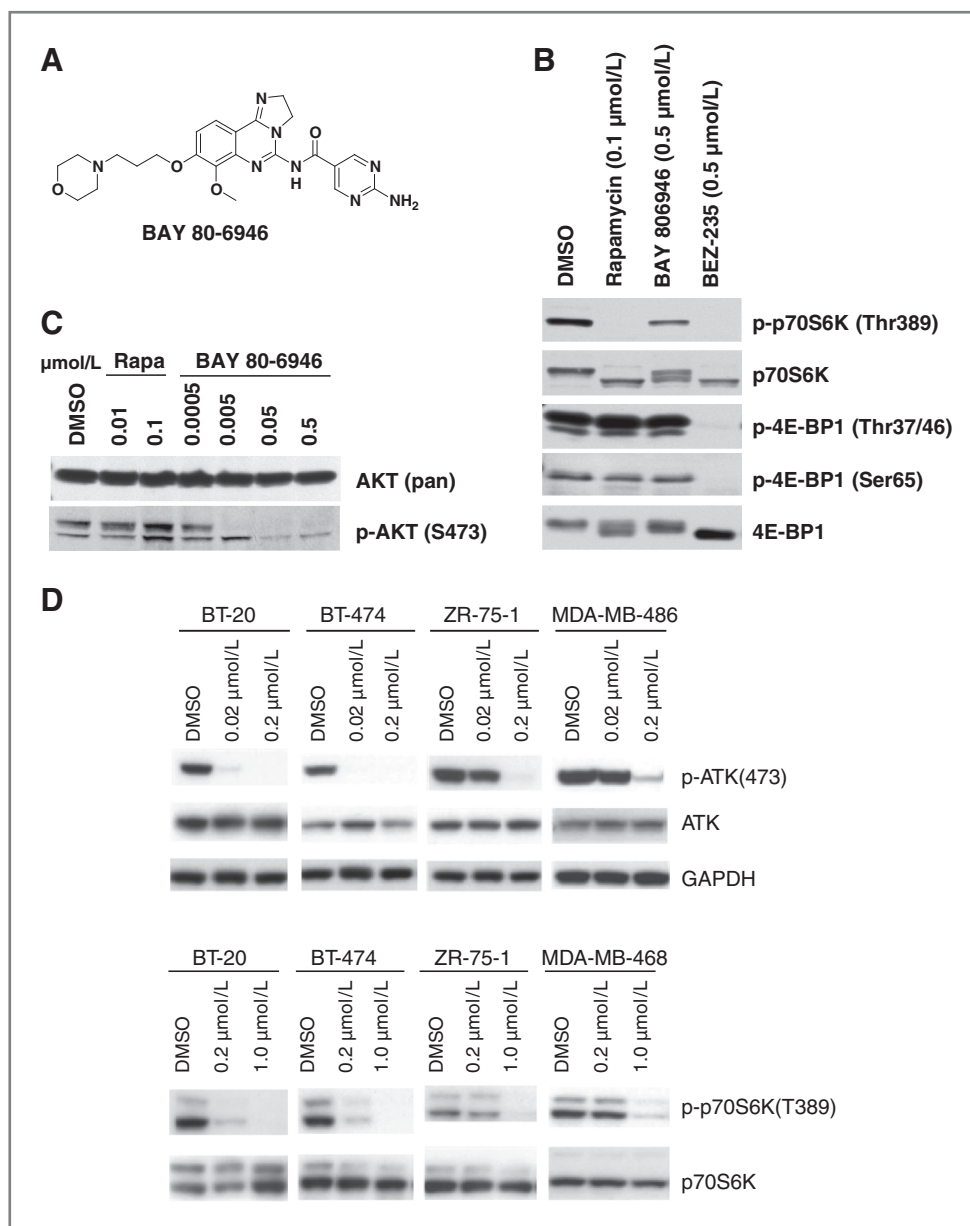


Figure 1. BAY 80-6946 chemical structure (A) and pathway inhibition by BAY 80-6946 and other PI3K pathway inhibitors in cells. Cells were treated with rapamycin or BAY 80-6946 at the indicated concentration for 2 hours. Western blot analysis of cell extracts was conducted with antibodies against p-AKT-S473/total AKT, p-p70S6K(T389)/total p70S6K, p-4E-BP1(Thr37/46), and p-4E-BP1(Ser65)/total 4E-BP1. B and C, Western blot analysis with ELT3 cells treated with BAY 80-6946 and other PI3K pathway inhibitors. D, Western blot analysis of AKT and p70S6K phosphorylation in breast cancer cell lines with *PIK3CA* mutation (BT-20 and BT-474), *HER2* overexpression (BT-474), *PTEEN* deletion mutation (ZR-75-1 and MDA-MB-468) at the indicated concentrations of BAY 80-6946.

fingerprint techniques at German Collection of Microorganisms and Cell Cultures (DSMZ) after two to four passages of the original stock from the providers. An aliquot of the substock was used for the studies described here. The cell lines were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum.

Biochemical lipid kinase assays

The effect of BAY 80-6946 on PI3K α , PI3K β , and PI3K γ activity was measured by the inhibition of ^{33}P incorporation into phosphatidylinositol as described in Supplementary Materials and Methods.

The effect of BAY 80-6946 on PI3K δ was determined by Upstate/Chemicon using their proprietary PIPProfiler assay system that uses homogeneous time-resolved fluorescence to measure inhibition of kinase activity. The effect of BAY 80-6946 on PI4K-II, PIP $_4$ -5K, and PIP $_5$ -4K was measured using assay conditions nearly identical to those for measuring PI3K activity, except that the p110 protein was replaced with either recombinant PI4K-II protein or PIP $_4$ -5K protein, respectively. In addition, PIP $_4$ (Echelon) and PIP $_5$ (Matreya) were used as the lipid substrate in place of phosphatidylinositol in the PIP $_4$ -5K and PIP $_5$ -4K assays, respectively.

Assessment of pAKT and p-4E-BP1 levels

Levels of pAKT (T308), pAKT(S473), and p-4E-BP1 (T70) in the presence or absence of PI3K inhibitors were quantitatively determined using AlphaScreen SureFire assays (PerkinElmer) or by Western blot analyses. Briefly, the effects of PI3K inhibitors on the basal levels of pAKT and p-4E-BP1 in KPL4 cells were measured after 2 hours of treatment with PI3K inhibitors. To determine the inhibition of lysophosphatidic acid (LPA)-stimulated phosphorylation of AKT and 4E-BP1, PC3 cells were cultured in serum-free medium overnight, then incubated with PI3K inhibitors for 1 hour before being stimulated with 50 $\mu\text{mol/L}$ LPA for 10 minutes. Determination of pAKT and p-4E-BP1 levels was conducted according to the manufacturer's instructions.

Proliferation assays

Cell proliferation was determined using the CellTiter-Glo Luminescent Cell Viability Kit (Promega) as described in the Supplementary Data.

Apoptosis assays

BT-20 or BT474 breast tumor cells were treated with test compounds for 24 hours. Activation of caspase-9 was determined using the Caspase-Glo9 Assay Kit (Promega). Phosphorylation of p53 at Ser15 and PARP cleavage was measured using the MSD Apoptosis Kit (Cat # K15102D-2) at 48 hours after treatment.

