A Novel, Selective Inhibitor of Fibroblast Growth Factor Receptors That Shows A Potent Broad-spectrum of Anti-tumor Activity in Several Tumor Xenograft Models

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Abstract

The fibroblast growth factor receptors (FGFRs) are tyrosine kinases that are present in many types of tumor cells as well as endothelial cells, and play an important role in tumor cell growth, survival, and migration, and also in maintaining tumor angiogenesis. Over-expression of FGFRs or aberrant regulation of their activities has been implicated in many forms of human malignancies. Therefore, targeting FGFRs represents an attractive strategy for development of cancer treatment by simultaneously inhibiting tumor cell growth, survival, and migration, and also tumor angiogenesis. Here, we describe a potent, selective small molecule FGFR inhibitor, 2-{4-[(E)-2-{5-[(1R)-1-(3,5-dichloropyridin-4-yl)ethoxy]-1H-indazol-3-yl}ethenyl]-1H-pyrazol-1-yl}ethanol, designated as LY2874455. This molecule is active against all four FGF receptors with a similar potency in biochemical assays. It exhibits a potent activity against FGF/FGFR-mediated signaling in several cancer cell lines and shows an excellent broad-spectrum of anti-tumor activity in several tumor xenograft models representing the major FGF/FGFR relevant tumor histologies including lung, gastric, multiple myeloma, and bladder cancers with a well defined pharmacokinetic/pharmacodynamic (PK/PD) relationship.
exhibits a 6-9-fold in vitro and in vivo selectivity on inhibition of FGF- over VEGF-mediated target signaling in mice. Furthermore, LY2874455 did not show VEGFR2-mediated toxicities such as hypertension at efficacious doses. Currently, this molecule is being evaluated for its potential utility in the clinic.

**Introduction**

There exist four different fibroblast growth factor (FGF) receptors (FGFRs) in the cell: FGFR1, FGFR2, FGFR3, and FGFR4 (1). FGFR signaling activity is mediated through FGFs, their natural ligands (2). The binding of FGF to FGFR leads to the receptor dimerization and subsequent activation of downstream signaling pathways. Activated FGFR stimulates tyrosine phosphorylation and activation of a number of signaling molecules including FGFR substrate 2 (FRS2) (1, 3). The phosphorylation of FRS2 leads to activation of the Grb2/Sos1 complex and subsequent activation of the MAPK pathway (3, 4). This signaling pathway contributes to FGFR-mediated cell proliferation and migration (1, 5). The activation of the FRS2 downstream pathway, PI3K, is involved in cell motility (6) and survival (1). Thus, the FGF/FGFR signaling pathway is important for many biological processes critical to tumor cells.
Aberrant regulation of the FGF/FGFR pathway has been implicated in many forms of human malignancies. FGFR and FGF are often over-expressed in numerous cancers, and their expression correlates with poor prognosis (7-13). Activating mutations in the FGFR kinase domain have been found in several types of tumors including breast, NSCLC, bladder, gastric, prostate, colon, and multiple myeloma (5, 12). Genomic amplification of FGFR locus has also been detected in patients with breast, gastric, lung, and multiple myeloma cancers (5, 10-12). Over-expression of FGFR or FGF has also been found in many different types of tumors including bladder, multiple myeloma, prostate, and lung cancers (5, 12-15) and shown to lead to tumor formation in animals (16-18). Cell lines that ectopically over-express FGF or FGFR display transformed phenotype and grow as tumors in nude mice (12, 16-21). In addition to their roles in tumor formation and progression, FGF and FGFR are also key regulators of angiogenesis (2, 22-24), especially during tumor growth. Up-regulation of FGF/FGFR signaling activity also leads to resistance to anti-angiogenic and other chemo-therapies (12, 25-28). Together, the FGF/FGFR pathway is essential to cancer cells and inhibition of this pathway therefore offers potential clinical utilities for treating various types of cancers.

Many of the published FGFR inhibitors retain significant activity against VEGFR2 (29-31). VEGF/VEGFR2 based anti-angiogenic therapies are approved for the treatment of several types of cancers (26, 32, 33). However, the anti-angiogenic therapies are associated with side effects such as hypertension and
bleeding (34-37). FGFR signaling plays a functional role in specific tumor types as well as their angiogenesis. In order to avoid the side effects of the anti-angiogenic therapies, we sought to develop an orally bio-available, FGFR dominant kinase inhibitor lacking significant activity against VEGFR2 in vivo. To this end, we developed a potent FGFR inhibitor, LY2874455 that demonstrates robust dose dependent efficacy in multiple preclinical tumor models. Therefore, LY2874455 exhibits a potential clinical utility for treating various forms of human malignancies.

