**ABSTRACT:**

2-(1H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d]pyrimidine (GDC-0941) is a novel small molecule inhibitor of the phosphatidylinositol 3-kinase (PI3K) pathway currently evaluated in the clinic as an anticancer agent. The objectives of this study were to determine in vitro whether GDC-0941 was a substrate of P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp1) and to investigate the impact of these transporters on the pharmacokinetics, brain penetration, and activity of GDC-0941 in FVBn mice (wild-type) and Mdr1a/b(−/−), Bcrp1(−/−), and Mdr1a/b(−/−)/Bcrp1(−/−) knockout mice. Studies with Madin-Darby canine kidney cells transfected with P-gp or Bcrp1 established that this compound was a substrate of both transporters. After administrations to mice, GDC-0941 brain-to-plasma ratio ranged from 0.02 to 0.06 in the wild-type and Bcrp1(−/−) mice and was modestly higher in the Mdr1a/b(−/−) mice, ranging from 0.08 to 0.11. In contrast, GDC-0941 brain-to-plasma ratio in Mdr1a/b(−/−)/Bcrp1(−/−) triple knockout mice was 30-fold higher than in the wild-type mice. The plasma clearance of GDC-0941 was similar in wild-type and all knockout mice, ranging from 15 to 25 ml/(min·kg) in the wild-type mice and from 18 to 35 ml/(min·kg) in the knockout mice. Exposure after oral administration was comparable in the four strains of mice. The PI3K pathway was markedly inhibited in the brain of Mdr1a/b(−/−)/Bcrp1(−/−) mice for up to 6 h postdose, as evidenced by a 60% suppression of the phosphorylated Akt signal, whereas no inhibition was detected in the brain of wild-type mice. The concerted effects of P-gp and Bcrp1 in restricting GDC-0941 access and pathway modulation in mouse brain may have implications for the treatment of patients with brain tumors.

**Role of P-Glycoprotein and Breast Cancer Resistance Protein-1 in the Brain Penetration and Brain Pharmacodynamic Activity of the Novel Phosphatidylinositol 3-Kinase Inhibitor GDC-0941**

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**INTRODUCTION**

2-(1H-Indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-thieno[3,2-d]pyrimidine (GDC-0941) (Fig. 1) is a novel small molecule inhibitor of the phosphatidylinositol 3-kinase (PI3K) pathway currently evaluated in Phase I studies as an anticancer agent (Sarker et al., 2009). It was shown to be selective for the class I (α, β, δ, γ) PI3K when screened against a panel of 228 kinases (Folkes et al., 2008). GDC-0941 is considered to be a “pan-inhibitor” of class I PI3K isoforms with IC_{50}s of 0.003, 0.033, 0.003, and 0.075 μM against p110α, β, δ, and γ, respectively, and potently inhibits the phosphorylation of Akt in PC3-NCI (prostate) and MCF7.1 cells (breast), with IC_{50}s ranging from 0.028 to 0.037 μM. It is also able to inhibit the proliferation of MCF7.1 and PC3-NCI cells with IC_{50}s of 0.72 and 0.28 μM, respectively (Folkes et al., 2008).

The PI3K pathway is one of the most frequently activated pathway in tumors, with mutations in one of its components detected in up to 30% of human cancers (Luo et al., 2003). It is also thought to play a critical role in the development of glioblastomas (Hartmann et al., 2005). Activating mutations of the p110α subunit or loss of the phosphatase and tensin homolog have been identified in 70 to 80% of malignant gliomas (Koul et al., 2010). Thus, PI3K represents an attractive therapeutic target for brain tumors. However, treatment of central nervous system (CNS) tumors is extremely challenging because of difficulty in reaching the brain and tumors with anticancer agents. Although the blood-brain barrier (BBB) may be disrupted in most brain tumors (Huse and Holland, 2010), which allows some exposure to the compounds, spreading edges of neoplasms are frequently shielded by an intact BBB and the protective function of efflux transporters (de Vries et al., 2006). Thus, effective treatment of primary brain tumors or brain metastases can only be achieved with molecules that are able to cross the BBB and circumvent efflux.

The objectives of this study were to determine in vitro whether GDC-0941 was a substrate of the ATP-binding cassette transporters P-glycoprotein (P-gp) and breast cancer resistance protein 1 (Bcrp1) and to assess in vivo the impact of these transporters in potentially limiting the brain penetration of GDC-0941. Both P-gp and Bcrp1 are efflux transporters, members of the ATP-binding cassette family, expressed on the...
apical side of the BBB. We measured the modulation of the PI3K pathway in the brain of wild-type (FVBn) and triple knockout [Mdr1a/b(−/−)/Bcrp1(−/−)] mice by quantitating the levels of the downstream pharmacodynamic (PD) marker pAkt.