Xenograft models

The *in vivo* antitumor efficacy of BAY 80-6946 was assessed in athymic nude rats or nude mice using xenograft models of human tumors. Animals were housed

according to institutional guidelines with access to food (pelleted diet) and water *ad libitum*. Studies with patient-derived tumors were conducted in EPO GmbH or in Oncotest GmbH with written informed consent from each patient and the approval from local ethical committees. Tumor xenografts were generated by harvesting cells from mid-log phase cultures and injecting subcutaneously into the right flank of each rat and mouse. BAY 80-6946 was dissolved in a PEG400/acidified water (0.1 N HCl, pH 3.5; 20/80, v/v) or 5% mannitol vehicle. Treatment was initiated when all animals in an experiment had established tumors. BAY 80-6946 was administered every second day, every third day, or weekly via an intravenous bolus at the indicated doses. Tumor dimensions and body weights were recorded twice weekly starting on the first day of treatment. Tumor volumes were calculated using the equation $(l \times x \times w^2)/2$, where l and w refer to the larger and smaller dimensions collected at each measurement. Antitumor efficacy was determined as a function of tumor growth inhibition (% TGI). TGI was calculated by the equation $[1 - (T/C)] \times 100$, where T and C represent the mean size of tumors in the treated (T) and control (C) groups, respectively. In addition, treatment responses were evaluated by means of clinically used Response Evaluation Criteria in Solid Tumors (RECIST) criteria; response rates (RR) were calculated as the percentage of animals with a complete or partial response (PR). The assessment of the pharmacodynamic effects of BAY 80-6946 in tumor specimens is described in the Supplementary Data.

Pharmacokinetics analysis

To determine the pharmacokinetic properties of BAY 80-6946, a solution of BAY 80-6946 was prepared in PEG400/pH 3.5 water (20/80) and administered as an intravenous bolus dose in the tail vein at 2 mL/kg for rats and 10 mL/kg for mice. Each time point has a group of 3 animals. Blood was collected at 5, 15, and 30 minutes and at 1, 4, 7, 24, and 30 hours after administration for pharmacokinetic analysis. Samples for tumor versus plasma exposure and pharmacodynamics biomarker analysis were taken at 24, 48, 72, and 96 hours.

Results

BAY 80-6946 is a highly selective and potent pan-class I PI3K inhibitor and does not exhibit significant cellular mTOR inhibition

BAY 80-6946 represents a novel proprietary imidazolinoquinazoline class of PI3K inhibitors identified through high-throughput screening of Bayer's compound library and further optimized with respect to drug-like characteristics (Fig. 1A). BAY 80-6946 potently inhibited the catalytic activity of the class I PI3K α , β , γ , and δ isoforms with IC $_{50}$ values of 0.5, 3.7, 6.4, and 0.7 nmol/L, respectively. It showed significantly weaker activity against mTOR with an IC $_{50}$ of 45 nmol/L. In contrast, 1 $\mu\text{mol/L}$ BAY 80-6946 did not inhibit PI4K-II, PIP $_4$ -5K, PIP $_5$ -4K, or an additional

220 kinases in the Millipore kinase panel (inhibition <30%), indicating that BAY 80-6946 is a specific PI3K inhibitor with more than 2,000-fold selectivity against other lipid and protein kinases, except for mTOR. To further evaluate the selectivity of BAY 80-6946 against PI3K versus mTOR kinase, rat ELT3 cells, which exhibit a PI3K-independent activation of mTORC1 due to TCS2 deficiency, were used. Complete inhibition of PI3K-mediated AKT phosphorylation was clearly shown at a concentration of 5 nmol/L (Fig. 1B), whereas BAY 80-6946 showed only a minor reduction of PI3K-independent mTORC1-mediated phosphorylation of S70S6K levels and no effect at all on p-4E-BP1 at a concentration of 500 nmol/L (Fig. 1C). In contrast, rapamycin at 100 nmol/L completely inhibited p-p70S6K and the dual PI3K/mTOR inhibitor BEZ-235 at 500 nmol/L completely blocked the phosphorylation of both p70S6K and 4E-BP1, indicating potent activities directly against mTOR kinase. These results suggest that BAY 80-6946 has a 100- to 1,000-fold cellular selectivity of PI3K versus mTOR signaling and is, therefore, not a dual PI3K/mTOR inhibitor in cells.

Biochemically, BAY 80-6946 showed differential potency against the class I PI3K isoforms. To show its cellular activity against each individual isoform, the activity of BAY 80-6946 in inhibition of PI3K α - and PI3K β -mediated activation of downstream effectors of the pathway was examined. Previous studies have shown that p110 α is essential for the signaling driven by *PIK3CA* mutations and/or oncogenic RTKs, whereas p110 β is the major isoform in mediating PTEN-deficient and GPCR signaling (24). KPL4, a breast carcinoma cell line that carries a *PIK3CA*-activating mutation and overexpresses HER2, was selected to assess the cellular PI3K α activity. PI3K β activity was measured using LPA-stimulated AKT phosphorylation in PTEN-null PC3 prostate tumor cells. To validate these assays, TGX-221, a PI3K β -specific inhibitor, was tested in KPL4 and PC3 cells. TGX-221 potently inhibited LPA-stimulated AKT phosphorylation in PC3 cells with single-digit nmol/L IC₅₀ values, whereas it was completely inactive at 1 μ mol/L in blocking AKT phosphorylation in KPL4 cells (Table 1). These data confirmed previous reports and validated the assays by showing that the phosphorylation of AKT in KPL4 cells is not driven by PI3K β , whereas LPA-stimulated AKT phosphorylation in

PC3 cells is a PI3K β -dependent and PI3K α -independent process (25). In KPL4 cells, BAY 80-6946 reduced basal levels of AKT phosphorylation at both Thr308 and Ser473 with IC₅₀ values of 0.4 and 0.6 nmol/L, respectively (Table 1). BAY 80-6946 also reduced pAKT levels in PC3 cells with activity similar to that of TGX-221, albeit at seven to 20 times higher concentrations than those required in KPL4 cells. This is consistent with biochemical data showing that BAY 80-6946 inhibits both α and β PI3K isoforms, but is more potent against the α isoform. In line with this observation, BAY 80-6946 was also approximately 10 times more potent in blocking AKT phosphorylation in breast cancer cell lines with *PIK3CA* mutations (e.g., BT-20, BT-474, and KPL4) than in those with loss of PTEN (e.g., ZR-75-1, MDA-MB-468) assessed by Western blot analysis in Fig. 1D.