Materials and Methods

Biochemical filter-binding assays for detection of FGFR phosphorylation activities

Reaction mixtures contained 8 mM Tris-HCl (pH 7.5), 10 mM HEPES, 5 mM DTT, 10 µM ATP, 0.5 µCi ³³p-ATP, 10 mM MnCl₂, 150 mM NaCl, 0.01% Triton X-100, 4% DMSO, 0.05 mg/mL poly(Glu:Tyr) (4:1, average MW of 20-50 kDa, Sigma), and 7.5, 7.5, and 16 ng of FGFR1, FGFR3, and FGFR4, respectively and were incubated at room temperature (RT) for 30 minutes followed by termination with 10% H₃PO₄. The reaction mixtures were transferred to 96-well MAFB filter plates that were washed 3x with 0.5% H₃PO₄. After air-drying, the plates were read with a Trilux.

Cell based Acumen and AlphaScreen SureFire™ assays for detection of FGF9- and FGF2-induced Erk phosphorylation (p-Erk) in bladder cancer and HUVEC cells, respectively.
All cell lines used in this paper were not authenticated. After overnight growth, RT-112 cells (DSMZ) were washed, incubated in RPMI16409 containing 20 mg/mL BSA at 37°C for 3 hours, and treated with LY2874455 at 37°C for 1 hour followed with addition of FGF9 (500 ng/mL) (R&D) for 20 minutes. The plates were fixed with formaldehyde (3.7%) followed with washing 3x with PBS and incubation with cold methanol at −20°C for 30 minutes. The plates were washed 3x with PBS and incubated at 4°C overnight with shaking. After addition of p-Erk antibody (Cell Signaling), the plates were incubated at RT for 1 hour, washed, and then incubated with Alexa Fluor 488 (Invitrogen). The plates were read after addition of propidium iodide with an Acumen Explorer (TTP LabTech).

After overnight growth in EBM medium, HUVEC cells were washed and incubated in the same medium (1.5% serum and 20 mg/mL BSA) at 37°C 5% CO2 for 3 hours. The plates were incubated for 1 hour after addition of LY2874455 and then FGF2 (50 ng/mL, Sigma) for 15 minutes. After removing the medium and adding a lysis buffer (TGR Biosciences), the plates were incubated at RT for 10 minutes with shaking. The lysates were transferred to a 384-well plate (NUNC) filled with 10 µL of reaction mixture (TGR Biosciences). The plates were sealed, incubated at RT for 2 hours, and read using an EnVision06.

**FGF2- and VEGF-induced tube formation assays**

After overnight growth in MCDB-131 (Invitrogen), adipose-derived stem cells (ADSC, 50,000/well) and human erythroid colony-forming cells (ECFC, 5,000
/well) were incubated at 37°C for 3-4 hours. Then, FGF2 or VEGF (50 ng/mL each) (Biosource) was added followed by addition of LY2874455. After incubation at 37°C for 4 days, the medium was removed and ice cold 70% ethanol was added followed with washing 3x with PBS at RT for 20 minutes. The plates were incubated at 37°C for 1 hour after addition of the antibody against the human CD31 (R&D Systems) and washed 3x with PBS followed by addition of the antibody against smooth muscle actin labeled with Cy3 (Sigma). The plates were incubated at RT for 1.5 hours and washed 3x with PBS followed by addition of Alex Fluor-488 donkey anti-sheep IgG secondary antibody and Hoescht in PBS. The plates were incubated at RT for 30 minutes, washed 3x with PBS, and read using an Arrayscan.

**MSD ELISA based detection of phosphorylated FRS2 (p-FRS2) in cancer cells**

SNU-16 and Kato-III (KCLB) cells after grown in RPMI1640 overnight were treated with LY2874455 at 37°C for 1 hour. After removing the medium, cells were lysed (MSD lysis buffer). After centrifugation, the supernatant was collected. The detection of p-FRS2 was performed accordingly to the manufacturer's recommendations.

**Western blotting assay for detection of phosphorylated FGFR2 (p-FGFR2) in gastric cancer cells**
SNU-16 and Kato-III cells were grown, treated, and processed as described above. The resulting lysates were analyzed by SDS-PAGE. After the transfer, the nitrocellulose membrane was blocked TBS buffer (5% dry milk) and p-FGFR antibody (Cell Signaling). The membrane was blocked with a HRP-conjugated secondary antibody (Cell Signaling) in TBS (5% BSA), developed in SuperSignal Western Pico Chemiluminescent Substrate (Pierce), and exposed to a Biomax Xar Film (Kodak).

The preparation of SNU-16 tumor xenograft lysates was the same as described above for p-FRS2. The level of p-FGFR in tumors was detected as described above for detecting p-FGFR from KATO-III and SNU-16 cancer cell lines.

**Cell proliferation assay**

Cells (2000/well) were first grown in RPMI for 6 hours and treated with LY2874455 at 37°C for 3 days. The cells were stained at 37°C for 4 hours and then solubilized at 37°C for 1 hour. Finally, the plate was read at 570 nm using a plate reader (Spectra Max Gemini XS).