Materials and Methods

Chemicals. GDC-0941 and N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-(ethyl)-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-azidocarbamoyl (GF120918) were synthesized by Genentech, Inc. (South San Francisco, CA). All solvents used in analytical assays were from Thermo Fisher Scientific (Waltham, MA) and were of analytical or high-performance liquid chromatography grade. Fumitremorgin C (FTC) was purchased from Alexis Biochemicals (San Diego, CA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

Pharmacokinetic Studies. Male FVBn (wild-type), Mdr1a/b(−/−), and Bcrp1(−/−) and Mdr1a/b(−/−)/Bcrp1(−/−) knockout mice were obtained from Taconic Farms (Germantown, NY). The mice weighed between 20 and 27 g at the beginning of the study. GDC-0941 was administered either intravenously (IV) through the tail vein or by oral gavage. The IV dose was prepared in 30% sulfobutylether-β-cyclodextrin, whereas the oral formulation was 0.5% methylcellulose/0.2% Tween 80 (MCT). The three knockout strains were studied on separate days (Studies 1, 2, and 3), each time with a control group of wild-type mice as outlined in Table 2. In each study, the animals received GDC-0941 IV and orally (PO) at 2.5 and 10 mg/kg, respectively. Terminal blood samples (0.2 ml) were collected from each mouse by terminal cardiac puncture at 0.033, 0.25, 0.5, 2, 6, and 9 h post-IV dose and at 0.083, 2, 6, and 9 h post-PO dose. At each time point, an individual mouse was sacrificed in half for PD analysis and GDC-0941 concentration measurement. The samples were stored at −70°C and analyzed for GDC-0941 total concentration as described previously. For PD analysis, cell extraction buffer (Invitrogen, Carlsbad, CA) containing 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na3PO4, 2 mM Na2VO3, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate was supplemented with phosphatase, protease inhibitors (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride and added to frozen brain tissues. Brains were homogenized with a small pestle (Kontes Glass Company, Vineland, NJ), sonicated briefly on ice, and centrifuged at 20,000g for 20 min at 4°C. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL). Proteins were separated by electrophoresis and transferred to nitrocellulose membranes (Invitrogen). LcIor Odyssey Infrared detection system (Licor, Lincoln, NE) was used to assess and quantify protein expression. PI3K pathway markers were evaluated by immunoblotting using antibodies against pAkt (Ser473) and total Akt (Invitrogen and Cell Signaling, Danvers, MA, respectively). Inhibition of pAkt (%) in triple knockout mice treated with GDC-0941 was calculated by comparing pAkt signal with that measured in untreated mice. An E_{max} model was used to fit the pAkt inhibition data using Prism 5 (GraphPad Software Inc., San Diego, CA).

Results and Discussion

GDC-0941, a PI3K inhibitor currently evaluated in Phase I as an anticancer agent, was tested in vitro in MDCK cells overexpressing P-gp or Bcrp1. The bidirectional transport across the cell monolayers was studied in the presence and absence of inhibitors of P-gp or Bcrp1. The efflux ratios (P_{app, A/B}/P_{app, A-A}) of GDC-0941 in the MDR1-MDCKI and Bcrp1-MDCKI were 27 and 80, respectively. The addition of the inhibitors GF120918 or FTC reduced these ratios to 1.5 and 1.7, respectively, confirming that GDC-0941 was a substrate of both P-gp and Bcrp1 (Table 1).