BAY 80-6946 is a potent inhibitor of tumor cell proliferation and shows activity in a subset of human cancer cell lines with *PIK3CA* mutations and/or overexpression of HER2

BAY 80-6946 potently inhibited cell proliferation in a panel of human tumor cell lines, many of which exhibited constitutively activated PI3K signaling resulting from, for example, somatic mutations in *PIK3CA* and *PTEN* (Fig. 2A). Antiproliferative activity was evident across multiple tumor types. Of note, several breast cancer, endometrial cancer, and hematologic tumor cell lines were particularly sensitive to BAY 80-6946, with IC₅₀ values less than 10 nmol/L. This profile may correlate with the strong activity of BAY 80-6946 against PI3K α and PI3K δ . To further understand the correlation between sensitivity and molecular features, BAY 80-6946 was profiled in a broader panel of breast cancer cell lines. Indeed, BAY 80-6946 had mean IC₅₀ values of 19 nmol/L against cell lines with *PIK3CA*-activating mutations ($n = 9$) and 17 nmol/L against HER2-positive cell lines ($n = 7$; Fig. 2B and C), whereas the activity in *PIK3CA* wild-type and HER2-negative cells was about 40-fold less potent (average IC₅₀ = 774 nmol/L; $n = 11$). This result is in line with the potent biochemical and cellular mechanistic activity against PI3K α and further indicates that *PIK3CA* mutation and HER2 overexpression are potential biomarkers to predict the sensitivity of breast cancer cells to BAY

Table 1. Effect of BAY 80-6946 and comparators on phosphorylation of AKT and 4E-BP1 in KPL4 cells and LPA-stimulated PC3 cells

Compound	IC ₅₀ (nmol/L) in KPL4			IC ₅₀ (nmol/L) in LPA-PC3		
	p-AKT (308)	p-AKT (473)	p-4E-BP1	p-AKT (308)	p-AKT (473)	p-4E-BP1
BAY 80-6946	0.4	0.6	4.6	7.8	10	34
GDC-0941	6.1	22	151	7.1	23	152
TGX-221	>1,000	>1,000	>1,000	2.1	6.1	33

NOTE: Inhibition of cellular levels of phospho-AKT at S473, phospho-AKT at T308, and phospho-4E-BP1 at T70 by indicated PI3K inhibitors in KPL4- or LPA-stimulated PC3 cells was determined using AlphaScreen Kits from PerkinElmer.

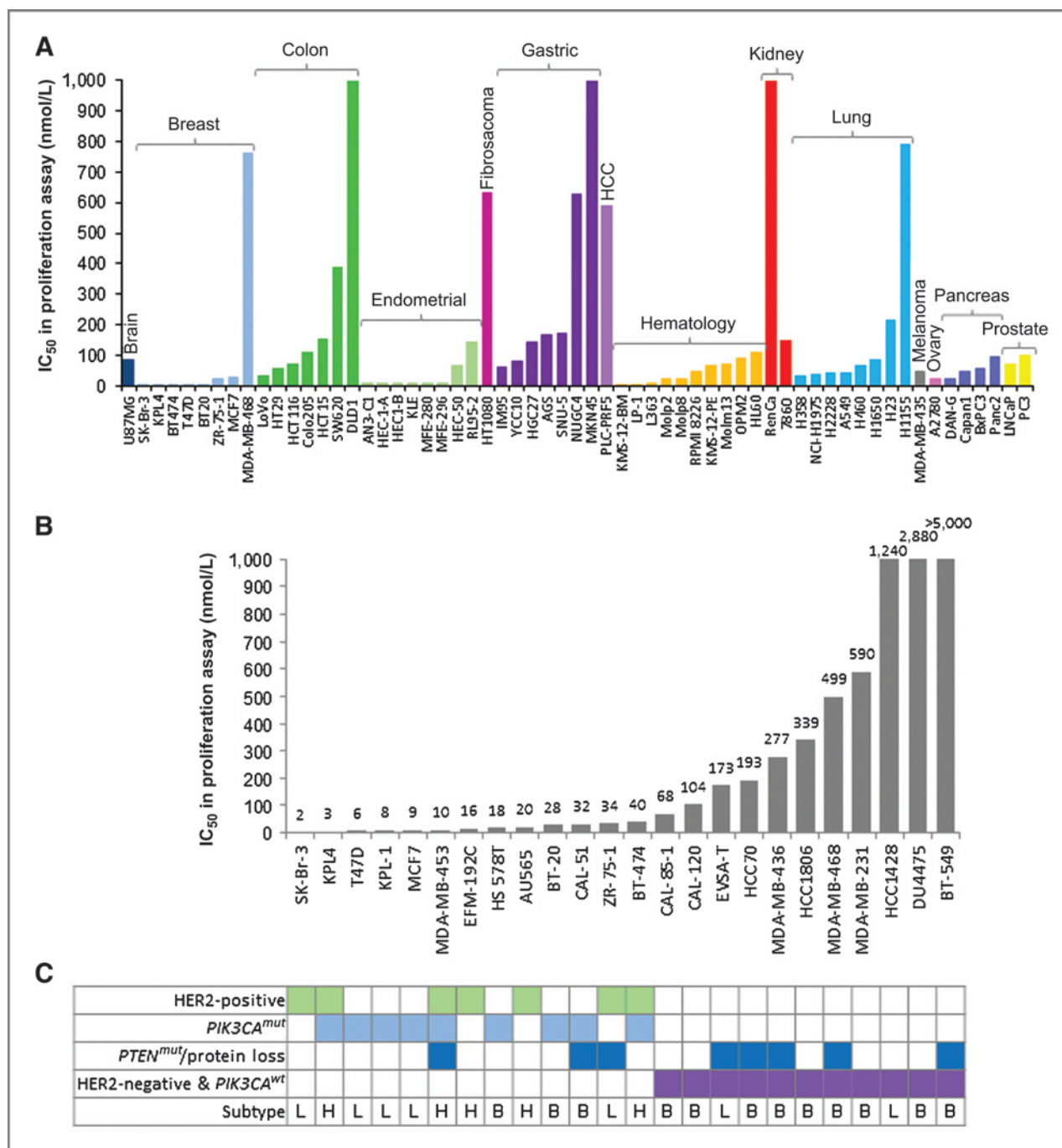


Figure 2. Antiproliferative IC₅₀ values of BAY 80-6946 in cancer cell lines from various indications with gene mutation status (A) and from breast cancer with genetic, protein expression, and histopathologic characteristics (B). C, the *PIK3CA* gene mutation status and HER2 expression in breast cancer cell lines shown in B. B, basal-like breast cancer cell lines; blank, without indicated characteristics; H, Her2-positive breast cancer cell lines; L, luminal-type breast cancer cell lines; HCC, hepatocellular carcinoma.

80-6946. In contrast, there is no clear correlation between the sensitivity and loss of PTEN. In addition, BAY 80-6946 was active in inhibiting proliferation of tumor cell lines resistant to the anti-HER2 agents trastuzumab and lapatinib, such as T47D (*PIK3CA*-mut), ZR-75-1 (PTEN-null), and MCF7 (*PIK3CA*-mut) with IC₅₀ values of 6, 24, and

27 nmol/L, respectively. The potent activity in hematologic tumor cell lines (*PIK3CA* wild-type) may associate with the potent PI3Kδ activity of BAY 80-6946. Taken altogether, these observations show that BAY 80-6946 has antiproliferative activity against a subset of human cancer cell lines, particularly those tumor cells with PI3K and/or

RTK alterations, such as *PIK3CA* mutation and/or over-expression of HER2 and may offer the potential to overcome resistance to anti-HER2 therapies.