**MSD based in vivo target inhibition (IVTI) assays for measuring FGF2-induced Erk and VEGF-induced VEGFR2 phosphorylation in mouse heart tissues and also p-FRS2 in tumors**
Female nude mice (CD1/nu/nu) were acclimated for 1 week prior to treatment. Animals were administered with LY2874455 formulated in 10% Acacia by oral gavage. Two hours after dosing, the animals were IV-injected with mouse FGF-2 (6 µg/animal, Biosource) and sacrificed 10 minutes post injection. Animal heart was homogenized in cold lysis buffer (Boston BioProduct) containing phosphatase inhibitors (Sigma). After centrifugation, the supernatants were collected and analyzed using MSD® phospho-Erk ELISA (MSD) to determine tissue p-Erk level. The inhibitory activity of LY2874455 against VEGFR2 was assessed as described above except VEGF (6 µg/animal) (R & D) and MSD® phospho-Kdr ELISA (MSD) were used. The ELISA procedures were the same per manufacturer’s recommendation (MSD) except that 0.2% SDS is added to the lysis buffer. TED_{50} or TEC_{50} and TED_{90} or TEC_{90} was defined as the dose or the concentration necessary to achieve 50 and 90% inhibition at this time point, respectively.

SNU-16 and OPM-2 tumor xenograft tissues were homogenized in a Tris lysis buffer (MSD) containing beads (MP Biomedicals). The lysate preparation and p-FRS2 determination were carried out as described above.

To determine compound exposure, plasma samples were prepared and analyzed using a Liquid Chromatography/Mass Spectrometer-Mass Spectrometer system (Applied Biosystems). Pharmacokinetic parameters were calculated using WatsonTM LIMS (Thermo Electron).
Assessment of effects on blood pressure

Four male rats (Sprague-Dawley CD/IGS) per group were dosed with vehicle (1% hydroxyethylcellulose, 0.25% polysorbate 80 and 0.05% Dow Corning antifoam 1510-US in purified water) on day-1 and LY2874455 (1, 3, and 10 mg/kg) on day 0. On day -1, at least 120 minutes of control data was collected following vehicle administration. On day 0, data was collected for approximately 20 hours beginning after the last animal was dosed.

Tumor Xenograft models for assessing efficacy of LY2874455

RT-112, OPM-2 (DSMZ), SNU-16 cells, and NCI-H460 cells (ATCC) were grown as described before. The cells (RT-112: 2x10⁶/animal; OPM-2: 10⁷/animal; SNU-16: 10⁶/animal; and NCI-H460: 3x10⁶/animal) were mixed with matrigel (1:1) and implanted subcutaneously into the rear flank of the mice (female, CD-1 nu/nu from Charles River for RT-112, OPM-2 & NCI-H460 and female, SCID from Charles River for SNU-16). The implanted tumor cells grew as solid tumors. To test the efficacy of LY2874455 in these models, the animals were orally dosed with approximately 1mg/kg (TED₅₀) or 3 mg/kg (TED₉₀) of LY2874455 in 10% Acacia once (QD) or twice a day (BID) after tumors reached approximately 150 mm³. The tumor volume and body weight were measured twice a week.

Results

Discovery of LY2874455
LY2874455, (R)-(E)-2-(4-(2-(5-(1-(3,5-Dichloropyridin-4-yl)ethoxy)-1H-indazol-3yl)vinyl)-1H-pyrazol-1-yl)ethanol, is a type I pan FGFR kinase inhibitor (Figure 1A). On the basis of the x-ray crystal structures obtained on structurally related compounds bound to the FGFR3 protein kinase domain construct (Figure 1B), the core indazole binds in the ATP pocket and forms key hydrogen bonds with the carbonyl of glutamate 230 (2.9 Å) and the NH of alanine 232 (3.1 Å). The vinyl pyrazole moiety extends from the indazole 3-position towards solvent, making important hydrophobic interactions. The benzylic ether extends down into a hydrophobic pocket making a hydrogen bond between the pyridyl N and asparagine 236 (3.1 Å), with the chiral benzylic methyl group appearing to reinforce the preferred conformation. This model suggests that LY2874455 is likely an ATP competitive type of molecule that inhibits FGFR activity via its occupation of the ATP-binding pocket of the enzyme. Consistent with this, biochemical and cellular studies demonstrate that LY2874455 indeed inhibits FGFR autophosphorylation activity (see below).

**Biochemical characterization of the purified FGFR proteins and the inhibitor, LY2874455**

To assess the ability of LY2874455 to inhibit FGFRs, we cloned the cDNA corresponding to the cytoplasmic/kinase domain of each human fgfr gene, expressed their gene products in SF9 insect cells as N-terminal His-tagged proteins: FGFR1 (AA 406-822), FGFR2 (AA407-821), FGFR3 (AA405-806); and
FGFR4 (AA 399-802), and purified the proteins to apparent homogeneity by using immobilized metal affinity chromatography followed by size exclusion chromatography (data not shown). We then developed a filter-binding assay for each purified protein. These assays, using poly(Glu:Tyr) and $^{33}$p-labeled ATP as substrates, measure autophosphorylation and phosphate transferring activities of each enzyme (Materials and Methods). Using these assays, we kinetically characterized each FGFR. We showed that the purified FGFR1, 2, 3, and 4 proteins had the $K_M$ values of 37.1, 47.1, 123.8, and 167 µg/mL for poly(Glu:Tyr), respectively, which correspond to 0.74-1.85, 0.94-2.35, 2.48-6.19, and 3.34-8.35 µM, respectively, since the poly(Glu:Tyr) have an average MW of 20-50 kDa (Sigma). Next, we determined the $K_M$ values of FGFR1, 2, 3, and 4 proteins for ATP to be 36.7, 10.2, 59.9 and 12.3 µM, respectively.