To assess the impact of the efflux transporters P-gp and Bcrp1 on the absorption, disposition, and brain penetration of GDC-0941, this compound was administered to male wild-type, Mdr1a/b(−/−), Bcrp1(−/−), and Mdr1a/b(−/−)/Bcrp1(−/−) mice IV (2.5 mg/kg) and PO (10 mg/kg). Plasma clearance was low to moderate, relative to the hepatic blood flow (Table 2), and was similar between the knockout strains and the wild-type control mice in each of the three separate genotypes.
studies, suggesting that P-gp and Bcrp1 did not play a major role in the systemic clearance of GDC-0941. There was no difference in exposure after PO administration of GDC-0941 between each knockout strain and their respective wild-type control mice, suggesting that, at the dose used, P-gp and Bcrp1 did not limit the intestinal absorption of the compound (Table 2). This result is likely due to the high passive permeability of GDC-0941, allowing it to bypass intestinal efflux. Although the formulation contained Tween 80, which was reported to inhibit P-gp (Zhang et al., 2003), the similar exposure observed in the Bcrp1(−/−) and wild-type mice strongly suggests that this surfactant did not have any detectable impact on GDC-0941 absorption. Overall, P-gp and Bcrp1 did not appear to play major roles in the absorption and elimination of GDC-0941 in these studies. Brain GDC-0941 levels were also measured, and the brain-to-plasma ratios determined in wild-type and Bcrp1(−/−) mice were low and ranged from 0.02 to 0.06, which may be attributed to blood contamination of brain tissues (Table 3). The brain-to-plasma ratio increased 3- to 4-fold in the Mdr1a/b(−/−) mice, compared with the wild-type mice, and ranged from 0.08 to 0.11 (Table 3). This result still represented a very modest increase in brain penetration. In contrast to the limited changes observed in the Mdr1a/b(−/−) and Bcrp1(−/−) mice, the brain-to-plasma ratio of GDC-0941 increased approximately 30-fold in the Mdr1a/b(−/−)Bcrp1(−/−) mice, compared with the wild-type mice, and ranged from 0.5 to 1. This combined effect of P-gp and Bcrp1 on a dual substrate, in the absence of marked individual impact of each of the transporters, is consistent with reports on lapatinib (Polli et al., 2009), topotecan, and imatinib (de Vries et al., 2007; Zhou et al., 2009) and suggests that the two transporters work in concert to limit the entry of GDC-0941 into the brain. As described by Kodaira et al. (2010), this combined effect is likely independent of any interaction between P-gp and Bcrp1.

The total brain concentrations reached in the triple knockout mice after a 10 mg/kg PO dose prompted further investigations. In a follow-up study, GDC-0941 was administered PO to triple knockout and wild-type mice at doses of 50 and 100 mg/kg, which were efficacious doses in subcutaneous xenograft models (data not shown). Blood samples and brains were collected at 1 and 6 h postdose. GDC-0941 plasma and brain concentrations were measured, as well as the brain levels of pAkt, a downstream PD marker of the PI3K pathway. The brain-to-plasma ratios measured in this experiment (data not shown) were similar to those determined in the PK study described above. A marked (up to 80%) concentration-dependent suppression of the PI3K pathway was observed (Fig. 2) in the brain of triple knockout mice. These results clearly indicated that inhibition of
basal Akt phosphorylation by GDC-0941 could be detected in brain with intact BBB. Because brain tumors often rely on activated PI3K, it is likely that pAkt inhibition would be even more readily detectable in gliomas and may affect tumor growth. The free fraction of GDC-0941 in mouse brain (0.009; D. Feng, unpublished data), determined as described by Kalvass et al. (2007), was used to calculate the unbound concentrations in the brain of Mdr1a/b(−/−)/Bcrp1(−/−) triple knockout mice (Fig. 2B). The relationships between pAkt inhibition and free GDC-0941 brain concentrations could be described by an E_{max} model, allowing the estimation of an in vivo IC_{50} of 0.041 μM (Fig. 3), which is consistent with the in vitro IC_{50} of 0.028–0.037 μM of this compound (Folkes et al., 2008). This result also suggests that GDC-0941 CNS entry was not limited once efflux mechanisms were absent. No difference in basal pAkt level was observed between the untreated wild-type and triple knockout mice (Fig. 2A), implying that the PI3K signaling pathway was not altered in the brain of the triple knockout mice. No PD modulation was detected in the brain of wild-type mice (Fig. 2A), in agreement with the poor brain penetration of GDC-0941 due to efflux in that strain. Although brain PD measurements were not performed in the PK study, minimal pAkt suppression would be expected in Mdr1a/b(−/−)/Bcrp1(−/−) mice. The highest concentration of GDC-0941 observed in that strain (0.35 μM at 0.5 h; Table 2) would correspond to a free brain concentration of ~3 nM, which is approximately 10-fold lower than the IC_{50} for pAkt inhibition. This assessment may have implications for the treatment of patients with brain tumors. GDC-0941 brain penetration is efficiently limited by P-gp and Bcrp1 in wild-type mice that possess an intact BBB. However, potent suppression of the PI3K pathway may be achieved if the efflux mechanisms are inhibited or absent, as in the triple knockout mice, or most likely if the BBB loses its integrity, as observed in certain gliomas (Liebner et al., 2000).

In summary, P-gp and Bcrp1 play a concerted role in mice in limiting GDC-0941 access to the brain, which leads to a 30-fold increase in brain-to-plasma ratio in triple knockout animals compared with wild-type mice. In addition, potent PD modulation was observed when GDC-0941 was allowed to access the brain. These findings may have implications for the treatment of brain tumors with this compound as well as potentially other kinase inhibitors, and the results also highlight the importance of considering both BCRP/Bcrp1 and P-gp early in the design and optimization of brain-targeting compounds to overcome efflux at the BBB.

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