BAY 80-6946 induces apoptosis in a subset of tumor cell lines that are resistant to lapatinib and trastuzumab

The PI3K/AKT pathway promotes tumor cell survival and prevents apoptosis by phosphorylation and inhibition of proapoptotic mediators such as caspase-9 and FOXO. BAY 80-6946 potently induced nuclear localization of FOXO1A with an EC_{50} less than 1 nmol/L (Supplementary Fig. S2) and apoptosis in a subset of tumor cell lines, including BT20, BT474, and ZR-75-1 breast cancer cells (26). To investigate the apoptosis pathways activated by BAY 80-6946, a set of studies were carried out with anti-HER2-resistant *PIK3CA*-mutated BT20 breast cancer cells. BAY 80-6946 significantly increased caspase-9 activities following 24-hour incubation with 2- and 3-fold inductions in caspase-9 activity at 20 and 62 nmol/L, respectively (Fig. 3A). These values were comparable with

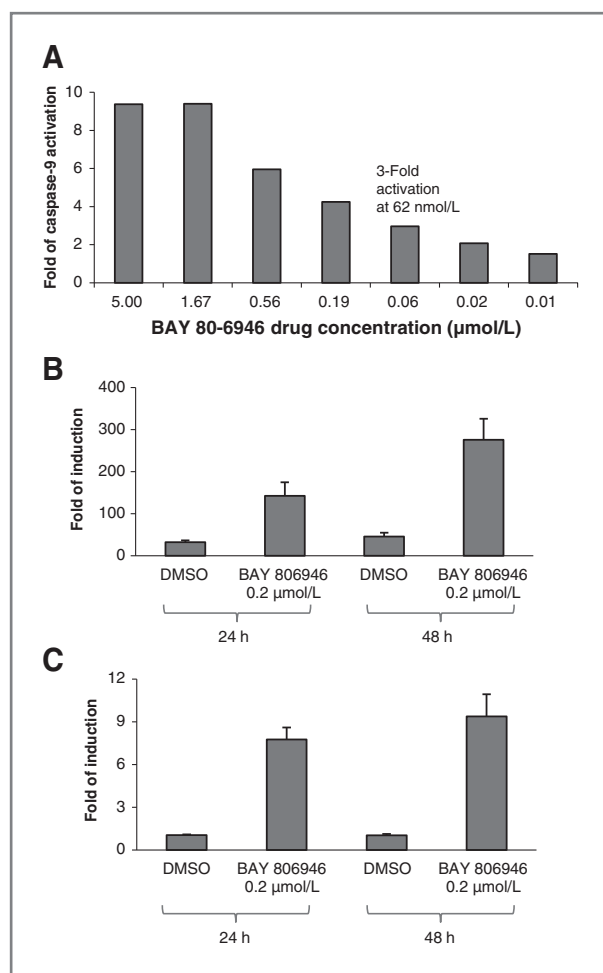


Figure 3. Induction of apoptosis in BT-20 (*PIK3CA*^{P539R, H1047R}) lapatinib-resistant breast cancer cells. A, activation of caspase-9. B, levels of phospho-p53 at Ser15. C, levels of cleaved PARP.

the IC_{50} of 6 nmol/L for antiproliferative activity in the same cell line. The effect of BAY 80-6946 on caspase-9 activation was even stronger than that of paclitaxel, whereas rapamycin was inactive at 1 μmol/L (Supplementary Fig. S3). Caspase-9 activation leads to cleavage of several procaspases, which in turn cleave cellular targets including PARP, thereby impairing DNA repair. DNA damage is reflected by the level of p53 phosphorylation at Ser15. Consistent with the induction in caspase-9, BAY 80-6946 at 200 nmol/L also increased levels of phosphorylated p53 at Ser15 (Fig. 3B) and cleaved PARP (Fig. 3C). Caspase-9 activation by BAY 80-6946, lapatinib or the combination of the two agents was further investigated in the anti-HER2-sensitive breast cancer cell line BT474. Interestingly, although lapatinib effectively inhibited tumor cell proliferation with an IC_{50} of 21 nmol/L (data not shown), it was incapable of inducing caspase-9 activation at a concentration up to 10 μmol/L (Supplementary Table S1). In contrast, BAY 80-6946 induced caspase-9 activation with an EC_{50} of 340 nmol/L. Interestingly, when BAY 80-6946 was combined with lapatinib, the same degree of caspase-9 activation could be reached with significantly less levels of BAY 80-6946 (e.g., 61 nmol/L) and lapatinib (e.g., 184 nmol/L) with combination indices of 0.20 to 0.29, across all concentrations tested (Supplementary Table S1). Taken together, these findings showed that BAY 80-6946, as a single agent, could induce cell death in both anti-HER2-sensitive and -resistant, PI3K-mutant breast tumor cells at clinically achievable concentrations (27). Combination with lapatinib further synergistically sensitized tumor cells to apoptosis.

Preclinical pharmacokinetic and pharmacodynamic properties

BAY 80-6946 has a high plasma-free fraction across all species tested (35% in rats, 14% in mice, 33% in dogs, and 16% in humans). The pharmacokinetic profile of BAY 80-6946 was evaluated following administration of single or multiple intravenous doses in nude rats (Supplementary Fig. S4). BAY 80-6946 exhibited very large volume of distribution ($V_{ss} = 32$ L/kg), high plasma clearance (3.95 L/kg·h) and long $T_{1/2}$ (6.0 hours). Similar exposure and other pharmacokinetic parameters were observed following multiple administration (every second day \times 5), suggesting that there is no accumulation of compound or changes in metabolic processes. In comparison with the pharmacokinetic in rats, BAY 80-6946 had a higher plasma clearance (16 L/kg·h) and a shorter $T_{1/2}$ of 0.7 hours. The V_{ss} was large (12.9 L/kg) in mice (Supplementary Table S2).

To investigate pharmacokinetic/pharmacodynamic relationship, tumor samples were collected from nude rats bearing H460 human non-small cell lung carcinoma (NSCLC) xenografts and treated with a single 6 mg/kg i.v. bolus dose. The concentrations of BAY 80-6946 and pharmacodynamic markers in tumors were assessed. As shown in Fig. 4A, BAY 80-6946 levels were approximately 100-fold higher in the tumor than in plasma at 48 hours