To determine its inhibitory activity, we tested LY2874455 in the filter-binding assays developed. We showed that LY2874455 inhibited FGFR1, 2, 3, and 4 in a dose-dependent manner with IC$_{50}$ values of 2.8, 2.6, 6.4, and 6 nM, respectively. Thus, these biochemical results suggest that LY2874455 is a pan FGFR inhibitor with a similar potency for each enzyme.

To assess its biochemical selectivity, LY2874455 was tested in a VEGFR2 time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Cerep, Poitiers, France). LY2874455 appeared to inhibit VEGFR2 with an IC$_{50}$ of approximately 7 nM. Since this assay uses a universal artificial peptide substrate and a truncated VEGFR2 enzyme with its cytoplasmic domain deleted (Cereps),
the selectivity of LY2874455 against VEGFR2 was further assessed by using the cellular and in vivo assays (see below).

**Cellular activity of LY2874455**

On the basis of the biochemical results, LY2874455 appears to be a potent pan inhibitor of FGFRs. To assess its ability to inhibit the target in the cell, we attempted to develop cell based assays that directly measure the inhibition of FGFR phosphorylation in the cell. However, our extensive efforts towards developing these types of p-FGFR assays failed due to the lack of high quality antibodies against p-FGFRs. We next explored the possibility of developing assays that can measure the inhibition of the downstream signaling activity of FGFR. To this end, we developed two high throughput cellular assays that measure the inhibition of FGF2- and FGF9-induced Erk phosphorylation in HUVEC and RT-112 cells, respectively (Materials and Methods). The HUVEC and RT-112 cell lines are shown to express FGFR1 (2 & data not shown) and FGFR3 (38), respectively. Using these assays, we showed that LY2874455 potently inhibited the Erk phosphorylation induced by FGF2- and FGF9 in both cell lines in a dose-dependent manner with average IC$_{50}$ values of 0.3-0.8 nM (Figure 2A). To establish that the inhibition of Erk phosphorylation by LY2874455 is due to its inhibition of FGFR in the cell, we tested this molecule in two gastric cancer cell lines, SNU-16 and KATO-III, which contain a high level of p-FGFR2 due to the amplification of fgfr2 in the cells (8, 39). LY2874455 indeed inhibited FGFR2
phosphorylation in SNU-16 and KATO-III cells with estimated IC$_{50}$ values of 0.8 and 1.5 nM, respectively (Figure 2B). In addition, LY2874455 inhibited the phosphorylation of FRS2, an immediate downstream target of FGFR in these cell lines, again with a similar potency of 0.8-1.5 nM (Figure 2C). Together, these results suggest that LY2874455 inhibits FGFR in the cell.

To further establish that LY2874455 specifically inhibits FGFR in the cell, we tested this molecule in a cell proliferation assay to assess its ability to inhibit different multiple myeloma cancer cell lines, some of which (KMS-11 and OPM-2) carry a FGFR3 chromosomal translocation, resulting in the over-expression of FGFR3 (40, 41). In this study, we also used L-363 and U266, known to contain little or no FGFR3 (41). We confirmed by a Western blotting analysis that KMS-11 and OPM-2, but not L-363 and U266, contained a relatively high level of p-FGFR (data not shown). The relative IC$_{50}$ values of LY2874455 for KMS-11, OPM-2, L-363, and U266 were determined to be 0.57, 1.0, 60.4, and 290.7 nM, respectively. Thus, the presence of the FGFR3 chromosomal translocation in multiple myeloma cancer cell lines renders them significantly more susceptible to inhibition by LY2874455. Consistent with this finding, we have also shown that the presence of fgfr2 amplification in gastric cancer cell lines renders them significantly more susceptible to inhibition by LY2874455 as compared to those with little or no FGFR2. For example, SNU-16 and KATO-III with a highly amplified fgfr2 are over 1000-fold more sensitive to inhibition by LY28774455 than NUGC-3 and SH-10-TC in which no fgfr2 amplification was observed (data not shown).
Therefore, these results suggest that LY2874455 inhibits FGFR in the cell and that its anti-proliferative effects are directly linked to the inhibition of FGFR signaling.