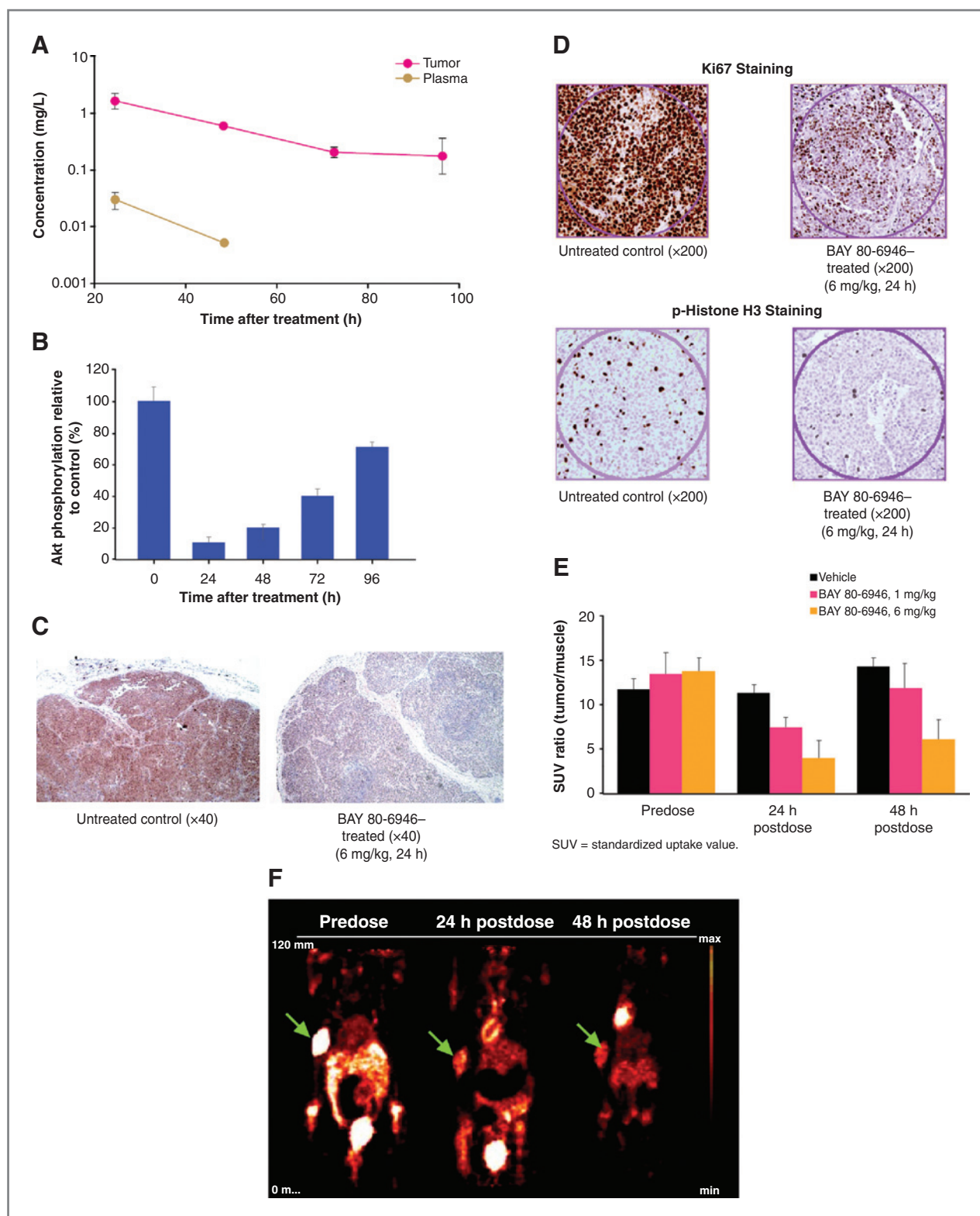


Figure 4. Pharmacokinetic/pharmacodynamic evaluations in H460 human NSCLC xenografts in nude rats. **A**, plasma and tumor concentrations of BAY 80-6946 formulated in PEG400/acidified water (0.1 N HCl, pH 3.5; 20/80, v/v) following a single intravenous administration of 6 mg/kg (3 animals/group). **B**, tumor pAKT (S473) levels following a single intravenous administration of BAY 80-6946 at 6 mg/kg (3 animals/group). **C**, pAKT staining (S473) at 24 hours after treatment with BAY 80-6946 and in untreated controls. **D**, Ki67 staining at 24 hours after treatment with BAY 80-6946 and in untreated controls. **E**, dose-dependent inhibition of FDG tumor uptake by BAY 80-6946. **F**, FDG-PET images before and after treatment with a single 6 mg/kg i.v. dose of BAY 80-6946.

and drug clearance from the tumor occurred more slowly than from plasma. This high tumor exposure might be explained, in part, by the basicity and large V_{ss} of the drug and acidic environment in tumors. The levels of pAKT-Ser473 in tumors showed more than 90% inhibition at 24 hours after intravenous dosing and remained suppressed for 48 to 72 hours (Fig. 4B). Strong reduction of pAKT levels in tumors was observed as early as 1 hour after intravenous dosing (data not shown). The potent inhibition of tumor AKT phosphorylation correlates well with the high level of tumor exposure of BAY 80-6946, suggesting a strong pharmacokinetic/pharmacodynamic relationship. The inhibition of AKT phosphorylation in response to BAY 80-6946 was further confirmed by immunohistochemical analysis with pAKT staining (Fig. 4C). Compared with vehicle control, tumors treated with 6 mg/kg of BAY 80-6946 for 24 hours showed 65% and 75% reduction in Ki67 and phosphohistone H3 staining, respectively, indicating that tumor cells were arrested at the G_0 -phase of the cell cycle (Fig. 4D). Rats bearing H460 xenografts were also evaluated by ^{18}F fluoro-2-deoxy-D-glucose-positron emission tomography (FDG-PET). BAY 80-6946 inhibited tumor uptake of FDG in a dose-dependent manner, with sustained inhibition evident over 24 to 48 hours after a single intravenous administration of 6 mg/kg (Fig. 4E and F). Taken together, these results suggest that *in vivo*, BAY 80-6946 reached high and prolonged exposure in tumors, which resulted in potent inhibition of the PI3K pathway exhibited by marked reduction in AKT phosphorylation and FDG uptake, as well as cell-cycle arrest in G_0 in the H460 NSCLC xenograft model in rats.

BAY 80-6946 is highly efficacious in rat and mouse tumor xenograft models following intravenous administration

BAY 80-6946 was well tolerated at all doses and schedules tested in the studies described here without producing any lethality. The maximum-tolerated dose (MTD) in rats was defined as 6 mg/kg. At the MTD, a maximum mean body weight loss of 6% to 10% occurred during the first few days of dosing and then consistently returned to the normal range by the end of the dosing period. The MTD in mice was more than 14 mg/kg with a every second day dosing schedule.

BAY 80-6946 was highly efficacious in a variety of human tumor xenograft models derived from different tumor indications that exhibit an activated PI3K pathway. The drug displayed robust antitumor activity in the rat KPL4 tumor xenograft model, which is an estrogen-independent HER2-positive breast carcinoma that carries a somatic *PIK3CA* mutation. BAY 80-6946 was administered at 0.5 to 6 mg/kg i.v. every second day for a total of five doses starting on day 14, following tumor cell implantation. On day 25, 3 days after the last dose, TGI rates of 77%, 84%, 99%, and 100% were observed with BAY 80-6946 at doses of 0.5, 1, 3, and 6 mg/kg, respectively (Fig. 5A). Complete tumor regression was shown in 10 of 10 rats in the 3 and 6 mg/kg groups, and all rats

remained tumor free at the termination of the study on day 73. Tumor growth delays more than 25 days were observed in the 0.5 and 1 mg/kg dose groups.