Several reported FGFR inhibitors have a dominant activity against VEGFR2 as compared to FGFRs (29-31). At efficacious doses, these molecules also retain many of the class effects associated with VEGFR2/VEGFR blockade including hypertension and bleeding (34-37). We therefore wanted to further assess LY2874455 with regard to its cellular and in vivo selectivity towards FGFRs vs VEGFR2. Using the FGF2- and VEGF-induced tube formation assays, we evaluated the ability of LY2874455 to inhibit these growth factors-induced tube forming activities. As shown in Figure 2D, LY2874455 inhibited the FGF2- and VEGF-induced tube forming activities with IC$_{50}$ values of 0.6 and 3.6 nM, respectively. Thus, these results demonstrate that LY2874455 is significantly more potent (=6-fold) at inhibiting the FGF2- than VEGF-induced tube forming activity. This finding is further confirmed by the results of the in vivo target inhibition and toxicology studies (see below)

**In vivo target inhibition (IVTI) activity of LY2874455**

On the basis of the cellular assay results that LY2874455 appears to be a potent and selective FGFR inhibitor, we want to further confirm that this molecule inhibits the target in an in vivo setting. As discussed above, due to the lack of high quality antibodies against p-FGFR that could be used for cellular assay development, we ruled out the possibility of developing an IVTI assay that
directly measured FGFR phosphorylation in animal tissues. To this end, we have explored several alternative approaches including the development of an assay based on the detection of the downstream signaling molecules of FGFR, and subsequently developed for the first time a robust IVTI assay that measures FGF-stimulated Erk phosphorylation in the heart tissues of mice (Materials and Methods). Using this assay, we showed that LY2874455 exhibited a potent, dose-dependent inhibition of FGF-induced Erk phosphorylation in the heart tissues of mice with estimated TED_{50} and TED_{90} values of 1.3 and 3.2 mg/kg, respectively (Figure 3A). In this study, we also examined the relationship between the exposures or concentrations of LY2874455 in the plasma and the inhibition of Erk phosphorylation. The results of this study showed that LY2874455 also exhibited a concentration-dependent inhibition of FGF-induced Erk phosphorylation with estimated TEC_{50} and TEC_{90} values of 6.2 and 28.4 nM, respectively (Figure 3B). To assess the in vivo stability of this molecule, LY2874455 was tested in mice at 3mg/kg (TED_{90}). This molecule inhibited 99.7, 95.5, 76.9, 45.3, and -23.3% of FGF-induced Erk phosphorylation 1, 2, 4, 8, and 24 hours, respectively after dosing (data not shown). Finally, to further confirm these mouse IVTI findings, we tested LY2874455 in a rat heart IVTI assay. Consistent with the results obtained from the mouse IVTI assay, LY2874455 also exhibited a potent IVTI activity with an estimated TED_{50} of 0.39 mg/kg (data not shown). Together, the results of these studies show that LY2874455 potently inhibits the FGFR signaling activity in vivo.
These findings were further confirmed by the results of the in vivo efficacy and toxicity studies (see below).

On the basis of its differential inhibition of FGF- and VEGF-induced tube forming activities, we wanted to further assess the VEGFR2 selectivity of LY2874455 in vivo. To this end, we also developed an IVTI assay that measures the VEGF-induced VEGFR2 phosphorylation in the heart tissues of mice (Materials and Methods). When tested in this assay (Figure 3C), LY2874455 exhibited a concentration dependent inhibition of VEGFR2 phosphorylation with estimated TEC$_{50}$ and TEC$_{90}$ values of 36 and 252 nM, respectively, as compared to the TEC$_{50}$ (6.2 nM) and TEC$_{90}$ (28.4 nM) obtained in the FGF-induced Erk phosphorylation assay. The results of this study show that LY2874455 is again much more (6-9-fold) potent at inhibiting the FGF- than VEGF-induced signaling activity in vivo (ie: VEGF p-VEGFR2 TEC$_{50}$/FGF p-Erk TEC$_{50}$ = 36 nM/6.2 nM = 6, and VEGF p-VEGFR2 TEC$_{90}$/FGF p-VEGFR2 TEC$_{90}$ = 252 nM/28.4 nM = 9).

The VEGF/VEGFR2 based anti-angiogenic therapies are associated with hypertension in the clinic (34-7). We therefore wanted to further assess whether treatment of animals with LY2874455 could also lead to a dose-dependent increase in blood pressure. When rats were dosed with LY2874455 at 1 and 3 mg/kg, which is 2.6 and 7.7-fold over the TED$_{50}$ (0.39 mg/kg) obtained in the rat heart IVTI assay, respectively, there were no significant changes observed in blood pressure (Figure 4 A & B). However, when rats were dosed with LY2874455 at 10 mg/kg, which is 25.6-fold over the TED$_{50}$, there were significant increases
observed in arterial pressures (Figure 4C). The peak of this increase in blood pressure occurred approximately between 4 and 8 hours after dosing (Figure 4C). These results suggest that the VEGFR2 activity was inhibited in vivo by LY2874455 at 10 mg/kg, but not at 1 and 3 mg/kg. Therefore, a treatment based on this molecule may avoid the class effects of the anti-VEGF/VEGFR2 based therapies in the clinic. Taken together, these in vivo and in vitro studies establish that LY2874455 is a potent FGFR-dominant inhibitor.

**In vivo efficacy of LY2874455 in different tumor models**

To assess its potential clinical utility, we evaluated the in vivo efficacy of LY2874455 in different tumor models that are dependent on or relevant to FGF/FGFR biology. By testing the molecule in these FGF/FGFR relevant tumor models, we also hope to identify the specific types of tumor histologies that can be effectively targeted by the molecule, thereby used as a basis for patient selection in the clinic. To this end, we identified and characterized several appropriate cancer cell lines with altered FGFR or FGF levels: RT-112 (over-expressing FGFR3) (38), SNU-16 (amplified FGFR2) (39), OPM-2 (over-expressing a mutant FGFR3 due to a chromosomal translocation) (40, 41), and NCI-H460 (a high level of FGF2) (42) and subsequently used these cell lines to establish tumor xenograft models for assessing efficacy of the molecule. As shown in Figure 5, LY2874455 exhibited a rapid, robust dose-dependent inhibition of tumor growth in all four models tested. Importantly, this molecule caused a significant
regression of tumor growth in the RT-112, SNU-16, and OPM-2 tumor models especially when dosed at 3 mg/kg BID (twice a day) (Figure 5A, B, & D.). Also, LY2874455 exhibited an excellent PK/PD relationship as demonstrated by its dose-dependent inhibition of the tumor growth at TED_{50} and TED_{90} (1 and 3 mg/kg, respectively). When tested in the RT-112 tumor xenograft model on an intermittent dosing schedule (BID one week-on and one week-off or BID two days-on and two days-off), LY2874455 was also efficacious (data not shown). Taken together, the results of these in vivo efficacy studies demonstrate that LY2874455 exhibits a potent broad-spectrum of anti-tumor activity in a number of tumor models that represent the major types of tumor histologies in the clinic.

To assess whether the inhibition of tumor growth by LY2874455 is due to the inhibition of the target in the cell, we analyzed the tumor samples that had been treated with the molecule for the effects of the molecule on p-FGFR and p-FRS2 levels. LY2874455 exhibited a dose-dependent inhibition of FGFR2 phosphorylation in SNU-16 tumor xenografts as compared to that in the vehicle control tumors (Figure 6A and B.). Similarly, this molecule also inhibited FRS2 phosphorylation in a dose-dependent manner (Figure 6C.). The inhibition of FGFR2 and FRS2 phosphorylation by LY2874455 is well correlated with its attenuation of tumor growth (Figure 5A and Figure 6). Consistent with these findings, the inhibition of FRS2 phosphorylation by LY2874455 was also observed in OPM-2 tumor xenografts, but not in the vehicle control tumors, and that this inhibition is again well correlated with its attenuation of tumor growth (data not
shown). Thus, the results of these studies demonstrate that the attenuation of
tumor growth by LY2874455 is most likely due to its inhibition of the FGFR signaling
activity in the cell.

Discussion

In this study, we identified LY2874455 as a novel, potent FGFR inhibitor. This
molecule is a FGFR dominant inhibitor with a significantly lower VEGFR2 activity.
It is much more potent at inhibiting the proliferation of the different cancer cell
lines with increased FGF or FGFR expression than those with little or no FGFR
expression. In addition, it is a potent FGFR inhibitor in vivo and very efficacious in
several tumor models representing major tumor histologies in the clinic.
Furthermore, the inhibition of the FGF/FGFR signaling pathway activity by this
molecule is well correlated with its inhibition of tumor growth. Finally, this
molecule does not cause significant hypertension at its efficacious dose. In light
of these results, the identification of this potent FGFR dominant inhibitor may
lead to the development of novel anti-cancer therapies for many patients:
especially those with the histologies relevant to the FGF/FGFR signaling pathway,
and also those in which the anti-VEGF/VEGFR based therapies are
contraindicated.

There are several lines of evidence demonstrating that LY2874455 inhibits
the target, FGFR, in the cell and that this on-target inhibition leads to the robust
efficacy in several FGFR relevant tumor models. First, LY2874455 is much more
potent at inhibiting the proliferation of cancer cells with a significantly increased FGFR signaling activity as compared to those with a little or no FGFR signaling activity. Second, LY2874455 preferentially inhibits FGF-over VEGF-induced tube forming activity. Third, LY2874455 inhibited the growth of tumor xenografts derived from gastric and multiple myeloma cell lines carrying a highly amplified FGFR2 and an over-expressed FGFR3 due to a chromosomal translocation, respectively, and this growth inhibition is correlated with its inhibition of FGFR2 and FRS2 phosphorylation in the tumor cells. Fourth, to achieve a robust anti-tumor activity with a VEGFR2 inhibitor, it is well established that the inhibitor has to be administered at a dose that can yield a concentration at which the target VEGFR2 activity has to be inhibited >90% during the period of the study (43). In our IVTI studies, we showed that LY2874455 inhibited FGFR activity by 90%, but VEGFR2 activity ≤45% (data not shown) when dosed at 3 mg/kg for 2 hours. In an IVTI time course study, LY2874455 inhibited FGFR activity by ≈50% at 8 hours when dosed at 3 mg/kg. On the basis of these findings, it is reasonable to believe that the inhibition of VEGFR2 activity by LY2874455 would not exceed 45% at maximum, nor would last more than 4 hours (2x2) during a period of 24 hours with a twice daily dosing schedule. Furthermore, the administration of LY2874455 did not lead to a significant increase in blood pressure in rats when dosed at 1 or 3 mg/kg (2.6 and 7.7-fold over the TED\textsubscript{50} of 0.39 mg/kg, respectively), but at 10 mg/kg (25.6-fold over the TED\textsubscript{50} of 0.39 mg/kg). This finding again demonstrates that LY2874455, when administrated at an
efficacious dose (TED$_{50}$ or TED$_{90}$), does not significantly inhibit VEGFR2 activity. Taken together, these findings indicate that the inhibition of tumor growth by LY2874455 is likely due to its inhibition of the intended target, FGFR.