Treatment of BAY 80-6946 led to tumor stasis in nude rats bearing HCT-116 human colon tumors, which carry *PIK3CA* and *KRAS* mutations. BAY 80-6946 was administered at 3 and 6 mg/kg i.v. every second day for a total of five doses, producing TGI of 75% and 88%, respectively, and tumor growth delays of 10 and 11 days, respectively (Fig. 5B, data not shown).

BAY 80-6946 was also effective in xenograft models established in nude mice with Lu7860 erlotinib-resistant, patient-derived NSCLC and MAXF1398 patient-derived luminal breast tumor models. BAY 80-6946 was administered at a dose of 14 mg/kg every second day for a total of 10 days, producing TGI rates of 88% in the NSCLC model (Fig. 5C) and 71% in the breast cancer model (Fig. 5D).

Alternative dosing schedule of intravenous twice a day once weekly for BAY 80-6946 also shows antitumor activity

The antitumor activity of BAY 80-6946 was also evaluated using a weekly dosing schedule, in which two doses of BAY 80-6946 were administered on the first day of the week. In the rat HCT-116 colorectal xenograft model, the TGI achieved with BAY 80-6946 at a dose of 9 mg/kg twice a day once weekly was equivalent to that with the 6 mg/kg dose given every second day for 10 doses (Fig. 6A). BAY 80-6946 at a dose of 6 mg/kg twice a day once weekly was efficacious with a TGI of 64%, comparable with the activity of docetaxel at 5 mg/kg every second day given for three doses.

Activation of PI3K has been shown to cause intrinsic and/or acquired resistance to chemotherapy. BAY 80-6946 was tested in combination with paclitaxel in Lu7343, a patient-derived *PIK3CA* (E545K)-mutated squamous cell NSCLC model in nude mice (Fig. 6B). Paclitaxel was given at doses of 15 or 25 mg/kg on days 14, 21, and 28. BAY 80-6946 was administered weekly at 10 mg/kg twice a day on days 15, 22, and 29 (24 hours after each paclitaxel dose). Treatment of BAY 80-6946 at 10 mg/kg/twice a day/week and paclitaxel at 15 mg/kg (60% of the MTD) alone showed only moderate efficacy with T/C% (treatment/control) values of 49% and 38%, respectively. Paclitaxel at the MTD (25 mg/kg) was effective and produced T/C% of 4% and 70% RR at the end of treatment on day 33 (Supplementary Table S3); however, tumor regrowth was observed immediately after stopping the treatment. Thus, on day 55, the RR in paclitaxel MTD group had dropped to 30% and tumors had progressed in 60% of the animals. The combination of BAY 80-6946 with both high and low doses of paclitaxel produced tumor regression, which was not only superior to either agent alone, but the combination with paclitaxel at the MTD also produced 100% RR with complete response (CR) in 5 of 10 and (PR) in 5/10 animals at the end of treatment. Importantly, in contrast with paclitaxel single agent at the MTD, the combination group showed a long-lasting tumor regression with 60% CR and

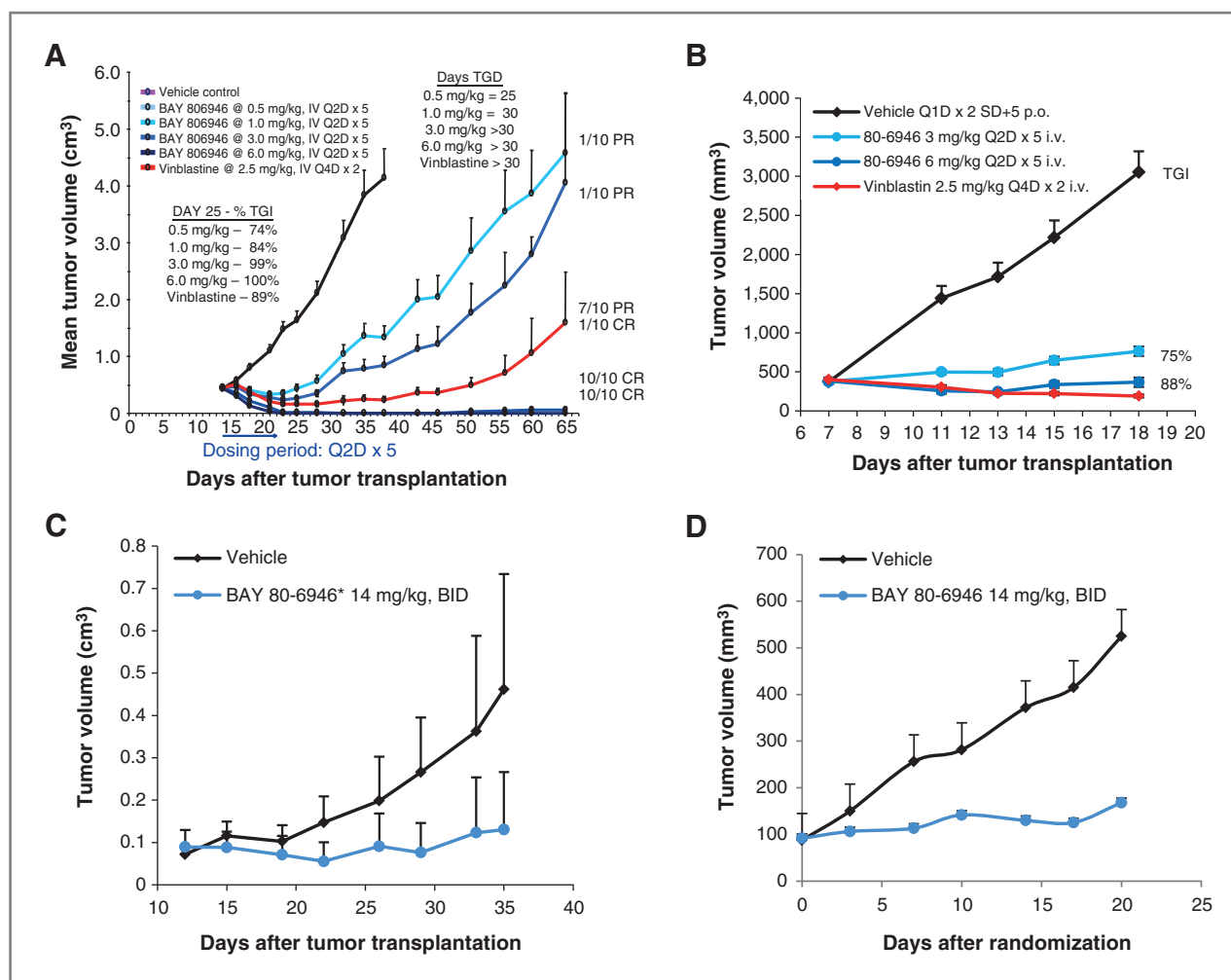


Figure 5. Activity of BAY 80-6946 in xenograft models using an every second day (Q2D) schedule. A, KPL4 breast cancer xenografts in nude rats (10 rats/group). B, HCT116 colon cancer xenografts in nude rats (10 rats/group). C, Lu7860 erlotinib-resistant, patient-derived NSCLC xenografts in nude mice (5 mice/group). D, MAXF1398 patient-derived luminal breast cancer xenografts in nude mice (6 mice/group). BAY 80-6946 was formulated in PEG400/acidified water (0.1 N HCl, pH 3.5; 20/80, v/v) in the studies described in A and B or in 5% mannitol vehicle in the studies described in C and D. Tumor volume in median \pm SEM was shown. p.o., orally; Q4D, every fourth day. *, $P < 0.05$; BID, twice a day.

40% PR on day 55 (22 days after stopping treatment). These findings suggest that BAY 80-6946, when combined with paclitaxel, synergistically improved tumor response rates and led to long lasting tumor regression even when dosed twice a day weekly over 3 weeks in mice.

Discussion

Many selective small-molecule inhibitors of PI3K have been identified, including selective pan-class I PI3K inhibitors, selective PI3K δ inhibitors, and dual PI3K/mTOR inhibitors. These new PI3K inhibitors are currently in the early stages of clinical development. Several pan-class I PI3K inhibitors, including BKM120 and XL147, have entered phase II trials for various advanced malignancies, such as castration-resistant prostate, endometrial, and breast cancer, and a p110 δ -selective PI3K inhibitor has entered phase III trials for lymphoma (28, 29). Although

the pan-class I inhibitors have activity against each p110 isoform, they may show preferential activity for specific members. It is likely that the various PI3K isoforms play different roles based on tumor type, somatic mutations, and/or previous therapy, as well as different toxicology profiles; therefore, understanding the pharmacologic profile of each PI3K inhibitor may be important for developing PI3K therapeutics for specific malignancies. Here, we present BAY 80-6946, a highly selective and potent pan-class I PI3K inhibitor, which has preferential activity against the p110 α and p110 δ isoforms, compared with p110 β and p110 γ , based on biochemical assays and PI3K α and PI3K β cellular assays. As it is difficult to compare the biochemical activity of the PI3K inhibitors against each p110 isoforms under physiologic conditions, we explored cellular assays in which the phosphorylation of AKT is driven by p110 α or p110 β , the two dominant isoforms expressed in solid tumors. The selectivity of BAY 80-6946 for the PI3Ks versus

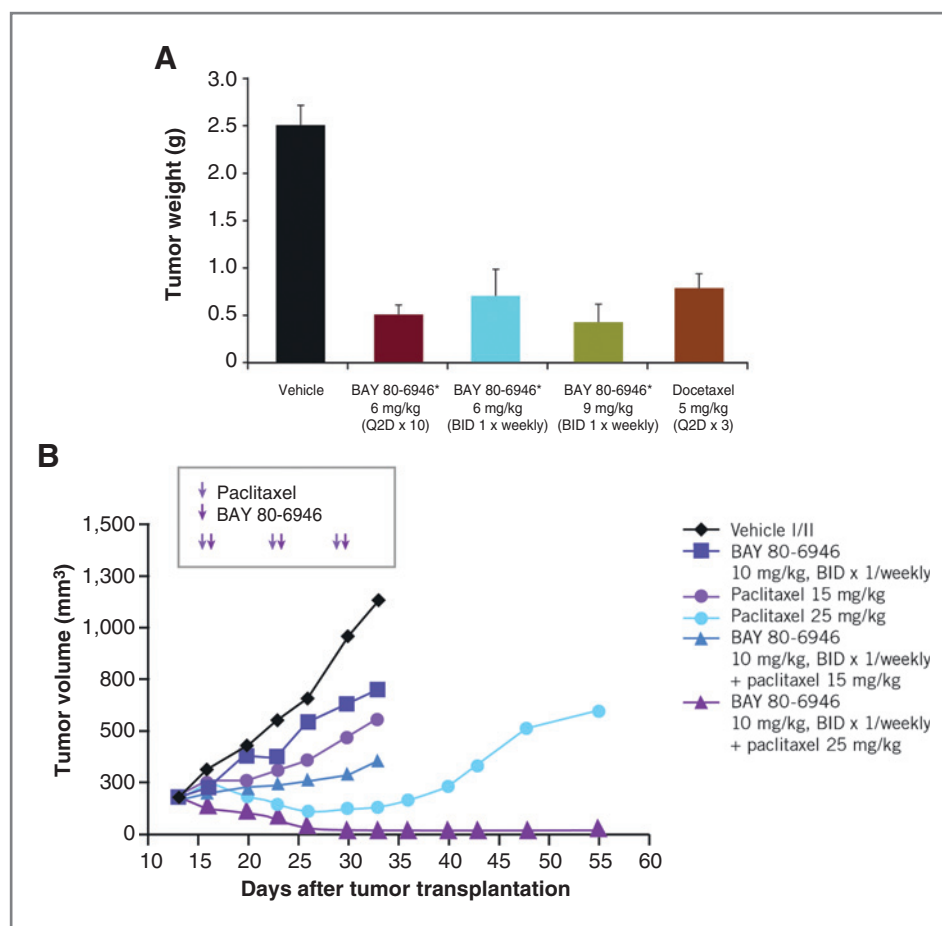


Figure 6. Activity of BAY 80-6946 in xenograft models using a twice a day (BID) once-weekly schedule. BAY 80-6946 was formulated in 5% mannitol vehicle. A, HCT116 colon cancer xenografts in nude rats (8 rats/group). Tumor weight at the end of the treatment was measured and presented in median \pm SEM. B, Lu7343 patient-derived NSCLC xenografts in nude mice (10 mice/group). Study was carried out in Epo GmbH. Tumor volume in median \pm SEM is presented. Q2D, every second day.

mTOR was evident in both the biochemical and cellular assays: BAY 80-6946 was 90-fold more active against PI3K α than against mTOR in biochemical assays and more than 250-fold more active in cellular assays using ELT3 cells with PI3K-independent constitutive activation of mTORC1 due to defective TSC2. This finding indicates that BAY 80-6946 is a highly selective and potent pan-class I PI3K inhibitor that does not exhibit significant cellular mTOR inhibition in contrast with BEZ-235 (30). In fact, based on the human pharmacokinetic data of BAY 80-6946, the plasma-free drug levels in human are significantly (>20-fold) below the concentration required for direct inhibition of mTOR kinase by BAY 80-6946 (27).

The greater activity of BAY 80-6946 against p110 α versus p110 β seen in the biochemical assays (IC₅₀, 0.5 vs. 3.7 nmol/L) was confirmed in tumor cells. First, BAY 80-6946 was 16- to 20-fold more potent in inhibiting AKT phosphorylation in *PIK3CA*-mutated and HER2-overexpressing KPL4 cells (PI3K α -driven p-AKT) compared with AKT phosphorylation in PTEN-null, LPA-stimulated PC3 cells (PI3K β -driven p-AKT). Second, BAY 80-6946 was about 10-fold more potent in inhibition of basal-level pAKT and p-p70S6K in cells with activated PI3K α (such as BT20 and BT474) than in cells with loss of PTEN where PI3K β is activated (ZR-75-

1 and MDA-MB-468; Table 1 and Fig. 1D). Interestingly, GDC-0941 showed equal potency in inhibition of cellular PI3K α and PI3K β activities (Table 1). This result for GDC-0941 is in line with previous reports by O'Brien and colleagues (31)).