The importance of the FGFR signaling pathway in the pathogenesis of diverse tumor types is well documented in the clinic as activating mutations in, and genomic amplification and over-expression of FGFRs or FGFs due to other genetic alterations have been reported in many different types of tumors (12). Therefore, the development of cancer therapies based on targeting the FGF/FGFR signaling pathway represents an attractive strategy and should have broad clinical implications to the treatment of various types of cancers. In this study, we show that LY2874455 is effective at inhibiting the proliferation of a variety of different cancer cell lines, especially those with elevated FGF or FGFR levels such as gastric, NSCLC, multiple myeloma, and bladder cancer cell lines. This molecule is also effective against OPM-2 which over-expresses FGFR3 and is also known to contain an activating mutation in its kinase domain (44). In addition, this molecule is potent at inhibiting the in vivo growth of tumor xenografts derived from these diverse cancer cell lines. In light of these findings, LY2874455 has the potential to be used as an effective anti-cancer agent for treating a variety of different types of cancers including, but not limited to those as described above and also other types of cancers with an aberrant FGF/FGFR signaling activity. As discussed above, its potential clinical utilities for different types of cancer patients are currently being evaluated in a phase 1 study.
The presence of genetic alterations in or aberrant regulation of the FGFR signaling pathway also enables us to develop a robust tailoring strategy aimed at identifying and selecting appropriate cancer patients who can most likely benefit from treatment with the molecule in the clinic. For example, patients with gastric cancers carrying an amplified \textit{fgfr2}, which can be identified via FISH (39), are predicted to respond to treatment with LY2874455 due to the extreme sensitivity of these cell lines to the inhibition by the molecule. Likewise, patients with multiple myeloma cancers carrying a FGFR3 chromosomal translocation can also be identified via FISH (45). Finally, the finding that LY2874455 effectively inhibits FRS2 phosphorylation in tumor xenografts derived from both gastric and multiple myeloma cell lines suggests that p-FRS2 can be used as a PD biomarker for assessing effects of LY2874455 on its intended target in the clinic. Using well defined patient populations in combination with the potential PD biomarkers may enhance the understanding of the mechanism of action of the molecule in the clinic and subsequently its clinical development.

Anti-angiogenesis based therapies are effective in the clinic, but also have well-characterized side effects, limiting the duration of exposure for many patients. Furthermore, over-expression of FGF2 or alterations in other signaling pathways has been implicated in the resistance to the anti-VEGF/VEGFR2 based agents clinically and pre-clinically (25-27, 46). The development of the resistance could pose a challenge to the use of these therapies in the clinic (25-27, 46). As discussed earlier, although several small molecule inhibitors of FGFR
are currently in the clinical development, in general, they retain significant VEGFR2 inhibitory activity. In contrast, our molecule is predominately a FGFR inhibitor. LY2874455 may be considered for treatment of patients who progressed on anti-VEGF/VEGFR2 based therapies. Although anti-angiogenic agents have demonstrated survival benefit in both the preclinical and clinical settings, some preclinical studies have suggested that they may have the potential to accelerate tumor metastasis (47, 48). FGFR inhibitors appear to have primary effects on tumor cell growth, but also may impact tumor-induced angiogenesis due to the important roles of FGFR in tumor and endothelial cells. This combined inhibitory effect warrants further testing in the clinic (46).
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References


Figure Legends

Figure 1.  A, Chemical structure of LY2874455, 2-{4-[(E)-2-{5-[(1R)-1-(3,5-dichloropyridin-4-yl)ethoxy]-1H-indazol-3-yl}ethenyl]-1H-pyrazol-1-yl}ethanol.  B, A binding model of LY2874455 in the ATP site of FGFR3.