Profiling of BAY 86-6946 indicated that *PIK3CA* mutations and HER2 overexpression might be effective predictive biomarkers for predicting the sensitivity of breast tumor cells to BAY 80-6946. Thus, BAY 80-6946 had approximately 40 to 45 times greater antiproliferative activity against breast tumor cells with *PIK3CA*-activating mutations and/or HER2 overexpression than against those with HER2-negative tumors with wild-type *PIK3CA* (Fig. 2C). In contrast, the mean IC₅₀ for GDC-0941 in *PIK3CA*-mutant and/or HER2-overexpressing cells compared with *PIK3CA* wild-type and HER2-negative cells showed only about a 2- to 3-fold difference in this study (data not shown) and in another report (31). The difference of the two pan-class I PI3K inhibitors in this aspect might be explained by the differential profiles in PI3K isoforms and kinase selectivity. BAY 80-6946 is a more potent PI3K α inhibitor compared with GDC-0941, but GDC-0941 has equally potent activity against both PI3K α and PI3K β . The higher sensitivity in cell lines harboring *PIK3CA*-activating mutations or amplification of HER2

was also reported recently with the highly selective allosteric AKT inhibitor, MK-2206 (32). Hence, sufficient selectivity against other kinases and potency in blocking PI3K α are indispensable for using *PIK3CA* mutation and HER2 overexpression as potential predictive biomarkers for PI3K pathway inhibitors.

Taken together, the differential isoform selectivity observed with BAY 80-6946 and other investigational PI3K inhibitors results in a differential antitumor profile (as described above for BAY 80-6946 versus GDC-0941). These findings suggest that the efficacy of the various PI3K inhibitors may differ across tumors with different genetic makeups. Understanding the preclinical profile of each PI3K inhibitor may help to guide its clinical development to the proper tumor type/subtype and also aid in formulating an optimal biomarker strategy for identifying patients most likely to benefit from treatment with a PI3K pathway inhibitor with a certain isoform profile. On the basis of these preclinical data, BAY 80-6946 was particularly active in a subset of breast cancer, endometrial cancer, and multiple myeloma cell lines, particularly those with *PIK3CA*-activating mutations and/or HER2 overexpression (Fig. 2).

Of note, BAY 80-6946 also potentially induced apoptosis in BT20 human breast cancer cells with *PIK3CA* mutation when it was tested as a single agent at a concentration similar to the IC₅₀ for blocking cell proliferation. A detailed analysis of the molecular features for apoptosis induction is described in another article (26).

BAY 80-6946 administered intravenously showed potent antitumor activity in multiple cell-line-derived and patient-derived xenograft tumor models. In line with the *in vitro* cellular data, the greatest efficacy was seen in xenograft models derived from tumor cells with *PIK3CA* mutations or HER2 overexpression. In the HER2-positive/*PIK3CA*^{mut} KPL4 xenografts, BAY 80-6946 monotherapy dosed intravenous at the MTD and 50% of MTD (6 and 3 mg/kg every second day) produced complete tumor regression that persisted after discontinuation of therapy. To our knowledge, CR in 100% animals in this model has not been achieved by any of to date investigational PI3K pathway inhibitors, including GDC-0941 (33), CH5132799 (34), and MK-2206 (35), when they were dosed at their respective MTD. BAY 80-6946 also exhibited potent antitumor activity in HT116 colon cancer (Fig. 5B) and H460 NSCLC (data not shown) xenografts with coexisting *PIK3CA* and *KRAS* mutations, with complete tumor stasis observed in these models. However, in these models with *KRAS* mutations, it seems necessary to administer BAY 80-6946 in combination with another active agent, such as a MAP-ERK kinase (MEK) inhibitor, to achieve tumor regression.

BAY 80-6946 exhibits unexpected higher and prolonged exposure in tumors compared with plasma concentrations after a single intravenous dose (Fig. 4A). It is plausible that the higher exposure in tumors led to marked and sustained reductions in tumor pAKT levels and FDG uptake (lasting for at least 48 hours). Our initial data

comparing p-AKT inhibition in tumors versus in blood monocytes indicated that BAY 80-6946 indeed suppressed p-AKT in tumors with better potency and over a longer time period (N. Liu; unpublished data). This result was consistent with the antitumor activity seen in xenograft models using a every second day schedule despite shorter plasma T_{1/2} in rat (6 hours) and in mice (1 hour). Interestingly, administration of BAY 80-6946 even on a twice a day once-weekly schedule in HCT116 xenograft model in rats resulted in a similar antitumor activity as the every second day schedule. Furthermore, when BAY 80-6946 was combined with other agents, such as paclitaxel (Fig. 6B), weekly dosing was sufficient to generate synergistic antitumor effects and to induce complete tumor regression in mice. This result indicated that maintaining constant inhibition of PI3K pathway might not be necessary especially when combining with paclitaxel. Hence, intermittent combination with targeted and chemo therapeutics could be a promising approach to synergistically enhance the antitumor efficacy and meanwhile, maintaining the safety profile in combination treatment. Currently, a series of pharmacokinetic–pharmacodynamic efficacy studies of tumor versus plasma and continuous versus intermittent dosing schedules are being conducted. These data will provide further insights to best explore PI3K inhibitor BAY 80-6946 in clinical development.

On the basis of its antitumor profile and unique physical–chemical and pharmacokinetic properties, BAY 80-6946 is currently being investigated in phase I trials dosed weekly intravenously in patients with advanced malignancies and in combination with paclitaxel as well as with an allosteric MEK inhibitor (36). Analysis of the upcoming clinical data will provide further insights on how the PI3K isoform profile together with pharmacokinetic properties impact on the antitumor efficacy of BAY 80-6946 in the clinic.

Disclosure of Potential Conflicts of Interest

S.M. Wilhelm has ownership interest (including patents) in Bayer HealthCare Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: N. Liu, B.R. Rowley

Development of methodology: N. Liu, B.R. Rowley, P.R. Fracasso, D.P. Wilkie

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Liu, B.R. Rowley, C.O. Bull, C. Schneider, A. Haeggebarth, C.A. Schatz, P.A. Fracasso, D.P. Wilkie, M. Hentemann

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Liu, B.R. Rowley, C.O. Bull, A. Haeggebarth, C.A. Schatz, P.R. Fracasso, W.J. Scott

Writing, review, and/or revision of the manuscript: N. Liu, C.A. Schatz, A. Haeggebarth, M. Hentemann, S.M. Wilhelm, W.J. Scott, D. Mumberg, K. Ziegelbauer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.R. Rowley, W.J. Scott, M. Hentemann, D. Mumberg

Study supervision: N. Liu, B.R. Rowley, K. Ziegelbauer

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preparation of this manuscript, the key contributor of this work, Dr. Bruce Rowley passed away from NSCLC on July 16, 2013. Bruce was truly an intelligent, solid, and creative scientist. We will be missing him on our way towards developing effective therapeutics for cancer patients.

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