Figure 2. Inhibition of FGF/FGFR- and VEGF/VEGFR2-mediated signaling activities in cells by LY2874455.  RT-112, HUVEC, KATO-III, and SNU-16 cells were grown and treated with LY2874455 at various concentrations (Materials and Methods).  After the treatment, cells were processed and analyzed for p-Erk, p-FGFR2, and p-FRS2 levels (Materials and Methods).  Adipose-derived stem cells
and human erythroid colony-forming cells were grown, induced with FGF2 or VEGF, and treated with LY2874455 at various concentrations (Materials and Methods). After the treatment, tubes formed were visualized and quantified. **A**, Inhibition of FGF2- and FGF9-induced Erk phosphorylation in HUVEC and RT-112 cells. **B**, Inhibition of FGFR2 phosphorylation in SNU-16 and KATO-III cells. **C**, Inhibition of p-FRS2 in SNU-16 and KATO-III. **D**, inhibition of FGF2 (▼) or VEGF (■) induced tube formation.

**Figure 3. Inhibition of FGF- and VEGF-induced Erk and VEGFR2 phosphorylation, respectively, in mouse heart tissues by LY2874455.** Mice were first treated with LY2874455 at various doses followed by tail vein injection of mouse FGF2 or VEGF (Materials and Methods). After the FGF2 or VEGF treatment, the heart tissues were collected and processed for the analysis of p-Erk and p-VEGFR2 levels by MSD ELISA assays (Materials and Methods). The plasma was also collected for the analysis of the exposures or concentrations of LY2874455 vs p-Erk or p-VEGFR2 levels (Materials and Methods). **A**, Dose-dependent inhibition of p-Erk formation. **B**, Exposure-dependent inhibition of p-Erk formation. **C**, Overlay of exposures vs inhibition of p-Erk and p-VEGFR2 formation.

**Figure 4. Effects of LY2874455 on blood pressure in rats.** Animals were treated and effects on blood pressure were assessed as described in (Materials and
Methods). Mean arterial blood pressure was measured in rats dosed with 1mg/kg (A), 3 mg/kg (B), and 10 mg/kg of LY2874455 (C).

Figure 5. Efficacy of LY2874455 in different tumor xenograft models. Mice were subcutaneously implanted with SNU-16, OPM-2, NCI-H460, and RT-112 (Materials and Methods). Once tumors grew to about 150 mm³, animals were randomly assigned (6-8 /group), and orally dosed with vehicle alone or LY2874455 at the indicated doses on an once a day (QD) or twice a day (BID) dosing schedule for 14-21 days (Materials and Methods). The growth of tumors was monitored and measured twice a week. A, SNU-16 tumor xenografts treated with LY2874455 (1.5 and 3 mg/kg QD or BID). B, OPM-2 tumor xenografts treated with LY2874455 (1.5 and 3 mg/kg BID). C, NCI-H460 tumor xenografts treated with LY2874455 (1 and 3 mg/kg QD or BID). D, RT-112 tumor xenografts treated with LY2874455 (3 mg/kg QD or BID).

Figure 6. Inhibition of FGFR2 and FRS2 phosphorylation in SNU-16 tumor xenografts by LY2874455. After the treatment with LY2874455 at various doses for about 2 weeks, the tumor xenografts derived from SNU-16 (see Figure 5A) were collected and processed. The levels of p-FGFR2 and p-FRS2 were measured by Western blotting and MSD ELISA analyses, respectively (Materials and Methods). A) Western blotting analysis of p-FGFR2 levels in the tumors vs. dose. B) The intensity of each band corresponding to p-FGFR2 from (A) was
quantified and plotted vs. dose. C) MSD ELISA analysis of p-FRS2 levels in the tumors vs. dose.
Fig. 1, Zhao et al.
Fig. 2 (Zhao et al.).

A  

% Inhibition

0.0001  1  0.0001  1  
LY2874455 (µM)  

HUVEC  
RT-112  

LY2874455 (nM)

B  

DMSO  0.098  0.195  0.391  0.781  1.562  3.125  6.25  12.5  25  50  100  

SNU-16  
KATO-III  

p-FGFR2  
β-actin  

p-FGFR2  
β-actin

C  

% Inhibition

0  20  40  60  80  100  

2874455 (nM)

Snu-16  Kato-III

D  

% Inhibition

-20  0  20  40  60  80  100  

Log [2874455 nM]

VEGF  FGF2

Fig. 2 (Zhao et al.)
LY2874455 (mg/kg)
TED\textsubscript{50} = 1.34 mg/kg
TED\textsubscript{90} = 3.18 mg/kg

LY2874455 (ng/ml)
TEC\textsubscript{50} = 2.76 ng/ml = 6.2 nM
TEC\textsubscript{90} = 13 ng/ml = 28.4 nM

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Fig. 3 (Zhao et al.)
Fig. 4. Zhao et al.
Fig. 5. Zhao et al.
Fig. 6. Zhao et al.
Molecular Cancer Therapeutics

A Novel, Selective Inhibitor of Fibroblast Growth Factor Receptors That Shows A Potent Broad-spectrum of Anti-tumor Activity in Several Tumor Xenograft Models

Genshi Zhao, Wei-ying Li, Daohong Chen, et al.

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